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DEVELOPMENT OF ISOPEROXIDASES ALONG THE GROWTH GRADIENT IN THE MUNG BEAN HYPOCOTYL

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Key Word Index—*Vigna radiata*; Leguminosae; mung bean; growth; cell walls; peroxidases.

Abstract—Cytoplasmic and wall bound peroxidases were extracted from successive segments of decreasing growth potential along the mung bean hypocotyl. Active wall bound peroxidases were present in the epidermis and external parenchyma layers at the end of the elongation phase. Two fast migrating anionic isoperoxidases covalently bound to the cell walls increased when the cell walls lost their plasticity. These isoenzymes were characterized by a high affinity for several peroxidase substrates and high thermal stability.

INTRODUCTION

Integration of diphenyl phenolic crosslink wall polymer subunits into the cell wall might limit the cell wall plasticity [1–3]. The formation of these diphenyl crosslinkages in muro might be catalysed by specific cell wall peroxidases [2–4]. Such crosslinkages might bind lignin [5], polysaccharides [6] or proteins [1–3, 7] to pectins or hemicelluloses [3, 8]. During the last few years, cell wall isoperoxidases involved in the oxidative polymerization of cinnamyl alcohols have been widely investigated [9–12]. Besides, cell wall peroxidatic activities were described in elongating tissues [8, 13–15] and their possible involvement in the cessation of growth was suggested [2, 3, 14, 16]. The aim of the present work was therefore to determine the possible relationships between cell wall peroxidases and growth processes along a growth gradient. This problem was investigated through parallel histochemical and biochemical approaches. Experiments were performed with excised mung bean hypocotyl segments, the growth characteristics of which are now well known [17–19]. Along this axis, the cell walls gradually lose their ability to react to acidic pH [17] owing to modifications of cell wall structure. We intended therefore to investigate the development of isoenzyme patterns and of cell wall peroxidase properties along this growth gradient.

RESULTS AND DISCUSSION

Distribution of peroxidatic activities along the mung bean hypocotyl

Three successive regions with decreasing growth potentials were investigated. Results represented in Fig. 1 reveal that in young cells, most of the peroxidase activity is located in the cytosol whereas in mature, fully expanded cells, 73% of the activity is bound to the walls.

Isoenzyme patterns

Isoenzymes present in each extract were separated by gel electrophoresis (Fig. 2). The ionically bound fraction (I) contained cationic and anionic isoperoxidases whereas especially anionic isoforms could be detected in all other fractions. The most striking modification observed along the hypocotyl was the development of two fast migrating anionic isoenzymes in the covalently bound fraction (C). The properties of this fraction were then further investigated with extracts isolated from young (I) and mature (III) cell walls.

Properties of covalently bound peroxidases

Lineweaver–Burk plots were constructed from initial velocity data. Apparent 'K_m' values were determined after linear regression analysis (Table 1). No significant differences could be observed for the soluble (CS) and ionically bound (I) fractions obtained from young and mature tissues. In contrast, the affinity of the covalently bound fraction for all tested chromogens [*p*-phenylenediamine-pyrocatechol (PPD-PC), trimethyl-

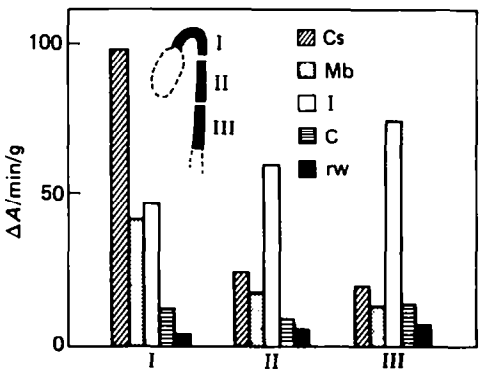

Fig. 1. Distribution of peroxidase activities along the mung bean hypocotyl. Activities as Δ *A* at 557 nm per min and per g dry material. I, II, III: successive hypocotyl segments. Cs, Cytosol; Mb, membranes; I, ionically bound fraction; C, covalently bound fraction; rw, residual cell walls (after pectinase–cellulase treatment).

Table 1. K_m values of peroxidase activities

	Enzymatic fractions					
	Cytoplasmic peroxidases		Cell wall peroxidases			
	CS		I		C	
Substrate	I	III	I	III	I	III
PPD-PC	2.5×10^{-3} M	1.4×10^{-3} M	2.5×10^{-3}	3×10^{-3}	7×1.10^{-3}	2.4×10^{-3}
TMB					3×10^{-6} M	0.4×10^{-6} M
Guaiacol					7×2.10^{-3}	2.1×10^{-3} M

The constants were estimated from double reciprocal plots. Enzymatic fractions (CS, I and C) were obtained from segments I and III.

