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Purification and Characterization of an Intracellular Peroxidase from *Streptomyces cyaneus*

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An intracellular peroxidase (EC 1.11.1.7) from *Streptomyces cyaneus* was purified to homogeneity. The enzyme had a molecular weight of 185,000 and was composed of two subunits of equal size. It had an isoelectric point of 6.1. The enzyme had a peroxidase activity toward *o*-dianisidine with a K_m of 17.8 μM and a pH optimum of 5.0. It also showed catalase activity with a K_m of 2.07 mM H_2O_2 and a pH optimum of 8.0. The purified enzyme did not catalyze Cα-Cβ bond cleavage of 1,3-dihydroxy-2-(2-methoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl) propane, a nonphenolic dimeric lignin model compound. The spectrum of the peroxidase showed a soret band at 405 nm, which disappeared after reduction with sodium dithionite, indicating that the enzyme is a hemoprotein. Testing the effects of various inhibitors on the enzyme activity showed that it is a bifunctional enzyme having catalase and peroxidase activities.

Peroxidases (EC 1.11.1.7) play an important role in the oxidation of a large number of aromatic compounds, including recalcitrant substances (5, 11, 12). Peroxidases widely occur in animals, plants, and microorganisms. Only a few reports of peroxidases from actinomycetes are available (19, 20, 24, 27). Several peroxidases of microbial origin which act as both peroxidases and catalases have been described. These enzymes have been detected in *Escherichia coli* (3), *Comamonas compransoris* (18), *Bacillus stearothermophilus* (15), and *Rhodopseudomonas capsulata* (9) and in *Streptomyces phaeochromogenes* (24), *Streptomyces griseus* (28), and *Streptomyces venezuelae* (13). An extracellular peroxidase from *Streptomyces viridosporus*, a lignocellulose-degrading actinomycete, has recently been isolated and characterized. The purified enzyme catalyzed Cα-Cβ bond cleavage of phenolic and nonphenolic arylglycerol-β-aryl ether lignin model compounds (20). *Streptomyces cyaneus* can also utilize graminaceous lignocellulose and degrade a nonphenolic arylglycerol-β-aryl ether lignin model compound (29, 30).

In this study, the intracellular peroxidase from *S. cyaneus* was isolated and purified to homogeneity to characterize its biochemical properties and its role in the degradation of a nonphenolic lignin model compound.

MATERIALS AND METHODS

Organism and culture conditions. *S. cyaneus* NCIB 12383 was grown in 1-liter flasks in a mineral salt medium containing, per liter, 1 g of yeast extract and 1 g of glucose. Medium (200 ml) was inoculated with a suspension of spores and mycelium and incubated for 30 h at 30°C on a shaker at 150 rpm.

Purification of the peroxidase. The cells from 200 ml of culture were harvested by centrifugation at 4,200 × *g* for 15 min, washed with 0.1 M Tris-HCl buffer (pH 7), and suspended in 30 ml of the same buffer. The cells were disrupted by treatment with a French pressure cell (American Instrument Co.) two times at 1,200 lb/in^2 (4°C) followed by centrifugation at 12,100 × *g* for 50 min at 4°C.

(i) Ammonium sulfate precipitation. Ammonium sulfate was added to the supernatant to 30% saturation. The supernatant was then centrifuged at 4,200 × *g* for 20 min. Ammonium sulfate was then further added to the supernatant to 80% saturation. After centrifugation at 4,200 × *g* for 20 min, the precipitated proteins were collected, dissolved in 0.1 M Tris-HCl buffer (pH 7), and dialyzed overnight at 4°C against the same buffer.

(ii) Anion-exchange chromatography. This crude protein preparation was applied to an anion-exchange Q-Sepharose column (1.5 by 15 cm; Pharmacia) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7) and eluted with a discontinuous NaCl gradient of 0.35, 0.4, 0.45, and 0.5 M. The fractions were assayed for peroxidase and catalase activities. The active fractions were concentrated by ultrafiltration through a PM-10 membrane (Amicon) and dialyzed overnight at 4°C against 0.1 M Tris-HCl buffer (pH 7). The dialyzed proteins were applied to an anion-exchange fast protein liquid chromatography (FPLC) column (Mono-Q, type HR 5/5; Pharmacia) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7). The proteins were eluted by a continuous NaCl gradient from 0 to 0.5 M in Tris-HCl buffer (pH 7).

(iii) Gel filtration. The active fractions from this column were concentrated and dialyzed as described above and then fractionated by gel filtration chromatography on a Superose 12 FPLC column (Pharmacia) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7) containing 50 mM NaCl.

