Purification and characterization of an extracellular phenol oxidase from culture filtrates of *Pyricularia oryzae*¹

Aisha Alsubaey, Hugh D. Sisler, and Benjamin F. Matthews

Abstract: Extracellular phenol oxidase activity was characterized and compared in *Pyricularia oryzae* wild-type and albino cell types to determine if this phenol oxidase was responsible for lack of melanization in the albino culture. Filtrates of the albino mutant Alb-5 showed activity similar to those of the wild type, while those of a buff mutant (Cp62) showed weak phenol oxidase activity. This indicated that the lack of melanization in the albino mutant was not due to an absence of phenol oxidase activity. The phenol oxidase isoform patterns from the wild type and two mutants were similar when analyzed by polyacrylamide gel electrophoresis. The slowest migrating isoform of phenol oxidase from wild-type *Pyricularia oryzae* was the major form and had a molecular mass of 380 kDa. The molecular masses of two of the minor forms were 220 and 130 kDa. The isoforms oxidized 1,8-dihydroxynaphthalene, the terminal metabolite in the polyketide pathway to melanin. The major phenol oxidase isoform was also present in extracts from albino mutants and the buff mutant. The major form was enriched by a combination of ammonium sulfate precipitation, DEAE-Sepharose column chromatography, and elution from preparative polyacrylamide gels. The enriched isoform of phenol oxidase separated into two forms after a second electrophoresis, indicating that these two isoforms interconvert. Analysis of both forms by sodium dodecyl sulfate – polyacrylamide gel electrophoresis indicated that both were composed of a single subunit with a molecular mass of 70 kDa. The enriched isoform preferred phloroglucinol as a substrate and had a Michaelis constant (Kₘ) of 19.3 mM for phloroglucinol and a pH optimum between 6 and 7.5.

Key words: phenol oxidase, laccase, *Pyricularia oryzae*, rice blast, melanin.

**Résumé** : L’activité de la phénol oxydase extracellulaire a été caractérisée, puis comparée, chez le *Pyricularia oryzae* indigène et des types de cellules albinos, pour déterminer si cette enzyme était responsable de l’absence de la mélanisation dans les cultures d’albinos. Des filtrats du mutant albinos Alb-5 ont présenté une activité similaire à ceux du type indigène, alors que ceux d’un mutant chamois, le Cp62, ont montré une faible activité phénol oxydase. Ceci indique que l’absence de mélanisation dans les cellules albinos n’est pas reliée à l’absence d’une activité phénol oxydase. Les profils isoformes de phénol oxydase du *Pyricularia oryzae* indigène dont la migration a été la plus lente s’est révélée être la forme dominante, avec une masse moléculaire de 380 kDa. Les masses moléculaires de deux des formes mineures ont été de 220 et 130 kDa. Les isoformes ont oxydé le 1,8-dihydroxynaphtalène, le métabolite terminal dans le sentier polykétide vers la mélanine. L’isoforme dominante de phénol oxydase a également été présente dans les extraits de mutants albinos et du mutant chamois. La forme dominante a été enrichie par une combinaison de précipitation au sulfate d’ammonium, chromatographie sur colonne de Sepharose-DEAE et élimination des gels de polyacrylamide préparatoires. Après une seconde électrophorèse, l’isoforme de phénol oxydase enrichie a été séparée en deux formes, ce qui indique que ces deux formes permettent. L’analyse de ces formes par électrophorèse sur gel de polyacrylamide avec dodécyl sulfate de sodium a indiqué qu’elles étaient composées d’une seule sous-unité de masse moléculaire de 70 kDa. L’isoforme enrichie a préféré le phloroglucinol comme substrat, et avait une constante de Michaelis (Kₘ) de 19,3 mM pour le phloroglucinol et un pH optimum entre 6 et 7,5.


