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Purification and characterisation of an extracellular peroxidase from white-rot fungus *Pleurotus ostreatus*

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A peroxidase was purified 98.3-fold from the culture filtrate of *Pleurotus ostreatus* with an overall yield of 12.4%. The molecular mass determined by gel filtration was found to be approx. 140 kDa. SDS-PAGE revealed that the enzyme consists of two identical subunits with a molecular mass of approx. 72 kDa. The p*I* value of this enzyme is approx. 4.3. The enzyme contains 41% carbohydrate by weight, and aspartic acid and asparagine (16.8%), and glutamic acid and glutamine (12.0%). The enzyme has the highest affinity toward sinapic acid and affinity towards various phenolic compounds containing methoxyl and *p*-hydroxyl groups, directly attached to the benzene ring. However, the enzyme does not react with veratryl alcohol and shows no affinity for nonphenolic compounds. The optimal reaction pH and temperature are 4.0 and 40°C, respectively. The catalytic mechanism of the enzymic reaction is of the Ping-Pong type. The activity of the enzyme is competitively inhibited by high concentrations of H_2O_2 and its K_i value is 1.70 mM against H_2O_2. This enzyme contains approx. 1 mol of heme per mol of one subunit of the enzyme. The pyridine hemochrome spectrum of the enzyme indicates that the heme of *P. ostreatus* peroxidase is iron protoporphyrin IX. The EPR spectrum of the native peroxidase shows the presence of a high-spin ferric complex with *g* values at 6.102, 5.643 and 1.991.

Introduction

Lignin is a three-dimensional polymer found abundantly in wood and herbaceous plants. It is composed of phenylpropane subunits interconnected by stable carbon-carbon and carbon-oxygen bonds. The chemical nature makes it resistant to microbial attack.

The most potent lignin-degrading organisms are white-rot fungi and the lignin-degrading enzyme systems have been studied quite extensively in *Phanerochaete chrysosporium* [1–4]. One of the enzymes, lignin peroxidase was purified and characterised [5–16]: The enzyme catalyses the H_2O_2-dependent oxidation of a variety of lignin model compounds by one-electron oxidation. The properties and the reaction mechanisms of the enzyme are well-characterised in *P. chrysosporium*. The occurrence of this kind of enzyme has been reported in *Trametes (Coriolus) versicolor* [17–19], *Phlebia radiata* [20,21], *Bjerkandera adusta* [22], *Polyporus ostreiformis* [23] and *Streptomyces viridosporus* [24].

However, enzymes of *Pleurotus* spp., involved in lignin degradation, were not purified yet, except veratryl alcohol oxidase [25] and phenoloxidase [26,27], even though these fungi have been reported as well-known lignin degraders [28–30]. In the present paper, we report the purification and the characterisation of a peroxidase from the culture filtrate of *P. ostreatus* and its possible implication in lignin degradation.

Materials and Methods

Organism and culture conditions. White-rot fungus, *Pleurotus ostreatus*, was obtained from Korean Forest Research Laboratories. For enzyme production, the organism was grown in 500-ml Erlenmeyer flasks, each of which contained 1% malt extract, 0.5% peptone, 0.5% yeast extract and 1% glucose supplemented with 100 ml of water, shaking at 28°C, and then, after 3 days, subsequently incubated at 28°C for 10 days without shaking.

Chemicals. S Sepharose, Sephacryl S-200HR, Sephacryl S-300 and molecular mass markers for gel

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filtration chromatography were purchased from Sigma, Protein-Pak DEAE 5PW column from Waters, Pharmalyte from Pharmacia and *N*-glycosidase F, *O*-glycosidase and molecular mass standards for SDS-PAGE from Boehringer-Mannheim. All other reagents used were of the highest quality generally available.

Enzyme assays. An aliquot of peroxidase was incubated in 3 ml of 20 mM sodium acetate buffer (pH 4.0) containing 20 mM pyrogallol and 0.1 mM H_2O_2. Its activity was determined at 30°C by monitoring the absorbance change at 430 nm due to the formation of purpurogallin from pyrogallol and using a molar extinction coefficient of 2470 $M^{-1}cm^{-1}$. The reaction was initiated by addition of H_2O_2. One unit (U) of enzyme activity was defined as the amount of enzyme which produced 1 μmol of purpurogallin per min per ml.

