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Heterogeneity and Regulation of Manganese Peroxidases from *Phanerochaete chrysosporium*

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Lignin and Mn peroxidases are two families of isozymes produced by the lignin-degrading fungus *Phanerochaete chrysosporium* under nutrient nitrogen or carbon limitation. We purified to homogeneity the three major Mn peroxidase isozymes, H3 (pI = 4.9), H4 (pI = 4.5), and H5 (pI = 4.2). Amino-terminal sequencing of these isozymes demonstrates that they are encoded by different genes. We also analyzed the regulation of these isozymes in carbon- and nitrogen-limited cultures and found not only that the lignin and Mn peroxidases are differentially regulated but also that differential regulation occurs within the Mn peroxidase isozyme family. The isozyme profile and the time at which each isozyme appears in secondary metabolism differ in both nitrogen- and carbon-limited cultures. Each isozyme also responded differently to the addition of a putative inducer, divalent Mn. The stability of the Mn peroxidases in carbon- and nitrogen-limited cultures was also characterized after cycloheximide addition. The Mn peroxidases are more stable in carbon-limited cultures than in nitrogen-limited cultures. They are also more stable than the lignin peroxidases. These data collectively suggest that the Mn peroxidase isozymes serve different functions in lignin biodegradation.

The white-rot fungus *Phanerochaete chrysosporium* produces H_2O_2 (13) and a lignin-degrading system in response to nutrient nitrogen (12, 24), carbon, or sulfur (10, 21) limitation. An integral part of this lignin-degrading system is extracellular peroxidases. These peroxidases can be divided into two catalytically distinct classes, the lignin peroxidases (16, 38) and the Mn peroxidases (20, 28). The lignin peroxidases catalyze the oxidation of nonphenolic aromatic substrates (26). The Mn peroxidases catalyze the oxidation of Mn^{2+} to Mn^{3+} (14). Mn^{3+}, in turn, can oxidize a large number of phenolic substrates.

The significance of these peroxidases in lignin biodegradation is well documented. The decomposition of lignin by ligninolytic cultures has been correlated to the appearance and level of the extracellular enzymes (10, 27). Mutants with low peroxidase activity also exhibit lowered total ligninolytic activity (17). These peroxidases have now been found in numerous lignin-degrading fungi (4). In one instance in which the enzyme activity has been difficult to detect, investigators have still found evidence for oxidation of peroxidase substrates in the extracellular fraction (43). Both the lignin and Mn peroxidases have been shown to catalyze the depolymerization of synthetic lignins and methylated native lignins in vitro (18, 44). Finally, H_2O_2, which is required for peroxidase activity, is also secreted by the fungus (13), and addition of catalase to ligninolytic cultures inhibits lignin degradation (10).

Of the 10 isozymes detected in the extracellular fluid of nitrogen-limited cultures by Kirk and coworkers (25), 6 are lignin peroxidases and 4 are Mn peroxidases. The roles of the various lignin peroxidase isozymes in lignin biodegradation are still not well understood. However, different roles for the lignin peroxidase isozymes are suggested on the basis of the existence of different genes encoding them as well as the observation that they are differentially regulated in response to environmental conditions (19). For example, it has previously been shown that the pattern of lignin peroxidase isozyme expression is highly dependent on the type of nutrient limitation (carbon or nitrogen) used to grow the fungus and to induce the ligninolytic system (19, 30).

In contrast to the lignin peroxidases, the molecular basis for the multiplicity of the Mn peroxidases is not well understood. Although two cDNAs encoding the Mn peroxidases have been isolated, these were from two different strains of *P. chrysosporium* (35, 37). In the present report, we demonstrate that the major Mn peroxidase isozymes are derived from different genes and that they are differentially regulated. This suggests different roles for each isozyme in lignin biodegradation. We also report here kinetic data comparing the three major Mn peroxidase isozymes from *P. chrysosporium*.

MATERIALS AND METHODS

Chemicals. Veratryl alcohol, guaiacol and vanillin were purchased from the Aldrich Chemical Company (Milwaukee, Wis.). Veratryl alcohol was purified by vacuum distillation. Vanillylacetone was synthesized as previously described (20, 34). All other chemicals were reagent grade and were used without further purification.

Growth of *P. chrysosporium*. *P. chrysosporium* BKM-F-1767 (ATCC 24725) was grown in 10-ml stationary cultures in 125-ml Erlenmeyer flasks as previously described (39). The cultures were flushed with 100% water-saturated dioxygen on day 3 of growth. Nitrogen-limited culture medium consisted of Basal III medium, as previously described (39), with 1.0% glucose and 1.1 mM ammonium tartrate. Carbon-limited culture medium was also Basal III medium, except that it contained 0.1% glucose and 11 mM ammonium tartrate. For RNA analysis, 100 cultures were harvested per day on days 1 to 6 of growth. The mycelium was separated from the extracellular fluid by passage through cheesecloth. The mycelium was immediately frozen in liquid nitrogen for RNA isolation. The pooled extracellular fluid was assayed for lignin and Mn peroxidase activity as described below.

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In experiments in which the Mn concentration was varied, cultures were grown under carbon or nitrogen limitation as described above, except that veratryl alcohol and additional trace elements were not added. Normal cultures contained 0.4 mM veratryl alcohol, 7× trace elements, and 203 μM $MnSO_4$. In these experimental cultures, the Mn concentration was varied so that the final concentration of $MnSO_4$ was 0, 6, 30, 119, or 237 μM.

For inhibition of protein synthesis, cycloheximide was added to normal nutrient-limited cultures that were set up as described above on day 3, 4, or 5 to a final concentration of 25 μM. Each time point consisted of three samples, and each sample consisted of three pooled flasks. Control flasks that had an equivalent volume of water added to the cultures were also assayed.

SDS-PAGE and Western transfer. Extracellular fluid (50 μl) from the day 1 to 6 cultures was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide) under denaturing conditions as described by Laemmli (29). Western immunoblots were visualized by using a rabbit polyclonal antibody affinity purified to isozyme H4 (35) and goat anti-rabbit antibody conjugated to alkaline phosphatase.

