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EFFECT OF GAMMA IRRADIATION ON PEROXIDASE ISOENZYMES DURING SUBERIZATION OF WOUNDED POTATO TUBERS

PAUL THOMAS* and HENRY DELINCÉE

Bundesforschungsanstalt für Ernährung, Engesserstraße 20, 7500 Karlsruhe, W. Germany

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; wound-healing; suberization; gamma irradiation; peroxidase; polyphenols; thin-layer isoelectric focusing; peroxidase isoenzymes.

Abstract—A comparison of peroxidase isoenzymes in skin, cortex and pith tissues of the potato tuber by thin-layer isoelectric focusing in Sephadex revealed major differences in the isoenzyme patterns. Wounding induced several-fold increases in the peroxidase activity which were correlated with the increased amounts of specific isoenzymes. The anodic and cathodic forms with high activity, normally present in large amounts in skin, were found to be preferentially synthesized in suberizing tissues, suggesting a functional role for peroxidase in the suberization process. Cycloheximide treatment prevented the rapid increase in the content and activity of these specific isoenzymes, which indicated that the increase in peroxidase is due to a *de novo* synthesis of the enzyme. Suberization is not inhibited by gamma irradiation at sprout-inhibiting dose levels.

INTRODUCTION

Wounding the tissue of a potato tuber induces a series of metabolic and cytological events which eventually lead to the healing of the wound [1]. This results from the deposition of suberin on the outer cell layers of the wounded surface and the formation of periderm below the suberized cells. Suberization plays an important role in preventing weight loss and decay of potatoes [2–4]. Gamma irradiation at sprout-inhibiting dose levels is known to prevent the formation of the wound periderm [5], but its effect on suberization is not well understood. The protective suberin is a polymer composed of fatty acids and phenylpropane derivatives [6], containing large proportions of phenolic materials [7], though the major components are fatty acids, fatty alcohols, hydroxy fatty acids and dicarboxylic acids [2]. Recent studies have indicated the presence of covalently attached ferulic acid in suberin preparations from potatoes and other root crops [7].

Peroxidase (EC 1.11.1.7 donor: H_2O_2 oxidoreductase) is known to be involved in the metabolism of phenolic compounds [8] and catalyses the oxidation of a large number of phenols and aromatic compounds which occur naturally in plant tissues [9]. A marked increase in peroxidase activity has been reported in sliced sweet potato roots [10] and this was shown to be due to *de novo* synthesis of the isoenzymes [11]. In potato tuber tissues, the wound-induced peroxidase isoenzymes were reported to be distributed in a highly specific spatial pattern [12].

The studies reported here were undertaken to find a possible functional relationship between wound-induced increase in peroxidase activity and suberization in potatoes, and to learn how gamma irradiation affects these processes. Based on thin-layer isoelectric focusing, evidence is presented for the preferential *de novo* synthesis during suberization of large amounts of certain peroxidase isoenzymes, which are normally present in the skin of the potato tuber.

RESULTS

The distribution of peroxidase activity and isoenzyme patterns in skin, cortex and pith tissues of the potato tuber are shown in Table 1 and Fig. 1, respectively. Large differences in activity in skin tissues were observed between the varieties Hansa and Sieglinde. In Hansa, skin contained 40–55 times more activity than pith or cortex tissues, while Sieglinde showed only *ca* 10 times more activity in skin as compared to pith or cortex. These differences in enzyme activity among the tissues were also reflected in the activities of the individual isoenzymes. By thin-layer isoelectric focusing a total of *ca* 30 isoenzymes with medium or low activity, comprising 4 groups, could be consistently observed in cortex and pith tissues of Hansa (Fig. 1). Group I (anodic) mainly consisted of 2 major isoenzymes with pI in the range 3.5–4, group II of 6–8 isoenzymes with pI of 4–6, group III of 9–11 isoenzymes with pI of 6–8.5 and group IV (cathodic) of 2–3 isoenzymes with pI in the range 8.5–9.5. In contrast, the skin pattern showed 3–4 anodic isoenzymes with pI

Table 1. Distribution of peroxidase activity in different tissues of the potato tuber*

Variety	Hansa	Sieglinde
Tissue		
Skin	167000	33600
Cortex	4340	3360
Pith	3030	2980

* Activity expressed as milli units/mg protein. Enzyme extraction and assay conditions are described under Experimental.