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Introduction

Pyricularia oryzae Cav. is an important pathogen in all major rice growing areas and causes rice blast disease. Although the most obvious symptom is leaf spot, the fungus attacks all aboveground parts of the plant. Under favorable conditions, the fungal spores germinate on the plant surface and develop appressorial structures from which infection pegs penetrate host cells within 8 h (Ou 1980). Several compounds are used to control rice blast. The most specific are nonfungitoxic antipenetrant compounds that interfere with melanin biosynthesis and are referred to as melanin biosynthesis inhibitors. Tricyclazole (5-methyl-1,2,4-triazolo[3,4-b]benzothiazole) is one such compound used in practical application to control the rice blast disease (Sisler 1986). When pathogenic strains of Pyricularia oryzae are exposed to these compounds, the ability of the appressorial structure to penetrate the epidermal layer or other barriers is inhibited (Ishida et al. 1969; Araki and Miyagi 1977; Chida et al. 1982; Woloshuk and Sisler 1982; Okuno et al. 1983; Inoue et al. 1984). Similar antipenetrant activity of tricyclazole has been reported with Colletotrichum lagenarium (Kubo et al. 1982) and Colletotrichum lindemuthianum (Wolkow et al. 1983). The antipenetrant activity of tricyclazole is correlated with the ability of the compound to block the polyketide pathway leading to fungal melanin biosynthesis in the appressorial wall of P. oryzae (Woloshuk and Sisler 1982; Woloshuk et al. 1980). Ultrastructure studies have revealed an electron-dense layer in walls of appressoria that are thickened at the base to form a reinforced region surrounding the periphery of the thin underwall in contact with the plant cuticle. This electron-dense layer is absent in walls of tricyclazole-treated appressoria (Woloshuk et al. 1983; Howard and Ferrari 1989). The importance of appressorial melanization for penetration is emphasized by the behavior of mutants genetically deficient in melanin biosynthesis. Buff mutants of Pyricularia oryzae that are nonpathogenic phenotypically resemble the tricyclazole-treated wild type and form unmelanized appressoria that lack the ability to penetrate epidermal barriers. The hyaline appressoria of albino mutants rarely penetrate epidermal barriers, but melanization and penetration capacity can be restored by adding either of the melanin precursors scytalone and 1,2-dihydroxynaphthalene (DHN) (Chida and Sisler 1987). In fact, pathogenicity of the albino strain Alb-5 can be fully restored observed in culture filtrates of an albino strain. Laccase activity in culture filtrates of a buff pigmented (nonvirulent) strain was similar to that in filtrates of the wild type, whereas the meta-diphenol oxidase activity was much weaker than in wild-type filtrates. The observation of Neufeld et al. suggests that meta-diphenol oxidase activity may be associated with normal pigmentation and pathogenicity of Pyricularia oryzae.

Extracellular laccase formation is thought to be part of the infective process for several fungi, including Pyricularia oryzae (Neufeld et al. 1958) and Botrytis cinera (Bar-Nun and Mayer 1989 1990).

Laccase gene or cDNA sequences have been determined from several fungi. The first report was for laccase from Neurospora crassa (Germann and Leric 1986; Germann et al. 1988). Subsequently, other sequences have been reported for Aspergillus nidulans (Aramayo and Timberlake 1990), Coriolus hirsutus (Kojima et al. 1990), Phlebia radiata (Saluemo et al. 1991), and Agaricus bisporus (Perry et al. 1993). A laccase-type of phenol oxidase enzyme (EC 1.10.3.2) has been detected in culture filtrates of Pyricularia oryzae (Reinhammar 1984). This phenol oxidase probably oxidizes excreted DHN to form black pigment present in fungal culture medium. One of these phenol oxidases may be the same enzyme that is involved in the melanization of appressorial walls.

This paper describes the extracellular phenol oxidases in culture filtrates of mutant and wild-type Pyricularia oryzae and the first detailed purification and characterization of the phenol oxidase present in culture filtrates of Pyricularia oryzae.

Materials and methods

Fungal cultures

Four strains of P. oryzae were used in this study: wild type (042), a pathogen of rice; a buff (brown) nonpathogenic mutant (C62); and the nonpathogenic albino mutants Alb-5 and Alb6, both of which were derived from wild type 042. These strains were kindly provided by Dr. Barbara Valent of the E.I. du Pont Co., Wilmington, Del.

