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Characterization and Localization of a Phenoloxidase in Mung Bean Hypocotyl Cell Walls[1]

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The occurrence of proteins able to oxidize polyphenols even in the absence of H_2O_2 was recently reported in mung bean (*Vigna radiata* L.) hypocotyl cell wall extracts (R. Goldberg, A. Chabanet, A.M. Catesson [1993] *In* K.G. Welinder, S.K. Rasmussen, C. Penel, H. Greppin, eds, *Plant Peroxidases: Biochemistry and Physiology*, pp. 296–300). Therefore, the possible presence of a laccase in the extracts was investigated using immunocytological and biochemical approaches. An enzyme catalyzing phenol oxidation in the presence of molecular O_2 was extracted and purified from the cell walls. This 38-kD cationic protein, like *o*-diphenoloxidases, was unable to oxidize *p*-diphenols or *p*-diamines. However, it cross-reacted with an anti-laccase antiserum and, like laccases, its activity was inhibited by *N*-cetyl-*N,N,N*-trimethylammonium bromide but not by ferulic acid salts. Immunolabeling data showed that the 38-kD oxidase was absent from all cellulosic cell walls. It was localized only in lignifying and lignified cell walls. This restricted localization suggests that this laccase-like phenoloxidase could participate in the lignification process but not in the primary wall stiffening, which develops in the epidermal and cortical tissues along the mung bean hypocotyl.

A role for peroxidases (EC 1.11.1.7) in primary cell-wall stiffening through the formation of phenolic cross-links is generally accepted (Fry, 1986). This process is very similar to that occurring in lignifying cell walls, where peroxidases are known to catalyze the oxidative polymerization of cinnamic alcohols. Recently, the participation of a nonhemic cell-wall oxidase (laccase or *p*-diphenoloxidase, EC 1.10.3.2.) has been suggested in the polymerization of lignin monomers (see Dean and Eriksson, 1994). In fact, the involvement of laccases in lignification was suggested as early as 1959 by Freudenberg and later denied by Harkin and Obst (1973a). During the last 2 years, a laccase purified from the medium of suspension-cultured cells of sycamore (Bligny and Douce, 1983) was shown to catalyze monolignol polymerization (Sterjiades et al., 1992, 1993). This laccase was co-localized with lignin precursors in the walls of the cultured cells as well as in the walls of stem epidermis and xylem of the same species (Driouich et al., 1992). Similarly, a laccase associated with lignification was described in pine xylem cell walls (Bao et al., 1993). These results challenged the hypothesis that peroxidases were the only enzymes participating in the polymerization of lignin monomers and suggested a possible cooperation between peroxidases and laccases in the lignification process (Sterjiades et al., 1993).

If cell-wall laccases are able to polymerize lignin phenolic monomers (Sterjiades et al., 1992, 1993), these enzymes may be involved in the formation of phenolic cross-links inside primary cell walls and, in turn, in the cell-wall stiffening process. During the course of our studies on this process along the mung bean hypocotyl, we reported the occurrence of three cationic peroxidases in the cell walls (Chabanet et al., 1993). These enzymes could oxidize various phenolic compounds even when H_2O_2 was omitted from the assays but, in this case, the reaction rate was conspicuously much lower. In addition, a protein fraction much more effective in the absence than in the presence of H_2O_2 was also extracted from the cell walls. The presence of a laccase-like enzyme in mung bean hypocotyl protein extracts was then suspected (Goldberg et al., 1993), since some of the hydrogen donors (PPD-PC, 4-MC, and SYR) used in the assays were also suitable substrates for PPO, which, according to Mayer and Harel (1979), include *o*-diphenoloxidases (EC 1.10.3.1) as well as *p*-diphenoloxidases (EC 1.10.3.2). The aim of this paper was to investigate the exact nature of this activity through biochemical and cytochemical approaches to determine whether a true laccase was present in the hypocotyl cell walls.

MATERIALS AND METHODS

Plant Material

Seeds of *Vigna radiata* (L.) Wilczek were soaked in tap water for 2 h, placed on moist vermiculites and covered with

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Abbreviations: CTAB, *N*-cetyl-*N,N,N*-trimethylammonium bromide; DOPA, dihydroxyphenylalanine; FER, isopropylammonium salt of ferulic acid; FPLC, fast protein liquid chromatography; 4-MC, 4-methylcatechol; PC, pyrocatechol; PPD, *p*-phenylene-diamine; PPO, polyphenoloxidase; SYR, syringaldazine.

a wet cloth. After 3 d at 26°C in the dark, seedlings with hypocotyls 45 mm (±5 mm) long were selected.