Northern blot analysis of RNA. Isolation of RNA from carbon-limited cultures of *P. chrysosporium*, Northern (RNA) blotting and hybridization were as described previously (35), with the exception that 10 μg of total RNA was loaded per lane instead of 1 μg of poly(A) RNA.

Purification of Mn peroxidase isozymes. An overproducing strain of *P. chrysosporium*, PSBL-1 (32, 41), was used for production of Mn peroxidases. Extracellular fluid from day 5 cultures was harvested and separated by using a fast protein liquid chromatography Mono Q column as described previously (25). Peaks containing isozymes H3 (pI = 4.9), (pI = 4.5), and H5 (pI = 4.2) were combined and dialyzed against 50 mM sodium succinate, pH 4.5. These isozymes were further purified by modifying the procedure of Glenn and Gold (15). The enzymes were subjected to chromatography on a blue agarose column (18 by 3 cm, ~100 ml volume; Cibracon Blue 3GA type 3000-L, Sigma) and eluted with 400 ml of a 0 to 0.4 M NaCl gradient in 50 mM sodium succinate, pH 4.5. Isozyme H4 was purified to homogeneity after this step; however, H3 and H5 required further purification. Preparative isoelectric focusing (IEF) was performed by using a Pharmacia LKB Multiphor II electrophoresis system as instructed by the manufacturer with Ultrodex as a support. Isozyme H3 was isolated from a gradient of pH 4.5 to 5.5; isozyme H5 was isolated from a gradient of pH 4 to 6. Gel fractions were eluted with 50 mM sodium succinate buffer, pH 4.5.

Analytical IEF gels. Analytical IEF was performed by using 5% acrylamide gels with a pH gradient of approximately 3.5 to 5.5. The gradient was made by mixing 0.9 ml of pH 4 to 6 ampholine and 0.6 ml of pH 3.5 to 5.0 ampholine in a total volume of 30 ml. All samples were dialyzed against water before being loaded on IEF gels.

Staining of IEF gels. All gels that were silver stained or Western blotted were first fixed in 0.16 M sulphosalicylic acid–0.7 M trichloroacetic acid for 30 min. Silver staining was carried out after the fixing step by the method of Merril et al. (31). Proteins were electrophoretically transferred to nitrocellulose for Western blots after the gels were fixed and soaked for 30 min in three changes of 12.3 mM Tris-Cl–96 mM glycine (pH 8.6) with 10% methanol and 0.001% SDS at 50°C. Subsequent visualization steps were similar to that of an SDS-PAGE Western transfer. Mn peroxidase activity in the gels was revealed by a color change due to the oxidation of phenol red as described previously (36).

cH4	A	V	C	P	D	G	T	R	V	T	N	A	A	C	C	A	F	I	P	L
H4	A	V	X	P	D	G	T	?	V	T	N	A	A	X	X	A	F	I	P	L
H3	A	T	X	P	D	G	T	K	V	N	N	A	A	X	X	?	F	I	P	L
H5	A	V	X	H	P	G	T	?	V	S	?	A	A	X	X	?	F	I	P	L
pulp	A	V	X	P	D	G	T	R	V	?	N	A	A	X	X	A	F	?	P	L

FIG. 1. N-terminal amino acid sequences of Mn peroxidases. N-terminal amino acid sequences of Mn peroxidase isozymes H3, H4, and H5; cDNA of H4 (cH4); and a Mn peroxidase isolated from *P. chrysosporium* grown on pulp (7).

Enzyme activity assays. One unit is defined as one micromole of product formed per minute. Lignin peroxidase activity was assayed by monitoring oxidation of veratryl alcohol as previously described (39). Mn peroxidase activity was measured by phenol red oxidation except as noted otherwise. Phenol red oxidation was assayed as described by Glenn and Gold (15) as modified by Pease et al. (36). Guaiacol and vanillylacetone oxidation by Mn peroxidase were assayed as previously described (33, 34). In the determination of K_m for vanillylacetone and Mn^{2+}, a cuvette with a 2-mm pathlength was used in order to assay higher concentrations of vanillylacetone. When determining the K_m for H_2O_2, 100 μM vanillylacetone and 100 μM $MnSO_4$ was used. For the experiments to determine the K_m for Mn^{2+}, 100 μM H_2O_2 and 200 μM vanillylacetone was used. Guaiacol oxidation was used to measure activity in experiments to investigate the protective effect of Mn. These incubations contained purified H4, 100 μM H_2O_2, and either 100 μM $MnSO_4$ or no Mn.

The H_2O_2 concentration was determined spectrophotometrically at 240 nm by using an extinction coefficient of 39.6 M^{-1} cm^{-1}.

RESULTS

Multiplicity of the Mn peroxidase isozymes. The three major Mn peroxidase isozymes, H3, H4, and H5, were purified by blue agarose column chromatography. Isozyme H4 was more than 95% pure by this procedure; isozymes H3 and H5 required further purification by preparative IEF. The N-terminal sequences of these three major isozymes are shown in Fig. 1. Also shown in Fig. 1 is the sequence of a Mn peroxidase isozyme isolated from *P. chrysosporium* growing on pulp (7) and the deduced amino acid sequence from cDNA λMP-1 (encoding H4) previously reported (35). All of the sequences are highly similar; however, distinct differences in the sequence (underlined in Fig. 1) clearly demonstrate that the Mn peroxidase isozymes are encoded by different genes.

