Plant Physiol. (1978) 62, 789–793

Time Course and Spatial Distribution of Phenylalanine Ammonia-Lyase and Peroxidase Activity in Wounded Potato Tuber Tissue[1]

Received for publication January 9, 1978 and in revised form July 8, 1978

ROLF BORCHERT
Department of Botany and of Physiology and Cell Biology, University of Kansas, Lawrence, Kansas 66045

ABSTRACT

The time course and spatial distribution of wound-induced activities of phenylalanine ammonia-lyase and peroxidase were determined to establish correlations between molecular and cellular aspects of the wound-induced pattern of cell differentiation in potato (*Solanum tuberosum* L.) tissue. A high correlation between peroxidase activity and suberization was observed. Peroxidase activity increased for several days after wounding. Peroxidase content of suberizing cells was more than 10 times higher than that of the immediately adjacent dividing cells. Suberizing and dividing cells contained different isoperoxidases. Neither time course nor spatial distribution of the wound-induced activity of phenylalanine ammonia-lyase was directly correlated with the wound-induced pattern of cell differentiation.

Cell differentiation, the differential acquisition of specific properties by cells of a previously homogeneous cell population, comprises temporal and spatial components. The characteristic time course, in which cell-specific molecular and structural components appear during cell differentiation, as well as its possible controls, are currently the object of intensive studies in many animal and plant systems. Except for theoretical considerations (*e.g.* 1, 7), little practical attention appears to have been paid to the analysis of the fact that during the formation of a pattern of cell differentiation, neighboring cells may acquire profoundly different biochemical capacities.

Wounding the storage tissue of a potato tuber activates a variety of metabolic processes (11) as well as a characteristic spatial pattern of cell differentiation which leads, within a few days after wounding, to the complete healing of the wound (5). Suberin, a co-polymer of fatty acid alcohols and phenylpropanoids (12), is deposited on the cell walls of the living cells adjacent to the wound surface, and cell division, accompanied by degradation of storage starch granules, occurs in one or two subperipheral cell layers (Fig. 1). To gain an understanding of the molecular controls involved in the establishment of this pattern of cell differentiation, the biochemical events which precede and accompany cell differentiation, that is, the correlations between the molecular and cellular aspects of cell differentiation, must be identified.

If a biochemical process is postulated to be specifically related to a certain type of cell differentiation, the following minimum requirements should be met: (*a*) it should occur exclusively in those cells undergoing cell differentiation; (*b*) its rate or activity should change significantly before or simultaneously with the observed cellular changes; (*c*) experimental induction or inhibition of cell differentiation should affect the biochemical events in parallel; (*d*) specific inhibitors of the molecular process should inhibit cell differentiation. Wound-induced DNA synthesis was found to be limited to those cell layers undergoing wound-induced cell division. It preceded mitosis by several hr, and inhibitors of DNA synthesis blocked cell division (5). Degradation of starch granules is confined to cells undergoing cell division and is prevented by inhibitors of DNA synthesis and mitosis (5). As suberization occurs only in the outermost living cells near the wound surface, it may be inferred that biosynthesis of suberin precursors is also limited to these cells.

Because most studies of metabolic activation in wounded tissues have dealt only with carbohydrate metabolism there is a scarcity of established correlations between molecular and cellular differentiation during wound healing. Carbohydrate metabolism is unlikely to be specifically correlated with cell differentiation, and often the study of the specific spatial distribution of the metabolic processes is omitted. The basic goal of this study is to illustrate the need for correlating time course and spatial distribution of biochemical activities with the observed pattern of cell differentiation.

In preliminary experiments we observed that cells of the outermost suberized cell layer of wounded potato tissue always contained about 10 times the activity of PAL[2] and PER than did the underlying tissue. As both enzymes have been postulated to control lignification (6, 9), we examined the correlation of their occurrence with the wound-induced pattern of cell differentiation in more detail.