Preparation of Enzymatic Fractions

Segments (2.5 cm long) were excised below the hook. About 300 g of hypocotyls were ground in 2 mM Na-K-Pi buffer, pH 6, with a Sorvall omnimixer (Roucaire, Vélizy, Villacoublay, France) and filtered through cheesecloth. The insoluble fraction was resuspended in the same buffer, incubated for 15 min in 0.1% Triton X-100, exhaustively washed with H_2O, and then suspended in distilled water. The homogeneity of the cell-wall suspension was checked with the electron microscope as previously reported (Goldberg et al., 1986). Proteins ionically bound to the walls were solubilized by two treatments of the walls with 0.05 M succinate buffer (pH 6) containing 1 M NaCl. After concentration by ultrafiltration on a PM 10 Amicon membrane, the protein extract was put on a CM-Sepharose CL 6B (Pharmacia, Uppsala, Sweden) column (20 × 1.7 cm) previously equilibrated with 0.01 M Na-K-Pi buffer, pH 6. The cationic proteins were eluted with a linear gradient of NaCl (0–0.3 M). Fractions (5 mL) were collected and analyzed for their peroxidase activity using PPD-PC as substrate. Appropriate fractions were pooled, dialyzed against H_2O, and concentrated by ultrafiltration. Four fractions were further submitted to FPLC on a Mono-S column (Pharmacia) equilibrated with 0.05 M Mes buffer, pH 6, with elution being effected with a NaCl gradient (0–0.2 M). Fractions (1 mL) were collected, their peroxidase activity was detected, and their isozyme composition was ascertained by native electrophoresis.

Enzymatic Assays

A wide range of aromatic compounds was used to discriminate between peroxidase, *p*-diphenoloxidase (laccase), and *o*-diphenoloxidase (catechol oxidase, EC 1.10.3.1) activities: (a) DOPA and 4-MC, *o*-diphenols oxidized both by *p*- and *o*-diphenoloxidases (Mayer and Harel, 1979; Walker and McCallion, 1980); (b) hydroquinone, a *p*-diphenol, and PPD, substrates for laccases but not for catechol oxidases (Bertrand, 1895; Walker and McCallion, 1980); (c) SYR and PPD-PC, convenient for both laccases and peroxidases (Harkin and Obst, 1973b; Imberty et al., 1985); (d) guaiacol (Van Loon, 1971) and ferulic acid salts (Pickering et al., 1973; Bao et al., 1993), reported to be oxidized by peroxidases and not by laccases. Oxidase activities were estimated both spectrophotometrically (Pang et al., 1989; Driouich et al., 1992) and polarographically (Bligny et al., 1986) in the presence or in the absence of H_2O_2. In polarographic assays, O_2 uptake was monitored with a Clark-type (Hansatech, King's Lynn, UK) oxygen electrode system; the O_2 concentration in the air-saturated medium at 25°C was taken as 240 μM; the incubation medium contained 50 mM Na-K phosphate buffer, pH 6.7, and 5 mM 4-MC or 2.7 mM PPD-PC.

Electrophoresis

Cationic isoperoxidases were separated by electrophoresis on a 7.5% polyacrylamide gel (15 mA, 2 h) using an LKB cell (Pharmacia) according to the method of Ornstein and Davis (1964). Activities were detected by incubating the gels in a

