REGULATION OF MANGANESE AND LIGNIN PEROXIDASE GENE EXPRESSION IN THE BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

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A thesis submitted to the faculty of the
Oregon Graduate Institute of Science & Technology
in partial fulfillment of the
requirements for the degree
Master of Science
in
Molecular Biology

October, 1995

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ABSTRACT

Regulation of Manganese and Lignin Peroxidase Gene Expression in the Basidiomycete *Phanerochaete chrysosporium*

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The expression of manganese peroxidase (MnP) in nitrogen limited cultures of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* is regulated by heat shock at the level of gene transcription. Nitrogen limitation and manganous ion (Mn^{II}) previously have been shown to regulate *mnp* gene transcription. Northern blot analysis demonstrates that a 45°C heat shock results in the accumulation of *mnp* mRNA even in cells grown in the absence of Mn. Heat shock induces *mnp* gene transcription in 4 or 5 day old cells and the mRNA is detectable after 15 minutes at 45°C. Maximum accumulation of *mnp* mRNA is observed one to two hours after transfer of cultures to 45°C. Two hours after heat shock-induced cultures grown in the absence of Mn are transferred back to 37°C, *mnp* mRNA is no longer detectable. Higher levels of *mnp* mRNA are obtained with simultaneous induction by Mn and heat shock than by either treatment alone. Neither MnP enzyme activity nor protein are detectable in heat-shocked cultures grown in the absence of Mn. However, higher MnP activity is found in the extracellular medium of cultures induced by both heat shock and Mn than is found in the medium of cultures induced by Mn alone. These results suggest that the putative HSEs found in the promoter region of the *mnp* genes are physiologically functional and that Mn may be required for a post-transcriptional step of MnP production under heat shock conditions.

The expression of MnP in nitrogen-limited cultures of *P. chrysosporium* is also regulated at the level of gene transcription by H_2O_2 and various chemicals, including ethanol, sodium arsenite, and 2,4-dichlorophenol. Northern (RNA) blot analysis demonstrates that the addition of 1.0 mM H_2O_2 to 5-day-old cultures grown in the

absence of Mn results in the appearance of *mnp* mRNA within 15 min. Higher levels of *mnp* mRNA are obtained with simultaneous induction by Mn and H_2O_2 than with H_2O_2 alone. As with heat shock induction, neither MnP activity nor associated protein is detectable in H_2O_2-induced cultures grown in the absence of Mn. However, simultaneous induction with Mn and H_2O_2 results in a 1.6-fold increase in MnP activity as compared with Mn induction alone. In the presence of Mn, purging of low-nitrogen cultures with 100% O_2, in contrast to incubation under air, results in an increase in the accumulation of *mnp* mRNA and a 13-fold increase in MnP activity on day 5. However, in contrast to the effects of H_2O_2 and heat shock, O_2 purging of Mn-deficient cultures results in negligible accumulation of *mnp* mRNA.

Western blot analysis using a polyclonal antibody to lignin peroxidase (LiP) isozyme H8 from *P. chrysosporium* demonstrates that LiP protein is detectable in the extracellular media of 5- and 6-day-old nitrogen-limited, but not nitrogen-sufficient cultures. Northern blot analysis demonstrates that *lip* mRNA is detectable from 5- and 6-day-old cells grown in nitrogen-limited, but not nitrogen-sufficient cultures. These results indicate that LiP expression is regulated at the level of gene transcription by nutrient nitrogen. Since lignin degradation by *P. chrysosporium* is derepressed by nitrogen starvation, it appears that lignin degradation and LiP expression are coordinately regulated in this organism. These results contradict a recent report which concluded that LiP protein expression is not regulated by nutrient nitrogen.[46]

CHAPTER 1

INTRODUCTION

Lignin is a complex, optically inactive phenylpropanoid polymer that constitutes 20-30% of extracted wood.[84] It is the second most abundant natural polymer, after cellulose; and since its presence in plant cell walls significantly retards the microbial depolymerization of cellulose, lignin degradation is a key biochemical step in the global carbon cycle.[18,23,84] White rot basidiomycete fungi are the only known organisms which are capable of degrading lignin extensively to CO_2 and H_2O.[35,54] The hymenomycete *Phanerochaete chrysosporium* is the best-characterized lignin degrading fungus.[12,35,54]

Phanerochaete chrysosporium degrades lignin during the secondary metabolic (idiophasic) phase of growth, the onset of which is triggered by depletion of nutrient nitrogen.[12,35,54] When cultured under ligninolytic conditions, *Phanerochaete chrysosporium* produces two extracellular peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), that, along with an H_2O_2-generating system,[35,54] are the major extracellular components of its lignin degradative system (LDS). Both LiP and MnP are glycoproteins of Mr~ 40,000-46,000, containing one mole of heme per mole of enzyme. Elucidation of the catalytic mechanisms of these enzymes;[35,54,111,113] the cloning, characterization, and expression of the genes encoding various isozymes;[31,33] and the recent determinations of the crystal structures of LiP and MnP[21,77,101] have increased our understanding of the biochemistry and genetics of this unique extracellular oxidative system.

MnP catalyzes the H_2O_2-dependent oxidation of lignin[112] and lignin derivatives[57] and a variety of phenolic lignin model compounds.[35,107,115] It has been demonstrated that Mn^{II} is the preferred substrate for MnP.[27,35,111,113,114] The enzyme oxidizes Mn^{II} to Mn^{III}, which diffuses from the enzyme surface and in turn oxidizes the phenolic substrate.

Organic acids, such as oxalate and malonate, which are produced by *P. chrysosporium*, activate the MnP system by chelating Mn^{III} to form stable complexes with high redox potentials[115] and by facilitating the dissociation of Mn^{III} from the enzyme.[111] MnP occurs as a series of isozymes encoded by several genes, and the sequences of three *mnp* cDNAs[71,72,78] and two *mnp* genomic clones (*mnp1 and mnp2*)[29,61] have been reported.

LiP catalyzes the H_2O_2-dependent oxidation of a wide variety of nonphenolic lignin model compounds and aromatic pollutants,[12,35,40,54,63,86,109,110] including synthetic lignin[39] via a mechanism involving the initial one-electron oxidation of susceptible aromatic nuclei by an oxidized enzyme intermediate to form a substrate aryl cation radical.[54,60,80,86] Genes encoding various isozymes of LiP have been cloned and characterized.[31]

1.1 Metal Ion Regulation of Gene Expression

Metals ions have structural and functional roles in numerous enzymes and are important components of many transcription factors (e.g. "zinc fingers").[24,96] Because many biological functions are dependent on metal ions, physiologically important concentrations of essential metal ions must be maintained within the cell. Low concentrations can result in lost biological function; high concentrations can result in enzyme inactivation and cell damage due to metal ion catalyzed oxidation of lipids, proteins and nucleic acids.[103]

Metal regulatory systems play important roles in essential metal homeostasis and in detoxification of heavy metals.[67,69,100,102] The two best-studied systems in prokaryotes are those regulating iron homeostasis[67] and mercury detoxification.[69,100] In eukaryotes the best-understood transcriptionally regulated system may be that regulating metallothionein biosynthesis.[44,102] However, it is becoming apparent that many other gene families also are regulated by metal ions.[69,102] Extensive work has been conducted on the translational regulation of ferritin synthesis and on transferrin receptor message stabilization by Fe.[13,69]

The response of cells to external chemical stimuli is mediated through specialized signal transduction systems. Two major types of systems, multi-component and single component, have been identified.[79] Multi-component systems consist of signal receptors, transducers and intracellular second messengers, culminating in the modification of the expression of specific genes or activities of specific enzymes.[79] Single component transducing systems consist of a single protein which acts as a signal receptor and transcription factor.

The iron, mercury and metallothionein systems illustrate three different mechanisms for metal regulation of gene transcription; however, each is a single component system wherein an intracellular protein acts both as a heavy-metal receptor and a trans-acting transcription factor.[69] The prokaryotic ferric uptake regulation (Fur) protein regulates a variety of genes involved in Fe homeostasis, acting as a Fe-responsive repressor.[67,69]

In bacterial Hg resistance systems, the transcriptional regulator, MerR, is both an activator and a repressor of the structural genes responsible for Hg detoxification. MerR is always bound to the cis-acting DNA sequences: in the absence of Hg, it represses gene transcription; however, in the presence of Hg, MerR binds Hg as a tricoordinate dimer and activates transcription of the Hg resistance operon.[69,100]

The heavy-metal binding protein, metallothionein (MT), occurs widely in nature. Although its primary function appears to be to protect eukaryotic cells from the toxic effects of heavy metals, in fungi there is evidence that MT also is involved in maintaining copper homeostasis.[37,66,102] In the best-studied system, the yeast *Saccharomyces cerevisiae*, transcription of Cu MT, encoded by the *cup*1 gene, is activated in the presence of Cu by binding of the Ace1 protein to cis-acting sequences in the promoter region of *cup*1. Ace1 is a soluble Cu-binding transcriptional activator structurally similar to MT.[102] In contrast, Cu regulation in prokaryotes appears to be mediated by a two component system involving phosphorylation of the transcription factor.[69] In the mammalian metallothionein gene promoter, multiple copies of metal response elements (MREs) with the core consensus sequence TGCPuCXC are the cis-acting sequences responsible for heavy metal induction of these genes.[102] Multiple homologous promoter elements are a common feature of eukaryotic gene regulation.[44,59]

Manganese is an essential trace element for all organisms.[87,93] Specific, high affinity, energy dependent uptake systems for Mn are known in bacteria[93] and fungi.[41] Mn is a required cofactor for a variety of redox and non-redox enzymes.[87] In addition to these roles, some evidence indicates that, like Ca and Mg, Mn can play a role as an enzyme activator.[87,118] While most of the metal-activated gene transcription systems described above utilize the metal as a transcription factor ligand, other more indirect mechanisms are possible. For example, Mn may be involved in the activation of a transcription factor by phosphorylation and Mn-dependent protein kinases have been reported.[94] As described above, Cu regulation in prokaryotes appears to involve transcription factor phosphorylation.[69] Metal ions are known to be involved in regulation of secondary metabolic processes.[116] Specifically, Mn is required for the synthesis of the fungal secondary metabolite patulin.[89]

1.2 Heat Shock Regulation of Gene Expression

The heat shock response is a pronounced alteration in metabolism, involving a transient rapid synthesis of HS proteins (HSPs) on exposure to hyperthermia and other environmental stresses, including respiratory poisons, oxidizing agents and ethanol.[59] This response has been found in every organism examined including bacteria, fungi, plants, and animals. It is generally agreed that the HS response provides protection against the toxic effect of stress; however, in most cases the specific mechanism of protection is unknown.