Table 1 compares the total amino acid compositions of isozymes H3, H4, and H5 with those of Mn peroxidases reported in the literature. Again, the data indicate a high degree of similarity between isozymes H3, H4, and H5. These results imply that they are very similar at the structural level. Further support for this comes from the fact that the antibody that was affinity purified to H4 cross-reacts with all three of the highly purified Mn peroxidase isozymes. This antibody preparation does not cross-react with lignin perox-

TABLE 1. Total amino acid composition of Mn peroxidase isozymes[a]

Amino acid	No. of amino acids			
	Isozyme H3	Isozyme H5	Isozyme H4	λMP-1 cDNA-H4
Ala	34	49	29	48
Arg(+)	10	6	11	11
Asn/Asp-	34	40	24	40
Cys	3	2	3	10
Gln/Glu-	24	25	25	32
Gly	62	45	51	29
His(+)	5	4	6	7
Ile	11	13	11	15
Leu	20	22	22	33
Lys(+)	3	8	1	12
Met	8	2	11	9
Phe	13	13	17	26
Pro	24	25	23	30
Ser	31	22	31	29
Thr	18	20	27	28
Trp	ND	ND	ND	1
Tyr	0	0	0	0
Val	18	17	17	22

[a] Determined at the Pennsylvania State University Biotechnology Institute. ND, not determined.

idase isozyme H8 (see Fig. 3 described below) or any other lignin peroxidase isozyme (data not shown).

Regulation of Mn peroxidases by nutrient limitation. Isozymes H3, H4, and H5 are the predominant Mn peroxidases produced by nitrogen-limited cultures (25). Figure 2 shows the time course of Mn peroxidase activity in carbon- and nitrogen-limited cultures. Mn peroxidase activity in nitrogen-limited cultures appears and peaks early in secondary metabolism (day 3) and then decreases thereafter, results which are similar to those of other investigators (30, 35, 37). The time course of expression of the Mn peroxidase activity in carbon-limited cultures differs from this in that the activity appears on day 3 and gradually increases throughout secondary metabolism, peaking on day 5 or 6. The level of expression also differs between the two nutrient-limiting conditions. The maximum activity in carbon-limited cultures is less than half of that observed in nitrogen-limited cultures.

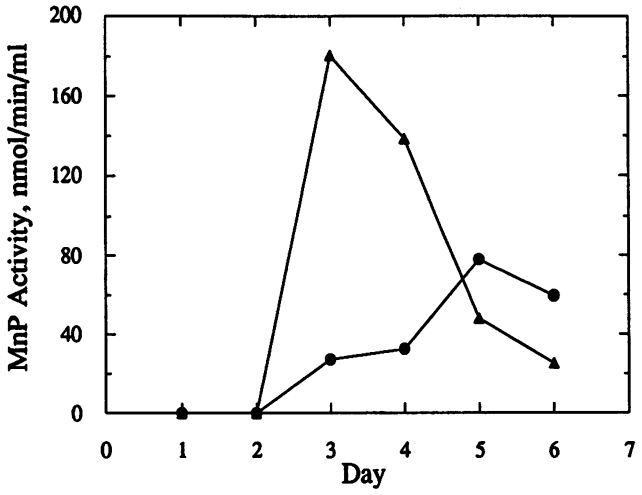

FIG. 2. Mn peroxidase activity profile from nutrient-limited cultures. Extracellular fluid from carbon- (●) or nitrogen (▲)-limited cultures was assayed for Mn peroxidase activity by phenol red oxidation as described in Materials and Methods.

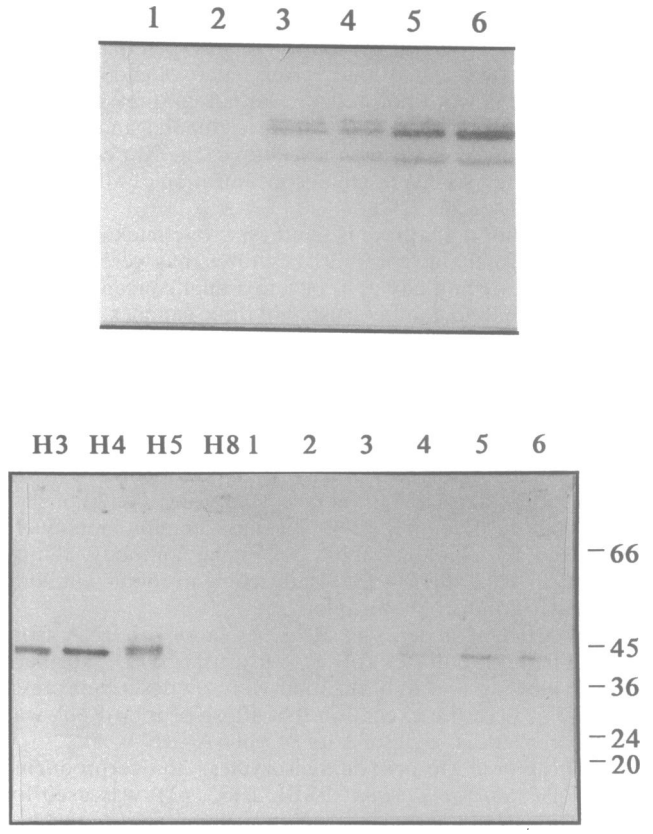

FIG. 3. Western blot analysis of Mn peroxidases present in day 1 to 6 extracellular fluid from carbon-limited cultures. The proteins in the extracellular fluid from day 1 to 6 cultures were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by using affinity-purified anti-H4. Lanes 1 to 6 contain 40 μl of extracellular fluid from day 1 to 6, respectively, and lanes H3, H4, H5, and H8 contain 0.1 μg of purified enzyme. The samples in the bottom panel are unconcentrated extracellular fluid, and those in the top panel are fivefold-concentrated extracellular fluid.

The increase in enzyme activity observed in carbon-limited cultures parallels an increase in the total Mn peroxidase protein as determined by Western blot analysis of SDS-polyacrylamide gels (Fig. 3). Because the H4 antibody reacts with all three major Mn peroxidase isozymes (H3, H4, and H5), the Western blot results can be used to quantitate these enzymes. An increase in the Mn peroxidase protein is observed from day 3 to 6 in carbon-limited cultures. This positive correlation between activity and protein level is similar to previous results with nitrogen-limited cultures, which showed that the activity level paralleled protein levels (35). The time of maximal expression differs between the nutrient-limited cultures; nitrogen-limited cultures exhibit an increase in protein levels up to day 3 and a subsequent decrease (35). These results for both carbon- and nitrogen-limited cultures indicate that the increase in enzyme activity is due to an increase in protein content and not to enzyme activation.

