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# Immunologic Relatedness of Extracellular Ligninases from the Actinomycetes *Streptomyces viridosporus* T7A and *Streptomyces badius* 252

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## ABSTRACT

Four isoforms of the extracellular lignin peroxidase of the ligninolytic actinomycete *Streptomyces viridosporus* T7A (ALip-P1, P2, P3, and P4) were individually purified by ultrafiltration and ammonium sulfate precipitation, followed by electro-elution using polyacrylamide gel electrophoresis. Three of the purified peroxidases were compared for their immunologic relatedness by Western blot analysis using a polyclonal antibody preparation produced in rabbits against pure isoform P3. The anti-P3 antibody was also tested for its reactivity towards a lignin peroxidase from the white-rot fungus *Phanerochaete chrysosporium* and another ligninolytic actinomycete *Streptomyces badius* 252. Results showed that peroxidases ALip-P1 through ALip-P3 are immunologically related to one another. The peroxidases of *S. badius,* but not the peroxidase of *P. chrysosporium,* also reacted with the antibody, thus indicating that the lignin peroxidases of *S. viridosporus* and *S. badius* are immunologically related. Based upon its specific affinity, lignin peroxidase isoform ALip-P3 of *S. viridosporus* was readily purified using an anti-P3 antibody affinity column.

**Index Entries:** Ligninases; Western blot analysis; affinity purification.

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## INTRODUCTION

The initial reactions of lignin degradation by ligninolytic fungi and actinomycetes are thought to be catalyzed by a class of extracellular enzymes, the lignin peroxidases, which utilize $H_2O_2$ to oxidize the lignin polymer (*1–3*). Extracellular peroxidases, such as lignin peroxidase (LiP), Mn(II)-dependent peroxidase from the white-rot fungus *Phanerochaete chrysosporium* (*1,2*), and lignin peroxidase ALip-P3 from the actinomycete *Streptomyces viridosporus* T7A (*4*), oxidize phenolic and nonphenolic low mol wt lignin substructure model compounds similarly, by catalyzing carbon-carbon bond cleavage between the $\alpha$ and $\beta$ carbons of phenylpropane sidechains. The enzymes also carry out a number of other oxidations (*2,4*). When lignin is the substrate, $C_\alpha$-$C_\beta$ cleavage results in substantial depolymerization of the polymer. Both the fungal and actinomycete enzymes are heme proteins. However, although a cation radical mechanism of action has been elucidated for the fungal lignin peroxidase (*1,2*), the mechanism by which the actinomycete enzyme functions has not been determined.

*S. viridosporus* produces four different extracellular peroxidases (ALip-P1, P2, P3, and P4) when grown on lignocellulose (*4*). These enzymes have been called isoforms (*4,5*), although they appear to have different substrate specificities (*4*). Of the four, lignin peroxidase isoform ALip-P3 has the widest substrate range and greatest oxidizing power, and it is the only one of the peroxidases that will oxidize chlorophenols, such as 2,4-dichlorophenol (*4*) The gene coding for this enzyme has recently been cloned into *Streptomyces lividans* (*5*). Recombinant ALip-P3-expressing *S. lividans* strains produce large amounts of the P3 enzyme, but not the other forms. This is an indication that other genes may code for the P1, P2, and P4 enzymes.

Another ligninolytic actinomycete, *Streptomyces badius* strain 252, has been shown to produce four extracellular peroxidases that appear very similar to those of *S. viridosporus* (*6*). Several other actinomycetes have also been shown to degrade lignin (*7–9*), and all of these actinomycetes degrade lignin similarly, by oxidizing and solubilizing the polymer, forming a water-soluble polymeric metabolite, Acid Precipitable Polymeric Lignin (APPL) (*3,10*). Thus, it would be reasonable to hypothesize that all of these ligninolytic actinomycetes produce similar lignin peroxidases, and that these peroxidases might also be mechanistically similar to fungal lignin peroxidases.

