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Purification, Characterization, and Coal Depolymerizing Activity of Lignin Peroxidase from *Gloeophyllum sepiarium* MTCC-1170

M. Yadav*, P. Yadav, and K. D. S. Yadav

Department of Chemistry, D.D.U. Gorakhpur University, Gorakhpur 273009, India; E-mail: drmeerayadav@rediffmail.com

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Abstract—Lignin peroxidase from the liquid culture filtrate of *Gloeophyllum sepiarium* MTCC-1170 has been purified to homogeneity. The molecular weight of the purified enzyme was 42 kDa as determined by SDS-PAGE. The K_m values were 54 and 76 μM for veratryl alcohol and H_2O_2, respectively. The pH and temperature optima were 2.5 and 25°C, respectively. Depolymerization of coal by the fungal strain has been demonstrated using humic acid as a model of coal. Depolymerization of humic acid by the purified lignin peroxidase has been shown by the decrease in absorbance at 450 nm and increase in absorbance at 360 nm in presence of H_2O_2. Depolymerization of humic acid by the purified enzyme has also been demonstrated by the decrease in the viscosity with time of the reaction solution containing humic acid, H_2O_2, and the purified lignin peroxidase. The influence of NaCl and NaN_3 and inhibitory effects of various metal chelating agents on the lignin peroxidase activity were studied.

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Key words: lignin peroxidase, *Gloeophyllum sepiarium*, veratryl alcohol, humic acid

Lignin peroxidase (EC 1.11.1.14) is a heme-containing enzyme and is characterized by its property of oxidation of high redox potential aromatic compounds such as veratryl alcohol, methoxy benzene, and non-phenolic lignin model dimers [1]. The catalytic cycle consists of the following three steps:

$$\text{LiP [Fe(III) P]} + H_2O_2 \longrightarrow \text{LiP-I [Fe(IV) P}^{+\bullet}\text{]} + H_2O, \quad (1)$$

$$\text{LiP-I [Fe(IV) P}^{+\bullet}\text{]} + RH \longrightarrow \text{LiP-II [Fe(IV) P]} + R^{\bullet}, \quad (2)$$

$$\text{LiP-II [Fe(IV) P]} + RH + 2H^{+} \longrightarrow \text{LiP [Fe(III)P]} + R^{\bullet} + H_2O, \quad (3)$$

where P stands for porphyrin; RH is organic substrate; LiP is lignin peroxidase; LiP-I is an oxyferryl cation radical above two electron oxidized state of LiP; LiP-II is an oxyferryl chemical species, one electron oxidized state of LiP. The radicals generated in steps II and III initiate secondary non-enzymatic reactions leading to products. Structural and functional aspects of lignin peroxidase have been studied [2, 3].

Lignin peroxidase is a biotechnologically important enzyme having applications in delignification of lignocellulosic materials [4] which are seen as an alternative to the depleting oil reserves; in the conversion of coal to low molecular mass fractions [5] which could be used as feed stock for the production of commodity chemicals; in biopulping and biobleaching [6] in paper industries; in removal of recalcitrant organic pollutants [7, 8]; and in polymerization [9, 10] in polymer industries.

The secretion of LiP by a large numbers of fungal strains has been reported [11-16], but LiPs of only a few fungal strains have been purified [1] and characterized. In this communication purification of LiP from the liquid culture filtrate of *Gloeophyllum sepiarium* MTCC-1170 grown in liquid culture medium amended with coir dust as a natural lignin substrate is reported. Enzymatic characteristics like K_m and pH and temperature optima have been determined, and the application of the enzyme in degradation of humic acid has been demonstrated.

MATERIALS AND METHODS

Chemicals. Veratryl alcohol (3,4-dimethoxy benzyl alcohol) and humic acid were from Aldrich (USA);

Abbreviations: LiP, lignin peroxidase.

* To whom correspondence should be addressed.

dimethyl succinate and nitrilotriacetate from Sigma (USA); protein molecular weight markers for SDS-PAGE from Bangalore Genei Pvt. Ltd. (India). Other chemicals were either from CDH or Loba Chemie (India) and were used without further purification.

Fungal strain and enzyme purification. *Gloeophyllum sepiarium* MTCC-1170 was purchased from the MTCC Center and Gene Bank Institute of Microbial Technology, Chandigarh. The fungal strain was maintained on Tien and Kirk [15] medium which consisted of glucose (10 g), malt extract (10 g), peptone (2 g), yeast extract (2 g), L-asparagine (1 g), KH_2PO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (1 g), thiamine-HCl (1 mg), and agar (20 g) dissolved in doubly distilled water (1 liter).

