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Purification and characterization of lignin peroxidases from *Penicillium decumbens* P6

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Summary

Peroxidases are essential enzymes in biodegradation of lignin and lignite which have been investigated intensively in the white-rot fungi. This is the first report of purification and characterization of lignin peroxidase from *Penicillium* sp. P6 as lignite degradation fungus. The results indicated that the lignin peroxidase of *Penicillium decumbens* P6 had physical and chemical properties and a N-terminal amino acid sequence different from the lignin peroxidases of white-rot fungi. The lignin peroxidase was isolated from a liquid culture of *P. decumbens* P6. This enzyme had a molecular weight of 46.3 KDa in SDS-PAGE and exhibited greater activity, temperature stability and wider pH range than those previously reported. The isolation procedure involved $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE-cellulose and CM-cellulose, gel filtration on Sephadex G-100, and non-denaturing, discontinuous polyacrylamide gel electrophoresis. The K_m and V_{max} values of this enzyme using veratryl alcohol as substrate were 0.565 mmol L^{-1} and 0.088 mmol (mg protein)$^{-1}$ min^{-1} respectively. The optimum pH of P6 lignin peroxidase was 4.0, and 70.6% of the relative activity was remained at pH 9.0. The optimum temperature of the enzyme was 45 °C.

Introduction

Lignin is a complex polymer consisting of phenylpropane units interconnected by a variety of carbon–carbon bonds and ether linkages (Adler 1977; Ramachandra *et al.* 1987). It is the main component of wood and lignite. In nature, lignin physically encrusts cellulose and is resistant to biodegradation (Kirk & Farrell 1987). Most active lignin degraders such as *Phanerochaete chrysosporium*, *Phlebia radiata*, *Trametes versicolor*, *Bjerkandera adusta*, *Chrysonilia sitophila*, *Streptomyces badius* and *Streptomyces flavovirens* belong to the basidiomycetes (Kirk & Farrell 1987; Blanchette 1991), though some of them are ascomycetes (Duran *et al.* 1987) or actinomycetes (Crawford *et al.* 1983; Ramachandra *et al.* 1988). In fungi, the biodegradation of lignin is an enzymatic procedure. The ligninolytic enzyme system consists mainly of manganese peroxidase, lignin peroxidase and laccase. Some evidence has shown that many ligninolytic fungi use a combination of any two from these three enzymes (Kuwahara *et al.* 1984; Kantelinen *et al.* 1989).

Lignin peroxidase (LiP; EC 1.11.1.14) is thoroughly investigated ligninolytic enzyme that was first discovered in the white-rot fungus *P. chrysosporium* (Glenn *et al.* 1983). The biochemistry and molecular genetics underlying the ligninolytic systems of *P. chrysosporium* are quite complex. Various LiP isozymes have been found, and the genome of *P. chrysosporium* contains at least 10 structurally related genes encoding LiP proteins, named as *lip*A to *lip*J respectively (Gaskell *et al.* 1994).

About 940 million tons of lignite are produced worldwide each year. In China, the conservative reserves of lignite are about 130.3 million tons, accounting for 13% of total coal resources. Converting this low grade coal to useful materials poses a significant problem both in China and worldwide. Since the typical structures of the original lignin are preserved in coal, some authors have designated lignite as demethylated and dehydrated lignin (Durie *et al.* 1960; Hayatsu *et al.* 1979; Hatcher 1990). Therefore, most of the main coal-biodegrading microorganisms are ligninolytic microorganisms.

In previous work, we obtained a lignite degradation fungus, *Penicillium decumbens* P6 (Yuan *et al.* 1999, 2000). We found that extracellular enzymes played an important role in lignite degradation (Yang *et al.* 2004), but the mechanism of degradation was not clear. Compared with the white-rot fungi, it is possible that LiP also play an important role in the biodegradation of lignite. However, very few data exist on the production and purification of lignin peroxidase or manganese peroxidase in *Penicillium* sp. (Laborda *et al.* 1999; Kumari *et al.* 2002). The present paper deals with the

lignin peroxidase excreted by *P. decumbens* P6, including the purification and characterization of this enzyme from liquid culture.

