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Purification and characterization of an intracellular catalase-peroxidase from *Penicillium simplicissimum*

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The first dimeric catalase-peroxidase of eucaryotic origin, an intracellular hydroperoxidase from *Penicillium simplicissimum* which exhibited both catalase and peroxidase activities, has been isolated. The enzyme has an apparent molecular mass of about 170 kDa and is composed of two identical subunits. The purified protein has a pH optimum for catalase activity at 6.4 and for peroxidase at 5.4. Both activities are inhibited by cyanide and azide whereas 3-amino-1,2,4-triazole has no effect. 3,3'-Diaminobenzidine, 3,3'-dimethoxybenzidine, guaiacol, 2,6-dimethoxyphenol and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) all serve as substrates. The optical spectrum of the purified enzyme shows a Soret band at 407 nm. Reduction by dithionite results in the disappearance of the Soret band and formation of three absorption maxima at 440, 562 and 595 nm. The prosthetic group was identified as a protoheme IX and EPR spectroscopy revealed the presence of a histidine residue as proximal ligand.

In addition to the catalase-peroxidase, an atypical catalase which is active over a broad pH range was also partially purified from *P. simplicissimum*. This catalase is located in the periplasm and contains a chlorin-type heme as prosthetic group.

Keywords: catalase-peroxidase; peroxidase; EPR; chlorin-type heme; *Penicillium simplicissimum*.

Catalases are ubiquitous enzymes which have been isolated from a broad range of procaryotic and eucaryotic organisms. Most catalases described so far are tetramers with molecular masses ranging over 220–270 kDa with each subunit containing a protoheme as prosthetic group. These typical catalases are active in the pH range 5–10 and are specifically inactivated by 3-amino-1,2,4-triazole.

More recently, some intracellular hydroperoxidases have been described that have properties deviating from the above enzymes. Enzymes of this new class of hydroperoxidases exhibit both catalase as well as significant peroxidase activity and are therefore called catalase-peroxidases. Besides their different catalytic behaviour these enzymes also differ in their reduction by dithionite, the narrow pH range for maximal activity, inactivation by hydrogen peroxide and their insensitivity to 3-amino-1,2,4-triazole. Most of the catalase-peroxidases are tetramers isolated from bacteria (Claiborne and Fridovich, 1979; Nadler et al., 1986; Hochman and Goldberg, 1991; Yumoto et al., 1990; Morris et al., 1992; Brown-Peterson and Salin, 1993). Those of *Bacillus stearothermophilus* (Loprasert et al., 1988), *Comamonas compransoris* (Nies and Schlegel, 1982), *Mycobacterium tuberculosis* (Diaz and Wayne, 1974) and *Streptomyces cyaneus* (Mliki and Zimmermann, 1992) exist as dimers. Furthermore, monomeric catalase-peroxidases have been purified from two halophilic archaeobacteria (Fukumori et al., 1985; Cendrin et al., 1994). Until now the tetrameric catalase-peroxidase from the

fungus *Septoria tritici* (Levy et al., 1992) is the only reported catalase-peroxidase of eucaryotic origin.

In this study we describe the purification and initial characterization of an intracellular catalase-peroxidase from the plectomycete *Penicillium simplicissimum*. When grown on veratryl alcohol, *P. simplicissimum* induces an intracellular vanillyl-alcohol oxidase which is a potential source of intracellular hydrogen peroxide (De Jong et al., 1992). During the purification of this flavoprotein oxidase, we copurified a hydroperoxidase with some unusual properties. Based on its catalytic and physical properties this enzyme is designated as a catalase-peroxidase. Furthermore, evidence is presented that, in *P. simplicissimum*, an atypical periplasmic catalase containing a chlorin-type heme as the prosthetic group is also expressed.

MATERIALS AND METHODS

General. 3-Amino-1,2,4-triazole, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), Coomassie brilliant blue R250, 3,3'-diaminobenzidine, peroxidase from horseradish and lysing enzymes from *Trichoderma harzianum* were obtained from Sigma. Acrylamide and bisacrylamide were from Serva. Phenyl-Sepharose CL-4B, Superose 6 HR 10/30, Superdex 200 HR 10/30, Superdex PG-200, Q-Sepharose HI-LOAD and the low-molecular-mass calibration kit for SDS/PAGE were products of Pharmacia. Hydroxyapatite was purchased from Bio-Rad. All other chemicals were of commercially available analytical grade.