HSPs have been categorized by their molecular weights and have been found to fit into three major groups: those with molecular weights of approximately 90 kd, (HSP90), those with molecular weights of approximately 70 kd, (HSP70), and a third group of proteins with molecular weights between 15kd and 30kd (small HSPs).[4] Normal, unstressed cells express low, constitutive, levels of HSPs and proteins that are similar to HSPs (HS cognates).[4] This observation suggests that HSPs also play roles in normal cellular processes.

Eukaryotic HSPs and heat shock-responsive proteins are regulated at the level of gene transcription, by trans-acting heat shock factors (HSFs) which bind to repeated heat

shock elements (HSEs) in the promoter regions of HS genes.[59,64] The palindromic consensus sequence of the HSE, C--GAA--TTC--G,[74] is conserved among a wide diversity of species.[59] Although multiple copies of the HSE are frequently present, at least one copy of the HSE has been found within the first 400 bases upstream of every eukaryotic heat shock gene analyzed.[4] Most heat shock genes contain several HSEs. The strength of a particular HSE seems to be dependent on the closeness of its match with the consensus sequence.[4] A match of 7/8 or 8/8 constitutes a strong consensus sequence, matches of 6/8 and 5/8 are considered weak. HSEs are commonly found in pairs or trimers with a characteristic spacing which suggests that proteins binding to these elements interact in a specific way. More recently, however, it was proposed[76] that the DNA sequences that specify HS induction are composed of contiguous arrays of inverted 5bp units, -GAA-,[2,119] as identified by genetic tests and sequence comparisons.

A trans-acting transcription factor (HSTF) that interacts with the HSE has been identified and genes encoding HSTFs have been isolated from *Schizosaccharomyces pombe* (fission yeast),[25] *Saccharomyces cerevisiae* (baker's yeast),[95,117] *Kluyveromyces lactis* (yeast),[45] *Drosophila melanogaster* (fruit fly),[14] and *Homo sapiens* (human).[88,90]

1.3 Mn and HS Regulation of *mnp* Gene Expression

The 5' untranslated regions of the *mnp*1 and *mnp*2 genes contain a TATAAA element, three inverted CCAAT elements (ATTGG), and a putative SP-1 site (GGGCGG). The *mnp*1 gene contains a putative AP-2 site (TGGGGA)[29] and the *mnp*2 gene contains a putative inverted AP-2 site (CCCACGGC).[61] Both the *mnp*1 and *mnp*2 promoters contain multiple putative consensus metal response elements (MREs), identical to the consensus MREs found in mammalian metallothionein genes[19,31] (Fig. 1-1). Importantly, the promoter regions of the *mnp* genes contain multiple HSEs similar to the consensus sequence C--GAA--TTC--G (Fig. 1-1). The HSEs match the consensus in six or seven of the eight positions.

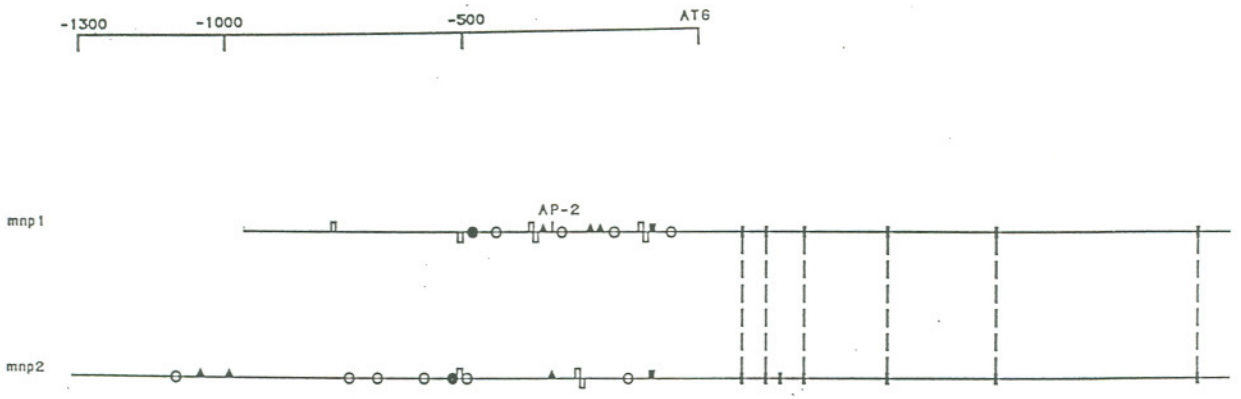


Fig. 1-1 Comparison of the structure of the *mnp*1 and *mnp*2 genes. The positions of the intron (▮), TATAA box (■), inverted CCAAT boxes (▲), HSEs (o), MREs (▯) SP-1 sites (•) and AP-2 site are indicated.

Accumulation of MnP activity in the extracellular medium of nitrogen-limited cultures depends on the presence of Mn^{II}, the substrate for the enzyme.[5,7,9,70] Western immunoblots confirm that MnP protein is expressed only in the presence of Mn, indicating that Mn is required for MnP synthesis.[9] In addition, *mnp* mRNA is detectable on Northern blots of RNA isolated from cultures grown in the presence but not in the absence of Mn, and only under conditions of nitrogen limitation.[7,61,78] Addition of Mn to 5-day old Mn deficient cultures results in detectable *mnp* mRNA within 40 min. and the amount of mRNA obtained is a function of the amount of Mn added, up to a final concentration of 180 μM. Although these studies were performed with *mnp*1 probes, we now have confirmed that *mnp*2 specific gene expression is likewise dependent on Mn.[61] In contrast to its effect on *mnp* gene transcription, Mn has no significant effect on mycelial dry weights, and on rates of nitrogen consumption.[5,9]

In addition, we previously demonstrated that the accumulation of *mnp* mRNA can be induced by heat shock in the absence of Mn.[29] In this study, we decided to further examine the effect of HS stress on *mnp* gene expression and MnP activity.

Possible interrelationships between heat shock and metal regulation of *mnp* gene expression are of interest. To our knowledge, the rat heme oxygenase gene contains the only other promoter in which both HSEs and MREs have been identified. This gene is known to be regulated by heat shock and heavy metals, as well as by heme, bromobenzene, and endotoxin.[65] It also has been shown that the *S. cerevisiae* metallothionein gene (CUP1) is regulated by heat shock via the HSTF and HSEs,[92] as well as by copper and silver via the Ace1 protein.[43]

Treatment with H_2O_2 or a variety of other chemicals can induce the heat shock response in both prokaryotes and eukaryotes.[15,17,68] Since lignin degradation is an oxidative process and *Phanerochaete chrysosporium* produces H_2O_2 as part of its extracellular lignin-degrading system, it is conceivable that oxidative stress, acting through the heat shock system, is a factor in the regulation of MnP expression.[29] For this reason, we decided to examine the effects of oxidative and chemical stresses on *mnp* gene expression and MnP activity.

1.4 Nitrogen Regulation of MnP and LiP

Addition of NH_4^+ or various nitrogenous compounds to ligninolytic cultures suppresses the degradation of lignin as well as production of the secondary metabolite veratryl alcohol.[22,54] However, there is some evidence that nitrogen regulation may be strain dependent[12] and that it may be affected by the available carbon source.[82,106] *lip*, *mnp*, and glyoxal oxidase (*glox*) mRNA have been detected from cultures of *P. chrysosporium* only after the nitrogen in the medium has been depleted and the organism has entered the idiophase.[7,51,105] Furthermore, *mnp* mRNA is detectable from nitrogen-deficient but not from nitrogen-sufficient medium.[78] These results demonstrate that LiP and MnP are regulated by nitrogen at the level of gene transcription.[31] There also is evidence to suggest that various LiP and MnP isozymes may be differentially regulated by nitrogen and carbon.[73,97] Additionally, several selections have been devised for mutants that are nitrogen deregulated for lignin degradation. One such selection utilizes polymeric dyes which can serve as substrates for the ligninolytic system.[26,32] The other selection is to mutagenize a lysine auxotroph and select for mutants that grow on a nitrogen-sufficient medium supplemented with an adduct of lysine and a lignin model compound.[104] In direct contrast to this previous work, a recent report suggests that LiP protein expression is not regulated by nutrient nitrogen.[46] Therefore, we decided to reexamine this question.

CHAPTER 2

HEAT SHOCK INDUCTION OF MANGANESE PEROXIDASE GENE TRANSCRIPTION IN *PHANEROCHAETE CHRYSOSPORIUM*

The white-rot basidiomycete *Phanerochaete chrysosporium* has been the focus of numerous studies on lignin degradation[12,35,54] and the degradation of aromatic pollutants.[11,38,108-110] Two families of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), along with an H_2O_2-generating system, are major components of this organism's extracellular lignin degradation system.[35,54]

MnP has been purified and extensively characterized.[27,55,111] In the presence of manganous ion (Mn^{II}), MnP catalyzes the H_2O_2-dependent oxidation of a variety of phenolic lignin model compounds[27,107] and the *in vitro* depolymerization of lignin.[112] The enzyme oxidizes Mn^{II} to Mn^{III} and the Mn^{III}, complexed with an organic acid such as oxalate or malonate secreted by *P. chrysosporium*, oxidizes the terminal phenolic substrate.[27,111,113] MnP occurs as a series of isozymes encoded by several genes and the sequences of three *mnp* cDNAs[71,72,78] and two *mnp* genomic clones (*mnp1 and mnp2*)[29,61] have been reported. Previously we reported that MnP is regulated by nutrient nitrogen at the level of gene transcription[78] and there is evidence that the MnP isozymes may be differentially regulated by carbon and nitrogen.[73] MnP activity is dependent on the presence of Mn^{II} in the culture medium[5,9] and *mnp* gene transcription is regulated by Mn.[7,9] In addition, we previously demonstrated that the accumulation of *mnp* mRNA can be induced by heat shock and that the promoter region of the *mnp1* gene contains putative heat shock elements (HSEs).[29,31] Herein, we use studies of MnP activity and Western and Northern blots to analyze the heat shock induction of *mnp* gene transcription.

