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Regulation of Peroxidase Transcript Levels in Liquid Cultures of the Ligninolytic Fungus *Pleurotus eryngii*

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A versatile peroxidase able to oxidize Mn^{2+} as well as phenolic and nonphenolic aromatic compounds is produced in peptone-containing liquid cultures of *Pleurotus eryngii* encoded by the gene *mnpl*. The regulation of its transcript levels was investigated by Northern blotting of total RNA. High-peroxidase transcripts and activity were found in cultures grown in glucose-peptone medium, whereas only basal levels were detected in glucose-ammonium medium. The addition of more than 25 μM Mn^{2+} to the former medium did not result in detectable peroxidase transcripts or activity. Potential regulators were also added to isolated mycelium. In this way, it was shown that high transcript levels (in peroxidase-expressing mycelium) were maintained on peptone, whereas expression was not induced in short-term incubation experiments. Similar results were obtained with Mn^{2+} ions. Strong induction of *mnpl* expression was caused by exogenous H_2O_2 or by continuous H_2O_2 generation during redox cycling of menadione. By the use of the latter system in the presence of Fe^{3+}, which catalyzes the reduction of H_2O_2 to hydroxyl radical, it was shown for the first time that the presence of this strong oxidant causes a rapid increase of the transcripts of a ligninolytic peroxidase. In conclusion, peptone and Mn^{2+} affect the levels of transcripts of this versatile peroxidase in culture, and reduced oxygen species induce short-term expression in isolated mycelium, probably via a stress response mechanism.

The ligninolytic basidiomycete *Pleurotus eryngii* degrades wheat lignin preferentially (26) under conditions used to treat straw in bio-semichemical pulping laboratory experiments (6). This fungus secretes laccase, aryl-alcohol oxidase (AAO), and peroxidase enzymes in liquid culture (16, 28, 30) and during lignocellulose solid-state fermentation (8), although different peroxidase isoenzymes have been identified under the two different growth conditions (9, 27, 28). Biochemical and molecular characterization revealed that they are versatile enzymes possessing catalytic properties of lignin peroxidase (LiP) and manganese peroxidase (or manganese-dependent peroxidase [MnP]) from *Phanerochaete chrysosporium* and other white-rot fungi. These properties include the ability to oxidize Mn^{2+}, substituted hydroquinones and phenols, veratryl alcohol, dimethoxybenzene, α-keto-γ-thiomethylbutyric acid, and phenolic or nonphenolic lignin model dimers (10, 19). Moreover, it has been found that these *Pleurotus* peroxidases have higher sequence and structural affinity with LiP than with MnP from *P. chrysosporium* but that their molecular structure includes an Mn^{2+} interaction site accounting for the ability to oxidize very low Mn^{2+} concentrations (9, 34).

All attempts to detect peroxidase activity in *Pleurotus* cultures grown under conditions similar to those used to produce *P. chrysosporium* LiP and MnP failed. However, the above-mentioned versatile peroxidases were purified from liquid cultures of different *Pleurotus* species when peptone was used as the N source (without Mn^{2+} addition) (7, 28, 35). These results suggest that not only are the *Pleurotus* peroxidases different from *P. chrysosporium* LiP and MnP in terms of catalytic properties and molecular structure but also that their expression is regulated in a different way. In the present study, regulation by N source, Mn^{2+}, and oxidative stress of the transcript levels of the unique ligninolytic peroxidase produced in peptone-containing liquid cultures of *P. eryngii* (34) was investigated by Northern blotting.

MATERIALS AND METHODS

Culture conditions. *P. eryngii* CBS 613.91 (IJFM A169) was grown in two N-sufficient media containing (wt/vol) 2% glucose, 0.2% yeast extract (Difco), and 0.5% peptone (Bacto Peptone [Difco]) (glucose-peptone medium), or ammonium tartrate (glucose-ammonium medium) (28). N-limited glucose-ammonium medium (containing 0.05% ammonium tartrate) was used in preliminary experiments. The effect of adding different Mn^{2+} concentrations to the above media was also determined. Finally, peptone was fractionated by molecular exclusion chromatography in Sephadex G15, and the resulting fractions were dried, weighed, and added to glucose-ammonium medium at concentrations corresponding to 5 g of peptone/liter. The results were compared with those obtained after the addition of 5 g of peptone or Casamino Acids/liter (Difco). In all cases the pH was adjusted to 5.5 after the addition of salts (0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7\ H_2O$), and cultures were incubated at 28°C and 180 rpm.

