Manganese peroxidase from *Phanerochaete crassa* WD1694

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Abstract

A manganese peroxidase from the white-rot fungus *Phanerochaete crassa* WD1694, that had exhibited very high ability to bleach unbleached kraft pulp, was purified and characterized. The MnP was purified by adsorption-desorption on DEAE-Sepharose CL-6B and FPLC on DEAE-Toyopearl. The purified MnP gave a single band at 48.3 kDa on SDS-PAGE and could be separated into four isozymes at extremely close pIs (pI 4.61, 4.59, 4.52, 4.50) by isoelectric focusing. The N-terminal sequences of the four isozymes were highly homologous and similar to those of the MnPs from *P. chrysosporium*. The enzyme oxidized 2,6-dimethoxyphenol (DMP) with and without Mn(II) but did not oxidize veratryl alcohol. The optimal pH of *P. crassa* WD1694 MnP was 3.0-4.0 and lower than that (4.5-5.0) of *P. chrysosporium* and *P. sordida* MnPs. Apparent $K_m$ values for oxidation of Mn(II) and DMP without Mn(II) were $35.8 \times 10^{-3}$ mM and 30.7 mM, respectively. These results showed that the MnP from *P. crassa* WD1694 was very similar to the MnPs from *P. chrysosporium* in terms of catalytic properties and N-terminal sequences.

Key words: manganese peroxidase, *Phanerochaete crassa*, purification, lignin biodegradation, white-rot fungi, biobleaching

INTRODUCTION

White-rot fungi are the predominant decomposers of lignin. Lignin is an aromatic polymer with the substituents connected by both ether and carbon-carbon linkages and constitutes 20-30% of woody plant cell wall. Lignin degradation by white-rot fungi is an oxidative and non-specific process. Manganese peroxidases (MnPs), lignin peroxidases (LiPs) and laccases (Lacs) are three families of enzymes that are implicated in the biodegradation of lignin. All the three enzymes catalyze the one-electron oxidation of phenolic substrates to phenoxy radicals that can undergo certain degradation reactions of lignin. However, the catalytic mechanism among the three enzymes is different (Kirk et al., 1987, Gold et al., 1993, Kirk et al., 1985, Gold et al., 1989). LiP has higher redox potential than MnP and Lac and it can also abstract single electrons from non-phenolic aromatic rings to form cation radicals. MnP generates Mn(III) from Mn(II) and H$_2$O, and Mn(III), in turn, can oxidize a variety of phenolic substrates to phenoxy radicals. In addition to the three families of enzymes, versatile peroxidases (VPs) with both LiP and MnP catalytic properties have been reported (Palma et al., 2000, Mester et al., 1998, Martinez et al., 1996, Sarkar et al., 1997, Wariishi, 2002, Kuwahara, 2002).

In the past decade many attempts to utilize lignin biodegradability of white-rot fungi in pulping and bleaching processes have been reported (Srebotnik et al., 1996, Reid et al., 1994, Paice et al., 1993, Kondo et al., 1994, Hiraï et al., 1994, Nishida et al., 1988, Iimori et al., 1994). We have selected *Phanerochaete crassa* WD1694 for biobleaching of kraft pulp (Takano et al., 2001). The strain had much higher ability to bleach UKP than well-studied white-rot fungi *P. chrysosporium* and *Trametes versicolor*. MnP was the major ligninolytic enzyme of *P. crassa* WD1694 during the cultivation of UKP and little Lac activity and no LiP activity were found in the fungal treated pulp. In this paper, the purification and characterization of MnP from *P. crassa* WD1694 was studied.

MATERIALS AND METHODS

Strain

*Phanerochaete crassa* WD1694 was obtained from collections of the Forestry and Forest Products Research Institute and maintained on potato dextrose agar (PDA) at 4°C.