For production of lignin peroxidase, the fungal strain was grown in medium containing (per liter) 10 g glucose, 1.32 g ammonium tartrate, 0.2 g KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 10 μg thiamine, and 1 ml of a solution containing (per liter) 3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1 g NaCl, 100 mg $FeSO_4 \cdot 7H_2O$, 185 mg $CoCl_2 \cdot 6H_2O$, 80 mg $CaCl_2$, 180 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $AlK(SO_4)_2$, 10 mg H_3BO_3, 12 mg $Na_2MoO_4 \cdot 2H_2O$, and 1.5 g nitrilotriacetate. The pH of the basal medium was adjusted to 4.5 with 20 mM dimethyl succinate.

Enzyme was prepared by growing the fungi in 100-ml conical flasks (totally 30) each containing 25 ml of sterilized culture medium amended with 0.5 g of natural lignin containing substrate coir dust. Each flask was inoculated with mycelia of 1 cm diameter under aseptic condition and the fungal culture was grown under stationary culture condition at 25°C in an incubator. On 5th day after inoculation, when lignin peroxidase activity reached maximum value, the cultures were pooled and filtered through four layers of cheese cloth, and then the culture filtrate (with a volume of 600 ml and activity of lignin peroxidase of 0.36 IU/ml) was concentrated (with an Amicon model 8200 concentration cell using PM10 ultrafiltration membrane with molecular weight cutoff value 10 kDa) to final volume of 10 ml. The concentrated enzyme was dialyzed against 1000-fold excess of 5 mM sodium succinate buffer, pH 5.5, overnight at 20°C. The dialyzed enzyme was loaded on a DEAE-cellulose column [17] (1 × 16 cm), which was pre-equilibrated with the same buffer. The column was washed with 50 ml of the same buffer, and the proteins were eluted by applying a NaCl gradient (0-200 mM). The active fractions were combined and concentrated with the Amicon concentration cell model 8200 and thereafter with model 3 using ultrafiltration membrane PM10. The concentrated enzyme was stored at 4°C.

SDS-polyacrylamide gel electrophoresis. The homogeneity of the enzyme preparation was checked by SDS-PAGE using the method of Weber and Osborn [18]. The separating gel was 12% acrylamide in 0.375 M Tris-HCl buffer, pH 8.8, and the stacking gel was 5% acrylamide in 0.063 M Tris-HCl buffer, pH 6.8. Proteins were visualized by silver staining. The molecular weight markers were phosphorylase (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa). The gel was run at a constant current of 20 mA [19].

Enzyme assay. The activity of lignin peroxidase was assayed by the method reported by Tien and Kirk [15] using veratryl alcohol as the substrate and monitoring the formation of veratraldehyde spectrophotometrically at λ = 310 nm using molar extinction coefficient 9300 $M^{-1} \cdot cm^{-1}$. The reaction solution (1 ml) consisted of 2 mM veratryl alcohol and 0.4 mM of H_2O_2 (the enzyme-saturating concentrations) in 50 mM sodium tartrate buffer, pH 2.5, at 25°C. The reaction was started by adding 50 μl of the enzyme solution of 0.203 IU/ml. The Hitachi U-2000 (Japan) spectrophotometer used was fitted with an electronic temperature control unit. The least count of absorbance measurement was 0.001 unit. The activity was expressed in international activity units (IU), which corresponds to 1 μmol/min.

Characterization. The K_m values and pH and temperature optima were determined using veratryl alcohol as the substrate and monitoring the formation of veratraldehyde spectrophotometrically as described in the enzyme assay section. For determination of K_m value for veratryl alcohol, steady-state velocities of the enzyme-catalyzed reaction at different concentrations of veratryl alcohol (0.2-2.5 mM) and fixed concentration of H_2O_2 (0.4 mM) were determined. The K_m value was calculated from the linear regression of the double reciprocal plot [20, 21]. A similar procedure was used for determining the K_m value for H_2O_2.

The pH optimum was determined by measuring the steady-state velocity of the enzyme-catalyzed reaction in solutions of different pH values (1.5-4.5) that were maintained by using 50 mM tartaric acid/disodium tartrate buffer and plotting the steady-state velocity versus pH of the reaction solution.

For determination of temperature optimum, steady-state velocity of the enzyme-catalyzed reaction was determined at different temperatures (15-35°C), and a plot of the steady-state velocity versus temperature was drawn.

