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Evidence for a new extracellular peroxidase

Manganese-inhibited peroxidase from the white-rot fungus *Bjerkandera* sp. BOS 55

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A novel enzyme activity was detected in the extracellular fluid of *Bjerkandera* sp. BOS 55. The purified enzyme could oxidize several compounds, such as Phenol red, 2,6-dimethoxyphenol (DMP), Poly R-478, ABTS and guaiacol, with H_2O_2 as an electron acceptor. In contrast, veratryl alcohol was not a substrate. This enzyme also had the capacity to oxidize DMP in the absence of H_2O_2. With some substrates, a strong inhibition of the peroxidative activity by Mn^{2+} was observed. Phenol red oxidation was inhibited by 84% with only 1 mM of this metal ion. Because DMP oxidation by this enzyme is only slightly inhibited by Mn^{2+}, this substrate should not be used in assays to detect manganese peroxidase. The enzyme is tentatively named 'Manganese-Inhibited Peroxidase'.

Ligninase; Manganese peroxidase; White-rot fungus; Lignin degradation; Manganese-inhibited peroxidase; *Bjerkandera*

1. INTRODUCTION

White-rot fungi are capable of depolymerizing lignin and metabolizing it to CO_2 and H_2O. The ligninolytic enzyme complex of the fungus *Phanerochaete chrysosporium* has been studied most extensively. Two extracellular peroxidases, ligninase (LiP) and manganese peroxidase (MnP), are thought to have an important role in the initial lignin degradation [1,2]. Both peroxidases were discovered in the extracellular fluid of this organism [3–5]. The mechanism of lignin model compound oxidation by LiP was elucidated in 1985 [6], but its exact function in lignin degradation is still a point of dispute [7,8]. Also there are conflicting reports as to the importance of MnP in the ligninolytic system [2,9].

Waldner et al. [10] and Nerud et al. [11] have detected extracellular peroxidase activity in the culture fluids of various white-rot fungi (*Bjerkandera adusta*, *Pycnoporus cinnabarinus* and *Dichomitus squalens*) that cannot be attributed to LiP or MnP. Moreover, the unknown peroxidase activity on ABTS or Phenol red was inhibited by Mn^{2+}.

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Abbreviations: DMP, 2,6-dimethoxyphenol; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DTT, dithiothreitol; LiP, ligninase (i.e. lignin peroxidase); MnP, manganese peroxidase; HRP, horseradish peroxidase; MIP, manganese-inhibited peroxidase.

In this study, we present the isolation and initial characterization of a manganese-inhibited peroxidase (MIP) from the white-rot fungus *Bjerkandera* sp. BOS 55.

2. MATERIALS AND METHODS

2.1. *Organism and culture conditions*

BOS 55 was isolated from forest soil samples with a selective medium containing hemp (*Cannabis sativa*) stem wood (0.2%), benomyl (15 ppm), guaiacol (0.01%) and agar (1.5%). BOS 55 was determined as a *Bjerkandera* sp. by the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands). The fungus was maintained on malt agar plates at 4°C. BOS 55 was cultivated under static conditions at 30°C in 5 l erlenmeyer flasks on hemp stem wood medium (500 ml containing 0.2% hemp stem wood in 10 mM dimethylsuccinate, pH 4.5). Cultures were inoculated with two cylindrical agar plugs (6 mm diameter) from the agar plates.

2.2. *Enzyme purification*

Medium from 10–12-day-old cultures was centrifuged at 20,000 × *g* for 30 min to remove mycelium. The supernatant was filtered through a 595½ filter (Schleicher & Schuell, Dassel, Germany) and concentrated on DEAE-Sepharose CL-6B column (25 × 2.8 cm). The adsorbed protein was washed with starting buffer (100 ml) and eluted with a linear gradient from 0 to 1 M NaCl in 10 mM sodium acetate, pH 5.5. Active fractions (250–350 mM NaCl) were pooled, washed with 10 mM sodium acetate, pH 5.5, and concentrated by ultrafiltration through a PM-10 membrane (Amicon, Rotterdam, The Netherlands). 2 ml of the concentrated fraction was purified with gel filtration. Using Sephadex G-100 gel chromatography with 20 mM sodium acetate/0.1 M NaCl, pH 5.5, buffer (47 ml/h, column 65 × 1.7 cm), one peak of activity was obtained. Active fractions were pooled, concentrated and washed as described above. Concentrated enzyme oxidized 2,6-dimethoxyphenol (DMP) with an activity of 0.037 absorbance units/(min·μl supernatant).

