# Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*

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**Abstract** A total of 11 manganese peroxidase isoenzymes ($MnP_1$–$MnP_{11}$) with isoelectric points (pIs) in the range of 4.58–3.20 were isolated from liquid- and solid-state cultures of the basidiomycete *Ceriporiopsis subvermispora*. In the presence of hydrogen peroxide, these isoenzymes showed different requirements for Mn(II) in the oxidation of vanillylacetone, *o*-dianisidine, *p*-anisidine and ABTS, whereas oxidation of guaiacol by any isoenzyme did not take place when this metal was omitted. $K_m$ values for *o*-dianisidine and *p*-anisidine in the absence of Mn(II) are in the range of 0.5–1.0 mM and 4.5–42.0 mM, respectively. Oxalate and citrate, but not tartrate, accelerate the oxidation of *o*-dianisidine, both in the presence and in the absence of Mn(II). MnPs from this fungus are able to oxidize kojic acid without externally added hydrogen peroxide, indicating that they can also act as oxidases. In this reaction, however, the requirement for Mn(II) is absolute.

*Key words:* Lignin degradation; Basidiomycete; Peroxidase; Manganese; Enzymatic oxidation

## 1. Introduction

Manganese-dependent peroxidase is an ubiquitous enzyme among ligninolytic fungi [1,2]. This enzyme catalyzes the oxidation of Mn(II) to Mn(III), which in turn can oxidize a variety of phenolic substrates, including lignin model compounds. The catalytic cycle of MnP is similar to that of other peroxidases [3,4]. First, hydrogen peroxide oxidizes the enzyme by two electrons to form compound I. The latter can be reduced by Mn(II) or a phenolic substrate to compound II, the one electron oxidized form of the enzyme. However, only Mn(II) can serve as a reductant of compound II to yield resting enzyme [3–5]. MnP oxidation capacity is more pronounced in the presence of chelators, which facilitate the reaction of Mn(II) with the enzyme and also stabilize Mn(III) in solution [5,6].

Usually, MnP activity is produced in the form of several isoenzymes [7–10], a phenomenon also observed in the case of the ligninolytic enzymes lignin peroxidase and laccase [1,2]. The significance of this multiplicity is not well understood, although in *Phanerochaete chrysosporium* it has been shown that the MnP isoenzymes are differentially regulated in response to nutrient limitation and culture age [11]. The highest number of MnP isoenzymes has been found in the white-rot basidiomycete *C. subvermispora* [12,13]. In liquid cultures in salt medium, this

fungus produces seven isoenzymes of MnP with pIs of 4.58–4.10, whereas when growing on wood chips, it produces four isoenzymes with pIs of 4.40–3.53 [14]. Amino-terminal sequences of several of these isoenzymes reveal mismatches that suggest the presence of more than one gene, while the consensus sequence shows clear homology to MnPs from other fungi [14].

Several approaches may be envisaged to investigate the purpose of isoenzyme multiplicity in ligninolytic fungi. One of them could be to study gene organization and gene expression under various culture conditions. Another suitable approach may be to conduct enzymological characterization of the isoforms, hoping to reveal differences that might have some significance for the process of ligninolysis. The present report summarizes some of the results obtained following the latter approach.

## 2. Materials and methods

### 2.1. Fungus

*C. subvermispora* strain FP-105752 was obtained from the Center for Forest Mycology Research (Forest Products Laboratory, Madison, WI). The fungus was maintained on agar slants of potato dextrose agar medium (Difco).

### 2.2. Cultivation

Liquid cultures (830 ml) of the fungus in salt medium [13] containing 11 ppm (195 $\mu$M) of $MnSO_4$ and 10 mM ammonium tartrate were grown at 30°C with agitation (120 rpm) in 2-l Erlenmeyer flasks. They were inoculated with 370 mg (dry weight) of homogenized mycelium [13]. Solid-state cultures were conducted in 2-l flasks containing 260 g of wood chips from radiata pine. Conditions of growth as well as enzyme extraction from the chips were as previously described [14].

