Characteristics of novel lignin peroxidases produced by white-rot fungus

Phanerochaete sordida YK-624

Hirofumi Hirai*, Mutsumi Sugiura, Shingo Kawai, Tomoaki Nishida

Department of Forest Resources Science, Faculty of Agriculture, Shizuoka University,

Shizuoka 422-8529, Japan

* Corresponding author. Tel & fax: +81-54-238-4853.

E-mail address: afhhirai@agr.shizuoka.ac.jp (H. Hirai).
Abstract

We characterized a lignin peroxidase (YK-LiP2) isolated from shaking culture inoculated with white-rot fungus *Phanerochaete sordida* YK-624. YK-LiP2 enzyme was identified and purified to homogeneity by anion-exchange chromatography and gel permeation chromatography. The molecular weight of YK-LiP2 was approximately 45 kDa, and the absorption spectrum of YK-LiP2 was almost the same as that of the LiP (Pc-LiP) from *P. chrysosporium*. Steady-state kinetics of veratryl alcohol (VA) oxidation by YK-LiP2 revealed an ordered bi-bi ping-pong mechanism, although the Pc-LiP oxidation of ferrocytochrome c obeys peroxidase ping-pong kinetics rather than ordered bi-bi ping-pong kinetics. Degradation of dimeric lignin model compounds by YK-LiP2 was more effective than that by Pc-LiP. Moreover, YK-LiP2 and YK-LiP1, which was previously isolated from static culture inoculated with *P. sordida* YK-624, oxidized VA under higher concentration of hydrogen peroxide (> 2.5 mM) although Pc-LiP could not oxidize VA in the presence of 2.5 mM hydrogen peroxide.

*Keywords*: *Phanerochaete sordida* YK-624; Lignin peroxidase; Ordered bi-bi ping-pong mechanism; Lignin substructure model compounds; Hydrogen peroxide
1. Introduction

The metalloenzymes, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, secreted by white-rot fungi from the class Basidiomysetes, are the only known enzymes being able to degrade the recalcitrant plant cell wall constituent lignin.

To degrade the aromatic polymer lignin, white-rot fungi secrete a large number of heme-containing peroxidase isozymes [1]. They fall into two families; one family is lignin peroxidase (LiP), and the other is manganese peroxidase (MnP) family. LiP is characterized by its high redox potential with hydrogen peroxide enabling oxidation of non-phenolic aromatic compounds [2, 3] and by its long range electron transfer pathway enabling oxidation of polymers such as lignin [4, 5]. MnP is able to oxidize Mn(II) to Mn(III) that it chelates, acting as a diffusing oxidizer [6]. Recently, a new versatile peroxidase, the third ligninolytic peroxidase described in fungi from the genera *Pleurotus* and *Bjerkandera*, has been characterized as a MnP-LiP hybrid peroxidase [7-9].

Several studies have identified efficient lignin-degrading fungi apparently lacking LiP [10-13]. *Phanerochaete sordida* is widely distributed and its potential for organopollutant degradation has been extensively studied [14, 15]. Particularly, the
strain \textit{P. sordida} YK-624 showed high bleaching ability of unbleached hardwood kraft pulp [16-18] and could degrade polychlorinated dibenzo-\textit{p}-dioxines and polychlorinated dibenzofurans [19]. Rüttimann-Johnson et al. characterized three \textit{P. sordida} MnP isozymes but failed to detect LiP or laccase under a wide range of culture conditions [13]. On the other hand, Rajakumar et al. have detected \textit{lip}-like genes in \textit{P. sordida} [20]. This result indicates that \textit{P. sordida} can produce LiP-like peroxidase.

