Oxidation of a non-phenolic lignin model compound by two *Irpex lacteus* manganese peroxidases: evidence for implication of carboxylate and radicals

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**Abstract**

**Background:** Manganese peroxidase is one of the Class II fungal peroxidases that are able to oxidize the low redox potential phenolic lignin compounds. For high redox potential non-phenolic lignin degradation, mediators such as GSH and unsaturated fatty acids are required in the reaction. However, it is not known whether carboxylic acids are a mediator for non-phenolic lignin degradation.

**Results:** The white rot fungus *Irpex lacteus* is one of the most potent fungi in degradation of lignocellulose and xenobiotics. Two manganese peroxidases (IlMnP1 and IlMnP2) from *I. lacteus* CD2 were over-expressed in *Escherichia coli* and successfully refolded from inclusion bodies. Both IlMnP1 and IlMnP2 oxidized the phenolic compounds efficiently. Surprisingly, they could degrade veratryl alcohol, a non-phenolic lignin compound, in a Mn2+-dependent fashion. Malonate or oxalate was found to be also essential in this degradation. The oxidation of non-phenolic lignin was further confirmed by analysis of the reaction products using LC–MS/MS. We proved that Mn2+ and a certain carboxylate are indispensable in oxidation and that the radicals generated under this condition, specifically superoxide radical, are at least partially involved in lignin oxidative degradation. IlMnP1 and IlMnP2 can also efficiently decolorize dyes with different structures.

**Conclusions:** We provide evidence that a carboxylic acid may mediate oxidation of non-phenolic lignin through the action of radicals. MnPs, but not LiP, VP, or DyP, are predominant peroxidases secreted by some white rot fungi such as *I. lacteus* and the selective lignocellulose degrader *Ceriporiopsis subvermispora*. Our finding will help understand how these fungi can utilize MnPs and an excreted organic acid, which is usually a normal metabolite, to efficiently degrade the non-phenolic lignin. The unique properties of IlMnP1 and IlMnP2 make them good candidates for exploring molecular mechanisms underlying non-phenolic lignin compounds oxidation by MnPs and for applications in lignocellulose degradation and environmental remediation.

**Keywords:** *Irpex lacteus*, Manganese peroxidase, Non-phenolic lignin, Veratryl alcohol, Dye decolorization, Carboxylate, Biofuel
Background

Lignocellulose is a renewable but recalcitrant resource for biofuels and bio-based chemicals [1]. In addition to cellulose and hemicellulose, lignin is one of the major components of lignocellulose. The complex lignin network contains phenolic and non-phenolic lignin substructures, with the latter constituting the major part. The white rot fungi (WRF) are regarded to be the best lignocellulose degraders, whose enzymatic systems have hence been an object of extensive studies [2]. WRF produce a range of lignin-modifying enzymes, which include lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase [MnP, or Mn(II):H2O2 oxidoreductase, EC 1.11.1.13], versatile peroxidase (VP, EC 1.11.1.16), laccase (Lac, EC 1.10.3.2), and dye-decolorizing peroxidase (DyP, EC 1.11.1.19) [3]. In addition, free radicals of different types such as hydroxyl radical (OH·), carboxylate anion radical (COO−), and superoxide radical (O2·−) generated by WRF are also implicated in lignocellulose depolymerization [4]. Among the lignin-modifying enzymes, LiP, VP, and DyP are capable of directly oxidizing non-phenolic lignin model compounds such as veratryl alcohol (VA), whereas MnP and Lac do not have this property [5]. Interestingly, however, MnP and Lac appear to be the most abundant lignin-modifying enzymes for many WRF [3]. This suggests that MnP and Lac may use an alternative mechanism(s) to oxidize the high redox potential non-phenolic lignin moiety.

It is well known that MnP can oxidize Mn(II) to Mn(III), which forms chelates with organic acids to directly attack the low redox potential phenolic lignin. During this process, unstable free radicals are formed, which tend to disintegrate spontaneously [6]. The chelated Mn(III) ions can also react with a certain co-oxidant (or mediator) to generate reactive radicals that can depolymerize the high redox potential non-phenolic lignin. Unsaturated fatty acids (UFA) and their lipid derivatives are such mediators that can be peroxidized to form highly reactive acyl and fatty acid peroxy radicals acting on the non-phenolic lignin [7–11]. Other mediators such as glutathione (GSH) may also be involved in the formation of thyl radicals, which are thought to be also involved in the degradation of recalcitrant compounds [12, 13]. However, whether organic acids, particularly those excreted by fungi, are implicated in degradation of high redox potential non-phenolic lignin and xenobiotics has never been clearly demonstrated.