### 2.3. Purification of MnP isoenzymes

Extracellular fluid from liquid cultures was precipitated with ammonium sulfate (60%) and the pellet was resuspended in 30 ml of 25 mM sodium acetate, pH 4.6. This solution was dialyzed extensively against water and then subjected to IEF in a Rotofor (Bio-Rad) using ampholites Bio-Lyte 3/5 (Bio-Rad). This step did not resolve individual MnP isoforms, but gave a broad peak of activity. The fractions were pooled and solid NaCl was added to a final concentration of 1 M to displace the ampholites. The solution was extensively dialyzed against 25 mM sodium acetate, pH 4.6, prior to preparative IEF conducted as indicated below. In turn, the protein extract obtained from wood chips was concentrated and fractionated by chromatography on Q-Sepharose as already reported [14]. MnP isoforms present in the pools from the rotofor and the Q-Sepharose column were then resolved by preparative IEF in separate gels as follows: 18–20 mg of protein from each pool were applied to 5% polyacrylamide gels (12 cm × 8 cm × 0.5 cm) that also contained 5% (w/v) glycerol, 300 $\mu$l of 0.1% riboflavin-5'-phosphate, 180 ml of 10% ammonium persulfate, 20 ml of TEMED and 2% (w/v) Bio-Lyte 3/5 (Bio-Rad). To facilitate gel formation, a fluorescent lamp was placed 5 cm above the tray containing the mixture. The anodic fluid (pH 3) contained, per liter of solution, 3.3 g of aspartic acid and 3.7 g of glutamic acid, whereas the catodic fluid (pH 10) contained, per liter, 4 g of lysine, 4 g of arginine and 120 ml of ethylenediamine. After applying the protein sample, the gels were run at 4°C: 180 min at 200 V, 60 min at 400 V and 20 min at 600 V. To visualize the MnP

bands, the gels were developed with 62 $\mu$M guaiacol as substrate and each band was excised with a razor blade. The corresponding activities were electroeluted overnight at 4°C in dialysis bag (cut off 3500 kDa) containing 2 ml of 50 mM sodium acetate, pH 4.6. Following the above procedures, 10–40 mg of protein (1–5 U) of each MnP isoenzyme were obtained in a final volume of ~1 ml.

### 2.4. Substrate oxidation experiments

Enzyme activity was measured at 30°C using a Shimadzu 160A UV-visible recording spectrophotometer. Reaction mixtures (1.0 ml), unless otherwise indicated, contained 100 mM sodium tartrate, pH 5.0, 0.1 mM $MnSO_4$, 0.05 mM hydrogen peroxide, 0.1 mM substrate and 0.02 U of MnP. 1 U of MnP activity is defined as the amount of enzyme required to oxidize 1.0 mmol of vanillylacetone per min. The wavelengths used to monitor the progress of the reactions with the various substrates are indicated in the figures or in the text. Initial velocities were recorded between 0 and 10 s. $K_m$ values for o-dianisidine and p-anisidine were determined with 0.015 and 0.007 U of MnP, respectively, using Eadie-Hofstee plots. In the experiments with kojic acid, the reactions contained 10 mM of this substrate, 100 mM sodium succinate, pH 5.0, as buffer and 0.06 U of MnP.

### 2.5. Other methods

Analytical IEF was performed using Servalyt Precotes 3–6 polyacrylamide gels from Serva [14]. SDS-PAGE was conducted as reported by Laemmli [15] and the protein bands were stained with a silver stain kit (Sigma). Protein concentration was measured by the method of Bradford [16]

## 3. Results

### 3.1. Isoenzyme properties

MnP isoenzymes from both liquid- and solid-state cultures of C. subvermispora were purified as described in Section 2. Seven isoforms were isolated from the cultures in salt medium, whereas four isoenzymes were fractionated in the extract deriving from the wood chips. Fig. 1A shows an analytical IEF gel of the former isoenzymes. In turn, Table 1 contains a list of the 11 isoforms with their corresponding pIs. Aliquots of the isoenzymes from liquid cultures were also subjected to PAGE-SDS to calculate their corresponding molecular masses. As shown in Fig. 1B, these isoforms exhibit a similar molecular mass of ~52.5 kDa. In contrast, the molecular mass of the individual isoenzymes from solid-state cultures is 62.5 kDa [14].