Gene regulation experiments. Studies on peroxidase transcript levels were carried out by including different compounds in the culture media or by adding them to 6-day-old mycelium from glucose-peptone or glucose-ammonium cultures which was separated by filtration, suspended in 20 mM sodium tartrate (pH 5), and incubated at 28°C and 180 rpm for up to 120 min after the addition of the potential transcription regulators. These included 5 g of peptone/liter, 100 μM Mn^{2+} (as $MnSO_4$), and 500 μM H_2O_2 (final concentration). Moreover, hydroxyl radical ($OH \cdot$) was generated in situ through redox cycling of 500 μM 2-methyl-1,4-naphthoquinone (menadione) in the presence of *P. eryngii* mycelium from 6-day-old cultures in glucose-ammonium medium, and 100 μM Fe^{3+} (17, 18). Peroxidase activity and *mnpl* mRNA were quantified as described below.

Enzymatic activities. Peroxidase activity was estimated by the formation of Mn^{3+}-tartrate complex (ε_{238}, 6,500 $M^{-1}\ cm^{-1}$) during the oxidation of 100 μM $MnSO_4$ in 0.1 M sodium tartrate (pH 5) containing 100 μM H_2O_2. One unit of enzymatic activity was defined as the amount of enzyme transforming 1 μmol of substrate per min.

Analysis of H_2O_2. H_2O_2 concentration was determined by using peroxidase and phenol red (31). The reaction mixture contained 0.01% phenol red, 2.5 U of horseradish peroxidase (Sigma, type II)/ml, and 0.1 M sodium phosphate buffer (pH 6). After 10 min, NaOH (0.2 M final concentration) was added, and the absorbance was read at 610 nm. Samples preincubated with 30 U of catalase (Sigma)/ml were used as blanks. A standard curve of H_2O_2 was prepared with dilutions of Perhydrol 30% (Merck) processed in the same way. The H_2O_2 concentration in the commercial solution was calculated from its absorbance at 230 nm (ε_{230}, 81 $M^{-1}\ cm^{-1}$).

RNA isolation and Northern analysis. After gene regulation experiments, mycelium was recovered by filtration, washed with distilled water, frozen, and

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stored at −80°C. It was disrupted in liquid N_2, and total RNA was isolated by using the Ultraspec RNA isolation system (Biotecx). RNA samples were solubilized in water and denatured in the presence of 40% formamide, 4% formaldehyde, 40 mM morpholinepropanesulfonic acid (MOPS) (pH 7), 10 mM sodium acetate, and 1 mM EDTA for 10 min at 65°C. Ten micrograms of each sample was electrophoresed overnight in 1.2% agarose-6% formaldehyde gels by using 40 mM MOPS (pH 7), 10 mM sodium acetate, and 1 mM EDTA. Gels were washed with water and transferred to nitrocellulose in 20× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate [pH 7]). RNA was cross-linked by using Stratalinker-UV. Then filters were hybridized in 5× SSC, 2.5× Denhardt's, 10% dextran sulfate, 20 mM sodium phosphate (pH 7.5), 50 μg of carrier single-strand DNA ml^{-1} and 50% formamide, at 42°C with probes labeled by using the *redi*prime DNA random labeling system (Amersham). Two probes were used in Northern blot analysis, as follows: the first corresponding to the 648-bp cDNA fragment from mRNA encoded by *P. eryngii* allele *mnpl2* (GenBank accession no. AF007224), which corresponds to the portion encoding Thr^{9}-Pro^{221} (34), and the second corresponding to a 12-kb *Eco*RI fragment of the 28S rRNA gene from *Drosophila melanogaster* included in pDm238 (33). The filters were sequentially hybridized with the *mnpl* probe and, after exhaustive washing removing labeling, with the *rRNA* probe. After each hybridization, the filters were washed (the final step consisted of 0.2× SSC, 0.1% SDS at 58°C), and both the europium screen of a PhosphorImager (Molecular Dynamics) and Kodak X-OMAT-AR-ray film were exposed (the latter for different periods of time). The films were scanned, and digital images were imported by the PhosphorImager software (program IQ) for processing and quantitation, together with the images obtained with this equipment. The *mnpl* mRNA values obtained were referred to the intensity of the signal of 28S rRNA in the same sample, which was used as an internal standard (to normalize differences due to sample loading, etc.). Moreover, an RNA sample corresponding to the highest production of *mnpl* transcripts (i.e., day 5 in peptone medium) was included in all the electrophoresis and (after normalization of the *mnpl* mRNA signal to rRNA) used as an external reference for the transcript levels, which were presented as percentages of the maximal transcript level obtained.