MATERIALS AND METHODS

Kennebec potatoes (*Solanum tuberosum* L., cv. Kennebec), grown locally or obtained by courtesy of the Red River Valley Potato Processing Laboratory at East Grand Forks, Minn., and stored at 3 to 4 C, were used throughout this work.

To analyze enzyme activities in different tissue layers, potatoes were cut with a knife into 1.5-cm slices, the surfaces of which were then smoothed with a Berkel meat slicer model 807. Slices were rinsed well in deionized H_2O and stored in moist chambers at 25 C. At the desired time after wounding, tissue samples comprising different cell layers were obtained in several ways. During the first 3 days after wounding, three consecutive 0.5-mm slices containing about four cell layers (Fig. 1A) were cut with the meat slicer from each surface of the thick slices. From about 4 days after wounding, the suberized surface layer of the slices could be removed with a spatula. It was rinsed immediately in water, and the next 0.5-mm layer was then cut with the meat slicer (Fig. 1B). Alternatively, suberin was removed from both surfaces of 1.5- to 2-mm potato slices (Fig. 1C), and suberizing and remaining cells, including the dividing cells, were assayed separately.

[1] Supported in part by National Science Foundation Grant BG-29313 and by a Biomedical Sciences Support Grant (PHS Award RR 07037) to the University of Kansas.

[2] Abbreviations: IPER: isoperoxidase; PAL: phenylalanine ammonia-lyase; PER: peroxidase.

Tissue was homogenized in 10 times its fresh weight of ice-cold 0.02 M borate buffer (pH 8.8) containing 5 mM bisulfite and filtered into a chilled aspiration flask. One aliquot of the filtrate was diluted with the same amount of 0.02 M borate buffer containing 25 mM mercaptoethanol and used immediately for the determination of PAL according to the method of Zucker (22). A second aliquot was diluted with the same amount of 0.1 M Na-acetate (pH 5.4) and frozen for later use in the determination of PER activity. The assay mixture contained 1 ml of 0.1 M acetate buffer (pH 5.4), 1 ml of 50 mM guaiacol, 1 ml of 30 mM H_2O_2, and 0.5 ml of enzyme solution. The enzyme extract was diluted to produce a change in O.D. of 0.5 to 0.8 in 4 min at 460 nm. A third aliquot was diluted with extraction buffer, frozen, and used for the determination of protein content by the method of Lowry *et al.* (14). To avoid interference of phenolics with the assay, proteins to be assayed were precipitated with 10% trichloroacetic acid, centrifuged, and redissolved in 1 N NaOH. As no significant differences in protein content between different tissue layers were observed, enzyme activities are expressed on a fresh weight base. As protein content was usually 10 to 12 mg/g fresh weight, enzyme activities can be converted to specific activity by dividing by 11.

Isoperoxidases (IPER) were separated electrophoretically in a polyacrylamide slab gel which permits the simultaneous separation of anodic (acidic) and cathodic (basic) IPER (4) in an LKB 2117 Multiphor apparatus. Gels consist of 3 cm of anodic and 7 cm of cathodic separating gels after Reisfeld *et al.* [17] 6% cyanogum, 0.4 M K-acetate (pH 4.3), 1% N,N,N′,N′-tetramethylethylene diamine, 0.14 NH_4-persulfate) separated by 1 cm of stacking gel (6% cyanogum, 0.06 M K-acetate [pH 6.7], 5 μg/ml riboflavin). The tray buffer contains 31.2 g/l β-alanine and is adjusted to pH 4.6 by glacial acetic acid. Tissue samples were frozen at the time of sampling and thawed just prior to electrophoresis. The crude sap was applied to the interphase between anodic separating and stacking gel by means of filter paper wicks holding 5 to 8 μl of sap. Electrophoresis was carried out at 200 v and 32 mamp for about 6 hr, until Cyt *c*, used as a marker protein, had arrived 1 cm from the cathodic edge of the gel. Gels were stained with 3-amino-9-ethyl carbazole according to Graham *et al.* (8) and fixed with methanol-acetic acid.