Penicillium simplicissimum (Oudem.) Thom. CBS 170.90 was maintained at 4°C on glucose/agar slants. The fungus was grown on veratryl alcohol as described before (De Jong et al., 1992). A 20-l fermentor was used for cultivation. Cells were harvested five days after inoculation by filtration over a cheese cloth, washed with 50 mM potassium phosphate pH 7.0 and stored at –20°C until use.

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Abbreviations. PCP, catalase-peroxidase from *P. simplicissimum*; PAC, atypical catalase from *P. simplicissimum*.

Enzymes. Catalase (EC 1.11.1.6); peroxidase (EC 1.11.1.7).

Enzyme purification. All purification steps were performed at 4°C and 0.5 mM phenylmethylsulfonyl fluoride was added to the buffers used in purification. After thawing, 300 g wet cells were resuspended in 20 mM potassium phosphate pH 7.5 and disrupted by passage through a chilled French press. The resultant homogenate was clarified by centrifugation and adjusted to 20% saturation with ammonium sulfate. After centrifugation the supernatant was applied to a phenyl-Sepharose column (33×2.6 cm) equilibrated with 20 mM potassium phosphate pH 7.5 containing 0.5 M ammonium sulfate. Following washing of the column, the catalase-peroxidase was eluted with 20 mM potassium phosphate pH 7.5. The active fraction was applied to a Q-Sepharose column (10×2.6 cm) equilibrated with 20 mM potassium phosphate pH 7.5. After washing, the enzyme was eluted with a linear gradient (0–1.0 M KCl in the starting buffer). Active fractions were concentrated and washed with 20 mM potassium phosphate pH 7.5 using an Amicon YM-30 ultrafiltration unit. The resulting enzyme preparation was then applied to a hydroxyapatite column (12×2.6 cm) equilibrated with 20 mM potassium phosphate pH 7.5 and eluted with the same buffer. In the final step the active fraction was concentrated by ultrafiltration to 8 ml and eluted over a Superdex PG-200 gelfiltration column (85×2.6 cm) equilibrated with 20 mM potassium phosphate pH 7.5. Active fractions showing optimal A_{407}/A_{280} ratios were pooled, concentrated by ultrafiltration and stored at –70°C.

Analytical methods. Catalase activity was routinely assayed spectrophotometrically at 25°C by following the decrease in absorption at 240 nm ($\epsilon_{240} = 43.6 \text{ mM}^{-1} \text{ cm}^{-1}$) of 10 mM H_2O_2 in 50 mM potassium phosphate pH 6.4. Peroxidase activity was measured spectrophotometrically in 50 mM potassium phosphate pH 5.4, 1.0 mM H_2O_2 and 0.5 mM 3,3'-diaminobenzidine. Furthermore 3,3'-dimethoxybenzidine ($\epsilon_{460} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$; Hammel and Tardone, 1988), guaiacol, 2,6-dimethoxyphenol ($\epsilon_{469} = 49.6 \text{ mM}^{-1} \text{ cm}^{-1}$; Wariishi et al., 1992) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) were used as aromatic substrates. 1 U is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol hydrogen peroxide min^{-1} under the assay conditions.

Absorption spectra were recorded at 25°C on an Aminco DW-2000 spectrophotometer. In reduction experiments, solutions were made anaerobic by alternate evacuation and flushing with argon. Enzyme reduction was achieved by adding sodium dithionite (final concentration 100 μM). EPR spectra were recorded on a Bruker ER 200D spectrometer with peripheral instrumentation and data acquisition as described before (Pierik and Hagen, 1991). Spectral simulation was done on an Intel 80486 based PC with a program written in FORTRAN (W. R. Hagen, unpublished). Ferrous PCP complexed with nitrite oxide was prepared as described by Cendrin et al. (1994) except that the sodium salts were added in buffer solution.