### 3.2. Requirement of Mn(II)

The MnP isoenzymes were compared with respect to their requirement of Mn(II) for the oxidation of different substrates. As expected, all the reactions proceeded more rapidly in the presence of this ion. However, dependence on Mn(II) varied among the isoenzymes and it was also less severe with certain substrates. Fig. 2 depicts representative reactions of different MnP isoenzymes with guaiacol (A), vanillylacetone (B), o-dianisidine (C) and ABTS (D). Requirement of Mn(II) for the oxidation of guaiacol was virtually absolute for all the isoenzymes. A high dependence on Mn(II) was also observed in the oxidation of vanillylacetone, particularly by the isoenzymes produced in liquid cultures ($MnP_1$-$MnP_7$) (Table 1). The reactions exhibiting the lowest demand for Mn(II) were the oxidations of o-dianisidine and p-anisidine (Table 1), which allowed the determination of $K_m$ values for these substrates in the absence of this metal (Table 1). Oxidation of ABTS also showed different requirements of Mn(II) by the various MnPs. A continuous measurement of the progress of the reaction with ABTS also revealed that oxidation of this substrate, both in the presence and in the absence of Mn(II), has a biphasic kinetics (Fig. 2).
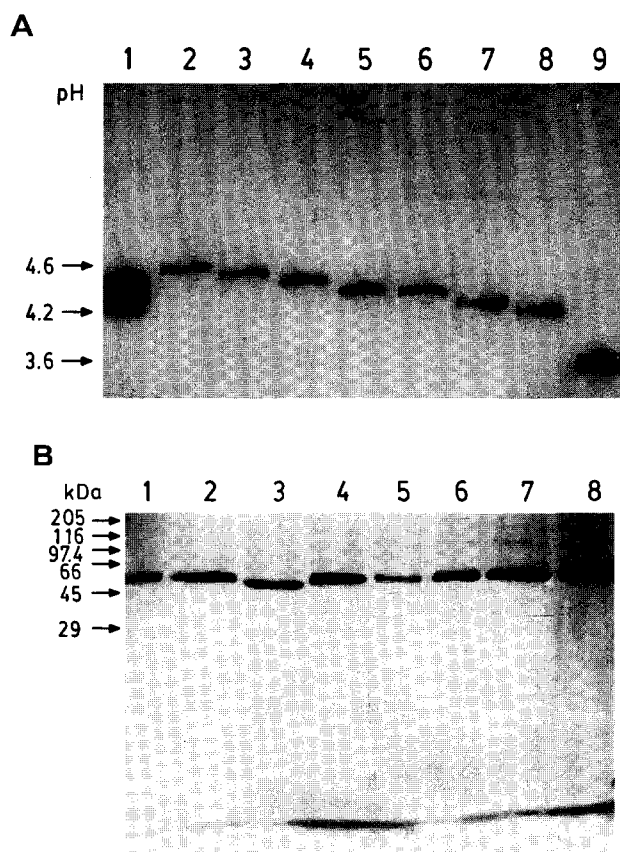


Fig. 1. (A) IEF of MnPs from C. subvermispora. Lane 1: pool of MnPs produced in liquid cultures in salt medium; lanes 2–8: individual isoenzymes from liquid cultures (pIs 4.58, 4.52, 4.49, 4.43, 4.31, 4.22 and 4.13, respectively); lane 9: pool of MnPs produced in cultures on wood chips. (B) SDS-PAGE (12% gel) of MnPs from liquid cultures, after preparative IEF. Lane 1: pool of MnPs; lanes 2–8: $MnP_1$ to $MnP_7$, respectively. In this experiment, 0.5 $\mu$g of protein were applied to each lane. Numbers at the left correspond to molecular mass markers (Sigma 6-H).

