Properties and Subcellular Distribution of Peroxidases of Onion Stem[1]

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Abstract

Peroxidases detected by cytochemical procedures are compartmentalized in several cell components of meristematic stem cells of onion including cell walls and vacuoles. In fractions, however, most of the activity is soluble suggesting that the peroxidases are released as organelles are disrupted. As the duration of homogenization is increased, activities of glucose-6-phosphate dehydrogenase, acid phosphatase, and peroxidases at pH 6.5 and 7.0 increase in the soluble fraction. The membrane-bound enzyme succinic dehydrogenase is not solubilized by homogenization. We conclude that the peroxidases may be compartmentalized within the cell, but are readily solubilized by conventional cell fractionation techniques.

Peroxidases comprise a complex of enzymes found in most plant and animal cells. They catalyze the oxidation of a wide range of donors in the presence of hydrogen peroxide (16, 21, 30). Peroxidases have been implicated in cell division (28), fatty acid oxidation (27), lignification (3, 13), and in the synthesis and breakdown of the plant hormone indoleacetic acid (6, 25). Additionally, peroxidases are a class of enzymes that can be located at the subcellular level by electron microscopy (8, 10, 22, 24). Thus, they are especially useful as 'marker' enzymes for cytological studies. The purpose of this study was to determine the subcellular distribution of peroxidase activities using a combined cytochemical and biochemical approach.

Materials and Methods

Green onions (*Allium cepa* L.) were obtained commercially. The stem axes were cut from the onions using a scalpel with a narrow blade as described previously (18).

For cytochemical studies, stem axes were diced with a razor blade in 2% glutaraldehyde prepared in 0.1 M sodium phosphate buffer, pH 7.6. Tissue pieces approximately 1 mm in diameter were transferred to fresh buffered 2% glutaraldehyde and fixed for about 30 min. To localize peroxidases, the fixed tissue was washed with 0.1 M sodium phosphate buffer, pH 7.6 (3 changes of 20 min each) and incubated with a solution containing reduced 3, 3'-diaminobenzidine (2 mg/ml) and 0.02% hydrogen peroxide in 0.05 M tris-HCL buffer, pH 7.6 for 1 hour at 26° C as modified from Graham and Karnofsky (8). The tissue was then washed with 0.1 M sodium phosphate buffer, pH 7.6 (3 changes of 20 min each) and post-fixed with 2% osmium tetroxide buffered with 0.1 M sodium phosphate, pH 7.6. The tissue was washed

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with buffer, dehydrated through an acetone series, embedded in Epon and prepared for electron microscopy as described previously (26). Thin sections were examined and photographed with a Phillips EM 200 electron microscope.

To prepare cell fractions, 20 g of the tissue were finely minced with a razor blade (on a glass surface over ice) in 10 ml of homogenization medium. The homogenization medium consisted of freshly prepared 0.1 M K_2HPO_4 and 20 mM EDTA in Millipore-filtered coconut milk, pH 7.4, and containing 0.5 M sucrose (9). An additional 10 ml of homogenization medium were added, and the homogenization was continued for 3 min with a mechanical homogenizer, the Polytron (19), operated at 5,000 rpm. The homogenate was filtered through Miracloth, a porous inert fabric, to remove unbroken cells and debris. The filtered homogenate was then centrifuged at 8,000 $\times$ *g* (7,000 rpm, Sorvall HB-4 rotor) for 15 min to remove nuclei, plastids, and mitochondria. The supernatant from this centrifugation was layered on a discontinuous sucrose gradient (29, Fig. 1) and centrifuged at 90,000 $\times$ *g* (26,000 rpm, Spinco SW-27 rotor) for 60 min. The materials collecting at the gradient interfaces were collected with a Pasteur pipette, re-suspended in homogenization medium, and pelleted by centrifugation at 90,000 $\times$ *g* for 45 min.

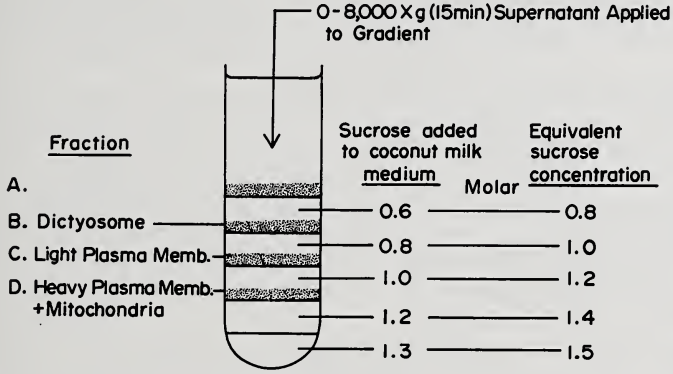


FIGURE 1. *Diagram of the discontinuous sucrose and coconut milk gradient used to isolate membrane fragments (29).*

In experiments to vary homogenization times, samples were removed at 30 sec intervals during Polytron homogenization. The samples were filtered through Miracloth and centrifuged for 30 min at 90,000 $\times$ *g* (26,000 rpm, Spinco SW-27 rotor) to remove cellular membranes. The supernatant was used in the assays for soluble enzymes of Fig. 9.