Coal depolymerization activity. Screening of the fungi for coal depolymerization was performed in Petri dishes containing Tien and Kirk medium [15], modified Kirk medium, or Czapek–Dox agar medium [22]. Tien and Kirk medium contained (per liter) humic acid (1 g), glucose (10 g), malt extract (10 g), peptone (2 g), yeast extract (2 g), L-asparagine (1 g), KH_2PO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (1 g), thiamine-HCl (1 mg), and agar (20 g). Modified Kirk medium contained (per liter) humic acid (1 g), glucose (0.2 g), yeast extract (0.05 g), dimethyl succinate (2.2 g), ammonium tartrate (0.5 g), KH_2PO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $CaCl_2$ (0.1 g), and agar-agar (28 g). Czapek–Dox agar medium consisted

(per liter) of humic acid (1 g), glucose (0.2 g), yeast extract (0.05 g), $NaNO_3$ (0.5 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.5 g), and agar-agar (18 g). Plates were inoculated with mycelia and were incubated at 25°C in the dark for two weeks. Decolorization of the dark brown agar around the fungal growth area was observed periodically. A pellet of diameter 1 cm taken from the decolorized zone was dissolved in 1 ml of doubly distilled water, filtered through a Millipore Millex-GS 0.22-μ filter unit (USA), and the filtrate was used for the assay of the enzyme.

The coal depolymerizing activity of the purified enzyme was assessed by measuring the decrease in absorbance at 450 nm and the increase in absorbance at 360 nm [22]. The reaction solution consisted of 200 μl humic acid (1 mg/ml in distilled water), 100 μl 0.4 mM H_2O_2 (freshly prepared), 200 μl 50 mM sodium tartrate buffer, pH 2.5, and 450 μl double distilled water and was maintained at 25°C. The reaction was started by the addition of 50 μl of the enzyme solution. Absorbance was observed at intervals of 5 sec at 450 and 360 nm. Absorbance versus time was plotted.

Enzymatic depolymerization of humic acid using purified lignin peroxidase was also studied by viscosity measurement. Time of flow of humic acid (5 mg) dissolved in 30 ml of double distilled water, which was treated with 1 ml of the enzyme solution (1.452 IU/ml) and 500 μl of H_2O_2 (4 mM) was noted at an interval of 30 min using a Tuan-Fouss viscometer [23] corrected to $\pm 0.0001 \cdot 10^{-3}$ N/(m^2·sec). Time of flow was measured using an electronic stopwatch (Racer, USA) with accuracy of ±0.01 sec. Density of the humic acid was determined using a single arm pycnometer [24] ($\pm 0.00001 \cdot 10^3$ kg/m^3). Viscosity of humic acid was plotted against time.

Inhibitor effects were studied under steady-state conditions at optimal temperature and pH values. To study the influence of NaCl on lignin peroxidase [25], the steady-state velocity of the enzyme reaction was measured at different concentrations of NaCl (0-300 mM). Concentrations of NaN_3 and metal chelating agents, namely EDTA, tetramethylethylenediamine (TEMED), ethylenediamine, and 2,2′-bipyridyl, were varied within 0-10 mM. The residual activity of the enzyme was determined as described above.

RESULTS AND DISCUSSION

The procedure for purification of the lignin peroxidase from the culture filtrate of *G. sepiarium* MTCC-1170 is summarized in Table 1, and the elution profile of the enzyme from the DEAE-cellulose column is shown in Fig. 1. The activity of lignin peroxidase is eluted in a single peak that coincides with one of the protein peaks, indicating that the enzyme should be relatively pure.

Figure 2 shows the SDS-PAGE analysis of the purified enzyme. The appearance of one protein band in lane *2* indicates that the enzyme is pure. The molecular weight of the purified enzyme calculated from the SDS-PAGE analysis was 42 kDa. This value of molecular weight of the purified lignin peroxidase lies in the range of molecular weights 40 to 48 kDa reported for the eight isoenzymes of *Phanerochaete chrysosporium* [15]. Thus, the purification of lignin peroxidase from the culture filtrate of *G. sepiarium* is simpler than the purification of lignin peroxidase of *P. chrysosporium*, the lignin peroxidase most extensively studied.

Michaelis–Menten and double reciprocal plots for the lignin peroxidase of *G. sepiarium* using veratryl alcohol and hydrogen peroxide as the variable substrates are shown in Figs. 3a and 3b, respectively. It is obvious from the figures that the purified lignin peroxidase from *G. sepiarium* followed Michaelis–Menten kinetics, and the calculated K_m values of the enzyme for veratryl alcohol and H_2O_2 were 54 ± 2 and 76 ± 2 μM, respectively. It follows from Table 2 that the K_m values of the lignin peroxidase of *G. sepiarium* for veratryl alcohol and H_2O_2 are in the same range as the values reported for lignin peroxidase of other fungal strains [16].

