Purification and Characterization of Isoperoxidases Elicited by Aspergillus flavus in Cotton Ovule Cultures

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ABSTRACT

Two anionic isoperoxidases were isolated from media of Aspergillus flavus-inoculated cotton (Gossypium hirsutum L.) ovule cultures and purified about 150-fold to apparent homogeneity by treatment with Cell Debris Remover and ion exchange chromatography on Accell QMA medium. These isoperoxidases were present in noninoculated cotton ovule cultures at low levels. The major activity peak (B) represented 90% of the recovered peroxidase activity and was electrophoretically homogeneous. The minor activity peak (A) was about 95% pure. Isoelectric focusing analysis showed that B was greater than 95% pure with respect to other peroxidase isozymes, while the enzyme in A was about 90% isozymically pure. Each isoperoxidase displayed a molecular mass of 56 kilodaltons by intercalation from denaturing gel electrophoresis. The B isozyme displayed a molecular mass of 55 kilodaltons by gel filtration chromatography. The pH optima for the cotton ovule isoperoxidases were similar, 5.0 for isozyme A and 6.0 for isozyme B. The isoelectric points for isozymes A and B were 4.2 and 4.4, respectively. Eugenol, guaiacol, and 3,3',5,5'-tetramethylbenzidine were good electron donor substrates, whereas 4-aminoantipyrine was a poor substrate. The absorption spectrum of the material in B revealed a major peak at 400 nanometers and a minor peak at 280 nanometers. The molar extinction coefficient at 400 nanometers (pH 7.0) was calculated to be $1.07 \times 10^{4}$ per square centimeter per mole. Amino acid analysis of isozyme B confirmed the acidic nature of this protein and identified a number of similarities to the anionic peroxidases from tobacco and potato. This glycoprotein was found to contain 12 to 14% sugar (by weight), mainly in the form of galactose and mannose.

Changes in peroxidase levels and isoform distribution are known to accompany higher plant processes, although in few cases have the isoforms involved been purified and characterized. Peroxidase has been implicated in a number of higher plant processes such as host defense mechanisms (29), cross-linking of hydroxyproline-rich glycoprotein monomers in cell walls (8), cross-linking pectic polysaccharides with phenolic acids in cell walls (7), lignification (5), and suberization (6). Peroxidase has been purified and characterized from a number of higher plant systems, including horseradish (26), tobacco (14), potato (6), turnip (17), and barley (25).

Developing cotton seeds are a primary target for the toxigenic fungus Aspergillus flavus Link. A nontoxic, fluorescent material, formed by oxidation of the fungal metabolite kojic acid by plant peroxidase(s), is associated with the cotton lint during the infection process (16). In cotton, peroxidase is localized in carpel tissue which surrounds the developing seed as well as in developing ovules and fibers (22). Infection by A. flavus significantly increases levels of peroxidase found in developing boll tissues and increases the diversity of isozymes produced (22).

In vitro cotton ovule cultures offer a convenient system for the study of cotton seed development (2). During an investigation of cotton ovule-specific proteins, it was observed that peroxidase was secreted into the surrounding medium of cultured ovule tissue (19). When challenged with A. flavus, cotton ovule cultures released increased levels of peroxidase into the medium (20), principally in the form of an anionic isozyme which is present at low levels in noninoculated ovule cultures. This anionic isoperoxidase would seem to be associated with a host defense response.

Because COP1 may play an important role in the interaction between A. flavus and developing cotton seed, the function and regulation of this protein is of interest. To develop protein-specific, immunological probes, as well as gene-specific probes, purification and characterization of the A. flavus-induced isoperoxidase(s) were required. This paper describes the purification and characterization of the fungal-elicited isoperoxidases from cotton ovule cultures. A preliminary report has been presented (21).

MATERIALS AND METHODS

Biological Material

Cotton (Gossypium hirsutum L. “Stoneville 208 glandless”) plants were maintained in standard greenhouse conditions (minimum night temperature of 20°C; maximum day temperature of 35°C). Cotton ovule cultures were initiated from fertilized ovules (2 DAF) and maintained in darkness at 30°C on the medium of Stewart and Hsu (28). Each culture flask contained ovules from one boll (24–32 developing cotton seeds) and about 35 mL of liquid medium (30 d after culture initiation).