Recently, we have isolated a novel lignin peroxidase (YK-LiP1) from \textit{P. sordida} YK-624 under static culture with Mn-deficient liquid medium [21] and LiP was involved in the biobleaching of manganese-less oxygen-delignified hardwood kraft pulp by \textit{P. sordida} YK-624 [22]. These results indicated that \textit{P. sordida} YK-624 is able to produce LiP isoenzymes extracellularly, and that these isoenzymes are involved in lignin biodegradation by \textit{P. sordida} YK-624. In the present study, we isolated a new LiP (YK-LiP2) from \textit{P. sordida} YK-624 under shaking culture, and these LiPs from \textit{P. sordida} YK-624 were characterized.

2. Materials and methods

2.1 Fungi
P. sordida YK-624 (ATCC 90872), which was isolated from rotted wood [16], and P. chrysosporium ME-446 were used in this study. These fungi were maintained on potato dextrose agar (PDA) slants at 4 centigrade degree.

2.2 Chemicals

4-Ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (dimer I) was synthesized by the modified method described by Kawai et al. [23]. Guaiacylglycerol-β-guaiacyl ether (dimer II) was purchased from Tokyo Chemical Industry, Japan. All other chemicals were also extra-pure grade, obtained from commercial sources, and used without further purification.

2.3 Enzyme preparation

P. sordida YK-624 was grown on PDA plates (d = 9 cm) for 3 days at 30 centigrade degree. In the present study, P. sordida YK-624 was cultivated under static and shaking incubations. On the static incubation, 2 disks (d = 8 mm) punched from the growing edge of mycelium were added to a 100-ml Erlenmeyer flask with 10 ml of Mn-deficient liquid medium [24] containing 1% glucose, 1.2 mM ammonium tartrate, and adjusted to pH 4.5. The culture was statically incubated at 30 centigrade degree for
7 days. On the shaking incubation, 40 disks (d = 8 mm) punched from the growing edge of mycelium were added to a 500-ml Erlenmeyer flask with 200 ml of Mn-deficient liquid medium [24] containing 1% glucose, 1.2 mM ammonium tartrate, 0.1% Tween 80, and adjusted to pH 4.5. The culture was incubated at 150 rpm at 30 centigrade degree.

After 3-days incubation, 6 mM of veratryl alcohol was added to the culture, and then incubated for 10 days. Each extracellular fluid was separated from the mycelia by filtrations with glass fiber filter paper GA-100 (Advantech, Tokyo, Japan) and membrane filters (pore size = 1, 0.45, and 0.2 micro meter; Advantech), and concentrated by ultrafiltration (10 kDa; Advantech). The each concentrated supernatant was adjusted to pH 5.5, and loaded onto a Mono Q HR 5/5 column (Amersham Biosciences Corp., Piscataway, NJ, USA) equilibrated with 10 mM acetate buffer (pH 5.5). Proteins were eluted with a linear gradient from 0 to 0.5 M NaCl (100 min) in the same buffer. The active fractions were collected and concentrated by ultrafiltration. The concentrated active fraction was applied to a column (2.0 by 50.0 cm) of Superdex 75 (Amersham Biosciences Corp.), which was equilibrated with 20 mM succinate buffer (pH 4.5) containing 0.1 M NaCl, with a flow rate of 0.5 ml/min. The active fractions from the Superdex 75 column were collected, and desalted by passage through a PD-10 column (Amersham Biosciences Corp.) equilibrated with distilled water. The final
products, each LiP-like peroxidase were designated “YK-LiP1 (from static culture)” and “YK-LiP2 (from shaking culture). LiP isozyme H8 (Pc-LiP) was purified from cultures of *P. chrysosporium* by the method described by Wariishi and Gold [25].

