Biochem. J. (1988) 254, 877–884 (Printed in Great Britain)

Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*

Marja-Leena NIKU-PAAVOLA,*‡ Eija KARHUNEN,* Päivi SALOLA* and Veijo RAUNIO†
*VTT, Biotechnical Laboratory, SF-02150 Espoo, Finland, and †National Public Health Institute, SF-00280 Helsinki, Finland

One oxidase (EC 1.10.3.2) and three lignin peroxidases (EC 1.11.1.–) were purified from the culture liquid of the white-rot fungus *Phlebia radiata* Fr. All the enzymes were glycoproteins. The oxidase had M_r 64000 and the lignin peroxidases I, II and III had M_r values 42000, 45000 and 44000 respectively. The lignin peroxidases were found to share common antigenic determinants: lignin peroxidases II and III were serologically indistinguishable and lignin peroxidase I was related but distinguishable. The oxidase did not share any immunological properties with the lignin peroxidases. Lignin peroxidases of *Phlebia* contain protoporphyrin IX as a prosthetic group. In the presence of H_2O_2 and an electron donor, veratryl alcohol, lignin peroxidases exhibit spectral shifts analogous to those of animal catalase (EC 1.11.1.6). *Phlebia* enzymes show optimal activity at pH 3–4.5 at 40 °C and are stable in the pH range 5–6. They modify Kraft lignin and phenolic compounds containing hydroxy and methoxy groups.

INTRODUCTION

Oxidative extracellular enzymes have been shown to be in part responsible for the degradation of lignin by the white-rot fungus *Phanerochaete chrysosporium* (Tien & Kirk, 1983). These enzymes have similarities to certain peroxidases (EC 1.11.1.5–EC 1.11.1.8) inasfar as the prosthetic group and reaction mechanisms are concerned (Tien *et al.*, 1986; Andersson *et al.*, 1987). Characterization of the gene coding for the major ligninolytic component of *Phanerochaete* revealed homology in the amino acid sequence of lignin peroxidase with the other peroxidases (Tien & Tu, 1987). Homology is found near the residues essential for peroxidase activity.

Among the characteristics of ligninolytic enzymes so far studied are the cleavage of aromatic rings in lignin model compounds (Leisola *et al.*, 1985) and degradative oxidation of non-phenolic compounds such as the environmental pollutant benzo[*a*]pyrene (Haemmerli *et al.*, 1986). The possible application of these enzymes in a range of different fields has stimulated much research into the biochemistry of the white-rot fungi.

Hitherto, most studies have concerned the enzymes of *Phanerochaete*, although the oxidative extracellular enzyme system is probably not identical in all white-rot fungi. Differences can be seen, for example, in the efficiency of lignin degradation (Hatakka & Uusi-Rauva, 1983) and in the pattern of degradation products (Ander *et al.*, 1980). In order to learn more about the biochemical function of ligninolytic enzymes in general, the purified enzymes of a number of different white-rot fungi should be studied.

The white-rot fungus *Phlebia radiata* has been shown to degrade lignin even more effectively than *Phanerochaete chrysosporium* under certain experimental conditions (Hatakka & Uusi-Rauva, 1983). The extracellular ligninolytic enzymes of *Phlebia* were shown to include three enzymes oxidizing veratryl alcohol and one oxidizing an oxidation–reduction indicator, ABTS stain (Niku-Paavola, 1987).

We present here the purification and properties of these four enzymes.

MATERIALS AND METHODS

Enzyme production

The organism used was *Phlebia radiata* Fr. strain 79 (kindly supplied by A. Hatakka, Department of Microbiology, University of Helsinki). Cultivations were performed in 1-litre batches in a laboratory bioreactor (Hatakka *et al.*, 1987). The low-nitrogen medium described by Fenn & Kirk (1979) was used with slight modifications. Vitamins and mineral salts were omitted, glucose and guaiacol (Merck, Darmstadt, Germany) concentrations were 2.8 mM and 0.8 M respectively and 0.05% (w/v) Tween 80 (Atlas, Essen, Germany) was included. Oxidase was produced with the use of this medium with a cultivation time of 3 days. For the production of lignin peroxidases the nitrogen supply was 0.38 g of yeast extract (Difco Laboratories, Detroit, MI, U.S.A.)/l and the cultivation time was 6 days.

Enzyme purification

Culture filtrates were frozen to −20 °C for at least 20 h. Thawed liquids were filtered through glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.) and equilibrated with 50 mM-sodium acetate buffer, pH 5.5. Proteins were adsorbed on 15 ml of Sepharose Q Fast Flow anion-exchanger (Pharmacia, Uppsala, Sweden) in 50 mM-sodium acetate buffer, pH 5.5. Unadsorbed proteins were washed with 100 ml of buffer. Lignin peroxidase I (L_I) was eluted with 120 ml of 0.13 M-NaCl in 50 mM-sodium acetate buffer, pH 5.5. A linear gradient of 0.18–0.3 M-NaCl in 180 ml of 50 mM-sodium acetate buffer, pH 5.5, was used to elute oxidase and lignin peroxidases II and III (L_{II} and L_{III}). Active fractions were dialysed against 50 mM-sodium acetate buffer, pH 5.5, and used for characterization of enzyme components.

Abbreviations used: ABTS, 2,2-azinodi-3-ethylbenzothiazoline-6-sulphuric acid; O_x, oxidase, L_I, L_{II} and L_{III}, lignin peroxidases I, II and III respectively.