Cultivation

The mycelium of *P. crassa* WD1694 was grown...
on PDA at 26°C. A total of 10 mycelial mats with a 6 mm diameter was inoculated into the medium (100 ml) containing 0.06% KH₂PO₄, 0.04% K₂HPO₄, 0.05% MgSO₄·7H₂O, 5x10⁻⁴% CaCl₂, 0.01% yeast extract, 1% glucose and 0.1% NH₄H₂PO₄ in a 300 ml Erlenmeyer flask and the flask was placed in a rotary shaker at 30 °C and 100 rpm for 4 days. The grown mycelium was homogenized. To a freshly prepared medium (100 ml) containing 0.005% MnSO₄ and 0.05% Tween 80 in addition to the medium described above in a 300 ml Erlenmeyer flask, 10 ml of the homogenate was inoculated. After the fungus was cultivated in a rotary shaker at 30°C and 100 rpm for 3 days, the culture filtrate was recovered by filtering off the hyphae.

**Purification step**

All the purification procedures were carried out at 4 °C. The pH of culture filtrate (2100 ml) was adjusted to 5.9 and the filtrate was mixed with an adsorbent ion-exchanger, DEAE-Sepharose CL-6B (20 ml), in a beaker. After the crude enzyme was extracted from DEAE-Sepharose CL-6B with 50 ml of 10 mM acetate buffer, pH 5.5, containing 0.5 M NaCl, the eluate was desalted, concentrated, and loaded on a DEAE-Toyopearl column in FPLC system (Waters 650). The enzyme was eluted with a linear 0-0.5 M NaCl gradient in 20 mM acetate buffer, pH 5.5, and fractions with MnP activity were collected, desalted, and concentrated.

**Enzyme assay**

MnP was assayed by measuring optical density at 270nm (ε=11.59 mM⁻¹cm⁻¹) and at 30°C on the basis of the oxidation of MnSO₄ (Wariishi, 1992). One unit of enzyme activity was defined as amount of enzyme that oxidize 1μmol of substrate per minute. The standard reaction mixture contained 50 mM malonate buffer, pH 3.5, 5 mM MnSO₄, 20μM H₂O₂, and proper concentration (around 0.07μg/ml) of MnP from *Phanerochaete* crassa WD1694. For determination of the substrate specificity of MnP, either 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) or 2,6-dimethoxyphenol (DMP) or veratrylalcohol was added to the reaction mixture and the oxidation was followed by monitoring optical density at 414 nm (ε=36 mM⁻¹cm⁻¹) for ABTS or at 470 nm (ε=49.6 mM⁻¹cm⁻¹) for DMP or at 310 nm (ε=9.3 mM⁻¹cm⁻¹) for veratryl alcohol, respectively (Mester et al., 1998, Moreira et al., 1997). Enzyme reaction was initiated by addition of H₂O₂.

**Characterization of MnP isozymes**

The molecular weight of MnP was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The MnP were further separated into four isozymes by isoelectric focusing (IEF) and their isoelectric points (pIs) were estimated.

**Activity staining**

A staining solution containing 2 mM β-naphtol, 2 mM 3-amino-9-ethylcarbazole, 100μM MnSO₄, 200μM H₂O₂ and 20% acetic acid in 80 mM acetic buffer (pH4.5) was prepared for the visualization of MnP activity. The IEF gel was put in the staining solution until the MnPs bands were stained and washed with the solution containing 25 % ethanol and 8 % acetic acid. The IEFgel was further rinsed with distilled water.

**Analytical method**

Amino-terminal sequence analysis of MnP isozymes was carried out at Sawady Technology Company by using the method of Edman.