The variation in the activity of the purified lignin peroxidase of *G. sepiarium* with variation in pH and temperature of the reaction solution is shown in Figs. 4 and

Table 1. Purification of lignin peroxidase from *Gloeophyllum sepiarium* MTCC-1170

Purification step	Volume, ml	Protein, mg/ml	Activity, IU/ml	Specific activity, IU/mg	Total protein, mg	Total activity, IU	Purification fold	Recovery, %
Filtrate	600	0.08	0.36	4.5	48.0	216.0	1	100
Concentration stage	10	0.70	8.00	11.4	7.0	80.0	2.5	37
Dialysis	16	0.45	5.22	11.6	7.2	83.5	2.6	39
DEAE-cellulose	15	0.05	1.45	27.9	0.8	21.8	6.2	10

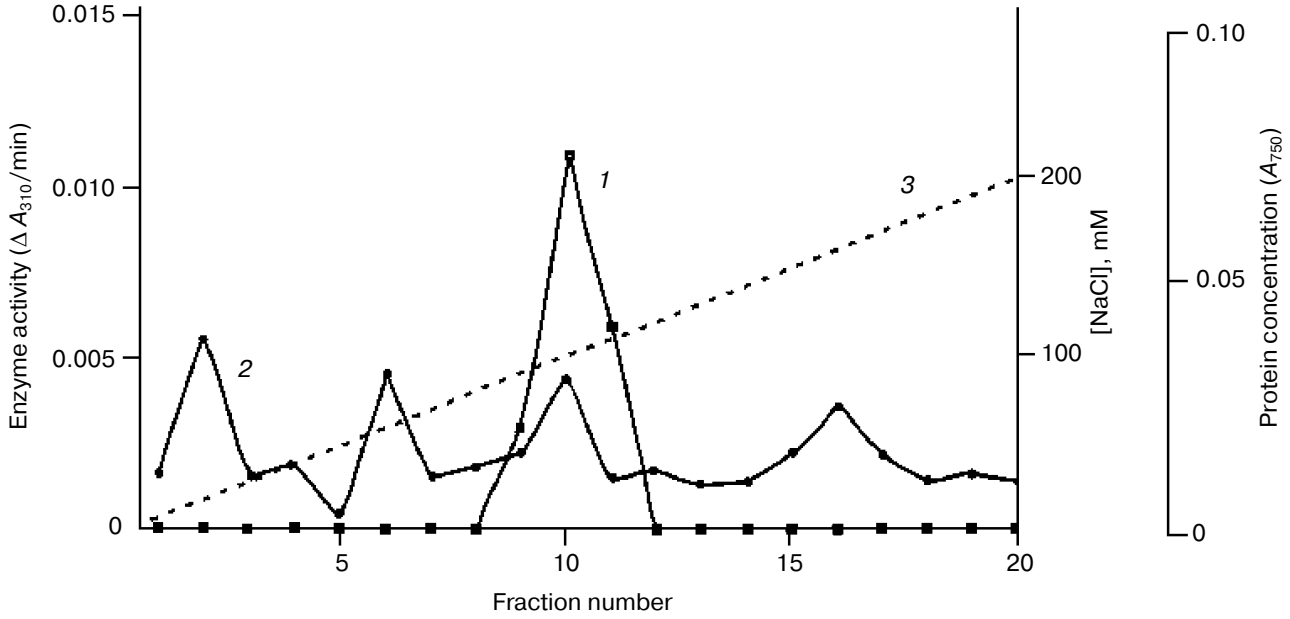

Fig. 1. Typical elution profile from DEAE-cellulose column of dialyzed crude preparation of enzyme: *1*) enzyme activity at 310 nm; *2*) protein at 750 nm; *3*) NaCl gradient. Fractions of 5 ml were collected.

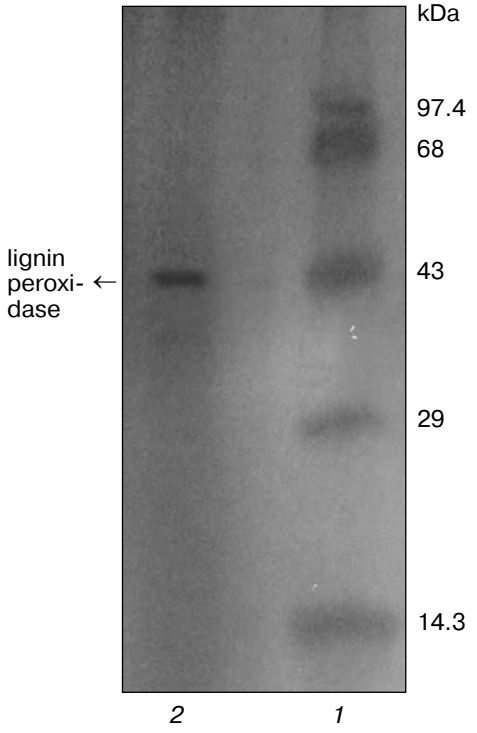

Fig. 2. SDS-PAGE in 12% polyacrylamide gel: *1*) molecular weight markers (from top): phosphorylase (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa); *2*) purified lignin peroxidase (2.5 μg).