2.1 Materials and Methods

2.1.1 Culture Conditions

P. chrysosporium strain OGC101[1] was maintained on slants as described previously.[34] The organism was grown at 37°C from a conidial inoculum in 20 ml stationary cultures in 250 ml Erlenmeyer flasks as described previously.[7,53] Cultures were incubated under air for 2 days, after which they were purged daily with 100% O_2. The medium was as previously described[7,53] with 2% glucose as the carbon source, 1.2 mM ammonium tartrate (limiting nitrogen) or 12 mM ammonium tartrate (sufficient nitrogen), and 20 mM sodium-2,2-dimethyl succinate (pH 4.5) as the buffer. As indicated, $MnSO_4$ was added to a final concentration of 180 μM.

2.1.2 Heat Shock

Cultures were heat shocked by transfer from a 37°C incubator to a water bath at the elevated temperature for various periods of time as indicated. The cultures then either were harvested immediately or transferred to a 37°C water bath for the indicated periods before harvesting.

2.1.3 RNA Preparation and Northern Blot Hybridization

Cells were filtered through Miracloth (Calbiochem), washed twice with cold distilled water, quick frozen in liquid nitrogen and stored at -80°C until use. The frozen cells were homogenized with TRI REAGENT (phenol, guanidine thiocyanate) (Molecular Research Center, Inc.) using acid-washed glass beads and a bead beater. The supernatants were extracted with chloroform and the RNA was precipitated with isopropanol, washed with 75% ethanol, and solubilized in diethyl pyrocarbonate-treated water. After spectrophotometric quantitation, equal amounts of total RNA (20-30 μg/lane) were electrophoresed on a denaturing (0.7 M formaldehyde, 1% agarose) gel. The RNA was transferred to Biotrace RP membranes and hybridized at 42°C with ^{32}P-labeled probes as described.[7] The *mnp1* cDNA[78] was used as a template for random-primed synthesis of ^{32}P-α-dCTP-labeled (DuPont-New England Nuclear) probes, using a Multiprime DNA Labeling Kit (Amersham).

2.1.4 MnP Enzyme Assays and Protein Analysis

A 50- to 100- µl aliquot of the extracellular medium was added to the reaction mixture (1 ml) consisting of 50 mM malonate (pH 4.5) and 0.5 mM $MnSO_4$. Reactions were initiated by the addition of H_2O_2 to a final concentration of 0.1 mM. Oxidation of Mn^{II} to Mn^{III} was measured by the increase in absorbance at 270 nm.[113] SDS-PAGE and Western blot analysis were carried out as previously described.[9]

2.2 Results

The induction of *mnp* gene transcription by heat shock and by Mn is shown in Fig. 2-1. Five-day-old nitrogen-limited, Mn-deficient cultures of *P. chrysosporium* were harvested at the indicated times following the addition of Mn or transfer to 45°C with or without concurrent addition of Mn. The accumulation of *mnp* mRNA was observed one hour after Mn addition or heat shock induction. The amount of *mnp* mRNA declined slightly after 3 hr at 45°C in Mn-deficient cultures and was no longer detectable after 18 hours at 45°C (data not shown). No *mnp* mRNA was detectable after heat shock of nitrogen-sufficient cultures (data not shown). Transfer of cultures from 37°C to 42°C or 49°C also resulted in *mnp* mRNA in the absence of Mn. However, maximum accumulation of *mnp* transcript was observed only after 3 h at 42°C, although the transcript was still detectable after 18 h. Significantly less *mnp* transcript was detected at all time points following a 49°C heat shock (data not shown). Therefore, all subsequent experiments utilized a 45°C heat shock. Nitrogen-limited cultures grown at 45°C from time zero accumulated *mnp* mRNA only in the presence of Mn (data not shown).

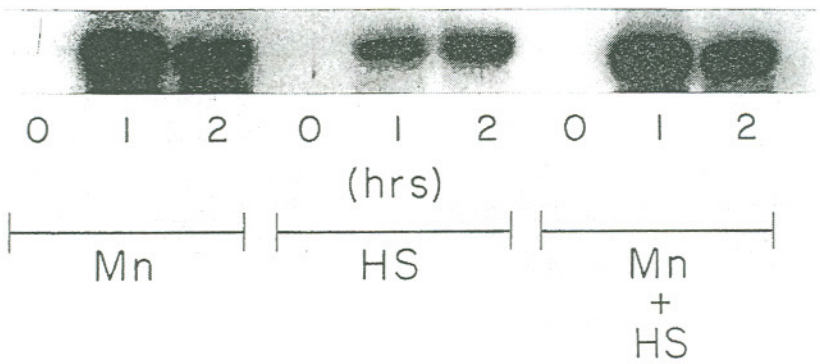


Fig. 2-1 Induction of *mnp* gene transcription by Mn and/or heat shock. Mn-deficient, nitrogen limited cultures were grown at 37°C. After 4 days Mn was added to a final concentration of 180 μM and/or cultures were transferred to a 45°C water bath. Cells were harvested immediately or after one or two hours. Total RNA was extracted, electrophoresed, transferred to a membrane and probed as described in the text.

The growth-stage dependence of heat shock induction of *mnp* mRNA in the presence or absence of Mn is shown in Fig. 2-2. Mn-deficient, nitrogen-limited cultures were grown for 3 through 7 days, after which they were subjected to a one hour heat shock at 45°C, with or without concurrent Mn induction. Heat shock induction of *mnp* mRNA was maximal on days 4 and 5 and declined on subsequent days (Fig. 2-2A). In contrast, *mnp* mRNA induction in cells treated concurrently with Mn and heat shock was maximal on day 5 and remained high on days 6 and 7 (Fig. 2-2B). On all days but day 3, more *mnp* mRNA was detected from cultures that were simultaneously induced with Mn. A time course for heat shock induction of *mnp* mRNA in five-day-old, nitrogen limited cultures is shown in Fig. 2-3. *mnp* mRNA was detected following a 15 minute 45°C heat shock, with maximum accumulation after 1 to 2 hours at 45°C. Again, accumulation of *mnp* mRNA appeared to be greater at all time points following concurrent Mn and heat shock induction.

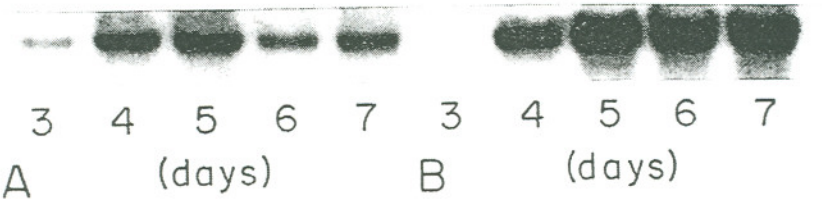


Fig. 2-2 Growth-stage-specific induction of *mnp* mRNA following heat shock in the presence or absence of Mn. Mn-deficient, nitrogen limited cultures were grown at 37°C for the indicated number of days, followed by a 45°C heat shock for one hour with (B) or without (A) simultaneous induction with Mn. Cells were harvested immediately and the total RNA was extracted and probed as described in the text.

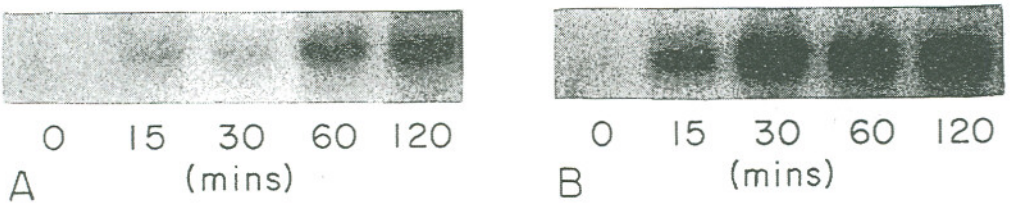


Fig. 2-3 Time course for the appearance of *mnp* mRNA following heat shock in the absence (A) or presence (B) of Mn. Mn-deficient nitrogen-limited cultures were grown at 37°C for 5 days, after which they were transferred to a 45°C water bath, with or without concurrent addition of Mn. Cells were harvested at the indicated times following transfer and total RNA was extracted and probed as described in the text.

A Northern blot of RNA extracted at various times during recovery from heat shock is shown in Fig. 2-4. Five-day-old Mn-deficient, nitrogen-limited cultures of *P. chrysosporium* were heat shocked at 45°C with or without simultaneous addition of Mn. After 1.5 hour at 45°C the cultures were returned to 37°C and harvested at the indicated times. As shown in Fig. 2-4A, the amount of *mnp* mRNA isolated from Mn-deficient heat shocked cultures decreased with time after return of the cultures to 37°C and was no longer detectable after two hours of reincubation at 37°C. In contrast, *mnp* mRNA isolated from cells simultaneously induced by Mn and heat shock remained abundant after 2 hours of reincubation at 37°C.

MnP activity and the production of MnP protein also were examined under heat shock in the presence and absence of Mn. Five-day-old nitrogen-limited, Mn-deficient cultures of *P. chrysosporium* were induced with Mn or were heat shocked at 45°C with and without simultaneous addition of Mn, for various periods of time. As shown in Fig. 2-5A, MnP activity was not detected in the extracellular medium of cells treated with heat shock alone. However, MnP activity in the extracellular medium of cells treated with both Mn and heat shock was higher at all time points than the activity in the medium of cultures treated with Mn alone. After 2 hours at 45°C MnP activity was 9-fold higher, and after 4 hours the activity was 4-fold higher, in cultures treated with both heat shock and Mn. Fig. 2-5B shows a Western blot of extracellular MnP protein from cells grown in nitrogen-limited Mn-deficient medium for 5 days at 37°C and then incubated for an additional 4 h at 37°C or 45°C in the presence or absence of Mn. At both temperatures, MnP protein was detected only in the extracellular medium of cultures incubated in the presence of Mn, although additional antibody-reactive bands were detectable in the medium from the heat-shocked cultures (Fig. 2-5B, lane 3). Similar results were obtained with Western blots of intracellular protein (data not shown).

