Purification and characterization of two lignin peroxidase isozymes produced by *Bjerkandera* sp. strain BOS55

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Abstract The white-rot fungus *Bjerkandera* sp. strain BOS55 excretes at least seven lignin peroxidase (LiP) isozymes. Two of these, LiP-2 and LiP-5 (molecular weight 40–42 kDa), were purified to homogeneity. Both isozymes had the same N-terminal amino acid sequence which showed strong homology with LiP isozymes produced by other white-rot fungi. The kinetics of both isozymes were similar. LiP-5 oxidized veratryl alcohol optimally only in the presence of H_2O_2 near pH 3.0 (16.7 U/mg) and LiP-2 did this below pH 2.5 (33.8 U/mg). Also at normal physiological pHs for fungal growth (pH 5.0–6.5) both isozymes were still active. Further characterization of LiP-2 and LiP-5 revealed that the K_m for H_2O_2 strongly decreased with increasing pH. As a result of this the catalytic efficiency (TN/K_m) calculated on the basis of the K_m for H_2O_2 in the oxidation of veratryl alcohol was constant over wide pH range.

Key words: *Bjerkandera* sp. strain BOS55; Lignin peroxidase; Purification; N-terminal sequence

1. Introduction

White-rot fungi can degrade nature's second most abundant polymer, lignin. This polyaromatic polymer gives woody plants their strength. White-rot fungi excrete peroxidases and H_2O_2-generating enzymes which work together to initiate lignin oxidation [2,3]. The most potent peroxidase having the capability to oxidize substrates with a high ionisation potential is lignin peroxidase (LiP) [9]. This enzyme uses small aromatic fungal metabolites such as veratryl alcohol (VA) [15,22], and 2-chloro-1,4-dimethoxybenzene [17,18] as a cofactor [19].

White-rot fungi have been used to degrade a large variety of organic pollutants such as polycyclic aromatic hydrocarbons (PAHs). A comparison of the PAH-degrading ability of several strains of white-rot fungi revealed that *Bjerkandera* sp. BOS55 was superior [4]. The latter strain also was shown to be among the best biobleachers [16]. *Bjerkandera* sp. BOS55 excretes LiP and manganese peroxidase (MnP) as the major oxidative enzymes [5,15]. This study concentrates on the purification and characterization of two lignin peroxidase isozymes from strain BOS55.

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2. Materials and methods

Bjerkandera BOS55 was cultured on 1 l nitrogen-rich glucose medium (5 g/l mycological peptone) as described earlier [5]. As an inoculum 10 agar plugs with BOS55 were used. After 20 days of culturing in the dark at 30°C, the extracellular culture fluid was harvested.

2.1. LiP activity measurement

LiP activity was measured by recording the increase in absorbance at 310 nm ($T = 30$°C) due to the oxidation of veratryl alcohol (VA) to veratraldehyde (VAD) by LiP ($\varepsilon = 9300$ M^{-1} cm^{-1}). The reaction mixture contained 100 mM sodium tartrate pH 3.0, 2 mM VA, LiP, and the reaction was initiated by the addition of H_2O_2 (final concentration 0.5 mM).

The K_m for H_2O_2 was determined with 2 mM VA in the incubation mixture. The K_m for VA and 2-chloro-1,4-dimethoxybenzene was determined using 0.5 mM H_2O_2 in the incubation mixture. The formation of the predominant product 2-chloro-1,4-benzoquinone from 2-chloro-1,4-dimethoxybenzene was measured at a wavelength of 255 nm ($T = 30$°C) using a molar extinction coefficient of 16 900 M^{-1} cm^{-1}.

Inactivation of LiP by H_2O_2 was studied by incubating LiP, sodium tartrate buffer pH 3.0 or 5.0, and 0.125 mM H_2O_2 at 30°C. To analyze the remaining LiP activity the complete mixture was mixed with VA (final concentration 2 mM) and H_2O_2 (final concentration 0.5 mM).

2.2. Aryl alcohol oxidase (AAO) and manganese peroxidase (MnP) measurements

AAO activity was measured at pH 6.0 (sodium acetate buffer 100 mM) using VA as a substrate. Formation of VAD was followed at a wavelength of 310 nm.