All steps of cell fractionation and centrifugation were at 0-4° C. Centrifugal forces are approximate and calculated to the middle of the tube.

To monitor peroxidase activity of isolated cell fractions, guaiacol in the presence of hydrogen peroxide was oxidized to form tetraguaiacol

(Fig. 2). The formation of tetraguaiacol was followed spectrophotometrically at 470 nm according to the procedure of Chance and Maehly (4). A problem with this method is that the orange-red color of the tetraguaiacol fades rapidly (15). To minimize the effects of the fading reaction on kinetic measurements, the initial velocity was estimated by extrapolation as illustrated by the dotted lines of Figure 3. To demonstrate the fading reaction, the enzyme was inhibited with potassium cyanide after color development (Fig. 3). Both the decrease in optical density after adding potassium cyanide and the departure from linear kinetics in the absence of cyanide was due to the fading reaction.

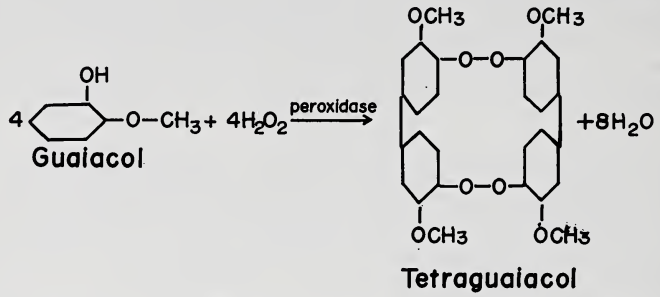


FIGURE 2. *Overall reaction of the guaiacol assay for peroxidase activity. In the reaction, 4 moles of guaiacol are oxidized in the presence of 4 moles of hydrogen peroxide to form one mole of tetraguaiacol which absorbs at 470 nm.*

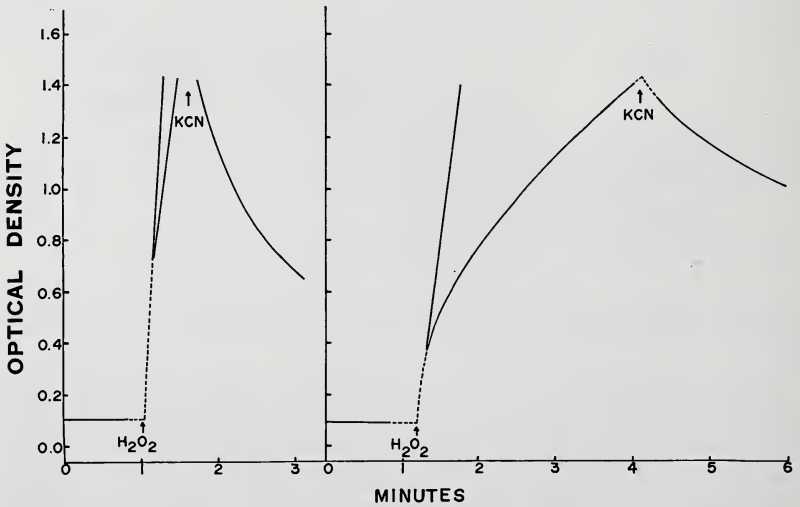


FIGURE 3. *Kinetics of tetraguaiacol formation and fading. The initial rate of absorbance change is shown by the dotted line extrapolated back to the time when the reaction was initiated by adding hydrogen peroxide. The gradual departure from linearity is due to fading of the chromogen. After color development, the enzymatic reaction was stopped by addition of cyanide to show the rapidity of the fading reaction. The departure from linearity due to fading was intensified under suboptimal assay conditions. Left: Near optimal conditions of 10 mM hydrogen peroxide and 14mM guaiacol, pH 7.0. Right: 1 mM hydrogen peroxide and 2.5 mM guaiacol, pH 7.0.*

The assay for glucose-6-phosphate dehydrogenase was reduction of NADP in the presence of glucose-6-phosphate (11). The reaction was followed spectrophotometrically at 340 nm.