‡ To whom correspondence should be addressed.

Lignin peroxidase activity was determined as the ability of the enzyme to convert veratryl alcohol (Fluka, Buchs, Switzerland) into veratraldehyde in the presence of H_2O_2 (Merck) (Tien & Kirk, 1984). The buffer used was 50 mM-glycine/HCl, pH 3.0, and the temperature was 20 °C. The reaction mixture contained 3.5 μmol of veratryl alcohol, 0.44 μmol of H_2O_2 and 1–10 μg of enzyme in 3 ml. The reaction was monitored during 10 min at 310 nm and activity was calculated as katals. ϵ for veratraldehyde is 9256 $M^{-1} \cdot cm^{-1}$. Oxidase activity was assayed with ABTS (Boehringer, Mannheim, Germany) as substrate (Werner *et al.*, 1970) in 50 mM-glycine/HCl buffer, pH 3.0, at a temperature of 20 °C. The reaction mixture contained 14 μmol of ABTS and 0.1–1 μg of enzyme in 3 ml. The reaction was monitored during 5 min at 436 m and activity was expressed as katals. ϵ for the product is 29300 $M^{-1} \cdot cm^{-1}$.

Protein concentration was determined by precipitating the preparations with 5% (w/v) trichloroacetic acid, dissolving the precipitate in 2% (w/v) Na_2CO_3 solution containing 0.4% NaOH and measuring A_{280}. Human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was treated in a similar way as a reference.

The homogeneity and molecular sizes of the purified enzymes were assessed by performing electrophoresis at pH 8.5 in 25 mM-Tris/192 mM-glycine/0.1% (w/v) SDS buffer at room temperature on 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS (Laemmli, 1970). Pharmacia low-M_r calibration mixture was used as reference. Proteins were localized by silver staining (Bio-Rad Laboratories, Richmond, CA, U.S.A.) (Merril *et al.*, 1981).

Antisera

Antisera were prepared against all the extracellular proteins from concentrated culture liquid and against the four purified enzymes.

During an immunization of 6 months, 1.0 mg of proteins emulsified with 1 ml of complete Freund's adjuvant (Difco Laboratories) was injected intracutaneously once a month into rabbits. The sera were collected and used without further purification for immunological tests.

Immunoelectrophoresis

Immunoelectrophoresis was performed on agarose gel in 50 mM-barbital buffer, pH 8.2, at room temperature during 45 min with a voltage gradient of 6 V/cm (Grabar & Burtin, 1964).

Glycoprotein test

Purified enzymes were treated with endoglycosidase H [endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) from *Streptomyces griseus* (Seikagu Kogyo Co., Tokyo, Japan)] and endoglycosidase F [endo-β-*N*-acetylglucosaminidase from *Flavobacterium meningosepticum* (NEN, Boston, MA, U.S.A.)] to eliminate the possible carbohydrate moiety. Purified enzymes (1–2 μg) were treated with 0.5 munit (12.5 ng) of endoglycosidase H in 125 mM-sodium citrate buffer, pH 5.0, at 37 °C for 24 h (Trimble & Maley, 1984). Ligninolytic enzymes were also treated with 125 munits of endoglycosidase F in 0.1 M-sodium phosphate buffer, pH 6.1, containing 50 mM-EDTA and 0.5% (v/v) Nonidet P-40 (Sigma Chemical Co.) at 37 °C for 24 h (Elder & Alexander, 1982).

After endoglycosidase treatment enzymes were electrophoresed on 10% (w/v) polyacrylamide gel in denaturing conditions (Laemmli, 1970) and electrophoretically transferred at pH 8.3 in 25 mM-Tris/192 mM-glycine/10% (v/v) methanol buffer for 3 h at 0.6 A (Towbin *et al.*, 1979) on to nitrocellulose filters (BA 85; Schleicher und Schüll, Dassel, Germany). The filters were treated with antisera (dilution 1:1000) against *Phlebia* proteins. Detection of the protein–antibody complex was carried out by using a second antibody linked with alkaline phosphatase (EC 3.1.3.1) (Proto Blot Promega Biotec, Madison, WI, U.S.A.). Phosphatase activity was assayed with 0.1 M-Tris/HCl buffer, pH 9.5, containing 0.1 M-NaCl and 5 mM-$MgCl_2$ and with 5-bromo-4-chloroindol-3-yl phosphate as substrate and Nitro Blue Tetrazolium (Promega) for colour development.

Enzyme characteristics

The effects of hydroxylamine, NaN_3, KCN, EDTA and NaF (all reagents from Merck) and of dithiothreitol (Sigma Chemical Co.) on enzyme activity were studied with the use of supplements in 0.1–0.001 mM concentrations. The effect of CO was studied by saturating the enzyme solutions with CO gas, corresponding to a concentration of 1 mM.

The absorption spectra of purified enzyme components were measured in 50 mM-acetate buffer, pH 5.5. The specific shifts were analysed by using supplements of 0.15 mM-H_2O_2 and 1.2 mM-veratryl alcohol added successively, as in the lignin peroxidase assay. The effects of 0.1 mM-dithiothreitol, -KCN and -NaN_3 were analysed separately.