2.4 Enzyme characterization

The activity of each peroxidase was determined by monitoring the oxidation of veratryl alcohol (VA) at 310 nm. The standard reaction mixture contained 1 mM VA, 20 mM succinate buffer (pH 3.0), and 0.2 mM hydrogen peroxide. One katal (kat) of peroxidase activity is the amount of enzyme producing 1 mol of veratraldehyde (9.3 mM⁻¹cm⁻¹) from VA per second. For some experiments, the concentration of hydrogen peroxide was changed (0.2-7 mM) to study the effect of hydrogen peroxide on the VA oxidation by each peroxidase (0.2 nkat). Protein concentration was determined according to Bradford [26] using bovine serum albumin as a standard. The molecular weight was determined by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gel) using marker proteins (low molecular mass calibration kit; Amersham Biosciences Corp.) and gel filtration through a Superdex 75 column using marker proteins (gel filtration LMW calibration kit; Amersham Biosciences Corp.).
2.5 Oxidation of various compounds by peroxidases

The oxidation of VA, anisalcohol, benzyl alcohol, 2,6-dimethoxyphenol, guaiacol, and Mn(II) by YK-LiP2 and Pc-LiP was determined. The oxidation of VA to veratraldehyde, anise alcohol to anisaldehyde (17.0 mM$^{-1}$cm$^{-1}$ at 285 nm), benzyl alcohol to benzaldehyde (1.8 mM$^{-1}$cm$^{-1}$ at 280 nm), 2,6-dimethoxyphenol to the coerulignone (49.6 mM$^{-1}$cm$^{-1}$ at 470 nm), guaiacol to the tetramer (26.6 mM$^{-1}$cm$^{-1}$ at 470 nm) was performed in 20 mM succinate buffer (pH 3.0). The oxidation of Mn(II) to Mn(III) was performed in 50 mM malonate buffer (pH 4.5), and monitored by the oxidation of 2,6-dimethoxyphenol (1 mM) at 470 nm. The reaction mixtures each contained reducing substrate (1 mM) and 0.2 mM hydrogen peroxide, and the reactions were carried out at 30 centigrade degree.

2.6 Steady-state kinetics of VA oxidation by peroxidases

$1/v$ versus $1/[VA]$ was plotted at several fixed concentrations of hydrogen peroxide. Reaction mixtures (1 ml) each contained peroxidase (0.07 nkat), VA (30-400 micro molar), and hydrogen peroxide (20-60 micro molar) in 20 mM succinate buffer (pH 3.0). The reactions were initiated by the addition of hydrogen peroxide at 30 centigrade degree.
2.7 Degradation of dimeric lignin model compounds

The reaction mixture (10 ml) consisted of 20 mM 2,2-dimethylsuccinate buffer (pH 4.5), 0.1 mM dimer I or II, 2.5 mM glucose, each peroxidase, and 0.25 U of glucose oxidase. The reactions were performed for 24 hours at 30 centigrade degree, and dimer I or II were assayed by high-performance liquid chromatography (HPLC).
3. Results

3.1 Production and purification of the peroxidase from P. sordida YK-624

*P. sordida* YK-624 produced LiP-like peroxidase, namely YK-LiP2, in Mn-deficient nitrogen-limited medium under shaking cultivation. Table 1 summarizes the result of purification of 970 ml of the extracellular culture fluid. After Superdex 75 chromatography, SDS-PAGE of the active fractions revealed that the protein consisted of a single band (data not shown). The overall enzyme yield was 39.6%, with a concomitant 2.9-fold purification. Specific activity of purified YK-LiP2, which was 414 nkat/mg, was almost the same as that of purified Pc-LiP (429 nkat/mg).

3.2 Physical properties

The enzyme exhibited a native molecular weight of approximately 45 kDa as determined by gel filtration on the Superdex 75 column (data not shown), and the molecular weight was also found to be 45 kDa with SDS-PAGE (data not shown). The spectrum of the peroxidase showed a typical peak for native state at 408 nm, and minor peaks at 504, 637 nm.
3.3 Catalytic properties

We analyzed the oxidation of monomeric substituted aromatic compounds by each peroxidase. Table 2 shows relative activities which are expressed as the percentage of activity for each substrate with respect to VA, following the determination of initial oxidation rate by each peroxidase. YK-LiP2 oxidized VA and a variety of substituted aromatic compounds but, of these, preferred to use VA as the electron donor. Reactivity of both peroxidases towards the different substrates was similar, and YK-LiP2 hardly oxidized Mn(II) in 50 mM malonate (pH 4.5) (data not shown).