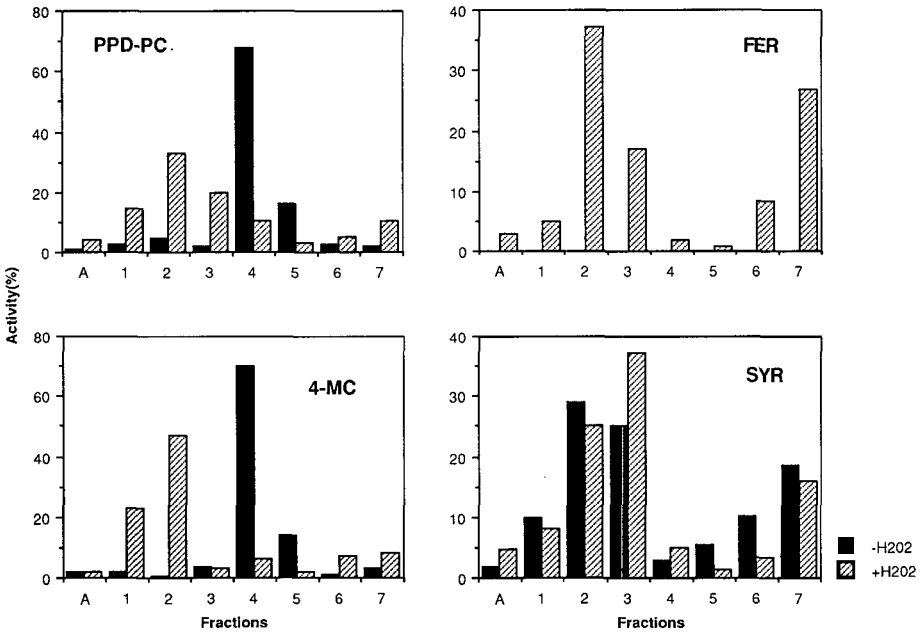

Figure 1. Distribution of oxidasic and peroxidasic activities in the anionic (A) or cationic (1–7) protein fractions obtained after chromatography of the proteins eluted with NaCl from mung bean hypocotyl cell walls on a Sepharose CL 6B column. Hydrogen donors were PPD-PC, FER, 4-MC, and SYR. Peroxidase activities were estimated in the presence of H_2O_2 (▨) and oxidase activities were estimated in the absence of H_2O_2 (■). Activities estimated spectrophotometrically are expressed as percentages of the total recovered activity.

Table I. *Distribution of oxidase activities among the different fractions recovered after chromatography of cell-wall proteins on CL-Sepharose CL 6B*

Activities as μmol O_2 min^{-1}.

	A	1	2	3	4	5	6	7
PPD-PC	0.66	1.7	3.7	4.6	151	19.7	14	5.1
4-MC				4.2	139	14.5	5.2	2.7

medium containing 7×10^{-4} M PPD, 9×10^{-3} M PC, 2.5×10^{-3} M H_2O_2, and 0.1 M Na-K-Pi buffer, pH 6.7.

SDS-PAGE was performed according to the method of Laemmli (1970) using a phast system (Pharmacia) operating at 15 mA (constant current) for 45 min. Mol wt standards obtained from Sigma France (St. Quentin Fallavier) were used to calibrate the gels. Proteins were detected by silver staining according to Blum et al. (1987).

Immunoblot Assay

Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979) and stained with Ponceau red (Coudriez et al., 1983). Nonfat dry milk (5% in PBS) was used for blocking the nitrocellulose transfer. Polyclonal anti-laccase antiserum was raised in rabbit according to Faye et al. (1986) by injecting deglycosylated laccase. This enzyme was isolated from suspension-cultured sycamore cells and further purified after trifluoromethane sulfonic acid treatment using SDS-PAGE and electroelution as previously described (Driouich et al., 1992). The antiserum was diluted (1:500) in TBS (0.1 M Tris-HCl, pH 7.5, 150 mM NaCl) for immunostaining of the blots. Antibody binding was visualized using anti-rabbit IgG alkaline phosphatase conjugate (dilution 1:500). We used a streptavidin-biotin amplification system (Amersham France, Les Ulis) according to the manufacturer's instructions.

Immunolocalization

Small pieces of tissues were cut either from the upper part (2 mm below the hook) or the lower part (20 mm below the hook) of the hypocotyl elongation zone. They were fixed in a mixture of 4% *p*-formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h. After several washes in buffer, tissue samples were dehydrated in a graded ethanol series and embedded in LR White medium resin. Ultrathin sections were collected on nickel grids and treated according to the following procedure: incubation in 0.1 M TBS, pH 7.6, containing 0.1% Tween 20 (TBST) for 15 min followed by 15 min in the same mixture plus 1% BSA (TBSTB); overnight incubation with rabbit immune serum used for immunoblot directed against deglycosylated *Acer pseudoplatanus* laccase (1:2000 in TBSTB); six washes, 5 min each in TBST, followed by a 15-min wash in TBSTB; 1-h incubation with 10 nm colloidal-gold-conjugated goat anti-rabbit IgG (Auroprobe EM, GAR 10, Jansen Biochimica, Noisy le Grand, France); a series of 5-min washes, six in TBST and six in distilled water. Control sections were incu-