A nontoxigenic strain of the fungus Aspergillus flavus Link NRRC 2061 was maintained on potato dextrose agar. Ovule cultures, 30 d postinitiation, were inoculated with conidia of A. flavus by means of a small, sterile brush and incubated at 30°C for 6 d. Maximum secretion of peroxidase activity into

1 Abbreviations: COP, cotton ovule peroxidase; CDR, Cell Debris Remover; TMB, 3,3′,5,5′-tetramethylbenzidine; AAP, 4-aminoantipyrine; IEF, isoelectric focusing; HRP, horseradish peroxidase.
the surrounding medium occurred 6 d after inoculation with the fungus. Axenic cultures of *A. flavus* did not produce extracellular (no secretion) peroxidases, when grown on either fungal (potato dextrose) or cotton ovule culture media.

**Peroxidase Isolation**

Media obtained from fungal-inoculated ovule cultures were collected by filtration *in vacuo*. The harvested ovule culture medium (500 mL) was treated with CDR (Whatman®), a weak anion exchange medium, at the rate of 1 g of CDR per 20 mL of ovule medium. The medium-CDR mixture was stirred for 30 min (25°C), and the liquid phase was separated from the solid phase by filtration *in vacuo*. The CDR “cake” (25 g) was immediately transferred to 100 mL of 0.05 M acetate buffer with 0.5 M NaCl (pH 5.5), and stirred for an additional 30 min. The buffer-CDR mixture was again filtered *in vacuo*, and the liquid filtrate represented the crude elicited anionic peroxidase fraction. It was important to harvest cultures before the growth medium turned cloudy, as such conditions resulted in a considerable loss of peroxidase activity.

**Peroxidase Quantitation**

The standard peroxidase assay used in this work has been described elsewhere (22). A linear relationship existed between the product (tetraguaiacol) concentration and the enzyme concentration.

To carry out kinetic studies, standard reaction mixtures were used, except that one substrate (H$_2$O$_2$ or electron donor) was held constant at an optimum concentration, while the other substrate was tested in a full concentration range, 0.6 to 6.25 mM for guaiacol and 0.2 to 2.0 mM for H$_2$O$_2$. The kinetic data were subjected to an Eadie-Hofstee treatment to derive the apparent $K_v$ values. The inhibitor study employed standard reaction mixtures with varying inhibitor concentrations. The pH optima data were obtained with a series of buffers in a pH range of 2.5 to 7.5, constructed from different ratios of the buffer components, 0.1 M citrate and 0.2 M Na$_2$HPO$_4$.

The reaction mixture used to assay TMB (Sigma) oxidation consisted of 0.87 mM TMB and 1 mM H$_2$O$_2$ in 50 mM acetate buffer (pH 5.0). Following addition of COP, the $A_{655}$ was recorded ($e_{655} = 3.9 \times 10^4$ cm$^2$ mol$^{-1}$) (9). The eugenol assay was performed as previously described (13). Assay conditions for AAP were described in the Worthington manual (1977).

**Protein Determinations**

Protein concentrations of crude COP preparations (medium and CDR-treated) were estimated by the bichinchoninic acid technique (27) with BSA as a standard. Protein concentrations in column fractions were initially monitored by $A_{280}$ as the fractions were collected. Protein concentrations in column fractions were estimated by the method of Waddell (30) with BSA as a standard.

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**Column Chromatography**

The fungal-elicited anionic COP was purified by chromatography on Accell QMA anion exchange medium (Millipore/Waters). The QMA medium was equilibrated with 20 mM phosphate (Na) buffer (pH 7.0), and packed to form a 2.5 x 17.5-cm column (bed volume of 86 mL). The column was washed with 20 mM phosphate buffer (pH 7.0). The crude COP preparation (acetate/NaCl fraction) was extensively dialyzed against 20 mM phosphate buffer and applied to the column. After sample application, the column was eluted (25°C) with 100 mL of phosphate buffer (80 mL/h), followed by 400 mL of a 0 to 0.3 M NaCl linear gradient in phosphate buffer. Final elution of the column was with 100 mL of 0.5 M NaCl in 20 mM phosphate buffer. The column fractions (7-mL) were monitored for protein ($A_{280}$) and peroxidase activity by the procedures described above.