RESULTS

Effect of peptone on peroxidase production and transcript levels. No peroxidase activity was detected in *P. eryngii* cultures grown in either N-limited or N-sufficient glucose-ammonium media. However, high activity was obtained in N-sufficient glucose-peptone medium. The two proteins with peroxidase activity, MnPL1 and MnPL2 (28), in peptone-containing cultures were found to be 99% identical variants encoded by two alleles of gene *mnpl* (GenBank accession no. AF007223 and AF007224) cloned from dikaryotic mycelium of *P. eryngii* (34). They represent a new type of peroxidase oxidizing both Mn^{2+} and aromatic substrates including typical LiP substrates. Recently, a second gene encoding peroxidase PS1 with similar catalytic properties (and 74% identity) was cloned from *P. eryngii* (9). It was found that both are differentially expressed, the two peroxidase variants encoded by gene *mnpl* being the only ones produced in liquid cultures, whereas the peroxidase PS1 was found during fungal growth on lignocellulosic substrates (9, 34). Southern blot experiments with the *mnpl* probe (data not shown) showed a unique hybridization band after digestion of *P. eryngii* DNA with *Eco*RI and *Eco*RV, suggesting that the probe was specific for a unique gene. This gene is different from that encoding *P. eryngii* peroxidase PS1 or *P. chrysosporium* LiP, as confirmed by the different hybridization pattern obtained with the *ps1* probe and the lack of hybridization signals with the *lpo* probe corresponding to the gene encoding LiP-H8 (as expected by the absence of LiP-type enzymes in *Pleurotus* species).

Taking into account the above results, the regulation of peroxidase MnPL production by peptone was studied by comparing the levels of transcripts in cultures grown in N-sufficient media (with peptone or ammonium as N sources) by Northern blot analysis with an *mnpl2* probe. The specificity of the probe and the high identity between *mnpl1* and *mnpl2* allowed us to monitor the levels of total *mnpl* transcripts in this study. Total RNA was isolated from mycelium during a 14-day incubation period, and the results of Northern blot hybridization are

