論文（Original Article）

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-Sepahrose 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 (pI4.61, 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 35.8×10^{-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_2O_2, 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, Martínez 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, Hirai 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℃.

Cultivation

The mycelium of *P. crassa* WD1694 was grown

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on PDA at 26℃. A total of 10 mycelial mats with a 6 mm diameter was inoculated into the medium (100 ml) containing 0.06% KH_2PO_4, 0.04% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, $5x10^{-4}$% $CaCl_2$, 0.01% yeast extract, 1% glucose and 0.1% $NH_4H_2PO_4$ in a 300 ml Erlenmeyer flask and the flask was placed in a rotary shaker at 30 ℃ and 100 rpm for 4 days. The grown mycelium was homogenized. To a freshly prepared medium (100 ml) containing 0.005% $MnSO_4$ 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℃ 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 ℃ . 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 $mM^{-1}cm^{-1}$) and at 20℃ on the basis of the oxidation of $MnSO_4$ (Wariishi, 1992). One unit of enzyme activity was defined to be amount of enzyme that oxidize 1μmol of substrate per minute. The standard reaction mixture contained 50 mM malonate buffer, pH 3.5, 5 mM $MnSO_4$, 20μM H_2O_2 and proper concentration (around 0.07μg/ml) of MnP from *P. 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 $mM^{-1}cm^{-1}$) for ABTS or at 470 nm (ε= 49.6$mM^{-1}cm^{-1}$) for DMP or at 310 nm (ε=9.3$mM^{-1}cm^{-1}$) for veratryl alcohol, respectively (Mester et al., 1998, Moreira et al., 1997). Enzyme reaction was initiated by addition of H_2O_2.

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 $MnSO_4$, 200μM H_2O_2 and 20% acetone in 80 mM acetate 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 $MnSO_4$ concentration on the production of MnP of the strain WD1694 were studied. MnP activity in the culture was measured spectrophtometrically as increase of ABTS oxidation. The maximal MnP activity was obtained on day 3 at 0.22 mM $MnSO_4$ (Fig. 1). The enzyme

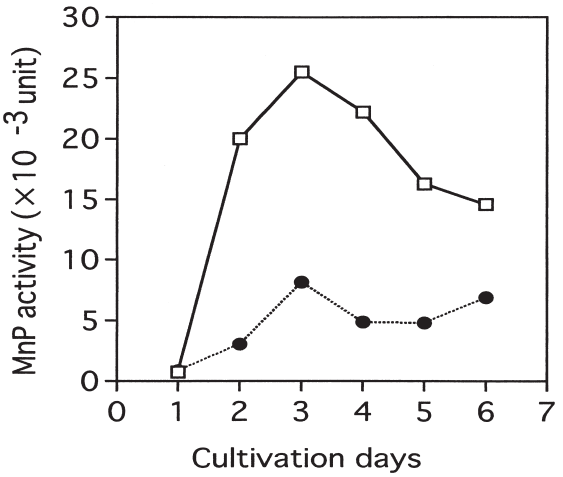

Fig.1 Production of MnP in the cultures containing $MnSO_4$.
MnP activity was measured in 1 ml of reaction mixture containing 50mM malonate buffer (pH3.5), 5mM $MnSO_4$, 0.02mM H_2O_2, 0.2m1 culture, 0.073mM ABTS. $MnSO_4$ 0.22mM (open square), 2.2mM (filled circle)

production was reduced significantly at the excess $MnSO_4$ 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 (A_{406}/A_{280}) 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.

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

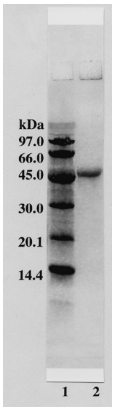

Fig.2 SDS-PAGE of the purified MnP from *P. crassa* WD1694.
Lane1, Molecular mass protein markers
Lane2, MnP from *P. crassa* WD1694

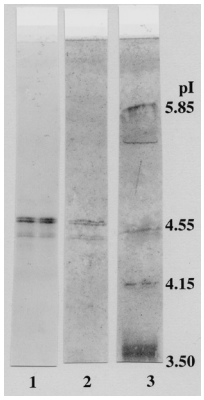

Fig.3 Isoelectric focusing of MnP isozymes from *P. crassa* WD1694.
Lane1, *P. crassa* WD1694 MnP isozymes stained with MnP active staining.
Lane2, *P. crassa* WD1694 MnP isozymes stained with Coomasie blue R.
Lane3, pI standars stained with Coomasie blue R.

isoelectric focusing into four isozymes with very close pIs (MnP 1: 4.61, MnP 2: 4.59, MnP 3: 4.52, and MnP 4: 4.50). All the four protein bands gave a positive activity staining of MnP (Fig. 3).

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℃ and was inactivated above 60℃. 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℃ 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.