The action of purified enzymes on monophenols and Kraft pine lignin (Indulin; AT West Waco, Covintong, VA, U.S.A.) were analysed in 50 mM-glycine/HCl buffer, pH 3.0, in the presence of 0.15 mM-H_2O_2 with lignin peroxidases, and in 50 mM-2,2-dimethylsuccinate (Fluka) buffer, pH 4.5, with H_2O_2 omitted with oxidase. The action of oxidase was also analysed on L-tyrosine (Merck) and DL-3,4-dihydroxyphenylalanine (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). The incubation mixture contained 10 μg of enzyme and 1 mg of substrate in 3 ml of buffer. The effects of enzyme were analysed after incubation at 20 °C for 10 min. The reaction mixtures were centrifuged and the supernatants were scanned from 190 to 600 nm to determine the differences between the untreated substrates and substrates treated with enzyme. H.p.l.c. analysis was performed with a Waters 6000A apparatus (Milford, MA, U.S.A.) equipped with a Lambda Max 480 u.v. detector. The column used was a 3.9 mm internal diam. × 15 cm one of Nova-Pak C_{18} (5 μm particle size; Waters). The eluent contained acetonitrile/water/tetramethylammonium hydroxide/phosphoric acid (20:80:1:1, by vol.). The run was performed at 40 °C and detection was by measuring A_{240}. The following monophenols were used as references and as substrates: catechol (Koch–Light, Colnbrook, Bucks., U.K.), coumaric acid (Fluka), coniferyl alcohol (Fluka), ferulic acid (Fluka), guaiacol (Merck), vanillin (BDH Chemicals, Poole, Dorset, U.K.), veratraldehyde (Fluka), veratryl alcohol (Fluka) and veratric acid (Fluka).

RESULTS

Under the nitrogen-limited cultivation conditions *Phlebia radiata* secreted three enzymes oxidizing veratryl alcohol and one oxidizing ABTS. On the basis of their

Table 1. Purification of *Phlebia* oxidase

Purification was performed from 1 litre of 3-day-old-culture liquid in which the activity of oxidase was 4.9 nkat/ml and that of lignin peroxidase was 0.007 nkat/ml. For experimental details see the text.

Purification stage	Volume (ml)	Protein (mg/ml)	Total protein		Activity		Total activity	
			(mg)	(% recovery)	(nkat/ml)	(nkat/mg)	(nkat)	(% recovery)
Culture filtrate	980	0.025	24.5	100	4.9	196	4802	100
Purified oxidase	40	0.23	9.2	38	99.0	430	3960	82

Table 2. Purification of *Phlebia* lignin peroxidases

Purification was performed from 1 litre of 6-day-old-culture liquid in which oxidase activity was 0.428 nkat/ml and that of lignin peroxidase was 0.744 nkat/ml. For experimental details see the text.

Purification stage	Volume (ml)	Protein (mg/ml)	Total protein		Activity		Total activity	
			(mg)	(% recovery)	(nkat/ml)	(nkat/mg)	(nkat)	(% recovery)
Culture filtrate	900	0.03	27	100	0.74	25.0	669	100
L_I	10	0.68	6.8	25	1.4	2	14	2
L_{II}	37	0.18	6.6	24	4.3	24	159	24
L_{III}	48	0.17	8.1	30	5.0	30	239	36
				Σ79				Σ62

chromatographic separation they were called lignin peroxidase I (L_I), oxidase (O_x), lignin peroxidase II (L_{II}) and lignin peroxidase III (L_{III}) (Niku-Paavola, 1987). To aid the purification, oxidase was separated from 3-day-old-culture filtrates, in which the lignin peroxidase activity was still low. The production of lignin peroxidases was enhanced when yeast extract was used as nitrogen source. After 6 days of cultivation the oxidase activity was low, whereas lignin peroxidase activities were high. The purification procedures are summarized in Tables 1 and 2. The purification increased the specific activity of oxidase 2-fold and the recovery of activity was 82%. The specific activities of L_{II} and L_{III} were not improved during the purification and that of L_I was even decreased. The total recovery of activity was 62% and that of total protein was 79%. The corresponding values for L_I were 2% and 25% respectively. Inactivation of lignin peroxidase during purification has also been reported for *Phanerochaete* enzymes (Tien *et al.*, 1986). The dissociation of haem in the purification conditions at pH 5.5 could be one reason for this. Fig. 1(*a*) shows that there are comparatively few proteins in the culture liquid; thus major increases in specific activities were not expected during purification.

Separated enzymes were shown to be homogeneous in denaturing SDS/polyacrylamide-gel electrophoresis. M_r values were 42000 for L_I, 64000 for O_x, 45000 for L_{II} and 44000 for L_{III}.

Endoglycosidase H treatment decreased the molecular sizes of all four *Phlebia* enzymes (Figs. 1*b* and 1*c*). Endoglycosidase F had a similar effect. The decrease in the M_r values was 5–14%. Western blotting was used to monitor the M_r values. This method is more sensitive than conventional protein staining methods and shows even minor changes quite accurately. Furthermore, antisera will react only with the antigens in question and not with the endoglycosidases used in hydrolysis.

Immunoblotting and immunoelectrophoretic analysis showed that O_x does not react with L_I antibodies and vice versa. O_x shows non-identity with L_{II} and L_{III} (Fig. 2*a*). L_I, L_{II} and L_{III} shared common antigenic determinants reacting with antibodies raised against the others. L_I shows partial identity with L_{II} and L_{III} (Figs. 2*b* and 2*c*). L_{II} and L_{III} are serologically indistinguishable (Fig. 2*d*).