Acid phosphatase was measured by incubating 20 mM *p*-nitrophenyl-phosphate, 50 mM tris-acetate, pH 4.5 and samples of fractions for 15 min. The liberation of *p*-nitrophenol was estimated from the increase in absorbance at 400 nm after addition of 0.2 N sodium hydroxide (2).

Succinic dehydrogenase (succinic-INT-reductase) was assayed by the colorimetric procedure of Pennington (23) with 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium (INT) as substrate.

All enzyme assays were at room temperature, 25-26° C, under conditions where product formation or disappearance of substrate was proportional to the time of incubation and to the quantity of protein present. Values were corrected for zero time and no-enzyme or boiled enzyme blanks. Protein was determined by the procedure of Lowry *et al.* (14).

Results

When onion stem was incubated for cytochemical detection of peroxidase activities, electron-dense deposits of oxidized diaminobenzidine were observed in the vacuole, on the tonoplast, and in the cell wall (Fig. 4). As in previous studies, including studies with onion (28), peroxidase activity was found to be greatest in young, dividing cells and least in older cells which no longer divided.

Filtered total homogenates were used to investigate the conditions for the peroxidase assay *in vitro* (Figs. 5-8). Figures 5 and 6 show the results of varying peroxide and guaiacol concentration. At 10 mM hydrogen peroxide and 14 mM guaiacol, activity was proportional to protein concentration over a wide range (Fig. 7). Two distinct pH optima, at pH 6.5 and pH 7.0, were found (Fig. 8) as well as shoulders at pH 6.0 and possibly also at pH 7.5. Using citrate buffer, activity was also observed over the pH range 3.5 to 6 with an optimum at 4.5 (data not shown).

During cell fractionation, peroxidase activity of the filtered homogenates was concentrated in the 90,000 $\times$ *g* supernatants (Table 1). In contrast to the cytochemical evidence, little activity was associated with any of the particulate fractions. Of the total activity of the filtered homogenates, 80 to 85% was recovered. Of the recovered activity, 3% was particulate and 97% was soluble.

To reconcile the cytochemical findings of compartmentalized peroxidases with the biochemical findings of soluble peroxidases, we studied enzyme solubilization as affected by varying times of homogenization (Fig. 9). Glucose-6-phosphate dehydrogenase, as an example of an enzyme found in the cytosol, and acid phosphatase, as an example of an enzyme thought to be in the vacuole (17), were released most rapidly and reached a maximum in the soluble fraction after 1 min of homogenization. Peroxidases were released more slowly and reached a maximum after about 3 min of homogenization. Peroxidase assayed