MnP activity was determined at a wavelength of 468 nm by measuring the oxidation of 2,6-dimethoxyphenol ($\varepsilon = 49\,600$ M^{-1} cm^{-1}). The incubation conditions were: 100 mM malonate buffer pH 4.5, 2 mM $MnSO_4$, 0.5 mM H_2O_2, 0.5 mM 2,6-dimethoxyphenol, and sample.

2.3. Purification of lignin peroxidase

The culture fluid was filtered through cheese cloth to remove fungal mycelium. $(NH_4)_2SO_4$ was added to the filtrate to 85% saturation at 0°C. The precipitated crude LiP fraction was recovered by centrifugation (10 000 × *g*, 0°C, 10 min). The pellet was resuspended in demi water and dialysed several times against 20 mM succinate pH 3.5 for two days at room temperature prior to using a FPLC cation exchange column (column material: Source 15S, Pharmacia, column dimensions: h = 7.0 cm, d = 1.5 cm). LiP was eluted using a salt gradient (0–400 mM NaCl in 30 min in 20 mM succinate pH 3.5, 3.0 ml/min). The LiP fraction was dialysed several times against 10 mM NaAc pH 6.0 at room temperature. The different LiP isozymes present in this fraction were separated using a FPLC anion exchanger (column material: Source 15Q, Pharmacia, column dimensions: h = 6.0 cm, d = 0.5 cm). The applied salt gradient increased from 10 mM to 450 mM NaAc pH 6.0 in 45 min at a flowrate of 1.0 ml/min.

Two collected LiP fractions were pure LiP isozymes as confirmed by the symmetrical peak eluting from the FPLC Source 15Q column. Furthermore, these fractions gave one single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide). The protein content was determined using the standard Lowry method with BSA as a standard.

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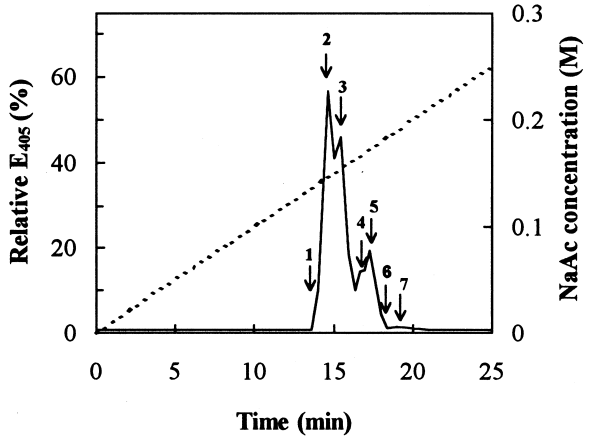

Fig. 1. The elution profile of LiP isozymes (1–7) produced by *Bjerkandera* sp. strain BOS55 from a Source 15Q FPLC column. The dashed line shows the salt gradient.

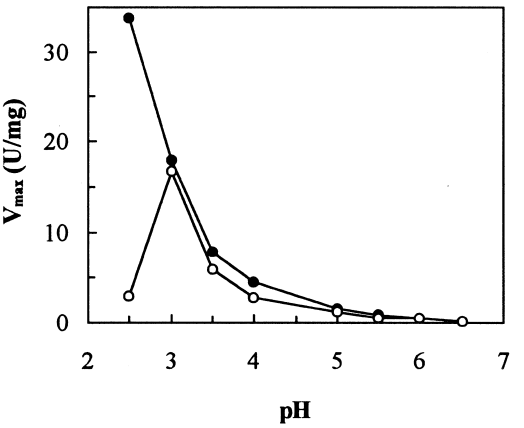

Fig. 2. The effect of the pH on the V_{max} (U/mg) of LiP oxidizing VA studied using both LiP-2 (●) and LiP-5 (○). Experimental conditions: 2.0 mM VA, 100 mM sodium tartrate pH 3.0–5.0/100 mM sodium acetate pH 5.0–6.5, $H_2O_2 = 0.5$ mM, and $T = 30°C$.

2.4. N-terminal sequence

The first 25 amino acids of the N-terminal sequence of both LiP isozymes were determined by Dr. R. Amons (Sylvius Laboratoria, Leiden, The Netherlands).