The activity in the major peak (B) was subjected to gel filtration chromatography to estimate the molecular mass of native COP. Bio-Gel P-100 (Bio-Rad) was equilibrated with 20 mM phosphate (Na) buffer (pH 7.0), and packed to form a 1.5 x 45-cm column (bed volume of 72 mL). The B activity of COP was extensively dialyzed against the phosphate buffer. After thoroughly washing the column with phosphate buffer, the COP sample was applied to the column and eluted with the same buffer. Column fractions (2-mL) were monitored for protein ($A_{280}$) and heme ($A_{400}$). The P-100 column was calibrated with the following molecular mass standards: BSA (67 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), and Cyt c (12.4 kD).

**Electrophoresis**

Analytical SDS-PAGE was performed generally according to the method of Laemmli (11). Gels (10%; 0.075 x 14 x 12 cm) were run at 10 mA (constant current) per gel at 15°C. Proteins were detected by silver staining (Bio-Rad kit). Protein molecular mass standards (low molecular mass) were obtained from Bio-Rad. Native IEF gels were performed according to a previously published procedure (22) and peroxidase activity was detected with 3-amino-9-ethylcarbazole (22).

**Sugar Analysis**

The sugar composition analysis of the major fungal-elicited COP was performed by Dr. F. Perini of the Department of Pharmacology, University of Michigan, Ann Arbor, using a previously published fluorometric procedure (24).

**Amino Acid Analysis**

The amino acid analysis of COP was performed by Dr. S. Rao in the Protein Analysis Center, Louisiana State University, Baton Rouge. Correction factors were applied for the destruction of the following amino acids: Ser, 10%; Thr, 6%; Tyr, 3%. The reported data (mol %) were averages of two runs.
RESULTS

Purification of Cotton Ovule Peroxidases

Initial treatment with CDR of media derived from fungal-stressed ovule cultures resulted in most of the peroxidase activity binding to the anion exchange medium. More than 95% of the peroxidase activity bound to the CDR in the low ionic strength condition (initial medium). Elution of the peroxidase-CDR matrix with 0.5 M NaCl effectively released the peroxidase activity, but most of the colored material (oxidized phenolics) was retained. Polyvinylpyrrolidone (insoluble) was an unsatisfactory adsorbent for elimination of unwanted pigmented metabolites in the fungal-stressed ovule medium.

Chromatography of the fungal-stressed anionic peroxidase fraction on the Accell QMA column resulted in two peaks of peroxidase activity (Fig. 1). The major activity peak eluted at about 80 mM NaCl in the salt gradient. Most of the protein was eluted in the later stages of the salt gradient (0.5 M NaCl). The QMA column yielded a 93% recovery of activity applied and a 65% recovery overall from the initial ovule medium (Table 1). The major peak of peroxidase was purified about 150-fold from the initial cotton ovule medium.

Analysis of Purified Peroxidases

Analysis of the major peroxidase peak (B, column fraction 40) by SDS-PAGE revealed a single band (Fig. 2) which corresponded to a molecular mass of 56 kD on a log molecular mass versus relative mobility plot (not shown). Thus, the protein in the major peak of activity was purified to apparent homogeneity. The material in the minor activity peak (A) was not homogeneous, as revealed by SDS-PAGE, but was still rather pure when compared to the sample applied to the column (Fig. 2). The protein material from the minor peak of activity also displayed an apparent molecular mass of 56 kD.

The isozyme purity of each activity peak obtained from the QMA column was ascertained by native IEF gels. While the material from peak A contained three discernible isozymes (Fig. 3), the most acidic one represented greater than 90% of the peroxidase activity. The smaller of the two minor isoperoxidases from peak A material was apparently due to a minor contamination by peak B material. The other minor peroxidase band in the A material corresponded to a major isoperoxidase observed in noninoculated ovule cultures (not shown). The major isozyme in the peak B material represented over 95% of the peroxidase activity in this fraction, there being only one additional minor isozyme (Fig. 3). Each peak obtained from the QMA column represented, essentially, a

| Table 1. Purification of COP from A. flavus-Inoculated Ovule Cultures |
|------------------|----------|---------|-------------|-----|
| Step             | Total Activity | Total Protein | Specific Activity | Purification |
|                  | units × 10^4 | mg       | units/mg    | Yield %   |
| Oval medium      | 2.68      | 250      | 1.072      | 1 100     |
| AcOH/NaCl        | 1.90      | 18.8     | 10.100     | 9.4 70.9  |
| Dialysate        | 1.87      | 12.2     | 15.300     | 14.3 69.8 |
| QMA, peak A      | 0.17      | 0.32     | 52.700     | 49.2 6.34 |
| QMA, peak B      | 1.57      | 0.97     | 162.000    | 151 58.6  |