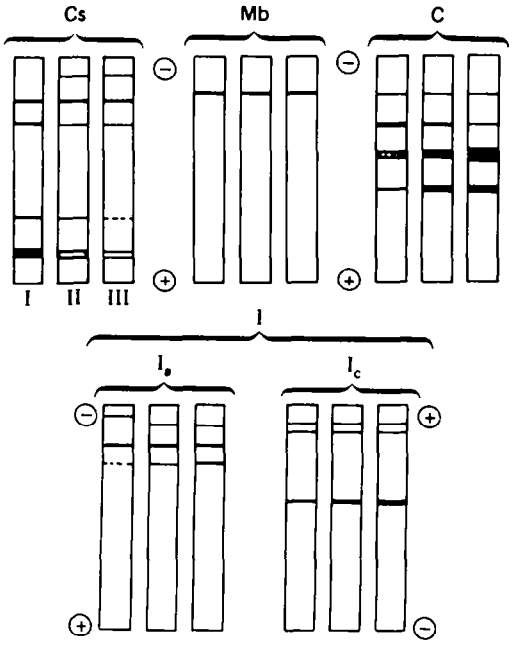

Fig. 2. Isoenzyme patterns of the peroxidase fractions obtained from the successive I, II and III segments. Peroxidases were separated by polyacrylamide disc gel electrophoresis. Cs, Soluble fraction; Mb, membrane bound fraction; C, covalently bound fraction; I, ionically bound fraction. Sc, Mb, C, I_a, Separation of anionic isoperoxidases; running buffer: Tris–HCl, pH 8.2. I_c, Separation of cationic peroxidases; running buffer: acetic acid–β-alanine, pH 4.5.

benzidine (TMB) and guaiacol] was greater in mature than in young cells.

Two different chemical inhibitors were tested. Potassium cyanide, known to act on the activity of cell wall peroxidases and diethyldithiocarbamate (DDC) reported to inhibit completely cell wall peroxidases [20–22]. Kinetic patterns obtained via Dixon plots [23] indicated competitive inhibition with both inhibitors for both enzymatic fractions and similar inhibition constants for enzymes isolated either from young or mature cell walls (Table 2). With DDC, a time lag occurred, the duration of which increased with inhibitor concentration. This behaviour is similar to the previously reported effects on other peroxidases [20, 21].

Table 2. K_i values for KCN and DDC

Enzymatic fraction	Inhibitor	
	KCN	DDC
I	3×10^{-6} M	1×10^{-4} M
III	3×10^{-6} M	1×10^{-4} M

The inhibition constants were estimated from Dixon plots. Enzymatic fractions: covalently bound enzymes solubilized from young (I) and mature (III) cell walls.

Heat inactivation assays were also performed with extracted enzymes. Thermal treatments were performed at 50, 70 and 80° during 1, 2, 5, 8 and 12 min. Two first order reactions were observed, as previously described for peroxidase in sweet corn [24] and poplar [20]. Therefore, two groups of isoperoxidases with different heat stabilities were probably present in the extracts, each being inactivated according to a first order reaction. The *D* values (decimal reduction time for inactivation of peroxidase activity) were calculated for each first order reaction and the thermal destruction coefficients (z) were obtained graphically by plotting the *D* values (on a logarithmic scale) against the temperatures. In the covalently bound fractions obtained from young (I) and mature (III) cell walls, the thermoresistant isoenzymes presented identical thermal denaturation coefficients (28°). In contrast, the thermolabile isoenzymes were more resistant in the fraction isolated from mature cell walls (III). Thermal denaturation coefficients for fractions I and III were respectively 65° and 46°.

Ion exchange chromatography of covalently bound fractions

Covalently bound fractions (C) isolated from young and mature cell walls were submitted to anion exchange chromatography on DEAE Sepharose CL 6B (Fig. 3). Three peaks of peroxidase activity were found. The first one (peak a) was important in young cell wall extracts and the two others in mature wall extract. Such data agree with