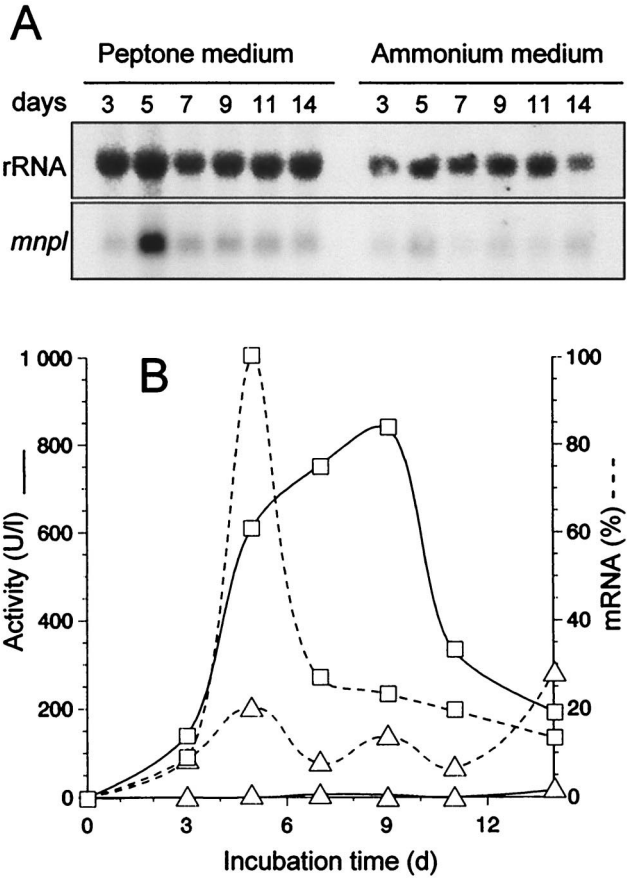

FIG. 1. Influence of N source (peptone versus ammonium tartrate) on the level of *mnpl* transcripts (dashed line) and peroxidase activity (continuous line) in N-sufficient cultures of *P. eryngii*. (A) Northern blot analysis of total RNA from mycelium samples with *mnpl2* cDNA and ribosomal DNA from *Drosophila melanogaster* as probes. (B) Time course of normalized *mnpl* mRNA levels (as percentages of the maximal level obtained, after normalization to the same rRNA in each sample) and Mn^{2+}-oxidizing peroxidase activity (MnP) estimated by formation of Mn^{3+}-tartrate complex in glucose-peptone (□) and glucose-ammonium (△) media.

shown in Fig. 1. The presence of peptone caused *mnpl* transcripts to peak after 5 days of growth (whereas only basal levels were found in the ammonium medium). Then the level of *mnpl* mRNA decreased to 20% of maximum in 2 days. A very similar profile was obtained for the daily increase of peroxidase activity. However, total extracellular activity reached a maximum level 4 days after the maximum of *mnpl* mRNA, suggesting peroxidase accumulation in the medium. No activity was detected in glucose-ammonium medium.

In order to investigate which components of peptone were involved in the stimulation of peroxidase activity, peptone was fractionated in Sephadex G15 (Fig. 2A). Six fractions were collected (I to VI), and the ability of each one to promote peroxidase activity was determined by adding it to glucose-ammonium medium used as a negative control. As shown in Fig. 2B, high levels of peroxidase could be obtained only with the highest-molecular-weight fraction, which represented more than 90% of total peptone weight but presented a comparatively low content of aromatic amino acids (as shown by the 280-nm profile), which were initially considered as potential peroxidase inducers. Lower-molecular-weight fractions or free amino acids had practically no effect on peroxidase activity

