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Peroxidases of *Neurospora*: purification and some properties of the heat-inducible and constitutive enzyme.

by

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ABSTRACT

Heat Inducible (HIP) and constitutive (CP) peroxidases were isolated from *Neurospora crassa*. HIP was isolated from heat shocked mycelium, and CP from a mutant overexpressing constitutive peroxidase. These enzymes were purified to near homogeneity, as judged by electrophoresis in denaturing gels, and some of their properties were investigated. For HIP and CP the relative molecular mass was found in the range of 116 kDa and 118-136 kDa, heat sensitivity 43 - 55°C and 48 - 58°C, pH optima 5.0 and 3.5 respectively. K_m, V_{max} and k_{cat} values for the reaction catalyzed by the CP with H_2O_2 as the variable substrate were ~22 μM, ~447 nmol/mg and ~0.3/sec, respectively, and for ABTS ~55 μM, ~453 nmol/mg, ~0.3/sec. For HIP with H_2O_2, K_m, V_{max} and k_{cat} were ~44 μM, ~6640 nmol/mg and ~10/sec respectively, and for ABTS the corresponding values were ~36 μM, ~5200 nmol/mg and ~8/sec. Neither of the enzymes utilized guaiacol.

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DEDICATION

To my parents Janusz and Halina

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INTRODUCTION

1. INTRODUCTION

All living cells display a rapid response to adverse environmental conditions, a phenomenon commonly designated as the stress response. The ability of organisms to sense and respond to unscheduled changes in the environment is crucial to their survival [Foster and Spector 1995]. Heat shock and oxidative stress are the two types of stresses that involve induction of peroxidase activity in *Neurospora crassa*, which is the subject of this study.

The heat shock response was first reported as a dramatic change in gene activity induced by a brief heat treatment of *Drosophila hydei* larvae [Ritossa 1964]. Until 1980 most attention was focused on the heat shock response in *Drosophila* where the heat shock proteins were first discovered. Similar findings with other eukaryotes and prokaryotes soon suggested that the response is an evolutionarily conserved genetic system which may be beneficial for the living cell [reviewed in Craig et al. 1993; Hartl 1996].

Heat shock proteins (HPSs), also described as "stress" proteins, are synthesized in response to a variety of treatments including sublethal hyperthermal exposure, oxidative stress, toxic metals, metabolic inhibitors and ethanol [Craig et al. 1993]. Many of the HSPs are present at low, but significant, level at physiologically relevant temperatures. Their induction upon heat shock is rapid and specific. The induction temperature reflects a stress condition for an organism such that HSPs are induced at a temperature which is above its optimal growth temperature. Specific induction of HSPs correlates with the subsequent development of thermotolerance, which is defined as a transient ability of the

cells subjected to high temperature to survive subsequent exposures to lethal temperatures. Heat shock proteins are produced as a result of a moderate stress, presumably to protect the organism from the lethal effects of a more severe stress. One of the common signals for HSP induction comprises the presence of abnormal or denatured proteins in the cells. Heat shock proteins are believed to have two major functions. First, to promote degradation of damaged proteins as some HSPs have proteolytic activity. Second, to act as molecular chaperones by preventing aggregation and mediating proper folding of nascent polypeptides, and in some instances in refolding of denatured proteins [Di Domenico et al. 1982; Craig et al. 1993].

The spectrum of HSPs synthesized upon a stress challenge is similar in many organisms. Stress protein families have historically been classified according to their average apparent molecular mass: HSP100, HSP90, HSP70, HSP60, HSP26 and HSP12 [Table 1]. In addition to stress-induced members, many proteins homologous to HSPs are synthesized constitutively, suggesting that important cellular functions may be performed by these proteins under normal circumstances. The functional significance of the heat shock response is evident from the magnitude and speed of the process. The ability of the cells to shift their metabolism rapidly towards synthesis of HSPs suggests that they may depend on this phenomenon for survival. On the basis of experiments conducted by Mager et al. (1993) in yeast, the shift from 23°C to 30°C does not result in any major alteration in protein synthesis, but a shift to 36°C does. The latter also leads to protection from death due to subsequent extreme (52°C) heat treatment.

Designation			Cellular Localization	Function
Yeast	**Bacteria**	**Higher eukaryotes**		
HSP150			secretory	unknown
HSP104			nocle(ol)us	stress tolerance
HSP83/90			cytosol/nucleus	chaperone
HSP70	HSP40, HSP70	HSP72, HSP73, HSP40	cytosol	protein transport
ssa1, ssa2, ssa3, ssa4			cytosol	chaperone
ssb2			unknown	unknown
ssc1 ssd1			mitochondria	chaperone
	DNAK	BiP	endoplasmic reticulum	chaperone
MGE1				
	GrpE			
HSP60	GroEL/GroES	HSP60/HSP10	mitochondria/cytosol	chaperone
HSP30			plasma membrane	unknown
HSP26			cytosol/nucleus	unknown
HSP12			cytosol	unknown
Ubiquitin			cytosol	protein degradation
TRiC	TRiC	TRiC	cytosol	chaperone
-Glyceraldehyde 3-phosphate dehydrogenase			cytosol	glycolysis
-Phosphoglycerate kinase			cytosol	glycolysis
-Catalase			cytosol	antioxidative defense

Table 1. Major stress proteins in yeast, bacteria and eukaryotic cells [Mager et al. 1993; Hartl 1996].

Recently, it has been reported that in yeast approximately 80 proteins are transiently induced as a result of a heat shock; twenty of these proteins are now classified as major HSPs [Lee and Park 1998]. Among these heat-induced proteins, HSP104 was found to be the most important thermotolerance related protein of *Saccharomyces cerevisiae*, enhancing survival after exposure to extreme heat or high concentrations of ethanol by more than 1000-fold. During recovery from stress conditions, HSP104 promoted disaggregation of heat-damaged proteins, enhancing their refolding by more than 500-fold. These observations led to the suggestion that HSP104 has the capacity to alter directly, or indirectly, the nature of aggregates during their formation when the rate of the protein unfolding exceeds the capacity of other chaperones to prevent the accumulation of aggregates [Glover and Lindquist 1998].

Stress Response of *Neurospora crassa*: Heat Shock and Oxidative Stress

Exposure of *N. crassa* to heat shock, oxidative stress and toxic metals causes rapid synthesis of heat shock proteins. It was shown that the high molecular mass heat-induced proteins include members of HSP70, HSP80 and HSP90 families of stress proteins [Kapoor and Lewis 1987]. The members of the HSP70 family are very highly conserved as they share ~50% amino acid sequence identity with each other. These proteins are presumed to bind to hydrophobic surfaces and maintain proteins in an unfolded conformation in order to facilitate the transport of such proteins across membranes and to promote their refolding [Chirico et al. 1988]. In *N. crassa*, HSP70 is the second most abundantly produced protein during hyperthermia. It is also an abundant

protein during normal growth and it is presumably located in the cytosol. HSP80 of *N. crassa*, the equivalent of mammalian HSP90, is the most abundant heat-inducible protein of *N. crassa*. It is also induced by ethanol and carbon starvation even at the normal growth temperature of 28°C, and it is localized in the cytosol [Roychowdhury et al. 1992]. The HSP90 family comprises cytosolic proteins in eukaryotic cells, shown to assist in protein folding by suppressing aggregation of denatured proteins and increasing the yield of correctly folded and functional proteins [Parsel and Lindquist 1993]. Another group of researchers [Bansal et al. 1991] performed experiments in which HSP90 was shown to play a role in thermotolerance in mammalian cells, where cell lines with reduced levels of this protein exhibited reduced survival at highly elevated temperatures.

As the subject of this investigation was purification and characterization of the heat-inducible and constitutive peroxidases of *Neurospora crassa* a brief description of oxidative stress is presented in the following.

The vast majority of organisms living on earth require oxygen. Animals, plants and many microorganisms rely on oxygen as an efficient means of energy production. This, however, started 2.5 billion years ago when cyanobacteria evolved oxidative photosynthesis and the Earth's atmosphere became more and more oxidative [McKay and Hartman 1991]. With an increase in the atmospheric level of molecular oxygen, a massive evolutionary selection pressure was imposed on existing, predominantly anaerobic organisms. This evolutionary pressure brought about not only the evolution of mechanisms for utilization of oxygen but also mechanisms for protecting cells against

reactive oxygen species [reviewed by Gille and Sigler 1995].

Although the oxygen molecule has a low reactivity, the toxic effects of oxygen are based on the formation of free radicals, and the formation of free oxygen radicals is a normal byproduct of aerobic metabolism [Gille and Sigler 1995; Demple 1991]. It is noteworthy that non-radical forms of oxygen (e.g. its excited singlet state or organic hydroperoxides, ROOH) are also fairly reactive.

Oxidative stress can be functionally defined as an excess of peroxidants in the cell. Active oxygen molecules have been shown to cause damage to DNA, RNA, proteins and lipids. Active oxygen species are produced as an inescapable byproduct of normal aerobic metabolism and their production is further enhanced by exposure to certain environments or by dietary or disease conditions. Oxygen toxicity results when the degree of oxidative stress exceeds the capacity of the cellular defense systems. Oxidative stress is strongly implicated in a number of diseases such as rheumatoid arthritis, inflammatory bowel disorders and atherosclerosis. It also is one of the important factors in mutagenesis and aging. Virtually all aerobic organisms have evolved complex defense and repair mechanisms to compensate for damaging effects of active oxygen [Farr et al. 1991].

The oxygen molecule in its ground state has two unpaired electrons. It is not very reactive due to the fact that the unpaired electrons have parallel spins and occupy different π antibonding orbitals. Therefore, to participate in oxidative reactions, oxygen first has to be activated to produce an unstable electron pair.

The reduced forms of molecular oxygen are mostly in an activated state and transition metals, which are capable of receiving or donating single electrons, play an

important role in oxygen-dependent reactions. The metals are usually localized in the active sites of various enzymes [Gille and Sigler 1995].

In the following, a brief account of reactive oxygen species including the superoxide radical, the hydroxyl radical and hydrogen peroxide along with the cellular defenses is presented.

Superoxide Radical

Active oxygen arises from an impressive array of environmental and endogenous mechanisms. Particularly, superoxide anion radical ($O_2^{\bullet -}$) seems to form easily [Farr et al. 1991]. The most important sources of it *in vivo* are the electron transport chains of the mitochondria, the endoplasmic reticulum and the chloroplasts. The reactivity of the superoxide radicals depends strongly on the nature of the medium. This radical is relatively highly reactive and stable in a hydrophobic environment while it has a lower reactivity and rapidly disappears in a hydrophilic environment [Gille and Sigler 1995]. There are three different spontaneous reactions responsible for the disappearance of superoxide radical in aqueous solution [Fridovich, 1989]:

$$HO_2^{\bullet} + HO_2^{\bullet} \longrightarrow H_2O_2 + O_2 \quad [1]$$

$$HO_2^{\bullet} + O_2^{\bullet -} + H^{+} \longrightarrow H_2O_2 + O_2 \quad [2]$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad [3]$$

These reactions are most rapid at acid pH values. It is noteworthy that each of the $O_2^{\bullet -}$ producing systems also gives rise to H_2O_2. In addition to nonenzymic reactions the other important sources of H_2O_2 are the reactions catalyzed by superoxide dismutase (SOD):

$$O_2^{\bullet-} + O_2^{\bullet-} \xrightarrow{CuZnSOD} H_2O_2 + O_2 \quad [4]$$

and several flavin-containing oxidases. For instance, the glycollate oxidase of plant peroxisomes oxidizes glycolic acid to glycoxylic acid with production of H_2O_2:

$$\text{glycollate} + O_2 \longrightarrow \text{glycoxylate} + H_2O_2 \quad [5]$$

A minor source of hydrogen peroxide production includes deamination of amino acids by amino acid oxidases, and oxidation of glucose in fungi [Schubert and Wilmer 1991].

Hydrogen Peroxide

Hydrogen peroxide can penetrate through all biological membranes and can therefore cause damage even in locations distant from its place of origin [Gille and Sigler 1995]. The special significance of hydrogen peroxide resides in its ability to generate reactive hydroxyl radical, $HO^{\bullet}$, and to form adducts (hydrogen bonded chelate structures which can carry and release H_2O_2) with many components of biochemical systems [Schubert and Wilmer 1991]. Hydrogen peroxide acts as an oxidizing agent and can directly inactivate enzymes such as glyceraldehyde-3-phosphate dehydrogenase via oxidation of thiol groups, oxidize pyruvate, and glyoxylate and react with keto acids [Gille and Sigler 1995]. Most importantly, H_2O_2 will react with reduced iron or copper ions to generate hydroxyl radicals in the Fenton reaction. Since $O_2^{\bullet-}$ will reduce both Fe^{3+} and Cu^{2+} and since its dismutation produces hydrogen peroxide, it is likely that when the intracellular concentration of $O_2^{\bullet-}$ increases, the concentration of H_2O_2 and $OH^{\bullet}$ will also rise [Farr et al. 1991; Lee et al. 1995]:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\bullet -} \quad [6]$$

$$2\,O_2^{\bullet -} + 2H+ \longrightarrow H_2O_2 + O_2 \quad [7]$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad [8]$$

The $OH^{\bullet}$ radical is one of the most reactive chemical species known. It reacts with a variety of molecules in the cell including sugars, amino acids, phospholipids and organic acids. The reactions take place immediately at the site of $OH^{\bullet}$ formation. The $OH^{\bullet}$ reaction products are less reactive, but they can still severely damage the cell by leading to lipid peroxidation, lesions to DNA and oxidation of proteins. In contrast to $O_2^{\bullet -}$ and H_2O_2, there is no enzymatic protection against $OH^{\bullet}$. For a molecule that can react with almost every other molecule in the cell, the prevention of its formation is of great importance [Gille and Sigler 1995].

H_2O_2 Toxicity

Hydrogen peroxide toxicity has been extensively studied by Imlay et al.(1988). This group of researchers found that exposure of logarithmically growing *E. coli* to H_2O_2 leads to two, kinetically distinguishable, modes of cell killing. Mode 1 killing is pronounced at low (1 to 3 mM) concentrations of hydrogen peroxide and is caused by DNA damage. Mode 2 killing requires a relatively high (> 20 mM) dosage; the site of the toxic injury has not been established. Differences in the kinetics of the production of these two classes of toxic damage indicate that they are not produced by a common mechanism. The damage that results in mode 1 killing is generated at a rate that is linearly proportional to the concentration of H_2O_2. Dose response in mode 2 killing, for

the generation of DNA lesions is more complex. It reflects some aspects of chemistry by which H_2O_2 is converted into an oxidant that is capable of damaging the DNA [Imlay et al. 1988].

The targets in DNA attacked by reactive oxygen species are the pentose sugar and base. In the case of deoxyribose, damage results in the generation of phosphoglycolate and 3' phosphates. In pyrimidines, damage results in production of thymine glycol, methyltartronylurea, urea, 5-hydroxymethyluracil, 4,6-diamino-5-formamidopyrimidine [Imlay et al .1988].

Single-stranded DNA breaks that contribute to mode 1 killing in DNA- repair deficient strains of *E. coli* result from the collapse of the deoxyribose ring after abstraction of a hydrogen atom. This process requires a powerful oxidant, and since the $OH^{\bullet}$ can be generated by monovalent reduction of hydrogen peroxide, this species could be a reasonable source of DNA damage. The chemistry of reduction of H_2O_2 was explored first by Fenton [Fenton 1894] and subsequently by Haber and Weiss [Haber and Weiss 1934] in their studies of the reductive decomposition of H_2O_2 by reduced metals represented by the following equation [Imlay et al. 1988; Demple 1991]:

$$Me^{N+} + H_2O_2 \longrightarrow Me^{(N+1)+} + HO^{\bullet} + H_2O \qquad [9]$$

Defenses Against Oxidative Stress

Although the $O_2^{\bullet-}$ free radical has only a limited reactivity in aqueous solutions, its fast and efficient elimination is of a great importance for the cell because it can give rise to much more deleterious oxygen species [Gille and Sigler 1995]. These defense

mechanisms involve antioxidant enzymes, such as superoxide dismutase (SOD), which catalyzes the dismutation of $O_2^{\bullet}$ to H_2O_2 and O_2, as well as catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides [Lee and Park 1998]. Three different kinds of SODs are known at present. They can be classified in two families: the copper-zinc superoxide dismutase (CuZnSOD), as well as the related manganese-containing (MnSOD), and the iron-containing (FeSOD) enzymes [Fridovich 1989].

The CuZnSOD is found in the cytoplasm of nearly all eukaryotes, a few prokaryotes and in the matrix of lysosomes and plant peroxisomes [Halliwell and Gutteridge 1991]. MnSOD is found in prokaryotes, in the mitochondrial and chloroplast matrix of eukaryotes. The FeSOD is found mainly in prokaryotes, protozoans and plants [Gille and Sigler 1995].

Another class of enzymes participating in the decomposition of reactive oxygen species (ROS) are the catalases. These enzymes catalyze the decomposition of H_2O_2 to oxygen and water, and belong to several types. The group referred to as typical catalases, isolated from animals, plants and microorganisms, contain protoheme IX and their molecular mass is in the range 225-270 kDa, and optimal activity range between pH 5.0 - 10. Atypical catalases occur in both prokaryotes and eukaryotes, with pH optimum at 6 - 6.5 and higher sensitivity to temperature and hydrogen peroxide.

Finally, the enzymatic protection against H_2O_2 is also provided by peroxidases. According to one classification, the heme-containing peroxidases are divided into microbial (yeast cytochrome c peroxidase, bacterial peroxidase-catalases, plant ascorbate peroxidases), fungal (lignin and manganese peroxidases, plant-type secreted peroxidases)

and plant peroxidases [Gazaryan et al 1995]. In another classification three superfamilies of peroxidases are recognized that are thought to catalyze the heterolytic cleavage of the peroxide O-O bond. Their catalytic residues and protein folds are different. One of the superfamilies constitutes heme peroxidases from plant, fungi and yeast, another comprises animal peroxidases and the third includes catalases from animals, plants fungi and yeast [Welinder 1991]. The plant / fungus / yeast superfamily contains invariant amino acid residues that are present in all presently known family members and must therefore be essential to either functional or structural integrity.

Plant, fungal and yeast peroxidases differ in several ways. Most importantly, their disulfide patterns are different: yeast peroxidase has no disulfide bridges, while fungal peroxidases have four putative disulfide bridges. In addition, mature fungal peroxidases possess an additional C-terminal domain of unknown function. The plant peroxidases oxidize and polymerize aromatic compounds, fungal peroxidases degrade lignin and yeast cytochrome c peroxidase directs electrons of toxic hydroperoxides into the electron transport chain of mitochondria [Welinder 1991].

The functions of the oxidative stress defenses go beyond the effect of rescuing an organism from reactive oxygen species. Those defenses help maintain genetic stability in the aerobically grown organisms. For example, SOD-deficient *E. coli* exhibits increased spontaneous mutagenesis during aerobic growth. Spontaneous mutagenesis is also strongly increased in *E. coli* with deletions in catalase genes and it is supressed upon overproduction of catalases [Demple 1991].

Peroxidases as Metabolic Enzymes

Extensive studies have been carried out by Paszynski et al. (1988), Gold et al. (1993), Mayfield et al. (1994) and Li et al. (1995) on peroxidases playing an important metabolic role in lignin degradation, as exemplified by the white rot fungi. Lignin degradation by *Phanerochaete chrysosporium* occurs in the secondary metabolic phase of growth which is triggered by nitrogen deprivation.

During this process, hydrogen peroxide is generated and it acts as a cosubstrate for two extracellular heme peroxidases: manganese peroxidase and lignin peroxidase, MnP and LiP, respectively [Brown et al. 1990]. Lignin peroxidases have been shown to oxidize aromatic substrates, while manganese peroxidases oxidize phenolic compounds in a hydrogen peroxide-dependent reaction [Jonsson et al. 1994]. Both enzymes exist in several isoforms and have been demonstrated in several species including *Trametes versicolor* [Johansson and Welinder 1994], *Coprinus cinereus* and *Coprinus macrorhizus*, *Arthromyces ramosus* [Kjalke et al. 1992], *P. chrysosporium* [Glenn and Gold 1986] (reviewed in Bogan and Lamar 1996).

So far lignin degradation has been most extensively studied in the latter species. This organism appears to employ a hierarchy of regulatory strategies for controlling MnP and LiP production. A summary of the inductive factors is presented in Table 2. It is apparent that multiple isoenzymes of manganese and lignin peroxidases are synthesized upon carbon and nitrogen limitation and an increased level of cAMP. Aromatic compounds induce LiP but not MnP, while heat shock, hydrogen peroxide, paracetic acid and ethanol induce transcription of only *mnp* genes. The *mnp* mRNA accumulates,

CONDITION	LiP isoenzymes	MnP isoenzymes
C-limitation	Only one (H2) isoenzyme induced: unstable transcript	Early induction of H4, then H3, H5: stable transcripts
N-limitation	H2, H8, H10 induced	All 3 isoenzymes present early
Increased level of cAMP	All isoenzymes induced	All induced
Aromatic compounds	All three induced	None
Manganese	None	Necessary for synthesis of MnP
Heat Shock	None	*mnp* mRNA accumulates, but no MnP activity
Paracetic acid, ethanol, sodium arsenite	None	*mnp* mRNA transcribed, no MnP activity without Mn.

Table 2. A list of inductive factors for manganese and lignin peroxidases (adopted from Li et al. 1995; Gold et al. 1993; Mayfield et al. 1994).

but manganese is required for generating enzymatic activity, implying post-transcriptional regulation.

