Thermally stable and hydrogen peroxide tolerant manganese peroxidase (MnP) from *Lenzites betulinus*

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Abstract A thermally stable and hydrogen peroxide tolerant manganese peroxidase (MnP) was purified from the culture medium of *Lenzites betulinus* by ion exchange chromatography, gel filtration and isoelectric focusing chromatography. The MnP purified from *L. betulinus* (L-MnP) has a molecular mass of 40 kDa and its isoelectric point was determined to be 6.2. The first 19 amino acids at the N-terminal end of the L-MnP sequence were found to exhibit 74% identity with those of a *Phlebia radiata* MnP. L-MnP was proved to have the highest hydrogen peroxide tolerance among MnPs reported so far. It retained more than 60% of the initial activity after thermal treatment at 60°C for 60 min, and also retained more than 60% of the initial activity after exposure to 10 mM hydrogen peroxide for 5 min at 37°C.

Key words: Manganese peroxidase; Hydrogen peroxide; Thermal stability

1. Introduction

Lignin, a complex and heterogeneous aromatic biopolymer in woody and herbaceous plants, is one of the most abundant natural polymers on earth. White rot fungi are primarily responsible for initiating the depolymerization of lignin in wood [3,6,10]. The extracellular lignolytic enzyme system of white rot fungi has been studied extensively in recent years. Lignin peroxidase, manganese peroxidase (MnP), and laccase are associated with the degradation of lignin. Several attempts to bleach hardwood kraft pulp by means of enzyme treatment have been reported. Arbeloa et al. [1] showed that treatment of unbleached kraft pulp with lignin peroxidase facilitated subsequent chemical bleaching. Bourbonnais and Paice [2] demonstrated that unbleached kraft pulp could be delignified with a laccase from *Trametes versicolor* in the presence of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate). We proposed a new method of biobleaching in which the enzyme (MnP) immobilized in a mesoporous material (FSM-16) and the pulp bleaching reaction are separated [17].

MnP is a heme containing enzyme that was first isolated from the extracellular medium of lignolytic cultures of the white rot fungus *Phanerochaete chrysosporium*, and it is considered to be a key enzyme in lignolysis by white rot fungi. MnP requires hydrogen peroxide as a cosubstrate and catalyzes the oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} complexed with an organic acid acts as a primary agent in lignolysis. Kondo et al. [12] reported that MnP could degrade residual lignin in kraft pulp. However, MnP is unstable compared to other peroxidases, e.g. horseradish peroxidase, and its stability at elevated temperatures and hydrogen peroxide levels must be increased before it can be used for an application such as pulp bleaching.

Kondo et al. [11] showed that the MnPs (G1 and G2) from *Ganoderma* sp. YK-505 were excellent as to thermal stability and hydrogen peroxide dependence compared with *P. chrysosporium* MnPs. Here we report the purification and further characterization of a thermally stable and hydrogen peroxide tolerant MnP from *Lenzites betulinus* (L-MnP). The N-terminal amino acid sequence of L-MnP is also reported.

2. Materials and methods

2.1. Fungal strain and culture conditions

White rot fungus strains were obtained from the Ministry of Agriculture, Forestry and Fisheries Genebank library. The wild type strains were isolated from white rotted wood or fruit bodies. All incubations were carried out at 25°C stationarily in the dark. In order to isolate and check the secretion of a lignin decomposing enzyme, agar plates containing 0.05% of Remazol brilliant blue R were used for prescreening. The white rot fungus strain was inoculated onto 10 ml potato dextrose culture medium in a 100 ml Erlenmeyer flask, and then cultivated for 7–10 days for precultivation. The culture was homogenized for 15 s, a mycelial solution being obtained. 10 ml of production culture medium (20 g glucose, 5 g peptone, 2 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, and 0.5 mM $MnSO_4$ per liter, pH 5.8) was added to a 100 ml Erlenmeyer flask, followed by inoculation of 100 μl of the mycelial solution and stationary cultivation. Finally, the MnP activity in the culture was measured at 270 nm as the change in absorbance due to Mn^{3+}.

2.2. MnP assays

The level of MnP activity was determined by monitoring the formation of the Mn^{3+}–oxalate complex at 270 nm at 37°C. The reaction mixtures comprised 2 mM sodium oxalate, 0.5 mM $MnSO_4$, and 0.1 mM hydrogen peroxide in sodium succinate buffer (pH 4.5). Assays were initiated by the addition of hydrogen peroxide. One unit of MnP was defined as the amount of enzyme producing 1 μmol of the Mn^{3+}–oxalate complex per minute.

