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Characterization of Manganese Peroxidase from *Pholiota aurevella*

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Abstract: Manganese peroxidase from *Pholiota aurevella* was purified to homogeneity using ammonium sulfate precipitation followed by Sephacryl S-300 HR, DEAE-Sepharose, Con A Sepharose and Mono Q HR 5/5 column chromatographies. The purified enzyme had a molecular weight of 40 kDa and N-terminal amino acid sequence exhibited more than 30% homology with the other manganese peroxidases from white rot fungi. The enzyme showed optimum pH and temperature at 5.5 and 45℃, respectively, indicating the enzyme showed higher optimum temperature than the other manganese peroxidases. It showed similar specificity against phenolic substrates and the presence of Mn^{2+} enhanced the enzyme activity greatly while Fe^{2+} completely inhibited the enzyme. Salt tolerance of the enzyme will make itself to be a potent candidate for the bioremediation of organic pollutants.

Key words: *Pholiota aurevella*, manganese peroxidase, characterization, substrate specificity

I　Introduction

Lignins occupy a unique place among the abundant renewable aromatic polymers and occur as lignocellulosic composites in the woody materials. Lignin degradation is affected by fungi especially the white rot basidyomycetes[1)]. Oxidative enzymes that commonly occurred in lignin degradation are lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and laccase (Lac, EC 1.10.3.2)[2~5)]. Due to their broad specificities, these enzyme systems find their applications in the xenobiotic degradation and also in other biotechnological applications[6~9)]. MnPs have been purified and characterized from white rot fungi[5,10)]. They are mostly measophilic enzymes in their thermal stabilities. As in the case of other lignin degrading enzymes, MnP also finds applications in different fields especially in pollution abatement, biopulping and biobleaching, lignin bioconversion via xenobiotic degradation and biotransformations of value added products such as biodegradable polymers. Application of these enzymes on pollution abatement is well documented[8,10)]. Most of the reported works on them are related to *Phanerochaete chrysosporium*[11)], but some white rot fungi containing the edible mushrooms are also exploited for enzyme production. Earlier work on the screening of different edible mushrooms for enzyme production suggested that *Pholiota aurevella* is one of the potent MnP producers[12)]. In this paper, the production of lignin degrading enzymes from *P. aurevella*, and the purification and characterization of MnP which is the major enzyme produced from the above fungus are described.

II　Materials and methods

1. Microorganism and culture conditions

Pholiota aurevella (IFO No. 30265), obtained from the Institute for Fermentation, Osaka was grown on a potato dextrose broth (24.0 g/l), yeast extract (5 g/l), pH 5.5. The fungus was cultured on a rotary shaker at 100 rpm and 25℃. After cultivation, the supernatant was separated by filtration using ADVANTEC No.2 filter and was used as crude enzyme solution.

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2. Estimation of biomass and soluble protein

Fungal biomass was estimated by dry weight and protein content was estimated by DC protein assay kit (BioRad Laboratories Inc) and also by measuring the absorbance at 280 nm assuming that 1mg/ml protein is 1.0.

3. Enzyme assays

Manganese peroxidase (MnP) activity was determined[13] at 468 nm by measuring the oxidation of 2,6-dimethoxyphenol (DMP). The reaction mixture contained 0.4 mM DMP, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2, in 50 mM sodium acetate buffer, pH 5.5, and enzyme solution. The enzyme reaction was carried out at 30℃. One unit of MnP activity was defined as the amount of enzyme which oxidizes 1 μmole of DMP in 1 min.

Laccase (Lac) activity was measured by monitoring the oxidation of ABTS (2,2'-azino-di-[3-diethylbenzothiazoline-6-sulphonic acid], Boehringer) at 420 nm[14] dissolved in 50 mM sodium succinate buffer, pH 5.5. One unit of laccase activity was defined as the amount of enzyme which oxidizes 1 μmol of ABTS in 1 min.

4. Purification of manganese peroxidase

Purification of MnP was carried out by ammonium sulate precipitation followed by chromatographies on Sephacryl S-300 HR, DEAE-Sepharose, Con A Sepharose and Mono Q HR 5/5 columns.

5. Effect of reaction pH and temperature on the MnP activity and stability

Buffers ranging from pH 3.0 to 8.0 were used with the substrate concentration of 0.4mM DMP. The reaction was carried out at 30℃ keeping the concentrations of other reagents constant.