General Catalytic Mechanism and Properties of Peroxidase Enzymes

All known peroxidases are are ferri-(Fe^{3+})-hemoproteins. Their substrates include hydrogen peroxide and alkyl peroxides. All known peroxidases are Fe^{3+} hemoproteins. These enzymes are brown in color and show characteristic optical spectra with peaks around 600, 500, and 400 nm representing the α, β and Soret bands, respectively, characteristic of high-spin, out-of-plane Fe^{3+} heme [Walsh 1989].

The reaction catalyzed by peroxidase includes two spectroscopically discrete intermediates, called compounds I and II, which are formed as follows. The resting enzyme is oxidized by two electrons as the first molecule of ROOH (alkyl peroxide) is split and a molecule of water is generated, while the RO fragment is retained in compound I [Walsh 1989]:

$$ROOH + ENZ\,(Fe^{3+}) \longrightarrow RO\text{-}COMPOUND\ I\,(Fe^{5+}) + H_2O \quad [10]$$

The second substrate molecule, an easily oxidizable species, AH_2 (phenols, alkyl amines, ascorbate, cytochrome c, glutathione), can now enter the reaction:

$$AH_2 + RO\text{-}COMPOUND\ I \longrightarrow AH_2\text{-}COMPOUND\ I + ROH \quad [11]$$

$$\longrightarrow COMPOUND\ II + AH^{\bullet} \quad [12]$$

AH_2 substrates donate one electron at a time, reducing compound I and generating compound II. Therefore a second molecule is needed to donate a second electron and regenerate ENZ-Fe^{3+} ready for another cycle [Walsh 1989]:

$$\text{COMPOUND II} + AH_2 \longrightarrow AH^{\bullet} + \text{ENZ}\,(Fe^{3+}) \quad [13]$$

Summarizing the last two steps:

$$2AH_2 + \text{COMPOUND I} \longrightarrow ROH + \text{ENZ}\,(Fe^{3+}) + 2\,AH^{\bullet} \quad [14]$$

$$2AH^{\bullet} \longrightarrow AH_2 + A \quad [15]$$

Similarly, in *P. chrysosporium* the peroxidase reaction is a two-electron oxidation-reduction, and the reaction occurs in three distinct steps [Gold et al. 1993]:

Lignin Peroxidases:

$$LiP + H_2O_2 \longrightarrow LiP(I) + H_2O \quad [16]$$

$$LiP(I) + Ar \longrightarrow LiP(II) + Ar^{+1} \quad [17]$$

$$LiP(II) + Ar \longrightarrow LiP + Ar^{+1} + H_2O \quad [18]$$

Manganese Peroxidases:

$$MnP + H_2O_2 \longrightarrow MnP(I) + H_2O \quad [19]$$

$$MnP(I) + Mn^{II} \longrightarrow MnP(II) + Mn^{III} \quad [20]$$

$$MnP(II) + Mn^{II} \longrightarrow MnP + Mn^{III} \quad [21]$$

In this scheme, P represents the native peroxidase molecule, P(I) is compound I, which is two oxidation equivalents above the native enzyme, and P(II) is compound II, which is one oxidation equivalent above the native enzyme [Kishi et al. 1994].

Both MnP and LiP are ferric, high spin, pentacoordinate heme proteins with the protein ligated to the heme via proximal histidines. LiP and MnP and CiP (*Coprinus*

cinereus peroxidase) are within the range 42 to 46 kDa in molecular mass, are glycosylated, with pI between 3.2 and 4.9. Their substrates vary depending on the step in fungal metabolism they catalyze. Some properties of the enzymes are listed in Table 3.

Oxidative Stress and Peroxidase of *N. crassa*

As mentioned in the preceding, a characteristic feature of *N. crassa* stress response is the strong induction of peroxidase activity in response to heat shock and oxidative stress. Besides heat shock and oxidative stress, heavy metal compounds (sodium arsenite, $CdCl_2$) are capable of peroxidase induction. It was also found that peroxidase activity was virtually undetectable in non-induced cells, but it appeared within 10 min of exposure to one of the above mentioned stressors [Kapoor et al. 1990]. Heat shock treatment of 60-h-old colonies for 1 h at 48°C resulted in a high degree of tolerance toward the normally lethal temperatures, while plates transferred directly to the lethal temperature showed virtually no surviving colonies.

In addition, considerable survival of colonies was noted for plates where *N. crassa* was pretreated with hydrogen peroxide. It was then suggested that accumulation of intracellular hydrogen peroxide or peroxides could account for some of the biological damage resulting from the heat shock [Kapoor et al. 1990].

All the treatments that ensured cell survival at lethal temperatures also led to the induction of peroxidase activity. Therefore, it has been suggested that there is a

PROPERTY	**ENZYME**		
	MnP	**LiP**	**CiP**
Mr	46,000	43,000	42,000
Carbohydrate content	17%	21%	varied: 1-7 residues
pI	4.2-4.9	3.2-4.0	3.5-3.8
Required for activity	Mn, H_2O_2	?	H_2O_2
Substrates	phenols, phenolic lignin compounds	nonphenolic lignin compounds, lignin polymer	phenols

Table 3. Some properties of peroxidases from *P. hrysosporium, Coprinus cinereus and Trametes versicolor* (adopted from Paszynski et al. 1986; Kjalke et al. 1992; Gold et al. 1993).

correlation between the induction of peroxidase acitivty and the acquisition of thermotolerance [Machwe 1996]. Earlier studies in our laboratory [Lin and Kapoor 1992] had also shown that the heat shock of *N. crassa* leads to an increased levels of superoxide anion in the culture medium. Therefore, it was proposed that a likely function for the heat-induced peroxidase is to effectively counteract the elevated intracellular levels of H_2O_2 and other peroxides thereby preventing lipid peroxidation and damage to membranes [Machwe 1996].

In the case of previous studies, a putative peroxidase gene was cloned and used as a probe in hybrid-arrested *in vitro* translation. A large polypeptide (~90 kDa) was identified that reacted with the polyclonal antisera raised against the heat-inducible peroxidase [Machwe and Kapoor 1993]. In addition, a ~10 kb transcript was demonstrated in heat-treated cells. It was present in the cells in significant amount even under normal non-stress conditions [Machwe 1996]. Moreover, it was found that *N. crassa* possesses a second peroxidase enzyme. This finding derives from experiments conducted in our laboratory which aimed at isolating mutations in the *hsp*70 gene of *N. crassa* by repeat-induced point mutations (RIP).

Several transformants were generated by electroporation of germinated conidia with a plasmid harbouring an incomplete copy of *hsp70* gene [Chakaborty et al. 1991]. One of the transformants (designated as E-45) exhibited a slow growth rate, low-temperature sensitivity and high levels of constitutive peroxidase and enhanced thermotolerance in the complete absence of the heat shock treatment [Machwe 1996]. Southern blot analysis of the genomic DNA demonstrated that in this transformant *hsp70*

DNA was ectopically integrated close to the peroxidase DNA. The level of peroxidase activity in heat-shocked E-45 was comparable to that developed by the heat-shocked wild type cells, but slightly reduced relative to that of non-shocked E-45 cells.

In this case too, Northern blot analysis revealed the hybridization of E-45 peroxidase transcript to the peroxidase gene probe from the wild type strain, suggesting that these two enzymes may be products of the same one or two closely related genes [Machwe 1996]. The nature of the transformation event resulting in increased peroxidase activity is not well understood.

Objectives

In view of the earlier results regarding the induction of peroxidase by heat shock, it was important to determine whether the heat-induced peroxidase, encountered in the wild type strain of *N. crassa* and the constitutively expressed peroxidase in E-45, represented the same or different enzymes. This study was undertaken with the objective of isolating and characterizing the stress-inducible and constitutive peroxidases of *N. crassa*. The results of experiments documented in the following show the two enzymes to be distinct as witnessed by their purification profile, relative molecular mass of the native protein, pH optimum and kinetic properties. This study provides information that can be used in the future to obtain an insight into the role of fungal peroxidases in defense against oxidative stress.

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Reagents used for this study were purchased from Sigma, BDH and Fisher Scientific. Chromatographic supplies were obtained from Sigma and Pharmacia. Nitrocellulose filters (0.22 μm) were from Millipore. Mini-Protean II Cell apparatus and Protein assay dye were from Bio-Rad. Centrifugal concentrators were obtained from Centricon (Amicon) and Filtron (Microsep).

2.2 METHODS

2.2.1 Growth and Heat Shock Treatment of *Neurospora crassa*

The wild type strain of *Neurospora crassa* (Fungal Genetics Stock Centre No 262) was grown in Vogel's minimal medium [Vogel 1956] with 2% sucrose as the carbon source. The fungus was grown in volume of 100 mL of liquid medium in 250 mL Erlenmeyer flasks for 15 hours on a rotary shaker, at 27°C at 150 rpm and the flasks were transferred to another rotary shaker at 48°C and heat-shocked for 75 min. Following heat shock treatment, the mycelium was vacuum filtered, lyophilized and stored at -20°C. *N. crassa* strain E-45, a transformant, was grown in 1L Fernbach flasks containing 500 ml of 2% Vogel's minimal medium [Vogel 1956] and 2% sucrose. The flasks were placed on a rotary shaker at 27°C with shaker speed of 150 rpm for 48 hours following which the mycelium was vacuum filtered, lyophilized and stored at -20°C.

2.2.2 Buffers

The buffers used for protein purification and characterization were:

1) 20 mM Tris-HCl with 20 mM $MgCl_2$ and 20 mM NaCl, pH 7.5;

2) 50 mM Acetate buffer with 200 mM NaCl, pH 7.5;

3) 50 mM M Citrate/100 mM Phosphate buffer, pH 5.0 and 3.5 and

4) 50 mM Potassium Phosphate buffer, pH 7.5.

2.2.3 Peroxidase activity Assay

The assay involved two peroxidase substrates 0.05% ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] and 1.5% H_2O_2. Peroxidase activity was tested using 1 mL of 0.05% ABTS in 50 mM citrate/100 mM phosphate buffer, 100 µL of protein sample and 10 µL hydrogen peroxide. The assay tubes were mixed gently and incubated for 20 min (262 strain) or 30 min (E-45) at room temperature. The absorbance was read at 415 nm in a Gilford model 250 Spectrophotometer.

Absorbance measurements required to determine molar extinction coefficient and enzyme spectrum were performed using Shimadzu (UV-2101 PC UV-VIS) Scanning Spectrophotometer. The absorbance reading was used to calculate the activity units, defined as unit change in absorbance of 1.0 at 415 nm in 20 min, and specific peroxidase activity defined as $\Delta A_{415\ nm}$/mg protein.

2.2.4 Polyacrylamide Gel Electrophoresis

2.2.4.1 Denaturing Gels

The gels were prepared essentially according the procedure of Laemmli (1970). Separating gels (10%) were prepared from a stock solution containing 30% acrylamide and 0.8% bis-acrylamide, and the lower gel buffer (1.5 M Tris-HCl, 0.4% SDS, pH 8.8), 0.05% TEMED (N,N,N'- Tetramethylenediamine) and 0.0025% APS (ammonium persulphate). The gel was poured between glass plates of the Mini-Protean II apparatus (Bio-Rad) and allowed to polymerize for 30 min. The stacking gel (5%) was prepared from the above acrylamide solution, with upper gel buffer (0.5 M Tris-HCl, 0.4% SDS, pH 6.8) and layered on top of polymerized separating gel. After 10 min of polymerization, the stacking gel was washed with tank buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3). The gels were run at 100 V through the stacking gel , and 180 V through separating gel. Protein Samples were added to at least 5 µL of sample buffer (12.5% upper gel buffer, 13% w/v glycerol, 2.3% SDS, 5% β-mercaptoethanol) and 2 µL of 0.01% Bromophenol Blue (prepared in sample buffer) was added to each protein sample and the samples were boiled for 5 min prior to loading. Following electrophoresis, the gels were stained in 0.1% Coomassie Brillant Blue R-250 prepared in 10% acetic acid and 30% methanol. Destaining was performed in 10% acetic acid and 30% methanol.

Gels were also subjected to **glycoprotein stain** [Segrest and Jackson 1972] following resolution by the above mentioned SDS-PAGE procedure. The SDS-PAGE gel was immersed in 12.5% trichloroacetic acid for 30 min, rinsed with water and soaked in

1% periodic acid prepared in 3% acetic acid for 50 min. Subsequently, the gel was washed several times with water (200 mL each time) and soaked in water overnight. The next day it was immersed in fuchsin sulfite stain for 1 hr. The stain was prepared as follows: 4 g of potassium metabisulfite, 5.25 mL concentrated HCl and 2 g fuchsin were dissolved in 500 mL distilled water and let stand for 2 hrs. The stain solution was then treated with activated charcoal (Darco G-60) for 15 min and filtered. The stain was intensively pink but clear. For gel staining the stain was diluted (1:3) in the destain medium consisting of aqueous solution of 0.5% potassium metabisulfite.

2.2.4.2 Non-denaturing Gels

Non-denaturing gels were prepared according to essentially the same procedure as denaturing gels. The modifications included omission of SDS and β- mercaptoethanol in the sample and tank buffers, as well as the addition of 20% w/v glycerol to the acrylamide stock solution. Acrylamide solutions were deaerated for 10 min before TEMED and APS were added. Protein samples were not heat denatured.

In order to visualize enzymatic activity, the staining solution included peroxidase substrates. Two solutions were prepared: one solution contained 50 mM citrate/100 mM phosphate buffer with 0.05% ABTS and 1.5% hydrogen peroxide. The second solution was prepared in 10 mM phosphate buffer with 2 mM o-dianisidine, 0.1 mM riboflavin, pH 7.2. The gels were soaked in the staining solutions for 20 min. The band containing active enzyme stained green with ABTS and brown with dianisidine.

2.2.5 Protein Determination

The Bio-Rad microassay was employed to estimate the protein concentration [Bradford 1976]. This assay was performed in 20 mM phosphate buffer (pH 7.2). Protein Sample (10-100 μL) was added to the buffer to final volume of 800 μL. Then, 200 μL of Bio-Rad dye were added and the solution was incubated for 5 min. The absorbance at 595 nm was recorded. A calibration standard was prepared using Bovine Serum Albumin (range of 1.25 to 15 μg/mL).

2.2.6 Preparation of Western Blots

Protein samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham) using a procedure similar to that described by Towbin *et al.*(1979). Prior to transfer, the membrane was soaked in the transfer buffer (20% methanol, 0.025 M Tris, 0.192 M glycine) for 15 min. The gel was placed on the pre-soaked membrane, inserted between two sheets of Whatman 3 mm paper and assembled in a plastic cassette. The cassette was then placed in the protein gel container filled with the transfer buffer and electro-transfer was performed for 1 hr at 100 V, at 4°C. When the transfer was complete, the membrane was recovered and placed in a paper towel to dry. In order to reduce non-specific binding, the membrane was immersed in 6% skim milk prepared in TBST (0.01 M Tris, 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) and placed on a rotary shaker for 2 hr. The blot was then washed three times with TBST (20 min each time) and treated with the primary antibody

(*Neurospora crassa* anti-peroxidase antiserum diluted 1:10,000 with TBST) for 1 hr. After this incubation three additional washes with TBST were performed (total of 20 min). Following these washes the blot was incubated for 20 min with the goat anti-rabbit horseradish peroxidase secondary antibody (Sigma) diluted 1:10.000 with TBST. The blot was then washed again with TBST for 30 min followed by a 1-min wash containing 1 mL of each ECL1 and 1 mL ECL2 (western blotting detection reagents, Amersham) and immediately the blot was exposed to X-ray film (Fuji Medical X-Ray Film) for 2-6 min.

2.2.7 Preparation of Anti-Peroxidase Antibodies

The antibodies used in this study were prepared previously [Machwe 1996]. A crude protein extract from heat-shocked mycelium was electrophoresed in non-denaturing polyacrylamide gels and stained for peroxidase activity. Gel slices showing the above activity were isolated and loaded on SDS-polyacrylamide gels and electrophoresed under denaturing conditions. The gel slices corresponding to the peroxidase polypeptides were isolated again, frozen and pulverized in liquid nitrogen, suspended in distilled water with an equal volume of Freund's complete adjuvant, and injected into 4-month old New Zealand rabbits. Booster doses were administered at 2-week intervals.

2.2.8 Preparation of Chromatographic Columns

2.2.8.1 QAE Sephadex

Five grams of dry material (Pharmacia) was suspended in 1L of double distilled water and allowed to hydrate for 4 hr. the supernatant was decanted and replaced with 0.5

M NaOH for 1 hr and the slurry was washed several times with water and suspended in 0.5 M NaCl for 1 hr. The water washes were repeated and finally replaced by Tris buffer (pH 7.5). After 1 hr the buffer was decanted and the slurry was poured into a 45 x 3.5 cm column which was washed with 5 x bed volume of Tris-HCl buffer.

2.2.8.2 Metal chelation column

Imino-diacetate-epoxy-activated agarose (Sigma) was thoroughly soaked 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, pH 3.0) resulting in charging the column with Fe^{2+}. The column was then equilibrated with 5 volumes of 50 mM acetate buffer (pH 7.5) containing 0.2 M NaCl.

2.2.8.3 Gel filtration

Dry gel (Biogel BP 100) was added to 10 x volume Tris-HCl buffer and allowed to hydrate for 12 hr. Following hydration, the supernatant was decanted, the solution was transferred to a filter flask and attached to a vacuum source. It was degassed for 15 min with occasional swirling of the flask. Two more volumes of the buffer were added, the solution was degassed again and the supernatant decanted. The procedure was repeated 3 - 4 times. Finally, the slurry was poured into the 52 x 1.2 cm column and 4 - 5 bed volumes of Tris-HCl buffer passed through it.

2.2.8.4 Mono Q (FPLC)

A Mono Q HR 5/5 column (Pharmacia) was washed with 20 mL of water (Milli Q) to remove ethanol, charged with the counter ion using 3 injections of 2 mL 0.5 M NaCl and equilibrated with 20 mL Tris-HCl, pH 7.5. Elution was performed using a discontinuous 0 - 0.5 M NaCl gradient at a flow rate of 0.75 mL/min and 1-mL fractions collected.

2.2.8.5 Gel filtration on Superose columns (FPLC)

Superose 6 and 12 (HR 10/30) columns were washed with 80 mL of water, and equilibrated with 80 mL of Tris-HCl buffer. The column was run at 0.2 mL/min and 0.4-mL fractions were collected. The Superose 12 HR 10/30 column was equilibrated and run exactly as Superose 6.

RESULTS

3.0 RESULTS

3.1 PURIFICATION OF THE HEAT-INDUCIBLE PEROXIDASE (HIP) FROM A WILD TYPE STRAIN OF *NEUROSPORA CRASSA*.

Freeze-dried mycelium (5g) of a wild type strain (FGSC No 262) was suspended in 100 mL of extraction buffer (0.05 M Tris-HCl, 0.1 mM EDTA, 50 μM β-mercaptoethanol, pH 7.5) and stirred continuously at 4°C for 30 min. After 20 min, two tablets of the Protease Inhibitor Cocktail (Complete, Boehringer-Mannheim) were added. The mycelial suspension was then homogenized in a Potter-Elvehjem homogenizer and centrifuged for 20 min at 15,000 g in a Sorvall RC-5 refrigerated centrifuge. The supernatant (crude extract) was subjected to ammonium sulfate precipitation. In the first step, 40% saturation was obtained and the precipitated proteins were pelleted and discarded. To the supernatant ammonium sulfate was added to a saturation of 65%. The pellet from this step was resuspended in 4 mL of Tris-HCl buffer and dialyzed extensively against the same buffer.

The dialyzed protein sample resulting from 65% precipitation with ammonium sulfate was loaded on the QAE Sephadex column. The column was washed with 120 mL Tris-HCl buffer to elute non-binding proteins, then developed with a linear elution gradient of 0 - 0.75 M NaCl (total of 360 mL). The fractions were collected at 4 min intervals (~ 5.5 mL/tube) and tested for peroxidase activity as described previously. As shown in Figure 1, peroxidase activity eluted in two peaks (P1 and P2): one in the flow-through fractions (P1), and the other (P2) at 0.46 M NaCl . The active fractions of P2

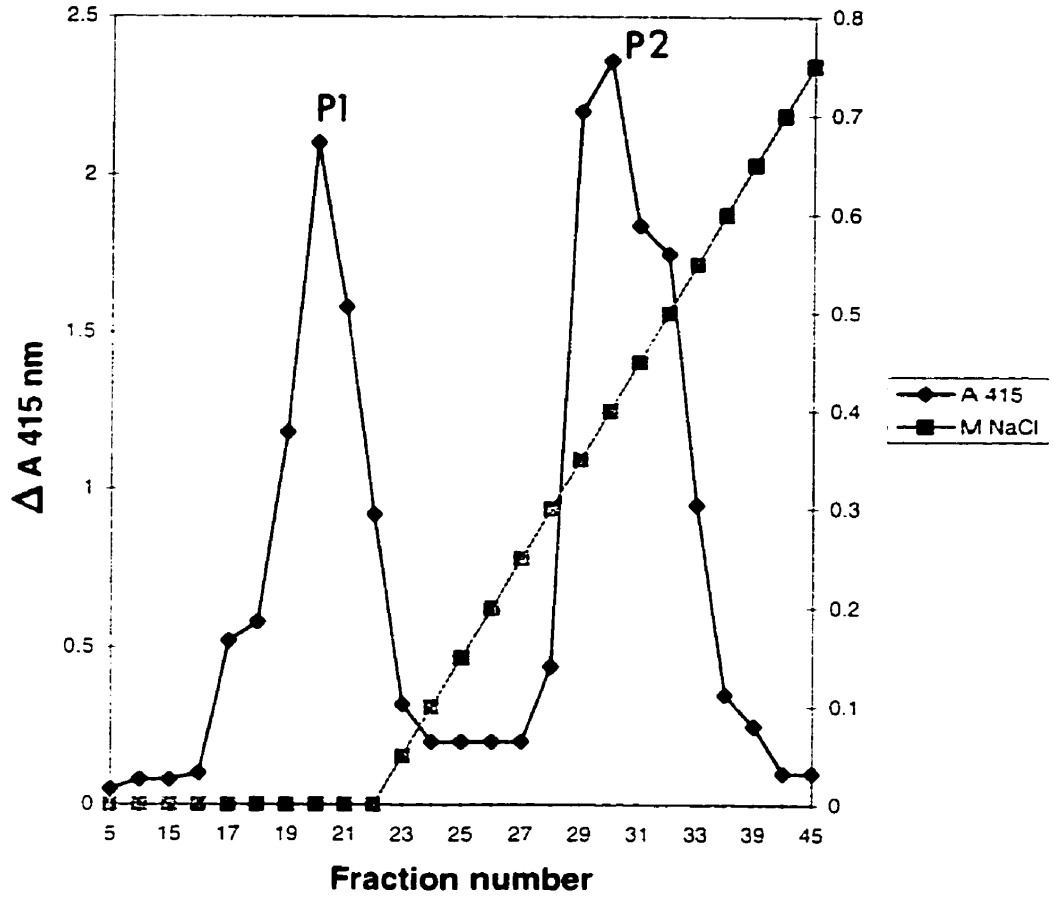


Figure 1. Fractionation of peroxidase on QAE Sephadex column (individual fractions were ~5.5 mL).