Enzyme purification. All procedures were performed at 4°C. The culture suspension was used for the enzyme purification after filtration through Whatman No. 1 filter paper. The aqueous solution was centrifuged to remove particles. Cold acetone (−10°C) was added to the clarified solution upto 65% saturation and the resulting precipitate was collected by centrifugation. After removing the acetone by nitrogen purging, the precipitate was resuspended in a minimal volume of 20 mM sodium citrate buffer ((pH 3.0), buffer A). The acetone fraction was applied to a S Sepharose column (2.8 × 18.5 cm). After the column was washed with 200 ml of buffer A, the enzyme fraction was then eluted with a linear concentration gradient of 0–0.25 M NaCl in the same buffer at a flow rate of 30 ml/h. The active enzyme fraction was applied to a Sephacryl S-200HR column (2.8 × 120 cm) equilibrated with 20 mM sodium phosphate buffer ((pH 6.0), buffer B). The enzyme was eluted with the same buffer at a flow rate of 30 ml/h. The pooled active fractions were further purified by the Waters Delta Prep 4000 chromatography system. After passing the active fractions through Protein-PAK DEAE 5PW column (2.15 × 15 cm), which was equilibrated with buffer B, the column was washed with 150 ml of buffer B and the enzyme was then eluted with a linear concentration gradient of 0–0.25 M NaCl in the same buffer at a flow rate of 5 ml/min.

Molecular mass determination. The molecular mass of native protein was determined by gel-filtration chromatography on a Sephacryl S-300 column. The column was calibrated with apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). SDS-PAGE was performed on polyacrylamide gels with concentrations ranging from 5 to 20%, according to the method proposed by Lambin [31]. As standard marker proteins, α_2-macroglobulin (170 kDa), phosphorylase *b* (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa) and trypsin inhibitor (20.1 kDa) were used. Coomassie brilliant blue G-250 was used for staining according to the method proposed by Neuhoff et al. [32].

Isoelectric focusing. Flat bed isoelectric focusing was carried out at 4°C for 3 h under the final setting of 1030 V and 4 W, using a 5% polyacrylamide gel containing 6.25% Pharmalyte of pH-range 4.0–6.5.

Determination of concentrations of protein and carbohydrate. The protein concentration was determined according to the method proposed by Lowry et al. [33], using bovine serum albumin as a standard protein. The carbohydrate content was determined according to the method proposed by Dubois et al. [34], using glucose as a standard carbohydrate. The contents of *N*-linked and *O*-linked glycan were determined using *N*-glycosidase F and *O*-glycosidase.

Amino-acid analysis. The amino-acid analysis was performed by reverse-phase chromatography after phenylisothiocyanate derivatisation. About 15 μg of enzyme was hydrolysed with 6 M HCl in vacuo at 105°C for 24 h, according to the method proposed by Bidlingmeyer et al. [35]. Amino-acid analysis was carried out with a reverse-phase high-performance PICO-TAG column.

Kinetic calculations. Rates of substrate oxidation were determined by means of spectrophotometry, using molar extinction coefficients of various substrates. The molar extinction coefficients determined in 50 mM sodium acetate buffer (pH 4.0) were 25460 $M^{-1}cm^{-1}$ at 316 nm for sinapic acid, 8360 $M^{-1}cm^{-1}$ at 256 nm for protocatechuic acid, 11460 $M^{-1}cm^{-1}$ at 307 nm for *p*-coumaric acid, 22160 $M^{-1}cm^{-1}$ at 318 nm for ferulic acid, 13480 $M^{-1}cm^{-1}$ at 295 nm for *o*-dianisidine, 21240 at 431 nm for phenol red, 5880 $M^{-1}cm^{-1}$ at 267 nm for gallic acid, 9820 $M^{-1}cm^{-1}$ at 270 nm for syringic acid, 10680 $M^{-1}cm^{-1}$ at 257 nm for vanillic acid, 18200 $M^{-1}cm^{-1}$ at 253 nm for *p*-hydroxybenzoic acid and 9300 $M^{-1}cm^{-1}$ at 310 nm for veratryl alcohol. For pyrogallol, the kinetic data were collected under the standard assay conditions. The K_m values were determined measuring initial velocity. All kinetic studies were performed at least three times and the kinetic data were fitted to hyperbola using the Michaelis-Menten equation. The best values were determined by a linear least-square regression analysis.