Expression of Mn peroxidases was also characterized by Northern blot analysis. Mn peroxidase-specific mRNAs from carbon-limited cultures were visualized with a radioac-

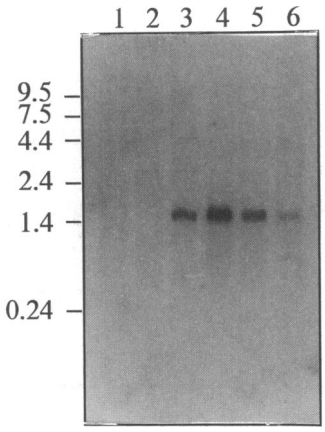

FIG. 4. Northern-blot analysis of total RNA from day 1 to 6 carbon-limited cultures by using the cDNA for H4 as a probe. Total RNA (10 μg) was subjected to electrophoresis on a 1.5% agarose gel containing 0.66 M formaldehyde and transferred to GeneScreen. The blots were hybridized with the λMP-1 cDNA *Eco*RI 1.3-kb insert. Lanes 1 to 6, day 1 to 6 RNA, respectively. Size markers are labelled in kilobases to the left of the blot.

tive λMP-1 cDNA probe, which encodes isozyme H4 (Fig. 4). In contrast to the Western blot and the enzyme activity profile, Mn peroxidase mRNA levels that hybridize to the λMP-1 probe appear on day 3, peak on day 4, and decrease thereafter. This indicates that the λMP-1 RNA levels do not correlate with the protein and activity levels. Previous data obtained for nitrogen-limited cultures showed that the RNA content paralleled both (35).

Stability of Mn peroxidase activity. One possible explanation for the difference in the mRNA levels and enzyme activity in carbon-limited cultures is that the Mn peroxidase is exceedingly stable. The turnover rate was examined by monitoring enzyme activity in the extracellular fluid after the addition of the protein synthesis inhibitor cycloheximide to cultures. Cycloheximide was added to nitrogen- and carbon-limited cultures on day 3, 4, or 5 of growth. Figure 5 shows the decrease in Mn peroxidase activity following cycloheximide addition on all three days. Mn peroxidase activity did not decrease significantly in carbon-limited cultures even beyond 72 h post-cycloheximide addition (Fig. 5A). Only when cycloheximide was added on day 5 was a decrease in activity observed (from 80 to 50 U/liter). These data indicate that the time of cycloheximide addition had no effect on the results, with no appreciable decrease in enzyme activity observed regardless of whether cycloheximide was added on day 3, 4, or 5. In contrast, a steady decline in activity over a short time was observed with nitrogen-limited cultures after cycloheximide addition (Fig. 5B). At the time of cycloheximide addition, enzyme levels were approximately 200 U/liter on days 3 and 4 and 50 U/liter on day 5. This activity decayed to near 0 within 48 h in all three cases. Half-lives of approximately 7.5, 2.4, and 3 h were determined when cycloheximide was added on days 3, 4, and 5, respectively. Control cultures that had water added instead of cycloheximide were analyzed concurrently with the experimental cultures and exhibited a normal Mn peroxidase activity profile. These data clearly show that the Mn peroxidases are more stable in carbon-limited cultures than in nitrogen-limited cultures. It also appears that the enzymes are less stable in older nitrogen-limited cultures.

Lignin peroxidase activity was also assayed in the same

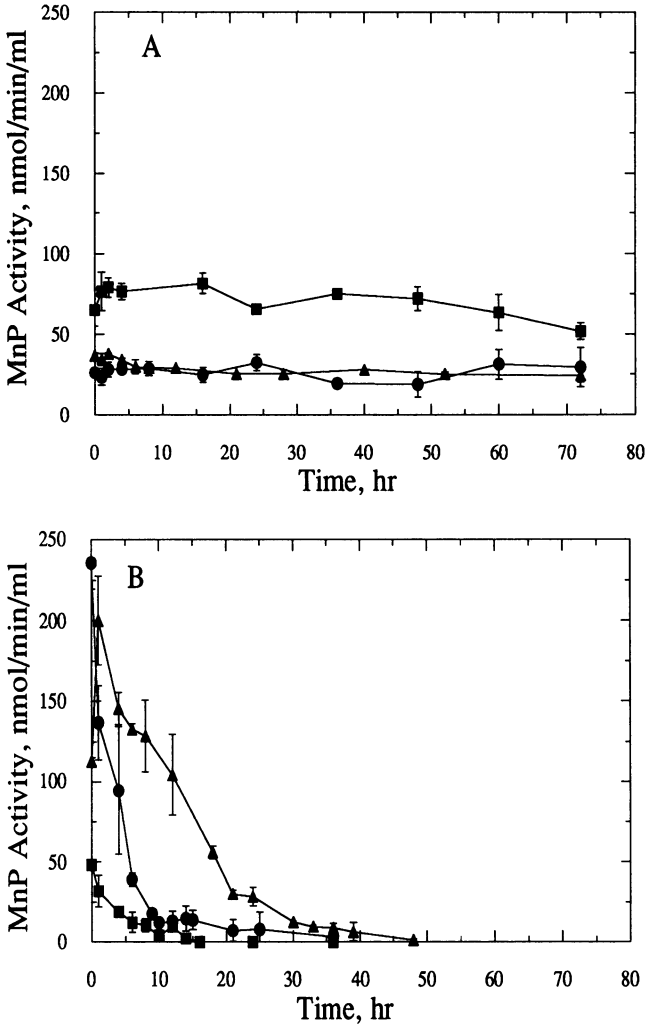

FIG. 5. Mn peroxidase activity in nutrient-limited cultures after addition of cycloheximide. Cultures were grown for 3 days (▲), 4 days (●), or 5 days (■), at which time cycloheximide was added to a final concentration of 25 μM. At specified times nine cultures were harvested in three sets of three. These samples were assayed for Mn peroxidase activity and averaged to make up one datum point. The error bars represent the mean plus and minus one standard deviation. (A) Carbon-limited cultures, (B) nitrogen-limited cultures.

cultures (data not shown). Unlike the Mn peroxidases, a decline in activity was observed in both nitrogen- and carbon-limited cultures. The activity was only detectable on day 3 in carbon-limited cultures and on days 4 and 5 in nitrogen-limited cultures. The calculated half-lives were approximately 3 h in carbon-limited cultures and 8 h in nitrogen-limited cultures. This is similar to results reported elsewhere (42).