3.4 Steady-state kinetics

The family of plots, $1/v$ versus $1/[VA]$, at various fixed concentration of hydrogen peroxide, yielded a set of parallel lines (Fig. 1), indicating a ping-pong mechanism for YK-LiP2 oxidation of VA. Secondary plot of y intercepts from Fig. 1 versus $1/[H_2O_2]$ shows a linear relationship but does not pass through the origin (Fig. 1, inset), suggesting that the reaction obeys the classical ordered bi-bi ping-pong kinetics.

3.5 Degradation of dimeric lignin model compounds

We determined the degradation ability of each peroxidase toward dimeric β-O-4
lignin model compounds. YK-LiP2 degraded both dimers more effectively than Pc-LiP, as shown in Fig. 2. YK-LiP2 (1.0 nkat) degraded 84% of phenolic dimer II whereas Pc-LiP (1.0 nkat) degraded it by 66% (Fig. 2). Moreover, 2.5 nkat of YK-LiP2 degraded 75% of non-phenolic dimer I, whereas 2.5 nkat of Pc-LiP degraded it by 49% (Fig. 2).

3.6 Effect of hydrogen peroxide on the VA oxidation by each peroxidase

We examined the effect of hydrogen peroxide, which is the oxidant of peroxidase, on the VA oxidation by YK-LiP1, YK-LiP2, and Pc-LiP (Fig. 3). The VA oxidation by Pc-LiP was drastically decreased by the increasing of the H₂O₂ concentration, and no VA oxidation was observed when 2.5 mM H₂O₂ was added to the reaction mixture. On the other hand, YK-LiP1 and YK-LiP2 indicated VA oxidation in 2.5 mM of H₂O₂ concentration, and the VA oxidations by YK-LiP1 and YK-LiP 2 were observed at 7 mM and 4 mM of H₂O₂ concentration, respectively.
4. Discussion

We have isolated novel lignin peroxidases (YK-LiP1 and YK-LiP2) from *P. sordida* YK-624. Reactivity of YK-LiP2 for various monomeric substituted aromatic compounds was also similar to that of Pc-LiP (Table 2). Same tendency has been observed in YK-LiP1 [21]. However, VA oxidation by YK-LiP2 obeyed ordered bi-bi ping-pong mechanism (Fig. 1). Moreover, VA oxidation by YK-LiP1 has obeyed a bi-reactant sequential mechanism [21]. On the other hand, Wariishi et al. indicated that ferrocytochrome c oxidation by Pc-LiP and Mn(II) oxidation by MnP from *P. chrysosporium* obeyed a peroxidase ping-pong mechanism [6, 27]. Thus, the catalytic mechanisms of LiPs from *P. sordida* YK-624 were different from that of LiP from *P. chrysosporium*, and these results suggest that these catalytic cycles of LiPs from *P. sordida* YK-624 are reversible although that of LiP or MnP from *P. chrysosporium* is irreversible [6, 27].

YK-LiP2 effectively degraded dimeric lignin model compounds, in contrast to Pc-LiP (Fig. 2). In our previous report [21], YK-LiP1 has degraded dimeric lignin model compounds more effectively than Pc-LiP, and the oxidation of sinapyl alcohol oligomer by YK-LiP1 has been faster than that by Pc-LiP. However, the oxidation rate
of sinapyl alcohol oligomer by YK-LiP2 was almost the same as that by Pc-LiP (data not shown). These results indicate that LiPs from *P. sordida* YK-624 has a higher affinity than Pc-LiP for dimeric lignin model compounds, and that YK-LiP1 possesses highest affinity for polymeric lignin in our tested peroxidases. These phenomena have not been observed in LiP from other white-rot fungi. In other words, these results suggest that LiPs from *P. sordida* YK-624 are involved in lignin biodegradation by *P. sordida* YK-624.