Native-PAGE was performed with 2 μl of the washed G-100 fraction on a Phast system (Pharmacia, Uppsala, Sweden).

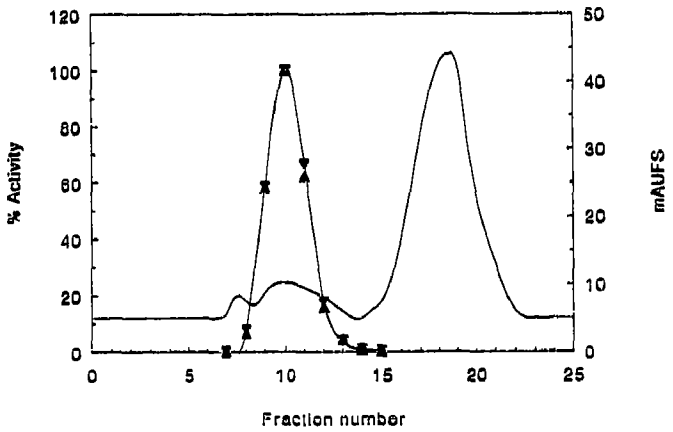

Fig. 1. Chromatography of *Bjerkandera* BOS 55 extracellular proteins on a Sephadex G-100 gel filtration column. Absorbance at 280 nm (–), oxidase activity with DMP (▲) and peroxidase activity with DMP (▼).

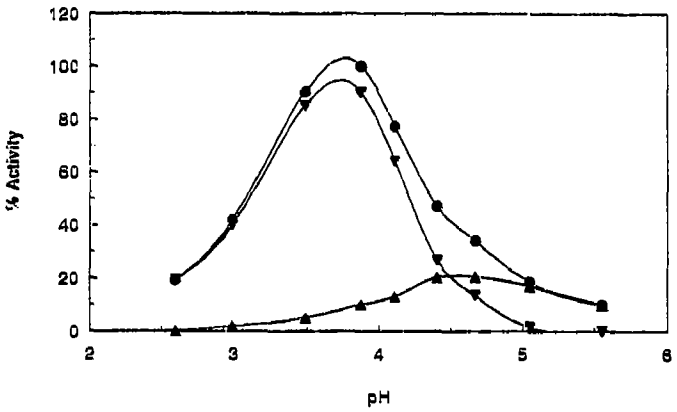

Fig. 3. pH-dependency of DMP oxidation rate. Mixtures contained 2 mM DMP in 0.1 M sodium tartrate buffers (oxidase activity (▲)) or mixtures contained 2 mM DMP and 0.4 mM H_2O_2 in 0.1 M sodium tartrate buffers (total activity (●)). Peroxidase activity (▼) was calculated by subtracting the oxidase activity from the total activity.

2.3. *Analytical methods*

Peroxidase activity was measured by the oxidation of Phenol red at 510 nm or DMP at 468 nm. The reaction mixture contained up to 300 μl culture broth or enzyme solution in 50 mM sodium tartrate, pH 4.0, and 0.01% Phenol red or 2 mM DMP. The reaction was started by the addition of 0.4 mM H_2O_2 in a total volume of 0.5 ml.

2.4. *Chemicals*

DEAE-Sepharose CL-6B and Sephadex G-100 are products of Pharmacia (Uppsala, Sweden). Horseradish peroxidase grade II (HRP), DTT and ABTS were from Boehringer (Mannheim, Germany). All other chemicals were commercially available and used without further purification.