Materials and methods

Fungal strain and preparation of crude enzyme

P. decumbens P6 (CGMCCNo.0866) was maintained on a yeast extract-malt agar (Lamar *et al.* 1990). Slants inoculated with P6 were incubated at 28 °C for 1 week and maintained at 4 °C. Spores from the slants were suspended in sterilized water and inoculated at concentration of 10^6 spores ml^{-1} into 500 ml flasks containing a 100 ml medium of 10 g glucose, 3 g malt extract, 3 g yeast extract, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $FeSO_4 \cdot H_2O$ in a litre of distilled water with pH 5.0 and sterilized at 115 °C for 30 min. Cultures were incubated with shaking at 28 °C for 7 days at 150 rev/min. Then liquid fermentation cultures were filtered. The filtrate was precipitated with an 80% saturated $(NH_4)_2SO_4$ solution and centrifuged at $10600 \times g$ for 15 min at 4 °C. The precipitate was dissolved in 100 ml distilled water and dialysed three times with 4 h in each time against 1000 ml 5 mM potassium phosphate buffer, pH 7.2 (PB) at 4 °C using dialysis tubing with a molecular weight cut off of about 8000 Da.

Protein concentration

Protein concentrations were determined by using the Bradford method and bovine serum albumin as the standard. LiP concentration in the column effluents was monitored by measuring the absorbance at 409 nm (Farrell *et al.* 1989).

Detection of peroxidase activity in P. decumbens P6

Non-denaturing discontinuous PAGE was used to analyse peroxidase enzymes (Ramachandra *et al.* 1987). After electrophoresis, the gel was treated for 10 min at 37 °C with a reaction mixture containing 10 mM caffeic acid (Sigma), 0.05 mM aminoantipyrine (Sigma), 4.0 mM hydrogen peroxide, 0.1 M potassium phosphate buffer (pH 7.0). Peroxidase bands stained red. Reactions were stopped by placing the gel in a solution of ethanol-water (1:1).

Enzyme assay

LiP activity was determined by monitoring the conversion of veratryl alcohol to veratryl aldehyde at 25 °C by hydrogen peroxide at 310 nm as described by Tien & Kirk (1984). One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of substrate per min.

Ion-exchange chromatography

The first step in peroxidase purification involved a DEAE-cellulose (Sigma) column (2.5 × 18 cm), which had been equilibrated with 5 mM PB (pH 7.2). Following elution of unbound material with the same buffer, the column was washed stepwise with 50, 150, 300, 500 mM and finally 1 M NaCl in 5 mM PB. The eluting solution was collected in fraction of 5 ml. Protein concentration and LiP concentration were determined respectively for each fraction by the absorption at 280 and 409 nm. Then the LiP activity was estimated as mentioned as above in each fraction with high absorption at 280 and 409 nm.

The active fractions were then loaded onto a CM-cellulose (Sigma) column (2.5 × 18 cm) equilibrated with 5 mM ammonium acetate buffer (pH 4.5). After the unadsorbed materials had been eluted by 300 ml 5 mM ammonium acetate buffer (pH 4.5), the adsorbed proteins were eluted with a linear gradient of 0–1 M NaCl in ammonium acetate buffer. Fractions of 5 ml were collected. A_{409} and A_{280} were measured for each fraction. LiP activity in each fraction with highest A_{409} and A_{280} was also assayed as mentioned above.

Gel filtration

The fraction on the CM-cellulose column with LiP activity was concentrated into 3 ml using ultra-filtration with a molecular weight cut off of 1000 Da and dialysed for 4 h against 500 ml 5 mM PB (pH 7.2) at 4 °C. Then the enzyme solution was supplied to a Sephadex G-100 (Sigma) column (2.6 × 100 cm) pre-equilibrated with 5 mM PB (pH 7.2) and eluted with the same buffer. Fractions of each 3 ml were collected. All the fractions with high A_{409} were pooled and concentrated and LiP activity was measured using non-denaturing, discontinuous PAGE (10% polyacrylamide gel) as described above. The bands with LiP activity were cut out respectively and the proteins recovered from the gel using an elution buffer (1% Triton X-100, 50 mM Tris-HCl, pH 9.5), shaken gently for 10 min and centrifuged at $4942 \times g$ at 4 °C. The supernatant was collected and concentrated by lyophilization.