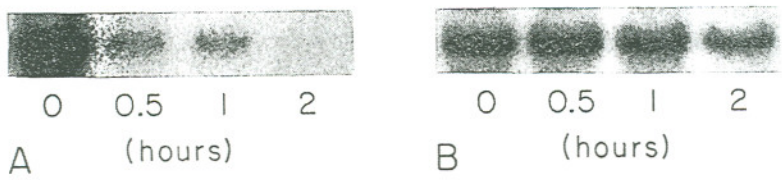


Fig. 2-4 *mnp* mRNA during recovery from heat shock in the presence and absence of Mn. 5-day-old, Mn-deficient, nitrogen-limited cultures were transferred to a 45°C water bath, with (B) or without (A) simultaneous addition of Mn. After 1.5 hour, the cultures were transferred to a 37°C water bath and harvested at the indicated times. Total RNA was extracted and probed as described in the text.

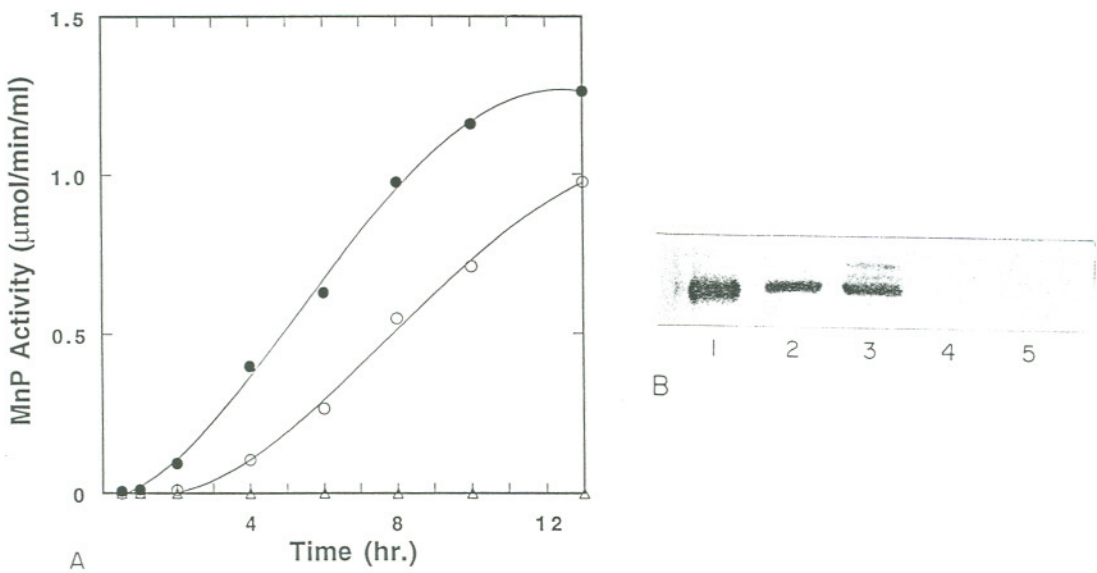


Fig. 2-5 MnP activity and immunoblot analysis of extracellular MnP following induction by Mn and/or heat shock. A. Mn-deficient, nitrogen-limited cultures were grown for 5 days at 37°C, followed by the addition of Mn [o], transfer to a 45°C water bath [Δ], or both [•]. MnP activity in the extracellular medium was assayed at the indicated times as described in the text. B. Cultures were as in (A) with the addition of an untreated control (lane 4). After 4 hours, samples of the extracellular medium were subjected to SDS-PAGE, electrophoretic transfer and immunodetection as described previously (4). Lane 1: purified MnP-1 isozyme; lane 2: 37°C with Mn; lane 3: 45°C with Mn; lane 4: 37°C without Mn; lane 5: 45°C without Mn.

2.3 Discussion

The lignin degradative system of *P. chrysosporium* is expressed during secondary metabolic (idiophasic) growth, the onset of which is triggered by limiting nutrient nitrogen.[12,54] Likewise, LiP and MnP activities are detectable in the extracellular medium only during the secondary metabolic phase of growth[31,54] and Northern blot analysis has demonstrated that LiP and MnP expression is controlled at the level of gene transcription by nutrient nitrogen.[78,105]

Previously we demonstrated that MnP expression also is regulated by Mn^{II}, the substrate for the enzyme, at the level of gene transcription,[7,9] and that the addition of Mn to nitrogen-limited Mn-deficient cultures on days 4, 5 or 6 results in detectable *mnp* mRNA within 40 minutes.[7] In the white rot basidiomycete *Dichomitus squalens*, MnP expression and lignin degradation also have been shown to be dependent on the presence of Mn.[75] Examination of the promoter region of the *P. chrysosporium mnp1* gene revealed the presence of 5 putative metal response elements (MREs)[7,29,31] identical to cis-acting sequences that are responsible for heavy-metal induction of mammalian metallothionein genes.[19] Interestingly, examination of the *mnp1* promoter region also revealed the presence of putative heat shock elements (HSEs) within 400 base pairs upstream of the *mnp1* translation initiation codon.[29,31] These sequences match the consensus HSE sequence, C--GAA--TTC--G,[59] in six or seven of the eight positions. Likewise, six putative HSEs are found within 1100 bp of the translation initiation codon of the *P. chrysosporium mnp2* gene.[31,61] Previously we reported heat shock induction of *mnp* mRNA, even in the absence of Mn, suggesting that the HSEs are physiologically functional.[29] In the present study, we examine the parameters affecting heat shock induction of *mnp* gene transcription.

As with Mn induction,[7] heat shock induction of *mnp* gene transcription occurs only under conditions of nitrogen limitation. Thus there appears to be a hierarchy of regulatory controls affecting MnP production. The addition of Mn to a final concentration of 180 μM at 37°C appears to result in more *mnp* mRNA than is induced by a 45°C heat shock (Fig. 2-1); however this may be accounted for, at least in part, by the transient effect of a 45°C heat shock on *mnp* mRNA accumulation as compared to the longer lasting effect of Mn induction (Fig. 2-4). Northern blot analysis shows no

obvious differences between the *mnp* mRNA induced by heat shock and that induced by Mn, although small differences in transcript size would not be detectable. Furthermore, the *mnp1* cDNA probe does not discriminate among the products of different *mnp* genes.

The growth stage specific induction of *mnp* gene transcription is similar for heat shock (Fig. 2-2A) and Mn induction,[7] with maximum induction occurring on days 4 and 5. In contrast, simultaneous induction with heat shock and Mn results in maximal levels of *mnp* mRNA on days 5 through 7 (Fig. 2-2B).

Although both heat shock and Mn induction result in maximal accumulation of *mnp* mRNA after one hour, heat shock induction results in detectable *mnp* mRNA within 15 min (Fig. 2-3), whereas Mn induced *mnp* mRNA is only detectable after 40 minutes.[7] This difference may be due to the relatively slow rate of Mn uptake as compared to the rapid increase in temperature achieved when small cultures are transferred to a 45°C water bath. However, this difference also may be a reflection of differing mechanisms of transcriptional activation. The heat shock response is known to be very rapid, with the activation and binding of heat shock factors (HSFs) to HSEs occurring within minutes of temperature elevation.[64] At present, little is known about the mechanism of Mn regulation of *mnp* gene transcription, although other eukaryotic metalloregulatory systems are known to involve metal binding transcription factors.[44,79]

Heat shock induced *mnp* mRNA decreases rapidly after cultures are returned to 37°C and the transcript is no longer detectable after 2 h at the lower temperature. This is typical of heat shock-responsive gene transcription.[64] In contrast, *mnp* mRNA remains at a high level in cells treated simultaneously with Mn and heat shock (Fig. 2-4B), presumably reflecting the continuing availability of Mn for activation of *mnp* gene transcription. However, it also is possible that Mn may affect *mnp* mRNA stability (see below).

Neither MnP activity nor MnP protein are detectable from cells that have been subjected to heat shock in the absence of Mn (Fig. 2-5). The presence of active MnP protein from cells heat shocked in the presence of Mn indicates that the elevated temperature is not a barrier to translation of *mnp* mRNA. It is possible that the heat shock induced *mnp* transcript differs in some way from the Mn-induced transcript and is not translatable, at least in the absence of Mn. The significantly higher MnP activity

found in the extracellular medium of cells simultaneously induced with Mn and heat shock as compared with cells induced with Mn alone (Fig. 2-5A) suggests that the heat shock induced transcript is translated in the presence of Mn. Furthermore, the largest difference in MnP activity is found after 2 hours at 45°C, possibly reflecting the more rapid induction by heat shock as compared to Mn (see above), and again suggesting that the heat shock induced transcript is translated in the presence of Mn. However, the additional higher molecular weight bands detectable by Western analysis of extracellular medium from cultures simultaneously induced with Mn and heat shock (Fig. 2-5B, lane 3) suggest that a 45°C heat shock may have other effects on MnP production, such as incomplete processing of carbohydrates or expression of alternative isozymes.

It is possible that Mn has a role in a post-transcriptional step of MnP production, in addition to its role as an inducer of *mnp* gene transcription. For example, Mn may have a role in *mnp* mRNA stability, although results from our laboratory indicate that the *mnp1* promoter region, and not the 3´ end of the *mnp1* gene, are required for Mn regulation.[28] The Mn requirement for MnP production under heat shock is under study in our laboratory.

We have demonstrated that MnP is regulated by heat shock as well as by Mn at the mRNA level. This appears to be a typical heat shock response in that it occurs rapidly, is relatively short lived (data not shown), and disappears upon return to normal temperatures. Presumably the *mnp* heat shock response is mediated via the HSEs found in the promoter regions of *mnp* genes, interspersed with inverted CCAAT sequences in an arrangement similar to that reported for the human and rodent heat shock protein (hsp) 70 promoters.[36] HSEs have not been found in the promoter regions of sequenced *lip* genes; nor is there evidence that LiP is regulated by heat shock.[31]

The physiological significance of heat shock regulation of *P. chrysosporium* MnP is unclear. The heat shock response was originally characterized as a universal response to increased temperature or other environmental stress, resulting in the expression of genes encoding the heat shock family of proteins (hsps). The *mnp1* gene[29] does not have a typical heat shock gene structure in that it contains introns and lacks the long 5´-untranslated leader sequence that is characteristic of genes encoding hsps.[59] However, other non-hsp heat shock responsive proteins have been identified, including a

peroxidase in *Neurospora crassa*.[50] This enzyme is induced by H_2O_2 and confers thermotolerance and tolerance to oxidative stress.[49] Treatment with H_2O_2 and other oxidative stresses can induce the heat shock response in both prokaryotes and eukaryotes.[68] Lignin degradation is an oxidative process and *P. chrysosporium* produces H_2O_2 as part of its extracellular lignin degrading system. It is conceivable that H_2O_2 or other active oxygen species produced during lignin degradation are involved in the regulation of MnP expression via the heat shock system.