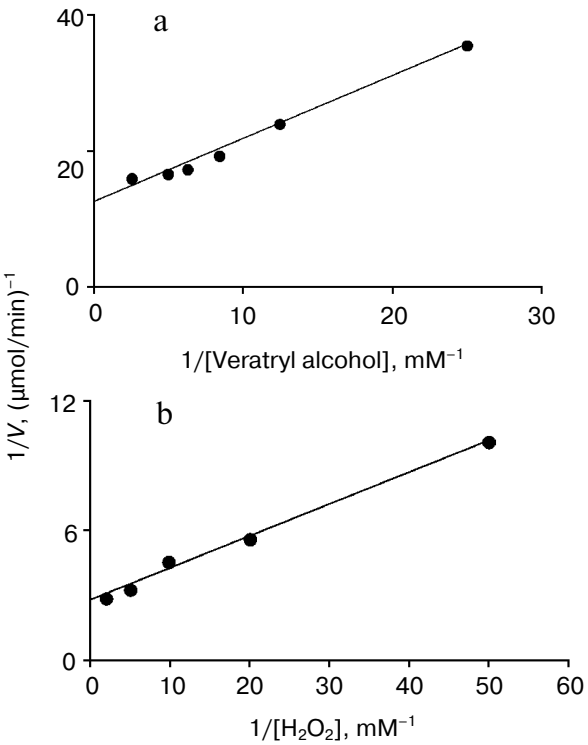

Fig. 3. Double reciprocal plots for the initial rate of lignin peroxidase of *Gloeophyllum sepiarium* versus veratryl alcohol (a) and H_2O_2 (b) concentrations. Reaction was performed in 1 ml of 50 mM sodium tartrate buffer, pH 2.5, at 25°C. Reaction mixture contained: a) 0.02-2.5 mM veratryl alcohol as the variable substrate and 0.4 mM H_2O_2; b) 0.006-0.6 mM H_2O_2 as the variable substrate and 2 mM veratryl alcohol.

Table 2. Temperature and pH optima and K_m values for lignin peroxidase using veratryl alcohol and H_2O_2 as the substrates

Fungal strains	K_m for veratryl alcohol, μM	K_m for H_2O_2, μM	Optimal temperature, °C	pH optimum
Phanerochaete chrysosporium [15]	60	80	26	3
Penicillium citrinum [16]	69	64	30	4
Fusarium oxysporium [16]	64	72	25	2.3
Aspergillus terreus [16]	60	80	22	2
*Gloeophyllum sepiarium**	55	75	25	2.5

* Present study.

5. The enzymatic activity is maximal at pH 2.5, and the enzyme retains more than 50% of its maximum activity in the pH range 2 to 3. The temperature optimum of this enzyme is 25°C. It follows from Table 2 that by the abovementioned parameters, the enzyme from *G. sepiarium* is similar to the peroxidases from *P. chrysosporium* [15], *Fusarium oxysporum* [16], and *Aspergillus terreus* [16], but differs from lignin peroxidase of *Penicillium citrinum* [16].

Figure 6 shows the mycelial growth of *G. sepiarium* on humic acid agar medium. The *G. sepiarium* decolorized the coal in modified Kirk medium [22] to maximum extent as compared to the Czapek–Dox agar medium [22] and Tien and Kirk medium [15]. Decolorization also occurred in the Czapek–Dox agar medium but to lesser extent. The color of humic acid-containing agar is dark brown, which changes to yellow around the fungal growth area. The bleaching effect was stable for three weeks at 25°C.

It has already been demonstrated [22] that the decolorization of agar medium containing coal humic acids is a suitable and easy method for the rapid detection of coal depolymerizing fungi. The bleaching effect around the growth area of fungi is due to a breakdown of high molecular mass coal fractions to low molecular mass fractions. It is assumed that fulvic acid-like compounds are formed during depolymerization of coal. The detection of lignin peroxidase activity inside the bleached growth area of the fungi indicates the role of lignin peroxidase of *G. sepiarium* in the depolymerization of coal.