All strains were maintained on rice-polishing agar medium (Tokousalides and Sisler 1978). Iwasaki’s medium (Chrysayi 1976), which contains yeast extract, was used as the standard liquid medium. Iwasaki’s medium, which contains yeast extract, was used as the liquid medium for most experiments (Roa and Suryanarayanan 1974). The glucose mineral salts medium of Courses and Sisler (1960), modified by doubling the concentration of the phosphate salts, was used for comparison with Iwasaki’s medium for phenol oxidase production in one set of experiments.

Liquid cultures were shaken at 220 rpm and 28°C. The hyphae were isolated on Whatman filter paper (No. 1) using a Buchner funnel with vacuum. Dry weights were determined after drying the hyphae at 80°C.

Phenol oxidase purification

The major phenol oxidase isoform was precipitated from filtrates of wild-type cultures with ammonium sulfate. Filtrate from 7-day-old wild-type cultures was brought to 70% saturation with solid ammonium sulfate at 4°C and allowed to stand overnight. The precipitate was collected by centrifugation at 9000 × g for 20 min at 4°C, resuspended in 10–20 mL of 10 mM Tris–HCl buffer (pH 8), and dialyzed 12 h against 4 L of the same buffer at 4°C with two buffer changes. The dialyzed preparation was centrifuged for 30 min at 26000 × g. Much of the black pigment was removed in this step. The clarified supernatants were pooled and concentrated in closed dialysis tubing.
surrounded by dry polyethylene glycol (average size 8 kDa) for several hours. The concentrated solution was stored at -20°C.

A 9-mL sample containing about 5.5 mg protein · mL⁻¹ was loaded onto a DEAE-Sepharose anion-exchange column (Pharmacia, Uppsala, Sweden) (1.5 × 40 cm) pre-equilibrated with 200 mL of 10 mM Tris–HCl buffer (pH 8). The column was washed with 200 mL of equilibration buffer. A linear gradient (400 mL) of NaCl (0–0.5 M), generated by a gradient mixer GM-1 (Pharmacia Products), was applied at a flow rate of 18 mL · h⁻¹ at room temperature. The appearance of laccase activity was monitored with a fast conductivity meter (YSI Scientific, Yellow Springs, Ohio), calibrated with standard solutions of 10 mM Tris buffer containing 0, 0.25, and 0.5 M NaCl. The protein content of each fraction was monitored at 280 nm.

Fractions containing laccase activity were pooled, concentrated and applied to a 9% polyacrylamide slab gel with a 5% stacking gel in a Protran I1 electrophoresis unit (Bio-Rad, Richmond, Calif.). Samples contained 20% glycerol and 10% bromophenol blue tracking dye. The protein was subjected to electrophoresis at 4°C in 10 mM Tris-glycine buffer (pH 8.9) until the dye migrated to 1 cm from the bottom of the gel (Matthews et al. 1989). Lanes at the gel edges were excised and the gel was transferred to nitrocellulose paper using a Bio-Rad Trans blot apparatus as per manufacturer’s instructions, at 60 V and 4°C for 2 h. The nitrocellulose was removed, air dried, and then soaked in a solution containing syringaldazine (4.4 μM) in 10 mM Tris buffer (pH 8). The locations of two major bands stained for phenol oxidase activity were marked and the nitrocellulose paper was aligned with the original preparative gel. Each area of phenol oxidase activity was excised separately, placed in individual dialysis tubing filled with Tris–borate buffer (pH 8.3) and placed in an ISCO model 750 electrophoretic concentrator (Lincoln, Nebr.) filled with the same buffer. The protein was electroeluted from the gel at 3 mA overnight. The buffer solution containing syringaldazine (4.4 μM) in 10 mM Tris buffer (pH 8). The extracellular phenol oxidase activities in culture filtrates of Pyricularia oryzae wild type 042, albino mutant Alb-5, and buff mutant Cp62 were assayed with 14 phenolic compounds (Table 1). Phenol oxidase from wild-type Pyricularia oryzae was used as the standard unit of phenol oxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of syringaldazine to its quinone form per minute at 25°C in 10 mM Tris–HCl buffer (pH 8), using a molar absorption coefficient of 65 000 for the product (Harkin and Obst 1973). Comparable results were obtained with either assay.