Analytical gelfiltration utilized Superose-6 HR 10/30 as well as Superdex 200 HR 10/30 columns. These columns were calibrated with 50 mM potassium phosphate at either pH 7.0 or pH 7.5, containing 100 mM sodium sulfate. Sedimentation-velocity measurements were performed essentially as described by Müller et al. (1979) using a MSE Centriscan 75 analytical centrifuge. Scanning wavelengths of 280 and 400 nm were used. The sedimentation coefficient ($S_{20,w}$) determined at 20°C in 50 mM potassium phosphate pH 7.0 was calculated from plots of $\ln r$ versus time and making appropriate viscosity corrections. The subunit molecular mass was determined by SDS/PAGE (Laemmli, 1970), using 12.5% (w/v) acrylamide. PAGE on native enzyme was performed on a Phast-system (Pharmacia). Polyacrylamide gels were stained for peroxidase and catalase activity as previously described (Goldberg and Hochman, 1989;

Hochman and Goldberg, 1991). Purified PCP to be used in the generation of antibodies was electroeluted from SDS/PAGE gels with recoveries of 50–60%. Rabbits were immunized by subcutaneous injection of 200 μg protein and a booster injection (150 μg) 14 days later.

Protoplasts were prepared by the method of Witteveen et al. (1992). Carbohydrate analysis was performed by the phenolic/sulfuric acid method using D-glucose as a standard (Ashwell, 1966). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS

Purification and subcellular localization of enzymes. Extracts of *P. simplicissimum* cells grown on veratryl alcohol exhibit, besides vanillyl-alcohol oxidase activity (De Jong et al., 1992), a relatively high catalase activity (150–250 U/mg). The activity in extracts of cells grown in glucose was considerably lower (50–100 U/mg) and no vanillyl-alcohol oxidase activity was present. Under both conditions no extracellular catalase could be detected.

Initial isolation of the hydroperoxidase from veratryl-alcohol-grown cells showed that almost half of the catalase activity was not retained on phenyl-Sepharose (Table 1). Subsequent purification of this fraction by Q-Sepharose and Superdex PG-200 resulted in a partially purified 'green' catalase, designated as PAC, showing a major band of about 60 kDa by SDS/PAGE (Fig. 1, lane C). The hydroperoxidase fraction retained by phenyl-Sepharose was further purified in three steps (Table 1). SDS/PAGE of this catalase-peroxidase (PCP) showed a protein band of 83 kDa and some minor impurities (Fig. 1, lane A). Antiserum to the purified PCP showed no cross-reactivity towards any protein in the partially purified PAC fraction (data not shown). This indicates that the two isolated hydroperoxidases are structurally different.

The presence of two catalases in *P. simplicissimum* was confirmed by catalase activity staining of protein subjected to PAGE. Extracts of cells grown on veratryl alcohol revealed two zones of catalase activity (Fig. 2, lane A), one of them weak and diffuse. In contrast, the purified catalase-peroxidase preparation (Fig. 2, lane B) showed only the diffuse component. This component also seen in extracts of protoplasts (Fig. 2, lane D) prepared from cells grown on veratryl alcohol was less diffuse. Staining of the same samples for peroxidase activity (Fig. 2, lane C and E) showed that the purified enzyme is also active as a peroxidase and therefore is a catalase-peroxidase. It can also be seen from Fig. 2 (lane B and C) that during purification a minor protein species is formed from the native PCP. Because this species is not observed in cell extracts (lane A) or protoplast preparations (lane E), the microheterogeneity of the purified enzyme may be due to chemical modification or limited proteolysis as noticed with the catalase-peroxidase from *Rhodospseudomonas capsulata* (Hochman and Shemesh, 1987). The second catalase band present in cell extracts (lane A) corresponds to the partially purified 'green' catalase. This catalase was not observed in the protoplast extract and did not show any peroxidase activity. Therefore it most probably represents a periplasmic catalase. Activity measurements on protoplast extracts also indicated that after digestion of the cell wall the specific peroxidase activity is comparable to that of extracts of normal cells while catalase activity decreases significantly (data not shown). Cells grown in glucose-containing media showed almost no peroxidase activity, indicating that the catalase activity of these cells is due to the low expression of catalase-peroxidase and a more constitutive expression of the periplasmic catalase.

Table 1. Purification scheme of catalase-peroxidase from *Penicillium simplicissimum*.