To obtain relative activities of different groups of IPER at different times after wounding, acrylamide gels were scanned with an EC910 densitometer at 500 nm, and integrator values for each absorption peak or group of peaks were reduced by integrator values for a corresponding section of nonstained gel.

RESULTS

As a first step in examining the relationship between wound-induced formation of PAL or PER and cell differentiation, the temporal and spatial pattern of both processes must be compared. Ideally, the time course of enzyme activity should be determined in each cell layer below the wound surface. As there are no suitable methods to measure the activities of PAL and PER histochemically, and mechanical separation of individual cell layers is impossible during the first days after wounding, the following methods were used:

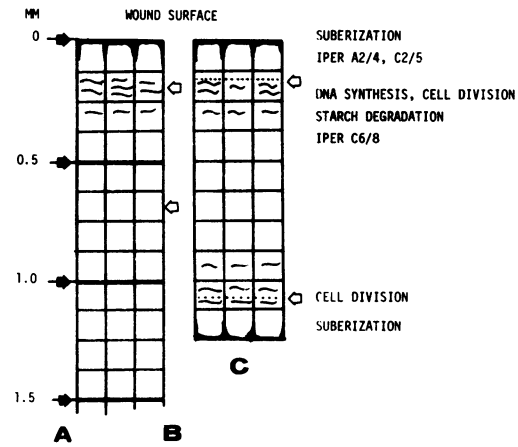

FIG. 1. Schematic representation of wounded potato tissue showing components of wound-induced cell differentiation pattern, location of tissue specific biochemical activities, and position of cuts (arrows) in different experimental techniques used to separate different cell types. A: three consecutive 0.5-mm slices cut from top of thick (1.5 cm) tissue slice; B: suberized layer and next lower 0.5-mm slice cut from thick slice; C: removal of suberized surface layers from 1.5-mm slice.

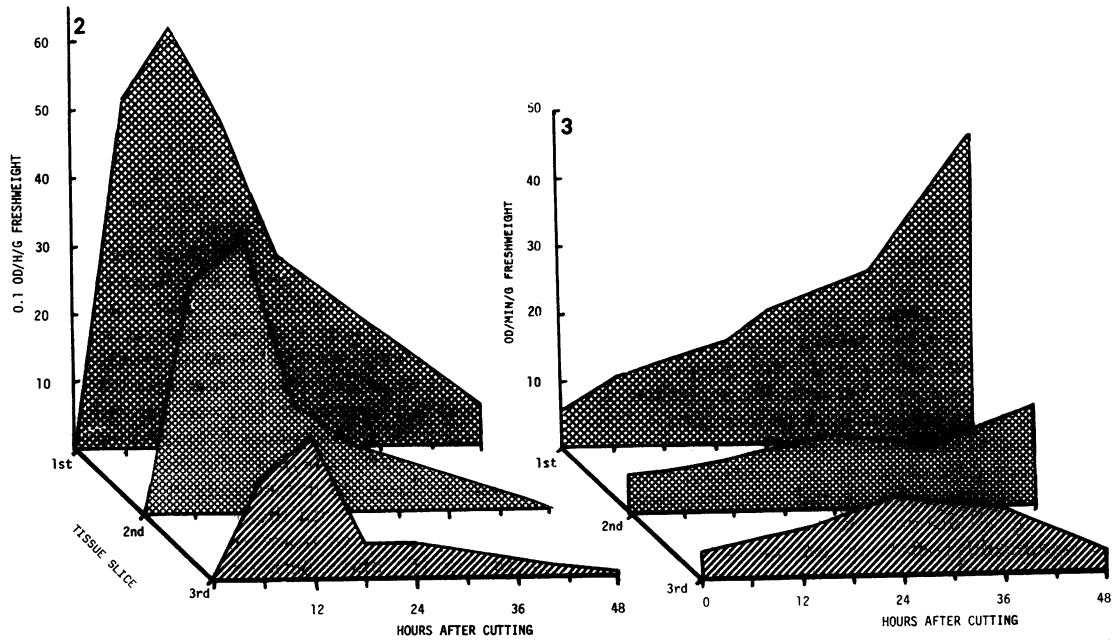

FIG. 2 and 3. Time course of phenylalanine ammonia-lyase (Fig. 2) and peroxidase (Fig. 3) in three 0.5-mm slices below the wound surface during the first 48 hr after cutting. Material was cut as shown in Figure 1A. PAL activity of 0.1 O.D./hr is equivalent to the production of 0.22 μmol cinnamic acid/hr.