### 3.3. Effect of oxalate

Oxalate, a metabolite secreted by some white-rot fungi [17–19], has been shown to be an efficient chelator in reactions catalyzed by MnP from P. chrysosporium [20,21]. Since we have also found oxalate in both liquid and solid cultures of C. subvermispora (unpubl. data), we decided to investigate if this compound would also activate MnPs produced by this fungus. These reaction mixtures were buffered with sodium succinate instead of sodium tartrate, since the former anion does not act as a chelator of Mn(III) [5,21]. As shown in Fig. 3A, oxalate activates the oxidation of o-dianisidine by $MnP_3$ in the presence of Mn(II), with an optimal concentration of 1 mM. Concentrations of oxalate up to 5 mM gave a reaction curve similar to that obtained with 1 mM (data not shown). Unexpectedly, oxidation of o-dianisidine by $MnP_3$ was also stimulated by oxalate in the absence of Mn(II), although in this case the optimal concentration of oxalate was 10 mM (Fig. 3B). Both reactions were also stimulated by sodium citrate (5 mM with or without Mn(II)). Similar results were obtained with isoenzyme $MnP_9$ (data not shown).
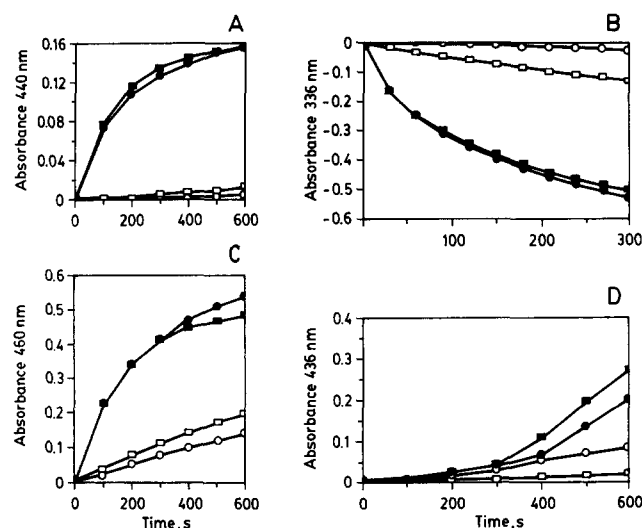
Fig. 2. MnP-catalyzed oxidation of guaiacol (A), vanillylacetone (B), o-dianisidine (C) and ABTS (D), in reactions lacking (open symbols) or containing (closed symbols) 0.1 mM Mn(II). Assays were conducted in 100 mM sodium tartrate, pH 5.0, and contained 0.05 mM $H_2O_2$, 0.01 mM substrate and 0.3–0.5 µg of MnP. Isoenzymes employed in the assays were: (A) circles, MnP7; squares, MnP10; (B) circles, MnP3; squares, MnP8; (C) circles, MnP7, squares MnP9; (D) circles, MnP3; squares, MnP9.
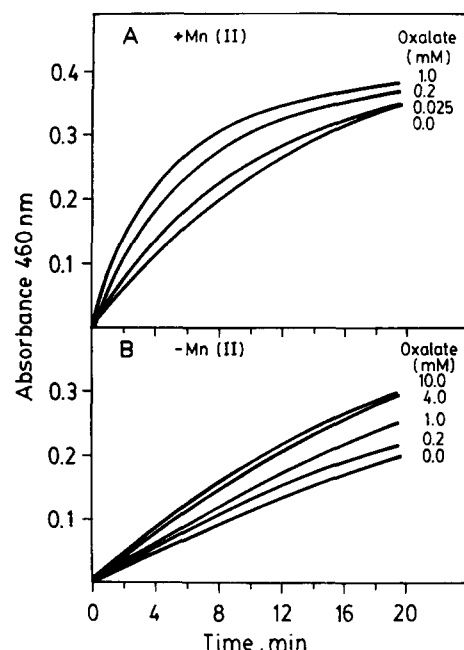


Fig. 3. Effect of oxalate on the oxidation of o-dianisidine catalyzed by MnP3, both in the presence (A) and in the absence (B) of externally added Mn(II), in reactions buffered with 100 mM sodium succinate, pH 5.0.