**RESULTS**

**Production, purification and characterization of MnP**

The effects of cultivation time and MnSO₄ concentration on the production of MnP of the strain WD1694 were studied. MnP activity in the culture was measured spectrophotometrically as increase of ABTS oxidation. The maximal MnP activity was obtained on day 3 at 0.22 mM MnSO₄ (Fig. 1). The enzyme production was reduced significantly at the excess MnSO₄ concentration. The MnP from the culture filtrate on day 3 was isolated and purified through the adsorption and desorption on DEAE-Sepharose CL-6B and FPLC on DEAE-Toyopearl. The purification steps are summarized in Table 1. The purified MnP had a RZ value (A₄70/A₂₈₀) of 3.67 and was a single band (molecular weight: 48.3 kDa) on SDS-PAGE (Fig. 2). The MnP could be separated on
Table 1. Purification steps of MnP from *P. crassa* WD1694.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (μunit)</th>
<th>Specific activity (μunit/mg)</th>
<th>Yield (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>1378</td>
<td>831.7</td>
<td>0.603</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-sepharose</td>
<td>5.61</td>
<td>634.6</td>
<td>113</td>
<td>76.3</td>
<td>187</td>
</tr>
<tr>
<td>DEAE-toyopearl</td>
<td>0.924</td>
<td>240.9</td>
<td>260.7</td>
<td>28.9</td>
<td>432</td>
</tr>
</tbody>
</table>

Fig. 2 SDS-PAGE of the purified MnP from *P. crassa* WD1694.
Lane 1, Molecular mass protein markers
Lane 2, MnP from *P. crassa* WD1694

Fig. 3 Isoelectric focusing of MnP isozymes from *P. crassa* WD1694.
Lane 1, *P. crassa* WD1694 MnP isozymes stained with MnP active staining.
Lane 2, *P. crassa* WD1694 MnP isozymes stained with Coomassie blue R.
Lane 3, pI standars stained with Coomassie blue R.

The thermal stability of MnP was examined by measuring the remaining activity after the enzyme was exposed at different temperatures for 30 min. The MnP was stable up to 40°C and was inactivated above 60°C. The optimal pH for MnP in 100 mM malonate buffer was 3.0-4.0. Concerning the pH stability, the remaining activity was measured after the enzyme was incubated with the buffer solution of various pHs at 25°C for 18 h. The pH stability was between 3.0 and 6.5 but the enzyme was inactivated below 2.5 and above 7.0.

The MnP from *P. crassa* WD1694 purified by FPLC was used for characterization of catalytic properties. The MnP was able to oxidize DMP and ABTS but not veratryl alcohol regardless of the presence of Mn(II).

The effects of concentration of various organic acids on the activity of MnP were evaluated as oxidation rates of ABTS (0.04% w/v) in the presence of Mn(II). A maximal activity was present in physiological concentration for oxalate and citrate, while a high activity level was kept in some concentration range up to 90 mM for malonate and lactate.

The Michaelis constants of *P. crassa* WD1694 MnP for Mn(II) and for DMP in both the presence and absence of Mn(II) were obtained from steady-state kinetic study (Table 3). The magnitude of *Km* values for DMP in the presence of Mn(II) and for Mn(II) was similar to those of MnPs from other white-rot fungi.

**DISCUSSION**

MnP, LiP and Lac are three major enzymes that oxidize and degrade lignin. In addition to these enzymes, VPs have been reported from Bjerkandera sp. BOS55 for MnP-LiP hybrid isozyme, *P. ostreatus* for MnP and *P. eryngii* for MnP1 and MnP2 (Palma et al., 2000, Mester et al., 1998, Martínez et al., 1996, Sarkar et al., 1997). VPs prefer manganese as substrates rather than aromatic compounds like MnPs, but also can oxidize unphenolic compounds like LiPs (Palma et al., 2000, Martínez et al., 1996, Sarkar et al., 1997). To distinguish MnPs and VPs, characterization of catalytic properties of enzymes are necessary. *Phanerochaete crassa* WD1694 MnP showed high peroxidase activity on Mn(II) and Mn(II)-mediated peroxidase activity on phenolic substrate (DMP) but could not oxidize non-phenolic substrate (veratryl alcohol) in the presence of Mn(II). The MnP could oxidize DMP in the absence of Mn(II), however, the *Km* value was 30.7 mM and was much higher than *Km* value of 7.75 × 10⁻³ mM in the presence of Mn(II). MnP compound I from *P. chrysosporium* can oxidize phenols in the absence of Mn(II) though MnP compound II
Manganese peroxidase from *Dactylorhiza* WD1694 were selected independently for pulp bleaching, are also the classic type MnPs as these from *Phanerochaete chrysosporium* (Hirai et al., 1994, Nishida et al., 1988, Rüttimann-Johnson et al., 1994, Matsubara et al., 1996). Unimportance of LiP on biological bleaching of brownstock has been reported for *T. versicolor* (Archibald, 1992). These results suggest that the oxidation ability of unphenolic compounds might not be critical on pulp bleaching.