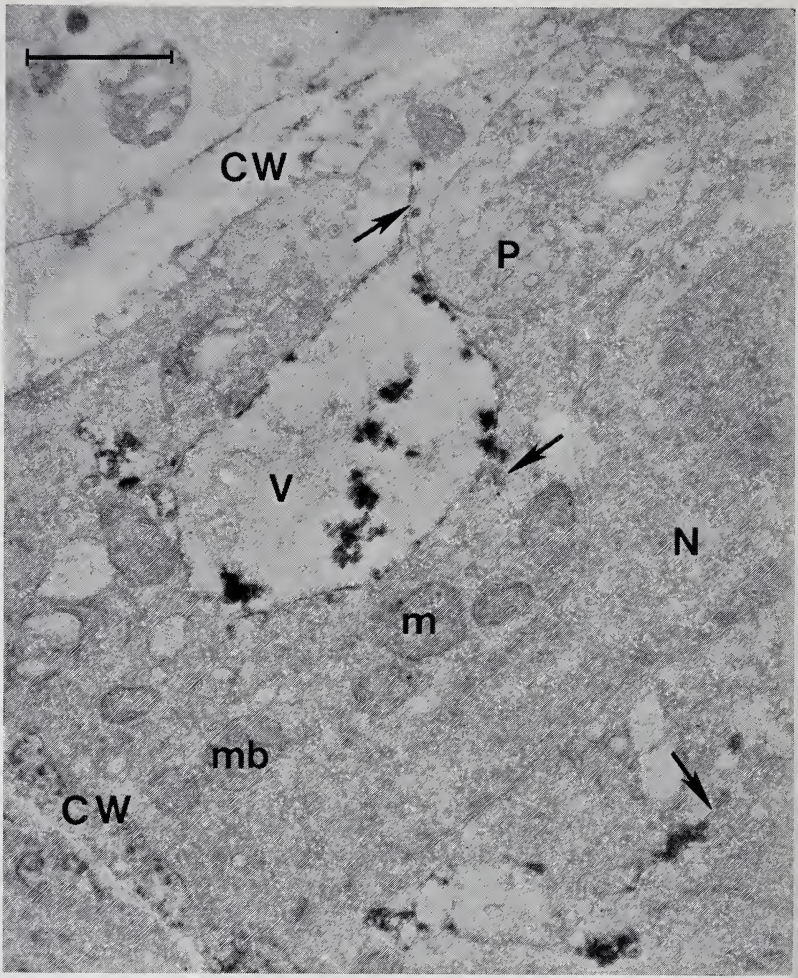


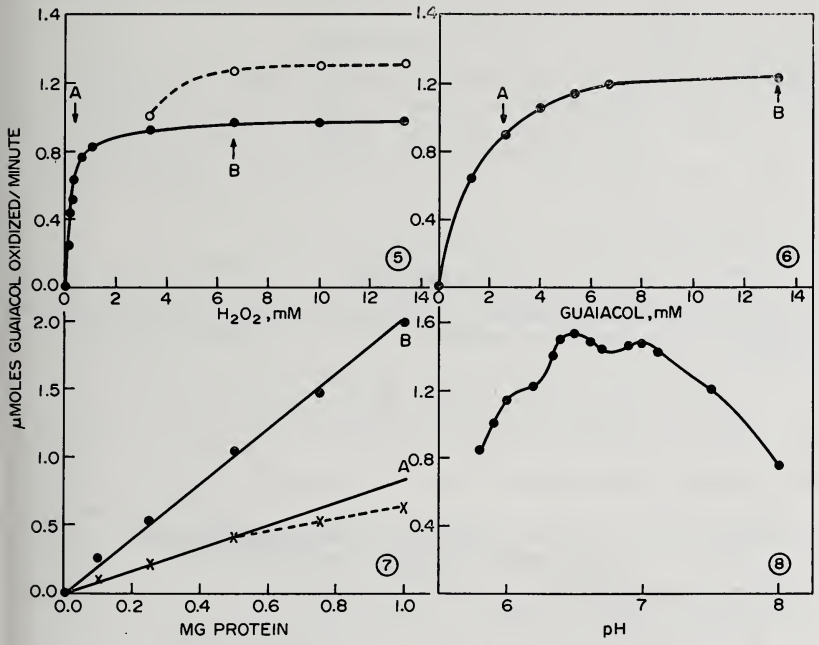
FIGURE 4. *Electron micrograph of a portion of a meristematic cell of onion stem fixed and incubated for peroxidase activity. Electron-dense deposits of oxidized 3,3'-diaminobenzidine were found in the cell wall (CW), vacuole (V) and in flattened cisternae resembling endoplasmic reticulum, which are continuous with the vacuole proper (arrows). In these cells, microbodies (mb) and mitochondria (m) lacked heavy deposits of oxidized diaminobenzidine. P=plastid. N=nucleus. Scale marker=1 u.*

at pH 6.5 was released more rapidly than the peroxidase assayed at pH 7.0. Succinic dehydrogenase, an enzyme of the inner membranes of mitochondria, was not solubilized by the homogenization procedure (Fig. 9).

Discussion

By cytochemical procedures, peroxidase activity has been found in mitochondria, endoplasmic reticulum, Golgi apparatus, cell walls and vacuoles of plant cells (7, 10, 24). The pH data, as well as those of

others (1, 5, 7, 12, 20), suggest multiple forms or isozymes of peroxidases; Evans (5) reported 12 isozymes for dwarf tomatoes. Our study was initiated to determine whether specific peroxidase isozymes were associated with specific cell components. If so, multiple forms of peroxidase would provide a set of marker enzymes which might help identify plant cell components isolated from cell homogenates. However, in the *in vitro* analyses, the peroxidase activity was largely solubilized by the cell fractionation procedures. No membrane fraction was significantly enriched in peroxidase, and 97% of the recovered activity was present in the post-microsomal supernatant (soluble fraction). This contrasted with the *in vivo* cytochemical findings and with the results for succinic-INT-reductase, a membrane-bound enzyme.