### 3.4. Oxidation of kojic acid

In the course of these studies, we observed that the compound kojic acid [22] is oxidized by MnPs from *C. subvermispora* in a Mn(II)-dependent reaction. Oxidation of kojic acid also takes place, although less rapidly, in the absence of hydrogen peroxide (Fig. 4). Addition of catalase to the latter incubation mixture produces a delay in kojic acid oxidation, strongly suggesting that hydrogen peroxide participates in the reaction as a transient intermediate (Fig. 5).

### 4. Discussion

The first challenge to conduct this work was to isolate the MnPs produced by *C. subvermispora*, free of cross contamination. Their fractionation by preparative IEF by the procedure described in Section 2 allowed the fulfillment of this objective.

More simplified protocols of preparative IEF had been successfully utilized by our group for amino terminal sequencing of MnPs [14], as well as for amino-terminal and substrate specificity studies of the laccases produced by the fungus [23].

As stated above, only compound I of MnP from *P. chrysosporium* can be reduced by phenolic compounds. Mn(II) is the exclusive reductant of compound II, hence its requirement for the completion of the catalytic cycle of the enzyme [3–5]. We have found that the requirement for Mn(II) by MnPs from *C. subvermispora* varies according to the substrate added to the reaction mixture and it is different for each isoenzyme. Thus, with vanillylacetone, the phenolic compound routinely used in MnP assays [24], the isoenzymes are activated 12.6–108-fold by Mn(II), with a more pronounced effect of the metal on the isoenzymes produced in liquid cultures (MnP1–MnP7) (Table 1). On the other hand, oxidation of p-anisidine and o-dianisid-

Table 1
Effect of Mn(II) on the activity of manganese peroxidase isoenzymes from *C. subvermispora* with various substrates

| Isoenzyme | pI | vi* + Mn(II)/ vi − Mn(II) vanillylacetone | vi + Mn(II)/ vi − Mn(II) o-dianisidine | $K_m$ (mM)** o-dianisidine | vi + Mn(II)/ vi − Mn(II) p-anisidine | $K_m$ (mM) p-anisidine |
|---|---|---|---|---|---|---|
| MnP1 | 4.58 | 108.4 | 4.3 | 0.65 | 17.3 | n.d. |
| MnP2 | 4.52 | 96.2 | 5.3 | n.d. | n.d. | n.d. |
| MnP3 | 4.49 | 96.3 | 5.9 | 0.52 | 13.7 | 32.08 |
| MnP4 | 4.43 | 66.0 | 5.9 | n.d. | 10.3 | 36.03 |
| MnP5 | 4.31 | 63.8 | 4.4 | n.d. | 10.1 | n.d. |
| MnP6 | 4.22 | 33.6 | 4.8 | n.d. | 9.2 | 41.80 |
| MnP7 | 4.13 | 42.0 | 4.5 | n.d. | 7.7 | n.d. |
| MnP8 | 3.53 | 12.6 | 2.7 | n.d. | 7.7 | 5.31 |
| MnP9 | 3.40 | 28.6 | 2.6 | 1.05 | 7.2 | 4.46 |
| MnP10 | 3.30 | 29.7 | 2.4 | 0.68 | 8.1 | 5.04 |
| MnP11 | 3.20 | n.d. | n.d. | n.d. | 9.7 | 6.44 |

*vi, initial velocity.
**$K_m$ were determined in the absence of externally added Mn(II).

ine proceed at significant rates in the absence of Mn(II), being the dependence on this metal also more evident with the isoenzymes from liquid cultures. The fact the MnPs from *C. subvermispora* can complete their catalytic cycle in the absence of Mn(II) has proved useful in the development of IEF gels, since in the sole presence of hydrogen peroxide, *p*-anisidine and *o*-dianisidine yield a red-brown and a pink color, respectively (data not shown). In contrast, oxidation of guaiacol by any isoenzyme in the absence of Mn(II) was negligible, two examples of which are shown in Fig. 2. To our knowledge, MnPs from *P. chrysosporium* have not been tested with *p*-anisidine and *o*-dianisidine as substrates, which would allow to discriminate between a general ability of these compounds to reduce compound II of any MnP vs. an intrinsic characteristic of MnPs from *C. subvermispora*.