* Present address: Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Bombay 400 085, India.

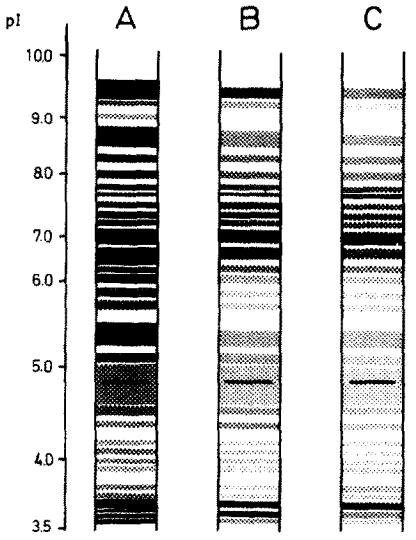

Fig. 1. Thin-layer isoelectric focusing of potato peroxidase (variety Hansa) on Sephadex G-75 containing 1% pH 2–11 ampholytes showing isoenzyme patterns in different tissues. Enzyme extracts were prepared from 5 g skin and 10 g each of cortex or pith. 20 µl of each extract were applied 6 cm away from the anode. Cathode at the top. The site of application is indicated. Enzyme detection with urea peroxide and *o*-phenylenediamine. A, skin (35); B, cortex (175) and C, pith (165). Values in parentheses indicate the amount of protein in µg contained in the respective samples.

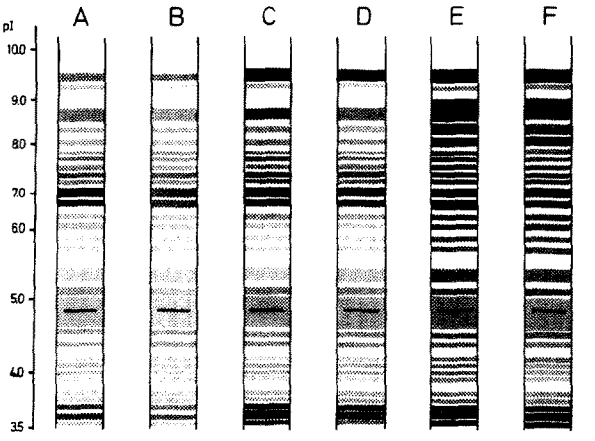

Fig. 3. Thin-layer isoelectric focusing of potato peroxidase (variety Hansa) showing isoenzyme development and pattern during suberization. 20 µl of each enzyme extract were applied 6 cm away from the anode. The site of application is indicated. Cathode at the top. A, Resting control tubers (170); B, resting irradiated tubers (145); C, control tuber halves suberized for 2 days (210); D, irradiated tuber halves suberized for 2 days (185); E, control tuber halves suberized for 11 days (165); F, irradiated tuber halves suberized for 11 days (130). Values in parentheses indicate the amount of protein in µg contained in the respective samples.

in the range 3.5–4 exhibiting very high levels of activity as compared with pith and cortex. Also the cathodic isoenzymes showed a high activity in the skin pattern. For the group III isoenzymes in the skin, a clear visualization could not be achieved because streaking of the zones occurred, possibly due to overloading or interference from phenolic constituents which were not completely removed by the purification procedures adopted in this study. Similar patterns were observed for the variety Sieglinde (not presented here).