Spectroscopic studies. UV absorption spectra of peroxidase were obtained by means of Shimadzu model UV-265 spectrophotometer (Japan). Spectra were obtained at 30°C using a 1-cm quartz cuvette containing enzyme dissolved in 0.1 M sodium phosphate buffer (pH 6.0). The heme content of the enzyme was determined by measuring the absorbance of heme-pyridine complex at 556.5 nm and calculating with its molar extinction coefficient of 33200 $M^{-1}cm^{-1}$, according to the method proposed by Appleby and Morton [36].

The EPR spectrum of native enzyme was measured on Bruker ESP 300S EPR spectrometer (Germany)

with 5 G modulation amplitude, 10 mW microwave power and 9.46 GHz frequency. Probe temperature was regulated with a helium cryostat equipped with a temperature-control unit. Temperature was maintained at approx. 9 K and 1,1-diphenyl-2-picrylhydrazyl was used as a *g*-marker.

Results and Discussion

Purification of peroxidase

The peroxidase was purified from the culture filtrate as summarised in Table I. The enzyme was purified 98.3-fold, with a recovery of 12.4%. The specific activity of the purified enzyme was 11.80 units per mg of protein.

Molecular properties

The apparent molecular mass of the purified enzyme was approx. 140 kDa as determined by Sephacryl S-300 column chromatography. On the other hand, when the enzyme was subjected to SDS-PAGE, a single band of approx. 72 kDa was found (Fig. 1), indicating that the peroxidase is composed of two identical subunits. The mobility of protein was not changed after SDS-PAGE in the absence of 2-mercaptoethanol, indicating that the dissociation of subunit was not dependent on a reducing agent. The molecular mass of this enzyme was quite distinct from those of other fungal peroxidases involved in lignin degradation, including lignin peroxidase and Mn(II)-dependent peroxidase of *P. chrysosporium*, which have been reported to be 41–42 kDa [5,7,16,37].

The peroxidase contains approx. 41% carbohydrate by weight, estimated according to the method proposed by Dubois et al. [34]. Molecular masses of the enzyme deglycosylated with *N*-glycosidase F, *O*-glycosidase and both enzymes were estimated to be approx. 59, 55 and 45 kDa, respectively (Fig. 2). From this result the contents of *N*-linked and *O*-linked glycan are calculated to be 18 and 23%, respectively. Its carbohydrate content is very high, compared to that of lignin peroxidase from *P. chrysosporium* (13%), which was reported by Tien and Kirk [5] and that of ligninase-I (21%) and that of peroxidase-M2 (17%) from *P. chrysosporium* BKM-F-1767, which were reported by Paszczynski et al. [37].

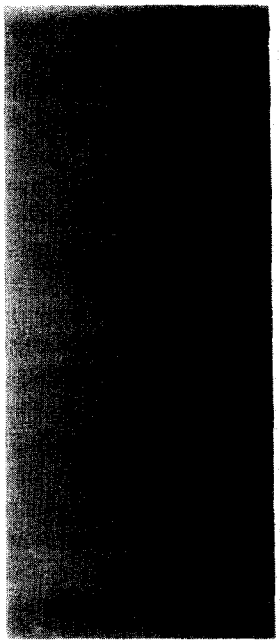

Fig. 1. SDS-PAGE of the *P. ostreatus* peroxidase. The gel concentration was 5–20%. Lane 1, purified enzyme; lane 2, molecular mass markers; α_2-macroglobulin (a, 170 kDa), phosphorylase *b* (b, 97.4 kDa), glutamate dehydrogenase (c, 55.4 kDa), lactate dehydrogenase (d, 36.5 kDa) and trypsin inhibitor (e, 20.1 kDa).