a. By means of a meat slicer, three 0.5-mm slices were cut from the surface of a wounded potato tuber. As the diameter of storage parenchyma cells is about 150 μm, these slices are about four cell layers thick, which means that the top slice contains both the suberizing and the dividing cell layers, and a distinction between enzyme activities in different cell types is not possible (Fig. 1A).

b. Three to 4 days after wounding, the suberized cell layer can be mechanically separated from the remaining tissue, because suberization fuses the surface layer of cells into a coherent, mechanically resistant skin, and cell walls of the underlying newly divided cells are weak. By removing suberin from the surface of a thick slice (Fig. 1B) or from the two surfaces of a 1.5-mm slice (Fig. 1C), the enzyme activities in the suberin layer and in the remaining tissue, including newly divided and nondividing cells, can be assayed separately. A combination of these two methods allows the assessment of the time course of total enzyme activity after wounding and of the distribution of enzyme activities between the two major types of differentiating cells.

c. Total PER activity in wounded potato tissue represents the sum of the activities of several isoperoxidases (IPER), which constitute specific markers of wound-induced cell differentiation, because certain IPER occur exclusively in either suberizing or dividing cells (2). Separation of IPER by acrylamide electrophoresis and subsequent quantification by densitometry permit the determination of the time course and distribution of relative IPER activity in the wound-induced differentiation pattern and can be related to the time course of total PER activity.

The time course of PAL in wounded potato tuber tissue shows the characteristic pattern described earlier (21) for potato discs aged in a shallow layer of buffer solution, namely a rapid rise in enzyme activity followed by an equally rapid drop (Fig. 2). However, probably due to our use of thick slices incubated in air, enzyme activity peaks as early as 12 hr after cutting, as compared to 24 hr in Zucker's experiments, and peak activities in the top tissue layer were consistently many times higher than those found by Zucker in thin discs aged in solution (9–22 *versus* 2–4 μmol cinnamic acid/g fresh weight), demonstrated a striking effect of variation in experimental conditions. The rapid increase in PAL activity occurs in all three slices below the wound surface, although with gradually declining intensity. Two days after wounding, PAL activity in the second and third layers has declined to very low activities (Fig. 2), and 5 days after wounding, activity in the inner cell layers is at most 1/10 of that in the suberizing cells of a tissue slice.

In contrast to PAL, the activity of PER in wounded tissue continues to increase for several days (Figs. 3 and 5). Most of the activity is concentrated in the four top cell layers (Fig. 3), and 4 days after wounding the PER activity of the suberizing layer is always 8 to 10 times higher than that of the underlying, nonsuberizing tissue. It has been shown earlier (2) that of the various wound-induced IPER the most cathodic bands (IPER C6/8) are found only in the dividing cells, whereas all others are confined to the suberin layer. While total PER activity increases for 4 or 5 days, the relative contribution of individual groups of IPER changes (Figs. 4 and 5). The increase in IPER C6/8 levels off after 3 days, and its relative contribution to total PER activity declines. Inversely, IPERs, located in the suberin layer are responsible for the over-all increase in activity and thus contribute an increasingly larger fraction of total activity. The relative composition of IPERs in the suberin layer changes, as groups A2/4, C4/5, and C2/3 appear at successive days.