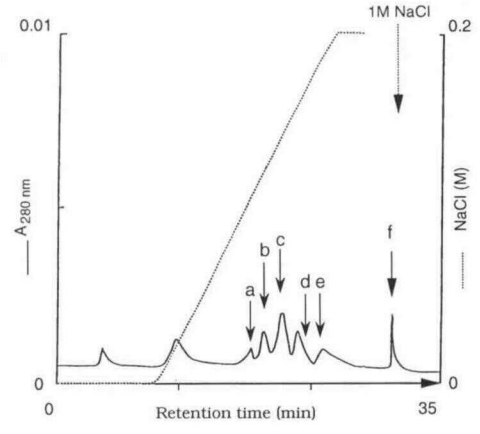

Figure 2. Purification of fraction 4 obtained after CM-Sepharose chromatography of the crude cell-wall protein extract. This fraction was submitted to FPLC on a Mono-S cation-exchanger column. Elution was performed with a linear 0 to 0.2 M NaCl gradient followed by a 1 M NaCl solution. Fractions of 1 mL were collected and assayed for their PPD-PC oxidase activity in the presence of H_2O_2. Arrows indicate the fractions able to oxidize PPD-PC.

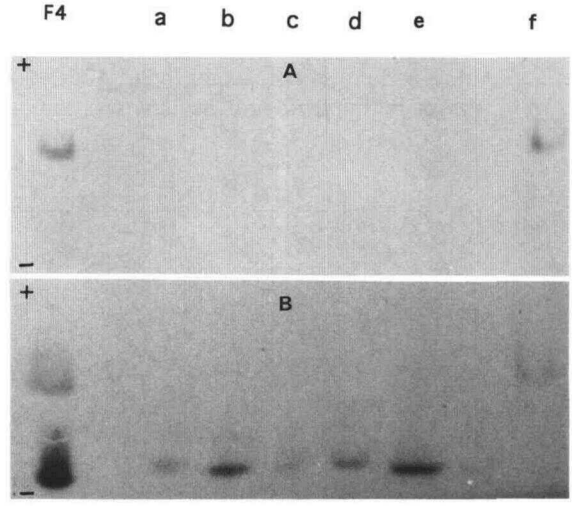

Figure 3. Isoenzyme patterns of fraction 4 (F4) before FPLC fractionation and of the active fractions (a–f) recovered from the Mono-S FPLC column. Running buffer was acetic acid-B-Ala, pH 4.5. Activities were detected by incubating the gels in Na-K-Pi buffer, pH 7, containing PPD-PC alone (A) or PPD-PC and H_2O_2 (B).

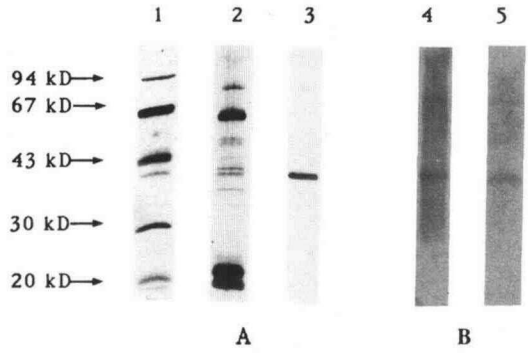

Figure 4. Analysis of purified mung bean oxidase with SDS-PAGE. A, Silver staining of the gel; B, western blot. Lane 1, Molecular mass markers (kD); lanes 2 and 4, fraction 4 (200 ng of protein) obtained after CM-Sepharose chromatography of the crude cell-wall protein extract; lanes 3 and 5, fraction f (35 ng of protein) obtained after FPLC (see Fig. 3).

bated in TBSTB alone instead of rabbit anti-laccase serum. All treatments were performed in the dark at room temperature. After incubation, improved contrast was achieved by overnight exposure to OsO_4 vapors followed by 2% uranyl acetate staining. Sections were viewed with either a Philips EM 300 or a Philips EM 400 (Eindhoven, The Netherlands) operating at 80 kV.