(with $\Delta A_{415\,nm}$ of at least 0.4) were pooled and subjected to precipitation with 80% saturation of ammonium sulfate. The pellet was recovered by centrifugation at 15,000 g, resuspended in 2 mL of Tris-HCl (pH 7.5) and dialyzed against the acetate buffer. The dialyzed sample was loaded on to a metal chelation column (prepared as described in Materials and Methods), and eluted with acetate buffer. Fractions of 1.5 mL were collected and assayed for peroxidase activity. The binding of HIP to metal chelation matrix appeared to be weak, the peak peroxidase activity eluting at 12 mL (Figure 2). Again, only fractions with activity of at least 0.4 were pooled and concentrated on Centricon filters (cut off 30 kDa) to a final volume of 2 mL.

The concentrated pool of metal chelation step was loaded on to BioGel BP 100 for the purpose of desalting the eluate and for buffer change. The column was equilibrated with Tris-HCl buffer and subjected to gravity flow, one-mL fractions were collected and tested for peroxidase activity. The active pool was centrifuged for 10 min at 15,000 g to remove residual particulate matter and applied to a Mono Q column. The elution method with non-linear gradient of 0-0.5 M NaCl was employed. Peroxidase activity eluted at 4.5% (of 0.5M) NaCl, corresponding to approximately 0.02 M, in a volume of 2 - 3 mL (Figure 3, 4 and 5). At this point, usually a single protein band was obtained. Occasionally low molecular weight impurities were present and in that case the sample was subjected to gel filtration as the last purification step.

Active Mono Q fractions were concentrated on Filtron filters (cut off 50 kDa) to a final volume of 200 µL. This sample was injected on a Superose 6 column, equilibrated with Tris-HCl and running at 0.2 mL/min. The fractions were

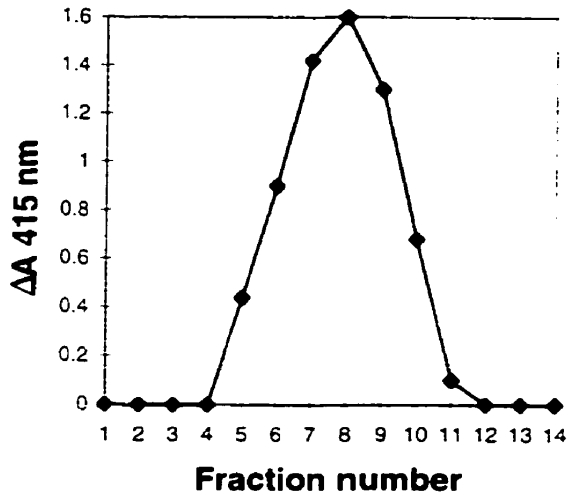


Figure 2. Elution profile of peroxidase activity from the metal chelation column (individual fraction~1.5 mL).

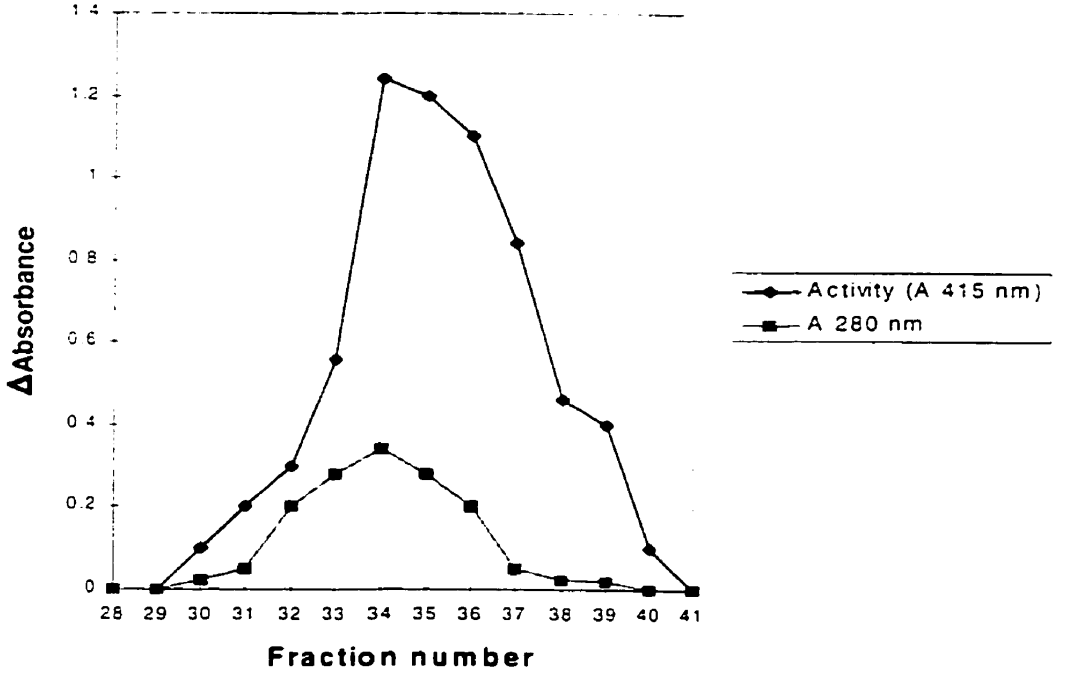


Figure 3. Elution profile of HIP from the MonoQ column (individual fraction 1 mL).

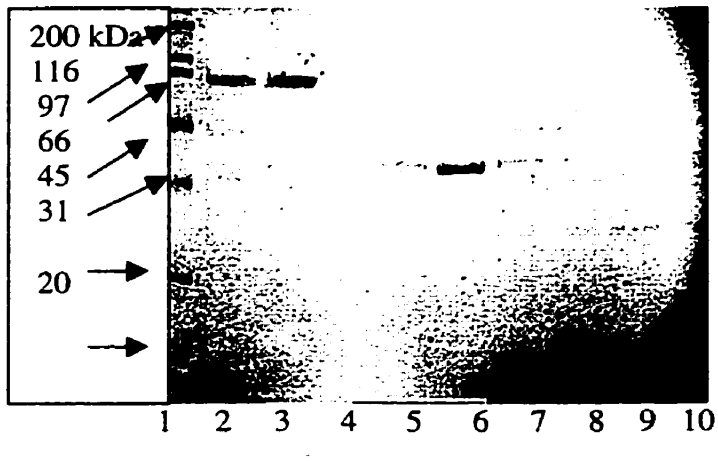


Figure 4. Fractionation of peroxidase activity by SDS-PAGE gel following MonoQ column. Lane 1, molecular weight markers; lanes 2-10 contain 15 µL of each of fractions 16 (lane 2) to 24 (lane 10).

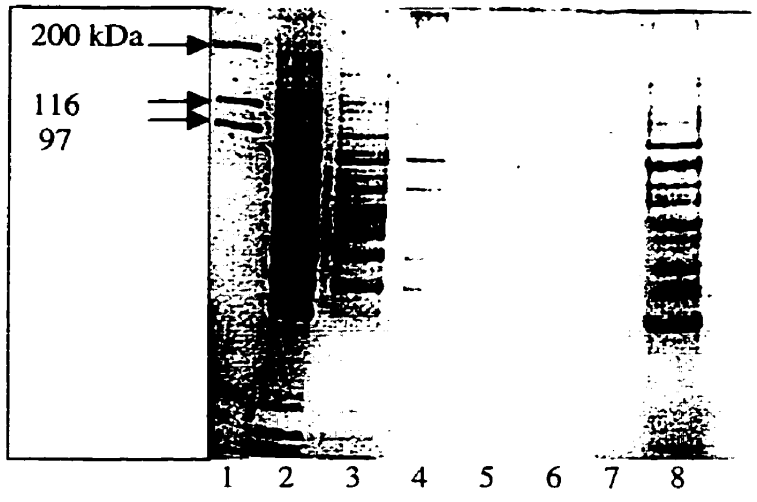


Figure 5. Summary of HIP purification steps. Protein separation by SDS-PAGE. Lane 1, molecular weight markers ; lane 2, crude extract of heat-shocked mycelium (2μL); lane 3, QAE Sephadex pool (10 μL); lane 4, metal chelation pool (5μL); lanes 5 and 7, no sample; lane 6, Superose 12 pool (20 μL), lane 8, E-45 crude extract (2μL).

collected in two minute intervals (0.4 mL each). The peak of peroxidase activity eluted in fractions 31-36 (Figure 6) corresponding to Ve=12.3 mL, Kav=0.34 and native molecular mass of 116 kDa.The pure, active fractions were pooled and concentrated on Filtron 50 to 0.4 mL volume and washed with citrate/phosphate buffer pH 5.0. This pool was stored at -20°C with 5% w/v glycerol prepared in phosphate buffer (50 mM, pH 5.0). The final purification fold was approximately 89-fold (Table 4).

3.1.1 Estimation of the Molecular Mass of Native HIP

Gel filtration on Superose 6 column was utilized to obtain an estimate of the apparent molecular mass of the native protein and also as a final purification step. Molecular mass determinations were performed by comparing an elution volume parameter (K_{av}) of HIP with the values obtained for several known calibration standards (Figure 6 and 7). Superose 6 column was equilibrated with Tris-HCl buffer, pH 7.5 and run at 0.2 mL/min. Fresh solutions of blue dextran (1 mg/mL) and calibration proteins (10 mg/mL) were prepared in the elution buffer. First, 400 µL of blue dextran solution was injected on the column to determine the void volume. It was estimated to be 6.2 mL. Next, 125 µL of each calibration standard were injected (described in legend to Figure 6) and absorbance at 280 nm and elution volume for each protein peak were recorded. K_{av} values were calculated from the formula: $K_{av} = Ve - Vo / Vt - Vo$

where Vo= void volume, Ve= elution volume, Vt= total volume*Vt = 24 mL (according to the Pharmacia Calatogue).

	Protein mg/ML	Activity units/mL	Specific Activity units/mg protein	Fold Purification	Vol (mL)	Total Activity	% Yield
Crude extract	7.2	0.69	0.09	1	100	66.9	100
QAE Seph Pool	0.47	0.23	0.48	5.3	192	44.16	65.3
Metal Chel. Pool	0.43	1.36	3.2	35.5	14	19.04	27.2
Mono Q	0.2	1.4	7.0	77.8	3	0.42	6.0
Superose 6 Pool	0.088	0.7	8.0	88.9	1.2	0.84	1.28

Table 4. Summary of Purification Steps of Heat Inducible Peroxidase. Total enzymatic activity= specific activity X total volume of protein; purification fold= specific activity (step n)/specific activity of crude extract; % yield= total activity (step n)/total activity of crude extract.

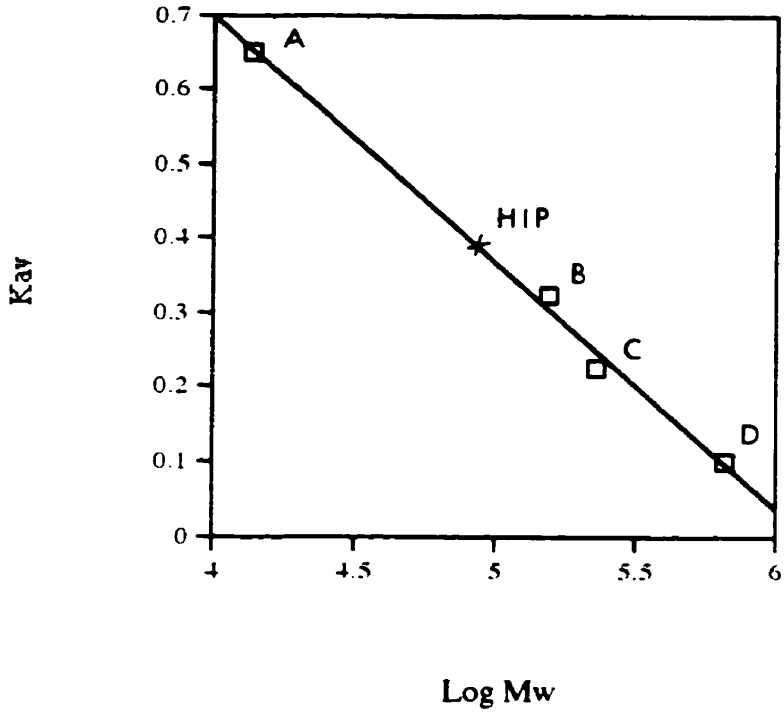


Figure 6 Estimation of molecular mass of the native heat-inducible peroxidase from Superose 6 column. Molecular mass standards used: A, ribonuclease (13,700); B, aldolase (158,000); C, catalase (232,000); D, thyroglobulin (669,000). The experiment was performed using 50 mM citrate/100 mM phosphate buffer (pH 5.0).

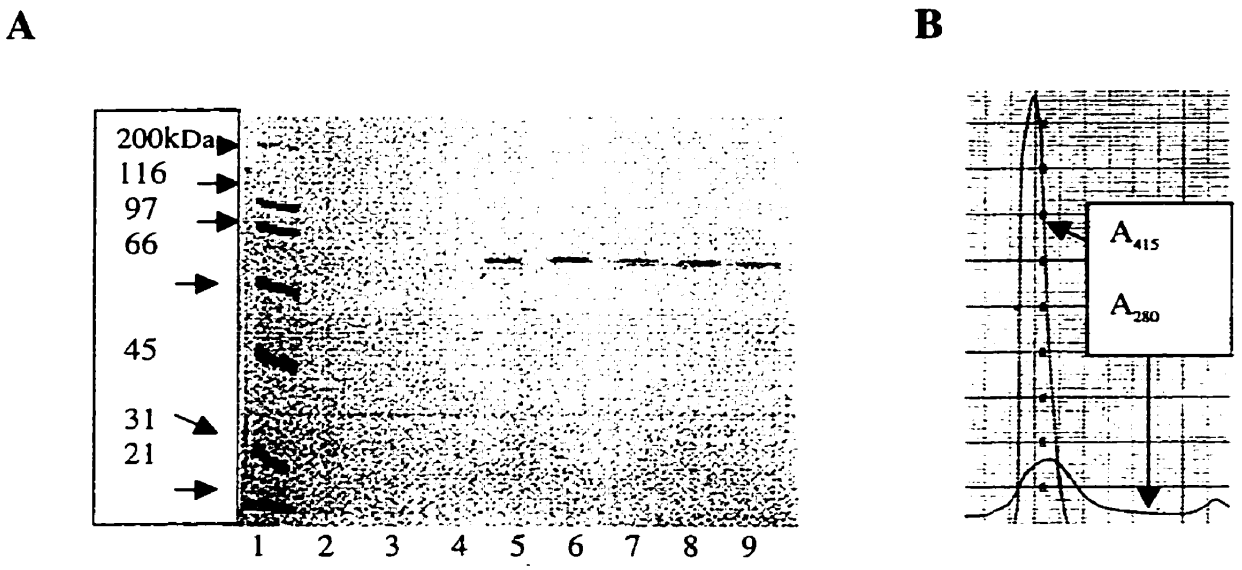


Figure 7. Fractionation of peroxidase activity on Superose 6. A, Resolution of proteins on SDS PAGE. Lane 1, moleculear weight markers; lanes 2-9 contain 20 µL samples of fractions 28 (lane 2) to 35 (lane 9). B, peroxidase activity ($\Delta A_{415\ nm}$) correlated with ΔA_{280nm}.

Logarithm of molecular mass of each protein standard was plotted against the appropriate K_{av} resulting in a standard curve, from which the molecular mass of native HIP was estimated to be 116 kDa

3.1.2 Identification of Hip Using Western Blots

As peroxidase activity eluted in two peaks from the QAE Sephadex column, it was necessary to characterize them by reaction with the antibody prepared against HIP. Therefore, a denaturing polyacrylamide gel was prepared as described in Materials and Methods. The protein samples included: Peak 1 (10 µL), Peak 2 (10 µL), crude extract (10 µL) and E-45 crude extract (10 µL). To each sample 10 µL of sample buffer and 2 µL of bromothymol blue tracking dye were added. The gel was run, transferred and subjected to Western Blot Protocol as described in Materials and Methods. As shown in Figure 8, the antibody against heat-inducible peroxidase recognized only one protein: the ~90-kDa peroxidase present in P2 and in the crude extract of the wild type strain. None of the proteins in the fractions of P1 or the mutant, crude extract of the E-45 reacted with the antibody. Based on this result, P2 was assumed to contain peroxidase activity resulting from the presence of HIP, and P1 was assumed to contain the constitutive peroxidase.

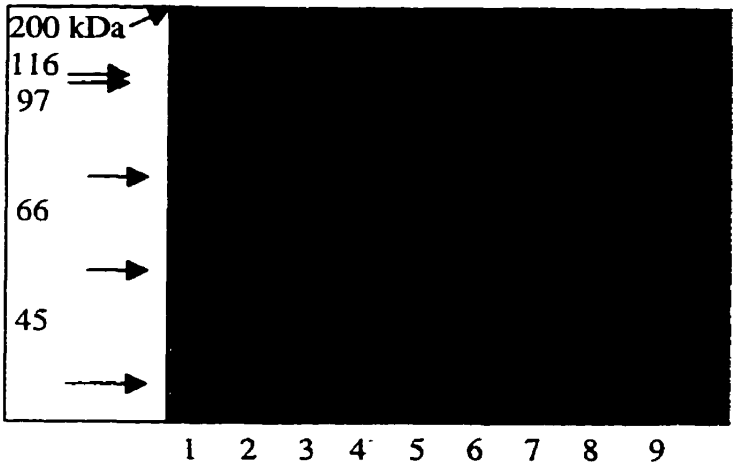


Figure 8. Western blot analysis of protein fractions following resolution by SDS-PAGE. Lane 1, molecular weight markers; lane 2, crude extract of heat shocked mycelium (18 µL); lane 3, peak 1 sample (10 µL); lanes 4, 6 and 7 no sample; lane 5, crude extract of E-45 (10 µL; lane 8, HIP from peak fraction eluted from the Mono Q column (10 µL); lane 9, crude extract of heat-shocked mycelium (10 µL).

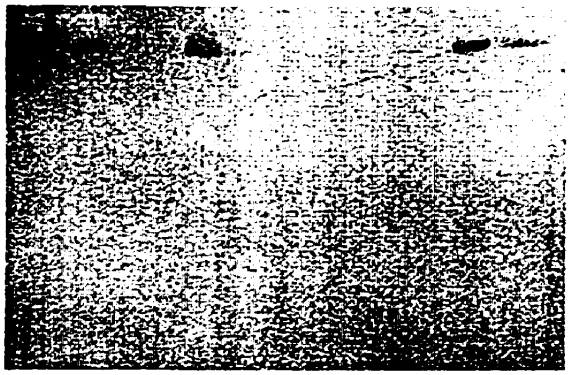
3.1.3 Visualization of Peroxidase Activity on Non-denaturing Gels

In order to monitor the peroxidase band during the purification procedure, non-denaturing gels were prepared as described in Materials and Methods. The protein samples included the following active pools: 30 μL of Mono Q, 40 μL of Superose 6. To each sample 20 μL of non-denaturing sample buffer and 2 μL tracking dye were added. The gels were run in non-denaturing tank buffer at 100 V for 140 min. Upon completion of electrophoresis, the gel was soaked in ABTS stain (20 min) followed by addition of 1.5% hydrogen peroxide (Figure 9A). Under the non-denaturing conditions, a single band showing peroxidase activity was detected. To confirm that the active band was due to one protein the gel was also stained in Coomassie Blue following the peroxidase stain (Figure 9B). A single band was visible as a result of this stain demonstrating the purity and identity of the protein.

3.1.4 Heat Sensitivity of HIP

The activity of HIP was studied as a function of temperature by heat treatment of enzyme samples and measurement of residual activity. As a heat-inducible enzyme, HIP was expected to be active at elevated temperatures. In order to check this, 10-μL aliquots of HIP were incubated at 20, 30, 37, 43, 48, 55 and 65°C for 10 min (pH 5.0). Following incubation, the samples were tested for peroxidase activity at room temperature. As seen in Figure 10, exposure of HIP to temperatures up to 43°C resulted in a ~2- fold increase of enzymatic activity, as

A



1 2 3 4 5 6 7 8 9 10

B

1 2 3 4 5 6 7 8 9 10

Figure 9. Electrophoresis of heat-shocked mycelium in non-denaturing gels. A, the gel stained for peroxidase activity as described in Materials and Methods; B, the same gel stained with Coomassie Blue. Lane 1, crude extract (10 µL); lane 4, QAE Sephadex peak 2 sample (10 µL); lane 9, Superose 12 fraction 34 (20 µL); lane 10, Superose 12 fraction 35 (20 µL); lanes 3, 4, 6, 7 and 8, no sample.