TABLE I

Purification of an extracellular peroxidase from P. ostreatus

Step	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Yield %)	Purification (-fold)
Culture filtrate	384	47.6	0.12	100	1
Acetone precipitation	126	42.6	0.34	89.5	2.8
S Sepharose chromatography	10.5	23.1	2.20	48.5	18.3
Sephacryl S-200HR chromatography	4.3	15.9	3.70	33.4	30.8
Protein-Pak DEAE chromatography	0.5	5.9	11.80	12.4	98.3

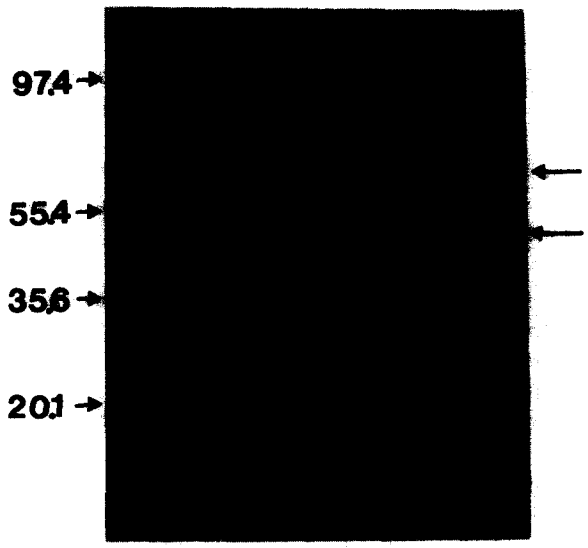

Fig. 2. Changes of electrophoretic mobility of the *P. ostreatus* peroxidase after SDS-PAGE after treatment of deglycosylating enzymes. Lane 1, native peroxidase; lane 2, peroxidase treated with *N*-glycosidase F; lane 3, peroxidase treated with *O*-glycosidase; Lane 4, peroxidase treated with both enzymes.

Amino-acid composition

The amino-acid composition of *P. ostreatus* peroxidase is given in Table II. The characteristic point of the amino-acid composition is that the contents of glutamic acid and glutamine (12.0%), and aspartic acid and asparagine (16.8%) in *P. ostreatus* peroxidase are higher than those in *Coprinus cinereus* peroxidase [38]. The N-terminal amino acid was not detected by Edman procedure, indicating that the N-terminus of this enzyme may be blocked, which is similar to the case of *C. cinereus* peroxidase [38].

Substrate specificity

This enzyme exhibited peroxidase activity for a wide variety of phenols and phenolic compounds. This activity was dependent on H_2O_2. The ability of the enzyme to catalyse oxidation of various aromatic compounds was investigated (Table III). This peroxidase has a high affinity towards phenolic compounds containing methoxyl and *p*-hydroxyl group, directly attached to the benzene ring, such as sinapic acid and protocatechuic acid. However, *P. ostreatus* peroxidase does not oxidise veratryl alcohol, which is a well-known substrate of various lignin peroxidases [5–16]. Also, this peroxidase shows no affinity for nonphenolic compounds, which are readily attacked by the lignin peroxidases [10].

Thus, it is suggested that the action mode of this enzyme is different from lignin peroxidase of *P. chrysosporium* [5–16] and *Streptomyces viridosporus* [24]. Garcia et al. [39] suggested that *P. ostreatus* produces another type of peroxidase which is not recognised by an antibody against lignin peroxidase originated from *P. chrysosporium*. In this respect, it is concluded that the peroxidase of *P. ostreatus* may play different roles in the lignin degradation.