3. Results

3.1. Purification of LiP isozymes

The purification results in Table 1 show that the culture broth contains a high proportion of contaminating peptides and proteins. Ammonium sulphate precipitation reduces this amount considerably and also removes peptides which interfere with the LiP assay [5]. This results in an apparent increase of total LiP activity of a factor 3.

During dialysis at pH 3.5 LiP was not as stable as anticipated, explaining the loss of activity observed after this step. In the cation exchange step, only lignin peroxidase (LiP) isozymes appear to bind to the column. The mixture of LiP isozymes is eluted as one single peak.

An anion exchange column was used to separate the different LiP isozymes. Fig. 1 shows the elution profile obtained with a FPLC Source 15Q column. The extinction at 405 nm shows the presence of seven heme proteins which were designated as LiP isozymes by measuring the H_2O_2 dependent veratryl alcohol (VA) oxidizing activity. The collected fractions LiP-2 and LiP-5 showed no contamination of aryl alcohol oxidase (AAO) and/or manganese peroxidase (MnP). Furthermore, both isozymes gave one band on SDS-PAGE. Finally, more than 1 mg of each pure LiP isozyme was obtained which was sufficient for further characterization.

3.2. Characterization of LiP isozymes

Both isozymes (LiP-2 and LiP-5) have the same molecular weight of approximately 40 to 42 kDa. Their A_{407}/A_{280} ratios were 5.3 and 4.2 respectively. The VIS-spectrum showed a strong absorbance at 407 nm and weak absorbances at 508 and 638 nm which is characteristic for native LiP isozymes.

Fig. 2 shows that LiP-5 oxidizes VA optimally near pH 3.0. The decrease in V_{max} at pH values below 3.0 suggests that the protein denaturates under these conditions. The pH optimum of LiP-2 is even below pH 2.5 (33.8 U/mg). Both isozymes could still oxidize VA at high pH values of 5.0 to 6.5 (V_{max} of LiP-2: 1.7–0.1 U/mg) which are of relevance in relation to normal fungal culture conditions. This differs from LiP from *Phanerochaete chrysosporium* which had no appreciable activity at pH values greater than 5.0 [20].

Table 2 shows that the K_m of LiP-2 and LiP-5 for veratryl alcohol (VA) increases by raising the pH from 3.0 to 5.0. The data suggest that VA binding to LiP is stronger at pH 3.0 than at pH 5.0. Since the turnover number (TN) of LiP-2 decreases from 11.1 s^{-1} at pH 3.0 to 1.1 s^{-1} at pH 5.0 and the K_m increases, the catalytic efficiency (TN/K_m) decreases from 1.2×10^5 at pH 3.0 to 5.8×10^3 M^{-1} s^{-1} at pH 5.0. These values are in the same range as those reported for purified LiP from *P. chrysosporium* [20]. Both LiP isozymes also oxidized the fungal metabolite 2-chloro-1,4-dimethoxybenzene. The K_m of this compound at pH 4.0 was in the same range as that for VA, but the V_{max} was about 10- to 20-fold lower.

Fig. 3 shows that the K_m for H_2O_2, measured using both the predominant isozyme (LiP-2) and the minor isozyme (LiP-5), strongly depends on the pH. The linear relationship between log K_m and pH suggest that a single acid group with a pKa above 5.0 is responsible for the binding of H_2O_2. At pH 2.5 the K_m (442 μM) of LiP-2 is about 60 times higher than at pH 5.0 ($K_m = 7.5$ μM). Comparison of the K_m values at pH

Table 1
Purification of two lignin peroxidase isozymes from the extracellular culture broth of *Bjerkandera* BOS55

Step[a]	Volume (ml)	Activity (U)	Protein (mg)	Spec. act. (U/mg)	Recovery (%)	Purification factor
CB	1860	446	5494	0.1	–	1
AS	48.4	1388	739	1.9	100	19
D	65.5	511	530	1.0	36.8	10
CE	4.64	166	32.1	5.2	12.0	52
AE LiP-2	4.6	28.3	1.7	16.7	2.0	167
AE LiP-5	4.6	25.7	1.4	18.2	1.9	182

[a]CB, culture broth; AS, $(NH_4)_2SO_4$ precipitation; D, dialysis; CE, cation exchange column; and AE, anion exchange column.