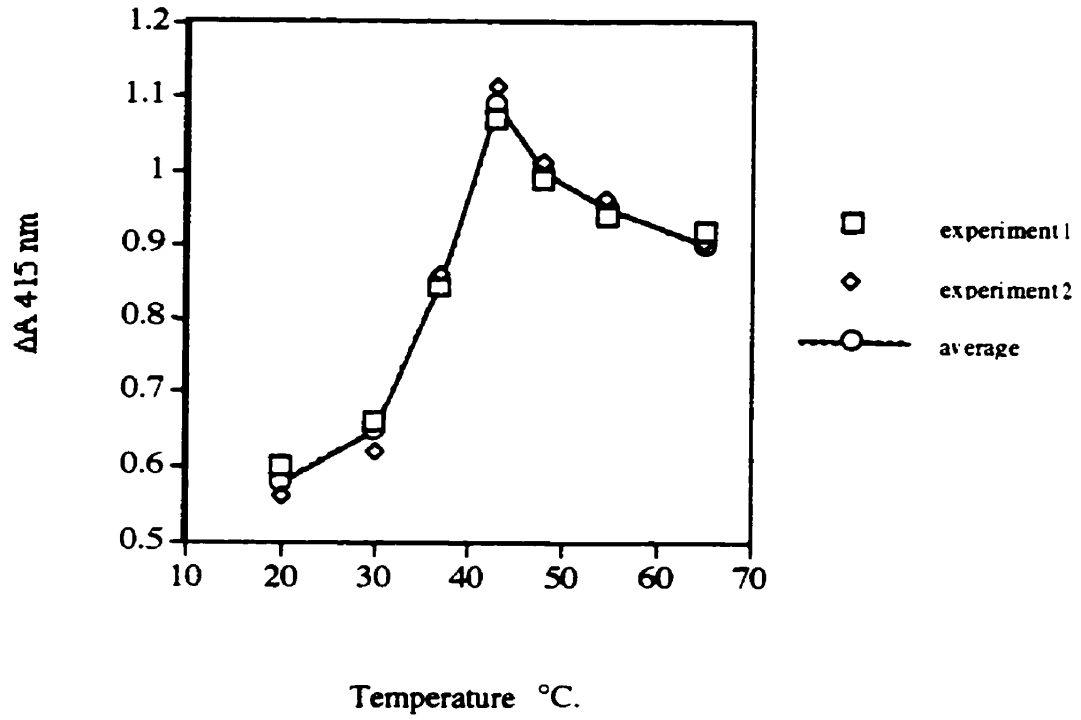


Figure 10. Heat sensitivity of HIP (pH 5.0).

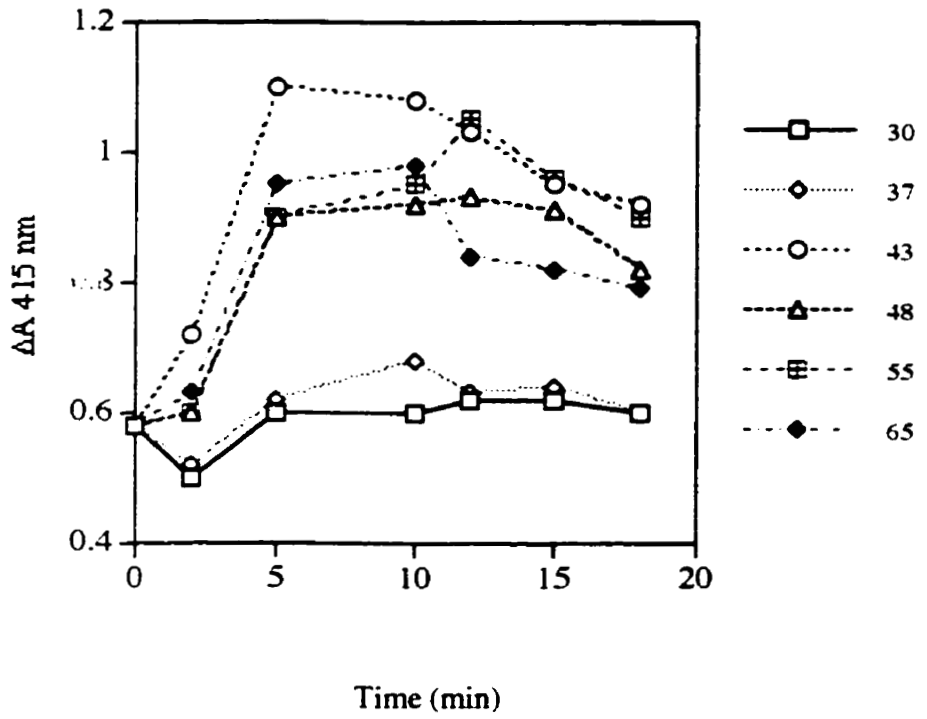


Figure 11. Heat stability of HIP. Samples of HIP were incubated at 30, 37, 43, 48, 55, and 65°C, then 10 µl aliquots were withdrawn, and tested for peroxidase activity. The experiment was performed in duplicate, the data points represent averages of two readings.

compared to specific activity at 20°C. Furthermore, the enzyme remained relatively stable up to 55°C. In this case,the specific activity was almost twice as high as that at 20°C.

As it was found that incubation of the enzyme at 43°C, 48°C (heat shock temperature), 55°C and 65°C resulted in an increase in activity, the ability of HIP to withstand elevated temperatures over extended periods of time was also investigated. Again, 10-µL samples were incubated at the above temperatures and tested for activity after 5, 10, 15, 20 min of incubation. As depicted in Figure 11, HIP exhibited the highest activity between 43 and 55°C but above that range the protein precipitated. Specific activity of HIP remained high and stable between 5 and 12 min of the incubation at temperatures between 48 - 55°C, while decreased only slightly after 18 min. These results indicate that HIP is stable at temperatures corresponding to heat shock for up to 12 min of heat exposure. Although the mechanism underlying increase in activity is not understood, it is possible that increased temperature induces conformational changes that result in enzyme activation.

3.1.5 Determination of pH Optimum of HIP

In order to further characterize the enzyme, the pH optimum had to be established to find conditions favoring maximum enzyme activity. The activity of HIP was tested in citrate/phosphate buffer prepared in the pH range 4.0 - 8.8. Samples of enzyme were added to 1 mL of buffer at the appropriate pH. To test peroxidase activity, 10 µL of ABTS (0.05 g/mL) and 10 µL of 1.5% hydrogen

peroxide were added as well. The samples were incubated for 20 min at room temperature. The optimum pH for HIP was determined to be 5.0. An increase (from 5.0 to 5.6 pH) of pH caused a 57% decrease in enzymatic activity of HIP. At pH lower than 4.6, the enzyme precipitated (Figure 12).

3.1.6 Kinetic Analysis of HIP

3.1.6.1 Determination of Molar Extinction Coefficient

Km and V_{max} are kinetic constants used to characterize the enzyme's substrate affinity and reaction velocity. Before these constants can be calculated, the molar extinction coefficient (ε) has to be determined. ε depends on the buffer used, its ionic strength, temperature and the wavelength at which the experiment is performed. In order to determine ε_{415} for a substrate which is oxidized in a chemical reaction, the absorption spectra of the reduced and oxidized forms of the substrate were obtained under conditions employed during measurement of the reaction rate.

The experiment was conducted in 50 mM citrate/100 mM phosphate buffer (pH 5.0) and at 25°C. Aliquots of ABTS were added to the citrate/phosphate buffer to achieve final concentrations of 0.015, 0.03, 0.045, 0.06 mM. As illustrated in Figure 13 the spectrum of reduced ABTS was recorded from 300 to 450 nm. Subsequently, 2 μL of horseradish peroxidase (0.5 mg/mL) and 15 μL of 1.5% hydrogen peroxide were added to completely oxidize the ABTS. Spectra were recorded until the reduced form was no longer visible and the peak representing

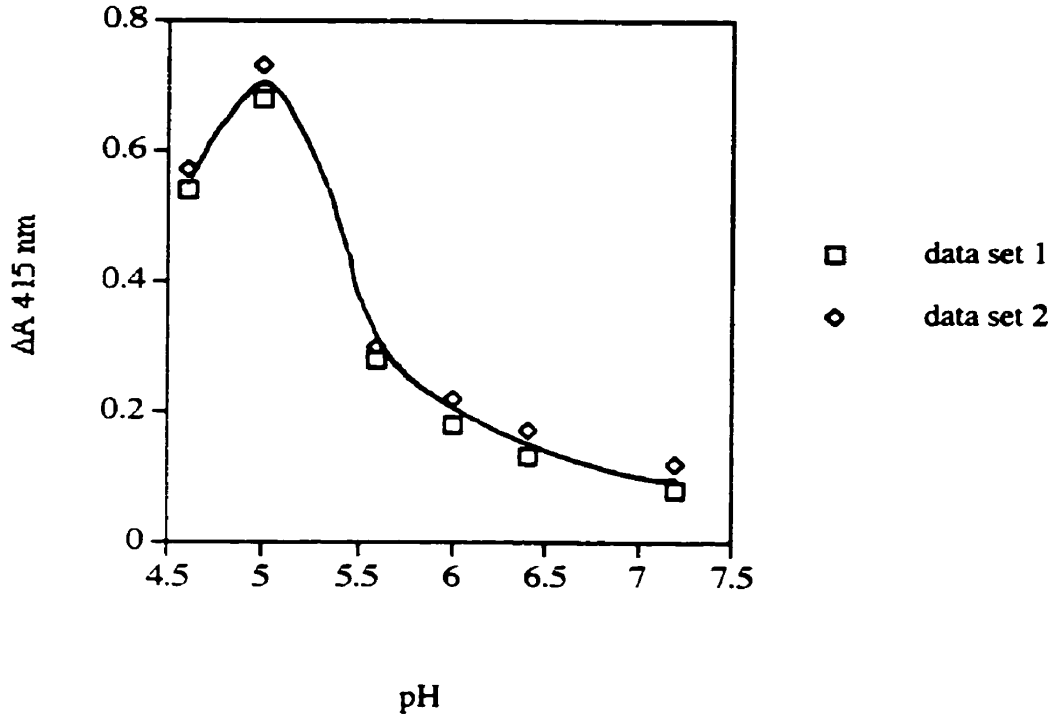


Figure 12. Determination of pH optimum for the heat-inducible peroxidase.

the oxidized form attained the maximum height. Absorbance at 415 nm of the oxidized form was recorded for each concentration of ABTS used and plotted against the concentration. As shown in Figure 14, a linear relationship between $A_{415\,nm}$ and the concentration of oxidized form of ABTS was obtained. The molar extinction coefficient was calculated from this graph using the formula:

$$\varepsilon_{415} = \Delta A_{415} / \Delta \text{conc x path (1 cm)} = 36\ mM^{-1}\ cm^{-1}$$

3.1.6.2 K_m, V_{max} and k_{cat} Determination

The quantities K_m, V_{max} and k_{cat} are characteristic for a given enzymatic reaction under a given set of conditions. These constants are normally calculated with the assumption known as the steady-state approximation. Under this assumption, HIP was characterized kinetically in reactions involving three substrates which are frequently used in testing peroxidases. The ability of HIP to oxidize H_2O_2, ABTS and guaiacol was investigated. First, concentration of ABTS was held constant at 0.5 mM (excess of the fixed substrate) and the concentration of H_2O_2 was varied from 0.62 to 124 μM. The concentration range was chosen based on earlier experiments conducted in order to establish preliminary K_m. This K_m value was an indication of the substrate range at which its ability to accelerate the reaction was greatest; therefore, the concentrations of the tested substrate were

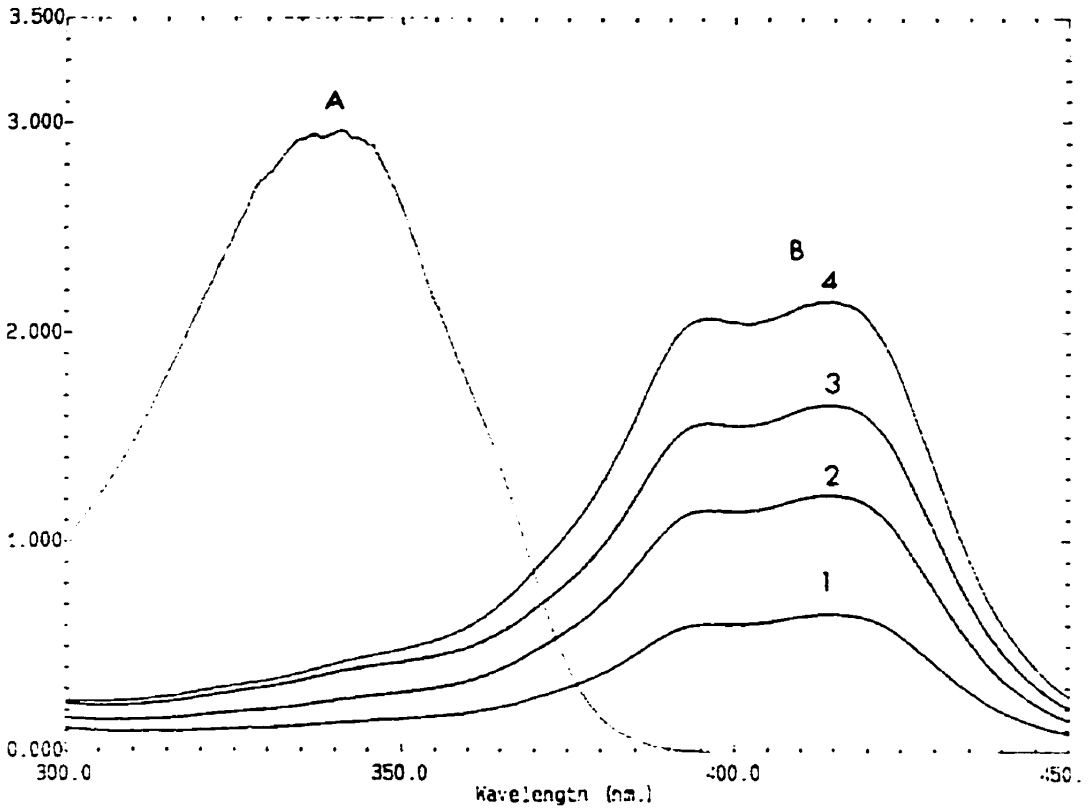


Figure 13. Spectrum of the reduced and oxidized form of ABTS: a. Absorption spectrum of the reduced form; b, Absorption spectrum of the oxidized form at four concentrations : 1. 0.015; 2. 0.03; 3. 0.045; 4. 0.06 mM. ABTS was oxidized with horseradish peroxidase in the presence of 15 µL of 1.5% hydrogen peroxide.

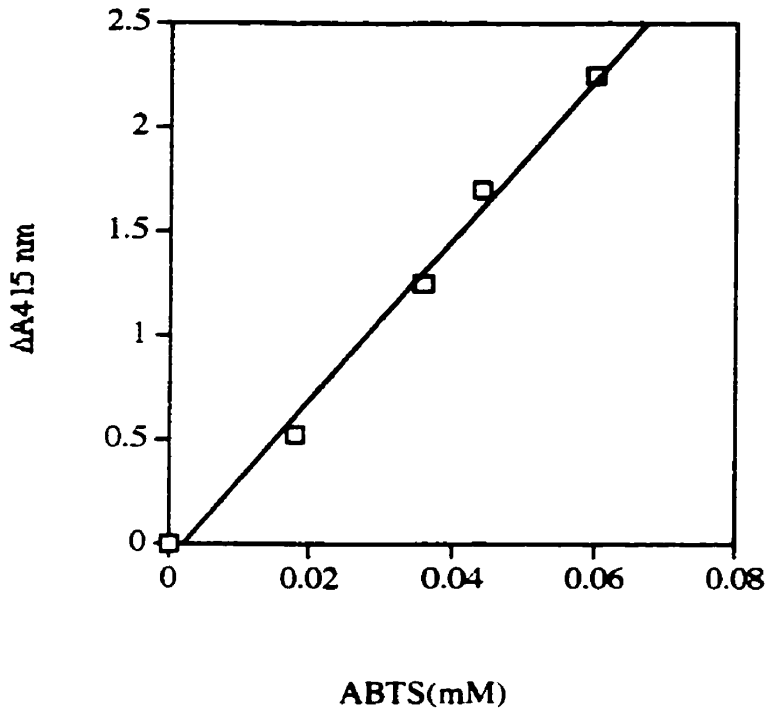


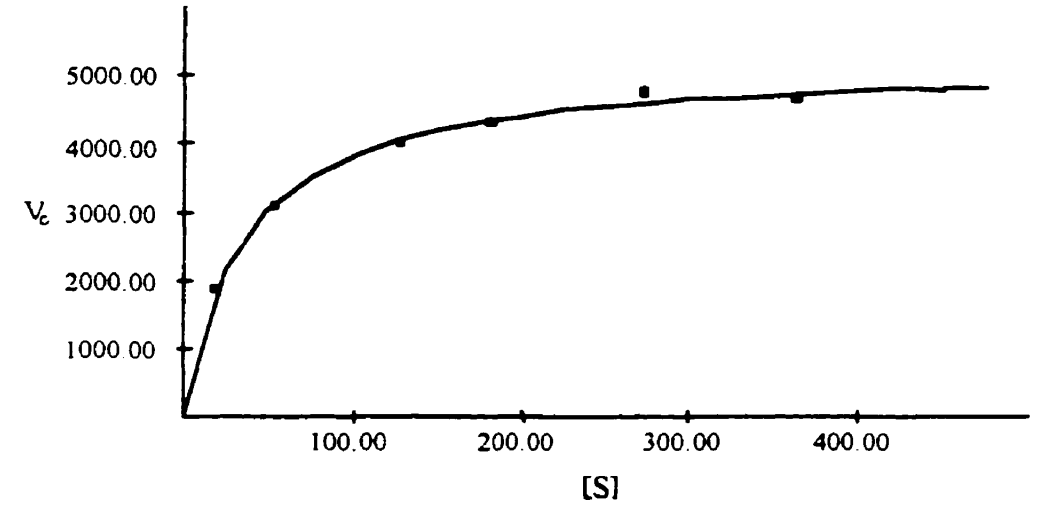
Figure 14. The relationship between the concentration and the $\Delta A_{415\ nm}$ of oxidized ABTS.

chosen to be below and above the preliminary K_m. To the 1-mL glass cuvette, 15 µL of pure enzyme (16.7µg/mL), 30 µl of ABTS and appropriate amounts of buffer and hydrogen peroxide were added. The experiment was conducted in a total volume of 1 mL, in 50 mM citrate/100 mM phosphate buffer pH 5.0 and at 25°C and absorbance at 415 nm was measured spectrophotometrically. The resulting data were analyzed using the ACS Enzyme Kinetics program. A hyperbolic curve was obtained applying the non-linear regression method and a straight line in the Eadie-Hofstee plot (Figure 15). The apparent K_m for ABTS as a substrate was determined to be 36 µM. V_{max} was calculated to be 5190 nmol/mg protein and k_{cat} was 7.8/sec.

Next, the experiment was conducted using the same conditions with fixed concentration of the other substrate, H_2O_2, at 0.26 mM and concentrations of ABTS were varied in the range of 18.2 to 364 µM. Six concentrations were tested in triplicate. To a 1-mL glass cuvette, aliquots of enzyme (15 µL), H_2O_2 (10 µL) and the appropriate amount of the same buffer were added and the change in absorbance during the reaction at 415 nm was recorded. The data plotted as above yielded a hyperbolic curve in the non-linear regression plot while a straight line was obtained in the Eadie-Hofstee plot (Figure 16). The following constants were calculated using the non-linear regression curve: K_M= 44 µM; V_{max}= 6640 nmol/mg and k_{cat}= 10/sec.

The third substrate tested was guaiacol, a compound considered to be a typical substrate for plant peroxidases [Ruttiman-Johnssoe et al. 1994]. It was prepared as 5 mM solution in 50 mM citrate/100 mM phosphate buffer (pH 5.0)

A



B

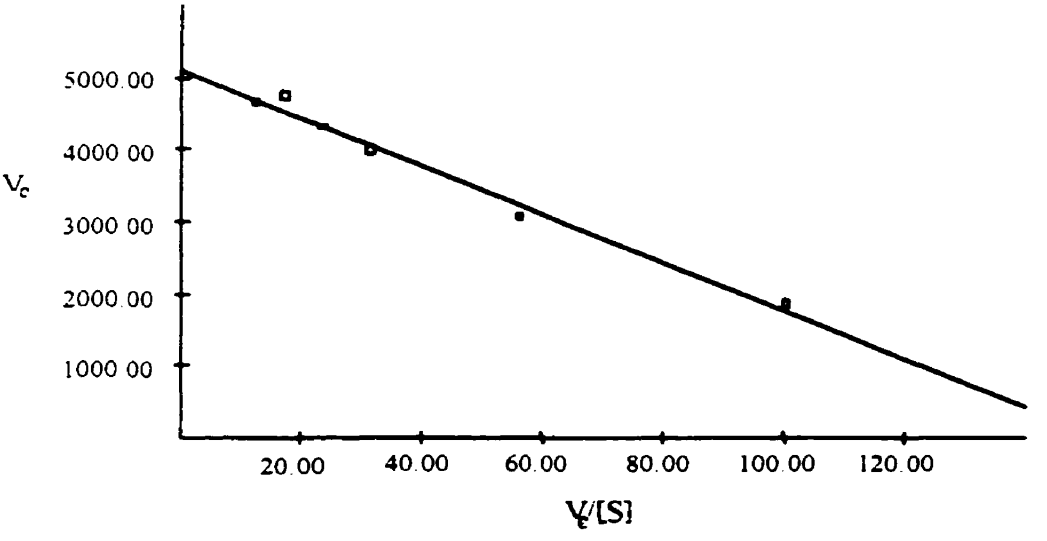


Figure 15. Determination of the K_m for HIP with ABTS as the variable substrate and hydrogen peroxide at saturating levels, using A, Non-Linear Regression plot; B, Eadie-Hofstee plot. The measurement of the increase of absorbance over time at 415 nm was recorded in triplicate for each of 6 concentrations of hydrogen peroxide and each data point represents an average of three readings. All reactions were performed in 50 mM citrate / 100 mM phosphate buffer (pH 5.0), temperature 25°C in total volume of 1 ml; enzyme concentration was 16.7 µg/ml.

with 25% glycerol. The enzymatic assay mixture was prepared with 15 μL of pure enzyme, 30 μL of H_2O_2 (30 mM) and aliquots of guaiacol solution to the final concentration in the range of 1-100 μM. No change in absorbance was recorded at 470, 450, and 418 nm. Therefore, guaiacol did not appear to be a substrate for HIP.