The white rot fungus *Ipex lacteus* has a strong potential in biopretreatment of lignocellulose as well as in biodegradation of xenobiotic compounds. *I. lacteus* appears to produce MnP as the main ligninolytic enzyme under tested conditions [14, 15]. *I. lacteus* CD2 is a strain isolated from Shennong Nature Reserve (Hubei, China) with outstanding capability in degrading lignin and dyes. Although a few MnPs have been purified from the *I. lacteus* cultures, it is not known how these enzymes are involved in destructing lignin and xenobiotics [16, 17]. Herein, we expressed two MnP genes from *I. lacteus* CD2 in *Escherichia coli* and successfully refolded them from inclusion bodies. We showed evidences that MnP-oxidized Mn(III) may chelate with a carboxylic acid and form radicals, which are further implicated in degradation of non-phenolic lignin and high redox potential dyes.

Results and discussion

**Gene cloning and sequence analysis of *IlMnP1* and *IlMnP2***

The MnPs of *I. lacteus* CD2 have been reported to play an important role in the biological pretreatment of lignocellulose and decolorization of synthetic dyes and even simulated textile wastewater [15]. However, the corresponding mechanism involved in lignin depolymerization and dyes decolorization was unclear. In the present study, two MnP genes (GenBank accession numbers KX620478 and KX620479), 1684 and 1622 bp, were identified in the genome of *I. lacteus* CD2 (Additional file 1), and their respective cDNAs were successfully obtained from the culture grown on BM medium. The *IlMnP1* and *IlMnP2* were interrupted by 11 introns and 10 introns, giving two open reading frames (ORFs) of 1077 and 1080 bp, respectively (Additional file 1). Deduced *IlMnP1* and *IlMnP2* contained 358 and 359 amino acid residues and harbored a signal peptide of 18 and 21 residues, respectively. Similar regulatory elements including TATA box, CAAT motif, CreA- and NIT2-binding sites, putative heat-shock element (HSE), and xenobiotic-responsive element (XRE) were discovered in the upstream region of both genes (Additional file 1). Carbon catabolite repression mediated by CreA or its orthologs was widely found both in ascomycetes [18] and basidiomycetes [19]. The presence of CreA-binding sites implied that the expression of the two MnP genes might be repressed by glucose.

**Refolding and purification of *IlMnP1* and *IlMnP2 expressed in *E. coli***

*Pichia pastoris* and *Trichoderma reesei*, the two popular microbial systems for large-scale production of commercial enzymes, were firstly used as the expressing host but the attempts to express *IlMnP1* and *IlMnP2* in these two microbes failed. *E. coli* was then chosen to express these two enzymes. Both *IlMnP* enzymes accumulated exclusively in the inclusion bodies, as had been reported previously for other MnP, VP, and LiP [20, 21]. The inclusion bodies were then solubilized using urea as described previously [20]. Multiple factors including pH, hemin, urea, GSSG, and refolding time are all critical for the successful refolding of peroxidases. By using a fast screening
method with 96-well microplates, the optimum pHs for refolding of both enzymes were determined to be pH 9.5 (Fig. 1a), which were the same as that for a VP from *Pleurotus eryngii* [22]. Alkaline pHs were favorable for the formation of thiolate anion, which was essential for the formation of disulfide bridges [22]. Note that both MnPs were predicted to have four disulfide bridges. Different urea concentrations were required for the maximal yield of active *I. lacteus* CD2 MnP1 (0.2 M) and *I. lacteus* CD2 MnP2 (0.5 M) in refolding (Fig. 1b). The requirements of MnPs from *I. lacteus* CD2 for urea were much lower than other Class II fungal peroxidases (up to 2 M) [21]. The reducing agents GSSG and DTT were also essential for the formation of disulfide bridges. As shown in Fig. 1c, the optimal GSSG/DTT ratios for the MnPs were 5:1 (0.5 mM GSSG versus 0.1 mM DTT). Although hemin was not necessary for the refolding of other Class II fungal peroxidase or the horseradish peroxidase, it was required for the refolding

![Fig. 1 Optimization of the refolding parameters for the recombinant *I. lacteus* CD2 MnP1 and *I. lacteus* CD2 MnP2. a pH. b Urea concentration. c GSSG concentration. d Hemin concentration. e Refolding time. All reactions were performed with 0.1 mg/mL of protein in 50 mM Tris–HCl buffer containing 5 mM Ca^{2+}, 0.1 mM EDTA, and 0.1 mM DTT at 15 °C.](image-url)
of II/MnP1 and II/MnP2 at an optimal concentration of 10 μM (Fig. 1d). Over the time course, the refolding of II/MnP2 significantly increased from 10 to 20 h to reach a plateau, while that of II/MnP1 decreased instead from 10 h (Fig. 1e).