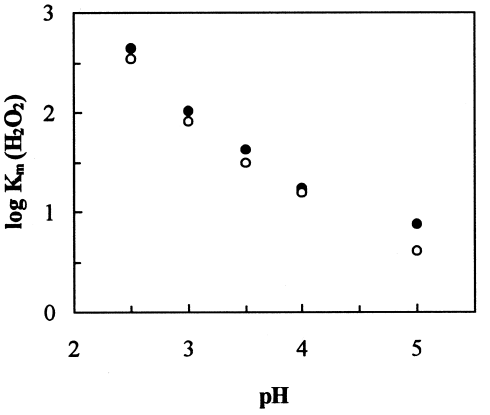

Fig. 3. The logarithm of the K_m for H_2O_2 of LiP-2 (●) and LiP-5 (○) at different pHs. Conditions: 2.0 mM VA, 100 mM sodium tartrate pH 3.0–5.0, and $T = 30°C$.

4.0 (LiP-2 = 17.2 μM, and LiP-5 = 15.7 μM) with literature data shows that these values are similar to those reported for LiP H1 and H8 produced by *P. chrysosporium*. The K_m for H_2O_2 of other *P. chrysosporium* LiP isozymes are higher, but in the same range [3].

The catalytic efficiency of LiP using H_2O_2 (TN/K_m) during the oxidation of VA is constant in a broad pH range from 3.0 to 5.0 (values were between 1.2 to 2.6×10^5 M^{-1} s^{-1}) which is comparable to LiP produced by *P. chrysosporium* [20].

3.3. Inactivation of LiP by H_2O_2

Lignin peroxidase uses H_2O_2 for its catalysis, but it is also known to be inactivated by H_2O_2 [21]. Fig. 4 shows that at pH 3.0 LiP-2 is almost instantly inactivated in the absence of VA whereas at pH 5.0 activity can still be detected after 15 min. The VIS-spectrum of the heme region (not shown) showed that LiPII and/or LiPIII [23] were present at least 20 min at pH 5.0. At pH 3.0, however, the heme was completely bleached within 3 min.

3.4. N-terminal amino acid sequence comparison

The N-terminal sequences (Table 3) were determined to allow comparison with literature N-terminal sequences of LiP isozymes produced by other fungi. The two sequences for LiP-2 and LiP-5 were identical, and showed a high degree of homology with the N-terminal sequence of the three LiP isozymes, LPO-1, LPO-2, and LPO-3, produced by *Bjerkandera adusta* [12]. Especially LPO-3 showed a high degree of homology; only one amino acid was different when the 12 N-terminal amino acids are compared.

Table 2
The kinetic parameters, K_m (μM) and V_{max} (U/mg) determined at different pHs using two fungal metabolites veratryl alcohol (VA) and 2-chloro-1,4-dimethoxybenzene

pH	*Bjerkandera* BOS55 LiP-2				*Bjerkandera* BOS55 LiP-5			
	Veratryl alcohol		2-Cl-1,4-DMB		Veratryl alcohol		2-Cl-1,4-DMB	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
3.0	89	15.9	n.d.	n.d.	59	13.9	318	2.0
4.0	123	7.5	106	0.4	107	5.6	198	0.5
5.0	190	1.5	–	–	131	0.9	–	–

n.d., not done; and –, 2-chloro-1,4-dimethoxybenzene was not oxidized by LiP at pH 5.0.
Conditions: Tartrate buffer (100 mM, pH 3.0–5.0), 0.5 mM H_2O_2, $T = 30°C$.

4. Discussion

Two different LiP isozymes have been purified to homogeneity. The N-terminal region showed high homology towards LiP isozymes produced by other white-rot fungi especially to those produced by *B. adusta* [12].

Both VA and H_2O_2 are extremely important in physiological oxidation reactions catalyzed by LiP. Native LiP reacts with H_2O_2 forming LiP compound I which can indirectly oxidize substrates by first oxidizing VA to the corresponding cation radical. This can in turn oxidize phenolics and organic acids [1,13]. Furthermore, VA may act as a substrate for LiP compound II allowing LiP to complete its catalytic cycle [10]. The combination of these two roles makes VA important in the LiP catalyzed oxidation reactions necessary for the degradation of polycyclic aromatic hydrocarbons (PAHs) and lignin.