In the present article, we report the immunological relatedness of *S. viridosporus* T7A lignin peroxidase ALip-P3 to two of the other peroxidase isoforms, to the four similar peroxidases produced by *S. badius* 252, and to a fungal lignin peroxidase (isoform H8) of *P. chrysosporium*. This comparison required the purification of the ALip-p3 protein from culture supernatants of *S. viridosporus*, followed by preparation of a polyclonal antibody

against pure ALip-P3 in rabbits. Then, Western blots were run to determine the relative reactivity of the anti-ALip-P3 antibody towards each of the other enzymes.

## MATERIALS AND METHODS

### Microorganisms

*S. viridosporus* T7A (ATCC 39115) was isolated from Idaho soil by D. L. Sinden (M.S. Thesis, Univ. Idaho 1979), whereas *S. badius* 252 (ATCC 39117) was isolated from soil by M. B. Phelan et al. (*11*). Stock cultures of strain T7A were maintained on yeast extract-malt extract-glucose agar (YEMED) (*12*), whereas strain 252 was maintained on Yeast Malt Agar [YMA; containing 10.0 g/L malt extract, 4.0 g/L yeast extract, and 20.0 g/L agar]. Spores from stock slant cultures were used as the inoculum in all experiments.

### Production and Purification of Lignin Peroxidases

For production of lignin peroxidases, spores from a single stock slant of *S. viridosporus* or *S. badius* were inoculated into 500 mL of production medium, which consisted of nitrogen-free mineral salts (*13*), plus 5.0 g/L glucose and 1.0 g/L each of the amino acids proline, asparagine, and glutamic acid. Shaking incubation was for 4–5 d. The culture supernatant was then collected by centrifugation, followed by vacuum filtration through Whatman #1 filter paper. This filtrate was concentrated approx 10- to 50-fold by ultrafiltration through an Amicon ultrafiltration cell (Amicon Corp., Beverly, MA) with a YM5 or PM-30 filter. Concentrated filtrates typically contained about 1.0 mg/mL protein. The lignin peroxidases present in the concentrated filtrate were precipitated from solution with ammonium sulfate. The protein that precipitated between 0–40% saturation was discarded, whereas that precipitating between 40–80% saturation was retained. This precipitate was collected by centrifugation (30 min, 7000 rpm). The pellet was resuspended in 3.0 mL of 0.1*M* Tris buffer at pH 7.0 and dialyzed against the same buffer at 4°C, or desalted on a Sephadex G-25M PD-10 gel permeation column (Pharmacia, Inc., Piscataway, NJ). The desalted solution obtained from the 40–80% saturation precipitate was a partially purified, concentrated mixture containing all four peroxidases from *S. viridosporus* or *S. badius*.

Further purification of the resuspended, dialyzed $(NH_4)_2SO_4$ cut employed preparative polyacrylamide gel electrophoresis, according to the method of Hames and Rickwood (*14*). A 10% total acrylamide resolving gel (pH 8.9) was used, with a 3.5% total acrylamide stacking gel (pH 6.8). Each slab was 2 mm thick. As much as 20 mg of total protein was layered on top of the stacking gel, without the use of wells. Electrophoresis was

carried out at 4°C and 25 mA, with cooling, until the sample reached the resolving gel. Then electrophoresis was continued at 35 mA until the dye front reached the bottom of the gel, and for an additional 4 h thereafter. A strip of the gel was then cut from each side and activity stained for peroxidase, using an L-Dopa oxidation activity stain (*12*). The ALiP-P3 band developed first. While the P3 band was still sharp, the gels strips were washed with water and lined up with the clean gel. A razor blade was used to cut a 1-mm-across strip from the gel, corresponding to the location of the P3 band. This strip was then cut into 1×1 cm pieces and loaded into the tubes of a BioRad electro-eluter (BioRad Corp., Richmond, CA). The elution buffer was 0.025*M* Tris—0.192*M* glycine (pH 8.8), and elution was for 10 h at 4°C and 8 mA/elution tube. After elution, the eluted protein was collected in the elution membrane cap in a volume of 0.5 mL. By cutting out strips corresponding to each peroxidase band, each of the four could be purified using this procedure.