Step	Volume	Protein	Activity	Specific activity	Yield
	ml	mg	kU	U/mg	%
Cell extract	600	2000	499	250	100
Ammonium sulfate	720	1700	352	210	71
Phenyl-Sepharose	90	270	150	550	30
Q-Sepharose	40	130	145	1120	29
Hydroxyapatite	8	53	99	1870	20
Superdex 200	7	18	52	2960	10



A B C

Fig. 1. SDS/PAGE results for purified hydroperoxidases from *P. simplicissimum*. Lane A, purified PCP; lane B, marker proteins (from top to bottom: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa); lane C, partially purified PAC.

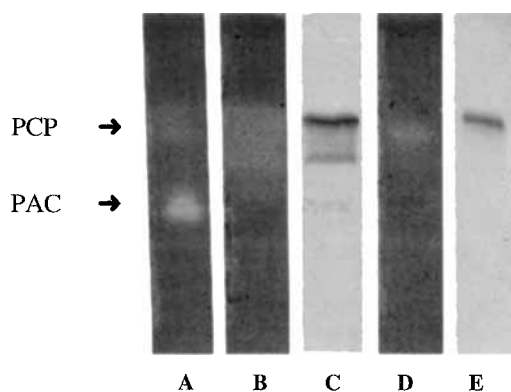


Fig. 2. Catalase and peroxidase staining of PAGE sections of different enzyme preparations from *P. simplicissimum*. Lane A, extract of cells grown in veratryl alcohol stained for catalase activity; lanes B and C, purified PCP stained for (B) catalase activity and (C) peroxidase activity; lanes D and E, extract of protoplasts of cells grown on veratryl alcohol stained for (D) catalase activity and (E) peroxidase activity.

Catalytic properties. The activity of purified PCP from *P. simplicissimum* is strongly pH-dependent (Fig. 3). The optimum for catalase is about pH 6.4, whereas the peroxidase activity is optimal around pH 5.4. This different dependency is a common feature for catalase-peroxidases. The apparent K_m for H_2O_2 of the catalase activity of PCP at pH 6.4 was 10.8 mM. At a substrate concentration of 10 mM, PCP has a specific activity of 2960 U/mg which is rather high when compared with other catalase-peroxidases.

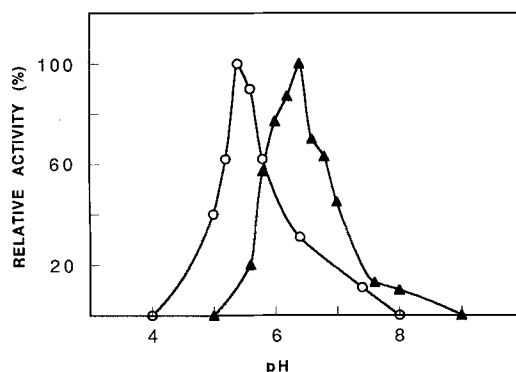


Fig. 3. pH dependence of the catalase and peroxidase activity of PCP. The catalase (\blacktriangle) and peroxidase (\circ) activity was determined in buffers of 50 mM potassium phosphate citric acid (pH 4.0–8.0) and 50 mM glycine (pH 8.0–10.5) at 25°C. The relative activity is plotted against pH. 3,3'-Diaminobenzidine was used as electron donor in the peroxidase assay.

Table 2. Peroxidatic activity of PCP with several aromatic compounds. ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).

Substrate	Detection wavelength	Specific activity
	nm	$\Delta A \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (U/mg)
ABTS	415	150
2,6-Dimethoxyphenol	469	77 (1.6)
3,3'-Diaminobenzidine	460	16
3,3'-Dimethoxybenzidine	460	28 (2.5)
Guaiaicol	450	7.5

PCP was also tested for its capability to oxidize certain aromatic compounds at pH 5.4 (Table 2). In addition to the compounds listed, NADPH, vanillyl alcohol and veratryl alcohol were also tested as electron donors. These compounds were not oxidized. The oxidation rate of 3,3'-dimethoxybenzidine is comparable with those of other catalase-peroxidases (Hochman and Goldberg, 1991; Levy et al., 1992). PCP is reversibly inhibited by potassium cyanide and sodium azide reaching 50% inhibition at 3 μM and 17 μM , respectively. PCP is not inactivated by 3-amino-1,2,4-triazole, a specific inhibitor of catalases (Margolish et al., 1960). Incubation of PCP for 60 min with 20 mM 3-amino-1,2,4-triazole had no effect.

