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CYTOCHEMICAL ASSAYS OF PEROXIDASE ACTIVITIES IN RELATION TO GROWTH AND DEVELOPMENT

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Introduction

By 1980, the literature contained more than 2,000 references to plant peroxidases (8), a list which has been expanded since then by many hundreds of new works. The data on peroxidases are primarily derived from biochemical and physiological studies, and concern protoplasts, suspension cell cultures, and biochemical fractions from whole plant tissues so yielding an array of data concerning isozyme patterns, sub-cellular localization and variation in activities (see this volume). It is through histological and cytochemistry, perhaps, that some shape can be given to these diverse forms of information. This article endeavours to initiate such a rationalization through cytochemical studies of peroxidase activities particularly related to xylogenesis in primary vascular tissue and to dormancy break in seeds. Immunocytochemistry will not be considered since although it forms an important approach to the study of peroxidases, it concerns only the localization of these enzymes and not their activities (which is the theme of this article).
Materials and Methods

Methods

The peroxidase cytochemist has a large list of available substrates for the study of this enzyme group (e.g. Table 1) and Table 1. Substrates available for a cytochemical analysis of peroxidase activities in plant cells and tissues.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Potentially Quantifiable</th>
<th>Light Electron Microscope</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Guaiacol</td>
<td>Yes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2. Benzidine</td>
<td>No</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3. Diaminobenzidine</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4. Syringaldazine</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5. 3-amino-9-ethylcarbazole</td>
<td>Yes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6. p-phenylene-diamine/pyrocatechol</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7. 2,7, fluorene-diamine</td>
<td>No</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8. Homovanillic acid</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

each of them is valuable to varying degrees. The details of these cytochemical reactions are given in the references to Table 1, and in Gahan (5). Of the substrates listed, benzidine has been banned from use in many countries through its carcinogenicity though diaminobenzidine (DAB) has been a better replacement yielding a reaction product which is coloured, electron dense and substantive to sites of production. This permits its use for both light and electron microscopy (11). Unfortunately both DAB and pyrocatechol/p-phenylenediamine yield products which are brown/black and so not readily quantifiable. In contrast, syringaldazine, 3-amino-9-ethylcarbazole and homovanillic acid are capable of yielding products which are either coloured or fluorescent and so potentially quantifiable. The problem with syringaldazine and 3-amino-9-ethylcarbazole is that though their end products may be quantified by integrating microdensitometry (5) having maximal absorption at a wavelength of 590nm, the reaction products do not bind readily to the sites of their production and permit diffusion. However, homovanillic acid offers a better approach in that the reaction involves the conversion of homovanillic acid monomers to dimers by peroxidase. The dimers are precipitated at the sites of production in the
presence of lead ions. The lead complex can be treated with either ammonium or hydrogen sulphide to give a readily visualisable product. Alternatively, although the autofluorescence of the homovanillic acid dimer is quenched in the presence of lead, a fluorescence may be restored by attaching either ethidium bromide or rhodamine B or G6 to the dimer, thus giving the feasibility of quantifying the activity of the enzyme (5).

Table 2. Controls to be performed with cytochemical reactions for peroxidase activity.

<table>
<thead>
<tr>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Omission of substrate (−H_{2}O_{2})</td>
</tr>
<tr>
<td>2. Heat inactivation of enzyme (5–15 min at 95°C)</td>
</tr>
<tr>
<td>3. Addition of 20–100 mM aminotriazole to block catalase activity.</td>
</tr>
<tr>
<td>4. Pretreatment of sections with buffered 0.05–0.075% catalase for 1–2 h to block endogenously produced H_{2}O_{2} production OR Addition of 2 mM sodium pyruvate to incubation medium.</td>
</tr>
<tr>
<td>5. Non-specific adsorption of DAB by treating sections with H_{2}O_{2}-free incubation medium, washing and placing sections in 3 x 10^{-5} M potassium ferricyanide for 5 min. to oxidize (and visualize) DAB.</td>
</tr>
<tr>
<td>6. Long washing periods in 0.5 N NaCl solution to remove wall-bound peroxidases.</td>
</tr>
</tbody>
</table>

Controls

Whichever of the methods (Table 1) are exploited, it is important to employ adequate control reactions to ensure the validity of the responses (for the main controls see Table 2). Clearly, the use of particular tissues may result in the need for additional controls e.g. the use of the homovanillic acid method with a fluorochrome will need to involve the control to check for non-specific fluorochrome binding to the section.