In addition to *mnp*, there are other known heat shock responsive genes that also are regulated by metal ions. Recently it was shown that the *Saccharomyces cerevisiae* metallothionein gene (CUP1) is regulated by heat shock via the heat shock transcription factor and HSEs[92] as well as by copper and silver via the Ace1 protein.[43] Both HSEs and MREs have been identified in the promoter region of the rat heme oxygenase gene which is known to be regulated by heat shock and heavy metals, as well as by heme, bromobenzene and endotoxin.[65] It has been suggested that heme oxygenase is involved in protection against oxidative stress via production of bilirubin.[98]

We are studying the effects of oxidative stress on *mnp* gene expression. We also are examining the relationships among heat shock, Mn and nitrogen regulation of *mnp* gene expression.

CHAPTER 3

REGULATION OF MANGANESE PEROXIDASE GENE TRANSCRIPTION BY HYDROGEN PEROXIDE, CHEMICAL STRESS AND MOLECULAR OXYGEN

The white-rot basidiomycete *Phanerochaete chrysosporium* degrades lignin[12,35,54] and a variety of aromatic pollutants[11,38,47,110] during the secondary metabolic (idiophasic) stage of growth. Two isozyme families of secreted peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), and an H_2O_2-generating system, constitute the known major components of this organism's extracellular lignin degradative system.[35,54]

In the presence of manganous ion [Mn(II)], MnP catalyzes the H_2O_2- dependent oxidation of a variety of phenolic lignin model compounds[35,107,113] and the in vitro depolymerization of lignin.[112] The enzyme catalyzes the oxidation Mn(II) to Mn(III). Mn(III), complexed with an organic acid such as oxalate secreted by *P. chrysosporium*, oxidizes the terminal phenolic substrate.[27,35,107,113] MnP occurs as a series of isozymes encoded by a family of genes and the sequences of three *mnp* cDNAs[71,72,78] and two *mnp* genomic clones (*mnp1* and *mnp2*)[29,61] have been reported.

Previously we reported that MnP is regulated by nutrient nitrogen at the level of gene transcription[78] and in *P. chrysosporium* and other white rot fungi, MnP activity is dependent on the presence of Mn(II) in the culture medium.[5,9,75,83] We also demonstrated that addition of Mn to *P. chrysosporium* cultures grown in the absence of Mn induces *mnp* gene transcription[7,9] and that the expression of MnP in nitrogen limited cultures is regulated by heat shock at the level of gene transcription.[8] Treatment with H_2O_2 or a variety of other chemicals can induce the heat shock response in both prokaryotes and eukaryotes.[15,17,68] Since lignin degradation is an oxidative process and *P. chrysosporium* produces H_2O_2 as part of its extracellular lignin degrading system, we

decided to examine the effects of oxidative and chemical stresses on *mnp* gene expression and MnP activity.

3.1 Materials and Methods

3.1.1 Culture conditions

P. chrysosporium strain OGC101 was maintained on slants as described previously.[34] The organism was grown at 37°C from a conidial inoculum in 20-ml stationary cultures in 250-ml Erlenmeyer flasks, as described previously.[9] Cultures were incubated under air for two days, after which they were purged daily with 100% O_2 for 10 min, unless otherwise noted. The medium was as described,[9,53] with 2% glucose as the carbon source, 1.2 mM ammonium tartrate (limiting nitrogen) or 12 mM ammonium tartrate (sufficient nitrogen), and 20 mM sodium 2,2-dimethyl succinate (pH 4.5) as the buffer. As indicated, $MnSO_4$ was added to a final concentration of 180 μM or cultures were heat shocked by transfer to a 45°C water bath. The effect of H_2O_2 on cell viability was measured by inoculating mycelial plugs onto agar plates containing growth medium[34] supplemented with various concentrations of H_2O_2 and comparing radial growth.

3.1.2 Chemicals

H_2O_2, sodium arsenite and cycloheximide were obtained from Sigma and peracetic acid and 2,4-dichlorophenol from Aldrich Chemical Co. H_2O_2 concentration was measured by the horseradish peroxidase method.[16]

3.1.3 RNA preparation and Northern blot hybridization

Cells were filtered through Miracloth (Calbiochem), rinsed twice with cold distilled water, quick-frozen in liquid nitrogen, and stored at -80°C. The frozen cells were disrupted and the RNA isolated by homogenizing in the presence of TRI reagent (Molecular Research Center, Inc.) as described previously.[8] After spectrophotometric quantitation, the RNAs (20 μg/lane) were denatured in the presence of 2.2 M formaldehyde and 50% formamide for 15 min at 68°C and electrophoresed in a denaturing (0.6 M formaldehyde-1% agarose) gel. The RNA was transferred to Magna NT membranes (MSI Inc.) and hybridized at 42°C with ^{32}P-labeled probes as previously

described.[7] The *mnp*1 cDNA[78] was used as a template for random-primed synthesis of [α-^{32}P]dCTP-labeled (Dupont-New England Nuclear) probes, using a Multiprime DNA Labeling Kit (Amersham). RNA blots were washed and exposed to Kodak XAR-5 X-ray film.

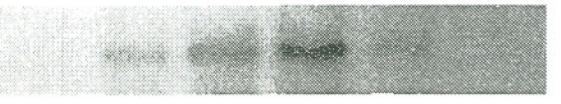
3.1.4 MnP enzyme assays and protein analysis.

50- to 100-µl aliquots of the extracellular medium were added to the reaction mixture (1 ml) consisting of 50 mM malonate (pH 4.5) and 0.5 mM $MnSO_4$. Reactions were initiated by the addition of H_2O_2 to a final concentration of 0.1 mM. Oxidation of Mn(II) to Mn(III) was measured by the increase in absorbance at 270 nm.[113] Intracellular protein preparations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis were carried out as previously described.[9]

3.2 Results

3.2.1 H_2O_2 induction of *mnp* mRNA

H_2O_2 was added to 5-day-old nitrogen-limited, Mn-deficient cultures of *P. chrysosporium* to final concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 mM. Cells were harvested after one hour. Accumulation of *mnp* mRNA was detected from cells exposed to H_2O_2 concentrations of from 0.25 mM to 1.5 mM with maximum accumulation at 1.0 mM. No *mnp* mRNA was detected in the absence of H_2O_2 or with H_2O_2 concentrations above 1.5 mM (Fig. 3-1). The concentration of endogenous H_2O_2 in the extracellular medium of 5-day-old, nitrogen-limited cultures was determined to be less than 4µM. Over a 24-hour period, radial growth of *P. chrysosporium* on plates containing 0.5 mM H_2O_2 was inhibited by less than 3% as compared to growth on plates lacking added H_2O_2. Plates containing 1.0 mM H_2O_2 exhibited less than 13% inhibition of growth over the same period, whereas 26% inhibition was observed at 2.0 mM H_2O_2 in the first 24 hours.



0 0.25 0.5 1.0 1.5 2.0

H_2O_2 (mM)

Fig. 3-1 H_2O_2 induction of *mnp* mRNA. H_2O_2 was added to 5-day-old nitrogen-limited, Mn-deficient cultures at the indicated final concentrations. Cells were harvested after 1 h and total RNA was extracted, electrophoresed, transferred to a membrane and probed as described in the text.

No *mnp* mRNA was detectable following H_2O_2 induction of nitrogen-sufficient cultures (data not shown). The growth-stage dependence of H_2O_2 induction of *mnp* mRNA in nitrogen-deficient cultures is shown in Fig. 3-2. Three- to seven-day-old Mn-deficient cultures were treated with 1.0 mM H_2O_2, with or without concurrent Mn induction, and cells were harvested after one hour. H_2O_2 induction of *mnp* mRNA in the absence of Mn was first detectable on day 4, maximal on days 5 and 6 and declined on day 7. *mnp* mRNA induction in cells treated concurrently with Mn and H_2O_2 was maximal on day 5 and declined on subsequent days. On days 4, 5, and 6, more *mnp* mRNA was detected from cultures that were simultaneously induced with Mn and H_2O_2 as compared with H_2O_2 induction alone.

A time course for H_2O_2 induction of *mnp* mRNA in 5-day-old, nitrogen-limited Mn-deficient cultures is shown in Fig. 3-3A. *mnp* mRNA was detected in cells harvested 15 min after induction with 1.0 mM H_2O_2 and continued to accumulate for at least two hours. Again, accumulation of *mnp* mRNA appeared to be greater at all time points following concurrent Mn and H_2O_2 induction (Fig. 3-3B).

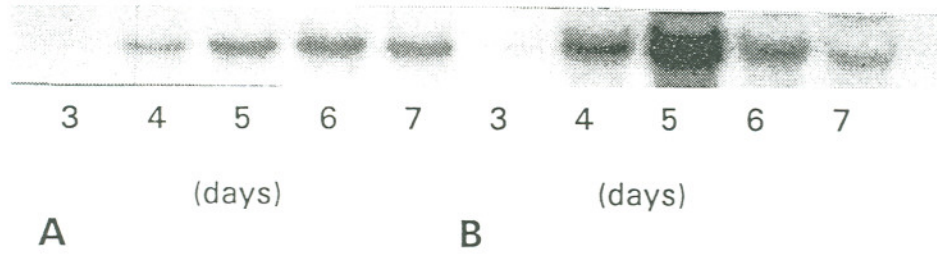


Fig. 3-2 Growth stage-specific induction of *mnp* mRNA by H_2O_2 in the presence or absence of Mn. Nitrogen-limited, Mn-deficient cultures were grown for the indicated number of days, after which 1.0 mM H_2O_2 was added with (B) or without (A) simultaneous induction with Mn. Cells were harvested after 1 h and total RNA was extracted and probed as described in the text.