Large-scale refolding of the II/MnP1 and II/MnP2 was conducted under the optimized conditions (0.5 mM GSSG, 0.1 mM DTT, 10 μM hemin, 5 mM CaCl2, and 0.1 mg/mL protein, 0.5 M urea for II/MnP1 or 0.2 M urea for II/MnP2, pH 9.5) for 10 h at 15 °C. The refolded proteins were further purified by anion exchange. Finally, yields of 28.2 mg and 13.3 mg of functional II/MnP1 and II/MnP2, respectively, per liter culture were obtained. Both enzymes showed a single band on SDS-PAGE gels, corresponding to the calculated molecular masses (Additional file 2). Moreover, the enzymes had an absorbance peak at 409 nm (Fig. 2), indicating that each MnP harbors a heme group [23]. The Rz (A407/A280) ratios of II/MnP1 and II/MnP2 were 1.0 and 2.4, respectively.

Optimal pH and temperature of II/MnP1 and II/MnP2

The optimal pHs of recombinant II/MnP1 and II/MnP2 were both pH 4.0 (Additional file 3a). When the pH was above 6.5, no activity was detected for both enzymes. This character was similar to that of native MnPs from I. lacteus strains CD2 (pH 3.0–6.0) and Fr. 238 (pH 3.0–7.6) and other fungi, which are all acidic MnPs (Table 1). The two MnPs varied in pH stability (Additional file 3b). At neutral pH, the II/MnP2 retained much more residual activity than II/MnP1. Interestingly, most native or recombinant MnPs from I. lacteus ever reported exhibit remarkable stability at neutral pH, while one MnP from Phanerochaete chrysosporium was inactive at near neutral pH (6.5) [24]. The optimal temperatures of II/MnP1 and II/MnP2 were both 60 °C (Additional file 3c). However, at 60 °C both enzymes quickly lost their activity within 10 min (Additional file 3d). The thermostability of the two recombinant II/Mnps were not as good as a natively purified MnP from I. lacteus CD2: the native I. lacteus CD2-MnP retained 93.2% of the initial activity after 1 h of incubation at 40 °C. At this temperature, II/MnP2 retained 80.5% activity, while II/MnP1 had only 13.1% left after 1 h of incubation [15]. This weakness in thermostability might be ascribed to the lack of glycosylation during heterologous expression in E. coli [25].

Biochemical analysis of II/MnP1 and II/MnP2 on Mn2+ and phenolic lignin model compounds

The KM values of II/MnP1 and II/MnP2 for Mn2+ were 193.8 and 152.2 μM, respectively, higher than those of native MnPs (17–49 μM) (Table 1). The kcat values of II/MnP1 and II/MnP2 were 7.1 and 6.6 s⁻¹, respectively. The structures and maximal absorbance wavelengths of the substrates (phenolic, non-phenolic lignin model compounds, and dyes) used in this study are listed in Table 2. II/MnP1 and II/MnP2 could oxidize two
phenolic substrates DMP and guaiacol as well as the substrate ABTS, with the specific activities significantly higher in the presence of Mn\(^{2+}\) (Table 3). Although \(I\)IMnP1 and \(I\)IMnP2 can directly attack phenolic lignin model compounds, both enzymes exhibited significant Mn\(^{2+}\)-dependent activity, which was commonly found in MnPs. For example, the \(k_{\text{cat}}\) of the native MnP from \(I.\ lacteus\) CCBAS238 on DMP in the presence of Mn\(^{2+}\)}

**Table 2 Lignin model compounds (LMC) and synthetic dyes used in this work**

| Class                  | Substrate | Structure | \(\lambda_{\text{max}}\) (nm) |
|------------------------|-----------|-----------|-------------------------|
| LMC: phenolic          | DMP       | ![DMP structure](image) | 470                     |
|                        | Guaiacol  | ![Guaiacol structure](image) | 465                     |
| LMC: non-phenolic      | VA        | ![VA structure](image) | 310                     |
| Other                  | ABTS      | ![ABTS structure](image) | 420                     |
| Dye: monoazo           | Remazol brilliant violet 5R | ![Remazol brilliant violet 5R structure](image) | 556                     |
| Dye: disazo            | Reactive back 5 | ![Reactive back 5 structure](image) | 596                     |
| Dye: anthraquinone     | Remazol brilliant blue R | ![Remazol brilliant blue R structure](image) | 600                     |
| Dye: indigo            | Indigo carmine | ![Indigo carmine structure](image) | 610                     |
| Dye: triphenylmethane  | Methyl green | ![Methyl green structure](image) | 640                     |
was 15.7 s⁻¹, 26.2-fold higher than that (0.6 s⁻¹) without Mn²⁺ [16]. The oxidation of phenolic substrates by MnPs was thought to be through one-electron oxidation involving the chelated Mn³⁺ ions [6].