The time course of peroxidase development in the suberizing layers of potatoes is given in Fig. 2. A 7- to 22-fold increase in specific activity, depending on the variety and treatments, was observed after 14 days of suberization. In both irradiated and un-irradiated tubers, the rate of increase in enzyme activity was slower during the first few days after wounding, irradiated tubers

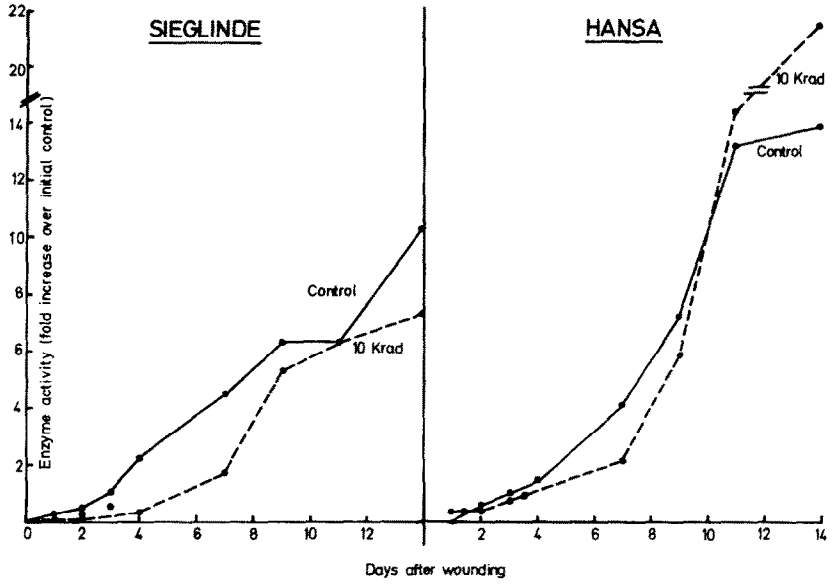

Fig. 2. The time course of peroxidase development in the suberizing layers of potato tuber tissue.

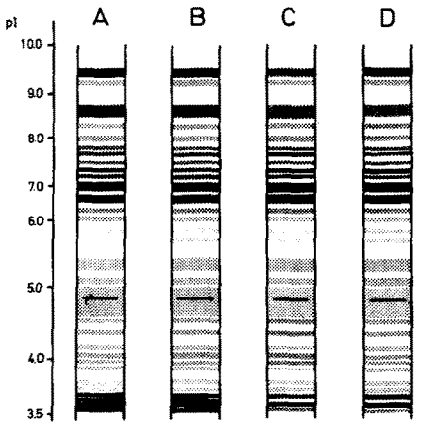

Fig. 4. Peroxidase isoenzyme pattern in resting and suberized potato tuber tissue (variety Hansa) with equal peroxidase activity applied. The site of application is indicated, 6 cm away from the anode. Cathode at the top. A, Irradiated tuber halves suberized for 3 days (30 220); B, control tuber halves suberized for 3 days (25 340); C, irradiated resting tuber (55 400); D, control resting tuber (45 385). Values given in parentheses show the amount of enzyme extract in µl and the content of protein in µg in samples when 1000 milli units each of enzyme was applied for focusing.

showing comparatively lower levels of activity than the un-irradiated ones. It is also evident that irrespective of treatments, the increase in peroxidase was greater in the variety Hansa than in Sieglinde.

The increase in peroxidase activity during suberization could be quantitatively correlated with the activity of specific isoenzymes, as illustrated in Fig. 3. The anodic and cathodic forms showed very marked increases in their activity as suberization progressed. In addition, an anodic isoenzyme normally occurring in the natural skin of the tuber appeared in the suberizing tissues.

In order to test further the possibility of new isoenzymes being formed during suberization, thin-layer isoelectric focusing of enzyme preparations from tuber halves suberized for specific periods was carried out applying equal amounts of peroxidase activity. It is evident from the isoenzyme patterns (Fig. 4) that one of the anodic forms which was not normally seen and another form only present in low amounts in cortical or pith tissues but present in high amounts in the skin, were newly formed in suberizing tissues. This difference in isoenzyme pattern was consistently observed in both varieties when tissues suberized for varying periods were compared with resting tissue.

In order to test whether the increase in peroxidase content and isoenzyme activity was due to a *de novo* synthesis of the enzyme or activation of a previously inactive form of the enzyme, potato discs treated with cycloheximide were allowed to suberize and their enzyme activities compared. The results presented in Table 2 and Fig. 5 show that the marked increase in peroxidase content and activity of the anodic and cathodic isoenzymes during suberization was prevented by cycloheximide treatment.