The data represented in Figure 5 were obtained with 1.5-mm slices of potato tissue, containing approximately 10 cell layers. Assuming that there are two layers of suberizing cells and eight layers of dividing and nondividing cells, the relative PER activity can be calculated for the different cell layers as follows from the data in Figure 5:

Activity (suberizing cells) = Activity (IPER A2/4 + C2/5)/2
Activity (remaining cells) = Activity (IPER C6/8)/8

Table I shows that the PER activity in the suberizing cells increases even more dramatically than apparent from Figure 5

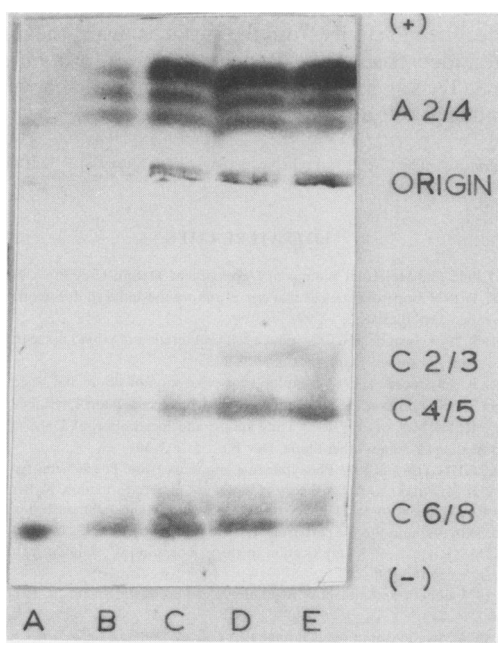

FIG. 4. Acrylamide electrophoresis of isoperoxidases in 1.5-mm slices of potato tuber tissue. A–E: changing IPER pattern during wound healing from 1st (A) to 5th (E) day after cutting.

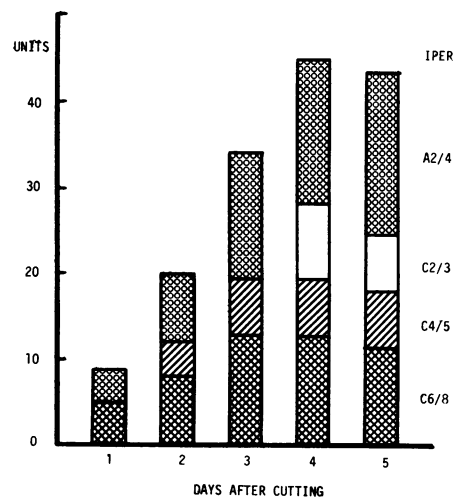

FIG. 5. Time course of total PER activity and of relative activities of various IPER groups in a 1.5-cm slice during 5 days after cutting. Enzyme activities given in units of relative density obtained from densitometric scanning.

Table I. Distribution of peroxidase activity in suberizing and non-suberizing cells of a 2 mm thick tissue slice

The data are calculated from Figure 5 as described in the text. Units are relative densitometer readings.

Day	Activity of IPER		Activity per cell		Ratio
	A2-4, C2-5	C-6	suberizing	non-suberizing	
1	4	5	2	0.63	3.2
2	12	8	6	1.0	6.0
3	22	13	11	1.63	6.7
4	32	13	16	1.63	9.8
5	32	12	16	1.5	10.7

and, within 4 days, leads to a value 10 times that of the remaining cells. This result agrees with earlier measurements of PER activity in suberizing and nonsuberizing cells.

DISCUSSION

There can be no doubt that the wound-induced activation of phenylpropanoid biosynthesis, including the biosynthesis of PAL, is a necessary prerequisite for the observed biosynthesis of chlorogenic acid (21) and the phenolic components of suberin (lignaceous materials) in wounded potato tissue. Figure 2 shows clearly that for several reasons wound-induced PAL activity is unlikely to be a specific control or cause of the establishment of the characteristic pattern of wound-induced cell differentiation. The initial increase in PAL activity extends rather deep into the tissue and far beyond the layers undergoing cell differentiation, *i.e.* correlation between the distribution of PAL activity and cell differentiation is poor. The wound-induced synthesis of chlorogenic acid, other phenolic compounds, and suberin continues well beyond the period of highest PAL activity, *i.e.* these processes can proceed in the presence of relatively low activity.