**Degradation of a non-phenolic lignin model compound by IlMnP1 and IlMnP2**

Surprisingly, the two MnPs could also oxidize the non-phenolic substrate VA albeit only in the presence of Mn²⁺ (Fig. 3a). No MnP has been reported previously to have VA-oxidizing ability, which was thought to be a unique catalytic feature of high redox potential peroxidases such as LiP and VP. These VA-oxidizing enzymes commonly have a tryptophan residue involved in VA binding near the heme-binding site [26]. Interestingly, neither IlMnP1 nor IlMnP2 bears such a characteristic tryptophan (aspartate for IlIMnP1 and alanine for IlIMnP2 at the corresponding position instead, Additional file 4), excluding the possibility that IlMnP1 and IlMnP2 are LiPs or VPs. The unusual catalysis of VA suggested that another mechanism must be involved in oxidation of VA by IlMnP1 and IlMnP2.

In order to confirm that IlMnP1 and IlMnP2 could oxidize the non-phenolic lignin model compound VA, the reaction products were further analyzed by HPLC as well as LC–MS/MS. A peak corresponding to veratraldehyde was clearly detected in reactions in the presence of IlMnP1 and IlMnP2, Mn²⁺, and malonate (Fig. 3a) or oxalate (Fig. 3b) but not with acetate, citrate, lactate, or succinate (Additional file 5). Figure 4 is a representative result of the LC–MS/MS analysis with the positive ionization mode, which was conducted with the IlMnP2 reaction product (IlMnP1 appeared to have the same pattern of VA oxidation but a lower peak in HPLC thus not included in MS/MS analysis). The veratraldehyde standard has a molecular weight of 166, thus producing daughter ions of 139 [M-28+H]+, 124 [M-43+H]+, and 109 [M-58+H]+ (Fig. 4a). These ions could also be observed in the MS/MS analysis of IlMnP2-VA reaction products, confirming that IlMnP2 could indeed convert VA into veratraldehyde (Fig. 4) [27].

The catalysis depends on the presence of both Mn²⁺ and a specific carboxylic acid since VA was not degraded when Mn²⁺ was absent (Fig. 3a) or if malonate was replaced by acetate, citrate, lactate, or succinate (Additional file 5). Acetate and succinate could not form complexes with Mn³⁺ [28]; therefore, no oxidation product was observed in the acetate or succinate buffer. Mn³⁺ can form chelates with the rest four organic acids. Mn³⁺-lactate/tartrate complexes were reported.

**Table 3 Substrate specificities of recombinant I. lacteus CD2 manganese peroxidases**

| Substrate | $E_{max}$ (M⁻¹ cm⁻¹) | Wavelength (nm) | $II$MnP1 (U/L) Mn²⁺ present | $II$MnP1 (U/L) Mn²⁺ absent | $II$MnP2 (U/L) Mn²⁺ present | $II$MnP2 (U/L) Mn²⁺ absent |
|-----------|-----------------------|------------------|-----------------------------|-----------------------------|----------------------------|-----------------------------|
| ABTS      | 36,000                | 420              | 920 ± 43                    | 480 ± 5                     | 933 ± 29                   | 160 ± 3                     |
| DMP       | 27,500                | 470              | 380 ± 6                     | 14 ± 1                      | 436 ± 10                   | 4 ± 0                       |
| Guaiacol  | 12,100                | 465              | 142 ± 10                    | 4 ± 0                       | 234 ± 5                    | 2 ± 0                       |

* The concentration of each substrate was 1 mM
to react rapidly with H$_2$O$_2$ to generate O$_2$ [28]. Therefore, we hypothesized that Mn$^{3+}$-lactate/citrate complexes was more apt to react with H$_2$O$_2$ than malonate/oxalate. However, this phenomenon was not detected for the malonate or oxalate buffer [28]. Moreover, it was reported that the carbon-centered radical and superoxide radical were generated from the oxidation of malonate/oxalate by Mn$^{3+}$ [29, 30]. The VA-oxidizing activities of IlMnP1 and IlMnP2 in malonate were higher than those in oxalate (Fig. 3b), and oxalate was also reported to be ineffective in supporting VA oxidation by MnP from *Lentinus edodes* [31]. These clearly indicated that both Mn$^{2+}$ and the carboxylate play an indispensable role in degrading the non-phenolic lignin model compound by IlMnP1 and IlMnP2.