Stability tests performed at 37°C showed that PCP is most stable at relatively high pH. At pH 5 all activity is lost within 5 min whereas no inactivation was observed between pH 7 and pH 9 at this temperature after a 10-h incubation. The enzyme remained fully active at pH 5 for more than 15 min at 25°C.

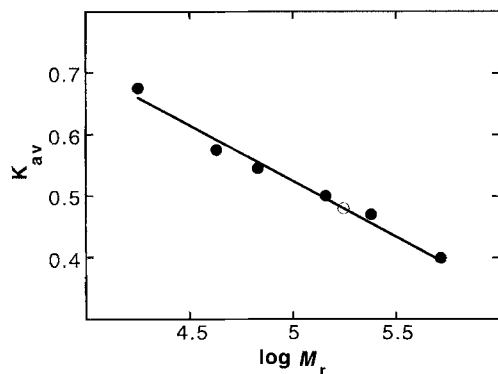


Fig. 4. Analytical gel filtration of PCP. The molecular mass relation to the partition coefficient (K_{av}) of the catalase-peroxidase of *P. simplicissimum* (○) as determined on a Superose 6 HR 10/30 column equilibrated in 50 mM potassium phosphate pH 7.0. The marker proteins: vanillyl-alcohol oxidase from *P. simplicissimum* (520 kDa; De Jong et al., 1992), beef liver catalase (240 kDa), yeast alcohol dehydrogenase (144 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and myoglobin (17.8 kDa) were run in parallel (●). Each calibration point is the result of at least two independent determinations.

The pH stability of PCP closely resembles that of the catalase-peroxidase from *Septoria tritici* (Levy et al., 1992).

The catalytic properties of the partially purified PAC were tested in some detail. This catalase has a very broad pH optimum for activity with more than 90% of the maximum activity remaining between pH 5.0 and 9.5. This characteristic is shared by typical catalases. An apparent linear relationship was found between the catalase activity and H_2O_2 concentration in the substrate range 0–20 mM. The activity of partially purified PAC was 6000 U/mg at a substrate concentration of 20 mM.

Physical properties. The molecular mass and the subunit composition of PCP were determined by SDS/PAGE, gel-permeation chromatography and analytical ultracentrifugation. As mentioned above, the subunit mass of PCP is 83 ± 3 kDa based on SDS/PAGE (Fig. 1). From analytical gel filtration under native conditions, an apparent molecular mass of 170 ± 10 kDa was determined (Fig. 4). This value was nearly independent of the type of column or buffer used (see Materials and Methods). Sedimentation velocity experiments at 0.1–0.5 mg/ml yielded single symmetric boundaries with a sedimentation coefficient, $s_{20,w} = 8.3 \pm 0.2$ S. These results indicate that the enzyme is a homodimer with an average molecular mass of about 170 kDa. This is in the same range as reported for other dimeric catalase-peroxidases.

PCP does not contain a significant amount of carbohydrate. In contrast, the total content of neutral sugar of the partially purified PAC is approximately 12%. These findings confirm the intracellular localization of both hydroperoxidases.

PCP shows a typical high-spin ferric heme spectrum with a maximum at 407 nm and two shoulders at 510 nm and 638 nm (Fig. 5). A value of 0.43 for the ratio A_{407nm}/A_{280nm} compares favorably with those reported for the bacterial catalase-peroxidases. Upon reduction with dithionite, the Soret band decreases and shifts to 440 nm with a new maximum appearing at 562 nm with a shoulder at about 595 nm. Addition of cyanide to the oxidized enzyme results in a shift of the Soret band to 423 nm and the appearance of an absorbance at 543 nm with a shoulder at 590 nm. These spectral properties closely resemble the optical characteristics of known catalase-peroxidases. The presence of a maximum at 543 nm for the cyanide complex suggests that the proximal heme ligand is a histidine (Hochman and Shemesh,