N-terminal amino acids sequence

The N-terminal amino acids sequence of four MnP isozymes from *P. crassa* WD1694 is listed with those of MnPs from other white-rot fungi in Table 2. In *P. crassa* WD1694, three MnP isozymes except MnP 2 had the identical 12 amino acids sequence of N-terminal and only difference between the three isozymes and MnP 2 was a N-terminal amino acid.

Catalytic properties of MnP

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 K_m 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 K_m value was 30.7 mM and was much higher than K_m 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

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

Name of the strain	N-terminal sequences													References
Phanerochaete crassa WD1694	MnP 1	A	V	X	P	D	G	T	R	V	N	E	A	This work
	MnP 2	T	V	X	P	D	G	T	R	V	N	E	A	This work
	MnP 3	A	V	X	P	D	G	T	R	V	N	E	A	This work
	MnP 4	A	V	X	P	D	G	T	R	V	N	E	A	This work
Phanerochaete chrysosporium	MP1	A	V	C	P	D	G	T	R	V	T	N	A	Pease et al., 1989
	H4	A	V	X	P	D	G	T	?	V	T	N	A	Pease et al., 1992
	PULP	A	V	X	P	D	G	T	R	V	?	N	A	Datta et al., 1991
	MnP1	A	V	C	P	D	G	T	R	V	S	H	A	Pribnow et al., 1989
Phanerochaete sordida	MnPI	A	V	X	S	Q	G	T	A	V	S	N	A	Rüttimann-Johnson et al., 1994
	MnPII	A	V	X	P	D	G	T	X	V	N	N	E	Rüttimann-Johnson et al., 1994
	MnPIII	A	V	X	P	D	G	T	A	V	P	S	T	Rüttimann-Johnson et al., 1994
IZU-154	MnP1	A	V	C	P	D	G	T	R	V	S	N	S	Matsubara et al., 1996
	MnP2	A	V	C	F	D	G	T	R	V	S	N	S	Matsubara et al., 1996
Bjerkandera sp. BOS1,2		V	A	C	P	D	G	V	N	T	A	T	N	Palma et al., 2000
Bjerkandera sp. BOS55		V	A	C	P	D	G	V	N	T	A	T	N	Mester et al., 1998
Pleurotus ostreatus		A	T	C	A	D	G	R	T	T	A			Sarkar et al., 1997
Pleurotus eryngii	MnP1	A	T	D	A	D	G	R	T	T	A	-	N	Martínez et al., 1996
	MnP2	A	T	D	D	D	G	R	T	T	A	-	D	Martínez et al., 1996
Trametes versicolor	MP1	V	A	C	P	D	G	V	N	T	A	S	N	Johansson et al., 1993
	MP2	V	A	C	P	D	G	V	N	T	A	T	N	Johansson et al., 1993
	MP5	V	A	C	P	D	G	V	N	T	A	S	N	Johansson et al., 1993

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

Substrate	K_m(mM)	V_{max}(unit)	V_{max} / K_m (UmM^{-1})
Mn(II)	35.8×10^{-3}	34.5	0.964
DMP with Mn(II)	7.75×10^{-3}	20.8	2.68
DMP without Mn(II)	30.7	7.55	2.45×10^{-4}

requires Mn (Ⅱ) to be reduced to native state (Gold et al., 1989, Wariishi et al., 1988). With respect to VPs, K_m values for Mn (Ⅱ)-independent oxidation of DMP are in the range of 41×10^{-3} to 950×10^{-3} mM and they are much smaller than that of MnP from *P. crassa* WD1694 (Palma et al., 2000, Mester et al., 1998, Martínez et al.,1996, Sarkar, 1997). Our results clearly show that the catalytic property of MnP from *P. crassa* WD1694 is classic type MnP similar as MnPs from *P. chrysosporium*.

The bleaching ability of *P. crassa* WD1694 depended on the MnP activity and no LiP activity was found during the cultivation (Takano et al., 2001). However, *P. crassa* WD1694 had higher ability to bleach UKP than *P. chrysosporium* that produce LiP, and *P. ostreatus* that produce VP. The MnPs from *Phanerochaete sordida* YK-624 and unidentified fungus IZU-154, both fungi were selected independently for pulp bleaching, are also 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.

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 *P. chrysosporium* (0.73mM) and *P. sordida* (0.2mM) (Bonnarme et al., 1990, 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 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 pIs 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 pI 4.50 to 4.61. These were focused in extremely close pIs compared with those for other fungal MnPs, *P. sordida* (pI 3.3, 4.2, 5.3), *P. chrysosporium* (pI 4.2, 4.5, 4.9), and IZU-154 (pI 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 pIs (Rüttimann-Johnson et al., 1994, Matsubara et al., 1996, Leisola et al., 1987). However, if the difference in pIs 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.

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Phanerochaete crassa WD1694 由来のマンガンペルオキシダーゼについて

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要　旨

高い未晒クラフトパルプ漂白能力を持つ白色腐朽菌 *Phanerochaete crassa* WD1694 の生産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- トヨパ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同性を示した。この酵素はマンガンの有無に関わらず 2,6- 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 2,6- 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 と相同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、バイオブリーチング

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