GSH and UFA have been reported to mediate oxidation of non-phenolic lignin compounds by MnPs through generation of highly active thiol and fatty acid peroxyl radicals, respectively [6, 13]. Note that carboxylic acids can also be oxidized by chelated Mn$^{3+}$ to generate radicals [6, 29]. We also noticed that the extent of VA oxidation by IlMnP1 and IlMnP2 improved when enzyme loading increased from 0.05 to 0.25 and then to 0.5 U/mL, particularly for IlMnP2 (Fig. 5a). Besides, the oxidation product veratraldehyde steadily increased with a linear relationship to the concentrations of VA (Additional file 6). However, whether the radicals generated in the MnP-Mn$^{3+}$-carboxylic acids system are able to attack the high redox potential non-phenolic lignin is not known from previous literature studies. Since Mn$^{3+}$ and a certain carboxylate (malonate and oxalate) are the two necessary components needed for IlMnP1 and IlMnP2 to degrade non-phenolic lignin compounds, it is now reasonable to infer that the ability of IlMnP1 and IlMnP2 to oxidize VA was actually through the action of radicals, which were generated by the reactions of MnP-oxidized Mn$^{3+}$ with malonate or oxalate. To gain some insights of VA oxidation by the two IlMnPs, the reactions in the malonate buffer were performed in the presence or absence of SOD (at a final concentration of 3000 U/
that the superoxide radical is at least partially responsi-
MnP2 (by 23.9%) (Fig. 5b). This indicated
Il25.3%) and
but partially inhibited the oxidation of VA by
MnP1, 965 and
1000 U/L in absence and presence of SOD, respectively; for
MnP2, 965 and
in a tartrate buffer
[37]. Note that the rate of dye decolorization was signifi-
cantly reduced in the absence of Mn2+ for all dyes. These
together support the notion that, like lignin degradation,
Mn2+ and a specific carboxylate such as malonate are
important constituents for efficient dye decolorization by
the two MnPs from I. lacteus CD2.

Conclusions
In this study, two manganese peroxidase genes were
cloned from the white rot fungus I. lacteus CD2. By
optimizing a variety of parameters, the E. coli-expressed
IIMnP1 and IIMnP2 were successfully refolded from
inclusion bodies. The recombinant IIMnP1 and IIMnP2
could oxidize a series of phenolic and even non-phenolic
lignin model compounds substrate VA. Mn2+ and a cer-
tain carboxylate (malonate or oxalate) are the two indis-

Application potential of IIMnP1 and IIMnP2 in decolorizing
dyes with different structures
IIMnP1 and IIMnP2 are capable of directly or indirectly
degrading phenolic and non-phenolic lignin compounds
with varying structures, which enlightens us to explore
if they also have the ability to decolorize dyes for envi-
ronmental remediation. A purified MnP from I. lacteus
CD2 has been reported to be able to efficiently decolor-
ize different types of dyes [15]. IIMnP1 and IIMnP2 also
exhibited strong ability to decolorize a broad range of
dyes including the azo dyes (RBV5R and RB5), anthraqui-
none dyes (RBBR), indigo dye (IC), and triphenylmethane
(MG) in the presence of Mn2+ and malonate (Fig. 5).
The decolorization of RBV5R and IC was the fastest: above
85% of the dyes (50 mg/L) could be decolorized by the
enzymes within 1 h (Fig. 6a, d). In contrast, the degrada-
tion of RBBR and MG was much slower; more than 90%
of the dyes could be decolorized after 5 h of incubation
(Fig. 6c, e). Nonetheless, the decolorization of MG by
IIMnP1 and IIMnP2 was much more effective than that
by the purified MnP from I. lacteus CD2 (32% decolor-
ized after 36 h of incubation [15]). The degradation of
RB5 was the slowest: 31.9 and 25.4% were decolorized by
IIMnP1 and IIMnP2, respectively, within 10 h (Fig. 6b).

RB5 is considered a specific substrate for VP but not oxi-
dized by the MnP of P. chrysosporium in a tartrate buffer
[37]. Note that the rate of dye decolorization was signifi-
cantly reduced in the absence of Mn2+ for all dyes. These
together support the notion that, like lignin degradation,
Mn2+ and a specific carboxylate such as malonate are
important constituents for efficient dye decolorization by
the two MnPs from I. lacteus CD2.

Methods
Strain and substrates
Irpex lacteus CD2 was isolated from Shennong Nature
Reserve (Hubei province, China) and preserved in the
Institute of Environment & Resource Microbiol-
y, Huazhong University of Science & Technology,
Wuhan, China. I. lacteus CD2 was maintained at 4 °C on
potato-dextrose agar (PDA) plate. Substrates 2,2′-azino-
bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS),
2,6-dimethylphenol (DMP), veratryl alcohol (VA), guai-
acol, and dyes with various structures including rema-
zol brilliant violet 5R (RBV5R), reactive black 5 (RB5),
remazol brilliant blue R (RBBR), methyl green (MG), and
indigo carmine (IC) were purchased from Sigma-Aldrich
(St. Louis, MO). The superoxide dismutase (SOD) was
purchased from Solarbio (Beijing, China). The structures

mL), which is a commonly used scavenger for superoxide
radical [32]. SOD had no inhibitory effect on the forma-
tion of Mn3+ (for IIMnP1, 1048 and 1053 U/L in absence
and presence of SOD, respectively; for IIMnP2, 965 and
1000 U/L in absence and presence of SOD, respectively)
but partially inhibited the oxidation of VA by IIMnP1 (by
25.3%) and IIMnP2 (by 23.9%) (Fig. 5b). This indicated
that the superoxide radical is at least partially responsi-
ble for VA oxidation by the two MnPs in presence of a
certain carboxylic acid. Interestingly, both malonate and
oxalate are known to be acids excreted by saprophytic
fungi including I. lacteus and P. chrysosporium [8, 28].
Our results suggest that I. lacteus may use its MnPs with
a particular organic acid(s) it excretes to co-operate in
degrading the more recalcitrant lignin.