The enzyme solution was incubated at the above pHs and at 30℃ for 30 minutes and the residual activity was measured.

The enzyme reaction was carried out at different temperatures ranging from 20 to 70℃. The buffered substrate with enzyme (pH 5.5) and Mn^{2+} was preincubated at the required temperature and then measured the absorbance change after the addition of H_2O_2 at the appropriate wavelength.

The enzyme solution was also exposed at pH 5.5 and above incubation temperatures for 30 minutes and the residual activity was measured.

6. Substrate specificity of MnP

Several phenol derivatives such as 2,6-dimethoxy phenol (DMP 468nm: $\varepsilon = 49{,}600\ M^{-1}cm^{-1}$), ABTS (2,2'-azino-di-[3-ethyl benzothiazoline-6-sulphonic acid] 420nm : $\varepsilon = 36{,}000\ M^{-1}cm^{-1}$), guaiacol (436 nm : $\varepsilon = 6{,}400\ M^{-1}cm^{-1}$), catechol (450nm: $\varepsilon = 2{,}211\ M^{-1}cm^{-1}$), pyrogallol (450nm: $\varepsilon = 4{,}400\ M^{-1}cm^{-1}$) were used at 0.4mM concentration in the reaction mixture.

7. Kinetic parameters of MnP in the presence and absence of Mn^{2+}

The effect of substrate concentration on the enzymatic activity in the presence and absence of Mn^{2+} was investigated. In the set of experiments, DMP at lower concentrations such as 0.025, 0.05, 0.1, 0.2 and 0.3mM, and at higher concentrations such as 3.0, 3.5, 4.0, 4.5, and 5.0mM was used as the substrate.

8. Effect of NaCl on MnP activity

Enzyme solution was mixed with equal amount of 50mM sodium acetate buffer pH 5.5 containing NaCl concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 M. Enzyme solutions after treating with different concentrations of NaCl as given above were kept 4℃ overnight and residual activities of MnP were determined.

III Results and discussion

1. Production of lignin degrading enzymes from *Pholiota aurevella*

Pholiota aurivella was cultured for 8 days and samples were taken at every 24 h for the analysis of pH change, biomass production and enzyme levels. The results were shown in Fig. 1. *P. aurevella* produced two lignin-degrading enzymes (MnP and Lac) at high level under the experimental conditions indicating that this fungus is a potent producer of these enzymes. White rot fungi especially the edible mushrooms prefer to secrete MnP and laccase, but not lignin peroxidase (LiP)[15,16]. In addition, the enzyme production pattern suggests that

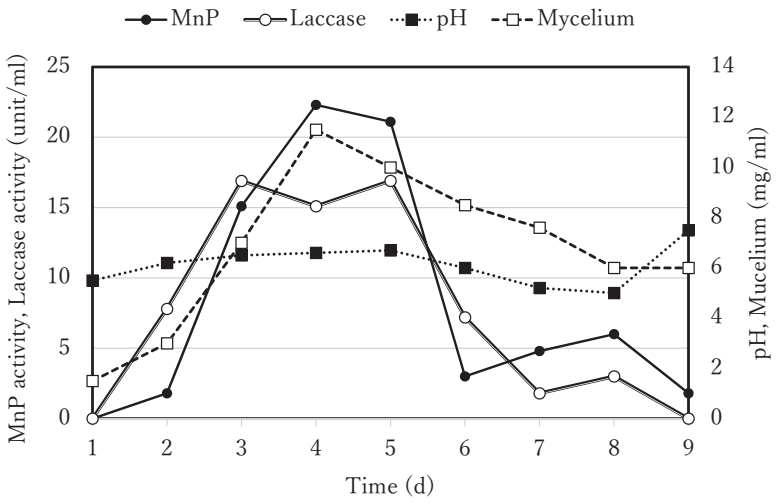

Fig. 1. Growth and enzyme production of *Pholiota aurivella*
During the cultivation of *P. aurevella*, pH, enzymatic activities and mycelium (dry weight) were determined.