(iv) Preparative IEF-agarose. Active fractions obtained by gel filtration chromatography were concentrated by ultrafiltration with a PM-10 membrane (Amicon) and then separated in a preparative isoelectric focusing (IEF)-agarose gel according to the method of Garfin (6).

Enzyme assays. (i) Peroxidase assay. Peroxidase activity was routinely measured as described previously by using *o*-dianisidine (3) and 2,4-dichlorophenol (1) as substrates at room temperature at pH 5.5.

To study the substrate specificity of the peroxidase, tetramethylbenzidine, 3-(dimethylamino)benzoic acid–3-methyl-2-benzothiazoline-1-hydrazone (MBTH), L-β-3,4-dihydroxy-phenylalanine, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid, syringaldazine, guaiacol, veratryl alcohol, benzidine, and the nonphenolic lignin model compound 1,3-dihydroxy-2-(2-methoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl) pro-

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pane were also tested. A Cα-Cβ bond cleavage of the lignin model compound was detected by measuring the UV spectra of the reaction products, which were identified by comparison with the spectra of authentic compounds.

One unit of peroxidase activity was defined as the amount of enzyme which converted 1 μmol of *o*-dianisidine per min (ε_{460} = 11,300 M^{-1} cm^{-1}) (4).

(ii) Catalase assay. Catalase activity was determined spectrophotometrically by measuring the decrease in the A_{240} of H_2O_2 or polarographically by measuring the O_2 produced by 100 μl of enzyme solution in a 1-ml reaction mixture from H_2O_2 or from sodium perborate (7) with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) at room temperature at pH 7. One unit of catalase activity was defined as the disappearance of 1 μmol of H_2O_2 per min (ε_{240} = 43.6 M^{-1} cm^{-1}) (8). The protein concentrations were determined by the method of Bradford (2).

Determination of M_r. The enzyme was chromatographed on a Superose 12 FPLC column (1 by 30 cm) which had been calibrated with standard proteins (calibration kit; Pharmacia). The proteins were eluted with 0.1 M Tris-HCl buffer (pH 7) containing 0.15 M NaCl, at room temperature. The M_r was also estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard protein markers (Bio-Rad Ltd.).

Gel electrophoresis. Native PAGE was performed with Phastgradient (8 to 25%) polyacrylamide gels (Pharmacia Phastsystem) and with 7.5% polyacrylamide gels (14). The pH of the separation gel was 7.6 because a pH higher than 7.8 resulted in artificial peroxidase bands in the peroxidase activity staining (3). SDS-PAGE was performed with Phastgradient (10 to 15%) gels (Pharmacia Phastsystem). Separation of the proteins by IEF was performed in IEF-Phastgels (pH 3 to 10) and in IEF-agarose gels (Pharmacia). The isoelectric point of the peroxidase was determined with standard protein markers (Bio-Rad Ltd.).

Protein bands were stained with silver nitrate (25). The peroxidase bands in native PAGE and IEF gels were detected by incubating the gels in a mixture of 1 mM 2,4-dichlorophenol or 10 μM *o*-dianisidine in the presence of 1.6 mM H_2O_2 in 50 mM succinate buffer, pH 5.5, for 15 to 30 min at room temperature.

A double staining (peroxidase staining followed by a ferricyanide negative staining) for native PAGE gels was performed according to the method of Wayne and Diaz (26), with the difference that *o*-dianisidine was used instead of diaminobenzidine for the peroxidase staining.

Enzyme inhibition. Assays for catalase and peroxidase were carried out in the presence of NaN_3, KCN, 3-amino-1,2,4-triazole, and hydroxylamine hydrochloride. The effects of dialysis against H_2O_2 and of treatment with ethanol-chloroform (cell extract–95% ethanol–chloroform, 10:5:3 [vol/vol/vol]) on the enzyme activity were estimated as described previously (17).

pH optimum. The effect of pH on peroxidase and catalase activities was measured over the pH ranges of 3.5 to 7 in 0.1 M potassium phosphate buffer and 7.5 to 10 in 0.1 M Tris-HCl buffer.

Spectral characterization of the enzyme. The oxidized and sodium dithionite-reduced spectra of the purified enzyme were recorded with a spectrophotometer (Kontron Uvikon 810), between 500 and 300 nm.