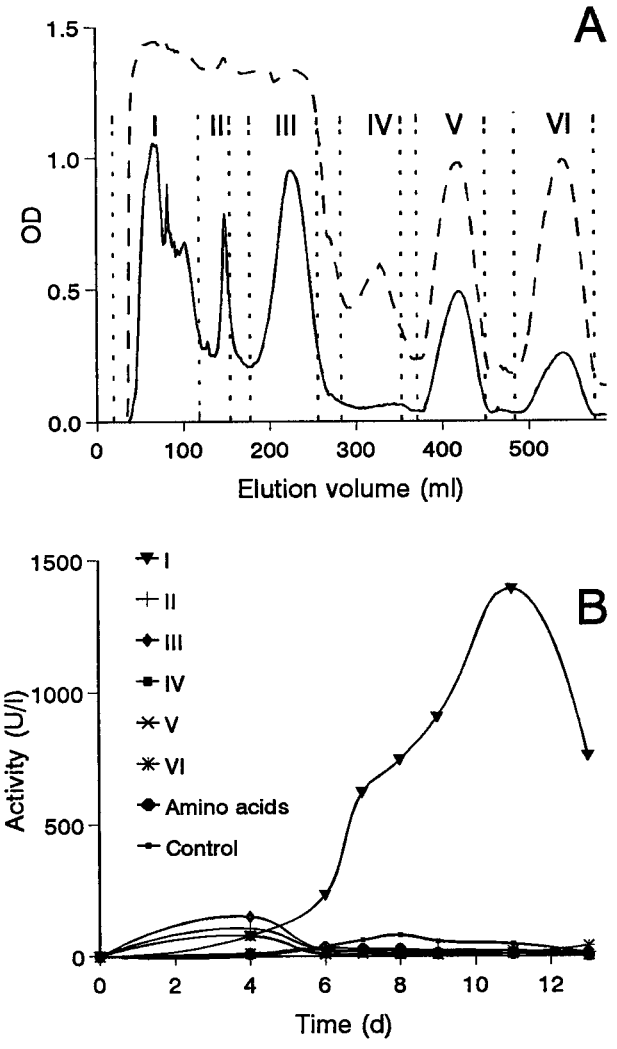

FIG. 2. Effect of peptone fractions on peroxidase activity in cultures of *P. eryngii*. (A) Peptone fractionation in Sephadex G15 (profiles at 205, dashed line, and 280 nm, continuous line, monitoring total and aromatic amino acids, respectively). (B) Peroxidase activity (estimated by formation of Mn^{3+} tartrate, MnP) after the addition of fractions I to VI, obtained during peptone fractionation (A) and free amino acids (5 g of Casamino Acids/liter from Difco) to glucose-ammonium medium used as a control (for each fraction, the amount obtained from 5 g of peptone was added to cultures, expressed in grams per liter).

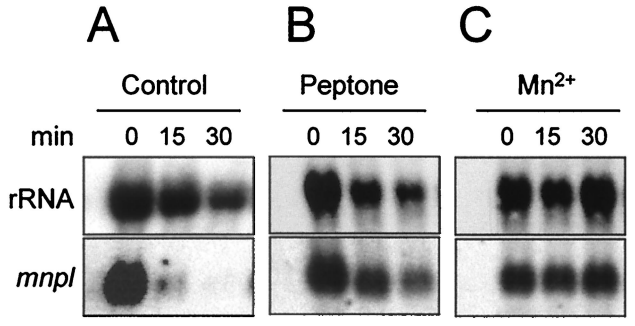

FIG. 3. *mnpl* mRNA levels maintained in peroxidase-expressing mycelium of *P. eryngii* after the addition of peptone (B) and Mn^{2+} (C) compared with the corresponding control, showing rapid decline of *mnpl* mRNA (A). Northern blot analysis of total RNA from samples of washed mycelium from glucose-peptone medium incubated for 30 min in the presence of 5 g of peptone/liter or 100 μM Mn^{2+} (in 20 mM sodium tartrate [pH 5]) and the corresponding control, with *mnpl2* cDNA and ribosomal DNA from *D. melanogaster* used as probes.

(although some short-term stimulation was observed with some of the peptone fractions).

The effect of peptone on *mnpl* mRNA levels was also investigated by using 6-day-old mycelium from glucose-peptone medium (Fig. 3A and B). Northern blot analysis showed that this mycelium contained relatively high levels of *mnpl* mRNA because of the strong induction obtained by using peptone medium. The mRNA level rapidly decreased during incubation in 20 mM sodium tartrate (pH 5) and was hardly detectable after 30 min. However, the decrease of *mnpl* mRNA was significantly slower when peptone was added to the isolated mycelium.

Effect of Mn^{2+} addition. The effect of Mn^{2+} on the levels of *mnpl* transcripts was first studied in liquid cultures with peptone or ammonium as N sources. Neither *mnpl* mRNA (Northern blotting) nor extracellular peroxidase activity was detected in glucose-peptone medium when Mn^{2+} concentrations 25 μM or higher were added (data not shown). In this medium, the highest levels of *mnpl* mRNA and peroxidase activity were obtained without added Mn^{2+} (the total manganese content in the peptone used, estimated by atomic absorption, was less than 0.5 ppm). Neither peroxidase activity nor *mnpl* mRNA levels were significant in glucose-ammonium medium with or without Mn^{2+}.

As in the case of peptone, studies were also carried out with isolated mycelium. Mn^{2+} (100 μM) was added to washed mycelium from 6-day-old cultures in media with ammonium or peptone as the N source (corresponding to noninduction and induction conditions, respectively). In the first case, Mn^{2+} exerted no effect, confirming the presence of peptone as a requisite for peroxidase production in liquid cultures of *P. eryngii* (data not shown). However, in the second case (Fig. 3C), the addition of Mn^{2+} maintained the initial levels of *mnpl* mRNA due to previous induction during growth in peptone medium, whereas *mnpl* mRNA declined rapidly in the control (Fig. 3A).

Effect of oxidative stress. The influence of reduced oxygen species on the expression of gene *mnpl* was studied. As shown in Fig. 4, induction was demonstrated by using mycelium isolated from N-sufficient ammonium medium, in which the gene is not expressed. H_2O_2 was added to washed mycelium to a final concentration of 500 μM, and samples were harvested after 15, 30, 60, and 120 min. Northern blotting analysis showed that the maximum accumulation of *mnpl* mRNA was after 1 h of incubation, when H_2O_2 was already exhausted.