RESULTS

Fractionation of Cell-Wall Peroxidase Activities

Ionically bound proteins were solubilized from mung bean hypocotyl cell walls and submitted to ion-exchange chromatography on CM-Sepharose CL 6B. As already reported (Chabanet et al., 1993) and illustrated in Figure 1, one anionic (A) and seven cationic fractions were recovered. The latter were successively obtained by elution with a linear gradient of NaCl (0-0.35 M) and numbered 1 through 7. Oxidation of different hydrogen donors such as PPD-PC, SYR, 4-MC, FER, and guaiacol by the different fractions was then estimated in the presence or absence of H_2O_2. The activity distribution over the eight fractions depended on the nature of the substrate used in the assay. Ferulic acid (isopropylammonium salt, FER) could be oxidized only when H_2O_2 was present in the assay, with fraction 2 exhibiting the maximal activity. Similar data were obtained with guaiacol (data not shown). For both ferulic acid and guaiacol, more than 50% of cell-wall peroxidase activity was recovered in fractions 2 and 3. With PPD-PC and 4-MC, the highest peroxidasic activity was also found in the same fractions (2 and 3), but these two substrates could also be oxidized in the absence of H_2O_2. In this case, oxidation rates were obviously lower than in the presence of H_2O_2, except with fraction 4, whose activity was higher when H_2O_2 was omitted. These data suggested the occurrence of a true oxidase activity in fraction 4 and also revealed that the isoperoxidases present in the other fractions were able to weakly oxidize PPD-PC and 4-MC at the expense of O_2. Finally, with SYR, the distribution of activities was not very different with or without H_2O_2, with fractions 2 and 3 being the most active.

Table II. *Kinetic constants of native and freeze-thawed phenoloxidase*

Activities were estimated using 4-MC and PPD-PC as substrates immediately after purification (A) or after 3 weeks at −25°C (B). K_m as 10^{-3} M. V_{max} as μmol O_2 min^{-1} μg^{-1} protein. Same conditions as in Figure 5.

Constant	Substrate	A	B
K_m	4-MC	12	5
	PPD-PC	0.6	0.8
V_{max}	4-MC	10	2.5
	PPD-PC	5	2.5

Polarographic estimations of oxidasic activities agreed with the spectrophotometric measurements performed without H_2O_2 (Table I). No oxidation of guaiacol or ferulic acid occurred, regardless of the enzymatic fraction used in the polarographic assays. With PPD-PC and 4-MC, fraction 4 was the most active, which strengthens the hypothesis regarding the presence of a phenoloxidase in this fraction. Experiments could not be carried out with SYR due to the poor solubility of this substrate and the low sensitivity of the O_2 electrode.

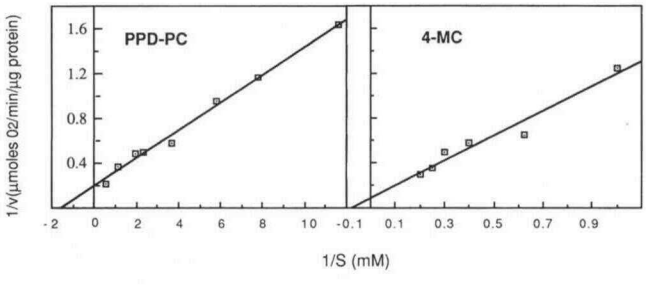

Figure 5. Double-reciprocal (1/v versus 1/S, where v = oxidation rate in μmol O_2 uptake per min per mg protein, and S = substrate concentration in mM) Lineweaver-Burk plots of the oxidation of PPD-PC and 4-MC by the purified mung bean cell-wall oxidase. Protein (16.4 ng) was used for the polarographic assays; oxidation rates were estimated during 1 min.

Purification of Fraction 4

Electrophoresis of fraction 4 obtained after chromatography on CM-Sepharose revealed the presence of several

Figure 6. (On facing page). Immunogold labeling of *Vigna* xylem. In the presence of anti-laccase antibody (A–C) the labeling is restricted to the cell walls, essentially to the secondary thickenings. Only a few scattered particles are present on the primary walls. A, Secondary wall formation in a differentiating vessel. B, Cell junction between two mature vessels and a parenchyma cell. C, Collapsing protoxylem element. D, Control section incubated in the absence of anti-laccase antibody. No labeling can be seen in this cell junction between two vessels and a parenchyma cell. P, Primary wall; Pa, parenchyma cell; S, secondary wall; V, vessel. Bars = 0.5 μm.