As mentioned in Section 1, reactions catalyzed by MnP are accelerated by chelators, which facilitate the interaction of Mn(II) with the enzyme and also stabilize Mn(III) in solution [5,6]. Maximal stimulation of MnPs from *P. chrysosporium* takes place at concentrations of oxalate in the range of 0.5–2 mM [20,25], similar to that found optimal for oxidation of *o*-dianisidine by MnPs from *C. subvermispora* in the presence of Mn(II) (1 mM). However, the latter reaction was also stimulated by oxalate in the absence of Mn(II), although at a higher concentration of this organic acid (10 mM). Due to the proposed roles for oxalate, this effect is difficult to explain. Although kinetic evidence obtained by Kishi et al. [20] indicate that chelators do not bind directly to the iron of the heme group, these authors do not preclude the existence of a binding site close to the heme. On the other hand, Sundaramoorthy et al. [26] have suggested that oxalate may interact with the enzyme by replacing the two water molecules located near the Mn(II)-binding site. If this direct interaction between MnP and oxalate does indeed take place by either one of these or even by another mode, it is conceivable that it may render the enzyme more susceptible to reduction by *o*-dianisidine. With respect to a similar activation observed with citrate, we do not know if this effect has any physiological significance, since this metabolite has not been detected by our group in cultures of *C. subvermispora*.

Ligninolytic fungi require an extracellular system to produce the hydrogen peroxide utilized as an oxidant by LiPs and MnPs. An enzyme called glyoxal oxidase that acts in conjunction with LiP and possesses a wide substrate specificity has been identified in *P. chrysosporium* [27,28]. The best substrates for this enzyme are glyoxal and methylglyoxal, although they are
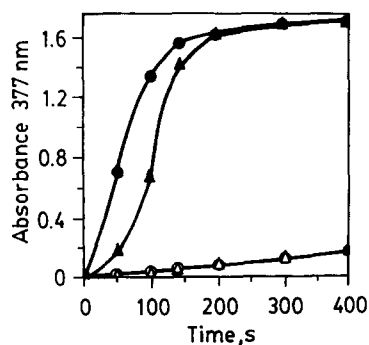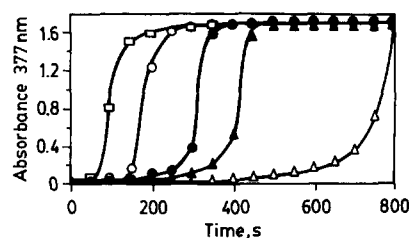


Fig. 5. Effect of catalase on the oxidation of kojic acid by MnP$_9$. Reaction mixtures contained 0 (□, 2 (○), 4 (●), 5 (▲) and 10 (△) $\mu$g of catalase (Mann Research Laboratories).

present in the extracellular fluid at concentrations considerably lower than their corresponding $K_m$ values. Either this enzyme or other oxidases able to use substrates such as glucose, galactose, methanol and aryl alcohols as substrates have not been found in cultures of *C. subvermispora* [13] (unpubl. data). For this reason, we are considering the possibility that MnP from *C. subvermispora* may act as an oxidase of certain metabolites present in the cultures, thus providing itself with the hydrogen peroxide required to oxidize the enzyme to compound I. We have shown that MnP from *C. subvermispora* can indeed oxidize a compound, in this case kojic acid, in the absence of hydrogen peroxide. Inhibition by catalase indicates that hydrogen peroxide is generated during the reaction, which could arise by scavenging by dissolved oxygen of radicals generated by auto oxidation of kojic acid [30] or by oxidation of this compound by traces of Mn(III) [31]. Although we have not detected kojic acid in cultures of *C. subvermispora*, oxalate could represent a putative candidate to act as a reductant of oxygen to form hydrogen peroxide, as suggested by other authors [25,29]. Studies are in progress to determine the actual involvement of oxalate in the generation of hydrogen peroxide in cultures of *C. subvermispora*.

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Fig. 4. Oxidation of kojic acid by MnP$_9$. (●): + H$_2$O$_2$, + Mn(II); (▲): −H$_2$O$_2$, + Mn(II); (○): + H$_2$O$_2$, −Mn(II) and (▲): H$_2$O$_2$, −Mn(II).

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