Regulation of Mn peroxidase isozyme profile by nutrient limitation. Another possible cause for the increase in the total Mn peroxidase activity despite a decrease in λMP-1 mRNA levels is that the mRNAs which encode the other Mn peroxidase isozymes do not cross-hybridize with the λMP-1 cDNA probe. Although a high degree of sequence similarity is observed in the N termini and the amino acid compositions are similar, the hybridization conditions used were very stringent and may be sufficient to discriminate among the

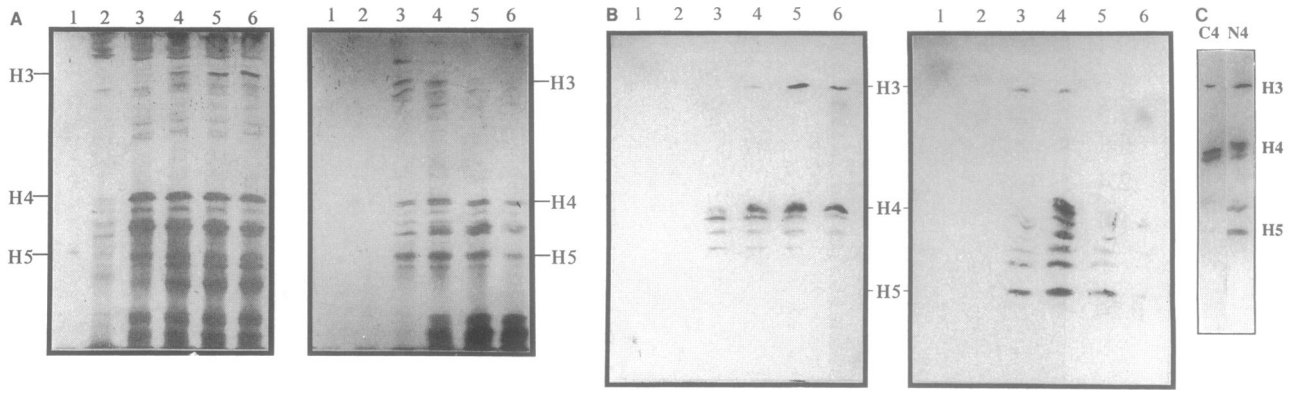

FIG. 6. IEF of extracellular proteins from carbon- and nitrogen-limited cultures. Extracellular fluid from carbon- or nitrogen-limited cultures was harvested and concentrated 32-fold, and 30 μl of sample was applied to each lane. (A) Silver-stained IEF gels; (B) IEF gels that have been Western blotted and visualized with anti-H4 antibody. Carbon-limited cultures are on the left and nitrogen-limited cultures are on the right. Lanes 1 to 6, day 1 to 6, respectively. The migration of the H3, H4, and H5 isozymes along with the pIs are indicated. (C) IEF gel stained for Mn peroxidase activity.

various RNAs. Because no other cDNAs encoding Mn peroxidases have been isolated from strain BKM-F-1767, the extent of cross-hybridization cannot be determined. Consequently, regulation of each individual isozyme cannot be examined by mRNA analysis. For this reason, we examined the effect of nutrient limitation on the Mn peroxidase isozyme profile at the protein level.

The proteins in the extracellular fluids of both carbon- and nitrogen-limited cultures were subjected to analytical IEF and visualized by silver staining (Fig. 6A), Western blotting with the Mn peroxidase antibody (Fig. 6B), and staining for activity by phenol red oxidation (Fig. 6C). In carbon-limited cultures, silver-stained and Western-blotted gels show that several Mn peroxidase protein bands appear early in the time course (day 3) and remain at a constant level throughout. On day 3, isozyme H4 (pI 4.6) appears to be the predominant isozyme. Later in the time course, a protein with a very high pI, isozyme H3 (pI 5.1), is abundantly expressed. The gel stained for activity confirms that the isozyme appearing late in the time course with a pI of 5.1 is active. This band corresponds to isozyme H3. There is no detectable isozyme H5 expressed in carbon-limited cultures. The gels of nitrogen limited-cultures show that isozymes H3, H4, and H5 (pI 4.2) are all present early in the time course and that the levels decline over time. The Western-blotted and activity-stained gels for both carbon- and nitrogen-limited cultures reveal that in addition to the three expected Mn peroxidase isozymes (H3, H4 and H5), there are a number of additional active Mn peroxidases present in both carbon- and nitrogen-limited cultures throughout the time course. In some of the bands (especially in nitrogen-limited cultures), intense bands shown on the Western blot (Fig. 6B) do not correlate with the intensity on the activity stain (Fig. 6C). This may be due to some cross-reactivity of the Mn peroxidase antibody or to low specific activity of certain isozymes.

Regulation of Mn peroxidase activity by Mn. In accordance with the results of others (2, 3), Fig. 7 shows that the addition of Mn causes an increase in total Mn peroxidase activity in both carbon- and nitrogen-limited cultures. The increase in activity is concentration dependent, yet saturable. Little or no enzyme activity is observed in the absence of Mn in both carbon- and nitrogen-limited cultures. Under both carbon- and nitrogen-limited conditions growth is not affected by the absence of Mn. The effect of Mn appears to