3. RESULTS AND DISCUSSION

In the extracellular fluids of 10–12-day-old cultures of *Bjerkandera* sp. BOS 55, a new peroxidase was found that oxidizes Phenol red. The purified peroxidase seems to be different from LiP and MnP. The new peroxidase showed no activity with veratryl alcohol, which is an indicative substrate for LiP. Moreover, the new peroxidase oxidized DMP which is not a substrate of LiP [12]. Although DMP is oxidized by MnP [12], the new enzyme is not stimulated by Mn^{2+} addition and, moreover, the oxidation of Phenol red is actually strongly inhibited by Mn^{2+}. For this reason, the new peroxidase is tentatively denominated as manganese-inhibited peroxidase (MIP). The fact that the activity was truly due to an enzyme was confirmed by observing the loss of all activity after 20 min of boiling. MIP was also able to oxidize DMP in the absence of H_2O_2. As shown in Fig. 1, the normalized peroxidase and oxidase activities in the different fractions from Sephadex G-100 gel chromatography were identical, indicating that the same enzyme was involved. To test this hypothesis, native-PAGE electrophoresis was performed. As can be seen in Fig. 2, both oxidase- and peroxidase staining with DMP occurred in the same protein band which confirms that the oxidase and the peroxidase activities originate from the same enzyme.

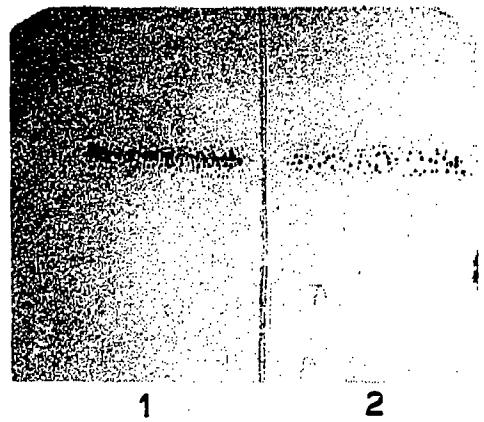

Fig. 2. Native-PAGE electrophoresis of G-100 fraction. Enzyme activity was detected by immersing half of the gel in 50 mM sodium tartrate, pH 4.0, containing 2 mM DMP and 0.4 mM H_2O_2 (1) and the other half in 50 mM sodium tartrate, pH 4.7, containing 2 mM DMP (2).

The occurrence of both oxidase and peroxidase activities in the same enzyme was also noted by Blaich and Esser [13]. Additionally, we found that even HRP displayed limited oxidase activity with DMP as substrate. It is known [16] that HRP has oxidase activity with dihydroxyfuramic acid. With Phenol red as substrate, MIP did not have detectable levels of oxidase activity. Extracellular fluid of *Bjerkandera adusta* [17,18] has both peroxidase and oxidase activity with veratryl alcohol. In that case two distinct enzymes are involved, lignin peroxidase and veratryl (aryl) alcohol oxidase.

The pH optima for the oxidation of DMP in the absence and presence of H_2O_2 were 4.5 and 3.8, respectively (Fig. 3). Almost the same pH optimum for peroxidase activity with Phenol red and DMP was found (Fig. 4). As can be seen in Fig. 3, the oxidase activity at pH 4.0 accounted for only 10% of the total activity with DMP as substrate.

Several substrates were tested for oxidation by MIP

Table I

Oxidation of various substrates by manganese inhibited peroxidase (MIP) and horseradish peroxidase (HRP)[a]

Substrate	λ (nm)	MIP		HRP		MIP:HRP[b]
		ACT[c] (ΔABS/(min·ml))	Mn INH[d] (%)	ACT[c] (ΔABS/(min·μg))	Mn INH[d] (%)	
ABTS	415	118.0	29.2	252.0	<5.0	0.47
DMP	468	37.0	<5.0	1.6	14.4	23.1
Phenol red	510	8.0	84.4	5.7	94.8	1.4
Guaiacol	450	8.0	9.4	10.3	<5.0	0.78
Poly R-478[e]	520	0.18	N.D.[f]	0.074	N.D.[f]	2.4

[a] Rates of oxidation were followed at 30°C at the wavelengths indicated in the table. The complete reaction mixture (0.5 ml) contained 10 μl MIP enzyme (G-100 fraction) or 0.1 μg of HRP, 400 μM H_2O_2 and 100 μg substrate.
[b] Ratio of the rate of oxidation of substrates by MIP over the rate of oxidation by HRP under the conditions used.
[c] Activity expressed as delta absorbance units per min with 1 ml of MIP or with 1 μg of HRP.
[d] Inhibition of MIP and HRP by Mn^{2+} (1 mM final concentration).
[e] In order to observe the reaction, 100 μl MIP and 2.0 μg HRP were added.
[f] Not determined.

and HRP, both in the presence and absence of Mn^{2+}. It was found that with both enzymes the oxidation of some substrates was inhibited by manganese (Table I). Because the protein concentration in the purified fraction was too low to assay, only a relative comparison of the MIP and HRP substrate oxidation rates can be made. The MIP/HRP activity ratios of most of the substrates were comparable within one order of magnitude. However, DMP formed an important exception, since the activity ratio on this substrate was from 10- to 49-times higher compared to the other substrates.