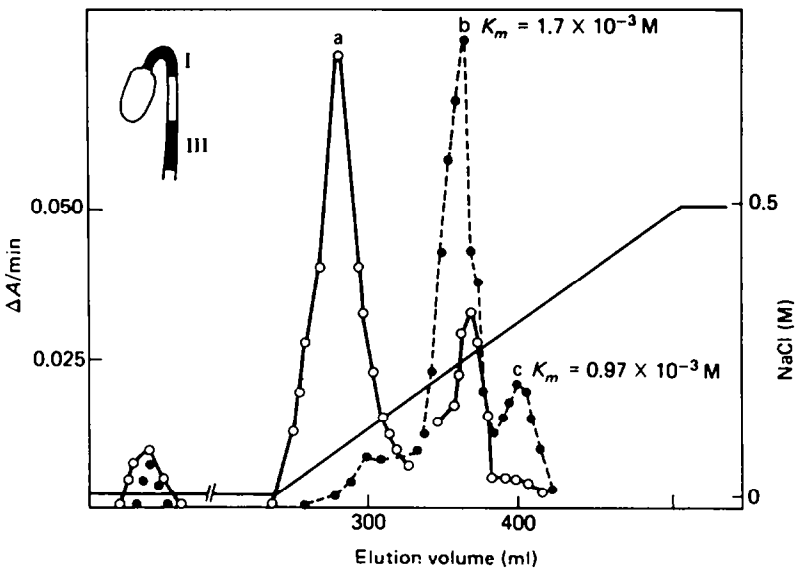

Fig. 3. Ion exchange chromatograms of covalently bound enzymes on DEAE-Sepharose CL-6B (20 × 1.7 cm). After loading, the column was washed with 0.01 M Na–K phosphate buffer pH 7.2 after which bound peroxidases were eluted by an NaCl gradient (0–0.5 M, 300 ml). Collected fractions were analysed for their PPD-PC oxidase activity expressed as Δ A at 557 nm per min and per 100 μl of each collected fraction. O——O Fraction solubilized from segments I; ● - - ● fraction solubilized from segments III.

the electrophoretic patterns depicted in Fig. 2. Optimal pH was about 6.9 for all three fractions. The three isoenzymes exhibit similar sensitivity to acidic pH (50% of maximal activity near pH 5.8) but the isoperoxidases 2 and 3 were less sensitive to alkaline pH than the first one (respectively 50% of the maximal activity at pH 8 and 7.5). The third eluted fraction presented the highest affinity for PPD-PC.

Influence of the substrate on the estimation of peroxidase activities

PPD-PC was used as routine chromogen in most of the above experiments because this substrate can be used for histochemical observations as well as for gel staining or rapid spectrophotometric estimations [25]. Peroxidase activities were also estimated in the different extracts with other substrates such as TMB, guaiacol and syringaldazine (Fig. 4). Important differences were then noticed. With all enzymatic fractions, the highest absorbances were obtained for oxidation of TMB, but with this chromogen, initial velocity was maintained for only 1 or 2 min. The proportion of soluble activity was higher with PPD-PC than with the other substrates. Distribution of peroxidase activity appears then to depend strongly on the nature of the substrate used in the assays. However, with all substrates tested, the proportion of wall bound enzymes increased along the hypocotyl.

Histochemistry

PPD-PC oxidase activity was detected in the phloem and pericyclic zone(cytoplasm and cell walls) all along the hypocotyl. In the outer layers of the cortical parenchyma and in the epidermis the distribution of the activity changed along the hypocotyl. In segment I, the cytoplasm was abundant and reacted strongly with the chromogen,

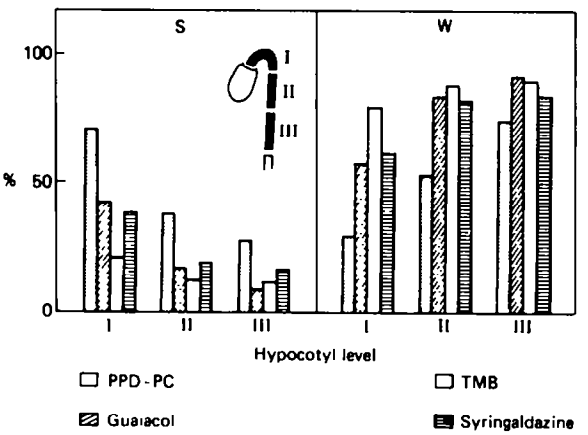

Fig. 4. Distribution of PPD-PC, TMB, guaiacol and syringaldazine oxidase activities in the three successive segments I, II and III. Soluble (S) and cell wall (W) activities were added and normalized to 100. Each kind of activity is expressed as a percentage of the total activity solubilized from each of the three hypocotyl segments.

whereas the cell walls gave little reaction. In segments II and III, the cytoplasm was restricted to a very thin layer surrounding a large vacuole; these observations may explain the important decrease of soluble activity occurring along the hypocotyl (Fig. 1). In these expanded cells, cell wall peroxidatic activity was obvious. In the parenchyma, this reaction was specially marked around the intercellular spaces. In parallel, thickness of epidermal cells decreased as previously observed at the electron microscope level [26]. Only few lignified vessels could be detected in the sections.