The amount of MnP and bleaching ability are known to have positive correlation ship (Hirai et al., 1994). Besides the catalytic difference, it is reported that MnPs and VPs have difference in N-terminal sequence and in manganese regulation on MnP production (Palma et al., 2000, Mester et al., 1998, Martínez et al., 1996, Sarkar et al., 1997). The optimal Mn (II) concentration for MnP production from *P. crassa* WD1694 was 0.22mM and was similar to those observed for *Phanerochaete chrysosporium* (0.73mM) and *P. sordida* (0.2mM) (Bonnarme et al., 1992, Rüttimann-Johnson et al., 1994). N-terminal sequences of the MnPs from *P. crassa* WD1694 were highly homologous with those from *P. chrysosporium, P. sordida* and IZU-154 (Table 2). They might have similar regulation system on MnP production that causes high bleaching ability of pulp.

The N-terminal sequences of MnP isozymes from *P. crassa* WD1694 had higher homology with the classic type MnPs as these from *P. chrysosporium* (Hirai et al., 1994, Nishida et al., 1988, Rüttimann-Johnson et al., 1994, Matsubara et al., 1996). Unimportance of LiP on biological bleaching of brownstock has been reported for *T. versicolor* (Archibald, 1992). These results suggest that the oxidation ability of unphenolic compounds might not be critical on pulp bleaching.

### Table 2. N-terminal sequences of MnPs and VPs from white-rot fungi.

<table>
<thead>
<tr>
<th>Name of the strain</th>
<th>N-terminal sequences</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bjerkandera</em> sp. BOS55</td>
<td>V ACPDGVNTATN</td>
<td>Palma et al., 2000, Mester et al., 1998</td>
</tr>
<tr>
<td><em>Trametes versicolor</em></td>
<td>MP1: VACPDGVNTASN, MP2: VACPDGVNTATN, MP5: VACPDGVNTASN</td>
<td>Johansson et al., 1993, Johansson et al., 1993, Johansson et al., 1993</td>
</tr>
</tbody>
</table>

### Table 3. Kinetic constants of MnP from *P. crassa* WD1694

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$(mM)</th>
<th>$V_{max}$(unit)</th>
<th>$V_{max}/K_m$(UmM)$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(II)</td>
<td>3.8×10$^{-4}$</td>
<td>34.5</td>
<td>0.964</td>
</tr>
<tr>
<td>DMP with Mn(II)</td>
<td>7.75×10$^{-4}$</td>
<td>20.8</td>
<td>2.68</td>
</tr>
<tr>
<td>DMP without Mn(II)</td>
<td>30.7</td>
<td>7.55</td>
<td>2.45×10$^{-4}$</td>
</tr>
</tbody>
</table>
type of MnPs from *P. chrysosporium* rather than versatile peroxidases (Table 2). Three of the four MnP isozymes of WD1694 had the identical 12 amino acids and only difference between the three isozymes (MnP 1, MnP 3, and MnP 4) and MnP 2 was a N-terminal amino acid. Similarity of N-terminal sequences among the four isozymes of *P. crassa* WD1694 was higher than that among the individual isozymes of the white-rot fungi such as *P. chrysosporium*, *P. sordida*, and IZU-154 (Table 2). Concerning the exact origin of the three MnP isozymes (MnP 1, MnP 3, and MnP 4) from *P. crassa* WD1694, further work is needed to clarify whether the multiple forms arise through post-translational modifications or are isozymes encoded by multiple structural genes. *Trametes versicolor* has possessed three MnP and two LiP isozymes expressed by multiple structural genes though they have common 10 amino acids sequence of N-terminal (Table 2).