Interestingly, while the pH optimum for LiP, VP, and
DyP in oxidizing VA is pH 3 or lower [5, 26, 33], IIMnP1
and IIMnP2 exhibited VA-oxidizing ability at pH 5, which
is also optimal for the mostly used T. reesei cellulases [34]
and similar to those of many other acidic plant cell wall
polysaccharides degrading enzymes [35, 36]. MnP, in the
presence of Mn2+ and a carboxylic acid mediator such as
malonate, may hence be used to formulate enzyme cock-
tails with cellulase and hemicellulase to simultaneously
deconstruct lignin, cellulose, and hemicellulose. Besides
the application potential in lignocellulose degradation,
IIMnP1 and IIMnP2 may also serve as good candidates
for further investigating the molecular mechanisms
underlying non-phenolic lignin depolymerization by
MnPs.

Application potential of IIMnP1 and IIMnP2 in decolorizing
dyes with different structures
IIMnP1 and IIMnP2 are capable of directly or indirectly
degrading phenolic and non-phenolic lignin compounds
with varying structures, which enlightens us to explore
if they also have the ability to decolorize dyes for envi-
ronmental remediation. A purified MnP from I. lacteus
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ize different types of dyes [15]. IIMnP1 and IIMnP2 also
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dyes including the azo dyes (RBV5R and RB5), anthraqui-
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(MG) in the presence of Mn2+ and malonate (Fig. 5).
The decolorization of RBV5R and IC was the fastest: above
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of the dyes could be decolorized after 5 h of incubation
(Fig. 6c, e). Nonetheless, the decolorization of MG by
IIMnP1 and IIMnP2 was much more effective than that
by the purified MnP from I. lacteus CD2 (32% decolor-
ized after 36 h of incubation [15]). The degradation of

valuable components in enzymatic degradation of the
non-phenolic lignin. It is proposed that radicals such as
superoxide radical formed in this carboxylate buffer sys-
tem are at least partially involved in degrading these high
redox potential lignin compounds. Besides, IIMnP1 and
IIMnP2 could also decolorize dyes of four different types,
whose efficiency also depended on the presence of Mn2+.
In summary, we demonstrated that the degradation of
non-phenolic lignin by MnP is not restricted to GSH or
UFA mediators but can expand to carboxylic acids, which
are excreted by fungi as a normal metabolite. The proper-
ties of IIMnP1 and IIMnP2 make them ideal candidates
for exploring molecular mechanisms underlying lignin
deconstruction by MnPs and potential players in formu-
lating efficient enzyme cocktails for lignocellulose degra-
dation and dye decolorization.
for the substrates, synthetic dyes, and non-phenolic lignin model compound are listed in Table 2.

Cloning and expression of IlMnP1 and IlMnP2

*Irpex lacteus* CD2 was grown for 5 days in the basal liquid medium [15]. Total RNA was extracted using the TRIZOL reagent (Invitrogen, Waltham, MA) according to the manufacturer’s instructions. The first strand cDNA was synthesized from the total RNA using the TransScript One-Step gDNA Removal and cDNA Synthesis Supermix with oligo (dT) (TransGen). Based on the 5′- and 3′-end sequences of the IlMnP1 and IlMnP2 structural genes, the MnP genes devoid of the sequences encoding the signal peptides were amplified with genespecific primers (as shown in Additional file 7). The PCR products were T-A ligated into pEASY-T3 (TransGen).
and then transformed into the E. coli Trans1-T1 to obtain pEASY-T3-IlMnP1 and pEASY-T3-IlMnP2.

The recombinant plasmids pEASY-T3-IlMnP1 and pEASY-T3-IlMnP2 were double-digested with BamHI/NotI and BamHI/XhoI, respectively, gel purified, and ligated into the pre-digested pET-28a(+) to obtain pET28a-IlMnP1 and pET28a-IlMnP2, and individually transformed into E. coli BL21 (DE3) competent cells. The cells harboring pET28a-IlMnP1 or pET28a-IlMnP2 were pre-cultured in LB medium supplemented with 50 μg/mL of kanamycin at 37 °C overnight with shaking at 200 rpm and used as the inocula of 200 mL LB medium. The cultures were grown at 37 °C for 3 h, followed by the addition of isopropyl-β-d-thiogalactoside (IPTG) to a final concentration of 1 mM for 4-h induction.