TABLE II

Amino-acid compositions of P. ostreatus peroxidase (POP) and Coprinus cinereus peroxidase (CiP)

Amino-acid residue	mol/100 mol	
	POP	CiP [a]
Asp/Asn	16.8	10.24
Glu/Gln	12.0	9.95
Phe	8.5	6.79
Leu	7.5	8.46
Ser	6.7	8.69
Pro	6.6	7.83
Gly	6.3	5.46
Thr	6.3	5.33
Ala	6.1	6.33
Arg	4.9	6.24
Val	4.5	5.25
Ile	3.9	4.68
Lys	3.5	1.52
Tyr	2.6	1.63
His	2.4	1.24
Met	1.4	2.68
Half Cys	n.d.	2.03
Trp	n.d.	0.94

n.d., not determined.
[a] Data from Ref. 38.

TABLE III

Substrate specificity of the peroxidase from P. ostreatus

Each value was obtained from at least three experiments in duplicate.

Substrate	Relative activity (%)
Sinapic acid	100.0
Protocatechuic acid	84.5
p-Coumaric acid	60.0
Ferulic acid	55.9
o-Dianisidine	48.7
Pyrogallol	41.6
Phenol red	34.7
Gallic acid	17.0
Syringic acid	10.2
Vanillic acid	2.1
p-Hydroxybenzoic acid	1.1
Veratryl alcohol	0.0

Effects of pH and temperature on the enzyme activity

The effect of pH on the peroxidase activity was examined at pH values ranging from 3.0 to 8.0. From the bell-shaped pH profile shown in Fig. 3A, the optimal pH for the peroxidase activity was estimated to be around 4.0. The enzyme was stable at 30°C for 1 h in pH range from 5.0 to 7.0 (Fig. 3B).

As shown in Fig. 4A, the optimal temperature for the enzyme activity was about 40°C under the standard assay conditions. Thermal inactivation was investigated by incubating the enzyme in 20 mM sodium acetate

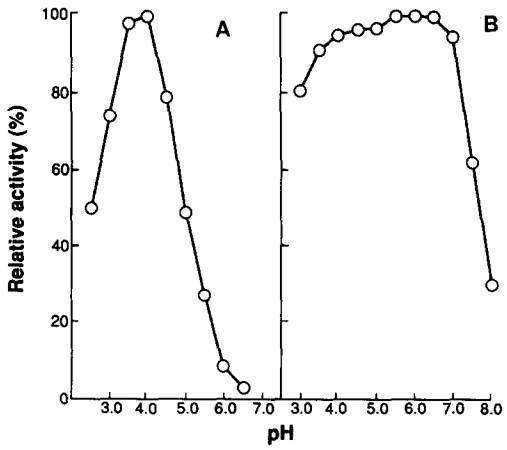

Fig. 3. Effect of pH on *P. ostreatus* peroxidase activity. (A), Enzyme activity was assayed at various pH values under the standard assay conditions. (B), The enzyme was incubated in 0.1 M citrate-phosphate buffer at the various pH values at 30°C for 1 h and the remaining activity was measured.

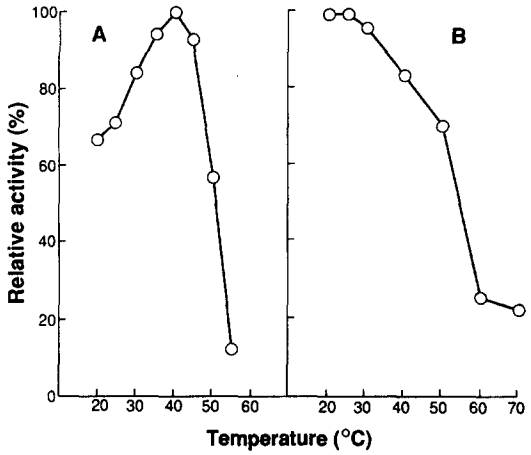

Fig. 4. Effect of temperature on *P. ostreatus* peroxidase activity. (A), Enzyme activity was assayed in 20 mM sodium acetate buffer (pH 4.0) at various temperatures. (B), The enzyme was incubated at various temperatures for 1 h and the remaining activity was measured.

buffer (pH 4.0) at a designated temperature for 1 h. The enzyme was stable up to 30°C (Fig. 4B).