Though MnP from *P. crassa* WD1694 showed these similar properties with MnP from *P. chrysosporium*, it showed difference in pls and molecular weight. Molecular weight of *P. crassa* WD1694 MnP was 48.3 kDa and was larger than that for *P. chrysosporium* (46 kDa), *P. sordida* (45kDa), and IZU-154 (43kDa) (Rüttimann-Johnson et al., 1994, Matsubara et al., 1996, Glenn et al., 1985). The four isozymes from *P. crassa* WD1694 existed in very narrow range from pl 4.50 to 4.61. These were focused in extremely close pls compared with those for other fungal MnPs, *P. sordida* (pl 3.3, 4.2, 5.3), *P. chrysosporium* (pl 4.2, 4.5, 4.9), and IZU-154 (pl 3.7, 4.5, 4.9) (Rüttimann-Johnson et al., 1994, Matsubara et al., 1996, Pease et al., 1992). Generally, MnPs isolated from a white-rot fungus exist as multiple isozymes with same molecular weight and different pls (Rüttimann-Johnson et al., 1994, Matsubara et al., 1996, Leisola et al., 1987). However, if the difference in pls has any significance is still unknown.

The optimal pH of *P. crassa* WD1694 MnP was 3.0-4.0 and was lower than that (4.5-5.0) of *P. chrysosporium* and *P. sordida* MnPs (Rüttimann-Johnson et al., 1994, Kishi et al., 1994). The difference in optimal pH with MnP from *P. crassa* WD1694 and MnP from other fungi was unexpected concerning other high similarities in catalytic properties and N-terminal sequences.

In conclusion, MnP from *P. crassa* WD1694 had similar catalytic properties and N-terminal sequences with classic type MnP like *P. chrysosporium*. The results suggest that the high ability in UKP bleaching of *P. crassa* WD1694 was depended on the high production of classic type MnP.

ACKNOWLEDGEMENT
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REFERENCES


要 旨
高い未熟クラフトバルブ漂白能力を持つ白色腐朽菌 Phanerochaete crassa WD1694 の産生するマンガ
ンペルオキシダーゼ (MnP) の精製と解析を行った。DEAE- セファロースと DEAE- トヨバールによって精
製された酵素は、SDS-PAGE で分子量 48.3kDa に単一バンドを示し、等電点電気泳動によって非常に近接
する等電点 (pI4.61, 4.59, 4.52, 4.50) を持つ 4 本のバンドに分離した。4 つの MnP アイソザイムの N 末端
アミノ酸配列はいずれも P. chrysosporium 由来のマンガンペルオキシダーゼのものと高い相関性を示した。
この酵素はマンガンの有無に関わらず 26-ジメトキシフェノールを酸化したが、ベラトリルアルコールは
酸化しなかった。P. crassa WD1694 由来の MnP の至適 pH は 3.0-4.0 であり、P. chrysosporium および
P. sordida の至適 pH4.5-5.0 より低かった。Mn(II) と 26-ジメトキシフェノールを基質とした場合の K_m 値は
それぞれ 35.8 × 10^3 mM と 30.7 mM であった。これらの結果より、P. crassa WD1694 由来の MnP は触
媒活性、N 末端アミノ酸配列とも P. chrysosporium 由来の MnP と相関性が高いことが明らかになった。

キーワード：マンガンペルオキシダーゼ、Phanerochaete crassa、精製、リグニン生分解、白色腐朽菌、
バイオプリーチング

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