SDS-PAGE

The purity and subunit molecular weight of purified enzyme was checked using SDS-PAGE (12% polyacrylamide gel). After electrophoresis, the protein bands were visualized by silver staining (Guo 1991). The molecular weights of proteins were estimated according to molecular weight standards (Sigma).

Properties of the LiP

The K_m and V_{max} values for the enzyme, using veratryl alcohol as substrate, were determined by a Lineweaver-Burk plot. Also, LiP activity was measured at 25 °C in

the pH range 3–9 (0.1 M NH_4OAc buffer at pH values of 3, 4 and 5. A 0.1 M phosphate buffer was used to assess pH 6 and pH 7, and 0.1 M Tris-HCl buffer used for pH 8 and pH 9). The LiP activity was also determined at various temperatures between 25 and 65 °C at optimal pH.

Amino acid sequencing

The purified LiP protein was subjected to SDS-PAGE using 12% separating gel. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane by blotting at 16 V for 30 min in a semi-dry transfer cell (Bio-Rad USA). After transfer, the membrane was stained and then washed extensively with Milli-Q water. The protein band was cut out and air-dried. The N-terminal amino acid sequencing was conducted using a 491 Protein Sequencer (ABI USA). The sequence of *P. decumbens* P6 LiP obtained was compared to other fungal LiPs in the sequence database by BLAST search.

Results

Detection and purification of LiP in P. decumbens P6

The excretion of LiP in liquid culture of *P. decumbens* P6 was evidenced by the results of selective staining of the enzyme in PAGE. It showed that *P. decumbens* P6 had two peroxidase isoenzymes, L1 and L2, in liquid culture (Fig. 1). The L1 isozyme was the major one.

With the ion-exchange chromatography on DEAE-cellulose, LiP activity was detected in 6 eluated fractions by absorption at 409 nm. They were an unadsorbed peak (D1) and five adsorbed peaks (D2–D6). Most of the lignin peroxidase activity was located in the two major protein peaks (D3 and D4) eluted with 150 and 300 mM NaCl. The D3 peak exhibited more LiP activity than the D4 peak.

In further purification of the D3 peak on the CM-cellulose column, the LiP activity was detected in two fractions: a small peak (C1) in the unadsorbed fraction and a big peak (C2) in the fractions between the elution volumn of 360–600 ml. These two peaks corresponded to the main protein peaks.

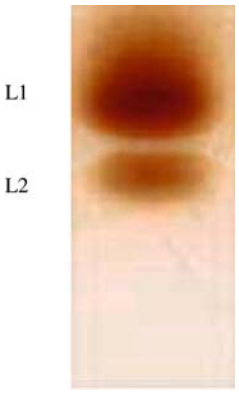

Figure 1. PAGE analysis of *P. decumbens* P6 liquid culture showing the existence of peroxidase activity. Seperation gel concentration was 10%. Enzyme were visualized by selective staining. The enzyme bands were marked as L1 and L2 and L1 was major band.

Fractions corresponding to the C2 peak were subsequently purified using a Sephadex G-100 column. One major peak and several small peaks appeared. The major activity was found in the first peak (S1), but there was an overlap zone between the peak S1 and the second peak (S2) while the collected S1 avoided S2.

The yields and specific activities of the chromatographic fractions are presented in Table 1. The purification efficiency of each method is shown in Fig 2. After purification, the specific activity had increased from 0.05 to 7.5 U/mg, demonstrating a 150.4-fold purification. The recovery of activity was 19.6%. The protein patterns in SDS-PAGE (Fig. 2) also showed that while the enzyme was getting purer, the number of protein bands was decreasing with the degree of purification.

The sample of combined peak S1 was purified finally by PAGE. Selective staining for peroxidase showed only one band with a high level of peroxidase activity. A total of 0.3 mg LiP was recovered from the gel.