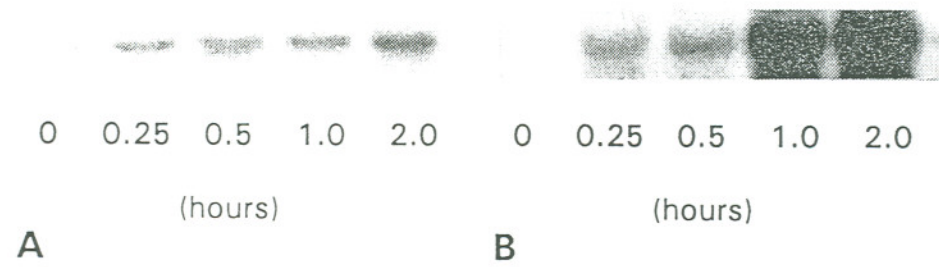


Fig. 3-3 Time course for the appearance of *mnp* mRNA following H_2O_2 induction in the presence and absence of Mn. 1.0 mM H_2O_2 was added to 5-day-old nitrogen-limited, Mn-deficient cultures with (B) or without (A) concurrent induction with Mn. Cells were harvested at the indicated times following the addition of H_2O_2 and total RNA was extracted and probed as described in the text.

MnP activity and the production of MnP protein were examined under H_2O_2 induction in the presence and absence of Mn. Triplicate 5-day-old, Mn-deficient nitrogen-limited cultures of *P. chrysosporium* were induced with 180 μM Mn, 0.5 mM H_2O_2 or both and MnP activity in the extracellular medium was assayed at various intervals over the following 48 h. MnP activity was not detected in the extracellular medium of cells treated with H_2O_2 alone. However, MnP activity in the extracellular medium of cells treated with both Mn and H_2O_2 was higher at all time points than MnP activity from cultures treated with Mn alone (Fig. 3-4A). At 35 h after induction, MnP activity was increased 1.6-fold by Mn plus 0.5 mM H_2O_2, as compared with Mn alone. Western blot analysis was performed on extracellular (Fig. 3-4B) and intracellular (data not shown) protein from cells grown in Mn-deficient, nitrogen-limited medium for 5 days, followed by an additional 4 h incubation with or without 1.0 mM H_2O_2 in the presence and absence of 180 μM Mn. MnP protein was detected only from cultures incubated in the presence of Mn.

3.2.2 Other chemical stresses

Five-day-old, Mn-deficient nitrogen-limited cultures of *P. chrysosporium* were incubated for 1 h prior to harvesting with the indicated final concentrations of each of the following chemicals: peracetic acid (1.0 mM), ethanol (10% v/v), sodium arsenite (1.0 mM), 2,4-dichlorophenol (0.5 mM) dissolved in N,N-dimethylformamide (1.65% v/v), and N,N-dimethylformamide alone (1.65% v/v). Addition of each of the above compounds resulted in the accumulation of detectable *mnp* mRNA (Fig. 3-5). Other organic peroxides such as t-butyl hydroperoxide and cumene hydroperoxide did not induce *mnp* mRNA at a concentration of 1.0 mM (data not shown). Cycloheximide added to a final concentration of 50 μg/ml did not prevent induction of *mnp* mRNA by either heat shock or H_2O_2. However, at that concentration, cycloheximide alone appeared to induce *mnp* gene transcription, resulting in several bands that probed with the *mnp1* cDNA but which were larger than the normally induced *mnp* transcript (data not shown).

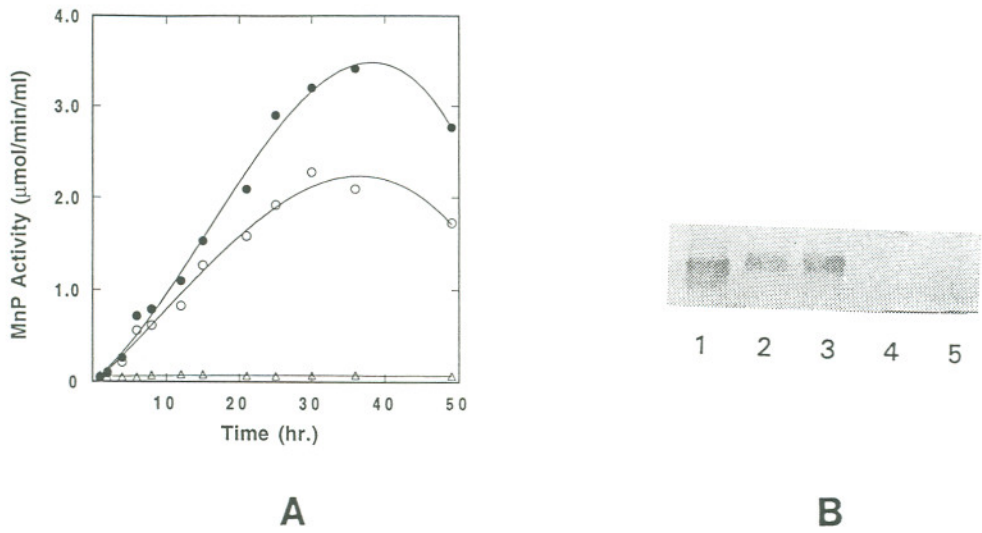


Fig. 3-4 MnP activity and immunoblot analysis of extracellular MnP following induction by Mn and/or H_2O_2. (A) Mn-deficient nitrogen-limited cultures were grown for 5 days followed by the addition of H_2O_2 alone (0.5 mM) (Δ), Mn alone (o), or H_2O_2 plus Mn (•). MnP activity in the extracellular medium was assayed at the indicated times as described in the text. (B) Cultures were prepared as described for panel A, with the addition of an untreated control (lane 5). After 4 h, samples of extracellular medium were subjected to sodium dodecyl sulfate-polyacryamide gel electrophoresis, electrophoretic transfer, and immunodetection as described previously (4). Lane 1, purified MnP isozyme 1; lane 2, with Mn; lane 3, with H_2O_2 (1.0 mM) plus Mn; lane 4, with H_2O_2 alone; lane 5, untreated control.

Fig. 3-5 Induction of *mnp* gene transcription by various chemical stresses. Five-day-old, Mn-deficient nitrogen-limited cultures were incubated with chemicals at the indicated final concentrations: lane 1, untreated control; lane 2, H_2O_2 (1.0 mM); lane 3, peracetic acid (1.0mM); lane 4, ethanol (10% v/v); lane 5, sodium arsenite (1.0 mM); lane 6, 2,4-dichlorophenol in N,N-dimethylformamide (0.5 mM); lane 7, N,N-dimethylformamide (1.65% v/v). Cells were harvested after 1h and total RNA was extracted and probed as described in the text.

3.2.3 Effect of O_2 purging

The effect of O_2 purging on MnP activity is shown in Fig. 3-6A. Cultures were grown in low nitrogen medium containing 180 μM $MnSO_4$. Daily purging with 100% O_2 for 10 min on days 3 through 6 resulted in an 8-fold increase in MnP activity on day 5 and at least a 4-fold increase at all time points, as compared with an air atmosphere. Daily purging with O_2 for 2 h resulted in a 13-fold increase on day 5 as compared to air alone.

The effect of O_2 purging on *mnp* gene transcription in the presence and absence of Mn is shown in Fig. 3-6B. Considerably more *mnp* mRNA was detected from 5-day-old Mn-supplemented cells that were O_2-purged for 2 h each on days 3 and 4, as compared with Mn-sufficient cultures incubated under air or O_2-purged for 10 min only on days 3 and 4 (compare lane 6 with lanes 4 and 5). Under the conditions used here, O_2-purging did not result in detectable *mnp* mRNA under Mn-deficient conditions (Fig 3-6B, lanes 1-3). However, prolonged exposure of the probed Northern blot resulted in a faint signal from Mn-deficient cultures that were O_2-purged for 2 h on days 3 and 4 (data not shown).

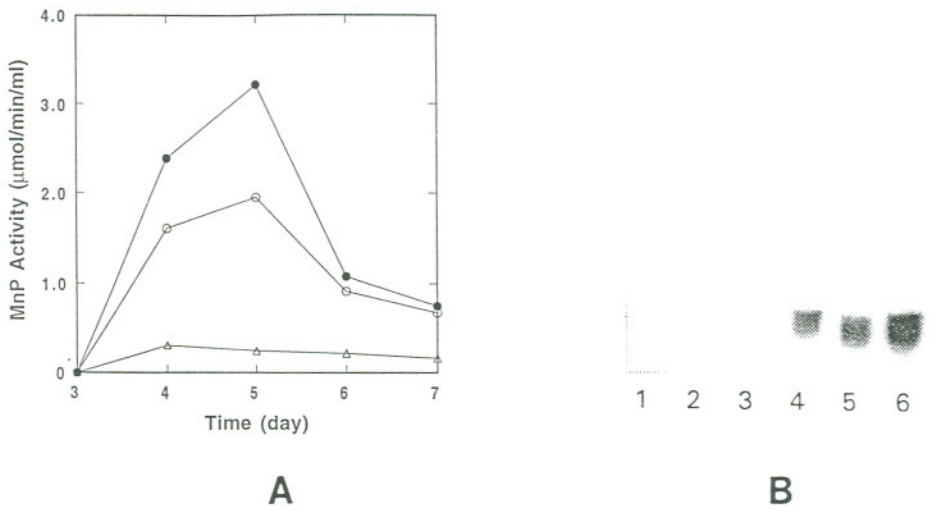


Fig. 3-6 Effect of O_2 purging on MnP activity and *mnp* gene transcription. (A) Nitrogen-limited cultures supplemented with 180 µM Mn were grown under air for two days after which they were purged daily for 10 min with 100% O_2 (o), purged daily for 2 h with 100% O_2 (•), or not purged (Δ). MnP activity in the extracellular medium was assayed on days 3 through 7 as described in the text. (B) Nitrogen-limited Mn-deficient cultures (lanes 1-3) and cultures supplemented with 180 µM Mn (lanes 4-6) were grown under air for two days, after which they were purged for 10 min with 100% O_2 on days 3 and 4 (lanes 2 and 5), purged for 2 hours with O_2 on days 3 and 4 (lanes 3 and 6), or not purged with O_2 (lanes 1 and 4). Cells were harvested on day 5. Total RNA was extracted and probed as described in the text.