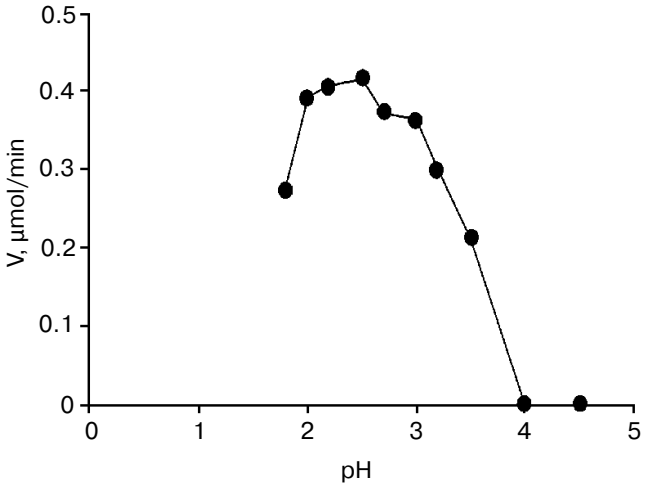

Fig. 4. pH profile of the activity. Reaction mixture (1 ml) consisted of 2 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 1.8-4.5, at 25°C.

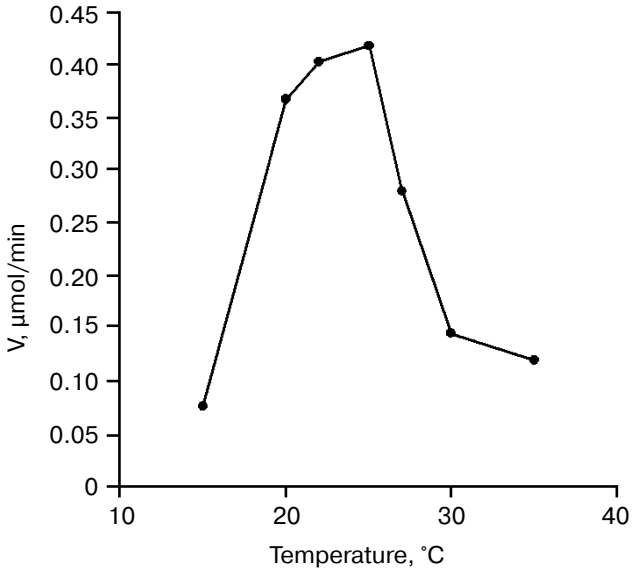

Fig. 5. Temperature profile of activity. Reaction mixture (1 ml) consists of 2 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5, at different temperature (15-35°C).

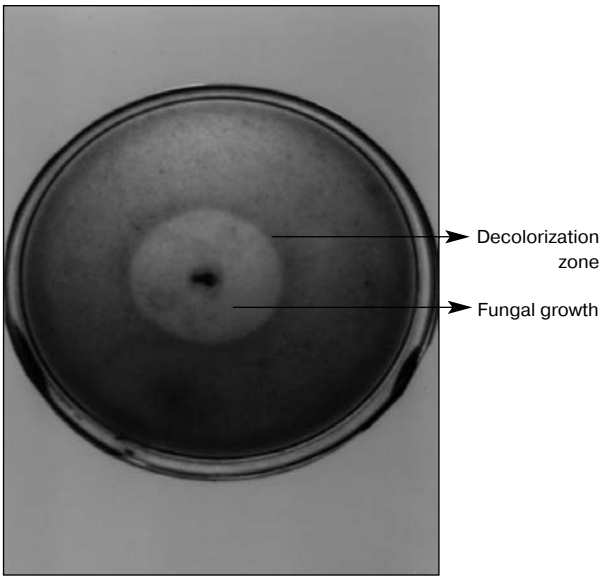

Fig. 6. Decolorization of humic acid by *G. sepiarium* in modified Kirk medium containing humic acid (1 g/liter).