2.3. MnP purification

Cultures were filtered through glass wool. 70% $(NH_4)_2SO_4$ was added to the resulting filtrate at 0°C. The resulting pellet was collected, resolved and dialyzed against 10 mM acetate buffer (pH 6.0). The resulting solution was loaded onto a DEAE-Sephacel (Amersham Pharmacia Biotech UK, Buckinghamshire, UK) column equilibrated with 10 mM acetate buffer (pH 6.0). The column was eluted with 200 mM succinate buffer (pH 4.5). After dialysis against 10 mM acetate

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buffer (pH 6.0), the active fraction was loaded onto a Mono Q column (Amersham Pharmacia Biotech UK) equilibrated with the same buffer. The protein was eluted with a Na-acetate gradient (0.01–0.7 M, pH 6.0). The active fraction was concentrated and then loaded onto a Superose 12 column (Amersham Pharmacia Biotech UK) equilibrated with 100 mM acetate buffer (pH 6.0). Finally, the active fraction was loaded onto a Mono P column (Amersham Pharmacia Biotech UK) equilibrated with histidine buffer (pH 7.0).

2.4. Electrophoretic analysis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 10% polyacrylamide gels (Tefco), with visualization by Coomassie blue staining. For determination of the MnP molecular mass, a low molecular mass calibration kit (Bio-Rad) was used.

2.5. Thermal stability

After adjusting the concentration to 10 U/ml, the medium was dialyzed against 10 mM succinate, pH 4.5. The medium was treated at each temperature for a predetermined time. The MnP activity was immediately measured after cooling. The activity of a non-treated sample was taken as 100%, and the residual activity under each condition was determined. All data are the values for at least four samples.

2.6. Hydrogen peroxide dependence

A reaction buffer comprising 1mM 2, 6-dimethoxy phenol (DMP), a predetermined concentration of hydrogen peroxide, 0.2–10 mM, 1mM $MnSO_4$, and 50 mM malonate buffer (pH 4.5) was used. The reaction was carried out for 1 min at 37°C. The difference in absorbance 469nm between a blank and a sample was measured. All data are the values for at least four samples.

2.7. N-terminal sequence analysis

The purified MnP was subjected to SDS–PAGE and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The band on the PVDF membrane was cut out and analyzed by the Edman method with a 473A sequencer (Perkin-Elmer Applied Biosystems).

3. Results

3.1. Screening

We investigated the thermal stability and hydrogen peroxide dependence of MnPs in the culture medium of 24 specimens of white rot fungi. Several specimens retained the full activity after thermal treatment at 50°C for 45 min. Among them, L-MnP showed high hydrogen peroxide dependence, i.e. more than 20% activity with 1 mM. Consequently, *L. betulinus* was chosen as a candidate for the production of stable MnP.

3.2. Purification and properties of L-MnP

For purification, mycelia were removed from *L. betulinus* culture medium (21st day) by filtration. The results of a typical purification of L-MnP are summarized in Table 1. The recoveries of activity in the salting-out and DEAE steps were both more than 80%. The DEAE fraction was loaded onto a Mono Q column, and three different active peak fractions were eluted with 0.17, 0.5 and 0.58 M NaCl (data not shown). The main active fraction was eluted with 0.17 M NaCl. Purified L-MnP was obtained by isoelectric focusing chromatography from the 0.17 M NaCl eluted fraction from Mono Q. The ratio of the MnP activity per A_{280} increased in proportion to the progress of purification. Finally, the RZ value of A_{405} per A_{280} was 4.29. The purified L-MnP was proved to give a single band in SDS–PAGE and its molecular mass was determined to be 40 kDa, as shown in Fig. 1. The isoelectric point of L-MnP was determined to be 6.2 by isoelectric focusing and the band of L-MnP was activated by Mn^{2+}.

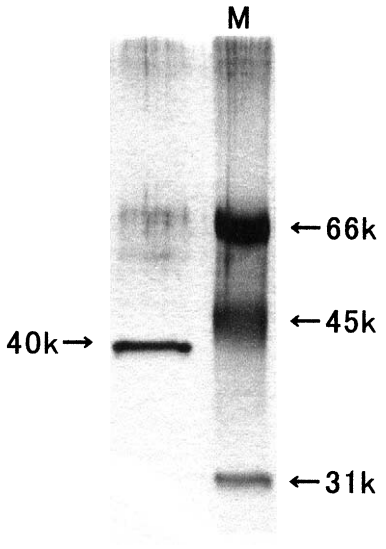

Fig. 1. SDS–PAGE of the purified MnP from *L. betulinus*. M, molecular weight markers.