Lignin peroxidases and oxidases were inhibited by conventional inhibitors of the respiratory chain, cyanide and azide, and also by the reducing agents dithiothreitol and hydroxylamine. Fluoride was shown to be an inhibitor of oxidase but not of lignin peroxidases. EDTA and CO affected lignin peroxidases but not oxidase.

The haemoprotein nature of *Phlebia* lignin peroxidases was seen in the absorption spectra of native enzymes and their derivatives (Fig. 3), which strongly resembled those of catalase (EC 1.11.1.6) (Nicholls & Schonbaum, 1963). Native L_{III} has a Soret peak at 408 nm and minor bands at 510 and 630 nm. The maxima for all the lignin peroxidases were found within ±5 nm of the corresponding peaks of L_{III}. Sequential addition of H_2O_2 and veratryl alcohol caused spectral shifts (Fig. 4) analogous to those of catalase peroxide Compounds I and III (Nicholls & Schonbaum, 1963). Oxidase did not show any absorption maxima at wavelengths longer than 300 nm.

The effect of the ligninolytic enzymes of *Phlebia radiata* on Kraft lignin is shown in Fig. 5. O_x and L_I most clearly modified lignin. This was seen in the absorption spectrum of the buffer-soluble fraction of lignin after the action of enzymes. In h.p.l.c. analysis, no specific monomeric component could be shown to be responsible for this

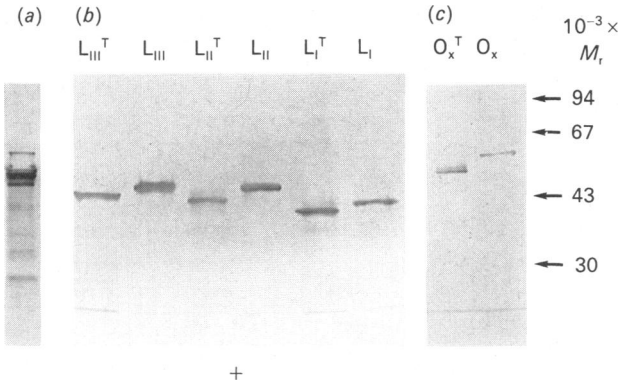

Fig. 1. Elimination of carbohydrates from *Phlebia* enzymes

(*a*) Culture liquid of *Phlebia*: 10 μg of protein from a 6-day-old culture. Separation was by SDS/polyacrylamide-gel electrophoresis at pH 8.5 with Tris/HCl buffer. Proteins were transferred on to a nitrocellulose filter at pH 8.3 with Tris/HCl buffer, and this was followed by incubation at pH 8.0 with Tris/HCl buffer with antiserum against all *Phlebia* proteins from 6-day-old culture liquid. The antiserum was diluted 1:1000. Thereafter incubation was with anti-(rabbit IgG) antibody conjugated with alkaline phosphatase. Immunocomplexes were detected by determining the activity of alkaline phosphatase at pH 9.5 with Tris/HCl buffer with 5-bromo-4-chloroindol-3-yl phosphate as substrate and Nitro Blue Tetrazolium for colour development. (*b*) Purified enzymes L_I, L_{II} and L_{III}, 1 μg in each case, compared with 1 μg of each treated with 12.5 ng of endoglycosidase H, L_I^T, L_{II}^T and L_{III}^T respectively. SDS/polyacrylamide-gel electrophoresis, immunoblotting and detection with anti-L_I serum were as indicated for (*a*). (*c*) Oxidase, O_x, and O_x treated with endoglycosidase H, O_x^T. SDS/polyacrylamide-gel electrophoresis, immunoblotting and detection with anti-O_x serum were as indicated for (*a*). Pharmacia low-M_r standards were separated and stained on the polyacrylamide gel before blotting, and their positions are indicated at the right-hand side of the Figure.