Preliminary results of thin-layer gel filtration of enzyme extracts from skin tissues and 9 day suberized layers showed 2 zones of activity corresponding to MWs of *ca* 37 000 and 75 000, while only the slower moving (i.e. lower MW) zone was observed in cortex and pith tissues. When the above zones (isolated by preparative gel filtration on a 40 cm-long plate) were further subjected to thin-layer isolectric focusing, major differences in their isoenzyme patterns were visible. The faster moving (higher MW) zone occurring in skin and suberizing tissues showed mainly 2–3 anodic isoenzymes with high activity and a diffuse low activity distribution over the higher pH range, whereas in the slow moving zone, 2 components, one cathodic with high activity and another anodic with low activity were recognized, with a spread activity distribution in between.

Table 2. Effect of cycloheximide treatment on peroxidase activity in suberizing potato tuber tissue

Treatment	Activity (milli units/mg protein)	Fold increase over initial control value
Control 0 day	2330	—
10 krad 0 day	1450	—
Control + cycloheximide 4 days	3840	0.65
10 krad + cycloheximide 4 days	4120	0.77
Control 4 days	30 700	12.27
10 krad 4 days	21 800	9.36

Enzyme extracts were prepared from 10 discs of 2 × 20 mm, treated with or without cycloheximide and incubated for 4 days at 15° as described under Experimental.

Fig. 6 shows the time course of formation of polyphenolic compounds in the suberizing tissues. During the first few days, accumulation of polyphenolics was comparatively lower in irradiated tubers; however, the values reached identical levels in both control and irradiated tubers towards the later periods of suberization.

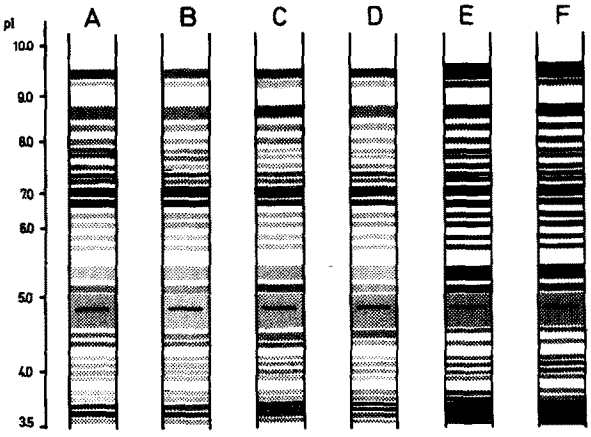

Fig. 5. Cycloheximide inhibition of peroxidase isoenzyme development during wound healing of potato tuber tissue (variety Hansa). The application site is indicated, 6 cm away from the anode. Cathode at the top. 20 µl each of enzyme extract were applied. A, Control resting tuber; B, irradiated resting tuber; C, discs from control tuber treated with cycloheximide (10 µg/ml) and suberized for 4 days; D, discs from irradiated tubers treated as in C; E, discs from control tubers suberized for 4 days without cycloheximide treatments; F, discs from irradiated tubers treated as in E.