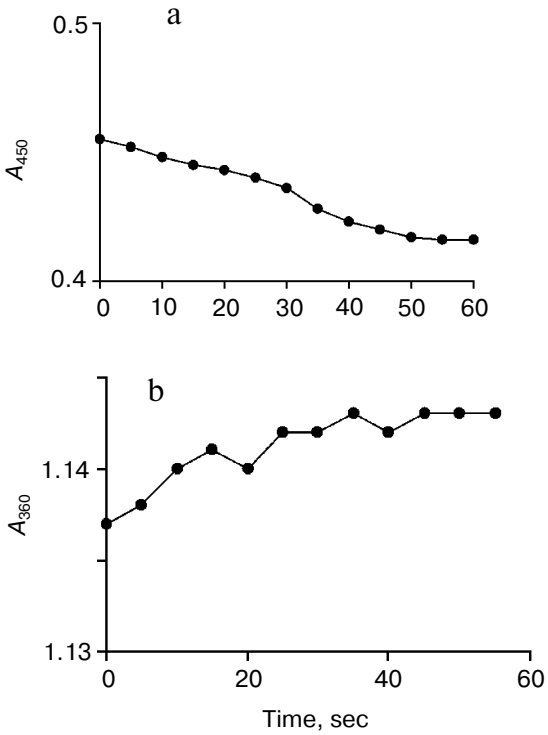

Fig. 7. Coal depolymerizing activity of the purified lignin peroxidase from *G. sepiarium* MTCC-1170 assayed by measuring (a) decrease in absorbance at 450 nm and (b) increase in absorbance at 360 nm. Reaction mixture consisted of 200 µl of humic acid (1 mg/ml in distilled water), 100 µl of H_2O_2 (0.4 mM), 200 µl of 50 mM sodium tartrate buffer, pH 2.5, and 450 µl of doubly distilled water and maintained at 25°C. The reaction was started by the addition of 50 µl of the enzyme solution.

The results of the studies on depolymerization of humic acid by the purified lignin peroxidase of *G. sepiarium* are shown in Fig. 7. Figure 7a shows the depolymerization of the dark brown humic acid fraction (decrease in absorbance at 450 nm) and Fig. 7b shows the formation of yellowish colored fulvic acid-like compounds (increase in absorbance at 360 nm) by purified lignin peroxidase of *G. sepiarium* which is also confirmed by decrease in viscosity of humic acid with respect to time as shown in Fig. 8. Thus, it has been shown that lignin peroxidase caused a disappearance of high molecular mass coal fractions and the formation of smaller ones. These investigations have clarified that the lignin peroxidase from *G. sepiarium* is involved in coal depolymerization. Wondrack et al. [26] first demonstrated the oxidizing effect of lignin peroxidase from *P. chrysosporium* on coal polymers in aqueous solution. The mechanism of breakdown is still to be investigated. Though the mechanism of enzymatic breakdown of coal humic acid by lignin peroxidase is not fully understood, lignin peroxidase is known to generate aryl cation radical by reaction with H_2O_2. This cation radical can induce depolymerization of coal humic acid.

The results of the studies of the effects of NaCl, NaN_3, and metal chelating agents on the activity of the lignin peroxidase of *G. sepiarium* are summarized in Table 3. Lignin peroxidase activity increased with increasing concentration of NaCl up to 150 mM and then decreased and became zero at 300 mM NaCl. The activity of lignin peroxidase decreased with increasing concentration of NaN_3 and became zero at 10 mM NaN_3. In case of metal chelating agents, the activity of the lignin peroxidase decreased and became zero at 8-10 mM concentration of

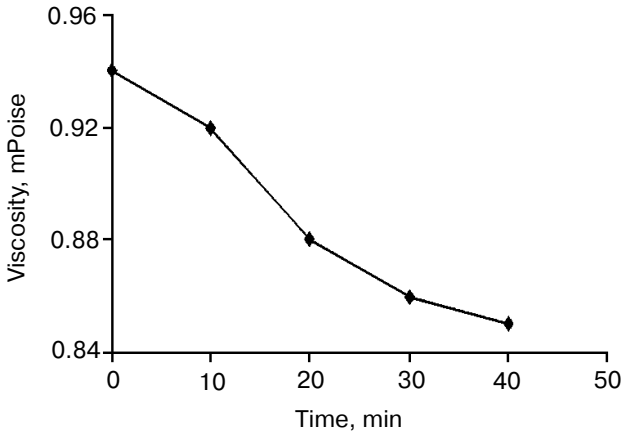

Fig. 8. Time course of changes of viscosity (η) of humic acid during incubation with purified lignin peroxidase from *G. sepiarium* MTCC-1170. The reaction mixture consisted of humic acid (5 g) dissolved in 30 ml of double distilled water, 1 ml of enzyme (1.45 IU/ml), and 500 µl of H_2O_2 (0.4 mM).