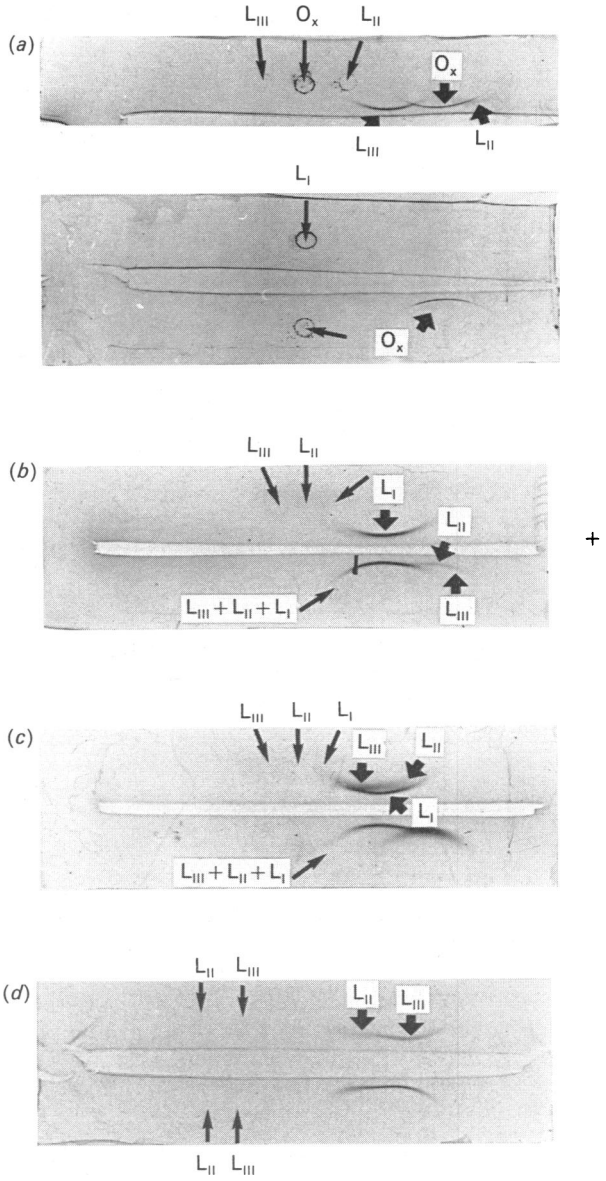

Fig. 2. Immunoelectrophoresis of *Phlebia* purified enzymes

Electrophoresis was in 50 mM-sodium barbital buffer, pH 8.2, on agarose gel. Protein staining was with Amido Black. Samples (1 μg) of purified oxidase, O_x, and lignin peroxidases L_I, L_{II}, L_{III} were applied as shown by thin arrows. Enzymes after electrophoretic separation and immunoprecipitation are indicated by thick arrows. Antisera were diluted 1:2 and applied into the horizontal wells after electrophoresis. (*a*) Antiserum against O_x; (*b*) antiserum against L_I; (*c*) antiserum against L_{III}; (*d*) antiserum against L_{II}.

modification. However, preliminary analysis of the insoluble Kraft lignin residues from the corresponding reaction mixtures indicated slight decreases in the molecular sizes of lignin treated with enzymes. T.l.c. analysis further confirmed the release of unidentified components from lignin by the action of enzymes. Monophenols containing extra methoxy or hydroxy groups were most affected by oxidase and lignin peroxidases. In h.p.l.c. analysis, this was seen both as the disappearance of the original substrate and as the appearance of new, unidentified, components (Fig. 6). *Phlebia* oxidase did not affect tyrosine or 3,4-dihydroxyphenylalanine.

DISCUSSION

Three lignin peroxidases and one oxidase were purified from the culture filtrate of the white-rot fungus *Phlebia radiata*. Under similar nitrogen-limited cultivation conditions *Phanerochaete* has been shown to secrete at least six lignin peroxidases (Kirk *et al.*, 1986) and various Mn-peroxidases affecting lignin model compounds (Kuwahara *et al.*, 1984). Oxidase has not been purified from *Phanerochaete* cultivations.

All the ligninolytic enzymes of *Phlebia radiata* were shown to be glycoproteins. Endoglycosidase H and F, by removing high-mannose heteroglycans, decreased the molecular sizes of *Phlebia* enzymes by 5–14%.

Lignin peroxidases of *Phanerochaete* are also glycoproteins, with neutral carbohydrate contents of 6% (w/w) (Renganathan *et al.*, 1985). *Polyporus* (Fåhraeus & Reinhammar, 1967), and *Neurospora* (Froehner & Eriksson, 1974) oxidases have been reported with 10–11% (w/w) carbohydrate.

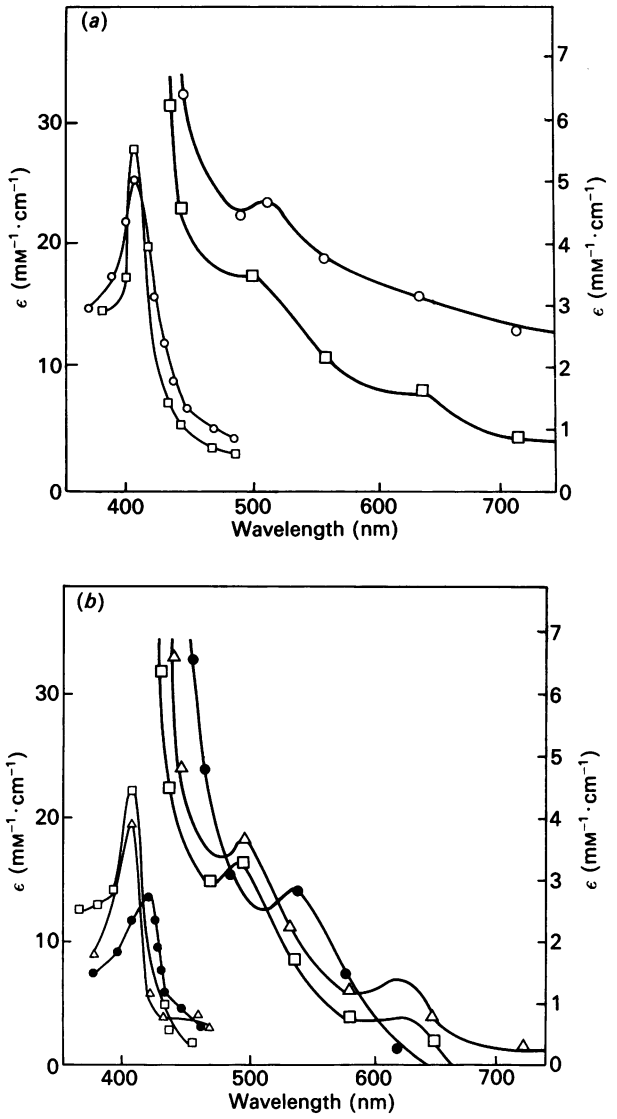

Fig. 3. Absorption spectra of L_{III} and its derivatives

(*a*) Spectra of 200 μg of L_{III} in 50 mM-sodium acetate buffer, pH 5.5, untreated (□) or modified by the addition of 0.1 mM-dithiothreitol (○). (*b*) Spectra of 100 μg of L_{III} untreated (□) or modified by the addition of 0.1 mM-NaN_3 (△) or KCN (●). Spectra were measured over the range 300–700 nm immediately after addition of the supplement at 20 °C.