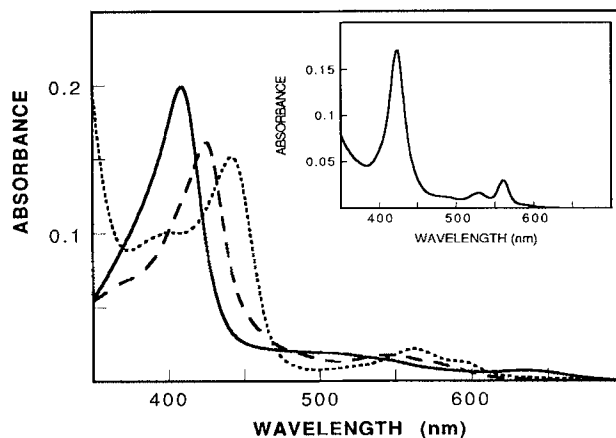


Fig. 5. Spectral properties of oxidized and reduced PCP. The spectra of PCP (0.24 mg/ml) in potassium phosphate pH 7.0 were recorded at 25°C; native enzyme (—); after the addition of 10 mM KCN (----); after addition of 0.1 mM sodium dithionite (.....). The inset shows the pyridine hemochrome spectrum of PCP after reduction with dithionite in 2.1 M pyridine and 0.1 M NaOH.

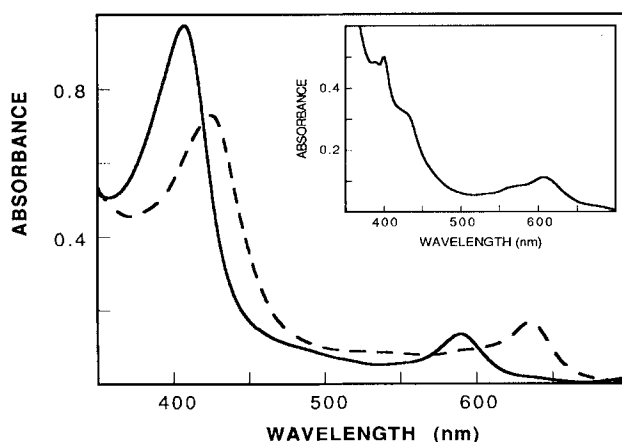


Fig. 6. Spectral properties of partially purified PAC. The spectra of PAC (2 mg/ml) in potassium phosphate pH 7.0 were recorded at 25°C; native enzyme (—); after the addition of 10 mM KCN (----). The inset shows the pyridine hemochrome spectrum after reduction with dithionite in 2.1 M pyridine and 0.1 M NaOH.

1987). The inset of Fig. 5 shows the absorption spectrum of PCP after treatment with pyridine/NaOH. The pyridine hemochromogen spectrum obtained is identical to that of protoheme IX.

The absorption spectrum of the oxidized form of PAC differed from that of PCP by having an additional maximum at 590 nm which explains the greenish color of the enzyme solution (Fig. 6). In contrast to PCP, PAC was not reduced by dithionite. This insensitivity towards dithionite is a general property of the typical catalases. Furthermore, the inset of Fig. 6 shows that the pyridine hemochromogen spectrum of PAC has a maximum at 606 nm with shoulders around 530 nm and 565 nm. This suggests that PAC contains a chlorin-type heme as prosthetic group (Jacob and Orme-Johnson, 1979). This unusual type of heme has been found in only a few proteins including tetrameric catalases from *Neurospora crassa* (Jacob and Orme-Johnson, 1979), *Klebsiella pneumonia* (Goldberg and Hochman, 1989) and the hexameric catalase of *Escherichia coli* (Loewen et al., 1993). In analogy with these enzymes, addition of cyanide to oxidized PAC results in a shift of the Soret band (425 nm) and the formation of a maximum at 636 nm (Fig. 6).

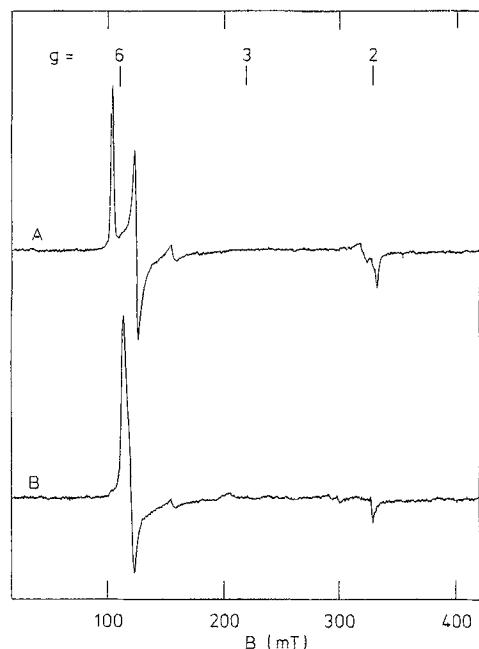


Fig. 7. High-spin ferric heme EPR spectra of PAC and of PCP. Trace A, 8 mg/ml PAC in 100 mM potassium phosphate pH 7.0; trace B, 10 mg/ml PCP in 100 mM potassium phosphate pH 7.0. EPR conditions: microwave frequency, 9.18 GHz, microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 14.5 K.