FIGURES 5-8. *Characteristics of the peroxidase of filtered homogenates of onion stem: 5) Peroxidase activity shown in relation to peroxide concentration at pH 7.0. The lower curve is at 2.5* mM *guaiacol. The upper curve is at 14* mM *guaiacol. 6) Peroxidase activity with increasing guaiacol concentration at pH 7.0 and 10* mM *hydrogen peroxide. 7) Peroxidase activity is proportional to protein concentration between 0 and 1 mg protein per assay for conditions of guaiacol and hydrogen peroxide marked by the letter B in Figures 5 and 6 and between 0 and 0.5 mg protein per assay for conditions marked by the letter A in Figures 5 and 6. 8) Peroxidase activity as a function of pH. The buffering system consisted of 0.01* M *sodium phosphate adjusted with hydrochloric acid. For each determination, the pH was measured at the beginning and at the end of the reaction and rmained constant ± 0.1 unit.*

To reconcile the *in vivo* and *in vitro* findings, a series of experiments was carried out in which the release of several enzymes was related to the degree of cell homogenization. The results show clearly that peroxidase was solubilized as the tissue was homogenized. The rate of

solubilization was sufficient to release over 50% of the peroxidase in soluble form by the time release of cytoplasmic (glucose-6-phosphate dehydrogenase) and vacuolar (acid phosphatase) contents was complete. The remaining peroxidase was apparently solubilized in subsequent homogenization and/or centrifugation steps to account for the lack of peroxidase in the purified membrane fractions.

TABLE 1. *Distribution of peroxidase activity among isolated cell fractions of onion stem.*

Fraction	Total Activity[1] (umoles/hr)	Specific Activity (umoles/hr/mg protein)
Filtered Homogenate	200	51
0-8,000 X g pellet	2.5	0.4
8-90,000 X g pellet	3.3	0.5
Gradient fractions: A	(0.4)[2]	0.3
B	(0.4)	0.3
C	(0.6)	0.3
D	(0.2)	0.3
90,000 X g supernatant	160	100

[1] % Recovery=81.8 ± 2.8.
% Activity of total recovered in particulate fractions=3.0 ± 1.8.

[2] Numbers in parenthesis refer to subfractions of the 8-90,000 X g pellet recovered from the sucrose gradient of Figure 1. Of the 3.3 units of activity applied to the gradient, 1.6 units were recovered which shows that 50% of the activity was lost or solubilized during the sucrose gradient procedure. All fractions were assayed by conditions A of Figures 5-7.

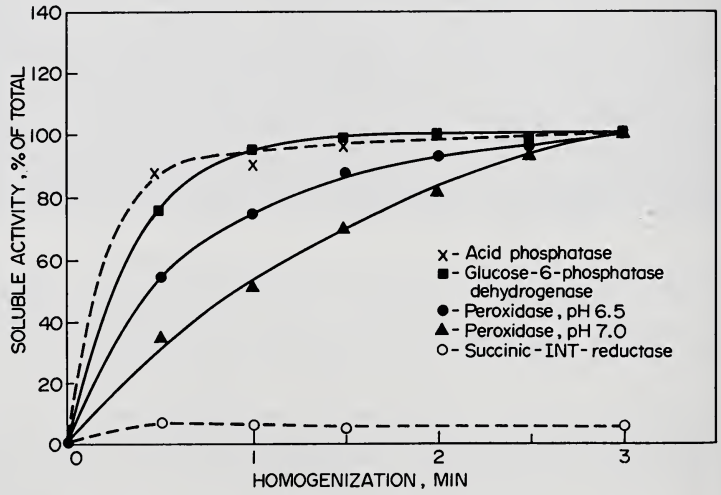


FIGURE 9. *Comparative rate of release of a cytoplasmic enzyme (glucose-6-phosphate dehydrogenase), a presumed vacuolar enzyme (acid phosphatose), an enzyme of the inner membranes of mitochondria (succinic-INT-reductase) and peroxidases at pH 6.5 and pH 7.0 as a function of time of homogenization with the Polytron operated at a constant speed of 5,000 rpm. Soluble activity was determined in the supernatant after centrifugation for 30 min. at 90,000 X g. Peroxidase was assayed according to conditions B of Figures 5-7.*

On the basis of the cytochemical and biochemical findings, we conclude that plant peroxidases are compartmentalized intracellularly but that the bulk of the activity is not tightly bound to membranes or at least is readily released from the membranes. The peroxidases are readily solubilized during cell fractionation, an observation that explains our *in vitro* results but adds a new order of complexity to an already complicated problem. Procedures such as glutaraldehyde fixation prior to fractionation to bind the peroxidase to the membranes or for cross-linking the enzymes within a compartment might provide an interim solution. In any event, it is clear that conventional methods of cell fractionation are unlikely to provide definitive information on the subcellular distribution of peroxidases in plants because of the rapid solubilization of the enzymes.

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