3.1.7 HIP Is a Glycoprotein

All of known fungal peroxidases are glycosylated proteins. It was, therefore, of interest to determine if HIP is also a glycosylated protein. To test this, SDS-PAGE gels were prepared and run as described in Materials and Methods. The protein samples included: molecular mass standards, 20 μL of crude extract of heat-treated wild type mycelium (5 mg/mL), 30 μL purified constitutive peroxidase, described in chapter 3.3 (10 μg/mL), and 30 μL of purified HIP (16.7 μg/mL). To each sample, 10 μL of denaturing sample buffer was added as well as 2 μL tracking dye.

Following electrophoresis, the gel was stained using the Fuchsin sulfite stain as described in Materials and Methods. As illustrated in Figure 17, HIP gave a positive glycoprotein stain (single pink colored band) corresponding to one of several positive bands in the crude extract of wild type *N. crassa* while CP does not seem to be a glycoprotein.

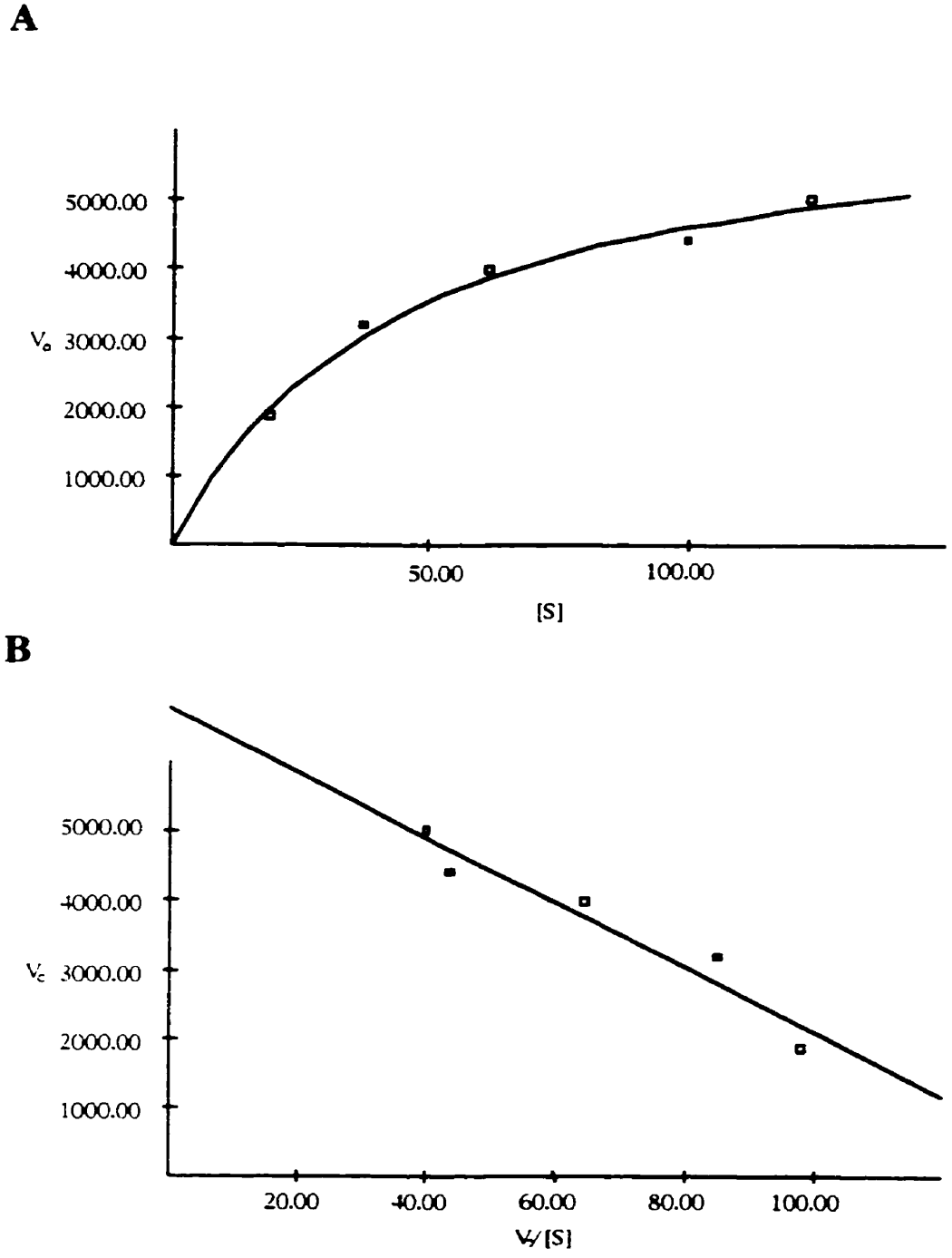


Figure 16. Determination of the K_m for HIP with H_2O_2 as the variable substrate using: A, Non-Linear Regression plot; B, Eadie-Hofstee plot. The increase of absorbance over time at 415 nm was recorded in triplicate for each of 5 concentrations of ABTS. The data points represent an average of three readings. All reactions were performed in 50 mM citrate / 100 mM phosphate buffer (pH 5.0), temperature 25°C, in total volume of 1 ml; enzyme concentration was 16.7 µg/ml.

3.1.8 Interaction of HIP with Hydrophobic Matrices

One of the methods that provided a high level of purification, but with significant loss of activity, was Hydrophobic Chromatography. In order to choose the optimal conditions, relatively hydrophilic Phenyl-Sepharose and strongly hydrophobic Octyl-Sepharose were tested. First, 2-mL Phenyl-Sepharose column (Pharmacia) was washed with 30% w/v aqueous solution of glycerol. The column was subsequently equilibrated with 50 mM phosphate buffer (pH 7.5), saturated to 30% with ammonium sulfate. A crude extract of wild type mycelium was dialyzed against 50 mM phosphate buffer (pH 7.5) containing 30% saturation of ammonium sulfate. A sample of 0.5 mL of the dialyzed pool was loaded on the Phenyl-Sepharose column and elution was carried out using a descending linear ammonium sulfate gradient (30-0%), at a flow rate of 0.5 mL/min. in a total volume of 30 mL.

Peroxidase activity eluted in fractions 35 to 38 corresponding to 8% saturation with ammonium sulfate (Figure 18). The active enzyme eluted as a doublet and represented approximately 90% of the total protein content. Next, Octyl-Sepharose chromatography was investigated. The chromatography matrix (1 mL) was equilibrated with phosphate buffer as above, and subsequently washed with the same buffer saturated to 60% with ammonium sulfate. A similarly prepared sample of crude extract saturated to 60% with ammonium sulfate was loaded on the column (0.5 x 8 cm) and eluted with a descending stepwise gradient of ammonium sulfate. Total elution volume was 20 mL using 4 mL each of: 60, 30,

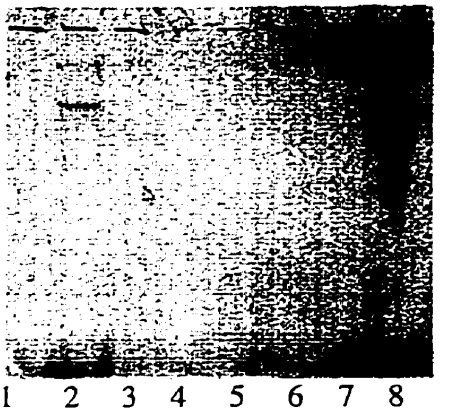


Figure 17. Separation of proteins on SDS-PAGE gel followed by glycoprotein stain. Lane 1, molecular weight markers; lane 2, purified HIP (20 µL); lane 5, purified CP (20 µL, 10 µg/mL); lane 8, crude extract (30 µL,5 mg/mL) of heat-shocked mycelium; lanes 3, 4, 6, and 7 no sample.

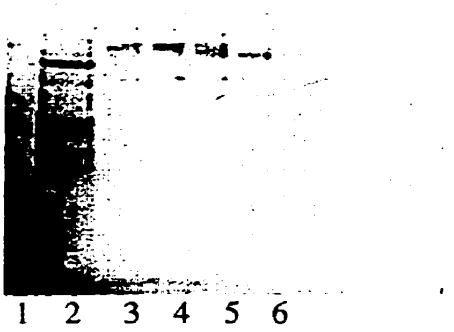


Figure 18. Peroxidase fractionation on Phenyl-Sepharose column (elution with ammonium sulfate). Lane1, molecular weight markers; lane 2, crude extact (2 µL); lanes 3-6 contain 15µL samples of fractions 35 (lane 3) to 38 (lane 6).

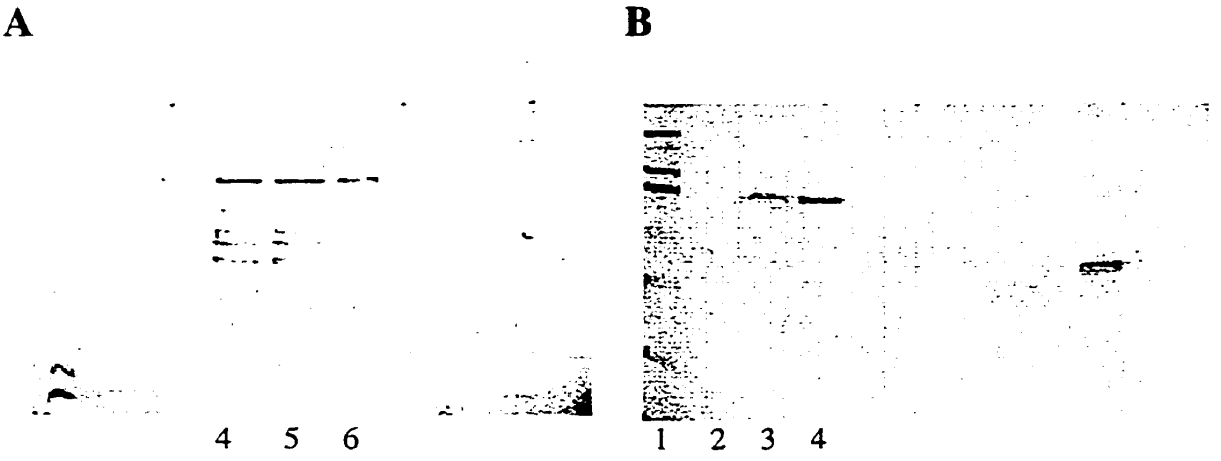


Figure 19. Fractionation of peroxidase activity on A, Octyl-Sepharose column (NaCl elution); lanes 4, 5 and 6 represent active Octyl-Sepharose fractions; B, active Octyl-Sepharose fractions applied to MonoQ column. Each lane contains 15 µL of corresponding fraction.

10, 5, and 0% ammonium sulfate. Peroxidase activity eluted at 0% salt but was detectable only after incubation at room temperature for 18 hr. It was suspected that a strong binding of the HIP to the matrix occurred, resulting in partial denaturation and unfolding of the protein. Therefore, to achieve weaker binding of the protein to the matrix, the less chaotropic salt (NaCl) was used.

The same column and sample were equilibrated with phosphate buffer containing 0.5 M NaCl. Elution was conducted using 4 ml of each salt concentration in phosphate buffer: 0.5, 0.3, 0.2, 0.1, 0.0 M NaCl and 0.0 M NaCl with 10% w/v glycerol and one-mL fractions were collected. Peroxidase activity eluted at 0.3 M NaCl and the enzyme appeared to constitute over 90% of the eluted protein as shown in the SDS-PAGE profile (Figure 19A). Concentrated enzyme from this preparation was loaded on a Mono Q column, elution being carried out as described in the preceding section. It appeared to be highly purified judging by the protein profile on SDS-PAGE (Figure 19B). Unfortunately, the specific activity was very low initially (0.2 units/mg) although it increased over time to 1.1 units/mg after 4 hr. In order to determine the incubation time that would result in maximum activity, the activity measurements were performed after 20 min, 60 min, 2 hr, 4 hr and 6 hr of incubation at 25°C. Peroxidase assays were conducted on fractions preincubated with 2 µL ABTS (0.05 g/mL) and compared to duplicate fractions incubated in its, absence but no difference was observed (data not shown). The above experiments were repeated several times using the same enzyme preparation, but the results were not reproducible. For instance, an increase in enzymatic activity was observed

consistently, however, the time required to reach the maximum value in different experiments varied from 4 to 18 hr.

In addition, the ability of Tween-20 and BSA to stabilize the protein was investigated. Three 0.5-mL fractions of the pooled elutate of Octyl-Sepharose, containing 0.3 M NaCl, were incubated at 25°C in the presence of Tween-20 and BSA along with the control with no additives. The three sets (100 µL aliquots) were assayed for peroxidase activity after 20 min and 6 hr (Figure 21). These attempts to stabilize the protein with BSA or Tween-20 proved to be unsuccessful as maximum activity regenerated in 6 hr was almost the same as in the control without any additives (Figure 21, A, B, C). Therefore it was apparent that neither BSA nor Tween-20 were effective in renaturation of peroxidase.

3.2 A 50/65 kDa PROTEIN WITH PEROXIDASE ACTIVITY

If the protease inhibitor cocktail was omitted during the initial steps of the purification procedure, a protein was isolated with peroxidase activity exhibiting a chromatographic behavior similar to that of HIP (Figure 22). The protein eluted at 0.5 M NaCl from the anion exchanger (DEAE cellulose), bound weakly to the metal chelation column (elution at approx. 12 mL) and fractionated on Superose 6 at a position corresponding to molecular mass of 130-148 kDa. Furthermore, it was shown to yield a single band upon electrophoresis in non-denaturing gels stained for peroxidase activity with ABTS, and when the same gel was subjected to Coomassie Blue stain (Figure 23). It seemed then, that there was a large protein with peroxidase

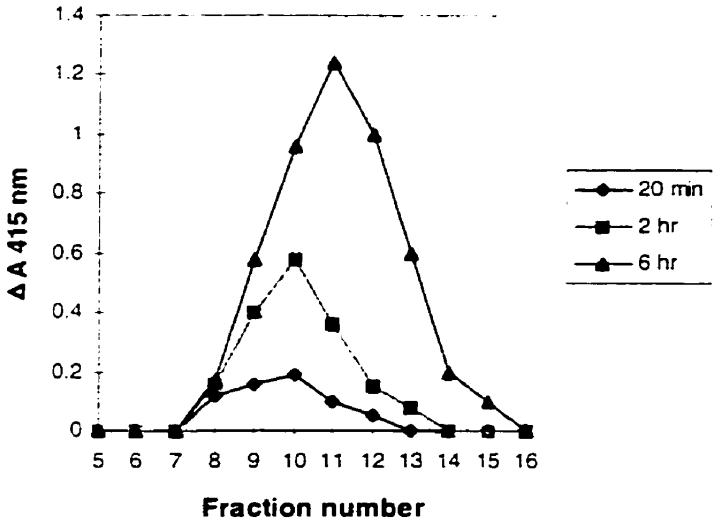
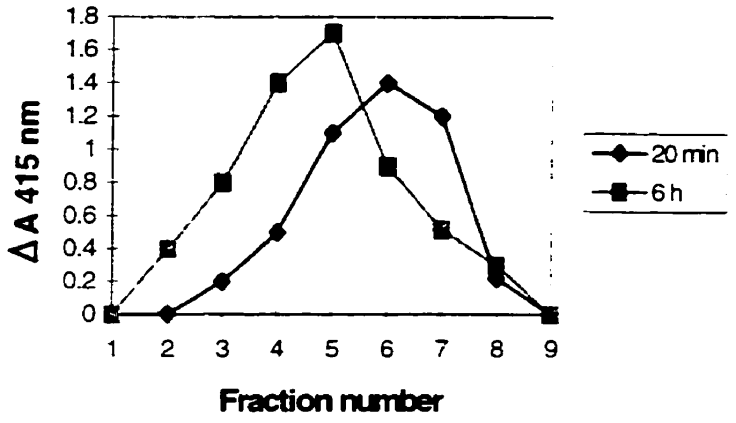
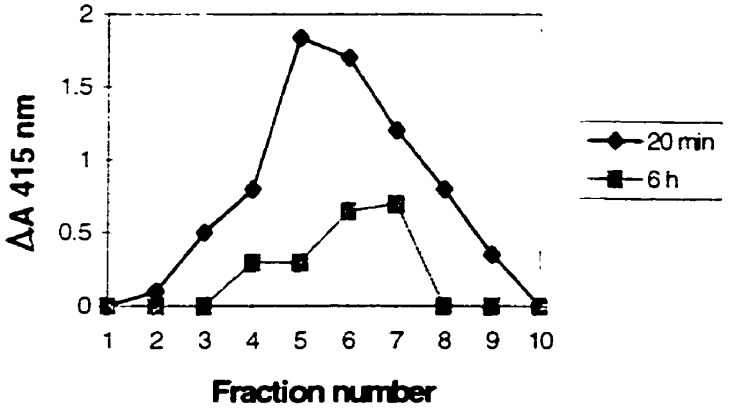


Figure 20. Peroxidase activity of fractions eluted from the Octyl-Sepharose column as a function of time of incubation at room temperature.

A


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ΔA 415 nm
1 2 3 4 5 6 7 8 9
Fraction number
20 min
6 h

B


2
1.5
1
0.5
0
ΔA 415 nm
1 2 3 4 5 6 7 8 9 10
Fraction number
20 min
6 h

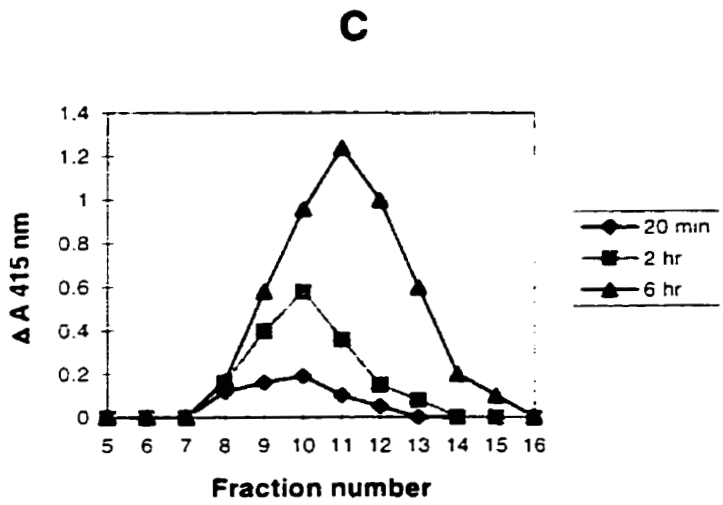


Figure 21. Peroxidase activity recovered after incubation with 1% BSA (A). 0.02% Tween-20 (B). no additives (C).

activity and its components were not separable in non-denaturing gels. Interestingly in resolution on SDS-PAGE, two bands, one corresponding to 50 kDa and the second corresponding to 65 kDa were seen consistently (in 12 purification cycles). As the two bands could not be separated chromatographically, it was likely that they constituted a two or three-component complex (designated as 50/65 kDa complex) in which probably one or both components exhibited peroxidase activity. On the other hand if protease inhibitors were included in the extraction media, the complex could no longer be isolated. It was therefore, concluded that the complex probably represented a product of partial proteolytic digestion with most of its original enzymatic activity intact. The steps leading to purification of the 50/65 kDa complex are summarized below (Table 5).

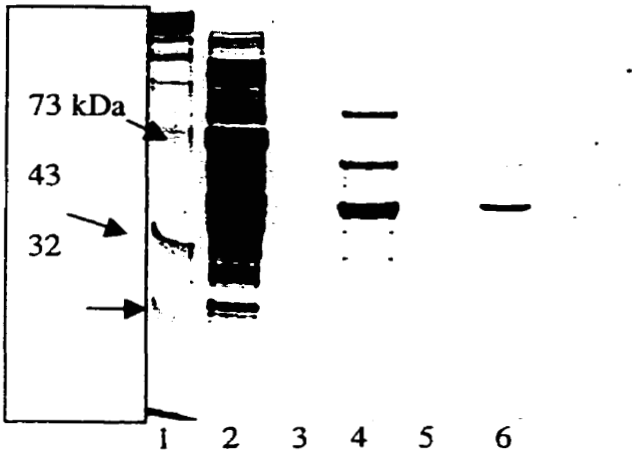


Figure 22. Resolution of the 50/65 kDa complex in SDS polyacrylamide gel. Lane 1, molecular weight markers; Lane 2, peak activity fraction eluting from gel filtration column (7 μL); Lanes 3 and 5 no sample; Lane 4, peak MonoQ column fraction (7 μL); Lane 6, Superose 6 peak fraction sample (40 μL).