3.3 Discussion

The lignin degradative system of *Phanerochaete chrysosporium* is expressed during secondary metabolic (idiophasic) growth, the onset of which is triggered by limiting nutrient nitrogen.[12,31,54] Likewise, LiP and MnP activities are detectable in the extracellular medium only during the secondary metabolic phase of growth[54] and Northern blot analysis has demonstrated that LiP and MnP expression is controlled at the level of gene transcription by nutrient nitrogen.[58,78,105] There also is evidence that various isozymes of LiP and MnP may be differentially regulated by carbon and nitrogen.[42,73,97]

We have been examining additional specific inducers of MnP expression and have shown that MnP is regulated by Mn(II), the substrate for the enzyme, at the level of gene transcription.[7,9] Both the *mnp1* and *mnp2* promoters contain multiple putative consensus metal response elements (MREs)[29,31,61] identical to cis-acting sequences that are responsible for heavy metal induction of mammalian metallothionein genes.[19] The *mnp1* and *mnp2* promoters also contain putative heat shock elements (HSEs)[29,31,61] and we recently reported on parameters affecting heat shock induction of *mnp* gene transcription and suggested that the HSEs are physiologically functional.[8] Neither MREs nor HSEs have been found in the promoter regions of sequenced *lip* genes.

In addition to thermal stress, a wide variety of chemical agents are known to induce heat shock proteins.[59,68,99] These factors include heavy metals, various organic compounds and oxidants.[68,85] Since lignin degradation is an oxidative process during which the fungus generates H_2O_2 as the cosubstrate for LiP and MnP, we examined the induction of *mnp* gene transcription by H_2O_2 and other chemical agents, as well as the effect of incubation under 100% O_2.

Northern blot analysis indicates that maximum accumulation of *mnp* mRNA occurs with H_2O_2 added to a final concentration of 1.0 mM (Fig. 3-1). No *mnp* mRNA is detectable when the cells are induced with 2.0 mM H_2O_2. Our results indicate that 1.0 mM exogenous H_2O_2 has only minimal effects on the growth of *P. chrysosporium* cultures. As with Mn induction[7,9,68,85] and heat shock induction[8] of *mnp* gene transcription, H_2O_2 induction of *mnp* gene transcription occurs only under conditions of

nitrogen limitation. Thus *P. chrysosporium* appears to employ a hierarchy of regulatory strategies for controlling MnP production.

The growth stage-specific H_2O_2 induction (Fig. 3-2) and the time course for H_2O_2 induction of *mnp* gene transcription (Fig. 3-3) are similar to parameters observed for heat shock induction of *mnp* mRNA,[8] suggesting that heat shock and H_2O_2 may be acting via the same or related mechanisms. In contrast, the additive effects of H_2O_2 and Mn induction (Figs. 3-2 and 3-3) or heat shock and Mn induction[8] suggest that Mn induces *mnp* gene transcription via a different mechanism.

No MnP activity or protein is detectable after H_2O_2 induction in the absence of Mn (Fig. 3-4). However, in the presence of Mn, more MnP activity is obtained after simultaneous induction with Mn and H_2O_2 as compared with Mn alone (Fig. 3-4A). A similar effect is seen with heat shock induction of MnP.[8]

Many other compounds are known to induce a heat shock-like response in a variety of systems.[68] In this study, peracetic acid, ethanol, sodium arsenite, 2,4-dichlorophenol and N,N-dimethylformamide were all found to induce *mnp* gene transcription (Fig. 3-5). Ethanol and arsenite are known to induce the synthesis of heat shock proteins in bacteria and fungi.[50,68] As with H_2O_2 and heat shock, no MnP protein was obtained with these inducers in the absence of Mn.

It is not yet clear why induction with heat shock or chemical stress does not result in MnP protein. The higher activity levels obtained with simultaneous induction by Mn and either heat shock[8] or H_2O_2 (Fig. 3-4A) suggest that the transcript induced by these agents is translatable in the presence of Mn and that the presence of H_2O_2 is not interfering with translation of the *mnp* mRNA. The *mnp* genes are not typical of genes transcribed during heat shock in that they contain 6 or 7 small introns and lack a long 5′ untranslated leader sequence.[29,61] Although Northern blot analysis shows no obvious differences between the *mnp* mRNA induced by either heat shock or H_2O_2 and that induced by Mn, small differences in transcript size, resulting from a variable transcription start site or incomplete processing, would not necessarily be detectable.

It is possible that Mn has a role in mRNA stability. However, our recent results indicate that only the region 5′ to the *mnp1* translation initiation codon is required for conferring Mn inducibility on a heterologous reporter gene.[28] It also is possible that Mn has an additional role in post-transcriptional processing of MnP. For example, Mn may be required for correct processing or translation of *mnp* message synthesized under heat shock or chemical or oxidative stress.

H_2O_2 recently was shown to activate the heat shock transcription factor in fibroblasts and this reaction was inhibited by an iron chelator, suggesting that the H_2O_2 effect may be mediated via the generation of hydroxyl radical by the Fenton reaction.[10] Alternatively, H_2O_2 may act indirectly, perhaps by causing membrane damage or the denaturation of preexisting proteins which, in turn, may result in a heat shock response.[3,17,30]

An intracellular peroxidase in *Neurospora crassa* also is known to be induced by heat shock and H_2O_2. Treatment with H_2O_2 leads to thermotolerance in this organism and it has been suggested that the induced peroxidase protects the cell from active oxygen species, including H_2O_2, that accumulate during heat shock and other stress.[50] It is not clear why *mnp* gene transcription is induced by H_2O_2. However, *P. chrysosporium* produces H_2O_2 as part of its extracellular lignin degrading system and induction of *mnp* by H_2O_2 or other oxidants produced during lignin degradation may be a factor in regulating the peroxide levels in this system. We are investigating the *mnp* promoter elements that are responsible for H_2O_2 and/or heat shock induction of *mnp* gene transcription.

Lignin degradation is an oxidative process and a high O_2 atmosphere enhances ligninolytic activity by *P. chrysosporium*.[52,53] We therefore examined the effect of O_2 on MnP activity and *mnp* gene transcription. As seen in Fig. 3-6A, purging with 100% O_2 for 10 min on days 3 and 4 results in a large increase in MnP activity as compared to growth under air. Increasing the duration of O_2 purging to 2 h results in a further increase in MnP activity. In the absence of Mn, O_2 purging results in only a negligible amount of *mnp* mRNA. However, in the presence of Mn, 2 h of O_2 purging on days 3 and 4 results in substantially more *mnp* mRNA on day 5, as compared with *mnp* mRNA from cells grown under air or purged for only 10 min (Fig. 3-6B). This suggests that

although the effect of O_2 is, at least in part, at the level of *mnp* gene transcription, it appears to be distinct from the mechanism of H_2O_2 induction. It is possible that O_2 is positively affecting Mn induction of *mnp* gene transcription. This is the first observation of an O_2 effect on *mnp* gene transcription and it may explain, at least in part, the positive effect of O_2 on lignin degradation.[52,53] We are continuing to investigate the effects of O_2 and other oxidants on MnP activity and gene transcription.

CHAPTER 4

NITROGEN REGULATION OF LIGNIN PEROXIDASE GENE TRANSCRIPTION

The white rot basidiomycete *Phanerochaete chrysosporium* degrades lignin[12,35,54] and a variety of aromatic pollutants[11,38,47,110] during the secondary metabolic (idiophasic) stage of growth, the onset of which is triggered by depletion of nutrient nitrogen. Two isozyme families of secreted heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), and an H_2O_2-generating system constitute the known major components of this organism's extracellular lignin degradative system.[35,54] Genes encoding various isozymes of LiP and MnP have been cloned and characterized.[31] *lip*, *mnp* and glyoxal oxidase (*glox*) mRNAs have been detected from cultures of *P. chrysosporium* only after the nitrogen in the medium has been depleted and the organism has entered idiophase.[7,51,105] Furthermore, *mnp* mRNA is detectable from nitrogen-deficient but not from nitrogen-sufficient medium.[78] These results have been taken as evidence that LiP and MnP are regulated by nitrogen at the level of gene transcription.[31] There also is evidence that various LiP and MnP isozymes may be differentially regulated by nitrogen and carbon.[73,97] Finally, there are several reports of the isolation of *P. chrysosporium* mutants that are nitrogen deregulated for LiP, MnP and GLOX production and lignin degradation.[6,56,70] In direct contrast to this previous work, a recent report suggests that nutrient nitrogen regulates LiP expression at the level of heme insertion into the apoprotein rather than at the level of gene transcription.[46] Therefore, we decided to re-examine this question using Northern and Western blot analysis.

4.1 Materials and Methods

4.1.1 Culture conditions

P. chrysosporium was maintained on slants as described previously.[34] The organism was grown at 37°C from a conidial inoculum in 20-ml stationary cultures in 250-ml Erlenmeyer flasks, as described previously.[9] Cultures were incubated under air for three days and then purged with 100% O_2. The medium was as described,[9,53] with 2% glucose as the carbon source, 1.2 mM ammonium tartrate (limiting nitrogen) or 12 mM ammonium tartrate (sufficient nitrogen), and 20 mM sodium 2,2-dimethyl succinate (pH 4.5) as the buffer. The media contained 0, 30, or 180 μM $MnSO_4$.

4.1.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were carried out as previously described.[9] The blot was probed with a polyclonal antibody to LiP isozyme H8 from *P. chrysosporium* strain OGC101[81] as previously described.[9]

4.1.3 RNA preparation and Northern blot hybridization

Cells were filtered through Miracloth (Calbiochem), rinsed twice with cold distilled water and quick-frozen in liquid nitrogen. The frozen cells were disrupted and the RNA isolated by homogenizing in the presence of TRI reagent (Molecular Research Center, Inc.) as described previously.[8] After spectrophotometric quantitation, the RNAs (20 μg/lane) were denatured in the presence of 2.2 M formaldehyde and 50% formamide for 15 min at 68°C and electrophoresed in a denaturing (0.6 M formaldehyde-1% agarose) gel. The RNA was transferred to Magna NT (MSI) membranes and hybridized at 42°C with ^{32}P-labeled probe as previously described.[7] The L18 cDNA[81] was used as a template for random-primed synthesis of [α-^{32}P]dCTP-labeled (Dupont-New England Nuclear) probes, using a Multiprime DNA Labeling Kit (Amersham). RNA blots were washed and exposed to Kodak XAR-5 X-ray film.

4.2 Results

A Western blot of the extracellular media from 5- and 6-day-old nitrogen-deficient and nitrogen-sufficient cultures is shown in Fig. 4-1. Whereas LiP protein was detected

in the extracellular medium of both 5- and 6-day-old nitrogen-deficient cultures at all Mn concentrations, no LiP protein was detectable in the extracellular medium of nitrogen-sufficient cultures.