After induction, the cells were harvested by centrifugation. The pellets were re-suspended in 50 mM Tris–HCl, 10 mM EDTA, and 5 mM DTT (pH 8.0). Lysozyme (Amresco, Solon, OH) was then added to a final concentration of 2 mg/mL and the cells were incubated on ice for 1 h. Then, 20 μL of DNase I (TransGen) was added and the incubation was continued on ice for 30 min. Subsequently, the cells were centrifuged at 12,000g for 30 min at 4 °C. No MnP activity could be detected from the supernatants. The cell debris was washed with 20 mM Tris–HCl, 1 mM EDTA, and 5 mM DTT (pH 8.0) twice, followed by incubation in 50 mM Tris–HCl, 8 M urea, 1 mM EDTA, and 1 mM DTT (pH 8.0) on ice for 1 h.

To optimize the parameters for recovering active enzyme from inclusion bodies, the refolding was performed in various conditions in a 200 μL volume using 96-well plates at 15 °C. A range of parameters including concentrations of urea, GSSG, and hemin and pH were varied during the refolding. The efficiency of refolding was indicated by the MnP activity. Based on the fast plate-screening result, large-scale refolding of MnPs was performed using the respective optimum parameters. After refolding, the crude enzymes were centrifuged at 12,000g for 10 min at 4 °C and the insoluble fractions were discarded. The supernatants containing the refolded MnP were concentrated through a 10 kDa cut-off centrifuge filter, followed by dialysis against 20 mM phosphate buffer, pH 6.0. The crude enzymes were further purified by a HiTrap Q HP anion exchange column (GE Health, Fairfield, CT) pre-equilibrated with the same phosphate buffer. The proteins were eluted with a linear gradient of 0–1.0 M NaCl, and fractions containing active enzymes were pooled.

Biochemical characterization of IlMnP1 and IlMnP2

The refolded IlMnP1 and IlMnP2 were first subjected to UV–visible spectroscopic analysis in the range of 230–800 nm in the 20 mM malonate buffer (pH 5.0). The MnP activity was measured by monitoring the oxidation of ABTS (ε290 = 36,000 M⁻¹ cm⁻¹) at 420 nm, in a buffer containing 50 mM malonate, 1 mM ABTS, 1 mM MnSO₄, and 0.1 mM H₂O₂ (pH 5.0 and 25 °C). For the Mn²⁺-independent activity assay, MnSO₄ was omitted. One unit (U) of enzyme activity was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per min at 25 °C [30]. For kinetic studies, the reactions were performed in the 50 mM malonate buffer (pH 5.0) at 25 °C using 10–4000 μM Mn²⁺ (in the presence of 0.1 mM H₂O₂) as the substrate by monitoring the formation of Mn₃⁺-malonate complexes (ε270 = 11,590 M⁻¹ cm⁻¹) at 270 nm [15]. The non-linear least square fitting method was used to calculate the Kₘ, kₙₐ₅, and kₙₐ₅/Kₘ parameters of the recombinant IlMnP1 and IlMnP2 using the GraphPad Prism 5 software.

To determine the pH optimum, the MnP activity on ABTS was determined in the 20 mM malonate buffer at a pH ranging from 3.0 to 7.0 at 25 °C. For temperature optimum, the enzymatic activity was measured in the 20 mM malonate buffer (pH 5.0) at a temperature from 20 to 80 °C. To evaluate the pH stability, IlMnP1 and IlMnP2 were individually incubated at different pH levels (3.0–7.0) for 1 h, and the residual activities were assayed as described above. For thermostability, IlMnP1 and IlMnP2 were incubated at 40–60 °C for 1 h with samples taken for activity measurement periodically. The residual activities were measured at its optimum pH and temperature.

The substrates specificities of IlMnP1 and IlMnP2 were studied for the oxidation of four different substrates ABTS, DMP, guaiacol, and VA in 50 mM pH 5.0 malonate and 0.1 mM H₂O₂ with or without 1 mM MnSO₄. Activities were calculated using absorption coefficients at the corresponding wavelengths.