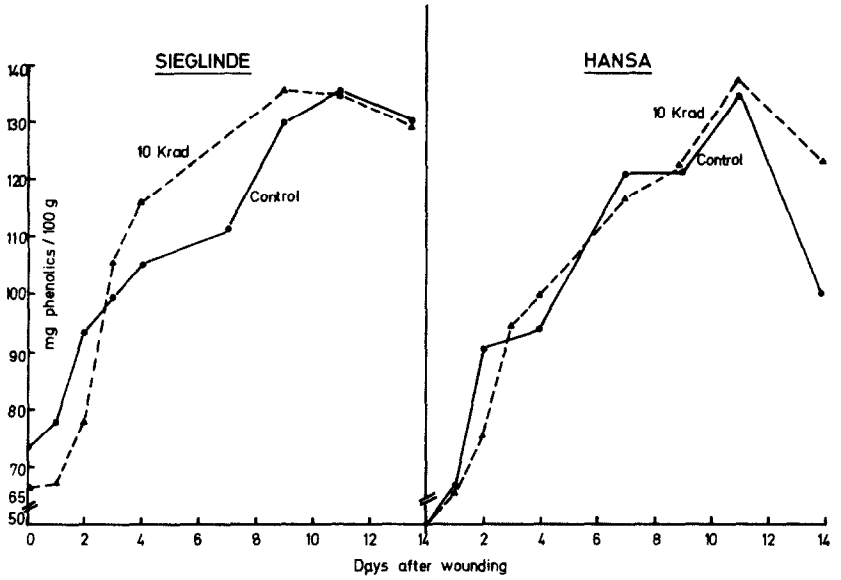

Fig. 6. The time course of the formation of polyphenolic compounds in the suberizing layers of potato tuber tissue.

Microscopic examination of stained tissue slices from both irradiated and un-irradiated tuber halves showed evidence of suberization as indicated by the blue staining of cell walls with *o*-toluidine blue while non-suberized cell walls stained purple.

DISCUSSION

During suberization of potato tuber tissues, there is a continued increase in peroxidase content lasting for several days. This can be correlated to a preferential synthesis of the anodic and cathodic isoenzymes, the major components in the isoenzyme pattern of the natural skin of the tuber. A comparison of the isoenzyme patterns of skin, cortex and pith, and that of suberizing tissues, revealed that no new isoenzymes other than those already present in the various tissues of the resting tuber are synthesized during wound-induced suberization. This is also clearly illustrated in Fig. 4 which shows the isoenzyme patterns of the resting and suberizing tuber tissue when identical peroxidase activity was focused. By this procedure, changes in the quantitative distribution of the isoenzymes can be well demonstrated.

It has been claimed that the combination of peroxidase isoenzymes extracted from various parts of a potato plant was specific for each organ and tissue, and all combinations were different from wounded tuber tissue [12]. However, the peroxidase isoenzyme pattern in the suberized layer was very similar to the skin pattern, the latter not having been examined in the above reported study. In another study [13, 14] about wound-healing of potato with different anatomical wound-reactions, the peroxidase content was also found to increase and 2–3 peroxidase isoenzymes appeared after wounding in addition to the 11 isoenzymes in the intact tissue. Here also, the skin pattern was not examined separately. The results of thin-layer gel filtration, though preliminary, strongly point to the formation of a higher MW form of peroxidase in suberizing tissues, which was also seen in extracts of the natural skin of the tuber.

The inhibition of the marked increase in peroxidase content and the activities of the anodic and cathodic forms during suberization by cycloheximide provides evidence that these isoenzymes are synthesized *de novo*. Using leucine-[^{14}C] incorporation and Blasticidin S, Shannon *et al.* [11] have demonstrated the *de novo* synthesis of peroxidase isoenzymes in sliced sweet potato tubers. A similar inhibition of wound-induced synthesis of peroxidase isoenzymes in potatoes by Phosphon and cycloheximide has been reported [15].

The skin of the potato tuber has been shown to consist of large amounts of suberin in addition to cellulose and lignin [16]. Since suberin is a polymer of fatty acids and phenylpropane derivatives [6], enzymes involved in the biosynthesis of the monomeric constituents and their subsequent polymerization must be activated upon wounding. The composition of the monomeric constituents of the lipid fraction of suberin formed at the wound periderm of potato tuber was shown to be identical to that of the suberin which covers the intact tuber [2, 17]. The presence of ω-hydroxy acid dehydrogenase enzyme from suberized potato slices has been demonstrated [18]. Accumulation of phenylpropanoids, along with increase in activities of associated enzymes, has also been reported in wounded potato tuber tissues [19–22]. The accumulation of polyphenolic compounds in the suberizing tissues observed in our studies points to an activation of the enzymes involved in the metabolism of phenolic compounds.

The observation that skin tissue possesses very high peroxidase activity, that the wound-induced peroxidase activity increases continuously as suberization progresses, and that the anodic and cathodic forms present in the skin are specifically synthesized in the suberizing tissue, would suggest a possible functional role for peroxidase in the suberization process. It is also likely that peroxidases are involved in the lignification of the cell walls since lignification is a common response to wounding or infection in plants [23, 24].