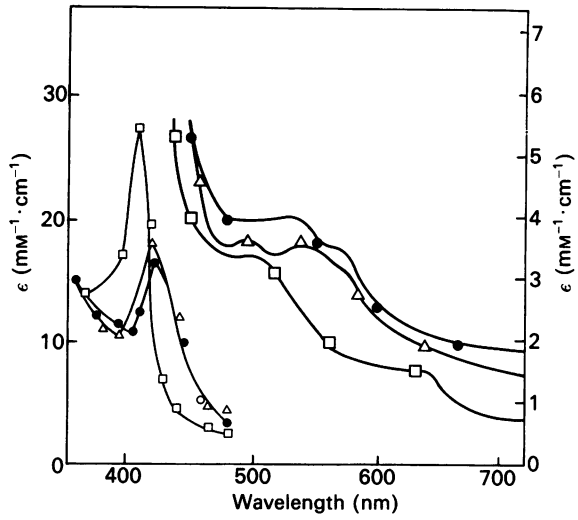

Fig. 4. Absorption spectra of L_{III} and its peroxide intermediates

Spectra of 200 μg of L_{III} in 50 mM-sodium acetate buffer, pH 5.5, untreated (□) or modified by the sequential addition of 0.15 mM-H_2O_2 (△) and 1.2 mM-veratryl alcohol (●). Spectra were measured immediately after addition of the supplement at 20 °C.

Lignin peroxidase I is serologically only partly identical with the other lignin peroxidases, L_{II} and L_{III}. The protein structure of L_I includes extra epitopes besides those in common with L_{II} and L_{III}. Serological similarities have recently also been found between the lignin peroxidases of *Phlebia* and those of *Phanerochaete* (Kantelinen *et al.*, 1988).

The spectra of native lignin peroxidases of *Phlebia* and their derivatives strongly resemble those of animal catalase (Nicholls & Schonbaum, 1963), which contains protoporphyrin IX as a prosthetic group. Minor differences among L_I, L_{II} and L_{III} were revealed in the shifts of the absorption spectra obtained by reducing and oxidizing agents. This could indicate differences in the state of oxidation or in the ligands in the prosthetic groups and hence explain the different ionic properties of *Phlebia* lignin peroxidases.

Spectral evidence shows that lignin peroxidases of *Phanerochaete* can achieve the five different redox states characteristic of conventional peroxidases (Tien & Kirk, 1984; Renganathan *et al.*, 1985; Tien *et al.*, 1986). With a molar equivalent of H_2O_2 lignin peroxidase is converted into the one-electron-deficient Compound II, which shifts instantaneously back to the ferric ground state when veratryl alcohol is added as an electron donor. The spectrum of *Phlebia* lignin peroxidase shows a different regeneration cycle. After addition of veratryl alcohol *Phlebia* lignin peroxidase remains in the oxidized state for a longer period (Fig. 4). In catalase-mediated reactions Compound II is only slowly converted into the ground state. The formation of Compound III as a consequence of unbalanced stoichiometry between the reactants is also a possibility: the step from Compound III to the resting enzyme is slow for all peroxidative enzymes.

Resonance Raman spectral studies (Andersson *et al.*, 1987) have shown that *Phanerochaete* lignin peroxidase more closely resembles horseradish peroxide (EC 1.11.1.7) than cytochrome *c* peroxidase (EC 1.11.1.5) or catalase (EC 1.11.1.6). The results obtained in the present work indicate that the lignin peroxidase of *Phlebia* is closer to catalase. However, definite conclusions concerning similarities between the *Phlebia* enzymes and other peroxidases require more data.

Phlebia oxidase is not a haemoprotein. It was shown to contain copper (atomic absorption analysis), although the spectral characteristics of known blue copper oxidases, including those of *Polyporus versicolor* laccase (Malkin & Malmström, 1970), were not exhibited. The featureless spectrum suggests the existence of copper as type 2 in *Phlebia* oxidase. Typical for type 2 copper in oxidase is inhibition by anions and tight binding with proteins. *Phlebia* oxidase was inhibited by anions and