Protein concentration was measured by the Pierce BCA protein assay (Pierce, Rockford, Ill.).

Analysis of phenol oxidase activity in crude samples

Enzyme extracts of wild-type 042 culture filtrates were analyzed by PAGE to determine the number of different isoforms of phenol oxidase present (Fig. 1A, lane 1). The gels were stained using silver nitrate (Heukeshoven and Denrick 1985). The native molecular mass was determined by comparison of the enzyme to high molecular mass standards (Pharmacia) (Andrews 1981).

A Phast gel electrophoresis system was used to determine the enzyme’s subunit composition and estimate molecular mass as described above, except that sodium dodecyl sulfate (SDS) buffer strips were used. Before application to the gel, the enzyme samples were placed in 25% SDS containing 5% v/v β-mercaptoethanol. The ratio of the samples to the SDS solution was 1:1. The mixture was boiled for 10 min and cooled on ice. SDS high molecular mass protein standards (Life Technologies Inc.) were used for molecular mass determination (Wyckoff 1977).

Phenol oxidase and protein assay

Phenol oxidase was assayed in duplicate for at least 3 min, measuring oxygen uptake using a YSI model 5300 biological oxygen monitor connected to an oxygen electrode (Clark type) at a temperature of 30°C. The assay contained 10 mM phloroglucinol in 0.1 M citrate-phosphate buffer (pH 7) unless otherwise noted. The assay mixture in the chamber was stirred steadily by a magnetic stirrer. The oxygen-dependent conductivity of the 0.1 M citrate-phosphate buffer was similar to that of water containing 12 mM KCl at 30°C. Thus we assumed an oxygen concentration in the buffer of 0.235 μmol · mL⁻¹. The linear drop in oxygen concentration was monitored for at least 3 min after the culture filtrate or enzyme extract was added. Corrections were made for auto-oxidation by assaying boiled enzyme or reaction mixtures lacking enzyme.

Phenol oxidase was also assayed using the syringaldazine assay, containing 44 μM syringaldazine in 10 mM Tris (pH 8). The change in absorbance at 520 nm was followed for 5 min at 25°C (Petroski et al. 1980). A standard unit of phenol oxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of syringaldazine to its quinone form per minute at 25°C in 10 mM Tris–HCl buffer (pH 8), using a molar absorption coefficient of 65 000 for the product (Harkin and Obst 1973). Comparable results were obtained with either assay.

Results

Phenol oxidase activity in culture filtrates

The extracellular phenol oxidase activities in culture filtrates of Pyricularia oryzae wild type 042, albino mutant Alb-5, and buff mutant Cp62 were assayed with 14 phenolic compounds (Table 1). Phenol oxidase from wild-type Pyricularia oryzae was used as the standard unit of phenol oxidase activity. Comparable results were obtained with either assay. The ability of these isoforms to oxidize a variety of para-, meta-, and ortho-diphenols and monophenols, as well as syringaldazine, a well-known activity stain specific for laccases (Harkin and Obst 1973; Leoniwicz and Grzywnowicz 1981), indicate that these are forms of extracellular laccases.
Purification were unsuccessful, as were attempts to desorb the noside or α-D-methyl glucoside (0-0.5 M) at pH 8 for further enzyme by lowering the pH to 3. The single band of activity representing the major isoform was excised from the gel after lamide gel electrophoresis. The single band of activity representing the major isoform was excised from the gel after agarose gel electrophoresis to determine the subunit composition of the isoforms.

The major isoform of phenol oxidase (Fig. 1A) and represents approximately 34% of the total phenol oxidase activity present in the crude extract. Numerous other proteins were also present in the extract (Fig. 1B, lane 1). The extract was further enriched for phenol oxidase activity by eluting the enzyme from a DEAE-Sepharose anion-exchange column (Fig. 2). A sharp peak of activity corresponding to the major form of phenol oxidase peak again separated into two bands on a gradient electrophoretic gel similar to those shown in Fig. 1A, lane 4. A second attempt by chromatofocusing using a pH gradient from 8.5 to 7.5 or a salt (NaCl) gradient from 0 to 0.5 M (data not shown). However, the two bands of phenol oxidase chromatographed as one sharp peak when monitored either by UV absorption or assay of laccase activity using syringaldazine as substrate. The fraction representing the single phenol oxidase peak again separated into two bands on a gradient electrophoretic gel similar to those shown in Fig. 1A, lane 4. A second attempt by chromatofocusing using a pH gradient from 7 to 4 resulted in a single, major coincident UV absorbance and activity peak (Fig. 3) similar to that obtained for the major phenol oxidase isozyme enriched by DEAE-Sepharose column chromatography (Fig. 2). However, the sharp peak of activity corresponding to the major form of phenol oxidase again separated into two distinct bands when subjected to electrophoresis.