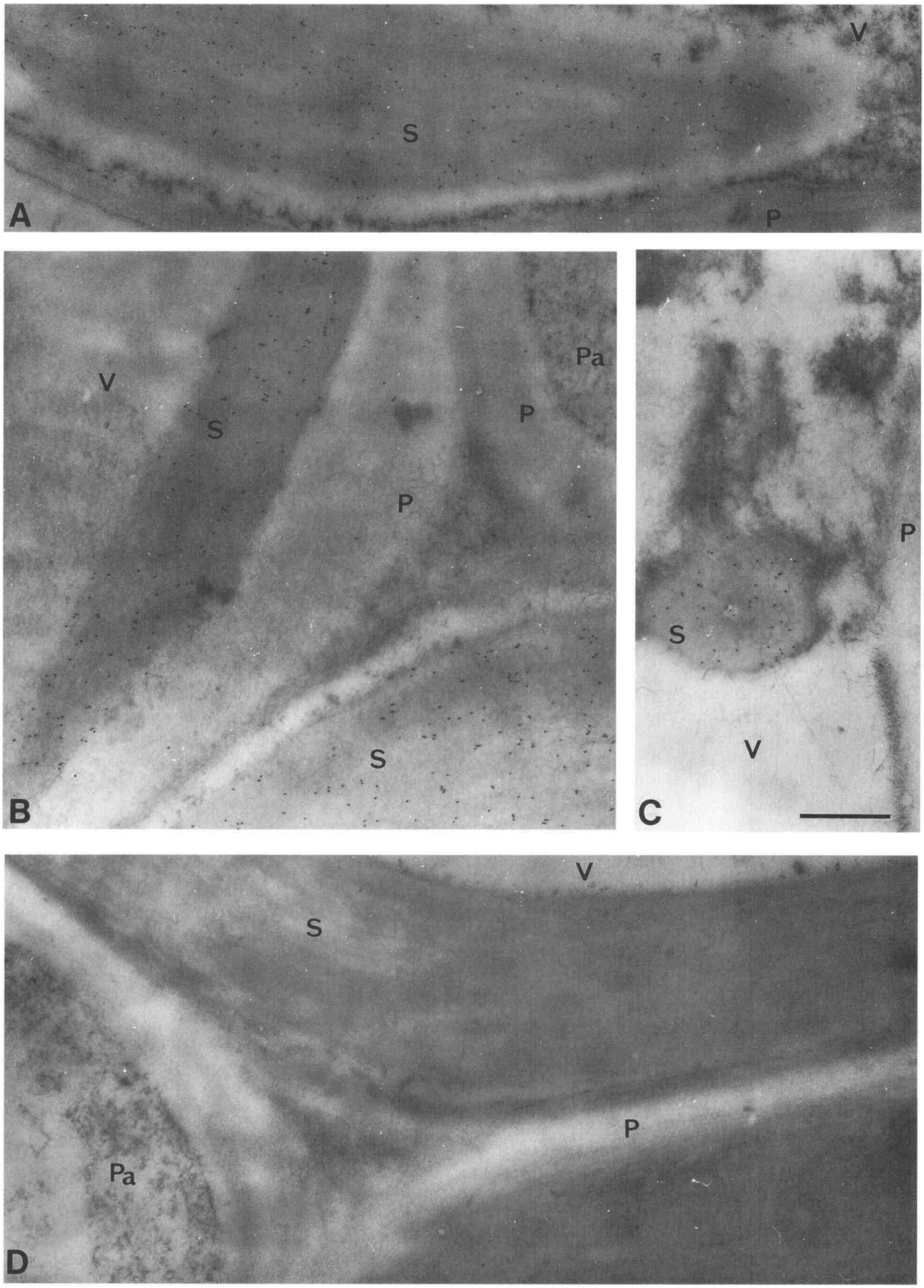

Figure 6. Legend on facing page.

stained bands when the gels were incubated in PPD-PC plus H_2O_2. An FPLC fractionation was then carried out on a Mono-S cation exchanger. Fractions oxidizing PPD-PC (arrows in Fig. 2) were submitted to electrophoresis. Activities were revealed by incubating the gels either in PPD-PC alone (Fig. 3A) or in PPD-PC plus H_2O_2 (Fig. 3B). Only the active fraction that eluted with 1 M NaCl (fraction f in Fig. 3) reacted more strongly in the absence than in the presence of H_2O_2, which suggested the occurrence of an oxidase activity. Moreover, SDS-PAGE of this fraction (Fig. 4, lane 3) revealed the presence of a single protein band of 38 kD, which cross-reacted (Fig. 4, lane 5) with an immunoserum prepared against a laccase extracted from sycamore suspension cultures and deglycosylated (Driouich et al., 1992).