## Affinity Purification of *S. viridosporus* Peroxidases

Peroxidase isoform P3 was purified by affinity chromatography on a column of rabbit-anti-P3 IgG covalently attached to CNBr-activated Sepharose CL-4B (Phramacia LKB Biotechnology, Piscataway, NJ). The IgG fraction of the crude antiserum against P3 was first purified by chromatography on Affi-Gel Protein A (Bio-Rad Laboratories, Richmond, CA), and then coupled to the activated matrix according to the manufacturer's instructions. Concentrated extracullular culture filtrate was treated with ammonium sulfate to 80% saturation, and the precipitated protein was pelleted by centrifugation at 27,000*g* for 10 min. The pellet was resuspended in 2.0 mL of 50 m*M* sodium phosphate (pH 7.0) and dialyzed against the same buffer. This preparation was then passed through a column of the affinity matrix (bed volume, 2.5 mL) three times. The column was then washed with 50 mL of the phosphate buffer. The bound protein was then eluted with 5 mL of 3*M* sodium iodide in the phosphate buffer. The affinity-purified peroxidase was then dialyzed against 50 m*M* ammonium acetate and lyophilized. This preparation was analyzed for purity by SDS-polyacrylamide gel electrophoresis, using β-mercaptoethanol as the reducing agent.

## Peroxidase Assays

Lignin peroxidase ALip-P3 was assayed with 2,4-dichlorophenol (2,4-DCP) as substrate (*4,5*). One unit of enzyme activity was defined as the amount of enzyme required for an increase in absorbance of 1.0 AU $min^{-1}$ at $A_{510}$. Peroxidase activity was also measured with L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma Chemical Co., St. Louis, MO) as substrate (*12*). Peroxidases were also characterized using nondenaturing, discontinuous polyacrylamide gel electrophoresis (PAGE) on vertical slab gels

(*12,14*). Peroxidase bands on the nondenaturing PAGE gels were developed by activity staining as previously described (*4,12*).

### Production of Antibody

A polyclonal antibody active against purified ALip-P3 enzyme was produced in rabbits. Electro-eluted P3 protein (25 μg) in Freund's incomplete adjuvant was used as the immunogen. A rabbit was injected with the preparation every 3 wk, and serum was collected 1 wk after each injection.

### Western Blot Analysis

Western blots were carried out on PAGE gels containing electrophoretically separated peroxidase bands. Protein (0.25–1.0 μg) was loaded onto a 7.5% mini-gel for PAGE separation. Proteins were transferred onto an Immobilon PVDF membrane (Millipore) according to the manufacturer's recommendations, using a PolyBlot model SBD-1000 transfer system (American Bionetics Inc.). Transfer was carried out for 30 min at 2 $mA/cm^2$. Then, the membrane was blocked with 5% (w/v) nonfat dry milk in Tris buffered saline (TBS: 20 m*M* Tris, 0.1*M* NaCl, pH 7.5) for 30–60 min with gentle agitation. The antibody binding assay was carried out by incubating the membrane overnight in 25 mL of primary antibody solution [1:1000 dilution, in a solution of 1% Bovine serum albumin (BSA) or nonfat dry milk + 0.05% Tween 20 in TBS]. Afterwards, the membrane was washed in a solution of 0.2% Tween 20 in TBS (3×; 5 min each) and then incubated overnight in 25 mL of secondary antibody (BioRad goat anti-rabbit IgG/alkaline phosphatase conjugate; 1:3000 dilution prepared in the same diluent as the primary antibody solution). Bands were visualized using a BioRad alkaline phosphatase color development reagent kit.