Oxidation of non-phenolic lignin model compounds by IlMnP1 and IlMnP2

VA was used in evaluating the abilities of IlMnP1 and IlMnP2 for degradation of the non-phenolic lignin compound. The degradation of VA was performed in 50 mM malonate buffer (pH 5.0) containing 1 mM VA, 1 mM MnSO₄, 0.1 mM H₂O₂, and 0.5 U/mL IlMnP1 or IlMnP2. In some reactions, the malonate buffer was changed to another carboxylate (acetate, oxalate, citrate, lactate, or succinate) buffer (pH 5.0) or MnSO₄ was omitted from the reaction. The effect of VA concentration (0.05–1 mM) on oxidation was analyzed in the malonate buffer (pH 5.0) for 48 h with 0.5 U/mL IlMnP1 or IlMnP2 in the presence of 1 mM Mn²⁺. The effect of enzymes loading and superoxide dismutase (3000 U/mL) on VA oxidation by IlMnP1 or IlMnP2 was analyzed in the malonate
buffer (pH 5.0) for 48 h in the presence of 1 mM Mn\textsuperscript{2+}. The ability of Mn\textsuperscript{3+} formation was evaluated by the oxidation of ABTS. The reaction proceeded at 30 °C for 48 h, and then the reaction products were analyzed by HPLC using a reversed phase C18-column (Eclipse XDB-C18, 4.6 mm × 250 mm, 5 μm). The elution condition was 0% Acetonitrile (ACN), 4 min; 0–60% ACN, 10 min; 60–100% ACN, 1 min; and 100% ACN, 5 min at a flow rate of 0.8 mL/min. The elution peaks were monitored at 310 nm. In order to confirm veratraldehyde as the oxidation product, LC–MS was also performed by coupling a Nexera UHPLC system to an AB-SCIEX 5600 Triple TOF mass spectrometer in positive and high-sensitivity mode.

Decolorization of dyes by IlMnP1 and IlMnP2

Five dyes of different structures were used to evaluate the decolorization capability of IlMnP1 and IlMnP2. The reactions were carried out at 30 °C in a total volume of 200 μL containing 50 mM malonate buffer (pH 5.0), 0.1 mM H\textsubscript{2}O\textsubscript{2}, 0.25 U/mL IlMnP1 or IlMnP2, and 50 mg/L of dye, with or without 1 mM Mn\textsuperscript{2+}. During the incubation, the color changes were periodically detected by measuring the optical density (OD) at 556 nm for RBV5R, 596 nm for RB5, 600 nm for RBBR, 610 nm for MG. The rate of decolorization was formed the experiments. XQ, HH, and YB analyzed the data. XQ and XYS wrote the manuscript and additional files.

Additional file 4. Amino acid sequence alignment of IlMnP1 and IlMnP2 with selected MnPs, VPs, and LiPs. Inverted triangle: the cysteines that might form disulfide bridges; diamond: the structural Ca\textsuperscript{2+}-binding residues; triangle: the active site histidine residues; hexagon: the acid residues forming the Mn\textsuperscript{3+} oxidation site; square: the cryptocyanin responsible for aromatic substrate oxidation. The GenBank accession numbers for these enzymes were: IlMnP1, KX652048; IlMnP2, KX652047; PoMnP2, KQ232043.1; PoMnP4, 4BM1; PoMnP5, KQ232043.1; PoMnP6, KQ232044.1; PoMnP-H4, P19136.1; AVP2, 2BOQ; PCLP-H2, P111542.2; PCLP-H8, AAB00798.1.

Additional file 5. The non-phenolic lignin model compound versatyl alcohol was not oxidized by either IlMnP1 or IlMnP2 as analyzed by HPLC. The enzymes (0.5 U/mL IlMnP1 and IlMnP2, respectively) were incubated with VA in the acetate, citrate, lactate, or succinate buffer (50 mM, pH 5.0) with 1 mM Mn\textsuperscript{2+} at 30 °C for 48 h.

Additional file 6. The product veratraldehyde steadily increased with a linear relationship to the concentrations of VA when oxidized by 0.5 U/ML IlMnP1 (a) or IlMnP2 (b). The reaction systems contained the malonate buffer (pH 5.0) and were incubated for 48 h in presence of 1 mM Mn\textsuperscript{2+}.

Additional file 7. Primers used in this study.

Abbreviations

WRF: white rot fungi; LiP: lignin peroxidase; MnP: manganese peroxidase; VP: versatile peroxidase; Lac: laccase; DyP: dye-decolorizing peroxidase; UFA: unsaturated fatty acids; GSH: glutathione; ORFs: open reading frames; HSE: heat-shock element; XRE: xenobiotic-responsive element; PDA: potato-dextrose agar; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DMP: 2,6-dimethylphenol; VA: versatyl alcohol; RBV5R: remazol brilliant violet SR; RBS: reactive black 5; RBBR: remazol brilliant blue R; MG: methyl green; IC: indigo carmine; SOD: superoxide dismutase; IPTG: isopropyl-β-D-thiogalactoside; ACN: acetonitrile.

Authors’ contributions

XZ, XYS, and BY conceived and designed the experiments. XQ and XHS performed the experiments. XQ, HH, and YB analyzed the data. XQ and XYS wrote the manuscript. YW and HL reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

All data supporting the conclusions of this article are included within the manuscript and additional files.

Consent for publication

All authors provide their consent for publication of their manuscript in Biotechnology for Biofuels.

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