Properties of the Purified Phenoloxidase

The phenoloxidase isolated after FPLC was then investigated. This protein, contrary to the purified peroxidase fractions, did not absorb light at 405 nm, which ruled out the possibility of the presence of a heme-containing protein. Kinetic constants were estimated polarographically using PPD-PC and 4-MC as substrates; all values were only apparent values since O_2 was limited under the experimental conditions. Lineweaver-Burk plots were constructed from initial velocity data (Fig. 5). The highest specific activity (V_{max}) was obtained with 4-MC, whereas the affinity of the enzyme toward PPD-PC was 10 times higher than toward 4-MC. Storage at −25°C markedly reduced V_{max} with both substrates but affected the K_m values differently (Table II). After 3 weeks of storage at −25°C the affinity toward PPD-PC remained quite unchanged, whereas it increased toward 4-MC. These data suggested that the enzyme probably contained different binding sites for the two substrates. Storage at −25°C, which rapidly decreased the oxidation rate, also induced a loss of the cross-reactivity toward anti-laccase antibodies.

This enzyme was able to oxidize PPD-PC, 4-MC, SYR, PC, and DOPA at the expense of O_2, with or without the addition of H_2O_2, but its activity was reduced when H_2O_2 was added to the assays. In contrast, the mung bean cell-wall oxidase was not able to oxidize guaiacol, routinely used as peroxidase substrate, or *p*-phenols like hydroquinone, or *p*-diamines like PPD.

The effects induced by different compounds reported to inhibit the activities of either PPOs (PVP, CTAB, and tropolone) or peroxidases and *o*-diphenoloxidases (ferulic acid salts) were checked polarographically. Oxidation of 4-MC was only weakly affected by ferulic acid: no inhibition occurred with 4 mM ferulic acid and only 14% occurred with 20 mM, a concentration that abolished the activity of the three cationic peroxidases purified from mung bean hypocotyl cell walls. All tested PPO inhibitors were effective: 0.1 mM tropolone was sufficient to inhibit the activity of the purified PPO completely, whereas an inhibition of about 33% was observed in the presence of 10^{-3} mM of this product. Furthermore, the mung bean oxidase activity was inhibited by 50% in the presence of 2.5 mM PVP or 10 mM CTAB, concentrations without any effect on peroxidase activities isolated from mung bean hypocotyls.

Immunolocalization of Wall-Bound PPO

Sections taken in the upper as well as in the lower parts of the hypocotyl elongation zone were investigated. In both cases, immunolabeling with anti-laccase antibody was restricted to the vessel elements and almost exclusively to their secondary walls, regardless of their age (Fig. 6). This was observed in differentiating metaxylem vessels (Fig. 6A), in fully mature functional vessels (Fig. 6B), and even in collapsing protoxylem elements (Fig. 6C). All these cell walls and only these walls were stained with phloroglucinol, a specific lignin reagent (F. Ermel and A.M. Catesson, unpublished data). Gold particles were never observed in the walls of other cell types, even in the epidermis. The absence of a positive reaction in the cytoplasm or in the vacuole confirmed that the enzyme was really cell-wall bound in intact hypocotyls. Control sections treated in the absence of anti-laccase antibody were always devoid of gold particles (Fig. 6D).

DISCUSSION

An enzyme catalyzing *o*-diphenol oxidation was extracted and purified from the cell walls of mung bean hypocotyls. It could be differentiated from peroxidases because: (a) it did not absorb at wavelengths around 405 nm; (b) it was able to use molecular O_2 to oxidize phenolic substrates and its activity did not increase in the presence of H_2O_2; (c) it did not oxidize either guaiacol or ferulic acid salts; and (d) it was highly sensitive to tropolone, which is both an effective copper chelator and a substrate for peroxidases (Kahn, 1985). The inhibitory effect of tropolone on mung bean phenoloxidase (complete inhibition at 0.1 mM) is stronger than what is currently reported for *o*-PPO, respectively 95% inhibition at 0.33 mM for *Vicia o*-PPO (Takahama and Oniki, 1991) and 75% at 0.2 mM for *Solanum o*-PPO (Kowalski et al., 1992). (e) Like most PPO, mung bean oxidase was very unstable and could not be stored without rapidly losing its native (i.e. active) configuration. After only 7 d at −25°C its activity was strongly decreased and the protein was no longer recognized by the anti-laccase antibodies. This lability seems to be a characteristic of copper enzymes, whereas ferric enzymes remain undamaged over a wide range of temperatures (Mayer and Harel, 1979). All these properties differentiate mung bean oxidase from a polyfunctional peroxidase able to use molecular O_2 such as the C_3 isoform, which is present in the same tissues (Goldberg et al., 1993).