A sequence of gel segments was excised parallel to and encompassing the band of activity representing the major phenol oxidase isozyme depicted between the arrowheads, Fig. 1B, lane 2) and electroeluted from the gel. The major isoform of laccase was enriched 194-fold (Table 2).

The enriched phenol oxidase isozyme was analyzed a second time by PAGE to judge its purity. Staining of the gel for phenol oxidase activity revealed that two isozymes were present (Fig. 1A, lanes 3 and 4), with no apparent, contaminating proteins (Fig. 1B, lanes 3 and 4). Attempts were made to separate these isozymes using an high pressure liquid chromatographic (HPLC) DEAE-cellulose analytical column with a pH gradient from 8.5 to 7.5 or a salt (NaCl) gradient from 0 to 0.5 M (data not shown). However, the two bands of phenol oxidase chromatographed as one sharp peak when monitored either by UV absorption or assay of laccase activity using syringaldazine as substrate. The fraction representing the single phenol oxidase peak again separated into two bands on a gradient electrophoretic gel similar to those shown in Fig. 1A, lane 4. A second attempt by chromatofocusing using a pH gradient from 7 to 4 resulted in a single, major coincident UV absorbance and activity peak (Fig. 3) similar to that obtained for the major phenol oxidase isozyme enriched by DEAE-Sepharose column chromatography (Fig. 2). However, the sharp peak of activity corresponding to the major form of phenol oxidase again separated into two distinct bands when subjected to electrophoresis.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild type</th>
<th>Albino</th>
<th>Buff</th>
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<tr>
<td>Phloroglucinol</td>
<td>4.06</td>
<td>3.43</td>
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<tr>
<td>Ferulic acid</td>
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<td>0.18</td>
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<tr>
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<td>0.07</td>
</tr>
<tr>
<td>DOPA</td>
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<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>α-Naphthol</td>
<td>0.33</td>
<td>0.18</td>
<td>0.01</td>
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<tr>
<td>Scytalone</td>
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<td>0.26</td>
<td>0.03</td>
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</table>

The data are the means of duplicate samples from two separate experiments.

All substrates were tested at a final concentration of 10 mM, except DHN and α-naphthol which were tested at 5 and 2.5 mM, respectively.

Molecular mass determinations

The molecular mass estimation of several isoforms of phenol oxidase was made using a native 10–15% gradient polyacrylamide gel and Pharmacia standards for high molecular mass proteins. The major form (Fig. 1, lane 4) had an average molecular mass of 480 kDa, the second form was 360 kDa, the third was 180 kDa, and the fastest migrating isoform was 70 kDa.

Purification of the major form of phenol oxidase

The major phenol oxidase isoform was partially purified by ammonium sulfate precipitation (Fig. 1A). The total activity increased, suggesting that an inhibitor of phenol oxidase may have been present in the crude extract. Numerous other proteins were also present in the extract (Fig. 1B, lane 1). The extract was further enriched for phenol oxidase activity by eluting the enzyme from a DEAE-Sepharose anion-exchange column (Fig. 2). A sharp peak of activity was eluted by 0.04–0.07 M NaCl. This peak of activity contained the major phenol oxidase enzyme from a DEAE-Sepharose anion-exchange column (Fig. 2). A sharp peak of phenol oxidase activity was eluted by increasing the pH to 3. The data are the means of duplicate samples from two separate experiments.

The major phenol oxidase isoform was purified further by preparative polyacrylamide gel electrophoresis. The single band of activity representing the major isoform was excised from the gel after staining for activity (the area is indicated with arrowheads, Fig. 1B, lane 2) and electroeluted from the gel. The major isoform of laccase was enriched 194-fold (Table 2).