EPR spectra confirmed that both PCP and PAC contain an $S = 5/2$ high-spin ferric heme. With both hemoproteins one rhombic signal was found with resonances at about $g = 2$ and $g = 6$ (Fig. 7, Table 3). The spectrum of PAC (Fig. 7, trace A) showed an increased rhombicity compared to the spectrum of PCP (Fig. 7, trace B) consistent with structural differences in ligation of the prosthetic groups. Interestingly, for both spectra the average value of g_x and g_y is significantly less than 6.0. This is indicative for quantum-mechanical mixing with a low lying $S = 3/2$ state, and has been noted for other peroxidases (Malttempo and Moss, 1976). The spectrum of PCP also shows a minor low-spin heme component with $g_z = 3.2$.

Nitrous oxide is a heme ligand which can be used to determine the nature of the proximal ligand of hemoproteins by EPR measurements. Because PAC can not be reduced by dithionite only the EPR spectrum of reduced PCP complexed with nitrous oxide could be obtained (Fig. 8). This protein has lost the signals in the $g = 6$ region and exhibits one with rhombic symmetry around $g = 2$ (Table 3) which is characteristic for low-spin ferrous heme NO complexes ($S = 1/2$). A triplet of signals is seen in the g_z region from hyperfine interaction with the ^{14}N nucleus ($I = 1$) of NO. An additional weaker hyperfine splitting of these three lines was observed which can be explained by the presence of an additional nitrogen coordinating to the ferrous ion (Yonetani et al., 1972). Simulation as a rhombic $S = 1/2$ spectrum with the presence of two nitrogen nuclei resulted in a very good fit (Fig. 8). This hyperfine coupling pattern indicates that in PCP the proximal ligand is most probably a histidine residue as suggested above.

DISCUSSION

In this study we describe the (partial) purification of two structurally distinctive hydroperoxidases from the plectomycete *Penicillium simplicissimum*, an intracellular catalase-peroxidase

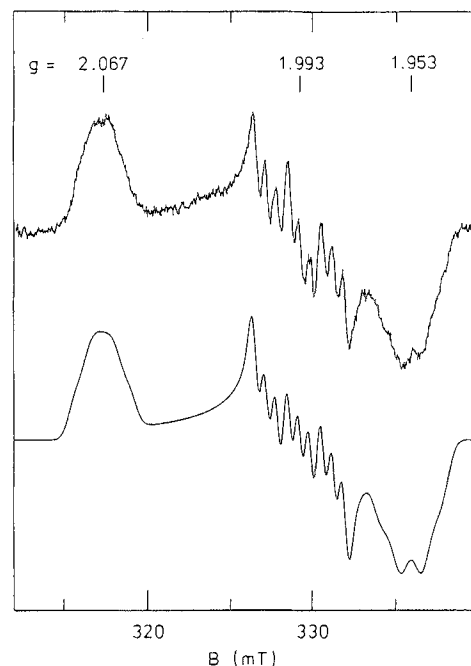


Fig. 8. EPR and simulation spectra of the NO derivative of PCP. PCP (7.1 mg/ml) in 100 mM potassium phosphate pH 7.0 was incubated for 10 min after addition of sodium nitrite and sodium dithionite under a strict argon atmosphere at ambient temperature. EPR conditions: microwave frequency, 9.18 GHz, microwave power, 8 mW; modulation frequency, 100 kHz; modulation amplitude, 0.13 mT; temperature, 100 K. The spectrum (upper trace) is an average of nine scans. The simulation (lower trace) is based on 100×100 molecular orientations and assumes a rhombic $S = 1/2$ spectrum with a Gaussian line shape in field space split by two nitrogen ($I = 1$) nuclei (cf. Table 3).