## RESULTS AND DISCUSSION

### Peroxidase Purification

The partially purified peroxidase preparations obtained by ammonium sulfate precipitation were highly active and could be further purified to separate the individual isoforms. The individual peroxidase isoforms of both *S. badius* and *S. viridosporus* readily separated from one another during polyacrylamide gel electrophoresis (Fig. 1). Thus, electro-elution allowed for the individual recovery of each enzyme from the gel. Isoform P3 from *S. viridosporus* T7A, for example, could typically be collected in pure form in up to 0.2 mg/mL, or 0.1 mg/0.5 mL volume in the elution membrane cap. After recovery, the protein migrated as a single band on nondenaturing polyacrylamide (PAGE) gels, as shown by electrophoresis and silver

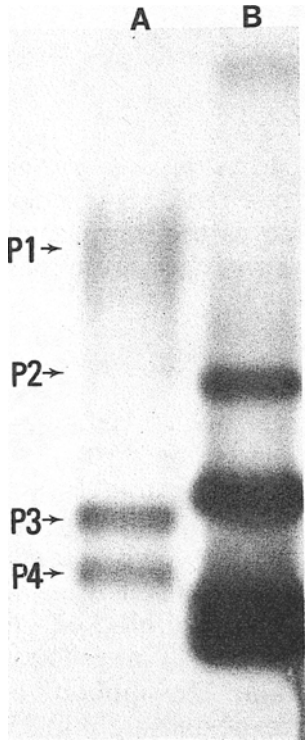


Fig. 1. Peroxidase activity stain of Native PAGE gel showing peroxidase isoforms of strains T7A and 252. Lane A—T7A, Lane B—252. 50 μg total protein loaded in each lane. T7A peroxidase isoforms are labeled.

staining. When ALip-P3 enzyme purity was checked by silver staining on SDS-PAGE gels, one band of 75,800 daltons was typically detected with unboiled samples (Fig. 2). Occasionally, a few contaminating proteins were detected, but, if so, they were always presnt in very low amounts. With boiling, on SDS-PAGE gels, the affinity-purified protein dissociated into two major bands of 67.1 kD and 37.1 kD (Fig. 3). A Ferguson plot (*14*) calculation of mol wt from the native gels gave an estimate of 134,000 daltons for the ALip-P3 protein. Using the L-Dopa oxidation assay, specific activity for the ALip-3 peroxidase of *S. viridosporus* is typically increased from 1.1 AU/min/mg in crude enzyme preparations to 60.0 AU/min/mg with pure enzyme, whereas with 2,4-dichlorophenol (2,4-DCP) as substrate, specific activity initially averages about 0.7 AU/min/mg, and the partially purified peroxidase averages 7.0 AU/min/mg. Table 1 shows typical results from a purification run starting with crude enzyme.

## Western Blot Analyses

The Western blot assays were varied such that the primary antibody used was the anti-ALip-P3 polyclonal antibody, and the proteins examined included the P1 through P4 peroxidases of *S. viridosporus* and *S. badius*, as

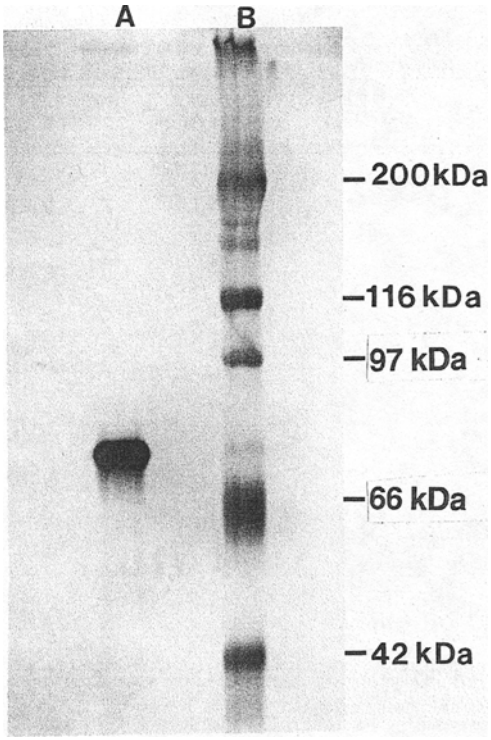


Fig. 2. Silver stain of purified ALip-P3 from T7A on 7.5% SDS-PAGE gel. Lane A—Electrophoretically purified ALip-P3 (unboiled, 1 μg), Lane B—mol wt standards.