Because of the frequently observed correlation between changes in the activity of PAL and in the rate of accumulation of flavonoids and other phenolics, PAL has been widely believed to control the biosynthesis of phenylpropanoids (6). Apart from the lack of correlation between cell differentiation and PAL activity described above, there are a number of studies in which no direct correlation between the level of PAL and the production of phenolic compounds in certain tissues was found (6, 16). In most studies the reported catalytic potential of PAL is much greater than required to synthesize the amount of phenylpropanoids accumulating in the tissue, which led to the conclusion that the synthesis of phenolics is usually not controlled by PAL, but by the amount of substrate, phenylalanine, available to the enzyme (16). This type of control fails to account for the cell-specific accumulation of certain phenylpropanoids during biochemical differentiation, which must be under the control of enzymes or substrates more specific for the biosynthetic pathways involved in the formation of cell-specific phenolics.

In contrast to the results obtained with PAL, correlations between the time course and spatial pattern of wound-induced PER and cell differentiation are excellent. (*a*) The specific occurrence of IPER C6/8 in dividing cells, and of all others in suberizing cells, as described earlier (2), has been confirmed. (*b*) Total PER activity in the suberizing cell layer increases for several days (Figs. 3, 4, and 5) and remains high thereafter, paralleling the time course of suberin synthesis, which starts at low intensity about 6 to 8 hr after wounding and continues at high rates for as long as 6 to 10 days after wounding (12; unpublished observations). (*c*) The increase in total PER activity in the suberized cell layer is due to the relative and absolute increase in the activity of those IPER which are characteristic of the suberin layer (Figs. 4 and 5; Table I). (*d*) If wounded potato tissue is washed in 0.01 M cyanide and subsequently stored in moist air containing 10% CO_2, suberization remains suppressed and surface cells of the wounded tissue proliferate (3). Preliminary experiments have shown that under these conditions IPER C6/8, but none of the IPER associated with suberizing cells, are induced (unpublished data).

The reported experiments thus satisfy three of the four conditions postulated in the introductory remarks as necessary for establishing good correlations between biochemical and cell differentiation. The fourth condition, inhibition of cell differentiation by a specific inhibitor of PER synthesis, cannot be tested at this time for lack of a suitable inhibitor.

As suberin contains a significant fraction of lignaceous materials, these observations are in agreement with the suggestion made by several authors, that peroxidases might be specifically involved in the oxidative polymerization of phenylpropanes into lignins (9, 13, 15). Nevertheless, there remain the questions as to the possible role of IPER C6/8 in the cytoplasm and cell walls of nonsuberizing dividing cells, the differential function of at least six different IPER in suberizing cells, and the control of the initiation of biosynthesis of individual IPER at different times after wounding (Figs. 3 and 4). We have observed (4) that a purified extract of IPER C6/8, the most basic IPER possessing an isoelectric point of more than pH 10.5, can be degraded *in vitro* to yield all other more acidic IPER found in extracts from suberizing cells, plus several others. A similar observation was made by Liu (13) with horseradish peroxidase. There is a possibility that IPER C6/8 may be the only IPER synthesized *de novo* after wounding, while all other IPER arise from C6/8 by posttranslational modification, possibly deamidation, in the suberizing cells. This suggestion agrees with our earlier observation that all IPER are charge isomers (unpublished data) and with literature reports indicating that all IPER are monomers and that genetic control of changes in IPER patterns related to tissue or organ development has been established only rarely (19). Another case of posttranslational control of a biochemical process closely related to wound-induced cell differentiation may be that of starch degradation in dividing cells. Available experimental evidence strongly suggests that starch granules in potato are degraded by phosphorylase, an enzyme which is present in high concentration in resting as well as wounded potato tuber tissue. Selective starch degradation in dividing cells must be controlled by the elimination of a barrier between enzyme and substrate, and not by *de novo* synthesis of the degrading enzyme.