In conclusion, these preliminary investigations revealed important modifications in cell wall peroxidatic fractions along the growth gradient of the mung bean hypocotyl. These changes were specially obvious in the epidermis. According to Roland *et al.* [26] the structure of this outer layer can be considered as the key of the cell plastic properties. We observed that in the epidermis, peroxidatic activity was restricted to cell walls of elongated cells. Moreover specific isoperoxidases developed concurrently with the decrease of cell wall plasticity. These covalently bound isoenzymes were isolated and characterized by their relatively high affinity for various chromogens. At the present time, the function of these enzymes is still uncertain and require further investigation. It would be specially interesting to test first their cross-linking ability.

EXPERIMENTAL

Plant material. Seeds of *Vigna radiata* (L.) Wilezek were soaked in tap water for 2 hr, placed on moist vermiculite and covered with a wet cloth. After 3 days at 26° in the dark, seedlings with hypocotyls 45 mm (± 5 mm) long were selected. Three successive segments were excised in the upper part of the hypocotyl as depicted in Fig. 1.

Preparation of enzymatic fractions. About 50 g fresh segments were ground in 2 mM Na-KPi buffer pH 6 with a Sorvall omnimixer. Enzymatic extractions were performed according to ref. [13] and [27] with an additional step. The soluble fraction was centrifuged for 20 min at 40 000 *g*; the supernatant was called 'cytosol fraction' (Cs), the pellet was homogenized in KPi buffer and called 'membrane fraction' (Mb).

Cell wall enzymes were fractionated into lightly- and strongly-bound enzymes, so called ionically (*I*) and covalently (*C*) bound enzymes, respectively solubilized by 1 M NaCl and cellulase–pectinase treatment; residual cell walls (rw) were put in KPi buffer. All enzymatic extracts were reduced to a small vol. in an ultrafiltration cell (membrane PM 10; Amicon).

Spectrophotometric assays. For *p*-phenylenediamine-pyrocatechol (PPD-PC) oxidation, the assay contained in 4 ml final vol.: 3.5×10^{-4} M PPD, 4.5×10^{-3} M PC, 1.125×10^{-3} M H_2O_2, enzyme extract (50 or 100 µl) and 0.1 M Tris–maleate buffer, pH 7. The oxidation time course was recorded at 557 nm. Oxidation of trimethylbenzidine (TMB) and guaiacol was followed spectrophotometrically according to ref. [25]; oxidation of syringaldazine was performed as in ref. [12]. Enzyme assays on residual cell walls were terminated by rapid suction.

Electrophoretic assays. The isoperoxidases were separated by electrophoresis on 7.5% polyacrylamide disc gel according to ref. [28]. The isoenzymes were stained by immersing the gels in an incubation mixture containing 7×10^{-4} M PPD, 9×10^{-3} M PC, 2.5×10^{-3} M H_2O_2 and 0.1 M Tris–HCl buffer, pH 7.6 in 6 ml final vol. [12].

Chromatographic assays. Covalently bound enzymatic extracts were submitted to ion-exchange chromatography on DEAE-Sepharose CL 6B (Pharmacia) columns (20 cm × 1.7 cm diam.) equilibrated with 0.01 M Na–KPi buffer, pH 7.2. Sample was loaded on the exchanger and the column was washed with 0.01 M Na–KPi buffer, pH 7.2. Bound peroxidases were eluted with an NaCl linear gradient (300 ml) at pH 7.2 (0–0.5 M). Fractions (5 ml) were collected and analysed for their PPD-PC oxidase activity. Appropriate fractions were pooled, dialysed against H_2O and analysed for their kinetic characteristics.

Heat stability. Heat treatments were performed on buffer solns heated to a specified temp. before injection of diluted enzymes. After defined incubation periods, the solns were rapidly cooled by immersing the tubes in ice-water. Samples were then assayed for their PPD-PC oxidase activity.

Histochemistry. Hand sections were made at different levels along the hypocotyls. The sections were collected in 0.1 M Tris–HCl buffer, pH 7.6, at room temp. and incubated for 5 min in the following medium: 7×10^{-4} M PPD, 9×10^{-3} M PC, 2.5×10^{-3} M H_2O_2 and 0.1 M Tris–HCl buffer, pH 7.6. The sections were then rinsed with H_2O and observed with the light microscope.

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