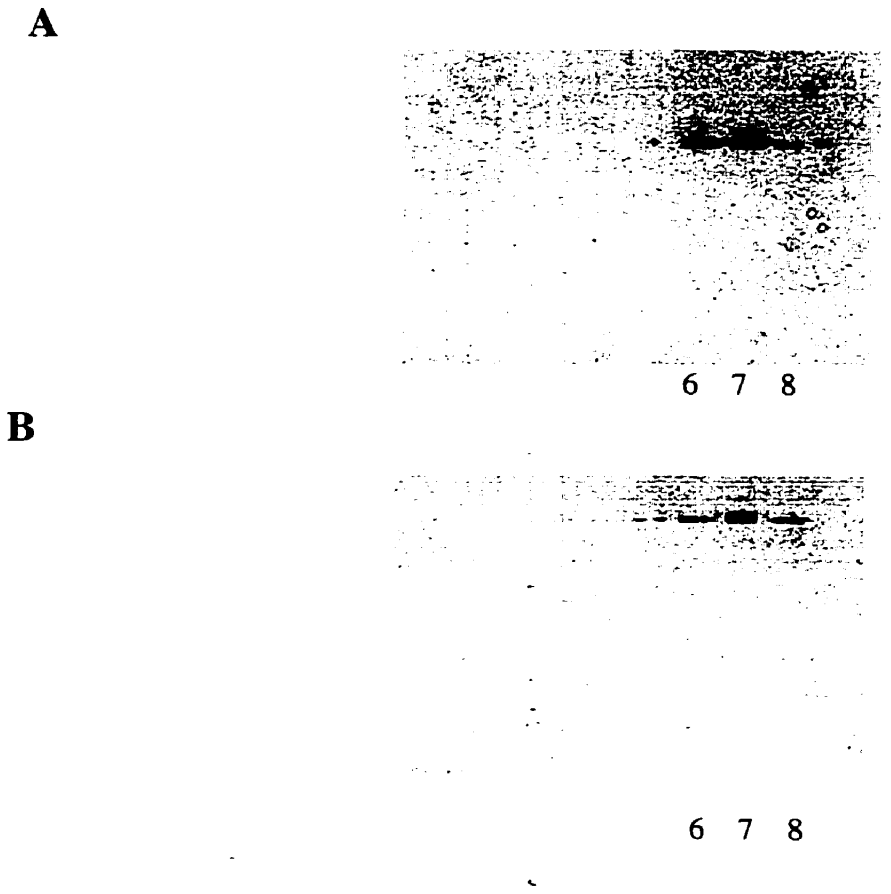


Figure 23. Electrophoresis of 50/65 kDa complex in non-denaturing polyacrylamide gels. A, gel stained with Coomassie Blue; B, the same gel stained with ABTS. Lane 6, 25 µL of the purified complex; lane 7, 45 µL of the same complex.

SAMPLE	Vol. (mL)	Activity ΔA_{415}/mL	Protein (mg/mL)	Specific Activity units/mg protein	Total Activity	Fold Purification	% YIELD
CRUDE	150	0.52	110	0.05	78	1	100
QAE SEPHADEX	88	0.71	12	0.06	65.2	12	80.1
METAL CHEL.	2.5	1.2	0.2	0.6	3	120	3.85
Mono Q	2	1.3	0.9	1.44	2.6	288	3.3

Table 5. Summary of purification steps of 50/65 kDa complex.

3.3 PURIFICATION OF THE CONSTITUTIVE PEROXIDASE

As described in the introduction, previous work from our laboratory had provided evidence for the existence of a constitutively expressed form of peroxidase. Question arose as to whether or not the constitutively expressed peroxidase activity, witnessed in the mycelium of the transformant E-45, was attributable to the same protein as the heat-induced peroxidase. To resolve this question, purification and characterization of peroxidase from E-45 was undertaken. A comparison of the properties of the two proteins with respect to the purification profile, native molecular mass, kinetic parameters, heat stability and pH optimum was conducted, as outlined in the following.

Lyophilized mycelium (5 g), was suspended in 75 mL of extraction buffer (0.05 M Tris-HCl, 0.1 mM EDTA, 50 μM β-mercaptoethanl pH 7.5) and stirred for 30 min at 4°C. At the end of that period, 2 tablets of Protease Inhibitor Cocktail (Boehringer Mannheim) were added. The mycelial suspension was subsequently homogenized using a glass Potter-Elvehjem apparatus and the homogenate was centrifuged at 15,000 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 precipitated proteins were removed by centrifugation as described above and the supernatant was subjected to the second precipitation step to achieve 80% saturation. The mixture was centrifuged again, pelleted proteins were redissolved in 4 ml extraction buffer and dialyzed against 20 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM NaCl (pH 7.5). The dialyzed protein sample was loaded on a Q Sepharose column that had been equilibrated with the same buffer. The bulk of the

protein did not bind to this column, emerging in the flow through fraction which contained peroxidase activity labelled as peak 1 (Figure 24). Following the elution of peak 1, a linear elution gradient (0 - 1 M NaCl) was applied to the column. At approximately 0.5 M NaCl, a second, minor peak of peroxidase activity was observed. Subsequently, the active fractions of peak 1 were pooled and the pooled sample of 80 mL was concentrated on Centricon 30 filters down to 6 mL volume and dialyzed against 2L of acetate buffer.

Aliquots of 2.5 mL of the Q-Sepharose dialyzed pool were loaded on the metal chelation column and eluted with acetate buffer (Figure 25 and 26). The constitutive peroxidase activity bound weakly to the metal chelation matrix. The peak of enzymatic activity eluted at approximately 16 mL. The active pool had a total protein concentration of 0.03 mg/mL and specific activity of 31.3 units/mg protein. It was concentrated to a volume of 200 μL on Centricon 30, centrifuged for 10 min at 15,000 g and loaded on a Superose 12 column equilibrated with 50 mM citrate/100 mM phosphate buffer (pH 5.0). The column was run at 0.2 mL/min and 2-min fractions were collected. Peroxidase activity was observed to elute between 13 and 13.6 mL (Figure 27 and 28). The active fractions were pooled and washed with the same citrate/phosphate buffer (pH 3.5) and stored at -20°C with 5% w/v glycerol. In order to estimate the molecular mass of the native protein, a Superose 12 column was calibrated with protein standards as described previously for HIP. By comparing the elution volume of the standards to the elution volume of the protein in question, the apparent molecular mass of this protein was

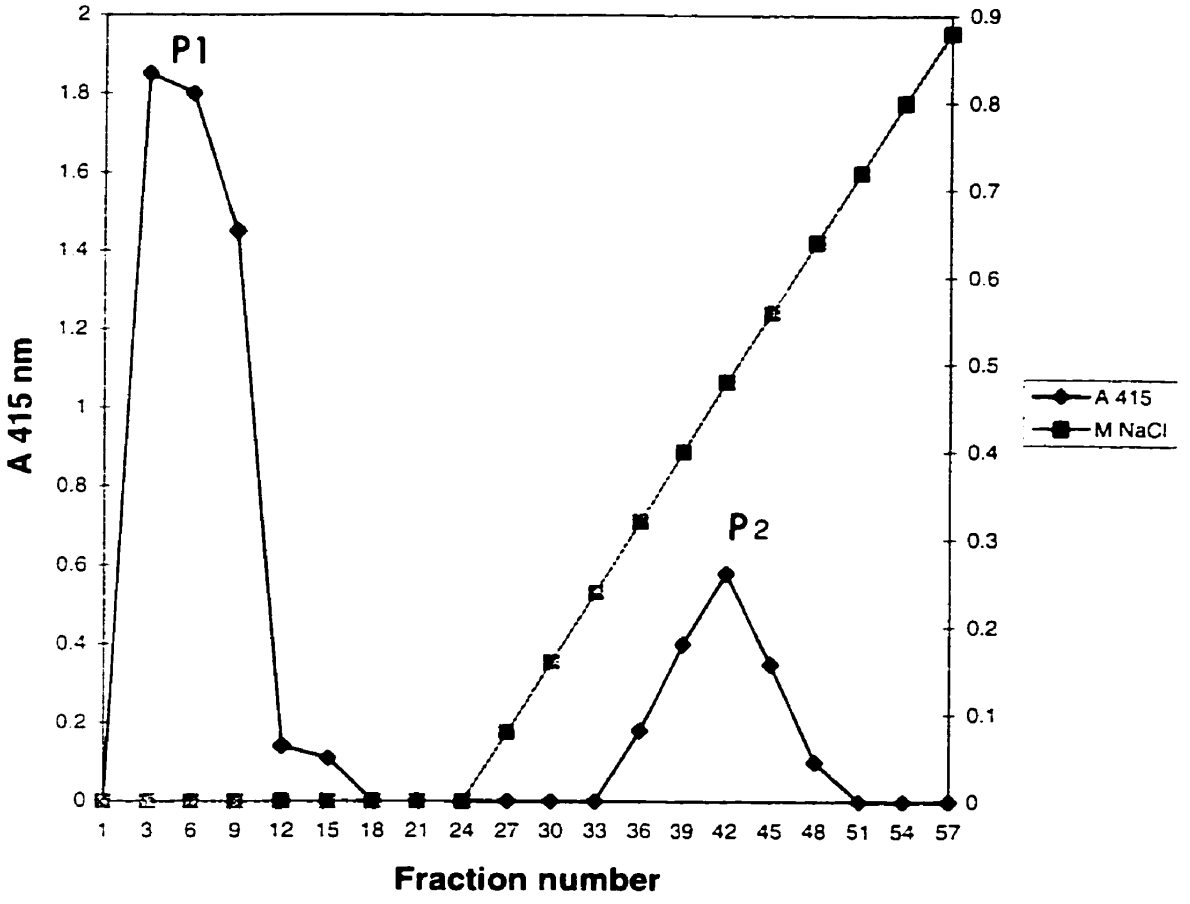


Figure 24. Fractionation of peroxidase activity on Q-Sepharose (individual fraction~5.5 mL).

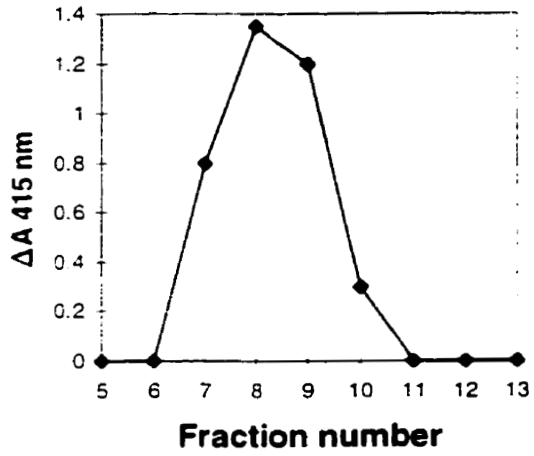


Figure 25. Fractionation of CP on metal chelation column.

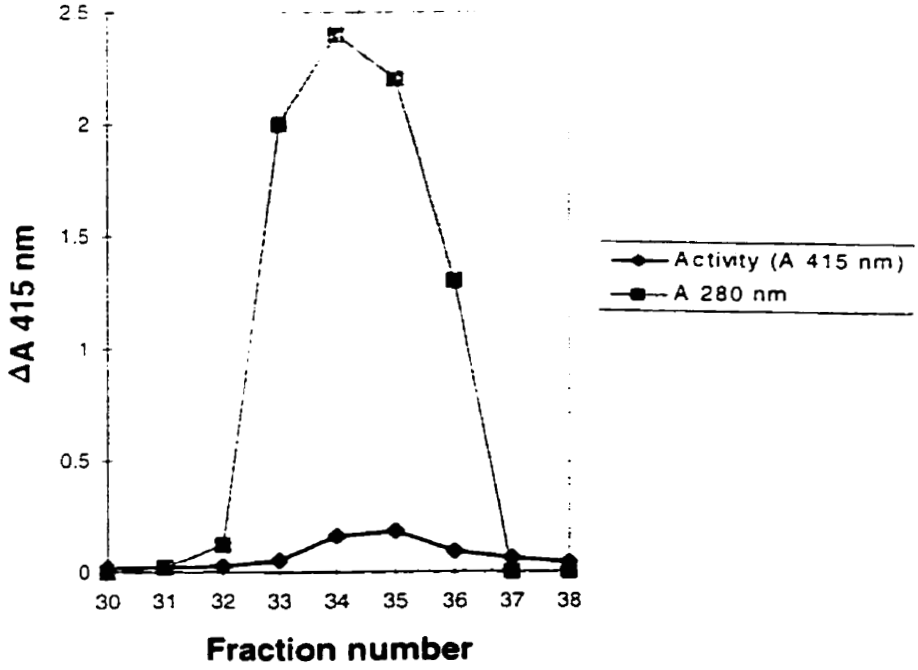


Figure 27. Fractionation of CP on Superose 12 column.

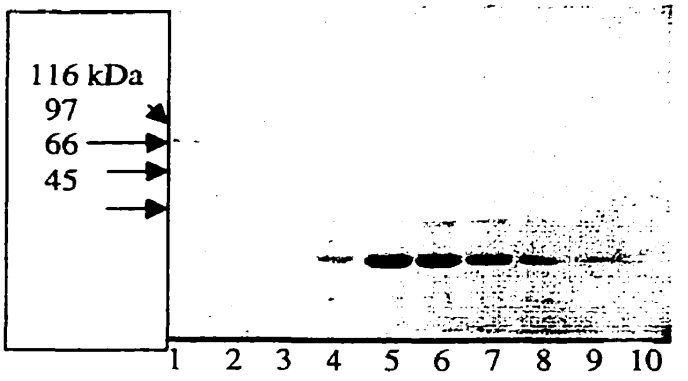


Figure 26. Fractionation of CP activity on metal chelation column; proteins were separated by SDS-PAGE. Lane 1, molecular weight markers; lanes 2-6 contain 15 µL samples of fractions 4 (lane 2) to 12 (lane 10).

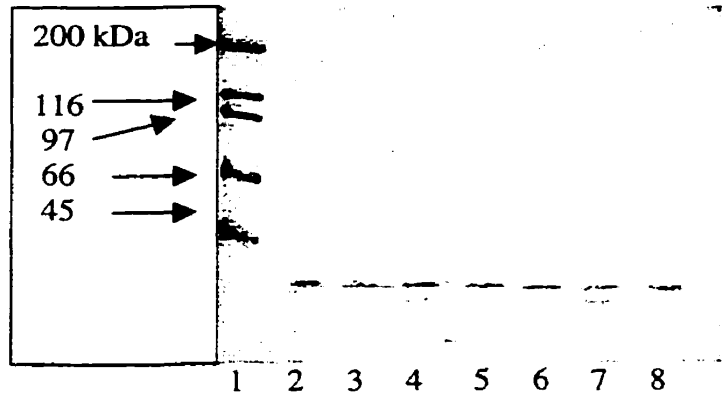


Figure 28. Fractionation of CP activity on Superose 12; proteins were separated by SDS-PAGE. Lane 1, molecular weight markers ; lanes 2-8 contain 20 µL samples of Superose fractions from different runs.

Sample	Protein mg/Ml	Activity (ΔA_{415}/mL)	Units /mg protein	Fold Purification	Vol (mL)	Total Activity	% Yield
Crude	13.5	1.72	0.13	1	48	82.56	100
Q Sepharose	0.04	0.8	20	153	80	64	77.5
Metal Chelation	0.03	0.94	31.3	241	1.2	11.3	13.7
Superose 12	0.01	2.9	145	115.4	2.0	5.8	7.0

Table 6. A summary of purification steps of constitutive peroxidase.

determined. The constitutive peroxidase activity eluted at Ve corresponding to approximately 118-136 kDa (Figure 29). The summary of purification steps is presented in Table 6.

3.3.1 Visualization of Constitutive Peroxidase Activity

Non-denaturing gels were prepared as described in Materials and Methods. The protein samples consisted of the enzymatically active pool eluted from Q Sepharose (15 μL) and Superose 12 pool (25 μL). To each sample the same volume of non-denaturing sample buffer was added, followed by loading on the non-denaturing gel. The gel was electrophoresed for 2.5 hr at 120 V. Upon completion of electrophoresis, the gel was stained for peroxidase activity as described in the section for heat-induced peroxidase. The gel was photographed and stained in Coomassie Blue. Constitutive peroxidase exhibited only one active band under non-denaturing conditions: this band corresponds to a band of the same mobility when stained in Coomasie Blue, indicating that the isolated protein was a peroxidase (Figure 30).

3.3.2 Heat Sensitivity of Constitutive Peroxidase (CP)

To compare it with the heat-inducible peroxidase, the activity of CP was studied as a function of temperature. In this experiment, aliquots of 20 μL of the purified enzyme (10 μg/mL suspended in 50 mM citrate/100 mM phosphate buffer pH 3.5) were incubated at temperatures ranging from 24 to 72°C. After 10 min of incubation, two 10-μL aliquots were added to test tubes containing 1 mL of assay buffer (50 mM citrate/100 mM phosphate) with ABTS (0.5mg/mL) and 10 μL of 1.5% hydrogen peroxide.

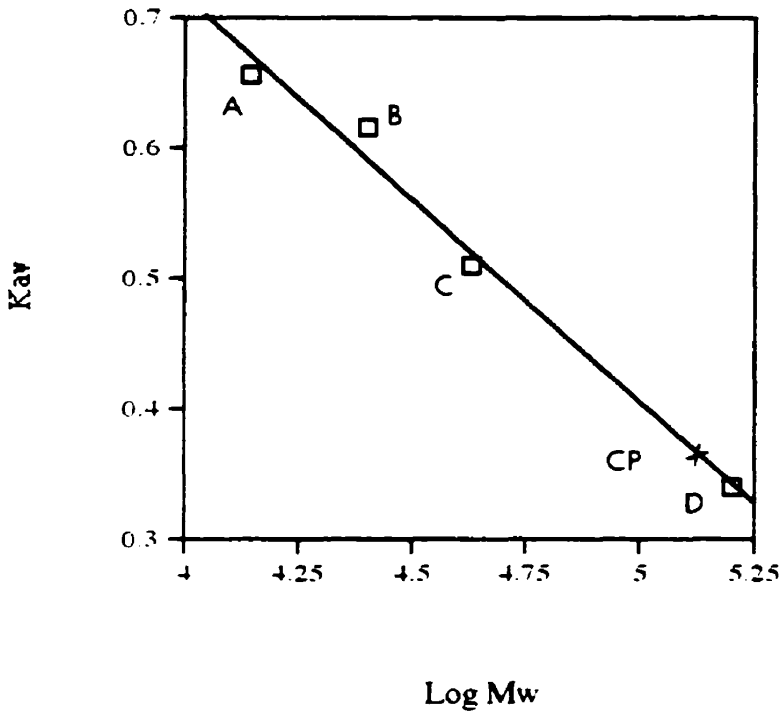


Figure 29. Estimation of molecular mass of the native constitutive peroxidase from Superose 12 column. Molecular mass protein standards used: A, ribonuclease (13,700); B, chymotrypsinogen A (25,000); C, ovalbumin (43,000), D, aldolase (158,000). The experiment was performed using 50 mM citrate/100 mM phosphate buffer (pH 5.0). The solutions of protein standards (10 mg/ml) were prepared in the same buffer and run two at a time (aldolase with ovalbumin and chymotrypsinogen with ribonuclease). The column was run at 0.2 ml/min. Void volume of 7.2 ml was determined using blue dextran (1 mg/ml).

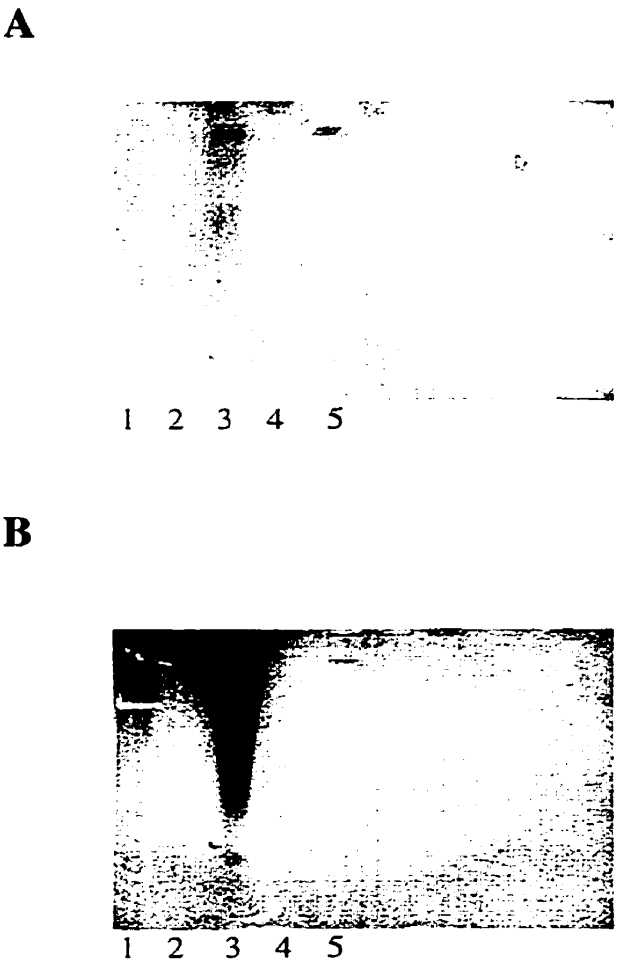


Figure 30. Resolution of the constitutive peroxidase activity in non-denaturing gels. A, gel stained for peroxidase with ABTS; B, the same gel stained with Coomassie Blue. Lane 1, crude extract (5µL); lanes 2 and4, no sample; lane 3, QAE Sephadex pool (15µL); lane 5, Superose 12 pool (20 µL).