The data presented in Table II show that DMP oxidation by MIP was markedly inhibited by sodium azide and potassium cyanide, which are both potent inhibitors of hemeprotein-catalyzed reactions. On the contrary, 1 mM of EDTA did not cause significant inhibition. In this respect, MIP is quite distinct from both LiP and MnP since several research groups report that these enzymes are highly inhibited by 1 mM EDTA [5,14]. Glenn and Gold [15] did not find inhibition of MnP by 1 mM EDTA when excess manganese was present.

The results of this study clearly indicate that aside from LiP and MnP, another peroxidase, MIP, is produced by white-rot fungi. Although we have only demonstrated the presence of MIP in one white-rot strain, the unknown peroxidase activity previously found in the extracellular culture fluids of various white-rot fungi [10,11] has striking similarities since the activity is also inhibited by Mn^{2+}. This seems to indicate that MIP is a ubiquitous enzyme of white-rot fungi. At present, it is not clear what role MIP plays in lignin degradation. Therefore, future studies should be directed at elucidating the function that MIP serves in the ligninolytic system of white-rot fungi. In any case, based on the fact that a third peroxidase has been iso-

Table II

Inhibition of initial DMP oxidation rates[a]

Inhibitor	% activity remaining
KCN	14.4
NaN_3	1.2
DTT	0.0
EDTA	97.6

[a] Mixtures contained 10 μl enzyme, 1 mM inhibitor and 2 mM DMP in 50 mM sodium tartrate, pH 4.0. After adding 0.4 mM H_2O_2 the total DMP oxidase activity was measured.

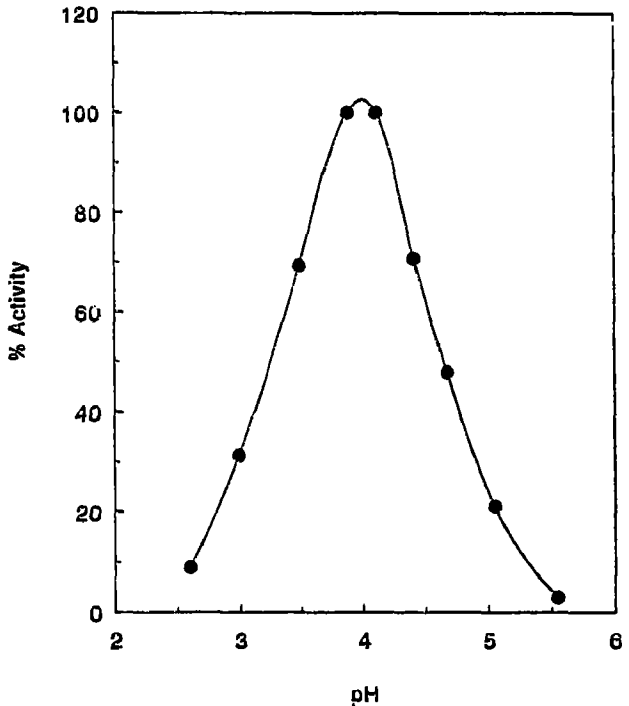

Fig. 4. pH-dependency of Phenol red oxidation rate. Mixtures contained 0.01% Phenol red and 0.4 mM H_2O_2 in 0.1 M sodium tartrate buffers.

lated from a white-rot fungus, the standard methods of measuring peroxidase activity in extracellular culture fluids must be reconsidered. This is particularly important for some substrates used for detecting MnP, such as DMP, which is also readily oxidized by MIP even in the presence of Mn^{2+}. Phenol red is a better substrate for distinguishing between MnP and MIP, since the oxidation is almost completely inhibited by Mn^{2+}. Furthermore, this study stresses once again that some peroxidases have phenol-oxidizing activity, which suggests that phenol oxidation in the absence of H_2O_2 is not really a suitable method for distinguishing laccases from peroxidases.

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