As shown in Fig. 3, LiPs from *P. sordida* YK-624 indicated VA oxidation under high concentration (> 2.5 mM) of hydrogen peroxide although no VA oxidation was observed in Pc-LiP in the presence of 2.5 mM of hydrogen peroxide. Pc-LiP has a typical peroxidase catalytic cycle but with several unique features, and the formation of a Pc-LiP compound III species from compound II occurs with considerably less hydrogen peroxide than is required with other peroxidases [28]. On the other hand, it is suggested that LiPs from *P. sordida* YK-624 do not form compound III easily since LiPs from *P. sordida* YK-624 indicated higher tolerance for hydrogen peroxide compared with LiP from *P. chrysosporium*.

In the present report, we isolated YK-LiP2 from shaking culture inoculated with white-rot fungus *P. sordida* YK-624. The characteristics of YK-LiP2 differed from that
of YK-LiP1 which has been isolated from static culture. Thus, *P. sordida* YK-624 has produced different LiP isozymes under different culture (static and shaking) conditions.

It is suggested that YK-LiP2 might be induced by physical stress such as shaking.

Further spectral, structural, and molecular biological studies aimed at the clarification of catalytic mechanism, the hydrogen peroxide-tolerance, and expressions of LiPs from *P. sordida* YK-624 are planned.
References


Table 1. Purification of YK-LiP2 from *P. sordida* YK-624 culture fluid.

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (nkat)</th>
<th>Specific activity (nkat/mg)</th>
<th>Yield (%)</th>
<th>Fold</th>
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<tr>
<td>Culture fluid</td>
<td>970</td>
<td>21.6</td>
<td>3134</td>
<td>145.1</td>
<td>100</td>
<td>1.0</td>
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<tr>
<td>UF concentration</td>
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<td>193.1</td>
<td>92.4</td>
<td>1.3</td>
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<td>6.7</td>
<td>2114</td>
<td>315.5</td>
<td>67.5</td>
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<td>1242</td>
<td>414.0</td>
<td>39.6</td>
<td>2.9</td>
</tr>
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</table>
Table 2. The oxidation of monomeric substituted aromatic compounds by each peroxidase.

<table>
<thead>
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<th>YK-LiP2 (%)</th>
<th>Pc-LiP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Anise alcohol</td>
<td>12.8 ± 1.1</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>8.8 ± 0.9</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>2,6-Dimethoxyphenol</td>
<td>65.9 ± 1.5</td>
<td>56.1 ± 1.3</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>12.3 ± 0.8</td>
<td>11.6 ± 0.8</td>
</tr>
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</table>

Note: Indicated dates are the mean and standard deviation of triplicate experiments.
**Figure titles**

Fig. 1. Lineweaver-Burke plots of VA oxidation by YK-LiP2. These reaction mixtures contained 20 micro molar (triangle), 30 micro molar (star), 40 micro molar (diamond), or 60 micro molar (square) hydrogen peroxide, respectively. Inset shows secondary plot of y intercept against inverse $[\text{H}_2\text{O}_2]$. Indicated dates are the mean and standard deviation of triplicate experiments.

Fig. 2. Degradation of dimer I (closed, solid line) and dimer II (open, dotted line) by YK-LiP2 (triangle) or Pc-LiP (diamond). Indicated dates are the mean and standard deviation of triplicate experiments.

Fig. 3. Effect of the concentration of hydrogen peroxide on the VA oxidation by YK-LiP1 (square), YK-LiP2 (triangle), and Pc-LiP (diamond). Indicated dates are the mean and standard deviation of triplicate experiments.
Fig. 1
Fig. 2

Decrease (%) vs. Peroxidase activity (nkat)