Fixation

In view of the tough treatment given to plant material during the reactions and subsequently if being prepared for electron microscopy, it may be necessary to fix the tissue. Unlike dehydrogenases which are almost completely inhibited by aldehyde fixatives, Hall et al. (13) showed that either 1% or 5% glutaraldehyde and either 1% or 5% formaldehyde fixation at 4°C for 2 hours gave only 14% or 12% and 7% or 8% inhibition of peroxidases in pea root tissues, respectively. Thus, whilst it will be necessary to check the level of their inhibition by fixation on other tissues, peroxidases appear to be relatively resistant to aldehydes.
A word of caution should be introduced for reactions with e.g. DAB, for electron microscopy since the reagents pass with difficulty into tissue blocks and often there is not complete penetration to the centre of the tissue (14).

Tissue preparation

The experiments cited in relation to xylogenesis of primary xylem were performed on unfixed, frozen sections of 5 days old root apices of Pisum sativum and to dormancy break and germination on unfixed, frozen sections of either dry or water-imbibed or GA3-imbibed seeds of Avena fatua and Lactuca sativa (6). After freezing the material (7) frozen sections were prepared with a Bright's automated cryostat at cutting speed setting 5 and section thickness setting 15 µm.

Peroxidase activity in root apices

Peroxidase activity in roots of Pisum sativum can be measured biochemically and localized cytochemically with either DAB or guaiacol as substrate using the light and electron microscopes. Peroxidase activity appears to be present in virtually all cell-types (14) and may be identified in cell walls, Golgi apparatus, vacuoles and intracellular spaces (14). The first three sites of localization are consistent with the idea of a ribosomal synthesis of peroxidase and a transfer to the Golgi apparatus for the attachment of carbohydrate molecules followed by transport to either the vacuole(s) or cell walls (1). The validity of the intercellular space as a site for peroxidase may be questionable in some instances; however, enzymes and non-enzymic molecules can pass into and out from cells and the presence of peroxidase activity on the outer surface of cell walls abutting the intercellular space may be real. The function of such activity is not clear.

An electrophoretic analysis of anionic peroxidases from roots of Pisum sativum (3,4) has shown nine isoenzyme bands using DAB as a substrate but only six bands using guaiacol, bands 3,5 and 7 only showing with DAB. A comparison of soluble and cell-wall bound enzymes in the cortex versus the stele showed an increasing number of bands (DAB) with increasing distance from the root cap junction. This change would appear to represent changes with the passage of time (ageing) with the production of more complex structural and physiological aspects of the tissues concerned. In particular, the cell wall-bound isoenzymes in the stele appear to involve only bands 1 and 2 whilst 3, 4, 5 and 7 are soluble and hence possibly related to the GERL system as well as or instead of the cell walls. Cell wall fractions from the stele must wait until a distance greater than 5 mm from the root cap junction before bands 8 and 9 appear at 15-20 mm.
There has been little further attempt to determine the roles of these enzymes, particularly the cell wall-bound enzymes of the stele. It is certain that at least one isoenzyme is involved with xylogenesis and in particular, with lignification (2). Preliminary studies using syringaldazine or homovanillic acids have been inconclusive. Both substrates have yielded a positive response from endodermal cells of embryos from red kidney bean seeds soaked in water for 24h, but no reactions have been seen in other vascular cell types. This may be due, in part, to the lability of the enzyme reaction product from unfixed sections, diffusing away from the sites of activity prior to being observed. Peroxidase activity might be expected to occur with syringaldazine within the first 5 mm from the root cap junction especially as the genetic programme for secondary cell wall production is available by 18-22 cells from the root cap junction (23) whilst increased UDP-P-D-glucose dehydrogenase activity occurs at about the same point (20). Further refinement of the methodology will be necessary to determine the moment of switch-on of this activity.