Characterization of LiP in P. decumbens P6

Enzyme recovered from the PAGE gel possessed a subunit molecular weight of 46.3 KDa (Fig 3). In analysis of veratryl alcohol oxidation at 25 °C, the K_m of LiP from *P. decumbens* P6 was 0.565 mmol/l and the V_{max} was 0.088 mmol (mg protein) min^{-1}, which was similar with to the values for white-rot fungal LiPs (Farrell *et al.* 1989; Glumoff *et al.* 1990; Rothschild *et al.* 2002). The optimum pH was 4 at 25 °C for the LiP of *P. decumbens* P6. At pH 9.0, 70.6% of the relative activity was still retained. The optimum temperature was 45 °C at pH 4. Enzymatic activity declined with increase or decrease of temperature. And LiP from *P. decumbens* P6 exhibited relatively high temperature tolerance, retaining 62.5% of the relative enzymatic activity at 55 °C.

Ten amino acid residues in the N-terminus of the 46.3 KDa band of LiP were sequenced and compared to other fungal peroxidase sequences (Table 2). *P. decumbens* P6 LiP had the conserve amino acid residues VLL as in fungal MnP and other peroxidases, but no conservative amino acid residues with other fungal LiPs.

Discussion

To reduce environmental damage from weathering and coal burning, biotechnological processes are needed to convert hard coal or lignite to clean, cost-effective energy sources or other useful materials. A microbial, enzymatic or enzyme-mimetic technology that can take place at moderate temperatures and pressures (Fakoussa 1992) would have great advantages compared to the current physical and chemical coal conversion technologies. Biocatalytic particles are also smaller than conventional catalytic particles and thus more efficient.

Table 1. Yields and specific activities during purification of extracellular lignin peroxidase from 10 L culture.

Fraction	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery of activity (%)
Culture filtrate	1287.6	60	0.05	1.0	100.0
$(NH_4)_2SO_4$ precipitation	621.4	49.9	0.08	1.6	83.2
DEAE-cellulose (D3)	18.2	38.6	2.1	42.8	64.3
CM-cellulose (C2)	6.8	25.3	3.7	74.2	42.1
Sephadex G-100 (S1)	1.6	11.7	7.5	150.4	19.6

The strain of *P. decumbens* P6 used in this study was isolated from coalmine soil in Inner Mongolia, China and could completely degrade Chinese lignite in less than 3 days in liquid culture or in 36 h on a plate colony. The products of degradation were humic acids and fulvic acids, both had obvious biological effects (Yuan *et al.* 1999, 2002). In addition, the characteristics of the degradation products changed distinctly (Yuan *et al.* 2000). Furthermore, P6 can excrete extracellular enzymes in the process of coal liquefaction (Yang *et al.* 2004).

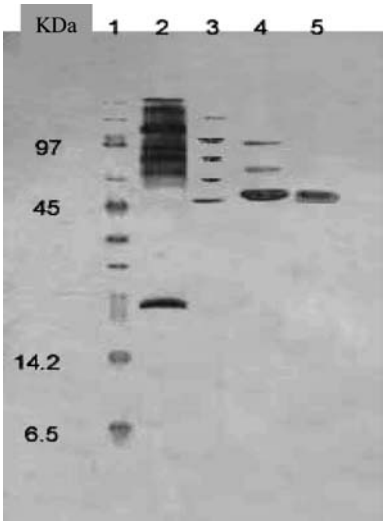

Figure 2. SDS- PAGE of proteins from *P. decumbens* P6 showing the purification efficiency at different stages. Lane 1: molecular weight standards; lane 2: crude enzyme; lane 3: D3 (from DEAE); lane 4: C2 (from CM); lane 5: S1 (from Sephadex G-100).

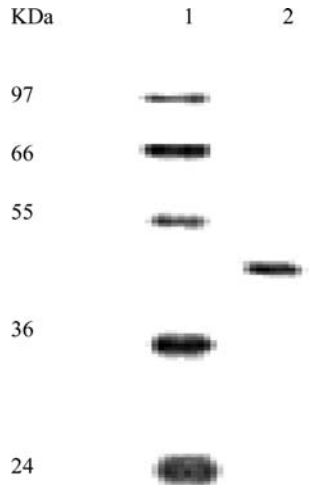

Figure 3. SDS-PAGE pattern of purified LiP 1. molecular weight markers; 2. purified LiP.