In parallel experiments, OH · was generated in situ by using a system based on the redox cycling of menadione in the presence of mycelium and Fe^{3+}. Northern blotting analysis of total RNA samples from mycelium samples collected during a 2-h incubation indicated that the response to this reduced oxygen species is very rapid (Fig. 5B). Maximal *mnpl* mRNA was detected in mycelium harvested 15 min after induction. Although the *mnpl* RNA levels decreased slightly after 60 min, they increased again in the second hour of incubation, consistent with the cyclic nature of the system enabling continuous production of OH · . When no Fe^{3+} was added (i.e., in *P. eryngii* mycelium incubated in the presence of 500 μM menadione), H_2O_2 from $O_2 \cdot^-$ dismutation accumulated, and the observed *mnpl* mRNA profile (maximum after 60 min) (Fig. 5C) was very similar to that obtained after the direct addition of 500 μM H_2O_2 (Fig. 4). No *mnpl* mRNA was detected after the addition of Fe^{3+} (Fig. 5D), and the levels in the control mycelium were very low (Fig. 5A).

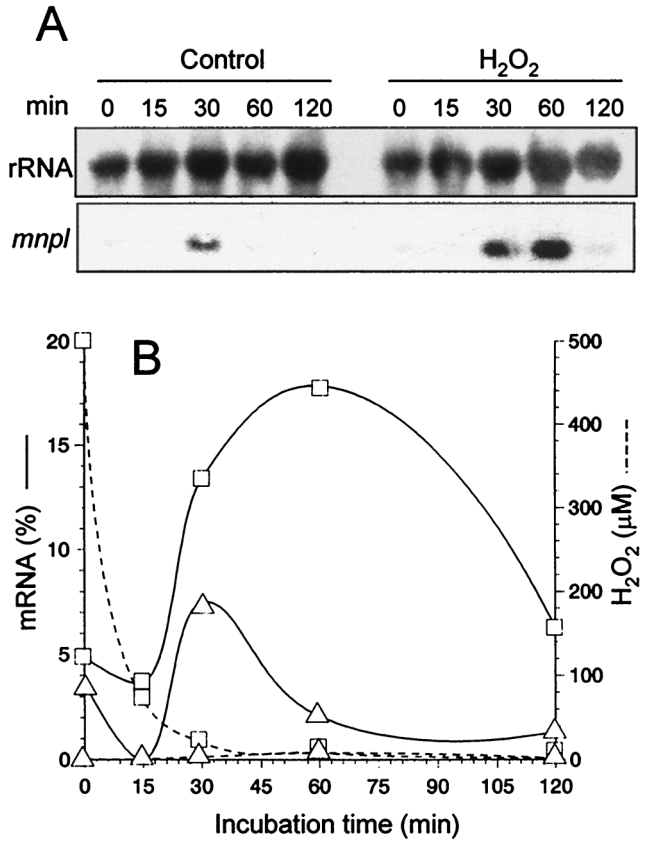

FIG. 4. Induction of *mnpl* transcription in the presence of H_2O_2. (A) Northern blot analysis of total RNA from samples of washed mycelium from glucose-ammonium medium incubated for 120 min after the addition of 500 μM H_2O_2 (in 20 mM sodium tartrate, pH 5) and the corresponding control (without inducer), with *mnpl2* cDNA and ribosomal DNA from *D. melanogaster* (control) used as probes. (B) Time course of normalized *mnpl* mRNA levels (as percentages of maximal transcript levels in peptone medium after normalization to same rRNA in each sample) in the presence (□) and absence (△) of H_2O_2 (evolution of H_2O_2 levels is also shown as dashed line).

