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Simultaneous Separation of Acidic and Basic Isoperoxidases in Wounded Potato Tissue by Acrylamide Gel Electrophoresis[1]

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ABSTRACT

Preparation and use of a newly developed pH 4.3 horizontal thin layer acrylamide gel which permits the simultaneous separation of acidic and basic isoperoxidases in up to 30 samples is described. Use of cytochrome *c*, horseradish peroxidase, and a purified potato isoperoxidase as internal standards for a range in isoelectric points of peroxidases from pH 3 to 11 is introduced to facilitate comparison of results obtained with different materials and different methods. Distribution of tissue-specific isoperoxidases in different cell layers of wounded potato (*Solanum tuberosum* L.) tissue is shown and their purification described. Evidence for the *in vitro* degradation of basic potato isoperoxidases resulting in more acidic forms similar to isoperoxidases occurring in wounded potato tissue is presented. The significance of this observation for the postulated differential function of different isoperoxidases is discussed.

The distribution of different groups of isoperoxidases (IPER)[2] induced in wounded potato tuber tissue has been shown to be highly correlated with the wound-induced pattern of cell differentiation (2, 3). Further analysis of this correlation between molecular and cellular differentiation is limited by the facts that the function of peroxidases in plant tissues is unknown, genetic control of variations in peroxidases associated with plant development in potato and most other systems has not been established (9), and attempts to improve methods for separation and identification of IPER have been initiated only recently (5–7). As a contribution to the improvement of methods for the analysis of IPER, we described here the simultaneous separation of acidic and basic IPER in an acrylamide slab gel, the use of internal standards and references, and the *in vitro* degradation of basic IPER, which raises questions as to the genetic control and possible function of multiple molecular forms of peroxidase.

MATERIALS AND METHODS

Cold (4 C)-stored potatoes (*Solanum tuberosum* L., cv. Kennebec) grown locally or obtained by courtesy of the Red River Valley Potato Processing Laboratory, East Grand Forks, Minn., were used throughout. To study wound-induced IPER, tubers were cut into 1.5-mm slices with a Berkel meat slicer model 707, rinsed three times in deionized H_2O, and stored moist at 25 C until sampled. The time course of IPER formation was determined by electrophoresis of samples which had been frozen at daily intervals after wounding To demonstrate tissue specificity of IPER, the suberin layers on both surfaces of a slice were removed with a spatula 4 days after cutting, and the juice from the ruptured cells was absorbed onto filter paper wicks for electrophoresis. The suberizing and remaining nonsuberizing cell layers were stored frozen and rethawed shortly before use in electrophoresis.

For purification of IPER, 1 to 2 kg of 5-day-old slices were ground and centrifuged in a Jupiter fruit juicer. Sodium bisulfite was added to the resulting juice as needed to prevent browning from oxidation of phenolic compounds. The remaining pulp was extracted once with 10 mM phosphate buffer (pH 7), recentrifuged, and the washing solution added to the original juice. The combined extracts were stored frozen until use. The remaining pulp, mostly starch and cell wall material, was washed repeatedly with several liters of water until the supernatant remained clear and had ceased foaming. The washed pulp was stored frozen.

Assay. IPER was assayed by the procedure described for horseradish peroxidase in the Worthington Enzyme Manual. The reaction mixture contained per ml: 83.3 μg of *o*-dianisidine (8.3 μl of 1% [w/v] solution in methanol), 30 μg of H_2O_2 (10 μl of 0.3% [w/v] solution in water), and 10 μmol of K-phosphate (pH 6.0). The assay mixture was incubated at 30 C and the reaction initiated by addition of an appropriate amount of enzyme. Increase in A_{460} was measured as a function of time in a Beckman model 25 spectrophotometer. One unit of IPER is defined as that amount of enzyme decomposing 1 μmol of H_2O_2 (represents an increase in A_{460} of 11.3)/min at 30 C. Specific activity is defined as units/mg protein.

Purification Procedures. Unless otherwise stated all operations were carried out at 4 C. For the purification of isozymes A 2/4 and C 3, 500 ml of extract from wounded potatoes was heated to 70 C and maintained at that temperature for 5 min before rapidly cooling to 4 C. The resultant precipitate was removed by centrifugation (15,000*g* for 30 min). The supernatant fluid was brought to 80% saturation with solid ammonium sulfate (297 g/530 ml), stirred for 3 hr, and centrifuged at 12,000*g* for 30 min. The precipitate was resuspended in 50 ml of 0.01 M K-phosphate (pH 7.0) and dialyzed against several changes of this buffer. After dialysis the enzyme solution was centrifuged (12,000*g* for 30 min); one half (42 ml) of the supernatant was applied to a column (2.5 × 60 cm) of DEAE-cellulose (Whatman DE50) previously equilibrated with 0.01 M K-phosphate (pH 7.0). The other half was applied to a similarly equilibrated column (2.5 × 50 cm) of CM-cellulose (Whatman CM-1). Each column was washed with 1 column volume of 0.01 M K-phosphate (pH 7.0) and subsequently eluted with a linear gradient consisting of 750 ml of the phosphate buffer in the mixing chamber and 750 ml of phosphate buffer containing 0.5 M NaCl in the reservoir. Five IPER peaks were