Figure 1. Peroxidase elution profile on Accell QMA anion exchange medium. The column was eluted with 20 mM phosphate buffer (pH 7.0), a 0 to 0.3 M NaCl linear gradient in phosphate buffer and, last, 0.5 M NaCl in the same buffer. Fractions were assayed for protein (A280), peroxidase activity, and ionic strength (conductivity).
Given that coefficient extinction at 280 nm COP was 1.25 μg; lane 2: COP peak B, 0.9 μg. Molecular mass protein standards are indicated in the right margin. Proteins were detected by silver staining.

Figure 2. SDS-PAGE analysis of ovule peroxidase fractions. Lane 1: precolumn COP sample, 4 μg; lane 2: COP, peak A, 1.25 μg; lane 3: COP, peak B, 0.9 μg. Molecular mass protein standards are indicated in the right margin. Proteins were detected by silver staining.

separate isoperoxidase.

Gel filtration chromatography of COP on a P-100 column yielded a Vc/Vo value of 1.16. Interpolation with this value from a log molecular mass versus Vc/Vo calibration plot (data not shown) gave an apparent molecular mass of 55 kD for COP.

Characterization of Isoperoxidases

The absorption spectrum of the peak B material was typical for a peroxidase protein. The spectrum revealed a minor peak at 280 nm and a major peak centered at 400 nm (Fig. 4). Given that the Mε of the isoperoxidase was 56 kD, the molar extinction coefficient (ε400, pH 7.0) was calculated to be 1.07 × 10^4 cm² mol⁻¹.

The physicochemical characteristics of the fungal-induced isoperoxidases were similar. The pH optima were 5.0 and 6.0 for isozymes A and B, respectively. The curves were similar and fairly broad between pH 5.0 and 6.0. The isoelectric points, as determined by interpolation from IEF gels, were 4.2 for isozyme A and 4.4 for isozyme B.

The kinetic parameters of isozymes A and B were similar. Apparent Km values (Table II) indicated that isozyme B had a greater affinity for both H₂O₂ and the electron donor substrate than isozyme A. The synthetic substrate TMB was a good substrate with COP (Table III), but the initial product was unstable above pH 6.0. Thus, it was critical to control pH for this substrate. Most of the natural peroxidase substrates, including lignin precursors, display very limited aqueous solubility, making their use in kinetic studies difficult. Eugenol (13) was an exception to this trend and proved to be an excellent substrate for COP. Unfortunately, the product of the eugenol reaction is insoluble, eliminating the possibility of obtaining apparent Km values. Both KCN and NaN₃ were potent inhibitors of the guaiacol oxidation. Potassium cyanide inhibited oxidation 50% at 0.75 μM, whereas NaN₃ inhibited 50% at 9.0 μM.

Figure 3. Isoelectric focusing analysis of elicited COP isozymes. The peroxidase profiles were done on a pH 3.0 to 7.0 IEF gel (5% acrylamide) and peroxidase-positive bands were detected with a 3-amino-9-ethylcarbazole-based activity stain. Lane 1: isozyme A, 35 units (guaiacol oxidative activity); lane 2: isozyme B, 40 units.

Figure 4. Absorption spectrum of COP, isozyme B. The sample concentration was 5.5 × 10⁻⁶ M in 20 mM phosphate buffer (pH 7.0), at 25°C.
Table II. $K_m$ Values for Purified COP (Isozymes A and B)

Kinetic data were subjected to an Edie-Hofstee treatment with linear regression. One substrate was added at saturation levels, while the second was varied (0.2–2.0 mW for H$_2$O$_2$; 0.6–6 mW for guaiacol) to collect rate data.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ Isozyme A</th>
<th>$K_m$ Isozyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td>mm</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>1.84</td>
<td>1.03</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>2.47</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Sugar composition analysis of COP (isoyme B) revealed that Gal and Man were the major saccharides, with lesser amounts of Xyl, Fuc, Ara, Glu, and GlcN (Table IV). Neutral saccharides represented 96% of the total sugars associated with COP. This sugar analytical procedure could not distinguish between Fuc and rhamnose residues. Calculations from this sugar analysis revealed a total carbohydrate content of 12 to 14%, by weight, of COP.