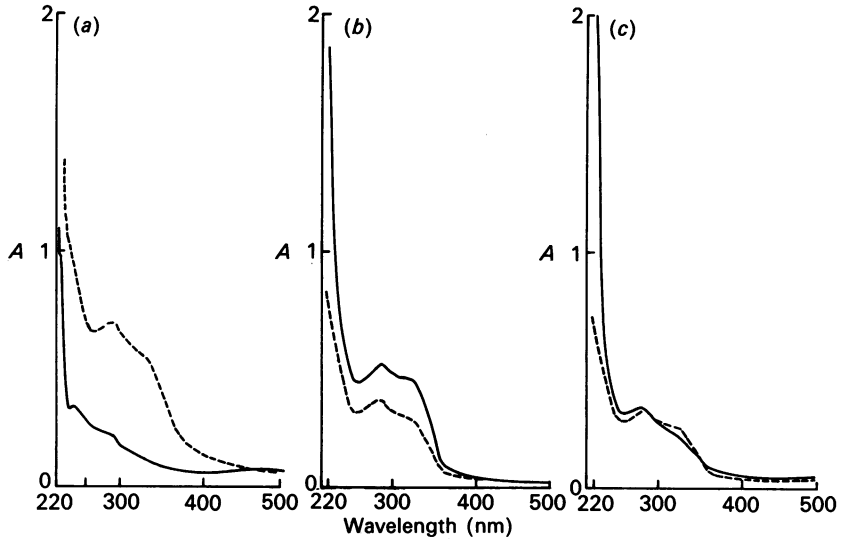

Fig. 5. Absorption spectra of Kraft lignin treated with ligninolytic enzymes

In each case 1 mg of Kraft lignin (M_r < 1000) was incubated with 10 μg of enzyme at 20 °C for 10 min. (*a*) Incubation in 50 mM-dimethylsuccinate buffer, pH 4.5, with O_x; (*b*) and (*c*) incubation in 50 mM-glycine/HCl buffer, pH 3, containing 0.15 mM-H_2O_2 with L_I (*b*) and with L_{II} (*c*). Spectra of the buffer-soluble fraction from Kraft lignin before (------) and after (——) enzyme treatment are shown.

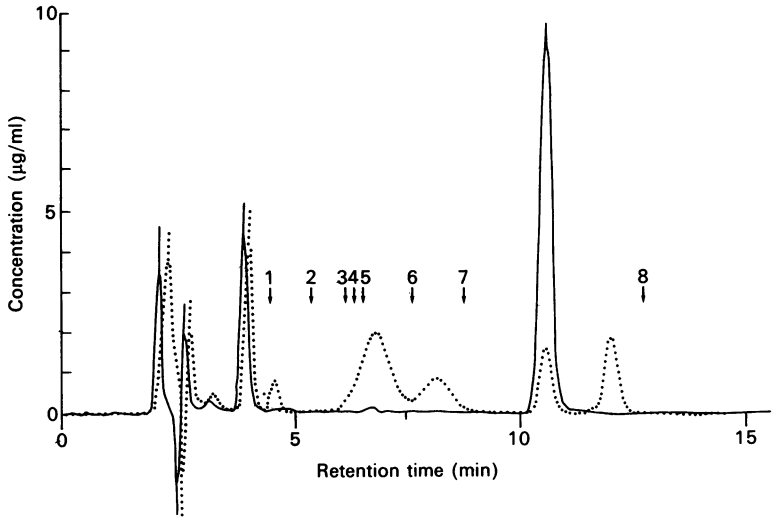

Fig. 6. H.p.l.c. analysis of guaiacol treated with *Phlebia* oxidase

Incubation conditions were as indicated for Fig. 5(*a*). Spectra of guaiacol before (——) and after (· · · · ·) treatment with O_x are shown. Retention of reference standards is indicated by arrows: 1, catechol; 2, veratryl alcohol; 3, coniferyl alcohol; 4, coumaric acid; 5, vanillin; 6, ferulic acid; 7, veratric acid; 8, veratraldehyde.

was not affected by EDTA. Further evidence for the Cu^{2+} form was insensitivity towards CO and inhibition by reducing agents. The absorption coefficients for type 2 copper compound are low, the limit of detection being 1 mM (Malkin & Malmström, 1970). Copper-containing extracellular laccases that lack the absorption spectra specific for *Polyporus laccase* have also been reported from *Agaricus bisporus* (Wood, 1980) and from *Schizophyllum commune* (de Vries *et al.*, 1986). *Phlebia* oxidase affected compounds containing a free phenolic hydroxy group. Such compounds included substituted *o*- and *p*-dihydroxybenzene, trihydroxybenzenes and *p*-phenylenediamine, but not tyrosine or 3,4-dihydroxyphenylalanine either separately or in combination. The reaction products obtained were generally coloured, suggesting the formation of quinones. The fate of O_2 was not analysed, but the reactions were strongly retarded when the incubation mixtures were purged with N_2. On the basis of these results *Phlebia* oxidase is considered a benzenediol:O_2 oxidoreductase from the EC group 1.10.3.2. *Phlebia* oxidase did not cleave the aromatic ring of its substrate, nor did it affect two substrates simultaneously. The enzyme cannot therefore belong to the EC groups 1.13 and 1.14 (oxygenases, monooxygenases and mixed-function oxidases).

The pH optima for activity of *Phlebia* enzymes are

pH 3 and 4.5 for lignin peroxidases and for oxidase respectively, whereas the stability optima are at pH 5–6. The lignin peroxidases of *Phanerochaete chrysosporium* are also active at acid pH values, the optimum being near pH 2 and no activity being shown at pH values greater than 5.0 (Tien *et al.*, 1986). Haemoproteins are readily dissociated into haem and apoprotein at pH values below 4.0, and thus the pH optima for lignin peroxidases of *Phlebia* and *Phanerochaete* are rather surprising. On the other hand, the formation of cation radicals essential for lignin degradation is favoured at acid pH values.