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[2] Abbreviations: IEF: isoelectric focusing; IPER-isoperoxidase; TEMED: N,N,N′,N′-tetramethylenediamine.

recovered from DEAE-cellulose. The first peak (*i.e.* material which was not adsorbed to the resin) contained primarily IPER C3; the A 2/4 isozyme was the only peroxidase activity in peak 5. Two IPER peaks were eluted from the CM-cellulose column. The first peak (*i.e.* not adsorbed) was predominantly A 2/4 whereas the second peak contained only C3.

For partial purification of IPER C6 wounded potato cell wall material, prepared as described above, served as the source. Extraction of 200 g (wet weight) of cell wall material with 400 ml 1 M LiCl in 0.05 M K-phosphate (pH 7.0) at 4 C with vigorous agitation yielded a soluble product containing primarily IPER C6. The extract was filtered through cheesecloth and the filtrate dialyzed against 0.05 M K-phosphate (pH 7.0). After dialysis the enzyme was filtered through glasswool to remove fine particles and the filtrate was chromatographed on CM-cellulose (2.5 × 60 cm) previously equilibrated with 0.05 M K-phosphate (pH 7.0). The column was washed with 4 column volumes of this buffer and IPER C6 was eluted with a linear gradient of 750 ml of 0.05 M K-phosphate (pH 7.0) in the mixing chamber and 750 ml of the same buffer containing 1 M NaCl in the reservoir. IPER C3 was recovered in the column void and C6 eluted early in the gradient.

Acrylamide electrophoresis of IPER was carried out in horizontal thin layer slab gels in an LKB Multiphor apparatus using a modification of the basic technique described by the manufacturer (LKB application note 306). The 25-cm-wide gels consist of 3 cm of anodic and 7 cm of cathodic separating gels separated by a 1-cm stacking gel and prepared as follows:

Separating Gel			Stacking Gel		
	Solution	Ratio		Solution	Ratio
A:	17.2 ml glacial acetic acid 48 ml 1 N KOH 8 ml TEMED 126.8 ml H_2O	1	D:	2.87 ml glacial acetic acid 48 ml 1 N KOH 0.46 ml TEMED 48.67 ml H_2O	1
B:	24 g acrylamide 1.20 g bisacrylamide 100 ml H_2O	1	B: E:	acrylamide 40 g sucrose 100 ml H_2O	2 4
C:	$(NH_4)_2S_2O_8$ 280 mg/100 ml	2	F:	5 μg/ml riboflavin	1

The tray buffer contains 31.2 g/l β-alanine adjusted to pH 4.6 by glacial acetic acid.

To pour such gels, 1.4-mm gasket about 10 cm longer than the circumference of the gel-supporting glass plates was cut from rubber tubing and clamped between the glass plates in such a way that the gasket ends overlapped about 10 cm at one short edge of the plates. While plates were standing with the long, anodic (upper) edge up, the cathodic running gel was poured with the "cathodic" (lower) gasket ending clamped between the plates and the gasket opening at the anodic side of the short edge. After solidification of the cathodic running gel under a 2-mm layer of water, the "cathodic" gasket ending was removed, plates were inverted, the "anodic" gasket ending was clamped in place, and the anodic running gel was poured through a gasket opening at the cathodic side of the short edge. After solidification of the anodic gel under water, the 1-cm-wide space between the running gels was filled with tray buffer, and the gel was stored cold until use. Before use the tray buffer was decanted, the stacking gel was poured and polymerized, the entire gel was cooled for 15 to 30 min at 4 C, the cover plate was removed from the gel, and 25 to 30 samples on filter paper wicks (1 × 1.5 × 4 mm holding 5–7 μl of extract containing approximately 70 μg of protein) were applied at the interphase between anodic running gel and stacking gel.

Except for their anodic and cathodic ends, gels were covered with clear plastic wrap and run in the Multiphor apparatus at 30 mamp and 180 to 200 v until Cyt *c*, used as cathodic tracking dye, had migrated to 1 cm from the cathodic end of the gel (about 6 hr). Gels were stained with 3-amino-9-ethyl carbazole according to Graham *et al.* (4) and fixed in methanol/acetic acid.