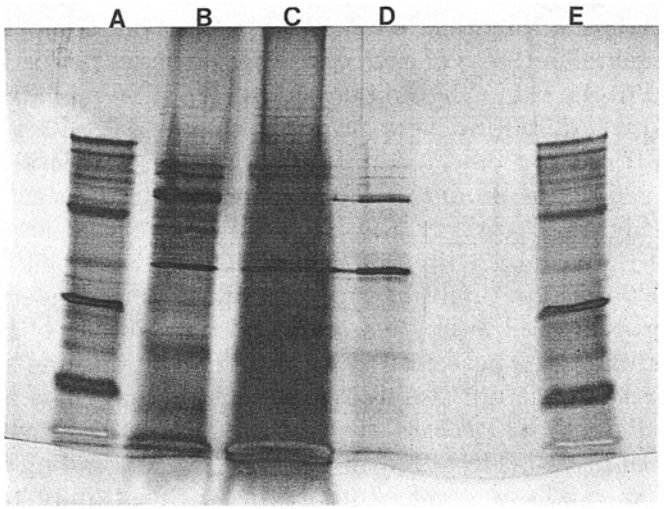


Fig. 3. Silver stain of 12% SDS-PAGE gel of affinity-purified ALip-P3 peroxidase showing subunits of 67.1 and 37.1 kD. Lanes A and E—mol wt standards, Lane B—Concentrated filtrate, 30 μg; Lane C—ammonium sulfate cut, 30 μg; Lane D—affinity-purified ALip-P3, 30 μg.

Table 1
Typical Results of the Purification Procedure Used to Purify the P3 Lignin Peroxidases from Culture Filtrates of *S. viridosporus* T7A and *S. badius* 252

| ENZYME PREPARATION | PROTEIN (mg/ml) | ACTIVITY (Au/ml/min) | SPECIFIC ACTIVITY (Au/mg/min) |
|---|---|---|---|
| *S. viridosporus* | | | |
| Crude Culture Filtrate | 0.24 | 0.17 | 0.70 |
| Ultrafiltered Concentrated | 0.39 | 2.13 | 5.52 |
| $(NH_4SO_4)$-Precipitated and Dialyzed | 0.72 | 5.03 | 7.00 |
| *S. badius* | | | |
| Crude Culture Filtrate | 0.77 | 0.29 | 0.37 |
| Ultrafiltered Concentrated | 1.38 | 1.05 | 0.76 |
| $(NH_4)SO_4$-Precipitated and Dialyzed | 1.09 | 2.92 | 2.68 |

*Activity is defined as the change in absorbance of the reaction mixture at 510 nm per minute per mL of enzyme preparation.

Assays were performed using the substrate 2,4-dichlorophenol as described previously (4,5).*

well as purified lignin peroxidase H8 of *P. chrysosporium* (kindly supplied by R. Farrell, Repligen Sandoz Research Corp., Lexington, MA). In some cases, dot blots were also performed with pure proteins eluted from PAGE gels. This combination of experiments allowed comparisons of the immunological relatedness of each of the *Streptomyces* peroxidases, one to another, and to the fungal lignin peroxidase. As shown in Fig. 4, the antibody, as expected, bound very strongly to the ALip-P3 protein of *S. viridosporus*. It did not bind to the fungal lignin peroxidase. In controls, the antibody did not bind to unrelated proteins, such as horseradish peroxidase (Sigma). However, the antibody was very sensitive for detection of ALip-P3 protein, with a positive reaction visible with 75 ng of P3 using a 1:3000 dilution of antibody. In contrast, when tested against the fungal H8 protein, no binding was detected with 100 ng of H8 at a 1:800 dilution of antibody. These results indicate that the fungal and bacterial lignin peroxidases are not closely related structurally. The antibody did bind to the P1 and P2 peroxidases of *S. viridosporus*, as well as to two peroxidases of *S. badius* to differing degrees (Fig 5). Binding to the latter, however, was quite weak in comparison to the affinity the antibody showed towards the *S. viridosporus* proteins. These results indicate that the lignin peroxidases of the two actinomycetes share some antigenic and, therefore, structural similarities.