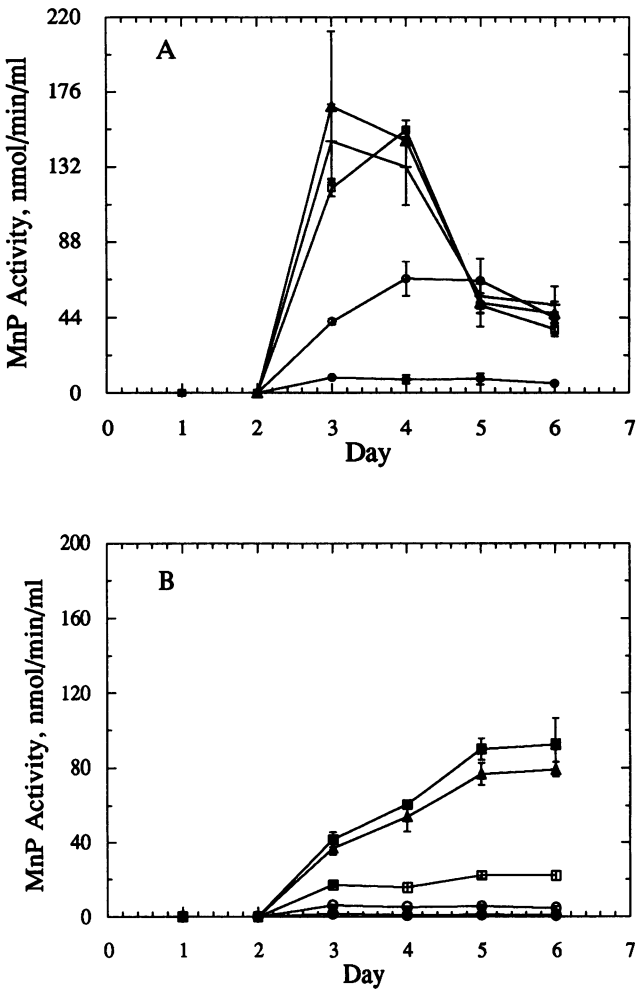

FIG. 7. Mn peroxidase activity profile from nutrient-limited cultures with various amounts of Mn. Cultures were grown under carbon (A) or nitrogen (B) limitation, and the $MnSO_4$ concentration was varied within the Basal III medium component to give final concentrations (in micromolar) as follows: ●, 0; ○, 6; □, 30; ▲, 119; and ■, 237. Cultures typically contain 203 μM $MnSO_4$ (see Materials and Methods).

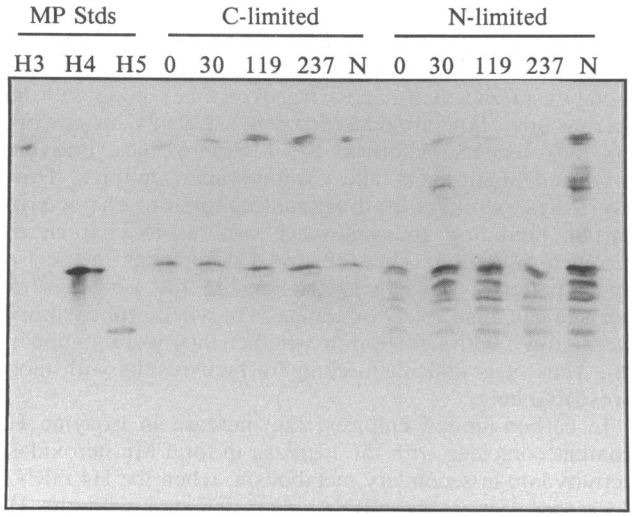

FIG. 8. Western blot analysis of Mn peroxidases from day 5 carbon-limited cultures and day 4 nitrogen-limited cultures grown with various concentrations of Mn. Proteins from the extracellular fluid of day 5 carbon-limited cultures and day 4 nitrogen-limited cultures containing various concentrations of $MnSO_4$, as indicated above the lanes, were separated by IEF and transferred to nitrocellulose and visualized with anti-H4. Mn peroxidase standards were isozymes H3, H4, and H5 as indicated.

be specific for the Mn peroxidases and not due to decreased tissue mass. Nitrogen-limited cultures are much more responsive to Mn than carbon-limited cultures. Maximal activity was obtained with 30 μM $MnSO_4$ for nitrogen-limited cultures, whereas 119 μM was required for carbon-limited cultures. (The activities shown in Fig. 7 are slightly lower than those shown in Fig. 2; slightly different media conditions were used as described in Materials and Methods.)

Regulation of Mn peroxidase isozyme profile by Mn. To determine the effect of Mn on the Mn peroxidase isozyme profile, the extracellular fluid of nitrogen- and carbon-limited cultures grown in the presence of various concentrations of Mn was subjected to IEF and visualized by immunodetection techniques (Fig. 8). The cultures were analyzed on the day of maximal activity, which was day 5 for carbon-limited cultures and day 4 for nitrogen-limited cultures. In carbon-limited cultures, isozymes H3 and H4 are the predominant isozymes and no isozyme H5 is detected (as observed in Fig. 6). Increasing the Mn concentration from 0 to 237 μM caused a much more dramatic increase in the isozyme H3 content than in the isozyme H4 content. The media typically used by most investigators (Fig. 8, lane N) contains 203 μM Mn but is also supplemented with other trace elements and veratryl alcohol. In agreement with enzyme activity measurements (Fig. 7), Western blots show a more intense response in nitrogen-limited cultures than carbon-limited cultures to $MnSO_4$ addition. Whereas the addition of 30 μM $MnSO_4$ causes a slight increase in proteins in carbon-limited cultures, a much more dramatic increase is observed in nitrogen-limited cultures. The increase is observed for all three of the major isozymes; however, it is difficult to decipher the relative response of each isozyme to $MnSO_4$ addition.

Kinetic properties of isozymes H3, H4, and H5. To address functional differences between the Mn peroxidases, the three major isozymes were kinetically characterized. All three isozymes have relatively high affinities for H_2O_2, exhibiting K_m values of 21 to 34 μM (Table 2). The K_m values for divalent Mn ions are also very low for all three isozymes; however, more substantial differences in the K_m values are observed for the isozymes. Isozyme H5 has the highest affinity for Mn^{2+}, with a K_m of 4 μM, and isozyme H3 has the lowest affinity with a K_m of 16 μM. The K_m values for the isozymes for vanillylacetone were similar, ranging from 22 to 31 μM. The kinetic constants of the three Mn peroxidase isozymes for phenolic substrates were also determined (Table 2). The k_{cat} values for guaiacol and phenol red are fairly similar for the isozymes, ranging from 39 to 66 s^{-1} for phenol red and 40 to 56 s^{-1} for guaiacol. Vanillylacetone is the substrate oxidized most rapidly by each of the isozymes, with the k_{cat} ranging from 76 s^{-1} for isozyme H5 to 97 s^{-1} for isozyme H3. For all three substrates, isozyme H3 exhibits the highest k_{cat} value and isozyme H5 has the lowest.