The enriched phenol oxidase isoform was analyzed a second time by PAGE to judge its purity. Staining of the gel for phenol oxidase activity revealed that two isozymes were present (Fig. 1A, lanes 3 and 4), with no apparent, contaminating proteins (Fig. 1B, lanes 3 and 4). Attempts were made to separate these isozymes using an high pressure liquid chromatographic (HPLC) DEAE-cellulose analytical column with a pH gradient from 8.5 to 7.5 or a salt (NaCl) gradient from 0 to 0.5 M (data not shown). However, the two bands of phenol oxidase chromatographed as one sharp peak when monitored either by UV absorption or assay of laccase activity using syringaldazine as substrate. The fraction representing the single phenol oxidase peak again separated into two bands on a gradient electrophoretic gel similar to those shown in Fig. 1A, lane 4. A second attempt by chromatofocusing using a pH gradient from 7 to 4 resulted in a single, major coincident UV absorbance and activity peak (Fig. 3) similar to that obtained for the major phenol oxidase isozyme enriched by DEAE-Sepharose column chromatography (Fig. 2). However, the sharp peak of activity corresponding to the major form of phenol oxidase again separated into two distinct bands when subjected to electrophoresis.

A sequence of gel segments was excised parallel to and encompassing the band of activity representing the major phenol oxidase isozyme depicted between the arrowheads, Fig. 1B, lane 2. The gel segments were numbered 1–5 from top to bottom and the protein was eluted and analyzed by PAGE (Figs. 4A and 4B). Again two bands of phenol oxidase activity were present (Fig. 4B), coinciding with two major bands of protein (Fig. 4A). Fractions containing one isozyme, the other, or both were analyzed by PAGE and SDS (Fig. 4C) gel electrophoresis to determine the subunit composition of the isoforms.

Table 1. Phenol oxidase activity in culture filtrates of 7-day-old cultures of *Pyricularia oryzae,*a

<table>
<thead>
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<th>Substrateb</th>
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<th>Albino</th>
<th>Buff</th>
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<td>Phenol oxidase activity</td>
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<td>Substrate</td>
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<td>µM O₂·mg dry weight⁻¹·h⁻¹</td>
<td>µM O₂·mL⁻¹·h⁻¹</td>
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<tr>
<td>Phloroglucinol</td>
<td>4.06</td>
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Fig. 1. PAGE separation of *Pyricularia oryzae* phenol oxidase isoforms. The gel in panel A was stained with silver nitrate, whereas the gel in panel B was stained for laccase activity with the substrate DHN. Lane M contain protein standards: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; bovine serum albumin, 67 kDa. Lane 1 shows separation of proteins in crude ammonium sulfate precipitate. Lane 2 shows protein bands after purification of ammonium sulfate precipitate by DEAE-Sepharose anion-exchange chromatography. Arrowheads on lane 2 in B shows gel area which was isolated by slicing with a razor. Lane 3 is a sample of protein eluted from a gel slice from lane 2.

Although several fractions possessed two bands of phenol oxidase activity as seen by PAGE, in each case a single band or subunit was present on the SDS 10–15% gradient gel. Thus, only one subunit of 70 kDa was present in the fractions analyzed whether the protein came from the upper band (Fig. 4C, lanes 1, 2, 3, and 4) or lower band (Fig. 4C, lane 5) of phenol oxidase activity from the original native gel. This indicates that the different higher molecular mass isoforms of phenol oxidase are multimers consisting of varying numbers of subunits.

When the two bands were excised separately from the gel, electroeluted, and examined by native, 10–15% gradient PAGE, both preparations exhibited the same two enzyme forms, suggesting that these two enzyme forms readily interconvert.

Activity of enriched phenol oxidase with various substrates

The enriched enzyme fraction showed about three times the activity or more with the substrate phloroglucinol (a metadiphenol) than with alternative substrates such as DOPA, α-naphthol, hydroquinone, or DHN (Table 3). These values are somewhat lower than those observed in crude culture filtrates.