Bjerkandera sp. strain BOS55 produces H_2O_2, but the physiological concentration is very low. In liquid media of *Bjerkandera* BOS55 the H_2O_2 concentration is relatively constant at 5 μM H_2O_2 [14]. For *P. chrysosporium* similar values have been reported [21]. These low H_2O_2 levels suggest that the fungal peroxidases should be very efficient in using their H_2O_2 at physiological pHs. As can be seen in Fig. 3 the K_m

Table 3
Comparison of the N-terminal amino acid sequence of LiP-2 and LiP-5 with those reported for selected LiP isozymes produced by other white-rot fungi

LiP isozyme	N-terminal sequence
BOS55 LiP-2	V-A-C-P-D-G-R-H-T-A-I-N-A-A-C-C-N-L-F-T-V-R-D-D-I[a]
BOS55 LiP-5	V-A-C-P-D-G-R-H-T-A-I-N-A-A-C-C-N-L-F-T-V-R-D-D-I[a]
B.a. LPO-3	V-A-C-P-D-G-R-N-T-A-I-N-A[b]
B.a. LPO-1	V-A-C-P-D-G-K-N-T-A-I-N-A-A-C-C-S-L-F-T-A-R-D-D-I[b]
B.a. LPO-2	V-A-C-P-D-G-K-N-T-A-I-N-A[b]
P.c. H1	V-A-C-P-?-G-V-H-T-A-S-N-A-A-C-C-A[c]
P.c. H2	V-A-C-P-D-G-V-H-T-A-S-N-A-A-C-C-A[c]
T.v. LP12	V-A-C-P-D-G-V-N-T-A-T-N-A-A-C-C-Q-L-F-A-V-R-E-D-L[d]

[a]This work.
[b]Adapted from [11].
[c]Adapted from [7].
[d]Adapted from [6,8].
BOS55, *Bjerkandera* sp. strain BOS55; B.a., *Bjerkandera adusta*; P.c., *Phanerochaete chrysosporium*; T.v., *Trametes versicolor*.

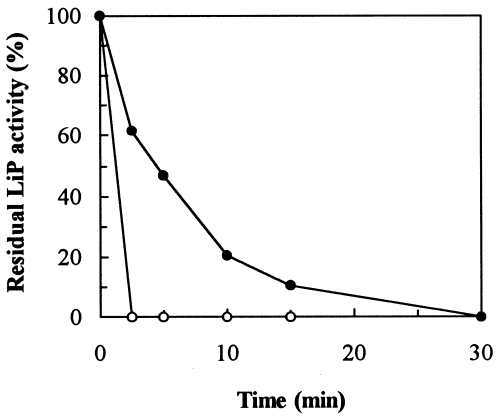

Fig. 4. Inactivation of the major isozyme LiP-2 at pH 3.0 (○) and 5.0 (●) in the presence of 0.125 mM H_2O_2 at 30°C. Residual activity was measured by mixing the incubated LiP with a solution of 2 mM VA, 0.5 mM H_2O_2 and 100 mM sodium tartrate pH 3.0.

for H_2O_2 at pH 5.0 are 7.5 μM (LiP-2) and 4.1 μM (LIP-5) respectively. In the whole range of pH values between 3.0 and 5.0 the catalytic efficiency of both LiP-2 and LiP-5, using H_2O_2 during the oxidation of VA, is more or less constant.

The logarithm of the K_m for H_2O_2 was found to be linearly dependent on the pH. This relation suggests that an ionizable group with a high pKa (above 5.0) is involved in the binding of H_2O_2.

At pH 5.0 to 6.5, far away from the pH optimum, VA is still oxidized by both LiP isozymes, meaning that under normal liquid BOS55 culture conditions the excreted LiP isozymes are also active. This suggests that LiP-2 and LiP-5 can participate in the extracellular degradation of organic pollutants such as PAHs.

The fungal metabolite 2-chloro-1,4-dimethoxybenzene [17,18] was a LiP substrate at pH values below 4.0. At pH 5.0, however, no appreciable activity could be detected. For this reason current research concentrates on the physiological role of 2-chloro-1,4-dimethoxybenzene and other chlorinated aromatic compounds.

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