The assay mixture was incubated for 30 min at 37°C and tested for peroxidase activity spectrophotometrically (Figure 31). Exposure to temperatures of up to 58°C resulted in a greater than 2-fold increase of enzymatic activity while at temperatures higher than 62°C enzyme inactivation was observed. The optimum enzymatic activity was established to be in the range of 48 - 58°C where a 2 to 2.5-fold increase in specific activity over that at 20°C. was observed. But as CP remained active up to 68°C. its heat stability over extended period of time was investigated. This time 100 µL aliquots of the same enzyme preparation were incubated at 48. 52. 58. 62 and 68°C. Every 5 min. two 10 µL samples were withdrawn and tested for peroxidase activity (Figure 32).

Approximately two-fold increase in enzymatic activity during the first ten min of incubation was observed for enzyme samples incubated at 52 and 58°C. At 58°C. the enzymatic activity decreased by 20% immediately after 10 min. but remained stable up to 20 min. Furthermore. at 52°C the enzyme remained active for up to 20 min of incubation with only a slight decrease in activity. Moreover. the enzyme samples incubated at 62 and 68°C did not exhibit the initial increase of activity and stable activity was observed at 62°C while at least 50% decrease was noted at 68°C.

3.3.3 Determination of pH Optimum of CP

In order that the assays for CP can be carried out under conditions favouring maximum enzyme activity. the pH optimum for enzymatic activity was investigated. The pH optimum of constitutive peroxidase was tested in the 50 mM phosphate buffer prepared in the range of 4.0 to 8.5 pH units. To 1 mL of buffer at the appropriate pH. 10

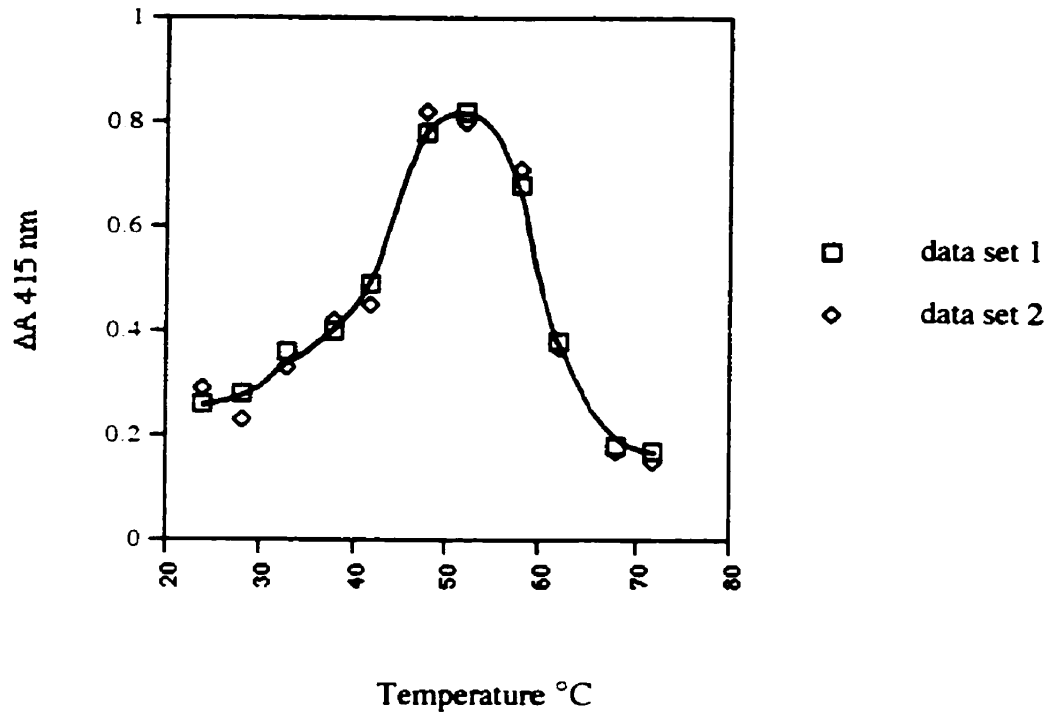


Figure 31. Heat sensitivity of CP (the experiment performed in pH 5.0).

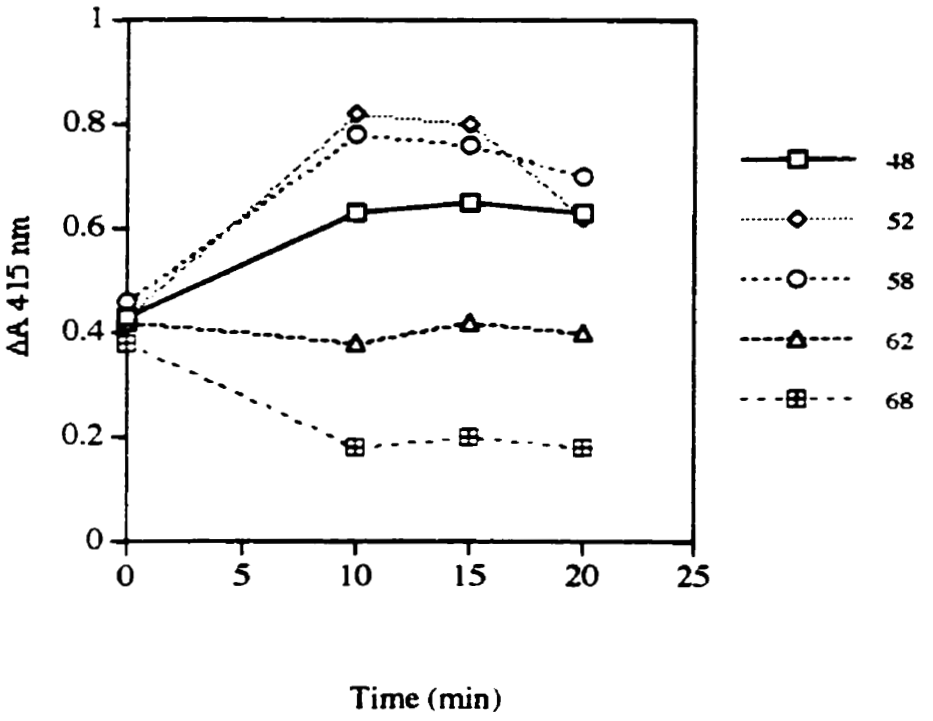


Figure 32. Heat stability of CP. Samples of CP were incubated at 48, 52, 58, 62 and 68°C , then 10 µl aliquots withdrawn, placed in 37°C water bath and tested for peroxidase activity.

μL of enzyme (10 μg/mL), 100 μL ABTS (0.5g/100 mL) and 10 μL of 1.5% hydrogen peroxide were added. After 30 min incubation at 37°C the samples were tested for peroxidase activity (Figure 33). The optimum pH for CP was determined to be 3.5. The increase from pH 2 to 3.5 caused an increase greater than 300% in activity. The enzyme remained relatively stable between pH of 3.0 and 4.0 with less than 10% decrease in activity, but it become completely inactive at pH 5.5.

3.3.4 Spectroscopic Analysis of Constitutive Peroxidase

As stated in the section on HIP, all known peroxidases are heme enzymes and they are characterized by the presence of a Soret band (around 410 nm) as well as α (around 550 nm) and β (around 500 nm) bands of the native enzyme spectrum (Figure 34). Another characteristics is the susceptibility of heme enzymes 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 in the enzyme. The absorption spectrum of the constitutive peroxidase was recorded on a Shimadzu Spectrophotometer using 60 μL enzyme (10 μg/mL) in 0.3 mL of 50 mM citrate/100 mM phosphate buffer pH 3.5 at 25°C. As illustrated in Figure 34, the spectrum of the native enzyme shows the following peaks: one (α) at 560 nm, one at 490 nm (β), and the most pronounced at 415 nm (Soret).

Next, the activity of CP was tested after addition of sodium azide as follows. Peroxidase assay mixture containing 10 μL of CP (10 μg/mL) in 300 μL of 50 mM citrate/100 mM phosphate buffer (pH 3.5) with ABTS (0.5 mg/mL) and 10 μL of 1.5% hydrogen peroxide was set up. Peroxidase activity of control samples was measured.

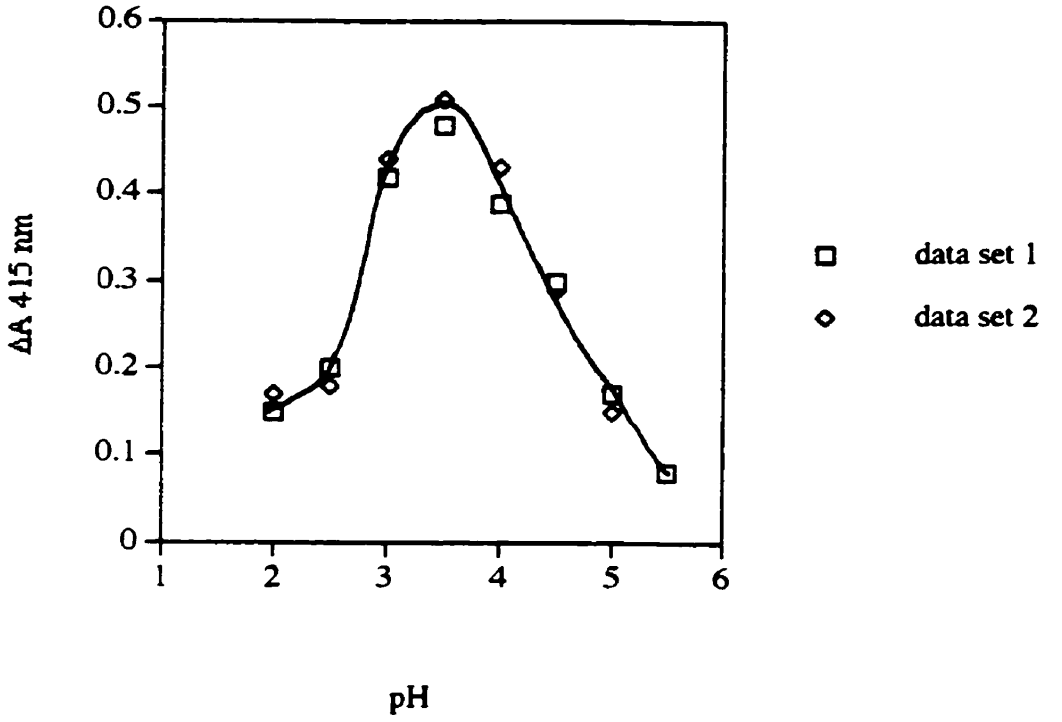


Figure 33. Determination of pH optimum of the constitutive peroxidase.

Then, aliquots of 0.02% sodium azide were added to a fresh assay mixture to a final concentration range of 0.125 to 0.5 mM. The samples were incubated at 25°C for 30 min and tested for peroxidase activity.

As shown in Figure 35, it is apparent that sodium azide inhibited the activity of CP at relatively low concentrations (0.125 mM). At 0.187 mM sodium azide, over 75% of peroxidase activity was abolished, while complete inhibition occurred at 0.5 mM sodium azide suggesting that CP is a heme enzyme. Next, the absorption spectrum of the enzyme following the addition of sodium azide was recorded along with the spectrum of the uninhibited enzyme in 100 mM acetate buffer (pH 7.0). Subsequently, to the same amount of enzyme, 98 µL of acetate buffer and 2 µL of 10 % sodium azide were added. The reaction mixture was incubated at 25°C for 2 min, following which the spectrum was recorded: a slight lowering of the peak at 415 nm was observed. The reaction was carried out with increasing amounts of sodium azide until the 415 nm peak was stable. The lowest point was reached at 0.625 mM of sodium azide and 3.125 mM and 6.35 mM did not reduce it any further (Figure 36).

3.3.5 Kinetic Analysis of CP

3.3.5.1 Determination of Molar Extinction Coefficient

The test was performed essentially as described previously, the only difference being the pH of the 50 mM citrate/100 mM phosphate buffer (3.5 instead of 5.0). The ε_{415} calculated for ABTS at pH 3.5 was 35.95 mM^{-1} cm^{-1} which is almost identical to that calculated for ABTS in buffer of pH 3.5 (35.85).

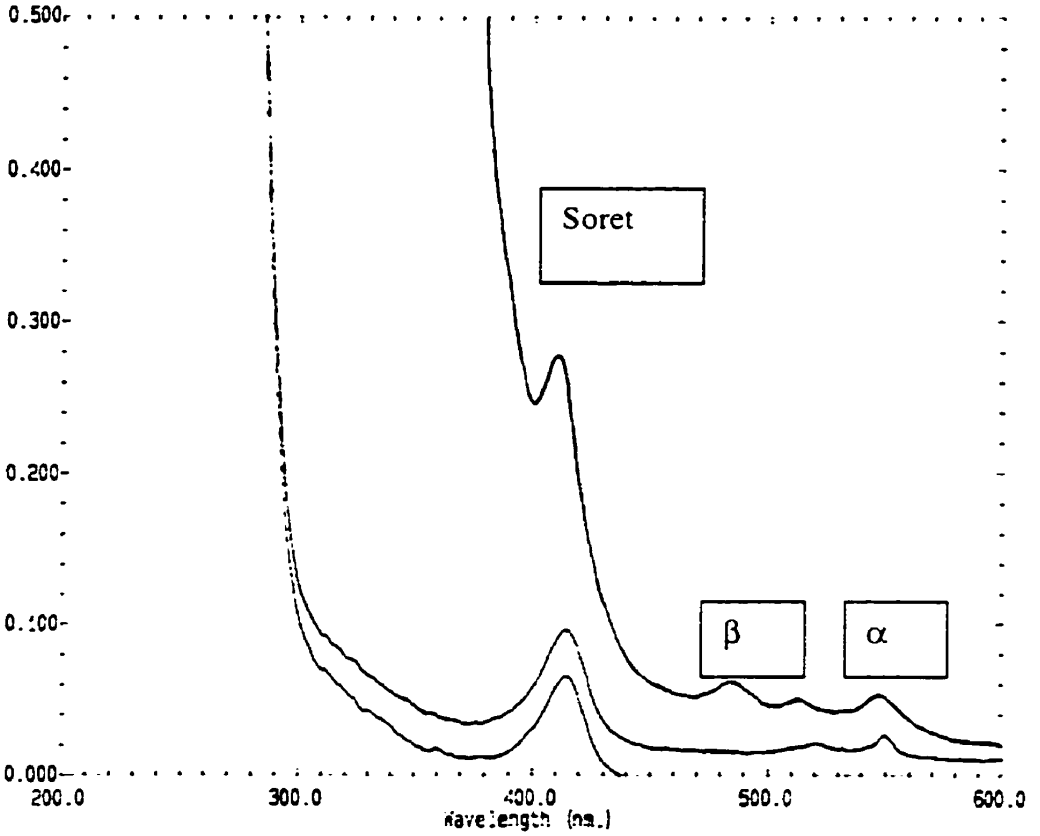


Figure 34. Absorption spectrum of the native CP. The experiment was performed using 0.6 μg enzyme in 50 mM citrate/100 mN phosphate buffer (pH 3.5), at 25°C.

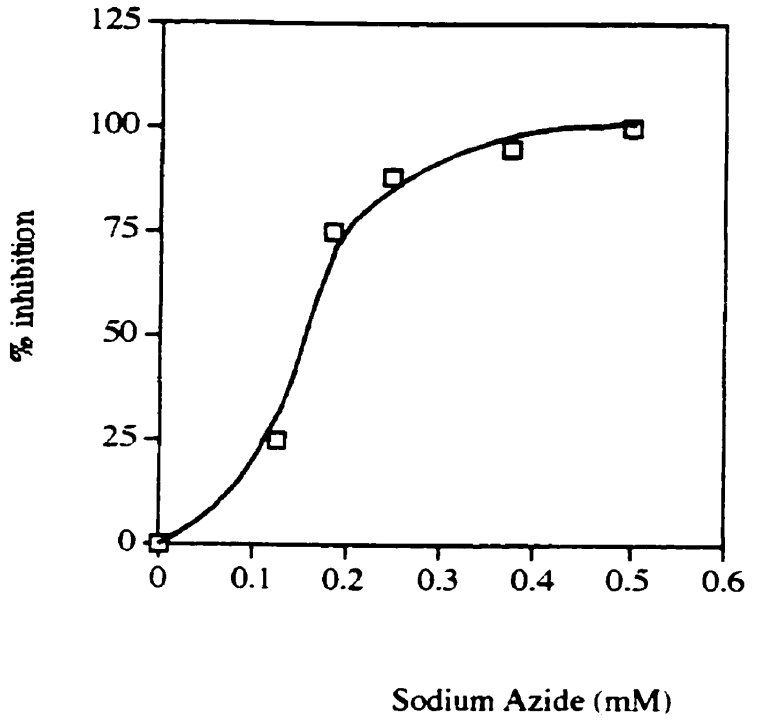


Figure 35. Inhibition of the constitutive peroxidase activity with sodium azide. The assay was performed in 50 mM citrate/100 mM phosphate buffer (pH 3.5), at 25°C.

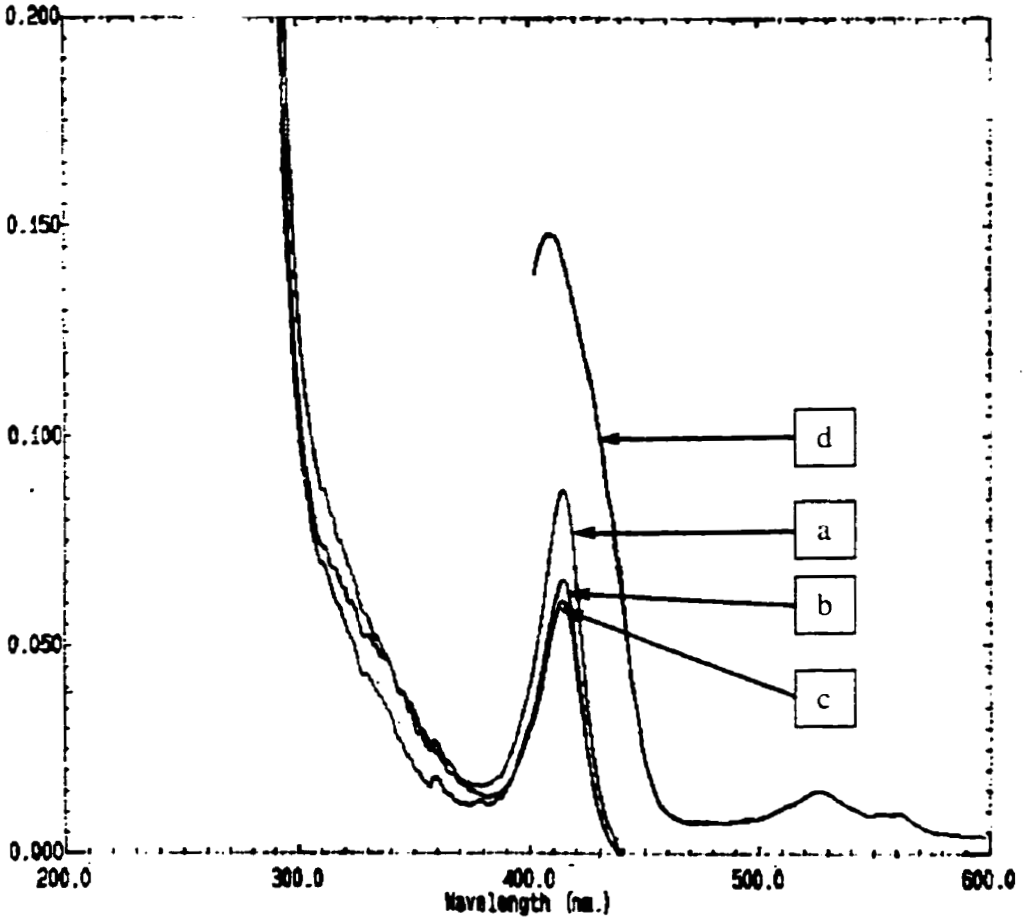


Figure 36. Absorption spectrum of constitutive peroxidase complexed with sodium azide. a. native enzyme spectrum: b. enzyme with 0.2 mM sodium azide: c. enzyme with 0.5 mM sodium azide: d. spectrum c magnified 2.5 times.

3.3.5.2 K_m and V_{max} Determination

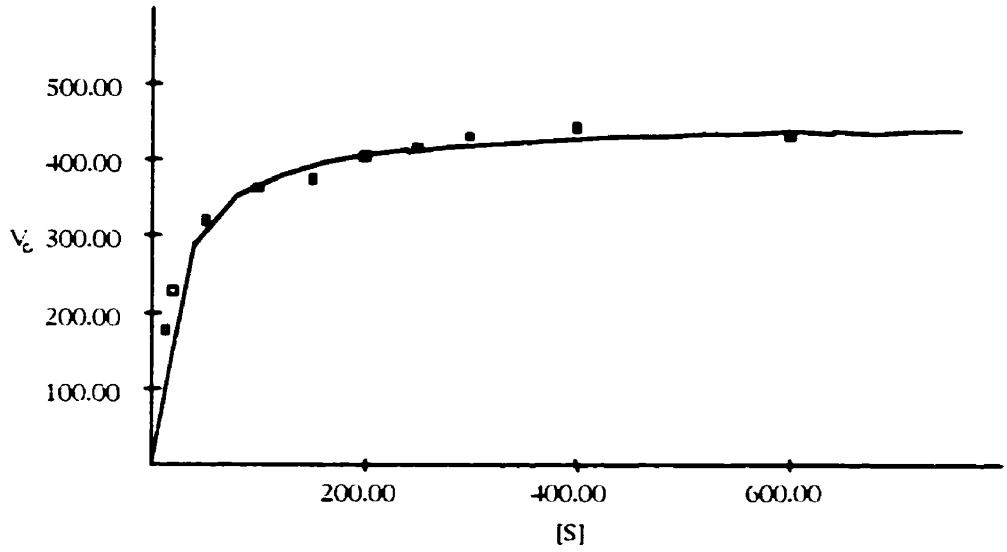
The ability of the CP to catalyze a two-substrate reaction was tested with ABTS and hydrogen peroxide, and with guaiacol and hydrogen peroxide. While testing ABTS as a substrate for CP, the reaction was carried out in 500 µL glass cuvette using 50 mM citrate/100 mM phosphate buffer (pH 3.5) with 15 µL of enzyme (10 µg/mL) at 25°C. To this solution 30 µL of 8 mM hydrogen peroxide was added. The change in absorbance was measured immediately and throughout the 2 min reaction time. The reaction was repeated with 10 different concentrations of ABTS (from 2.5 to 500 µM), while concentration of hydrogen peroxide was maintained constant at 0.5 Mm (10 x K_m for ABTS). The concentration range was chosen based on earlier experiments conducted in order to establish preliminary K_m. This K_m value was an indication of the substrate range at which its ability to accelerate the reaction was greatest; therefore, the concentrations of the tested substrate were chosen to be below and above the preliminary K_m.