Enzyme kinetics

The substrate phloroglucinol was used to study the kinetic behavior of the major phenol oxidase isoform of *Pyricularia oryzae*. From the reciprocal plot of various phloroglucinol concentrations (0.5–20 mM) versus velocity (data not shown), a \( K_m \) value of 19.30 mM was calculated. Phenol oxidase in this reaction utilized atmospheric oxygen to oxidize phloroglucinol to a quinone. The oxygen concentration was not limiting in the reaction mixture, as indicated by the failure of added oxygen to increase the rate of the reaction. The presence of the inhibitor EDTA (30 mM) changed the \( K_m \) and \( V_{max} \) values, indicating that EDTA acts as an uncompetitive inhibitor.

The pH profile of the activity of the major isoform of phenol oxidase with phloroglucinol as a substrate had an optimum range between pH 6 and 7.5 (data not shown).

Discussion

Culture filtrates of the albino mutant Alb-5 possessed phenol oxidase activity similar to those of the wild type Cp62. Thus, the lack of melanization in Alb-5 is not due to an absence of phenol oxidase activity. Culture filtrates of *Pyricularia oryzae* wild type showed phenol oxidase activity for a wide range of phenolic substrates. The filtrates oxidized the monophenol p-cresol; paradiphenols, e.g., hydroquinone and Dopa as well as the p-diamino compound PPDA; orthodiphenols, e.g., catechol; and metadiphenols, e.g., orcinol and phloroglucinol.

When grown on Iwasaki’s medium, which contains yeast extract, culture filtrates of the albino mutant Alb-5 showed approximately the same level of activity towards these substrates as those of the wild type, but those of the buff mutant Cp62 demonstrated weak phenol oxidase activity with most of the substrates tested. Low phenol oxidase activity was also observed for a buff mutant by Neufeld et al. (1958). The reason for low phenol oxidase activity of buff mutants is not known.

It is surprising that the tricyclazole-treated wild type demonstrated good phenol oxidase activity, because the buff mutants closely resemble the tricyclazole-treated wild type in respect to pigmentation, polyketide metabolism, and lack of pathogenicity (Woloshuk et al. 1980).

Neufeld et al. (1958) concluded that the metadiphenol oxidase in *Pyricularia oryzae* culture filtrates responsible for oxidizing phloroglucinol was distinct from the laccase enzyme responsible for oxidizing hydroquinone. The present study indicates, however, that this may not be the case because the major phenol oxidase isolated in this study oxidized both phloroglucinol and hydroquinone and exhibited preferred activity for phloroglucinol as compared with hydroquinone and other similar substrates. The metadiphenol oxidase described
Fig. 2. Elution profile of phenol oxidase activity from a DEAE-Sepharose column. Units of phenol oxidase activity (x), UV absorbance (■), and salt concentration (—) are indicated.

Fig. 3. Separation of 2 mL of the major phenol oxidase isofonn eluted from a DEAE-Sepharose column (Fig. 2) on a column of polybuffer PBE 94 gel (Pharmacia Fine Chemicals). Elution conditions: start buffer, 0.025 M imidazole—HCl (pH 7.4); elution buffer, polybuffer 74—HCl, pH 4 (Pharmacia Fine Chemicals). Flow rate, 20 mL/h. The peak of activity contained two distinct bands of phenol oxidase, which could be separated on a polyacrylamide gel. The enzyme eluted in the pH range between 6.57 and 5.99. ■, UV; —, pH; —, enzyme activity.

Table 2. Enrichment of laccase from Pyricularia Oryzae wild type 042 culture filtrate.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Laccase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume</td>
<td>mg · mL⁻¹</td>
</tr>
<tr>
<td>Crude cell-free extract</td>
<td>1000</td>
<td>0.78a</td>
</tr>
<tr>
<td>Ammonium sulfate, 70% saturation</td>
<td>9</td>
<td>5.53</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>1.69</td>
<td>0.36</td>
</tr>
<tr>
<td>Preparative gel</td>
<td>2.90</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*Pigments may have interfered in this protein determination, yielding an artificially high value.
Table 3. Activity of enriched laccase (major form) with different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>µmol O₂ consumed · h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloroglucinol</td>
<td>0.31</td>
</tr>
<tr>
<td>DOPA (10 mM)</td>
<td>0.09</td>
</tr>
<tr>
<td>a-Naphthol (2.5 mM)</td>
<td>0.07</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>0.06</td>
</tr>
<tr>
<td>DHN (5 mM)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

a Twenty microlitres of enriched laccase used contained an average of 12 µg protein.