Compared with *P. chrysosporium* and other white-rot fungi, P6 has obvious advantages in the biodegradation of lignite, such as being easy to grow and resistant to contamination and so on. Laborda *et al.* (1999) reported that *Penicillium* sp. could excrete extracellular manganese peroxidase in the processes of liquefaction/solubilization of Spanish coals, but their study only focused on the fundamental aspects of microbial coal liquefaction/solubilization involved in coal solubilization.

P. decumbens P6 can excrete peroxidase isoenzymes under liquid fermentation conditions. Peroxidase activity was detected in PAGE by selective staining. The optical absorption spectra of enzyme showed that P6 peroxidase had a Soret band (Yang 2004), which was the typical adsorption spectra of peroxidase (Glumoff *et al.* 1990). Furthermore, enzyme activity indicated that P6 peroxidase was different from aryl alcohol oxidase, which could oxidase aryl alcohol to produce H_2O_2 (Guillén *et al.* 1992; Gutiérrez *et al.* 1994). P6 LiP had high veratryl alcohol activity, without MnP activity, and enzyme activity had to be activated by H_2O_2. The comparation of N-terminal amino acid residues of P6 LiP with those of *P. chrysosporium*, *Ceriporiopsis subvermispora*, *Armoracia rusticana* and *Arabidopsis thaliana* peroxidase revealed that P6 LiP had the conserved peroxidase sequence VLL. All of these data confirmed that the purified enzyme from *P. decumbens* P6 was a peroxidase.

LiPs are the most investigated ligninolytic enzymes in white-rot fungi, but not in *Penicillium* sp. The subunit molecular weight range of white-rot fungal LiP was 38–47 KDa and that of MnP was 38–50 KDa (Fakoussa & Hofrichter 1999). P6 LiP had a subunit molecular weight of 46.3 KDa, that was within this range. Its pure enzyme turnover number was 2.3^{-1} and activity was 7.5 U/mg, 68.4 times than that of commercial LiP from whit-rot fungi (0.11 U/mg) (Fluka), indicating it had production potential.

Fakoussa & Hofrichter (1999) reported that the pH range for LiP was between 2.0–5.0, with an optimum somewhere between 2.5–3.0. The pH range for MnP was between 2.0–6.0, with an optimum between 4.0–4.5. In our case, the P6 LiP is different from other fungal LiP and similar to MnP, since the optimum pH of P6 LiP was 4.0. However, the P6 LiP had a wider range of pH.

Table 2. Comparison of N-terminal amino acid sequences of *Penicillium decumbens* P6 and other fungal peroxidase sequences.

Accession number	Strains origin and peroxidases	N terminal amino acid sequences									
	Penicillium decumbens P6 LiP	V	L	L	P	A	D	E	K	N	A
Q02567	*Phanerochaete chrysosporium* MnP 1	V	L	L	K	G	V	G	F	P	G
AAA62243	*Phanerochaete chrysosporium* MnP H3	V	L	L	K	G	V	G	F	P	G
A32630	*Phanerochaete chrysosporium* MnP H4	V	L	L	K	G	T	G	F	P	G
JC2579	*Ceriporiopsis subvermispora* MnP 1	V	L	L	K	G	V	G	F	P	G
Q42517	*Armoracia rusticana* Peroxidase N	V	L	L	D	G	T	N	S	E	K
Q9SZH2	*Arabidopsis thaliana* Peroxidase 43	V	L	L	S	A	A	H	T	I	G

At pH 9.0, 70.6% of the relative activity was still retained, coinciding with the lignite degradation phenomenon. P6 LiP also exhibited relatively high temperature tolerance, retaining 62.5% of the relative enzymatic activity at 55 °C. The optimum temperature of P6 LiP was 45 °C, higher than that reported for LiP in other researches (Fakoussa & Hofrichter 1999; Kumari *et al*. 2002).

Conclusively, our results confirmed the excretion of LiP by *P. decumbens* P6. The LiP we obtained from *P. decumbens* P6 was different from those produced by other fungi in the amino acid sequence, optimum pH and optimum temperature. These findings offered basic information for the utilization of this strain in the biodegradation of lignite.

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