Table 1. Purification of manganese peroxidase from *Pholiota aurevella*

	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Purification (-fold)
Culture supernatant	9,622	154.0	63	100	1
Ammonium sulfate fractionation	6,596	47.5	139	68.5	2.2
Sephacryl S-300	5,569	5.9	944	57.8	15.1
DEAE Sepharose	4,564	2.9	1,577	47.4	25.7
Con A Sepharose	3,081	1.6	1,879	32.0	30.0
Mono Q	1,953	1.0	1,987	20.3	31.8

When the concentrated protein from ammonium sulfate precipitation was loaded onto Sephacryl S-300 column which was equilibrated with 0.05M sodium acetate buffer containing 0.5M NaCl, pH 5.5, a single activity peak was observed.
The active fractions from the above were dialyzed against 50mM sodium acetate buffer, pH 5.5, and were loaded onto the DEAE-Sepharose column which was equilibrated with the same buffer. The adsorbed protein was eluted with decreasing pH gradient from pH 5.5 (50mM sodium acetate buffer) to pH 3.0 (0.1M acetic acid) with the combination of increasing NaCl gradient from 0 to 0.5 M.
The active fractions from this column were pooled, dialyzed against sodium acetate buffer, pH 5.5 containing 0.5M NaCl, 1mM $CaCl_2$, and $MnCl_2$, and $MgCl_2$, and concentrated by Ultrafiltration on YM10 membrane (Toyo Roshi) and then again loaded onto the Con A Sepharose column. After washing with the same buffer, the MnP activity was eluted with 0.2M α-methyl-D-glucoside.
The active fractions from affinity column were pooled, dialyzed against 50mM sodium acetate buffer, pH 5.5, and then loaded onto Mono Q HR 5/5 column. The active fraction was eluted with the NaCl gradient from 0 to 0.5 M.

there can be the presence of two MnPs, major one expressed in the stage of the growth (3d) and the minor one expressed in the later stage (7d).

2. Purification of MnP

Purification of MnP from the crude enzyme preparation was carried out using different steps including affinity chromatography as shown in Table 1. The purification step using Con A as a ligand is effective for the purification of the enzyme, indicating the enzyme contains oligosaccharide chains in the molecule. The overall yield was 20.3 % with a purification fold of 31.8.

3. Characterization of MnP

The purified MnP was subjected to SDS-PAGE and the results were given in Fig. 2. The protein migrated as a single band and the molecular weight was estimated to be 40 kDa. The molecular weights of MnPs are reported to be ranging from 40 to 50 kDa[17].

This protein was transferred to PVDF (polyvinilidene difluoride) membrane and its N-terminal amino acid sequence was determined (Fig. 3). The homology

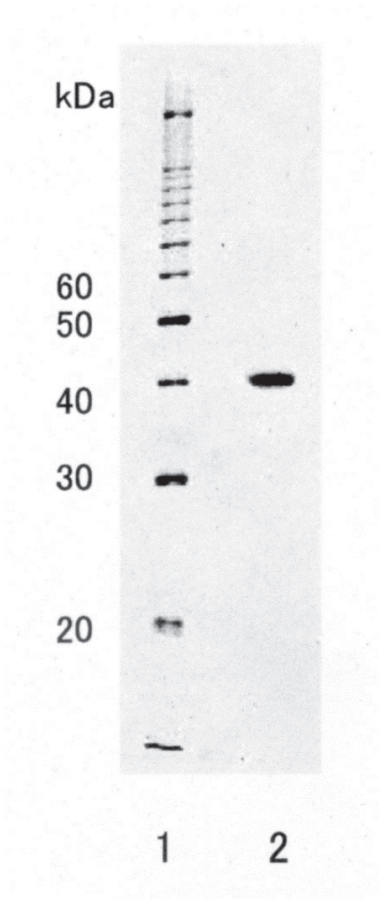

Fig. 2. SDS-PAGE of purified MnP from *Pholiota aurivella*
The purified protein was subjected to SDS-PAGE. The gel concentration was 12.5 %(w/v) and the protein markers were ranging from 10 to 100 kDa. After electrophoresis, the gel was stained with 0.25 %(w/v) CBB R250 and destained with a mixture of methanol/acetic acid/distilled water (3:1:6, v/v).

search revealed that it had more than 30% homology with the previously reported MnPs derived from white rot fungi such as *Pleurotus eryingii*, *Pleurotus ostreatus*, *Ganoderma applantum*, *Trametes versicolor*, etc.

Three Cys residues are conserved among all enzymes and these Cys residues are thought to be important because of the relation to the activity and/or the maintenance of enzyme structure. These three Cys residues are conserved at the same position of the primary sequence of the protein belonging to the same protein family.