3.3. Thermal stability

The residual activity of the purified MnP after thermal treatment at several temperatures is shown in Fig. 2. More than 90% MnP activity was retained after treatment at 55°C for 45 min. At 60°C, MnP obtained from *P. chrysosporium* was completely inactivated within 15 min, but L-MnP retained more than 60% of the initial activity even after 60 min treatment (Fig. 3).

Table 1
Purification of L-MnP

	Volume (ml)	A_{280}	A_{405}	Activity[a] (U/ml)	Activity/A_{280} [b]	Activity/A_{405} [c]	A_{405}/A_{208} [d]	Purification (fold)
Culture sup.	500	7.7	0.68	11	1	16	0.09	–
0–70% salting out	50	15.1	2.66	89	6	33	0.18	4.1
DEAE (pH 6→4.5)	15	7.28	2.18	279	38	128	0.30	26.8
Mono Q (pH 6.0→0.7M)	2	1.51	1.38	188	124	136	0.91	87
Superose 12	0.5	0.49	1.48	362	733	244	3.00	513
Mono P (pH 7.0→4.0)	1	0.139	0.596	125	899	210	4.29	629.5

[a]One unit of MnP is defined as the amount of enzyme producing 1 μmol of the Mn^{3+}–oxalate complex (monitored at 270 nm) per minute.
[b]Specific activity per A_{280}.
[c]Specific activity per A_{405}.
[d]The RZ value which was calculated by A_{405} per A_{280}.

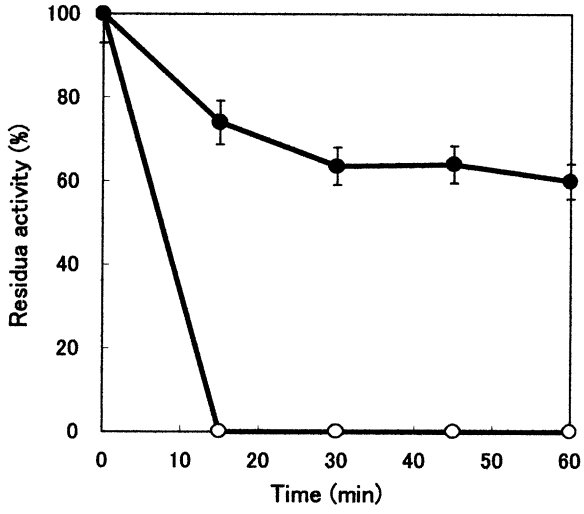

Fig. 2. Thermal stability of the purified MnP from *L. betulinus* at 60°C. ●, *L. betulinus*; ○, *P. chrysosporium*. All data are the mean values of at least four samples. The error associated with each point without an error bar is less than 10% of the value of the point.

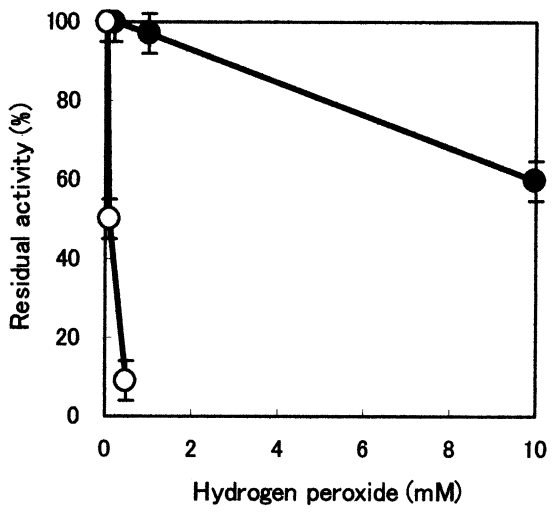

Fig. 4. Hydrogen peroxide dependence of the MnPs from *L. betulinus* (●) and *P. chrysosporium* (○). All data are the mean values of at least four samples. The error associated with each point without an error bar is less than 10% of the value of the point.

3.4. Hydrogen peroxide dependence

With respect to the hydrogen peroxide dependence of *P. chrysosporium* MnP, the optimal hydrogen peroxide concentration was 0.02 mM, and its activity was completely lost with 1 mM hydrogen peroxide. For L-MnP, the optimal hydrogen peroxide concentration was 0.2 mM, and the residual activity was 95% with 1 mM and 60% with 10 mM (Fig. 4).