Kinetic properties

Apparent K_m values of the peroxidase for pyrogallol and H_2O_2 were calculated from double-reciprocal plots. The K_m and V_{max} values for pyrogallol were 13.39 μM and 0.42 μmol/s under the standard enzyme assay conditions. Sinapic acid was one of the most efficient substrates (Table III). And the K_m and V_{max} values for H_2O_2 were 46.73 μM and 0.19 μmol/s, respectively. The mechanism of the oxidation of pyrogallol by the peroxidase was investigated measuring initial velocity. As shown in Fig. 5, the lines obtained from Lineweaver-Burk plots for various concentrations of H_2O_2 showed parallel lines, suggesting a Ping-Pong mechanism. At concentrations above 200 μM, H_2O_2 inhibited competitively the oxidation of pyrogallol by the enzyme (Fig. 6A) and the secondary replot of the

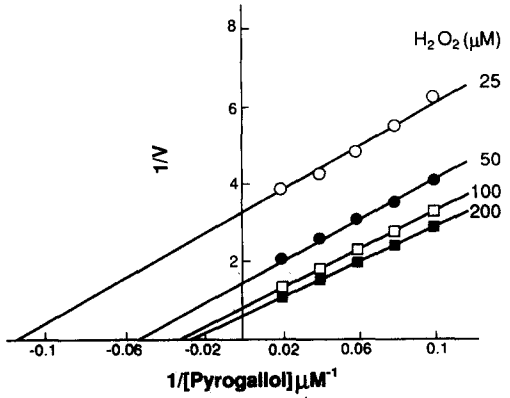

Fig. 5. Double-reciprocal plots of initial velocity vs. pyrogallol concentrations with various concentrations of H_2O_2.

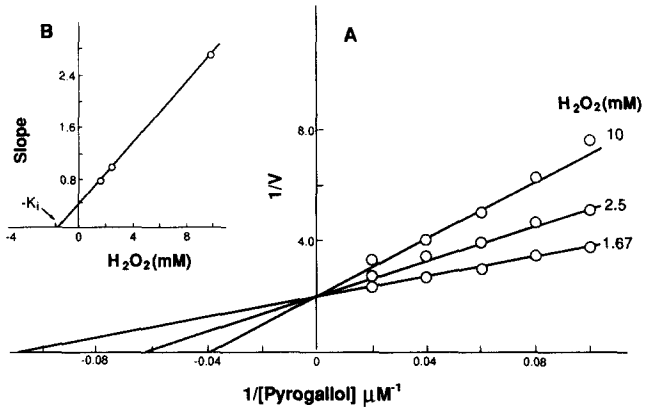

Fig. 6. Inhibition of *P. ostreatus* peroxidase by H_2O_2. (A), Lineweaver-Burk plot at various H_2O_2 concentrations and (B), secondary replot of the slopes vs. H_2O_2 concentration.

slopes vs. the reciprocal of H_2O_2 concentrations was linear and indicated a K_i value of 1.7 mM (Fig. 6B).

Identification of the prosthetic group

The absorption spectrum of the purified enzyme showed maxima at approx. 400, 510 and 640 nm and the ratio of A_{400}/A_{280} reached 1.25. The molar extinction coefficient of the enzyme was 223 $mM^{-1}cm^{-1}$ at 400 nm (Fig. 7). The addition of H_2O_2 to the enzyme solution results in a red shift from 400 and 510 to 420 and 525 nm, respectively, and absorbance decrease (Fig. 8). These spectral characteristics are very similar to Mn(II)-dependent peroxidase from *P. chrysosporium* [7] and suggest that the enzyme is a hemoprotein. This was verified by formation of a diagnostic pyridine-hemochromogen complex. The pyridine hemochrome spectra of the peroxidase gave absorbance maxima at