Lignin peroxidases of *Phanerochaete* require slightly higher temperatures (approx. 45 °C) for optimal activity (Farrell, 1986) than do *Phlebia* enzymes (40 °C).

Several soil bacteria and fungi metabolize aromatic compounds by performing successive hydroxylation, decarboxylation, demethylation and oxidation reactions that together result in cleavage of the aromatic ring (Cain, 1980). However, a separate enzyme is involved for each step. Lignin peroxidases of the white-rot fungus *Phanerochaete* have been shown to be capable of all the reactions necessary for ring cleavage in lignin model compounds (Umezawa *et al.*, 1986). The biochemical function of ligninolytic enzymes from white-rot fungi is difficult to study because the amount of protein secreted is low, 0.01 g/l of culture liquid. For comparison, the amount of extracellular protein can be as high as 20 g/l with industrial enzyme producers such as *Trichoderma reesei* (Enari, 1983). To obtain purified ligninolytic components for industrial purposes, the enzyme production of white-rot fungi must be much improved.

We are grateful to Anne Kantelinen, M.Sc., for providing the *Phlebia* culture liquids. The excellent technical assistance of R. Arnkil, M. Tikka and R. Suihkonen is greatly acknowledged. This work was financially supported by the Technology Development Centre, Finland.

REFERENCES

Ander, P., Hatakka, A. & Eriksson, K.-E. (1980) Arch. Microbiol. **125**, 189–202

Andersson, L. A., Renganathan, V., Loehr, T. M. & Gold, M. H. (1987) Biochemistry **26**, 2258–2263

Cain, B. R. (1980) in Lignin Biodegradation: Microbiology, Chemistry and Potential Applications (Kirk, T. K., Higuchi, T. & Chang, H.-M., eds.), vol. 1, pp. 21–60, CRC Press, Boca Raton

de Vries, O. M. H., Kooistra, W. H. C. F. & Wessels, J. G. H. (1986) J. Gen. Microbiol. **132**, 2817–2826

Elder, J. H. & Alexander, S. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4540–4544

Enari, T.-M. (1983) in Microbial Enzymes and Biotechnology (Fogarty, W. M., ed.), pp. 183–223, Applied Science, London

Fåhraeus, G. & Reinhammar, B. (1967) Acta Chem. Scand. **21**, 2367–2378

Farrell, R. L. (1986) Proc. Int. Conf. Biotechnology in the Pulp and Paper Industry 3rd, Stockholm, 61–63

Fenn, P. & Kirk, K. (1979) Arch. Microbiol. **123**, 307–309

Froehner, S. C. & Eriksson, K.-E. (1974) J. Bacteriol. **120**, 458–465

Grabar, P. & Burtin, P. (1964) Immuno-electrophoretic Analysis, pp. 3–29, Elsevier, Amsterdam

Haemmerli, S. D., Leisola, M. S. A., Sanglard, D. & Fiechter, A. (1986) J. Biol. Chem. **261**, 6900–6903

Hatakka, A. I. & Uusi-Rauva, A. K. (1983) Eur. J. Appl. Microbiol. Biotechnol. **17**, 235–242

Hatakka, A., Kantelinen, A., Tervilä-Wilo, A. & Viikari, L. (1987) Colloq. INRA **40**, 185–189

Kantelinen, A., Waldner, R., Niku-Paavola, M.-L. & Leisola, M. (1988) Appl. Microbiol. Biotechnol. **28**, 193–198

Kirk, T. K., Croan, S., Tien, M., Murtagh, K. E. & Farrell, R. L. (1986) Enzyme Microb. Technol. **8**, 27–32

Kuwahara, M., Glenn, J. K., Morgan, M. A. & Gold, M. H. (1984) FEBS Lett. **169**, 247–249

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Leisola, M. S. A., Schmidt, B., Thanei-Wyss, U. & Fiechter, A. (1985) FEBS Lett. **189**, 267–270

Malkin, R. & Malmström, B. G. (1970) Adv. Enzymol. Relat. Areas Mol. Biol. **33**, 177–244

Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) Science **211**, 1437–1438

Nicholls, P. & Schonbaum, G. R. (1963) Enzymes 2nd Ed. **8B**, 147–225

Niku-Paavola, M.-L. (1987) Colloq. INRA **40**, 119–123

Renganathan, V., Miki, K. & Gold, M. H. (1985) Arch. Biochem. Biophys. **241**, 304–314

Tien, M. & Kirk, T. K. (1983) Science **221**, 661–663

Tien, M. & Kirk, T. K. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 2280–2284

Tien, M. & Tu, C.-P. D. (1987) Nature (London) **326**, 520–523

Tien, M., Kirk, K. T., Bull, C. & Fee, J. A. (1986) J. Biol. Chem. **261**, 1687–1693

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Trimble, R. B. & Maley, F. (1984) Anal. Biochem. **141**, 515–522

Umezawa, T., Shimada, M., Higuchi, T. & Kusai, K. (1986) FEBS Lett. **205**, 287–292

Werner, W., Rey, M. G. & Wielinger, H. (1970) Z. Anal. Chem. **252**, 224–227

Wood, D. A. (1980) J. Gen. Microbiol. **117**, 327–338

Received 14 December 1987/30 March 1988; accepted 7 June 1988