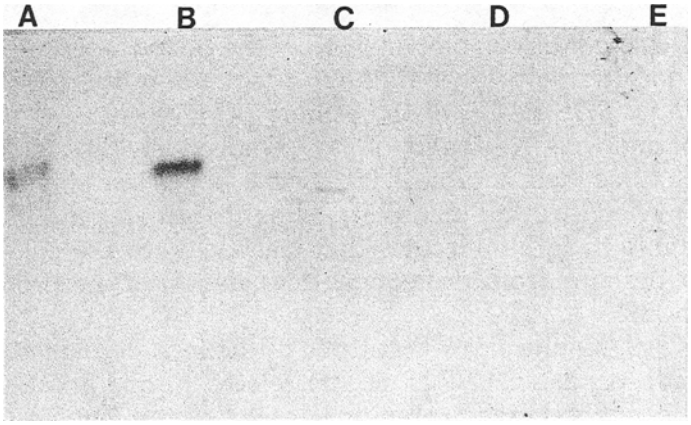


Fig. 4. Western blot showing binding of polyclonal antibody to electrophoretically purified T7A isoforms, but not lignin peroxidase H8 or bovine serum albumin (BSA). Lane A—isoform P1, 200 ng; Lane B—isoform P2, 200 ng; Lane C—isoform P3, 100 ng; Lane D—*P. chrysosporium* H8 peroxidase, 100 ng; Lane E—BSA, 1 μg.

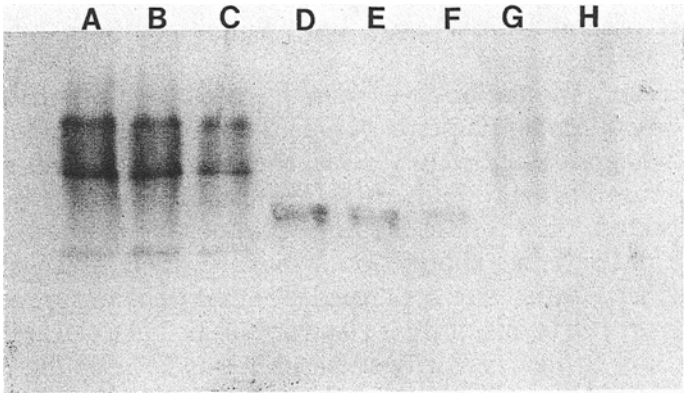


Fig. 5. Western blot showing binding of polyclonal antibody to T7A and 252 partially purified peroxidases and affinity-purified T7A peroxidase preparations. Lanes A-C—T7A crude enzyme, 1.0, 0.5, and 0.25 μg. Lanes D-F—252 crude enzyme, 1.0, 0.5, and 0.25 μg. Lanes G and H—affinity-purified T7A peroxidase; 0.2 and 0.1 μg.

## Antibody Affinity Column Purification of *S. viridosporus* Peroxidases

Native PAGE analysis showed that all four of the *S. viridosporus* peroxidases were bound by the Sepharose-linked antibody. The ALip-P3 protein bound strongly to the affinity matrix, whereas lesser amounts of the other isoforms also bound. ALip-P3 peroxidase could be readily separated from the other peroxidases by preferentially deadsorbing it from the

antibody affinity matrix with a 3*M* NaI solution. These results correlate with those of the Western blot analysis of the peroxidase isoforms, which indicated that the polyclonal antibody preparation had specificity for all four isoforms. SDS-PAGE of the affinity-purified material showed two subunits of mol wt 37.1 kD and 67.1 kD, respectively (Fig. 3). The affinity-purified enzyme had a typical UV-visible spectrum similar to that of ALip-P3 purified by other means (*4*). Minor bands appearing on gels may be attributable to IgG molecules that leached from the colulmn during elution, or perhaps from proteolytic degradation of the proteins during sample loading.