Using enzyme kinetics software a hyperbolic relationship was observed in the non-linear regression method: the apparent Km was determined to be ~23 µM, whereas the value for V_{max} was ~448 nmol/mg and k_{cat} was 0.3/sec (Figure 37).

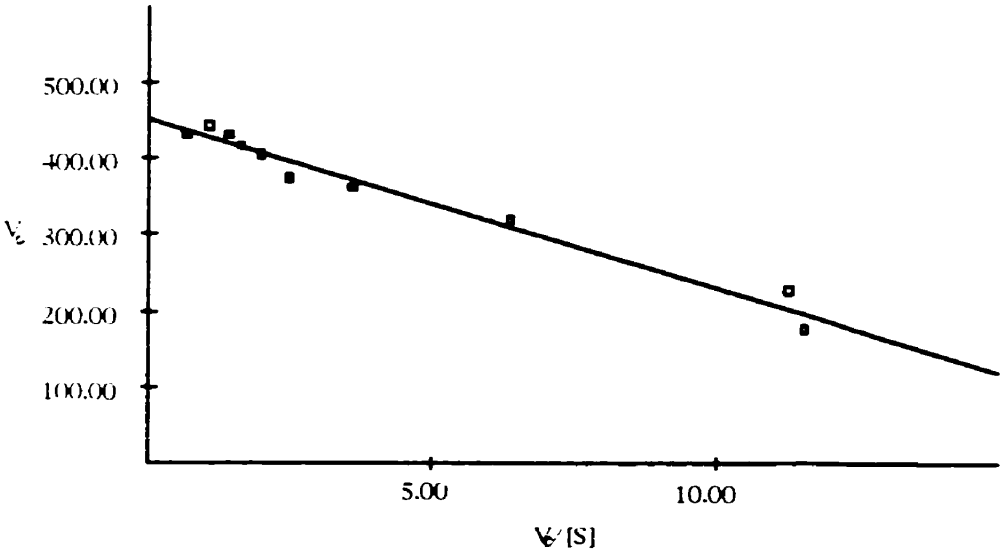
When H_2O_2 was tested as a variable substrate, a fixed concentration of ABTS (0.5 µM, ~ 10 x K_m for H_2O_2) was used along with 15 µL of enzyme (10 µg/ mL). Again a hyperbolic curve was obtained for the relationship between absorbance change and 1/[S] (non-linear regression). From this graph the values of K_m (~55 µM), V_{max} (~453 nmol/mg) and k_{cat} ~0.3/sec were calculated (Figure 38). Guaiacol was also tested for its suitability as a CP substrate. As in the case of HIP, guaiacol was not used as a substrate

by CP.

These studies demonstrated that two distinct forms of peroxidase are synthesized in *N.crassa* one of which is produced in response to stress. The constitutive form is virtually undetectable in the wild type strain but its presence is readily demonstrable in the transformant strain, E-45.

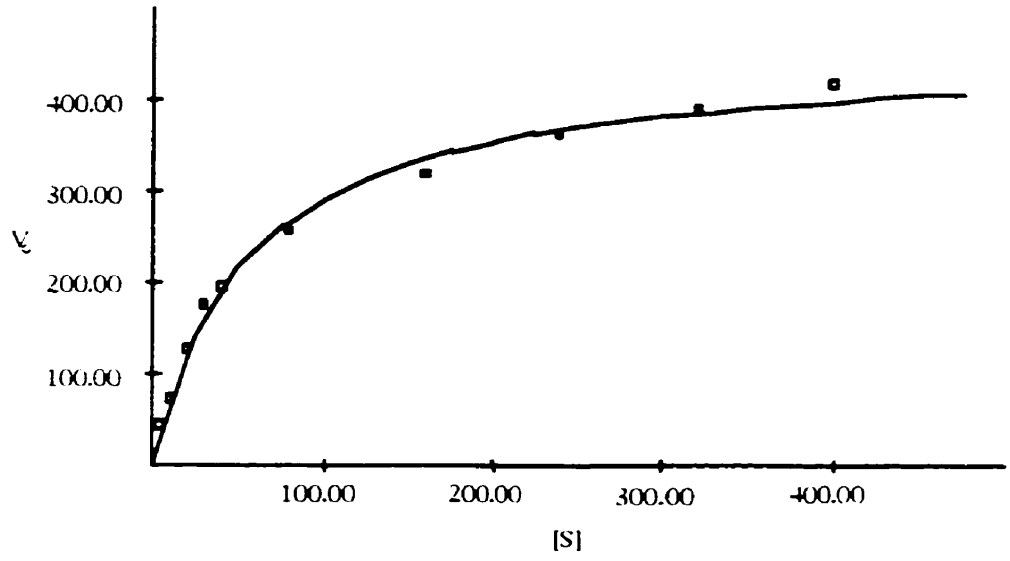


A

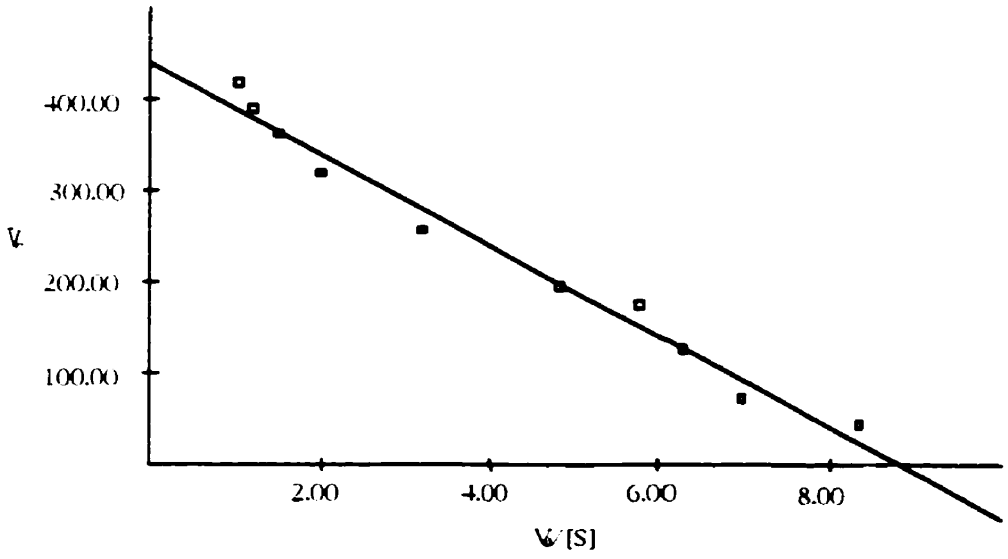


B

Figure 37. Determination of the K_{SI} for CP with ABTS as the variable substrate and hydrogen peroxide at saturating levels, using A, Non-Linear Regression plot; B, Eadie-Hofstee plot. The measurement of increase of absorbance over time at 415 nm was recorded in triplicate for each of 10 concentrations of ABTS and each data point represents an average of three readings. All reactions were performed in 50 mM citrate / 100 mM phosphate buffer (pH 3.5), temperature 25°C, in total volume of 0.5 ml; enzyme concentration was 10 µg/ml..



A



B

Figure 38. Determination of the K_M for CP with H_2O_2 as the variable substrate and ABTS at saturating levels, using A. Non-Linear Regression plot; B, Eadie-Hofstee plot. The measurement of increase of absorbance over time at 415 nm was recorded in triplicate for each of 10 concentrations of H_2O_2 and each data point represents an average of three readings. All reactions were performed in 50 mM citrate/100 mM phosphate buffer (pH 3.5), temperature 25°C, in total volume of 0.5 ml; enzyme concentration was 10 µg/ml.

DISCUSSION

4. CONCLUSIONS AND DISCUSSION

Upon oxidative stress many cellular functions are altered. The systems that are primarily affected are: antioxidant defenses, heat shock and chaperone proteins, translational apparatus, proteases and carbohydrate metabolism [Godon et al. 1998]. However, reactive oxygen species are also normal by-products of aerobic metabolism and they can damage a wide variety of cell components, leading to cell death. Therefore, it seems necessary for all aerobic organisms to employ specific defenses. In *Neurospora crassa* some of these defenses include peroxidase enzymes. I attempted to identify and purify two peroxidases: constitutive (CP) and heat-inducible (HIP) and to characterize them to determine similarities and differences between them.

The heat-inducible peroxidase was isolated from heat-shocked wild type *N. crassa*. The mycelial extract was fractionated by ammonium sulfate precipitation and applied to QAE Sephadex column. Peroxidase activity eluted in two peaks: peak 1 contained in the flow-through, and peak 2 emerging at ~0.46 M NaCl. The two peaks were further characterized in a reaction with the antibodies raised against heat-inducible peroxidase (Figure 8). Peak 2 enzyme was identified as the heat-inducible peroxidase, while peak 1 was not recognized by the antibody and it was suspected to be the constitutive peroxidase. This was further supported by the fact that peak 1 peroxidase activity isolated from *N. crassa* E-45 mutant (previously identified as a transformant overexpressing constitutive peroxidase), exhibited the same chromatographic behavior as peak 1 of the wild type fungus. Peak 1 peroxidase activity was, therefore, assumed to

contain mainly the constitutive peroxidase. The two enzymes were further purified employing metal chelation chromatography and gel filtration. The latter method allowed for molecular mass estimation. The native molecular mass of HIP was found to be ~116 kDa, and that for CP ~118 - 138 kDa. On SDS-PAGE. HIP appeared to be a 90 kDa protein, while CP migrates as a 38 kDa protein. As manganese and lignin peroxidases isolated from *P. chrysosporium* and *C.cinereus* exhibit various degrees of glycosylation (Table 3), it was interesting to determine whether HIP and CP were glycosylated. The presence of carbohydrate residues is known to effect protein migration in denaturing gels. To determine if this was the case, both enzymes were subjected to SDS-PAGE followed by specific stain for glycoproteins; HIP appeared to be a glycoprotein while CP did not (Figure 17). In such circumstances the estimation of HIP molecular mass from denaturing gels does not seem the most reliable method and gel filtration was assumed to be a better method. Therefore it is reasonable to conclude that HIP exists mainly as a monomer. Although CP did not appear to be glycosylated, there are factors that could increase its migration in denaturing gels. These factors include presence of significant amount of acidic residues as well as undetected carbohydrates. In view of that, moleculas mass estimation by gel filtration was also assumed to be more reliable than SDS-PAGE and it was concluded that the native CP was probably a trimer.

These peroxidases were also subjected to polyacrylamide electrophoresis in non-denaturing conditions followed by peroxidase stain. In both cases a single band of peroxidase activity appeared, confirming that the purified proteins had peroxidase activity.

Some other biochemical properties of the two enzymes were also investigated. For instance, the two enzymes were found to be different in their heat sensitivity and stability at high temperatures. The heat-inducible peroxidase was found to have an optimum activity between 43 and 55°C and to withstand this type of stress for up to 12 min. The constitutive peroxidase exhibited the highest specific activity between 48 and 58°C for at least 20 min. These findings suggest that CP is generally more stable during extended heat exposure and that it can still function at slightly higher temperatures than HIP.

Another difference was found in pH sensitivity of the two enzymes. The pH optimum for HIP was determined to be 5.0, while it was 3.5 for CP. HIP was also very sensitive to pH changes: a drop or an increase of 0.4 pH units caused at least 40% drop in specific activity of the enzyme. On the contrary, CP was still very stable in the same pH range (+/- 0.5 pH units) losing no more than 10% of the optimal activity (Table 7). This pH optimum range for peroxidase enzyme is not unusual. Manganese peroxidase isoenzymes I-III from white rot fungi have pH optimum between 3.75 and 4.5 [Ruttman-Johnson et al. 1994], while Korean-radish isoperoxidases are optimally active in the 5-6.5 pH range [Lee et al. 1991]. A plausible explanation for the low pH optimum of *Neurospora* peroxidases may be that the majority of the oxygen radicals are produced in the membrane-bound electron transport chains. Hence the pH in the immediate vicinity of the membrane is much lower than that in the cytosol as a result of the accumulation of H^+ [Gille and Sigler 1995]. Therefore, it would be beneficial for the cells to be able to mobilize an enzyme capable of neutralizing ROS at a low pH, to protect membranes

CHARACTERISTICS	ENZYME:	
	HIP	**CP**
heat sensitivity	43-55°C	48-58°C
stability to heat exposure	48-55°C (up to 12 min)	48-58°C (at least 20 min)
pH optimum	5.0	3.5
glycosylation	yes	no

Table 7. A summary of physical properties of *N. crassa* peroxidases.

from lipid peroxidation.

Manganese and lignin peroxidases isolated from *Phanerochaete chrysosporium* and *Coprinus cinereus* exhibit various degrees of glycosylation (Table 3). It was therefore, interesting to see whether HIP and CP were glycosylated as well. Both enzymes were subjected to SDS-PAGE followed by specific stain for glycoproteins. As a result of this stain, HIP appeared to be a glycoprotein while CP did not (Figure 17).

As most of the known peroxidases are heme enzymes, determination of the presence of heme was undertaken. The absorption spectrum of the native CP strongly suggests the presence of a heme prosthetic group [Glenn and Gold 1985; Welsh 1989]. The native peroxidase shows peaks at 560, 490 and 410 nm. These absorption maxima are very similar to those reported for horseradish peroxidase (HRP), indicating that the

prosthetic group of the peroxidase is iron protoporphyrin IX [Glenn and Gold 1985; Lin et al. 1998](Table 7).

Peroxidases as heme proteins bind small ligands such as azide. The binding rates and affinities for these types of ligands vary significantly in different heme proteins. It is due to different chemical environments found in the active sites of heme proteins resulting in their differential reactivity in living organisms [Lin et al. 1998]. *N. crassa* CP bound sodium azide attributable to enzyme inactivation, which indirectly supports the hypothesis that CP is a heme enzyme. But more importantly, the inhibited enzyme spectrum at pH 3.5 exhibited red shift in β and Soret bands as well as lowered absorptivity of the Soret. The same tendency with respect to Soret was found in other fungal peroxidases (Table 8) and in metmyoglobin [Lin et al. 1998]. The results of the spectral analysis and inhibition experiments show that CP is a heme enzyme.

The purified enzymes were also tested for their substrate binding affinity, reaction velocity and turnover number. As seen in Table 9, HIP has a lower K_m for hydrogen peroxide than the CP (36 μM). K_m is the concentration of the substrate that will permit the catalytic reaction to go at half its maximal velocity and it is higher for CP; K_m is usually also equal to the binding affinity of a sunbstrate of an enzyme. Maximal velocity of HIP using hydrogen peroxide as a substrate is ~15 times greater than that of CP. The k_{cat} for HIP was found to be ~10/s which is ~33 times faster than CP.

	Peak	*P. chrysosporium* manganese peroxidase	Horseradish peroxidase	*N. crassa* CP
Native enzyme	α	500	498	490
	β	500	498	490
	Soret	406	403	410
N_3^- complex	α	640	635	560
	β	540	530	520
	Soret	410	416	415

Table 8. A comparison of the spectral characteristics of CP with manganese peroxidase of *P. chrysosporium* and horseradish peroxidase [adapted from Glenn and Gold 1985].

The findings regarding the other substrate (ABTS) show similar trends. So, although the substrate affinity of HIP is lower than that of CP, the maximum velocity of HIP is more than 11 times greater. With respect to ABTS, HIP is again more efficient in catalyzing the modification of substrate; approximately 8 molecules are converted in to the product each second (k_{cat}= 7.78/sec vs. 0.302/sec) which is ~26 times faster than CP. An important feature of HIP that emerged from the above data is that HIP is a more efficient enzyme than CP, which supports the hypothesis that as a result of oxidative stress *N. crassa* induces a more potent peroxidase to alleviate the adverse condition.

Another characteristics of *N. crassa* peroxidases is the ability to utilize hydrogen peroxide and ABTS as substrates and the inability to use guaiacol. These characteristics resemble the substrate specificity of *P. chrysosporium* peroxidases (Table 9) and the peroxidases from the fungus *Phellinus igniarius* [Gazaryan et al. 1994]. Peroxidases with the above characteristics are placed within the plant/fungus/yeast superfamily of plant peroxidases [Welinder 1991].

It is strongly suspected that the two enzymes play an important role in protecting the cells from ROS. Heat-induced peroxidase is the more efficient enzyme which can probably offer protection from the consequences of heat shock, while CP parallels this function under normal conditions. This role is further supported by the remarkable heat resistance of these enzymes. Nevertheless, the cellular substrate of either of *N. crassa* peroxidases remains unknown.

Heat-inducible peroxidase has similar molecular mass (as seen on SDS-PAGE gels) to yeast Hsp104 and both proteins are also strongly heat-inducible. Hsp104 was

found to be necessary for cell survival at lethal temperatures while dispensable under normal growth conditions when its function is performed by Hsp70 and Ssa1p [Craig et al. 1993]. Heat-inducible peroxidase is probably not synthesized during uninduced growth: peroxidase activity is virtually undetectable in normally-grown cells. But contrary to HIP, Hsp104 is suspected to regulate one or more proteases and it was shown to disagregate heat-damaged proteins and to possess a hexameric structure that provides multiple sites for polypeptide substrate binding [Glover and Lindquist 1998].

HIP and CP may parallel the function of another compound involved in neutralizing ROS in fungi. It was found that DOPA (dihydroxyphenylalanine) and DHN (dihydroxynaphthalene) melanins produced by fungi are not essential for normal growth but function in protection of microbes against environmental stress. Melanins were found to offer protection from hydrogen peroxide (*Azotobacter salinestris*)[Page and Shivprasad 1995], hyperosmotic shock and elevated temperature (*Vibrio cholerae*)[Coyne and Al-Harthi 1992], and oxidative stress caused by UV irradiation [Kollias et al. 1992]. DOPA melanins themselves are stable radicals that scavenge and neutralize free radicals, and convert UV energy to heat. For this reason, they act as sponges for cytotoxic free radicals and prevent damage resulting from interaction of the free radicals with metals by binding metals and immobilizing them [Senesi et al. 1987]. In this way metal participation in Fenton reaction is prohibited. DHN melanins were also shown to provide protection against oxidizing agents such as permanganate, hypochloride and hydrogen peroxide [Jacobson et al. 1995]. Interestingly, DHN melanins also seem to supplement SOD function. They bind oxygen radicals mainly below 37°C while SOD does so above

	H_2O_2	ABTS	Guaiacol
***P. chrysosporium* wt MnP**	K_m= 42 μM[1]	ND	ND
	K_m= 41.7 μM[4]	ND	ND
	K_m= 21-34 μM[5]	ND	ND
Korean-Radish[3]	K_m= 0.77-2.5 μM	ND	K_m= 5.6-11.0 μM
***N. crassa* :**			
HIP	K_m= 44 μM .	K_m= 36 μM	
	V_{max}=6640nmol/mg	V_{max}=5190 nmol/mg	
	k_{cat}=10/s	k_{cat} =8/s	
CP	K_m= 54.85 μM	K_m= 22.32 μM	
	V_{max}=453nmol/mg	V_{max} =448nmol/mg	
	k_{cat} =0.3/s	k_{cat} =0.3/s	

Table 9. Kinetic constants of peroxidases from three fungi: 1, adopted from Kishi et al. 1996; 2, Glenn et al. 1986; 3, Lee et al. 1996 (isoenzymes A1-A3 and C3); 4, Mayfield et al. 1994; 5, Pease and Tien 1992 (isoenzymes H3-H5); ND, not determined.

that temperature [Jacobson et al. 1994]. In the functional aspect, HIP and CP seem to be quite similar to melanins. The two peroxidases neutralize hydrogen peroxide (presumably their major substrate) and therefore prevent it from causing lipid peroxidation and participation in Fenton reaction. Melanins, in turn, seem less specific than HIP and CP in neutralizing ROS and they have a wider range of ROS that can be scavenged. But their efficiency has not been determined so far. In addition to the wide range of substrates that melanins can neutralize, it may not be surprising that melanins can also be used as virulence factors by pathogenic fungi and protect the fungus from the host defenses [Butler et al. 1998].

More information and growing understanding of the systems involved in cellular defenses against ROS would allow us to appreciate the benefits these systems provide. Presently, the structure, cellular localization and *in vivo* substrate of both HIP and CP of *N. crassa* are still open for further research. Further studies are required to evaluate the contribution of these enzymes toward acquisition of thermotolerance by *N. crassa* cells.Those studies could focus on sequencing peroxidases and their genes which would allow tha classification of these enzymes in the appropriate family of peroxidases and thereby help understanding the molecular evolution of peroxidases.

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5. BIBLIOGRAPHY

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