The production of peroxide for the final step in lignin formation may be through the formation of NADH by wall-bound malate dehydrogenase activity, peroxide being generated from NADH either by a peroxidase or via the formation of superoxide ions (12) or by a cell wall-bound polyamine oxidase (19). Attempts to cytochemically localize cell wall malate dehydrogenase have not always been successful (10). This may be due either to a lack of malate dehydrogenase or to the nature of the cytochemical reaction. Dehydrogenases can be localized in plant cells using tetrazolium salts (5), but through a two-step reaction. The first step involves the action of the enzyme on substrate with a concomitant reduction of \( \text{NAD(P)}^+ \); the second step is the oxidation of \( \text{NAD(P)}^- \) via the tetrazolium salt with the formation of \( \text{NAD(P)}^+ \) and a coloured, water-insoluble formazan. The second step is catalysed by a pyridine nucleotide-tetrazolium reductase system which is tightly membrane-bound, such sites being demonstrable only in the cytoplasm and not in the cell wall. Thus, if present in the cell wall, malate dehydrogenase activity is unlikely to be localized by a tetrazolium reaction.

A number of studies have indicated that dormancy break has a primary involvement of the pentose phosphate pathway (6). Glucose-6-phosphate dehydrogenase is a far-from equilibrium enzyme forming a flux-generating control step of the pentose phosphate pathway, the cessation of which, in dormancy, is thought to be related to the complete reduction of all \( \text{NADP}^- \). Thus, reoxidation of the \( \text{NADPH} \) would result in the initiation of pentose phosphate pathway activity and hence dormancy break. Seeds of *A. fatua* obtained 6 months after harvest and exhibiting deep dormancy were imbibed with either water or 8.7 \( \times 10^{-3} \) M \( \text{GA}_3 \).
after dehusking the seeds. The water-treated seeds failed to germinate whilst all the GA$_3$ seeds germinated after treatment for 10 days at 10°C. A quantitative cytochemical study of the embryos of such seeds showed that glucose-6-phosphate dehydrogenase activity was doubled in the embryo, but not in the scutellum, this event occurring in advance of any change in cell size or cell number in the embryo i.e. before germination (6).

One suggested method by which the NADPH may be oxidized is through the action of glutathione peroxidase activity. Although a glutathione stimulated peroxidase activity can be observed in plant tissues, no selenium-containing glutathione peroxidase as found in animal tissues has so far been isolated from plants (15).

A cytochemical examination of peroxidase activity using DAB, homovanillic acid and p-phenylenediamine/pyrocatechol reactions on tissues from embryos of *A. fatua* and *L. sativa* (5) has shown a
similar localization pattern with the three substrates. Relatively little peroxidase activity is seen in the tissues which show a doubling of glucose-6-phosphate dehydrogenase activity in *A. fatua* (Fig. 1). In fact, in embryos of *L. sativa*, most peroxidase activity occurs in tissues in which it is difficult to demonstrate dehydrogenase activities (6). This may be due in part to the lack of dehydrogenase activity, but also to a malfunction of the pyridine-nucleotide-tetrazolium reductase system in those regions not showing G6PD activity. This, in turn, could reflect membrane damage by superoxide molecules derived during the desiccation of the seed, and leading also to the production of H$_2$O$_2$. Thus, the observed peroxidase activity may not necessarily be linked to dormancy-break, but instead may be needed to reduce the levels of H$_2$O$_2$ being generated at specific times during desiccation and imbibition of the seed.

**Acknowledgements**

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