DISCUSSION

The optimal conditions for peroxidase production in liquid cultures of *P. eryngii* were described previously (28). No significant activity was detected in cultures grown in glucose-ammonium medium, with the maximal activity being produced in low-manganese N-sufficient glucose-peptone medium. The stimulation of peroxidase levels by peptone has also been reported in other white-rot basidiomycetes (21). The above-mentioned conditions are different from those established for LiP and MnP production in *P. chrysosporium* cultures (14, 23, 36), in which the maximal activity of ligninolytic peroxidases is obtained in N-limited media containing glucose and ammonium tartrate, the highest MnP and LiP levels being obtained in high- and low-Mn^{2+} media, respectively. Subsequent studies demonstrated that LiP and MnP production in *P. chrysosporium* is regulated at the level of gene transcription by nutrient N (25, 32). Moreover, MnP of this fungus is regulated at the same level by Mn^{2+}, H_2O_2, chemical agents, O_2, and heat shock (only in N-limited cultures) (2–4, 15, 24, 29). Recently, differential expression of the three *mnp* genes in response to Mn^{2+} has been shown in *P. chrysosporium* (13).

The results obtained here demonstrate that the levels of transcripts of *P. eryngii* versatile peroxidase are controlled by N source, Mn^{2+}, and oxidative stress. The *mnpl* mRNA was present at very low levels in N-sufficient cultures in glucose-ammonium medium. This could be due to gene repression by this N source, but even under conditions involving a limited concentration of ammonium, no peroxidase activity was detected in *P. eryngii*. However, when ammonium was replaced by peptone, the induction of gene *mnpl* transcription was strong, and extracellular activity was detected. A similar effect on peroxidase activity was observed when the highest-molecular-weight peptone fraction was added at the same ratio as peptone. By contrast, the addition of free amino acids did not result in detectable peroxidase activity. These results suggest that the effect of peptone (in the culture medium) on peroxidase activity was due to peptides and not to free amino acids. A second effect of peptone added to peroxidase-expressing mycelium was the slower decline in the level of *mnpl* mRNA.

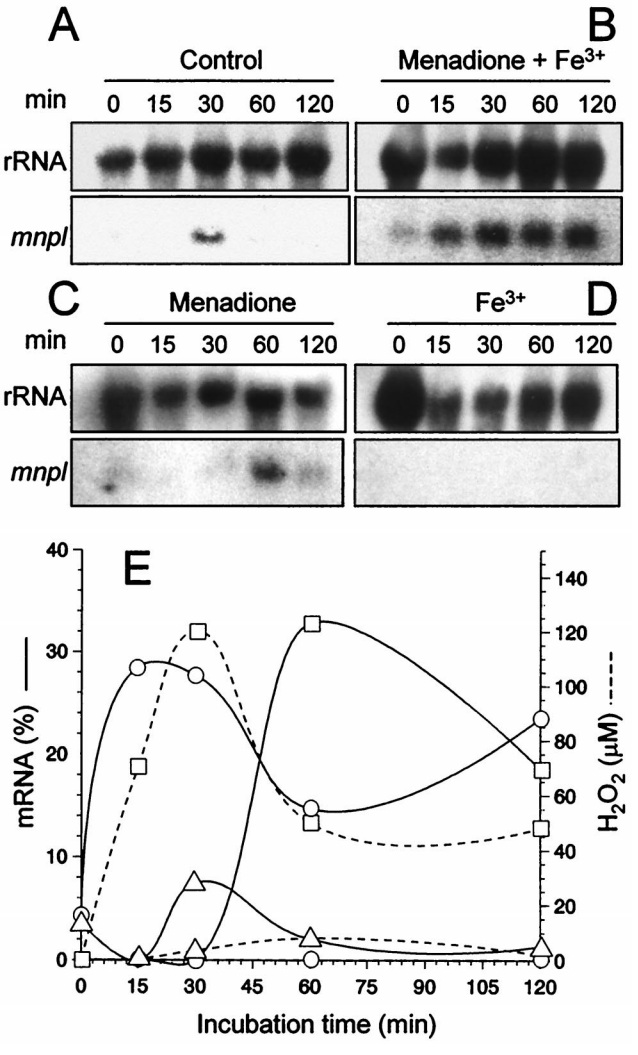

FIG. 5. Induction of *mnpl* transcription in the presence of OH · . (A to D) Northern blot analysis of total RNA from samples of washed mycelium from glucose-ammonium medium incubated for 120 min in the presence of 500 μM menadione and 100 μM Fe^{3+} generating OH · (B), 500 μM menadione generating H_2O_2 (C), 100 μM Fe^{3+} (D), and the corresponding control without the addition of the above-mentioned compounds (A), with *mnpl2* cDNA and ribosomal DNA from *D. melanogaster* (control) (all samples were incubated in 20 mM sodium tartrate, pH 5). (E) Time course of normalized *mnpl* mRNA levels (as percentages of maximal transcript levels in peptone medium after normalization to same rRNA in each sample) corresponding to B (○), C (□), and A (△). The H_2O_2 level is also shown (dashed line).