TABLE 2. Kinetic constants for Mn peroxidases[a]

Isozyme	K_m (μM)			k_{cat} (s^{-1})		
	Van	Mn	H_2O_2	Van	PR	Gu
H3	31	16	34	97	66	53
H4	24	9	32	91	41	56
H5	22	4	21	76	39	40

[a] Van, vanillylacetone; PR, phenol red; Gu, guaiacol.

Although essential for activity, H_2O_2 was inhibitory at high concentrations (Fig. 9). Phenol red was the most sensitive and vanillylacetone was the least sensitive to H_2O_2 inhibition. This H_2O_2 inhibition prevented accurate K_m and k_{cat} determinations for some of the substrates.

Protection of Mn peroxidase by Mn. Mn peroxidases are inactivated by H_2O_2. The ability of Mn to protect the enzymes from inactivation was investigated. When purified Mn peroxidase was incubated with H_2O_2 (100 μM) in the absence of Mn, a dramatic decrease in enzyme activity is observed over time (Fig. 10). The addition of $MnSO_4$ (100 μM) to the Mn peroxidase-H_2O_2 reaction mix resulted in a stabilization of the activity. There is no apparent decrease in activity over the same time course. This demonstrates that Mn^{2+} can protect the Mn peroxidases from inactivation by H_2O_2.

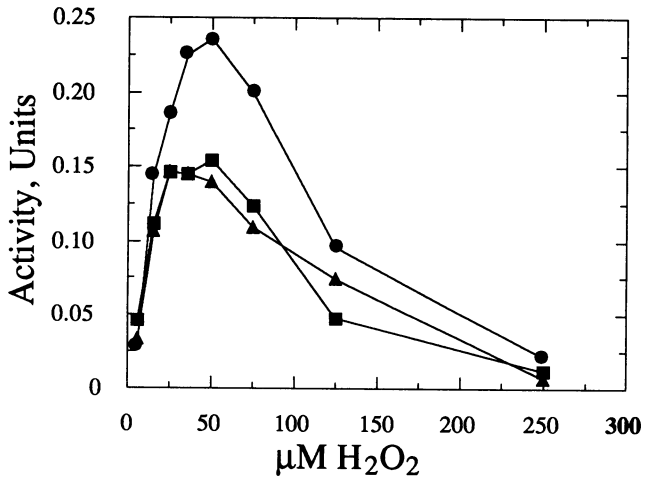

FIG. 9. Inhibition of Mn peroxidase activity by H_2O_2. Enzyme activity was monitored by phenol red oxidation for isozymes H3 (●), H4 (■), and H5 (▲).

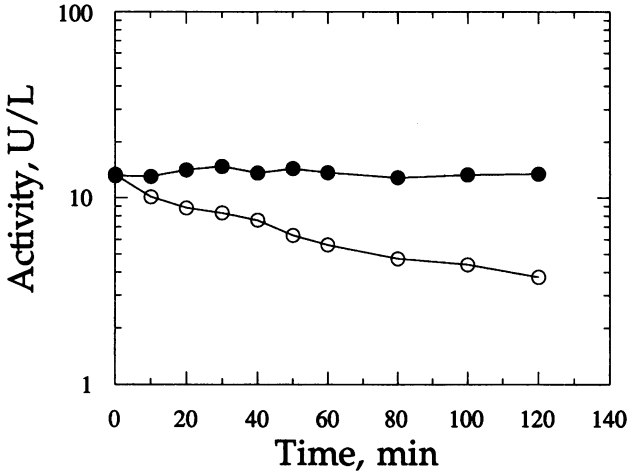

FIG. 10. Protection of Mn peroxidase activity by Mn^{2+}. Isozyme H4 (0.2 nM) was incubated with 100 μM H_2O_2 in the presence (○) or absence (●) of Mn^{2+} (100 μM). At specified times, enzyme activity was assayed by guaiacol oxidation.

DISCUSSION

The present study has focused on the multiplicity of the Mn peroxidase isozymes. We have characterized the enzymology and the regulation of these isozymes in carbon- and nitrogen-limited cultures. The Mn peroxidases, like the lignin peroxidases, are expressed as a family of isozymes (25, 30). The multiplicity of the Mn peroxidases was initially proposed to arise from differential protein processing of a single gene product (30). N-terminal amino acid sequence data presented here show that the Mn peroxidase isozymes are very similar; however, differences in sequences indicate that the isozymes are encoded by different genes. The existence of different genes encoding the Mn peroxidases would suggest different functions for each isozyme, and indeed our data are consistent with this suggestion.

Previous work demonstrated that the lignin peroxidase isozymes are regulated differently (19). For example, under carbon limitation, lignin peroxidase isozyme H2 is the predominant isozyme, whereas under nitrogen limitation, isozyme H2 is much less abundant. Isozyme H8, however, is the predominant isozyme under nitrogen limitation and cannot be detected under carbon limitation. The time of maximal enzyme expression is also different depending on the nutrient limitation used to induce the ligninolytic system. In carbon-limited cultures, lignin peroxidase activity appears early in secondary metabolism and then gradually decreases, but in nitrogen-limited cultures the activity appears later in the time course and gradually increases.