Chemicals. Q-Sepharose gel, *o*-dianisidine, 3-amino-1,2,4-triazole, hydroxylamine, and 4-aminoantipyrine were obtained from Sigma Chemical Co.; H_2O_2, NaN_3, tetramethylbenzidine, 3-(dimethylamino)benzoic acid, and MBTH were obtained from Fluka Chemical Co.; and 2,4-dichlorophenol was obtained from Aldrich Chemical Co. 1,3-Dihydroxy-2-(2-methoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl) propane, 4-ethoxy-3-methoxybenzaldehyde, and 4-ethoxy-3-methoxybenzoic acid were synthesized as described previously (22, 23, 30).

TABLE 1. Purification of the intracellular peroxidase and catalase activities from *S. cyaneus*

Purification step	Total proteins (mg)	Activity[a]	Total activity (U)	Sp act (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	62.02	P	460	7.4	100	1.0
		C	674	10.8	100	1.0
Ammonium sulfate precipitation	41.2	P	396	9.6	86	1.3
		C	490	11.9	72	1.1
Q-Sepharose	15.6	P	329	21.6	72	2.9
		C	217	14	32	1.3
Mono-Q	2.3	P	113	48.2	24.6	6.5
		C	140	60	20.8	5.5
Superose 12	0.6	P	99	165.2	22	22
		C	72	120	10.6	11
Preparative IEF	0.38	P	94	247	21.7	33.5
		C	64	168	9.5	15.5

[a] P, peroxidase; C, catalase.

RESULTS AND DISCUSSION

Enzyme purification. The crude intracellular protein preparation from *S. cyaneus* showed peroxidase activity when incubated with *o*-dianisidine or 2,4-dichlorophenol in the presence of H_2O_2 and also had catalase activity.

In some experiments, incubation of 1,3-dihydroxy-2-(2-methoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl) propane with the crude cell extract and H_2O_2 resulted in the cleavage of the lignin model compound, as indicated by the formation of 4-ethoxy-3-methoxybenzaldehyde. However, this activity seemed to be very unstable since it could be detected in only some of the crude cell-free preparations.

The peroxidase-catalase could be purified by anion-exchange and gel filtration chromatography followed by preparative IEF; a summary of the purification is given in Table 1. The peroxidase bound to the anion-exchange Q-Sepharose gel and was eluted as a single peak, with 0.35 to 0.4 M NaCl. Gel filtration chromatography on Superose 12 also resulted in one peak containing peroxidase and catalase activities. After these steps of purification, the enzyme preparation was still not homogeneous, as indicated by minor protein bands detectable on native PAGE gels. Attempts to improve the chromatographic separation were unsuccessful because the enzyme was rapidly inactivated at this stage of purification. Purification to homogeneity was obtained by preparative IEF in an agarose gel which yielded a pure enzyme preparation, as indicated by SDS-PAGE analysis (Fig. 1). The purification steps resulted in an enzyme preparation with a specific activity of 247 U mg^{-1}, a purification factor of 33.5, and a yield of 21.7% for the peroxidase activity.

M_r and isoelectric point. The molecular weight of the enzyme determined by SDS-PAGE was estimated to be

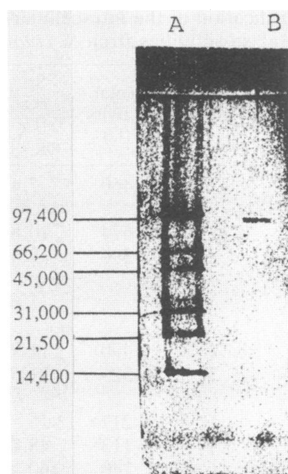

FIG. 1. SDS-PAGE of the purified peroxidase from *S. cyaneus*. Lanes: A, protein standard-molecular-weight markers, as indicated on the left; B, peroxidase purified by preparative IEF.

92,000. A molecular weight of 185,000 was obtained under native conditions by using gel filtration, indicating that the enzyme is a dimer consisting of two subunits of equal size. Similar dimeric enzymes with catalase and peroxidase activities from *B. stearothermophilus* (15) and *C. compransoris* (18) have been reported previously. The pI of the enzyme determined by IEF was estimated to be 6.1.

Effect of pH on the enzyme activity. The enzyme showed pH optima of 8.0 for catalase activity and 5.0 for peroxidase activity. Peroxidase-catalases from *S. phaeochromogenes* (24) and from *E. coli* (3) with similar pH optima have been described previously. In contrast, typical catalases are pH independent in the pH range of 5.5 to 10 (17).

Substrate specificity. During all steps of the purification, the enzyme preparation showed both catalase and peroxidase activities.