3.5. N-terminal sequence analysis

The N-terminal end of the 40 kDa band material of L-MnP was sequenced to the 19th amino acid residue and then compared to the other fungal sequences (Fig. 5). L-MnP was found to have consensus sequence CPDG at positions 3–6 and to exhibit homology with other MnPs, especially that from *Phlebia radiata* (74% identity).

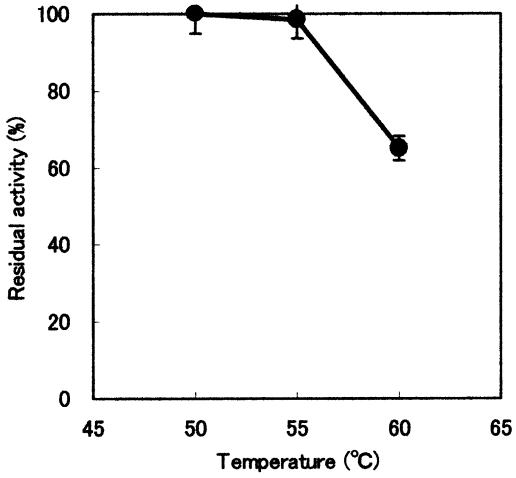

Fig. 3. Thermal stability of the purified MnP from *L. betulinus* for 60 min. All data are the mean values of at least four samples. The error associated with each point without an error bar is less than 10% of the value of the point.

4. Discussion

A thermally stable and hydrogen peroxide tolerant MnP was purified from the culture medium of *L. betulinus*, by anion exchange chromatography, gel filtration and isoelectric focusing. One MnP enzyme (L-MnP) was isolated to homogeneity by SDS–PAGE and partially sequenced. An increase in total MnP activity was observed with the purification steps. The p*I* value reported in this paper, 6.2, differs from those previously reported. Most p*I* values so far reported for MnPs are not as basic as that of L-MnP. The p*I* values for *P. chrysosporium* (4.2–4.9 [18]), *P. radiata* (3.8 [9]), *Pleurotus eryngii* (3.75 and 3.65 [14]), and *Rigidoporus lignosus* (3.5–3.7 [5]) are typical. The predominant band (40 kDa) for the MnP1 preparation we obtained was shown to be a MnP protein by N-terminal amino acid sequencing. MnPs have highly conserved N-terminal sequences (Fig. 5) and, within the group, BLAST search reveals that the first 19 residues of L-MnP show the highest similarity to those of the MnP from *P. radiata* (74%) [7]. The MnP is smaller than most other MnPs so far characterized. The enzymes from *Lentinula edodes* (44.6 kDa [4]), *P. chrysosporium* (46 kDa [13]), *P. radiata* (47–50 kDa [19]), and *Pleurotus eryngii* (43 kDa [14]) are all larger, but the extent to which any difference accounts for a difference in glycosylation is unknown.

We proposed the TSRS method (two-stage reactor system) for biobleaching involving MnP and mesoporous materials [18]. In this system, the Mn^{3+} generation step with MnP immobilized on mesoporous materials and the pulp bleaching

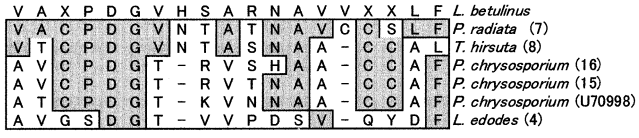

Fig. 5. N-terminal sequence of the first 19 amino acids of various MnPs[4,7,8,15,16]. The dark boxes show regions of sequence identity against L-MnP. Gaps denoted by dashes are included to optimize alignment.

step are separate. In order to produce the high concentration of the Mn^{3+} complex required for efficient pulp bleaching, it is useful to raise the thermal stability and hydrogen peroxide concentration in the MnP reactor. The purified L-MnP exhibited high thermal stability, more than 60% activity being retained after 60 min treatment at 60°C, and also exhibited high hydrogen peroxide dependence, more than 60% activity being retained even with 10 mM hydrogen peroxide. As previously reported, *Ganoderma* sp. YK-505 MnPs (G1 and G2) exhibited 100% MnP activity after treatment for 60 min at 60°C, but lower than 20% residual activity with 10 mM hydrogen peroxide [11]. It is thought that the purified L-MnP has the highest hydrogen peroxide dependence known so far.

Considering the unique characters of the purified L-MnP, it would be useful to realize efficient pulp bleaching and to extend the time for continuous use of an immobilized (MnP–mesoporous material) column. However, the level of MnP production by the fungi is not enough for mass production. In order to realize mass production of L-MnP, it is necessary to clone the gene and to develop the recombinant production methods for such a heme containing enzyme.

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