Table 3. Effect of inhibitors on activity of lignin peroxidase from *G. sepiarium* MTCC-1170

Inhibitor	Concentration, mM	Residual activity, %
Control	–	100
EDTA	2	24.9
	4	13.3
	6	1.6
	8	–*
TEMED	2	32.4
	4	19.1
	6	7.4
	8	–*
Ethylenediamine	2	43.3
	4	29.0
	6	9.1
	8	0.5
	10	–*
2,2′-Bipyridyl	2	40.8
	4	26.6
	6	7.4
	8	0.6
	10	–*
NaN_3	2	35.8
	4	23.3
	6	6.6
	8	0.4
	10	–*
NaCl	50	51.6
	100	66.6
	150	83.3
	200	54.1
	250	27.4
	300	–*

* Activity is not detected.

the chelating agents. Of note, the bidentate ethylenediamine and 2,2′-bipyridyl caused less inhibition compared to the quadridentate TEMED and hexadentate EDTA ligands. Extensive studies are needed to understand the mechanism of inhibition of lignin peroxidase activity by these agents.

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REFERENCES

1. Angel, T., and Martinez, A. T. (2002) *Enzyme Microb. Technol.*, **30**, 425-444.
2. Wong, D. W. (2008) *Appl. Biochem. Biotechnol.*, DOI 10.1007/s1210-008-8279-z.
3. Piontek, K., Smith, A. T., and Blodig, W. (2001) *Biochem. Soc. Trans.*, **29**, 111-116.
4. Catcheside, D. E. A., and Ralph, J. P. (1999) *Appl. Microbiol. Biotechnol.*, **52**, 16-24.
5. Eriksson, K. E., and Kirk, T. K. (1994) *FEMS Microbiol. Rev.*, **13**, 351-364.
6. Bumpus, J. A., Tien, M., Wright, D., and Aust, S. D. (1985) *Science*, **228**, 1434-1436.
7. Kwant, S. S., and Chang, J. K. (1998) *Biotechnol. Lett.*, **20**, 569-572.
8. Satwinder, S. M., Rajesh, G., Chand, P., and John, F. K. (1998) *J. Chem. Technol. Biotechnol.*, **73**, 292-296.
9. Yoshida, Y., Xia, Z., Takeda, K., Katsuta, T., Sugimato, K., and Funaoka, M. (2005) *Polym. Advanced Technol.*, **16**, 783-788.
10. Ward, G., Hadar, Y., Bilkis, I., Konstantinovsky, L., and Dosoretz, C. G. (2001) *J. Biol. Chem.*, **276**, 18734-18741.
11. Crawfold, D. L., and Crawfold, R. L. (1980) *Enzyme Microb. Technol.*, **2**, 11-22.
12. Stephen, B. P., Anna, L. P., Gavin, J. D. S., Kevin, D. H., and Adinaryana, R. (2005) *Mycol. Res.*, **109**, 115-124.
13. Shanmugam, V., and Yadav, K. D. S. (1996) *Ind. J. Exp. Biol.*, **34**, 1164-1165.
14. Shanmugam, V., and Yadav, K. D. S. (1997) *Ind. J. Microbiol.*, **37**, 105.
15. Tien, M., and Kirk, T. K. (1988) *Meth. Enzymol.*, **161**, 221-234.
16. Kumari, M., Yadav, R. S. S., and Yadav, K. D. S. (2002) *Ind. J. Exp. Biol.*, **40**, 802-806.
17. *Ion Exchange Chromatography: Principles and Methods* (1983) Pharmacia Fine Chemicals, Uppsala, Sweden.
18. Werber, K., and Osborn, M. (1969) *J. Biol. Chem.*, **244**, 4406-4412.
19. *Polyacrylamide Gel Electrophoresis: Laboratory Techniques* (1983) Laboratory Separation Division, Pharmacia, S-75182, Uppsala, Sweden.
20. Engel, P. C. (1977) *Enzyme Kinetics: The Steady State Approach*, Chapman and Hall, London.
21. Yadav, M., and Yadav, K. D. S. (2006) *Ind. J. Biochem. Biophys.*, **43**, 48-51.
22. Hafrichter, M., and Fritsche, W. (1996) *Appl. Microb. Biotechnol.*, **46**, 220-225.
23. Tuan, D. F. T., and Fuoss, R. M. (1963) *Ind. J. Phys. Chem.*, **67**, 1343.
24. Baur, N., and Lewin, S. N. (1995) in *Physical Methods of Organic Chemistry*, Vol. I, Pt. I, 3rd Edn. (Weissberger, A., ed.) Interscience, New York, pp. 131-190.
25. Li-Wen, W., Showalter, A. M., and Ungar, I. A. (1997) *Am. J. Botany*, **84**, 1247-1255.
26. Wondrack, L., Szanto, M., and Wood, W. A. (1989) *Appl. Biochem. Biotechnol.*, **20/21**, 765-780.