An alternative procedure is to cast a single gel consisting entirely of the 6% (pH 4.3) separating gel using a slot former such as is found in LKB 2117-601. (We made a completely reliable slot former by gluing 1- × 2- × 5-mm aluminum rods to 3-mm glass plates.) Up to 10 μl of sample could be applied to such slots and electrophoresed as above.

Isoelectric focusing (IEF) using the LKB Multiphor apparatus and LKB Ampholines in the pH ranges of 3.5 to 10 or 7.5 to 11 was carried out according to the manufacturer's instructions (LKB application note 75).

RESULTS

To illustrate the simultaneous separation of acidic and basic IPER, the increase in IPER during the first 5 days of wound-healing in potato tissue is shown in Figure 1, A through E. While anodic bands are sharp even in a homogeneous pH 4.3 acrylamide slab gel, use of a stacking gel is required to obtain sharp cathodic bands. As reported earlier (2, 3), the total IPER found in a 5-day-old slice of potato tissue can be subdivided into several cell specific groups. The most basic IPER (group C 6/8) occurs only in the cytoplasm and—ionically bound—in the cell walls of nonsuberizing, dividing cells (Figs. 2C and 3C). Suberizing cells from the periphery of the tissue contain high activities of the most acidic group A 2/4 and of group C 4/5 (Fig. 2A). Extracts from an entire slice (Figs. 1E and 2D) as well as cell sap released during separation of the suberized cell layer from the remaining tissue (Fig. 2B) contain the sum of the IPER found in suberizing and nonsuberizing tissue. An additional band (C2), absent in air-stored tissue, appears in tissue incubated in water (Fig. 1F). While IPER groups A 2/4, C 4/5, and C 6/8 in the pH 4.5 acrylamide gel can be rather safely identified as groups A 4/9, C 2/3, and C 4/5, respectively, in the pH 8.9 starch gel used in earlier work (Borchert 1974), the correlation between groups C 2/3 in acrylamide and A 2/3 in starch is less clear. It should be also noted that anodic IPER groups comprise a greater number of bands in the pH 4.3 gel. This difficulty of unequivocally identifying and characterizing different groups of IPER extracted from the same tissue but separated by different electrophoretic methods has been a recurrent problem in the investigation of IPER. As recently illustrated for the IPER of tobacco (7), the problem is compounded if IPER from different tissues analyzed by different laboratories are to be compared.

To alleviate this problem, we developed a set of internal standards or references, which facilitate comparison between different gels within our laboratory and, if commonly used, should also aid comparison of work carried out in different laboratories. As a small, strongly basic, colored heme protein, Cyt *c* combines several useful properties. It migrates to the cathode faster than the most basic potato IPER and because of its color it can serve as a cathodic tracking dye during electrophoresis. It reacts with peroxidase stains and thus constitutes a reference point at the cathodic end of the stained gel (Figs. 2E and 3D). Horseradish peroxidase has been thoroughly investigated as to its biochemical properties and electrophoretic behavior (5, 6, 10), and is commercially available in several purified forms. Our sample (Sigma P-8000) contains several IPER which at pH 4.3 are partly acidic, partly basic, and thus provide several additional reference points (Figs. 2E and 3D). Major fractions of acidic IPER can be easily purified by means of ion exchange chromatography (8, 10), and at least one purified IPER, preferably of intermediate isoelectric point (pI) should always be used as an additional reference, specific for a certain experimental material. We have isolated an acidic (A 2/4), intermediate (C 2/3), and basic (C 6/8) group of IPER (Fig. 3, A–C). A combination of Cyt *c*, horseradish peroxidase, and potato IPER C 2/3 thus provides reproducible and easily identifiable references for the entire spectrum of acidic and basic IPER.

When IPER were separated by IEF, potato IPER A 2/4 and the two acidic horseradish IPER migrated out of a pH 3.5 to 9.5 gel at the anodic end, *i.e.* their pI values are lower than pH 3.5 (compare ref. 5). Cyt *c* and IPER C 6/8 migrated out of a pH 7.5