To determine if *lip* genes were transcribed under conditions of nitrogen sufficiency, Northern blots of total RNA from 5- and 6-day-old cultures were probed with the cDNA corresponding to LiP isozyme H8 (L18).[81] Fig. 4-2 demonstrates that whereas *lip* mRNA was detected in nitrogen-deficient cultures at all Mn concentrations, no *lip* transcript was detectable in any nitrogen-sufficient culture.

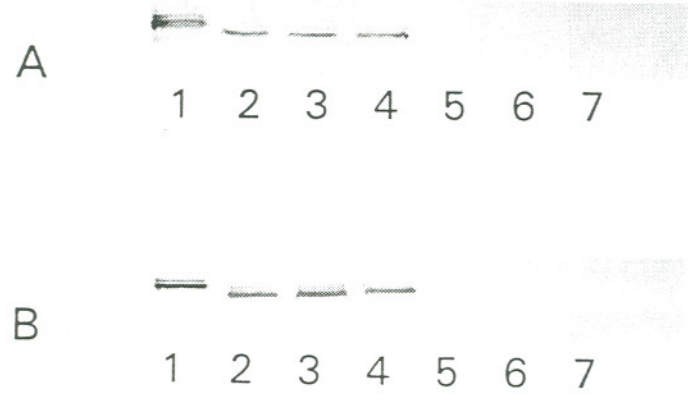


Fig. 4-1 Immunoblot analysis of extracellular LiP from nitrogen-limited and nitrogen-sufficient cultures. Samples of extracellular medium from 5-day-old (A) and 6-day-old (B) cultures were subjected to SDS-PAGE, electrophoretic transfer and immunodetection as described in the text. Lane 1, purified LiP isozyme H8; lanes 2-4, cultures contained 1.2 mM NH_4 tartrate and 0, 30 and 180 μM $MnSO_4$, respectively; lanes 5-7, cultures contained 12 mM NH_4 tartrate and 0, 30 and 180 μM $MnSO_4$, respectively.

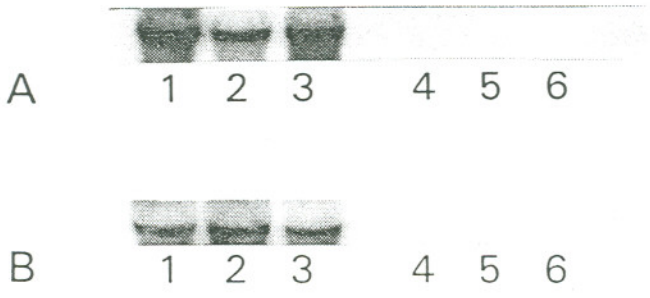


Fig. 4-2 Detection of *lip* mRNA from nitrogen-limited and nitrogen-sufficient cultures. 5-day-old (A) and 6-day-old (B) cultures were harvested and total RNA extracted, electrophoresed, transfered to a membrane and probed as described in the text. Lanes 1-3, cultures contained 1.2 mM NH_4 tartrate and 0, 30 and 180 μM $MnSO_4$, respectively; lanes 4-6, cultures contained 12 mM NH_4 tartrate and 0, 30 and 180 μM $MnSO_4$, respectively.

4.3 Discussion

The results presented here indicate that *P. chrysosporium lip* is regulated by nitrogen at the mRNA level. These results are in agreement with previous work indicating that gene transcription of *lip* isozyme H8 from strain BKM-F-1767 is regulated by nitrogen at the level of gene transcription.[97,105] These results also are in agreement with a large body of evidence indicating that lignin degradation by *P. chrysosporium*,[12,31,35,52,53] although not necessarily by other white rot fungi,[12,48,54,75] is regulated by nitrogen limitation. *mnp* mRNA from *P. chrysosporium* also is detected only under conditions of nitrogen depletion[7,78] and several other components of this organism's ligninolytic system appear to be coordinately regulated with LiP and MnP production during secondary metabolism. These include GLOX production[51] and synthesis of the secondary metabolite veratryl (3,4-dimethoxybenzyl) alcohol.[91] It has been suggested that activator protein-2 (AP-2) sequences present in the promoter regions of many *lip* genes, as well as the *mnp1* gene, may be involved in the regulation of gene transcription by nitrogen deficiency.[20]

The results presented here are in contrast to a recent report which concluded that *lip* isozyme H8 mRNA is produced in nitrogen-sufficient as well as nitrogen-deficient cultures of *P. chrysosporium*.[46] In that study, a small amount of *lip* mRNA was detected from nitrogen-sufficient cultures and a large amount from nitrogen-deficient cultures by reverse transcription and polymerase chain reaction. However, dot blot analysis indicated substantially more *lip* mRNA from nitrogen-sufficient cultures as compared with nitrogen-deficient cultures. Contamination of the mRNA used on the dot blots with genomic DNA is a possible explanation for this discrepancy. The authors of that study also claim that, although no LiP activity was detectable in the extracellular medium of nitrogen-sufficient cultures, FPLC elution profiles indicated the presence of LiP apoprotein. However, no attempt was made, using antibodies for example, to verify that the FPLC peaks corresponded to LiP. Furthermore, it is highly unlikely that LiP apoprotein would elute on FPLC at the same position as the holoenzyme, as claimed in that study. The results presented in Fig. 4-1 indicate that no LiP protein is present in the extracellular medium of nitrogen-sufficient cultures of *P. chrysosporium*, strongly contradicting the assertions in reference 46.

The results shown in Figs. 4-1 and 4-2 further demonstrate that appearance of *lip* mRNA and protein is independent of the concentration of Mn in the medium. We showed previously that *mnp* gene transcription and protein synthesis are dependent on the presence of Mn in the culture medium, with maximum *mnp* gene expression occurring at 180 μM Mn.[7,9] Our earlier results also indicated that Mn had little effect on LiP activity and no effect on veratryl alcohol production.[9] Our results here confirm that in 2,2-dimethylsuccinate buffer, Mn concentrations ranging from 0 to 180 μM have no apparent effect on either *lip* gene transcription or protein synthesis. We are continuing our studies on the regulation of *lip* and *mnp* gene transcription by nitrogen and other factors.

CHAPTER 5

CONCLUDING REMARKS

5.1 Summary of Research

The goal of this work has been to contribute to our understanding of the regulation of manganese and lignin peroxidase gene expression in the lignin-degrading basidiomycete *Phanerochaete chrysosporium.*

We have demonstrated that *mnp* gene transcription is regulated both by heat shock and by chemical oxidants, and other stresses, in addition to Mn ion and nitrogen.

We have also demonstrated that LiP expression is regulated at the level of gene transcription by nutrient nitrogen.

5.2 Future Directions

(1) Since no MnP protein is produced under HS in the absence of Mn, the HS-induced *mnp* transcript and a possible post-transcriptional Mn requirement should be examined. The following experiments should be carried out to help elucidate this process.

a. Since it is possible that the *mnp* transcript is not correctly processed at 45°C, in vitro translation (to determine whether the HS-induced mRNA is translatable), S1 nuclease mapping (to compare the transcriptional start sites of Mn and HS induced *mnp* mRNAs), intron probes (to determine whether the *mnp* transcripts are processed identically) and PCR (to clone the 3' ends of the *mnp*1 and *mnp*2 cDNAs for comparison with our sequenced cDNAs of *mnp*1 and *mnp*2) could be used to compare the *mnp* mRNAs induced by independent mechanisms.

b. Results with our homologous expression system in which transcription of the *mnp*1 gene is regulated by the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter[33,62] indicate that MnP protein is synthesized and secreted normally during primary metabolism in the absence of Mn. Furthermore, our *mnp*1 promoter-orotidylate decarboxylase (ODase) reporter construct, in which transcription of the ODase gene is regulated by the *mnp*1 promoter, has a pattern of ODase expression similar to that of endogenous MnP and both ODase and MnP proteins are synthesized and secreted normally during secondary metabolism only in the presence of Mn. This suggests that a Mn requirement for translation could reside in the 5' untranslated region of the *mnp* gene, or could be a general requirement for processing or translation of HS/chemical stress-induced transcripts. Since HS expression of ODase is difficult to determine, the Mn requirement might be examined using a fusion of the *mnp*1 promoter-lip2 coding region to determine whether active LiP is produced with HS induction in the absence of Mn. Constructs in which the 5' untranslated region of the *mnp*1 gene is replaced with the 5' untranslated region of the *lip*2 gene also will be used to analyze Mn and HS regulation.

c. The effect of Mn on HSPs could be examined to determine whether Mn has a general effect on the expression of HSPs.

(2) The cis-acting sequences involved in HS/H_2O_2 regulation could be identified.

a. The *mnp*1 promoter-truncated *mnp*1 coding region (reporter) gene construct and site-directed mutagenesis of putative HSEs will be used to determine if these sequences are involved in HS/H_2O_2 regulation of *mnp* gene transcription.

b. Deletion analysis of the *mnp*1 promoter could be used to identify the sequences necessary for full regulation of gene transcription by HS/H_2O_2 and these sequences could be incorporated into a heterologous promoter to verify function.

(3) The differential regulation of individual *mnp* genes by specific and global factors could be examined.

Both our *mnp*1 promoter-truncated *mnp*1 coding region (as reporter) construct and our *mnp*2 promoter-truncated *mnp*2 coding region (as reporter) construct, which give one wt *mnp* band and one specific shortened *mnp*1 or *mnp*2 band respectively on Northern blot under Mn or HS/H_2O_2 induction, could be used to study the differential regulation of individual *mnp* genes by Mn and HS/H_2O_2 under conditions of nitrogen and carbon limitation by quantitative Northern blot analysis.

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BIOGRAPHICAL NOTE

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The author's publication list includes the following:

Brown, J. A., D. Li, M. Alic, and M. H. Gold. 1993. Heat Shock Induction of Manganese Peroxidase Gene Transcription in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 59:4295-4299. (Chapter 2)

Li, D., M. Alic., J. A. Brown., and M. H. Gold. 1995. Regulation of Manganese Peroxidase Gene Transcription by Hydrogen Peroxide, Chemical Stress, and Molecular Oxygen. Appl. Environ. Microbiol. 61:341-345. (Chapter 3)

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