b An average of duplicate measurements from three separate enzyme samples.

by Neufeld et al. (1958) is almost certainly the major phenol oxidase described in the present study. They reported a pH optimum of 7–7.5, which is consistent with a pH optimum of 6.0–7.5 for the major phenol oxidase examined in the present study. The optimal pH for phloroglucinol oxidation by *Podospora anserina* laccases II and III is between 7.5 and 8.5 (Hoffmann and Esser 1977). With *Polyporus versicolor*, the apparent pH optimum for oxidation of phloroglucinol was found to be much higher than for oxidation of several other phenols (Fahraeus and Ljunggren 1961). Moreover, Neufeld et al. (1958) reported no consistent success in separating “metadiphenol oxidase” from laccase by thermal inactivation, column chromatography with various adsorbents, and paper electrophoresis.

The phenol oxidase in this study has a preference for phloroglucinol as compared with hydroquinone and other substrates, indicating it is a metadiphenol oxidase. This appears to be the major phenol oxidase in culture, but there may be minor phenol oxidases present, which do not have this substrate preference. On the other hand, this major phenol oxidase may change its preference for oxidation of phloroglucinol relative to other phenols through enzyme conformation or subunit composition. It has been shown, for example, that the substrate specificity of laccase I of the fungus *Podospora anserina* is expanded to include several metadiphenols after freezing and thawing (Shanel and Esser 1971). This is believed to be due to structural changes in the polymeric configuration of laccase I rather than to dissociation into monomeric forms. Laccase II and III of *Podospora anserina* are low molecular mass, monomeric forms with physical properties that resemble the subunit of laccase I, but they typically oxidize metadiphenols not oxidized by unmodified laccase I (Shanel and Esser 1971).

In addition to the major phenol oxidase activity mentioned above, we detected two other minor zones of phenol oxidase activity after separation of proteins in ammonium sulfate precipitates by PAGE. All three regions demonstrated laccase-type activity, since they readily stained with DHN, DOPA, PPDA, hydroquinone, and syringaldazine. Apart from estimating the molecular mass, the two minor phenol oxidases were not studied further.

The major phenol oxidase with an apparent average molecular mass of 380 kDa behaves as though it interconverts between two forms. It is postulated that these are two forms of the same enzyme. The molecular mass of the SDS-denatured enzyme is 70 kDa. This suggests that the major phenol oxidase of *Pyricularia oryzae* contains five to six subunits. The difference in the molecular mass of the two native protein bands may be due to a difference in the number of aggregated subunits. Multiple forms due to different degrees of aggregation have been reported for phenol oxidases from various sources, especially for catechol oxidases, a similar group of enzymes which act on orthodiphenols (Mayer and Harel 1979; Harel and Mayer 1968; Jolley and Mason 1965; Smith and Krueger 1962).

Laccase I of the fungus *Podospora anserina*, with an average molecular mass of 390 kDa, is thought to be composed of four to five subunits. These subunits are equal in molecular mass and dimension to monomeric laccases II and III of the same fungus, which have respective molecular masses of 70 and 80 kDa (Molitoris et al. 1972). Wood (1980) reported that the laccase of *Agaricus bisporus* behaved as a single molecular species on chromatographic and centrifugation analysis, but showed multiple enzymatically active forms on gels after...
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apparent molecular mass differences of these forms may be due 
to minor charge differences on fractions of the population of 
...of the Km values of laccases with this 
substrate. Neufeld et al. (1958) reported a Km value of 1 mM 
using a culture filtrate of Pyricularia oryzae. Laccases II and 
III of Podospora anserina have Km values of 13 and 
0.15 mM, respectively (Hoffmann and Esser 1977). The 
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since EDTA works as a chelator of metals, including copper, 
which is an essential component for laccase enzyme oxidation– 
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