An investigation of the effect of Mn^{2+} on transcript levels indicated no peroxidase activity in peptone-containing cultures at Mn^{2+} concentrations over 25 μM. The addition of Mn^{2+} to mycelium grown in glucose-ammonium medium had no effect on the expression of gene *mnpl*. A peroxidase has recently been described in *Trametes versicolor* whose transcript levels are repressed by low concentrations of Mn^{2+} in the culture medium (11). On the other hand, the results obtained after the addition of Mn^{2+} to peroxidase-expressing mycelium of *P. eryngii* suggested that Mn^{2+} could also be implicated in the stabilization of *mnpl* mRNA. The stabilization of mRNA by metals has been reported for ferredoxin I from the cyanobacterium *Synechococcus* sp. (1).

The interest of studying the effect of reduced oxygen species on the transcript levels of ligninolytic peroxidases is related to the oxidative nature of lignin biodegradation (22). This process requires H_2O_2 (12) as a cosubstrate of peroxidases or a precursor of OH·, which can be directly involved in lignin attack (20). As demonstrated in the present study, both H_2O_2 and OH· can also be involved in the induction of ligninolytic peroxidases. The action of H_2O_2 (500 μM) was demonstrated by using *P. eryngii* mycelium from glucose-ammonium medium. After an *mnpl* mRNA maximum, the induction effect disappeared, because most H_2O_2 was destroyed by the mycelium. A positive effect of H_2O_2 on the transcript levels of *P. chrysosporium mnp* has been reported (24).

The effect of OH· on peroxidase transcript levels had not been previously shown, although it was suggested that some cell responses to the oxidative stress produced by exogenous H_2O_2 could be mediated by OH· (5). In the present study, this strong oxidant was generated by menadione added to fungal mycelium in the presence of Fe^{3+}. Quinone redox cycling involving mycelium-associated reductases provided a continuous supply of $O_2\cdot^-$ (17, 18). This radical dismutase generates H_2O_2, which is reduced by Fe^{2+} (from Fe^{3+} reduction by semiquinone or $O_2\cdot^-$), yielding OH· (Fenton-type reaction). OH· formation has been confirmed under these experimental conditions (18), and the reaction mechanism was supported by the formation of H_2O_2 when only menadione was added to the fungal mycelium. Using the above-described system, we showed for the first time that OH· elicits the transcriptional expression of a ligninolytic peroxidase, probably via a stress response mechanism. It is interesting that stimulation of *P. eryngii* peroxidase activity (in glucose-peptone medium) has been observed in the presence of sublethal doses (0.05 to 0.1 mg/ml) of several toxic compounds which can also induce stress response, such as α-amanitin and hycanthone, as well as with actinomycin D (data not shown). Even though the response observed is indirect, it is notable that the presence of OH· triggers the expression of gene *mnpl* faster than the addition of H_2O_2. The undetectable levels of H_2O_2, which is reduced in a Fenton-type reaction, support the notion that mainly OH·, and not H_2O_2, was involved in gene induction response under the experimental conditions used. The possibility of an effect of the semiquinone, either in the presence of Fe^{3+}, resulting in the formation of OH·, or in its absence, resulting in the formation of H_2O_2, cannot be completely ruled out. However, this aromatic radical tends to auto-oxidize, as revealed by the reduction of Fe^{3+} in the first case (data not shown) and by the formation of H_2O_2 in the second. In the latter case, the response was very similar to that previously obtained with exogenous H_2O_2, suggesting that peroxide is involved. The rapid peroxidase induction in the former case also suggests induction by a stronger chemical oxidant, as formed in the Fenton-type reaction.

Finally, it should be mentioned that the promoter region of gene *mnpl* includes some putative response elements (34) which could be involved in the above regulation of transcript levels of the new ligninolytic peroxidase produced by *P. eryngii*. Additional studies are necessary to elucidate this and other aspects of ligninolytic peroxidase regulation in these white-rot fungi.

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