Table 3. EPR parameters of ferric PAC/PCP and the ferrous NO derivative of PCP. The parameters for the Fe(II)-NO complex were determined in the computer simulation of Fig. 8. The other g -values were obtained from the spectra recorded in Fig. 7.

Enzyme	Form	g_x	g_y	g_z	
PAC	high-spin ferric	5.41	6.48	1.98	
PCP	high-spin ferric	5.60	5.90	2.00	
	low-spin ferric	n.d.	n.d.	3.22	
ferrous-NO		2.067	1.953	1.993	
		Value	Other values		
			x	y	z
			mT		
PCP ferrous-NO	line width	0.7	0.7	0.38	
	hyperfine splittings from:				
	NO	1.0	1.2	2.05	
	histidine	0.65	0.65	0.65	

(PCP) and a periplasmic atypical catalase (PAC). The first catalase-peroxidase of eucarotic origin was recently purified from the fungus *Septoria tritici* (Levy et al., 1992). This catalase-peroxidase resembles PCP by its spectral and catalytical properties. A major difference between the two enzymes is their oligomeric structure. Whereas PCP from *P. simplicissimum* is a homodimer of 170 kDa, the catalase-peroxidase from *S. tritici* is a

homotetramer with a molecular mass of 244 kDa (Levy et al., 1992). Spectral properties indicate that both eucaryotic catalase-peroxidases contain histidine as proximal heme ligand which is a common feature of catalase-peroxidases and all heme-containing peroxidases. In this study EPR experiments confirmed the presence of a histidine as proximal ligand in PCP.

Sequence similarity studies have revealed that bacterial catalase-peroxidases consist of two domains which have similarities with yeast cytochrome *c* peroxidase (Welinder, 1991). Thus catalase-peroxidases appear to have evolved from an ancestral peroxidase and are therefore members of the plant peroxidase superfamily. As PCP is the second fungal catalase-peroxidase described, it seems likely that this type of hydroperoxidases is not restricted to a bacterial peroxidase family as proposed by Welinder (1992) but forms a more widespread family consisting of both procaryotic and eucaryotic members.

In addition to a catalase-peroxidase, a catalase is also present in *P. simplicissimum* which is active over a broad pH range and can not be reduced by dithionite. This catalase contains an uncommon chlorin-type heme. This type of prosthetic group has recently been extensively studied (Bracete et al., 1994) and has been found only in three other catalases.

The presence of multiple catalases has been observed in other fungi. Three different catalases were detected in *Neurospora crassa* (Chary and Natvig, 1989) and four in *Aspergillus niger* (Witteveen et al., 1992). In *A. niger* two catalases are located peripheral to the outer cell membrane. Furthermore, cell extracts of the fungal wheat pathogen *S. tritici* contained, in addition to a catalase-peroxidase, two additional catalases (Levy et al., 1992). Eucaryotic catalases are commonly located in peroxisomes but have also been found in the periplasm of the ligninolytic fungus *Phanerochaete chrysosporium* (Forney et al., 1982) and the cell wall of the plectomycete *A. niger* (Witteveen et al., 1992). Furthermore, it is known that catalases can be excreted by fungi, especially by *Penicillia* and *Aspergilli*, in high quantities (Chaga et al., 1992; Nishikawa et al., 1993). In *P. simplicissimum* an atypical catalase is located outside the cell membrane. This 'green' catalase presumably eliminates external hydrogen peroxide.

The PCP of *P. simplicissimum* may be involved in detoxification processes. It is highly expressed when grown on veratryl alcohol which coincides with the induction of the flavoprotein oxidase, vanillyl-alcohol oxidase (De Jong et al., 1992). Induction of both enzymes suggests that the hydrogen peroxide formed by the flavoprotein is either destroyed or used for some catabolic process by the catalase-peroxidase. Preliminary results suggest that both PCP and vanillyl-alcohol oxidase are located in peroxisomes which is a further indication that the physiological functions of these enzymes are linked. A similar peroxisomal oxidase/peroxidase couple is present in methylotrophic yeasts. In these organisms methanol oxidase and catalase both oxidize methanol to form formaldehyde using oxygen and hydrogen peroxide, respectively, as electron acceptor (Kawaguchi et al., 1989). However, the question of whether PCP *in vivo* is mainly active as a catalase or a peroxidase and its relation to vanillyl-alcohol oxidase remains to be elucidated.

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