The overall results show that three of the four peroxidases produced by *S. viridosporus* are probably closely related to one another, since the anti-ALip-P3 antibody readily bound to each of them. Their different binding affinities, however, show that there are distinctive differences between the proteins. They may be isoforms of the same enzyme, but express different substrate specificities, oxidation/reduction potentials, carbohydrate contents, and/or mol wt. If so, each may serve a related, but different function in lignin degradation by *S. viridosporus*. The quantitatively dominant P3 enzyme is the only one of the four thus far confirmed to oxidize lignin; therefore, additional research is needed to confirm a lignin degradation role for the others.

As expected, the antibody prepared against lignin peroxidase ALip-P3 did not react with lignin peroxidase H8 from *P. chrysosporium*. Although both enzymes are catalytically similar, they are clearly distinct from one another immunologically and, therefore, probably structurally as well. Also as expected, the immunological relatedness of the peroxidases of *S. viridosporus* and *S. badius*, though low, was confirmed. This was predicted, since *S.* badius produces four peroxidases that behave similarly to those of *S. viridosporus* in terms of their electrophoretic behavior, relative abundance, and substrate specificities. In addition, both of the *Streptomyces* solubilize lignin in a similar way. Additional research must be done in order to compare the lignin peroxidases of the two *Streptomyces* at the level of protein structure. The ability to purify easily relatively large amounts of individual lignin peroxidases using antibody affinity techniques will allow us to begin comparing properties, such as *N*-terminal amino acid sequences, carbohydrate contents, subunit structures, and so on, of these ligninases almost immediately. These comparisons will in turn provide information that will help us assess the relative functions of the different peroxidases in the degradation and solubilization of lignin by *Streptomyces*.

## ACKNOWLEDGMENTS

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## REFERENCES


1. Kirk, T. K. and Farrell, R. L. (1987), *Ann. Rev. Microbiol.* **41,** 465–505.
2. Shoemaker, H. E. and Leisola, M. S. A. (1990), *J. Biotechnol.* **13,** 101–109.
3. Zimmermann, W. (1990), *J. Biotechnol.* **13,** 119–130.
4. Ramachandra, M., Crawford, D. L., and Hertel, G. (1988), *Appl. Environ. Microbiol.* **54,** 3057–3063.
5. Wang, Z., Bleakley, B. H., Crawford, D. L., Hertel, G., and Rafii, F. (1990), *J. Biotechnol.* **13,** 131–144.
6. Adhi, T. P., Korus, R. A., and Crawford, D. L. (1989), *Appl. Environ. Microbiol.* **55,** 1165–1168.
7. McCarthy, A. J. (1987), *FEMS Microbiol. Rev.* **46,** 145–163.
8. McCarthy, A. J. and Broda, P. (1988), *J. Gen. Microbiol.* **130,** 2905–2913.
9. McCarthy, A. J., Paterson, A., and Broda, P. (1986), *Apppl. Microbiol. Biotechnol.* **24,** 347–352.
10. Crawford, D. L., Pometto, A. L. III, and Crawford, R. L. (1983), *Appl. Environ. Microbiol.* **45,** 898–904.
11. Phelan, M. B., Crawford, D. L., and Pometto, A. L. III. (1979), *Can. J. Microbiol.* **25,** 127–1276.
12. Ramachandra, M., Crawford, D. L., and Pometto, A. L. III. (1987), *Appl. Environ. Microbiol.* **53,** 2754–2760.
13. Pridham, T. G. and Gottlieb, D. (1948), *J. Bacteriol.* **56,** 107–114.
14. Hames, B. D. and Rickwood, D. (1981), *Gel Electrophoresis of Proteins: A Practical Approach.* IRL Press, Oxford.