Amino acid analysis of COP (isoyme B) revealed high levels of Asp, Glu, Gly, Ser, Thr, and Ala (Table V). In addition, Pro and the hydrophobic residues Val, Ile, Leu, and Phe were present in considerable abundance. The acidic nature of COP was confirmed by the high levels of acidic residues (Asp, Glu). Tryptophan analysis was not conducted.

**DISCUSSION**

Peroxidase is a major component of a relatively simple mixture of proteins secreted into the surrounding medium of cotton ovule cultures (23). Fungal-induced stress results in the elevated secretion of essentially one isoperoxidase, which is present at low levels in noninoculated cultures (20). By selecting the proper conditions for the cotton ovule system, an enrichment of the fungal stress-associated isoperoxidases was obtained.

Use of the preparative agent CDR simplified the purification of the elicited anionic COP. This material effectively and quickly separated the cationic protein fraction from the fraction of interest (anionic), which contained 95% of the peroxidase activity. In addition, CDR also effectively removed much of the colored material (oxidized phenolics) from the crude starting material (medium). Such oxidized plant secondary metabolites are often associated with irreversible losses in enzyme activities and anomalous protein profiles on PAGE.

Table III. Comparison of COP (Isozyme B) Specific Activities for Different Substrates

Units are defined as $\mu$M/min. Kinetic details are given in text.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity units/mg</th>
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<tbody>
<tr>
<td>Guaiacol</td>
<td>$1.61 \times 10^5$</td>
</tr>
<tr>
<td>TMB</td>
<td>$1.52 \times 10^5$</td>
</tr>
<tr>
<td>AAP</td>
<td>$1.56 \times 10^5$</td>
</tr>
</tbody>
</table>

The properties of the crude ovule peroxidase preparation both eased the purification process and complicated its analysis. The thermostability of COP (19) allowed column procedures to be conducted at ambient temperatures (25°C). However, accurate estimation of protein concentrations in crude samples proved to be difficult. The crude peroxidase contained significant concentrations of low molecular mass materials (perhaps oxidized phenolics and peptides) which yielded false positive reactions with the Lowry assay. COP did not seem to bind well to dye-containing reagents (Coomassie G-250), a problem common with glycoproteins. The Waddell assay (30) was specific and sensitive when utilized for the analysis of column fractions, although care was required in the selection of buffers, and crude fractions contained materials which caused interference. The bicinchoninic assay (27) was more reliable for use with crude preparations, although there was dialyzable material in these samples which yielded positive values in the assay (Table I).

The absorption spectrum for COP confirmed the presence of a maximum at 400 nm, as well as a small peak at 280 nm. A peak in the 400 nm region of the absorption spectrum is diagnostic for the presence of an iron-containing cofactor. Peroxidase typically contains a heme moiety (protohemin IX, 26). The absorption spectrum for COP provided additional evidence that this protein is a member of the peroxidase family.

The molecular mass of 56 kD for COP is higher than molecular mass values reported for most plant peroxidases. These molecular mass values include: horseradish, 40 kD (26); potato, 47 kD (6); turnip, 33.4 kD (18); barley, 44 kD (25); and tobacco, 37 kD (12). An anionic molecular mass 98 kD wall peroxidase and a cationic molecular mass 58 kD wall peroxidase have been reported in maize seedlings (10). In addition, ethylene-inducible 33 kD (cationic) and 60 kD (anionic) peroxidases have been reported in cucumber cotyledons (1). There appears to be a wide range of molecular masses observed for peroxidases in higher plants. Cotton ovule peroxidase apparently exists as a monomer in solution, as the molecular mass obtained from gel filtration chromatography corresponded closely to the molecular mass obtained from SDS-PAGE.