To study the substrate specificity of the peroxidase, different kinds of hydrogen donor substrates were incubated with the Superose 12-purified enzyme preparation. Among these compounds, only *o*-dianisidine (10 μM), 2,4-dichlorophenol (1 mM), tetramethylbenzidine (8.3 mM), and 3-(dimethylamino)benzoic acid–MBTH (10 μM) were used as substrates by the peroxidase and gave relative activities of 100, 80, 45, and 60%, respectively. The K_m of the peroxidase with *o*-dianisidine as the substrate was 17.8 μM, and the K_m of the catalase activity was 2.07 mM H_2O_2. Syringaldazine, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid, L-β-3,4-dihydroxy-phenylalanine, diaminobenzidine, guaiacol, benzidine, veratryl alcohol, veratric acid, veratraldehyde, 3-methoxy-4-ethoxybenzoic acid, and 3-methoxy-4-ethoxybenzyl alcohol were not used as substrates by the peroxidase. The purified enzyme did also not cleave the lignin model compound 1,3-dihydroxy-2-(2-methoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl) propane. In contrast, a purified extracellular peroxidase from *S. viridosporus* was able to degrade both phenolic and nonphenolic arylglycerol-β-aryl ether lignin model compounds. This peroxidase also uses 2,4-dichlorophenol as a substrate (20).

Effect of inhibitors on enzyme activity. The effect of several specific peroxidase and catalase inhibitors was studied, and the results are shown in Table 2. Neither peroxidase nor catalase activity was inhibited by 200 mM 3-aminotriazole.

TABLE 2. Effect of inhibitors on peroxidase and catalase activities

Reagent	Concn	% Inhibition	
		Peroxidase	Catalase
3-Aminotriazole	200 mM	0	0
NaN_3	50 μM	20	100
NaN_3	50 mM	100	100
KCN	10 μM	35	100
KCN	25 μM	100	100
Hydroxylamine	50 μM	100	100
2-Mercaptoethanol	1 mM	100	100

This compound is an inhibitor for typical catalases without having any effect on peroxidase activity (16). In the presence of 50 μM NaN_3, the catalase activity was completely inhibited, whereas the peroxidase activity retained 80% of its initial value and was completely inhibited only in the presence of 50 mM NaN_3 (Fig. 2). These results are in agreement with those reported for the catalase-peroxidase from *S. phaeochromogenes* (24) and *Bacillus cereus* (10). The enzyme was inhibited by a mixture of ethanol and chloroform and by dialysis against 2 mM H_2O_2. These properties have been described previously for catalase-peroxidase enzymes (17). In contrast, typical catalases are not inhibited by these treatments. Both enzyme activities were also inhibited by 1 mM 2-mercaptoethanol. Thiol compounds also act as catalase inhibitors (21). A double staining of the active enzyme on native PAGE gels, consisting of a peroxidase activity staining followed by a ferricyanide negative staining, was performed. This staining can be used to differentiate between typical catalase and catalase-peroxidase enzymes (26). The results showed a dark-blue-colored band on the PAGE gel after the double staining, indicating that this enzyme is a catalase-peroxidase. The spectrum of the purified enzyme showed a soret band at 405 nm, which disappeared after reduction with sodium dithionite. The inhibition of the enzyme by KCN, NaN_3, and hydroxylamine, known as typical hemoprotein inhibitors (17), also indicated that the enzyme from *S. cyaneus* is a hemoprotein. The results show that the purified enzyme from *S. cyaneus* is a single enzyme with peroxidase and catalase activities. This intracellular

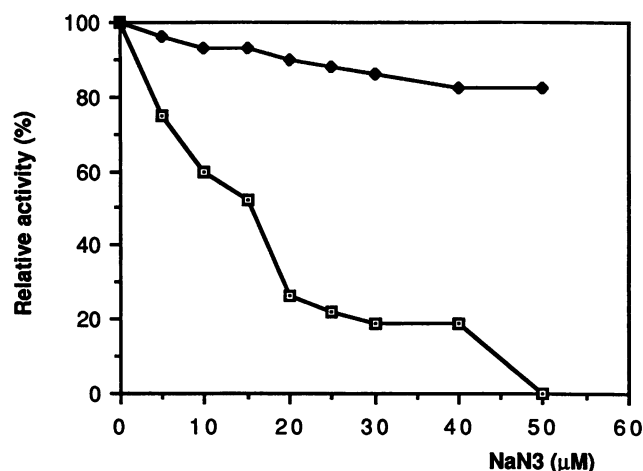

FIG. 2. Effect of NaN_3 on catalase (□) and peroxidase (◆) activities of the purified enzyme from *S. cyaneus*.

enzyme is not responsible for the cleavage of the nonphenolic lignin model compound 1,3-dihydroxy-2-(2-methoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl) propane, which is degraded by whole cells of the strain (30).

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