4. Effect of pH and temperature on activity and stability of MnP

MnP was most active at pH 5.5 and this enzyme is stable for 30 min around its optimum pH. The optimum temperature of MnP is 45℃ and the enzyme was stable up to 40℃. MnPs are susceptible to thermal inactivation due to their relatively low affinity to Ca^{2+} ions, which are required for its stability and activity[18]. The optimum temperature of the enzyme is higher than those of MnPs from white rot fungi (22℃-40℃)[19] but heat stability is almost the same as those of MnPs [20].

5. Substrate specificity of MnP

Different phenolic substrates such as DMP, ABTS,

Pholiota aurivella	VTCANGKTXT-NAVCCPLFDXVDFLQ
Pleurotus eryngii	VTCATGQTTA-NEACCALFPILDDIQ
Pleurotus ostreatus	VTCATGQTTA-NEACCALFPILEDIQ
Ganoderma lucidum	ATCANGKTTA-NDACCVWFDVLDDIQ
Trametes versicolor	VACXDGYNTATNAACCQLFXVRDDIQ
Ceriporiopsis subvermispora	VTCSDG-TVVPDSMCCDFIPLAQDLG
Phanerochaete chrysosporium	ATCPDG-TKVNNAACCAFIPLAQXLQ
Dochomitus squalens	TVCSDG-TVVPDSVCCEFVPLAQALQ
Tylospora fibrillose	ATCPDG-TKVNNAACCAFIP

Fig. 3. Comparison of N-Terminal amino acid sequences of MnPs from various origins including *Pholiota aurevella*
The SDS-PAGE was conducted for MnP as given above. The protein in the gel was transferred to PDVF membrane by electroblotting using semi dry transfer cell equipment (Bio-Rad Laboratories Inc) at 15 V for 2 h. The transferred proteins in the membrane were stained with 0.1 %(w/v) CBB R250 and destained with 50 %(v/v) methanol. The transferred protein was sequenced using a protein sequencer G1000A (Hewlett Packard Corp.) along with standard PTH amino acids. Identical amino acids among all and majority of enzymes are indicated as open and underlined characters, respectively.

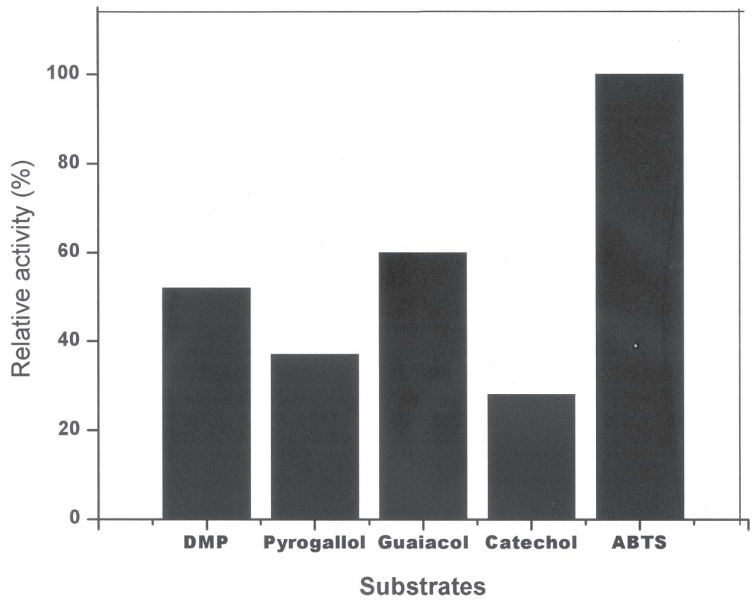

Fig. 4. Substrate specificities of manganese peroxidase from *Pholiota aurivella*
Different phenol derivatives such as dimethoxy phenol (DMP 468 nm: ε = 49,600 M-1cm-1), ABTS (420nm : ε = 36,000 M-1cm-1), guaiacol (436 nm : ε =6,400 M-1cm-1), catechol (450 nm: ε = 2,200 M-1cm-1), pyrogallol (450 nm: ε = 4,400 M-1cm-1) were taken at 0.4 mM concentration in 50 mM sodium acetate buffer, pH 5.5 and used as substrates.