However, if the mung bean oxidase can be considered as a PPO sensu lato (as defined by Mayer, 1987), it is rather difficult to discriminate between catechol oxidase (*o*-PPO) and laccase (*p*-PPO) in this instance. The mung bean phenoloxidase indeed exhibits some characteristics of each kind of enzyme. Like a laccase this enzyme: (a) is localized only in lignifying cell walls, whereas *o*-PPOs are considered to be chloroplastic enzymes (Mayer, 1987); (b) oxidizes SYR, which is a substrate of both peroxidases and laccases (Harkin and Obst, 1973b); (c) cross-reacts with anti-laccase antiserum; (d) is not inhibited by ferulic acid, whereas *o*-PPOs have a specific "inhibitor site" for ferulic acid (Mayer and Harel, 1979; Walker and McCallion, 1980); and (e) can be inhibited by CTAB, whereas *o*-PPOs are not inhibited (Walker and

McCallion, 1980; Mayer, 1987). However, laccases are known to oxidize both *o*- and *p*-diphenols and the mung bean oxidase acts only on *o*-diphenols. Moreover, unlike *Acer* laccase (Sterjiades et al., 1993), mung bean oxidase does not oxidize guaiacol or ferulic acid.

In addition, this cell-wall oxidase is a rather small polypeptide of 38 kD as estimated from SDS-PAGE, unlike the laccases isolated from *Acer* (100–110 kD, Driouich et al., 1992) and from *Pinus* (90 kD, Bao et al., 1993). However, while investigating sycamore cell-wall laccase, Driouich et al. (1992) reported that their anti-laccase antiserum reacted also with a smaller polypeptide (around 58 kD) extracted from stem tissues. The fraction 4 recovered from the CM-Sepharose column also contained traces of a 58-kD protein that cross-reacted slightly with the laccase antiserum (Fig. 4, lane 4), but this faint band was not detected in the purified phenoloxidase fraction (Fig. 4, lane 5). Besides, like *Rhus* and *Pinus* laccases (O'Malley et al., 1993), the mung bean phenoloxidase is a cationic protein, in contrast with sycamore laccases, whose isoelectric points range from 4 to 5.2 (Sterjiades et al., 1992). Taking into account the similarities in properties and localization between the mung bean phenoloxidase and laccases already purified from higher plants, the former might be considered as a laccase-like enzyme in spite of its inability to react with *p*-diphenols or *p*-diamines.

The mung bean oxidase exhibited a higher specificity for PPD-PC than for 4-MC. Moreover, the K_m values for these substrates were differently affected after storage at −25°C, decreasing with 4-MC and increasing with PPD-PC. Mung bean oxidase, as already described for some peroxidases (Ortiz de Montellano et al., 1993), might present different binding sites for the two substrates with different sensitivities to storage at low temperature.

Immunolabeling data showed that in the mung bean hypocotyl, the laccase-like phenoloxidase was restricted to lignifying or lignified xylem cell walls and thus cannot be involved in the stiffening process of cellulosic cell walls. The presence of a laccase in the epidermal cell walls of *A. pseudoplatanus* branches (Driouich et al., 1992) was probably not related to primary cell-wall stiffening but to the later deposition of lignin-like compounds in these walls. The labeling observed in mung bean hypocotyl xylem cell walls that also exhibit true peroxidasic activity (Goldberg et al., 1993) agrees with the hypothesis, suggesting that both peroxidases and phenoloxidases might participate in the polymerization of lignin monomers (O'Malley et al., 1993; Sterjiades et al., 1993; Dean and Eriksson, 1994). The phenoloxidases involved in this process could be either true laccases or, as in mung bean hypocotyl, a laccase-like phenoloxidase exhibiting some properties of true laccases but also some of catechol oxidases.

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