From the time course of peroxidase activity during suberization, polyphenolic accumulation and the changes

in the isoenzyme patterns, it is concluded that suberization proceeds rather slowly in gamma-irradiated tubers during the initial few days after wounding, though the differences between irradiated and un-irradiated samples were not apparent after 2 weeks of the suberization period. Staining of tissue slices with *o*-toluidine blue provides further proof that suberization is not prevented by gamma irradiation.

EXPERIMENTAL

Plant material. Potatoes of the varieties Hansa and Sieglinde were obtained soon after harvest and stored at 4°. Washed, dried tubers were irradiated in air with 10 krad in a Gamma-cell 220 (Atomic Energy of Canada, Ltd.) at a dose rate of 360 krad/hr. 6–7 days after irradiation, tubers of uniform size were sliced in half transversely and allowed to suberize at 15° under high humidity. After specified periods of suberization (1, 2, 3, 4, 7, 9, 11 and 14 days), 2 mm thick slices below the wound surface, including the suberized layer, were cut with a meat slicer. 10 g of such tissue pooled from 3 slices and freed of natural skin layer were used for enzyme extraction.

Enzyme extraction. Diced tissues frozen in liquid N_2 were extracted twice with chilled (−25°) Me_2CO. The Me_2CO extracts were used for estimation of total phenolics [25]. The dry powder (4°, overnight) was stirred for 45 min at 4° with 400 ml of cold 0.02 M K-Pi buffer, pH 7, containing, 1% polyvinylpyrrolidone (PVP-40), 0.01 M ascorbic acid and 1% Triton X-100. Partially purified enzyme prepns were obtained as described previously [26].

For studying cycloheximide inhibition of peroxidase synthesis, 10 discs (2 × 20 mm) prepared from the pith were soaked in an aq. soln of cycloheximide (10 μg/ml) for 40 min. The discs were lightly blotted and incubated at 15° in a 20 l. desiccator lined with moist filter paper. After specified periods of incubation, enzyme extracts were prepared as above. Discs incubated without cycloheximide served as a control.

Peroxidase activity was measured in triplicate from each of the samples by a slightly modified procedure of ref. [27]. The increase in *A* by the peroxidation of *o*-dianisidine was measured in an Eppendorf Enzyme Analyzer Model 5080 equipped with a computer and printer. The assay system was: wavelength 436 nm; half-micro glass cuvettes with 1 cm light path; final vol. 0.6 ml; temp. 25°; concn in test, 0.01 M K-Pi buffer pH 6; 1 mM H_2O_2; 0.33 mM *o*-dianisidine. The reaction was initiated by the addition of 10–50 μl of an appropriately diluted enzyme extract. The results are printed out as milli units and one unit of peroxidase activity is that amount of enzyme decomposing 1 μmol of peroxide per min at 25°. The enzyme activity is expressed as milli units/mg protein.

Protein was estimated by the biuret method [28].

Focusing. Thin-layer isoelectric focusing was performed essentially as described previously [26, 29], using pH 2–11 'Servalyt' carrier ampholytes (Serva, Heidelberg, FRG) and Sephadex G-75 Superfine (Pharmacia, Uppsala, Sweden) on 20 × 20 cm glass plates, the separation distance being 15 cm. Enzyme extracts (10–40 μl) were applied to the gel layer, 6 cm away from the anode with the aid of 15 × 15 mm microscopic cover slips. Focusing was carried out initially at 200 V for 2 hr followed by 600 V for 2 hr at 4°. pI values at 25° were estimated by comparison with pH marker proteins. Peroxidase activity was detected by the paper print technique using 1% urea peroxide and 1% *o*-phenylene-diamine [30]. Thin-layer gel filtration was performed as described earlier [31].

For qualitative evaluation of suberization, fresh hand sections were cut from suberized tuber halves, stained with 0.05% toluidine blue in water and examined microscopically [15].

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