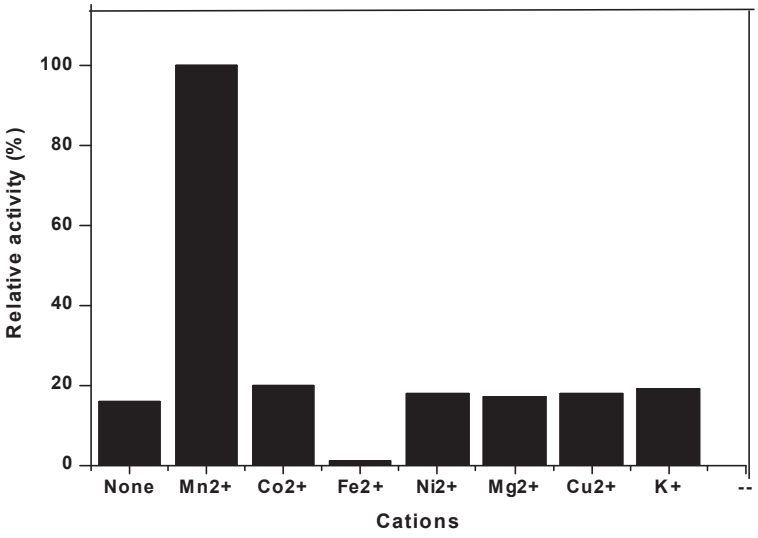

Fig. 5. Effect of cations on the activities of manganese peroxidase from *Pholiota aurevella*
Instead of $MnCl_2$, different chlorides of metals like $CoCl_2$, $FeCl_2$, $NiCl_2$, $MgCl_2$, $CuCl_2$, KCl were used at the concentration of 0.1mM and DMP was used as substrate.

pyrogallol, guaiacol, and catechol were used for investigation of the substrate specificity of MnP. The results were given in Fig. 4. The enzyme showed its maximum affinity towards ABTS followed by guaiacol, DMP, pyrogallol, and catechol as in the case of MnPs from other white rot fungi[11].

In the absence of Mn^{2+}, the Km value of DMP increased (11.79 mM) whereas in the presence of 0.1 mM Mn^{2+}, Km value decreased significantly (0.104 mM). Similar results are reported in the case of MnP from *Phanerochaete crassa* WD1694[17] and *P. chrysospolium* indicating MnP from *P. aurivella* is similar to the enzymes from these basidiomycetes in terms of catalytic properties.

Table 2. Effect of various organic acids on manganese peroxidase activity from *Pholiota aurevella*

Organic acid	Relative activity (%)
oxalate	74
succinate	28
maleate	7
malonate	91
citrate	33
glycolate	54
lactate	89
malate	100
gluconate	37
tartrate	49
phosphate	17
acetate	4

Sodium salts of different organic acids listed above were used at the concentration of 50mM at pH 4.5. The oxidation of DMP was monitored by the standard MnP assay conditions.

6. Effect of cations on MnP activity

When individual cations were added to the reaction mixture, Mn^{2+} gave the maximum activity and the other cations such as Co^{2+}, Ni^{2+}, Mg^{2+}, Cu^{2+}, K^{+}, showed less than 20% of the activity exhibited by Mn^{2+}. In addition, Fe^{2+} inhibited the enzyme almost completely. (Fig. 5)

7. Effect of organic acids on MnP activity

DMP oxidation in the different organic acids was monitored. The activity of the enzyme was enhanced by the addition of organic acid chelators like malate, malonate, lactate and oxalate that stabilize the Mn^{3+} complex of the organic acids. At 50mM concentration, malate showed the maximum activity followed by malonate, lactate, oxalate, glycolate, and tartate as shown in Table 2.

When the oxidation of DMP monitored with different chelating organic acids by MnP, according to the stability of chelator-Mn^{3+} complex, various enhancement of the activities were observed. Earlier reports suggested that oxalate, which was secreted by the fungus, and other organic acid chelators, such as malonate, stabilize the enzyme-generated Mn^{3+} [21,22] and these Mn^{3+}-chelate complexes moved away from the enzyme to oxidize phenolic substrates, including lignin, lignin model compounds and aromatic pollutants.

8. Influence of NaCl on MnP activity

The residual activities of the enzyme treated with different concentrations of NaCl showed 100% activities with 0.1M NaCl, followed by other concentrations from 0.2M to 0.5M which retained more than 80% activities. This results indicate that the MnP from this fungus is a salt tolerant and it is an advantage for the application of the enzyme for xenobiotic degradation even in the sea water.

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