The lignin and Mn peroxidases are not expressed at the same time during secondary metabolism in carbon- or nitrogen-limited cultures. This demonstrates that the two classes of peroxidases are not coordinately regulated (30, 35). Data presented here show that within the Mn peroxidase isozyme family, each isozyme is also differentially regulated. This was revealed by comparison of the isozymes produced in carbon- versus nitrogen-limited cultures as well as by comparison of isozymes produced within carbon- and nitrogen-limited cultures at different times during secondary metabolism. In the extracellular fluid of carbon-limited cultures, isozyme H4 is the predominant isozyme, little if any isozyme H5 is produced, and isozyme H3 is not detected until late in secondary metabolism, at which time it is abundantly expressed. In the extracellular fluid of nitrogen-limited cultures, isozymes H3, H4, and H5 are all present at approximately the same time (abundant early in secondary metabolism and then decreasing over time) along with numerous other Mn peroxidase isozymes. Leisola and coworkers (30) also characterized the Mn peroxidase isozymes produced in nitrogen- and carbon-limited cultures. However, these workers used agitated cultures to characterize carbon limitation and stationary cultures to characterize nitrogen limitation. They observed differences in the isozyme patterns; however, variations in the isozymes expressed can arise solely on the basis of whether the cultures were grown with agitation or whether they were stationary (6). Thus, it is difficult to compare their results with those presented here.

In carbon-limited cultures, the increase in isozyme H3 content coincides with the increase in total Mn peroxidase activity late in secondary metabolism, when the H4 mRNA decreases. Since the mRNA level detected with the H4 cDNA probe is decreasing while Mn peroxidase activity and protein levels are increasing, it suggests that H3 mRNA does not strongly hybridize to the λMP-1 cDNA probe which encodes isozyme H4. This is somewhat surprising in light of the similarity between the N-terminal sequences of these two isozymes. However, we have recently isolated other cDNAs encoding Mn peroxidases which only weakly hybridize to λMP-1, implying that the genes may differ enough to prevent cross-hybridization under stringent conditions.

In addition to differences in the isozyme profile observed between carbon- and nitrogen-limited cultures, differences were also observed in enzyme stability. After cycloheximide addition, a half-life of less than 12 h was determined for the Mn peroxidase activity in nitrogen-limited cultures. In contrast, less than 20% loss of activity was observed in 72 h for carbon-limited cultures. The basis for this stability is not clear. Extracellular proteases produced by *P. chrysosporium* (8) may account for the rapid turnover in nitrogen-limited cultures (9). However, these proteases appear during early primary metabolism and much later in secondary metabolism, when carbon is depleted from the medium. Although low protease activity is detected during days 3 to 5 (8), it may account for the rapid turnover in nitrogen-limited cultures. It is also not surprising that a higher rate of enzyme turnover is observed during nitrogen limitation because this is when cellular nitrogen pools are under flux. Another factor which could facilitate the rapid turnover of Mn peroxidases in nitrogen-limited cultures is heme destruction by H_2O_2. Similar to lignin peroxidase (40), H_2O_2 inactivates Mn peroxidase at high concentrations, and reducing substrates, such as Mn^{2+}, can protect the enzyme from inactivation. For H_2O_2 inactivation of the enzyme to account for the difference in stability of the Mn peroxidases in carbon- and nitrogen-limited cultures, the H_2O_2 content would have to be much higher in nitrogen-limited cultures than in carbon-limited cultures. Because of difficulties in the measurement of H_2O_2 levels in vivo, it is difficult to test this hypothesis. However, some indirect evidence would suggest that this is true. A proposed end product of Mn peroxidase-catalyzed oxidation of Mn^{2+} is MnO_2 (14, 22, 23). MnO_2 has been proposed to be the chromophore which causes the browning of the cultures (14). Although both carbon- and nitrogen-limited cultures produce Mn peroxidases, only nitrogen-limited cultures turn brown (producing MnO_2). This would be consistent with higher levels of H_2O_2 produced by nitrogen-limited cultures than by carbon-limited cultures. Inactivation by H_2O_2, in

turn, may make the enzymes more susceptible to proteolysis.

It is difficult to assess the physiological significance of the greater stability of the Mn peroxidases relative to the lignin peroxidases in both carbon- and nitrogen-limited cultures. If this stability is intrinsic to the isozyme and is also observed on decaying wood, it would suggest that Mn peroxidase activity is required at a greater distance from the growing hyphae. This actually has been observed in immunogold detection of Mn peroxidases in decayed wood (5).

Bonnarme and Jeffries (1) and Brown et al. (3) reported that the addition of Mn to cultures caused an increase in Mn peroxidase activity. This was attributed to enhanced rates of transcription as determined by hybridization to one of the Mn peroxidase cDNAs (3). Our results here are in accord with these findings, with an increase in Mn peroxidase activity being observed in both nitrogen- and carbon-limited cultures with increasing concentrations of Mn^{2+} in the media. We have further demonstrated that the increase in Mn peroxidase activity upon Mn^{2+} addition can be attributed to a coordinated increase in all isozymes already present. However, the response to Mn^{2+} addition is not the same for all isozymes. A greater increase in isozyme H3 content than in H4 content was observed upon addition of $MnSO_4$ to carbon-limited cultures. This is consistent with differential regulation of the Mn peroxidase isozymes and suggests that they are not controlled by the same regulatory elements.

If the Mn peroxidase isozymes served different functions in lignin biodegradation, kinetic differences among the Mn peroxidase isozymes would be expected. Indeed, more than a fourfold variation is observed in k_{cat} and K_m values for the substrates H_2O_2, Mn, and vanillylacetone. This variation in kinetic constants is of the same order of magnitude as that observed with the lignin peroxidase isozymes and lignin model compounds (11). Although these kinetic differences do not reveal the role of each isozyme in lignin biodegradation, they do suggest that each isozyme has a preferred substrate in lignin biodegradation.

In conclusion, the results presented here clearly demonstrated the complexity of the Mn peroxidase isozyme family. We have shown that the major isozymes H3, H4, and H5 are not derived from a single gene. The isozymes are not identically regulated, suggesting different functions for these isozymes. The number of active Mn peroxidases may be greater than just these three isozymes. Our IEF gels demonstrate the presence of numerous bands that cross-react with the antibody to isozyme H4 and exhibit phenol red-oxidizing activity. This suggests that the bands are not inactive, degraded proteins but indeed represent other, as yet unidentified, active Mn peroxidase isozymes. The role of the specific Mn peroxidase isozymes, as well as the lignin peroxidase isozymes, in the complicated process of lignin biodegradation is still not known and poses a challenging scientific question. Further molecular and enzymological studies, ongoing in many laboratories, will help unravel this complicated process.

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