The two COP isozymes displayed similar apparent $K_m$ values, indicating similar substrate specificities. The apparent
α values for guaiacol observed for COP were similar to those observed for tobacco peroxidase \(A_e\) (15). The substrate specificity for the major isozyme (COP B) indicated that guaiacol, TMB, and eugenol were good substrates, while AAP was a poor substrate. A similar substrate specificity was observed with potato peroxidase (6). The potent inhibition by KCN and NaN\(_3\) provided corroborating evidence that COP is an electron transfer protein.

The COP’s glycosidic nature was suspected due to its thermostability and susceptibility to periodate oxidation (19). Sugar analysis confirmed, on a quantitative level, this glycosidic nature. Of the reported purified plant peroxidases, HRP(c) and turnip(c) peroxidase have been completely characterized, with respect to carbohydrate composition. HRP contains Man and GlcN in the highest levels, with lesser amounts of Fuc, Xyl, and Ara (3). The sugar composition of turnip peroxidase is similar to that of HRP, with the exception of higher Ara levels in the turnip enzyme (18). The COP sugar composition was similar to both of the above peroxidases with respect to the low-frequency sugar residues, although Glu is not present in either HRP or turnip peroxidase. However, a significant deviation occurred with respect to Gal, which occurred with the greatest frequency in COP but is not present in the aforementioned cationic peroxidases. The functional significance of these differing carbohydrate compositions is not fully understood. Since COP is a secreted protein, its carbohydrate composition may be linked to a transport mechanism for the processed enzyme. Sugar moieties presumably provide water solubility for the apoenzyme, although the importance of this property would depend upon the enzyme’s site of action in planta.

The amino acid analysis confirmed the acidic nature of COP. A comparison of amino acid compositions for COP and other characterized anionic peroxidases, as well as HRP(c), is presented in Table V. In addition to the acidic residues (Asp, Glu), COP contained higher levels of Ser, Thr, Ala, and Gly residues than the other peroxidases. Histidine residue number (3–4) appears to be conserved within the anionic family of peroxidases. At least one His presumably functions as a ligand for the iron in the heme cofactor. Arginine is also apparently conserved with respect to the anionic peroxidases. A similar number of Tyr residues occurred in both anionic and cationic peroxidases. Only one disulfide bridge is apparently present in COP, whereas the other peroxidases have two or more such structures. A similar residue frequency of Lys occurred in the anionic peroxidases detailed. It is interesting to note that, if the hydrophobic residue numbers for Ile, Leu, and Phe are summed as a group, there appears to be a conservation trend within this group of anionic peroxidases (COP, 59; tobacco, 61; potato, 56). Perhaps this subset of residues forms a hydrophobic core within the peroxidase molecule. It is not surprising to observe the high degree of conservation between tobacco and potato peroxidases, since both of these proteins are derived from solanaceous plants. The amino acid composition for COP seems to support the observation that an anionic peroxidase from a given plant species is more closely related to other anionic peroxidases than to a cationic peroxidase from the...
same species (4). Direct comparisons of amino acid compositions between COP and other peroxidases are difficult to make due to the larger size of the cotton protein, but the observed trends are interesting.

The physiological role of these A. flavus-elicited isoperoxidases remains an interesting question. Certainly, their strong correlation with fungal-induced stress would seem to implicate them in a host defense role. The substrate specificity of COP was similar to that reported for potato peroxidase, which is believed to be involved in suberization of potatoes. Perhaps COP performs a similar function. Since COP is secreted into the surrounding culture medium (19), it may be secreted into the extracellular space around the developing seed in planta where it functions in the lignification of the seed coat. Peroxidase has been demonstrated to participate in seed coat lignification of malvaceous weed species (5).

In relatively few instances have peroxidases linked to specific metabolic responses been purified and characterized. The data presented in this paper represent an additional case of characterization of a physiologically significant peroxidase which may play an important role in the plant-fungal interaction. While this protein has the general characteristics associated with the peroxidase family, specific properties of COP may allow the protein to function in the appropriate arena.

The availability of pure COP will allow the development of immunological, protein-specific probes for use in immunocytolocalization studies and will aid in the development of gene-specific probes. Cloning the gene for COP will enable us to determine the factors which regulate the synthesis of this enzyme during normal seed development and to examine the effects of fungal-induced stress upon these genes.

ACKNOWLEDGMENTS

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LITERATURE CITED


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