This study illustrates the need for caution in making assumptions about the control of wound-induced biochemical processes in potato tuber tissue and their possible correlations with cell differentiation. While increases in metabolic activities of wounded potato tissue have been considered to result from wound-induced gene derepression (10), the above examples show that biochemical processes highly correlated with wound-induced cell differentiation may well be controlled at the posttranslational level. Direct correlations between biochemical processes and cell differentiation should be postulated only if there are significant, stepwise differences between the various cell types of the pattern of cell differentiation, as shown to exist for IPER, DNA synthesis, starch degradation, and suberization. Biochemical activities showing a gradual decline in activity from the surface toward the inner layers of the wounded tissue, like PAL, respiration, protein synthesis, and others (18, 20), are unlikely to be specifically correlated with wound-induced cell differentiation.

Acknowledgments The careful and reliable assistance of J. Sluss and P. Ketchum is acknowleged.

LITERATURE CITED

1. Bonner J 1965 The Molecular Biology of Development. Oxford Univ Press, New York
2. Borchert R 1974 Isoperoxidases as markers of the wound-induced differentiation pattern in potato tuber. Dev Biol 36: 391–399
3. Borchert R 1977 Cyanide and CO_2 induce cell proliferation in potato tissue in the absence of applied plant hormones. Plant Physiol 59: S-119
4. Borchert R, CJ Decedue 1978. Simultaneous separation of acidic and basic isoperoxidases in wounded potato tissue by acrylamide gel electrophoresis. Plant Physiol 62: 794–797
5. Borchert R, JD McChesney 1973 Time course and localization of DNA synthesis during wound-healing of potato tuber tissue. Dev Biol 35: 293–301
6. Camm EL, GHN Towers 1973 Phenylalanine ammonia-lyase. Phytochemistry 12: 961–973
7. Gierer A, H Meinhardt 1972 A theory of biological pattern formation. Kybernetik 12: 30–39
8. Graham RC, U Lundholm, JJ Karnovsky 1965 Cytochemical demonstration of peroxidase activity with 3-amino-9-ethyl carbazole. J Histochem Cytochem 13: 150–154
9. Harkin JM, JR Obst 1973 Lignification in trees: indication of exclusive peroxidase participation. Science 180: 296–298
10. Kahl G 1973 Genetic and metabolic regulation in differentiating plant storage tissue cells. Bot Rev 39: 274–299
11. Kahl G 1974 Metabolism in plant storage tissue slices. Bot Rev 40: 263–314
12. Kolattukudy PE, BB Dean 1974 Structure, gas chromatographic measurement, and function of suberin synthesized by potato tuber slices. Plant Physiol 54: 116–121
13. Liu E 1971 The isozymic forms of peroxidase found in the horseradish plant. PhD thesis. Michigan State Univ, East Lansing

14. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
15. Mäder M, A Nessel, M Bopp 1977 On the physiological significance of the isozyme groups of peroxidase from tobacco demonstrated by biochemical properties II. pH-optima, Michaelis constants, maximal oxidation rates. Z Pflanzenphysiol 82: 247–260
16. Margna U 1977 Control at the level of substrate supply—an alternative in the regulation of phenylpropanoid accumulation in plant cells. Phytochemistry 16: 419–426
17. Reisfeld RA, UJ Lewis, DE Williams 1962 Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 282–283
18. Rosenstock G, H Lange, G Kahl 1969 Wechselbeziehungen zwischen CO_2—Gaswechsel, Volumen und Oberfläche bei Gewebefragmenten von Speicherparenchym. Z Pflanzenphysiol 60: 173–186
19. Scandalios JG 1974 Isozymes in development and differentiation. Annu Rev Plant Physiol 25: 225–258
20. Zimmerman HJ, G Rosenstock 1977 Die Lokalisation traumatisch induzierter Stoffwechselprozesse beim Speichergewebe der Kartoffelpflanze. Z Pflanzenphysiol 82: 144–160
21. Zucker M 1965 Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol 40: 779–784
22. Zucker M 1968 Sequential induction of phenylalanine ammonia-lyase and a lyase-inactivating system in potato tuber disks. Plant Physiol 43: 365–374