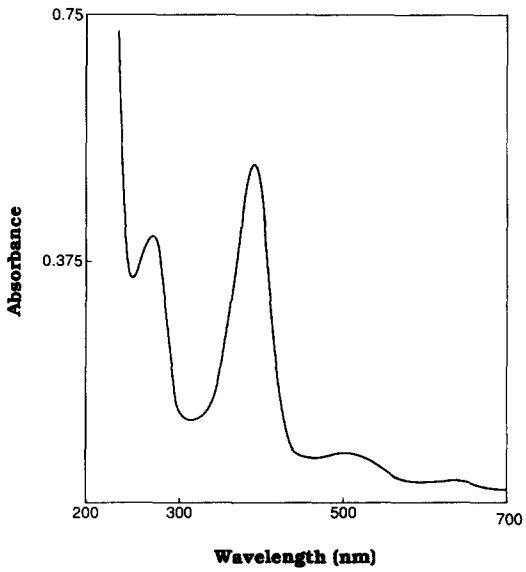

Fig. 7. Absorption spectrum of *P. ostreatus* peroxidase in 0.1 M sodium phosphate buffer (pH 6.0).

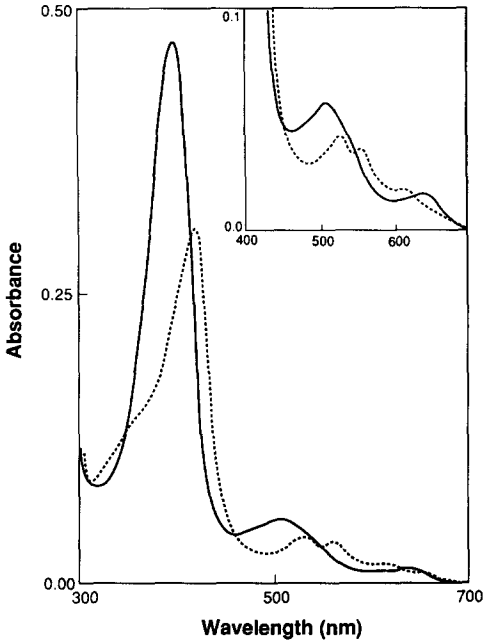

Fig. 8. Absorption spectra of *P. ostreatus* peroxidase. 240 μg enzyme was dissolved in 0.1 M sodium phosphate buffer (pH 6.0). The oxidized form (·····) was obtained by adding 0.1 mM H_2O_2 to the reduced form (———). The inset shows the enlarged spectrum.

approx. 418, 525 and 556 nm. It was reported that the pyridine hemochrome of Mn(II)-dependent peroxidase determined under the same conditions showed identical spectrum, indicating that the heme of *P. ostreatus* peroxidase is iron protoporphyrin IX.

On the basis of the molar extinction coefficient of 33 200 $M^{-1} cm^{-1}$ for the pyridine hemochrome at 556.5 nm [36], the heme content was calculated to be approx. 1 mol per mol of one subunit of the enzyme.

The EPR spectrum of native peroxidase at approx. 9 K is shown in Fig. 9. The g values for the enzyme are 6.102, 5.643 and 1.991, and these are characteristic of a high-spin ($S = 5/2$) ferric state. The EPR spectrum is distinct from that of high-spin pentacoordinate ferric lignin peroxidase [6], horseradish peroxidase [40], cytochrome-*c* peroxidase [40] and catalase [41]. These results led to the suggestion that the heme environment of this peroxidase is different from that of any other peroxidase.

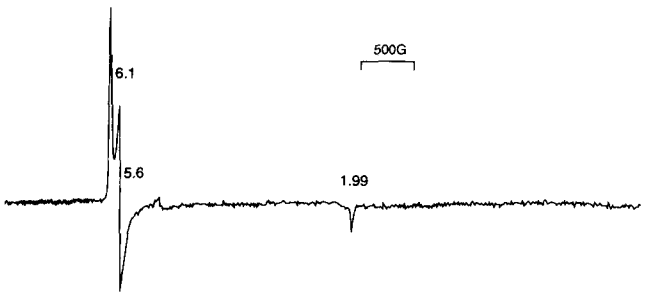

Fig. 9. X-band EPR spectrum of *P. ostreatus* peroxidase. Experimental conditions were described in Materials and Methods.

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