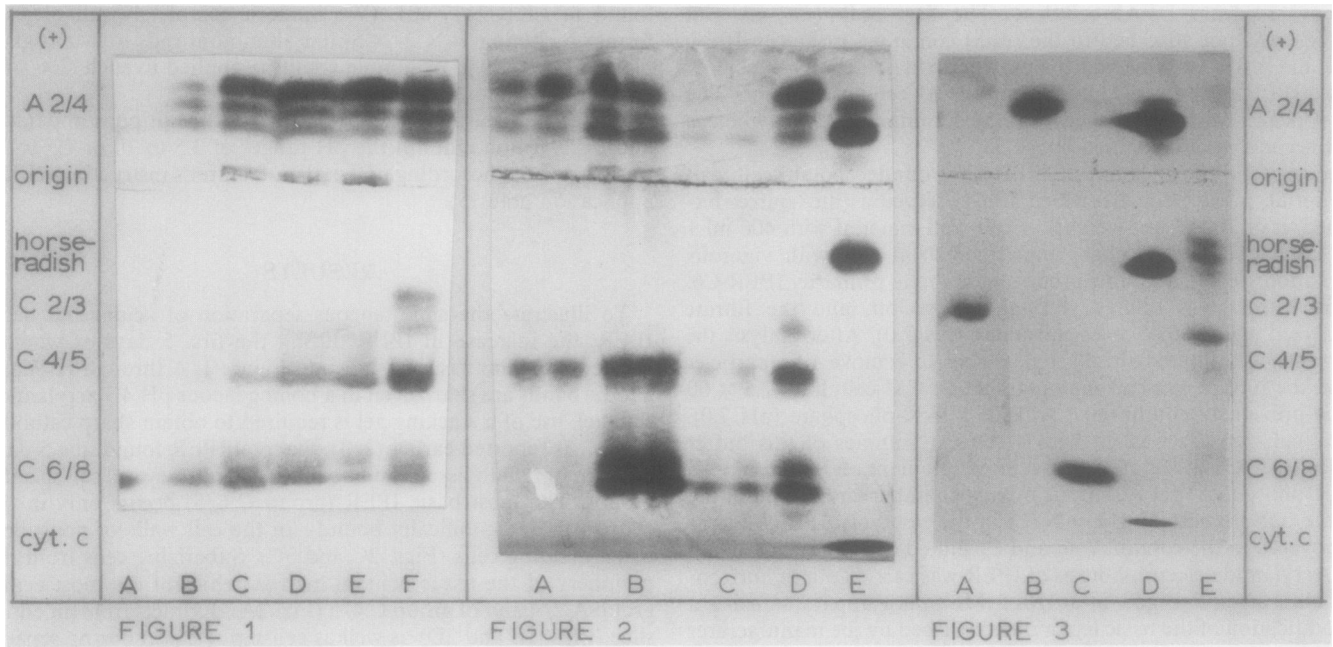

FIG. 1. Time course of appearance of IPER in a 1.5-mm slice of potato tissue, incubated in air, from the 1st (A) to the 5th (E) day after cutting; F: 5-day-old tissue aged in a shallow layer of water. Designation of IPER bands is given at left side of gel. Electrophoresis of isoperoxidases was carried out in a horizontal thin layer acrylamide gel at pH 4.3. Samples were applied on filter paper wicks at the "origin." Stacking gel has been removed prior to photographing gels.

FIG. 2. Separation of tissue-specific IPER from 1.5-mm potato slices by acrylamide electrophoresis. A: extract from suberized peripheral cell layer removed on 4th day after cutting; B: cell sap released during removal of suberin layer; C: extract of remaining, nonsuberized cells; D: extract of entire 1.5-mm slice (compare Fig. 1E); E: reference containing horseradish peroxidase (Sigma P-8000; upper three bands) and Cyt *c* (lower band near cathodic end of gel).

FIG. 3. Electrophoretic separation of purified IPER from wounded potato tissue. A: IPER C3 and B: IPER A 2/4 isolated by ion exchange chromatography from press juice of wounded potato tissue; C: IPER C6 from cell wall extract (extracted with 1 M LiCl at pH 7, dialyzed, and concentrated); D: reference containing horseradish peroxidase and Cyt *c* (Fig. 2E); E: degradation products of IPER C6 (Fig. 3C) after ammonium sulfate precipitation and chromatography on CM-cellulose at pH 9.

to 11 gel at the cathodic end, indicating pI values higher than pH 10.5. It is because of this wide range in the pI of IPER that the entire set of IPER cannot be satisfactorily separated in either an anodic or cathodic disc gel, or by IEF.

Purification of potato juice containing all IPER (Fig. 1E) by means of a combination of ion exchange chromatography on DEAE-cellulose and CM-cellulose consistently failed to yield IPER more basic than C3, *i.e.* basic IPER were somehow lost in the purification process and IPER C3 was obtained in a higher proportion of total IPER than present in the original extract. We resorted to cell walls as a source of basic C 6/8 isozyme (Fig. 3C). When IPER C 6/8 was washed from cell walls with salt solutions of high ionic strength (1 M), dialyzed, and then absorbed to CM-cellulose at pH 9, the eluate lacked activity of C 6/8, but contained bands (Fig. 3E) similar to those found in 5-day-old tissue (compare Fig. 1F). If the pH used in CM-cellulose chromatography was lowered (pH 8) *in vitro* degradation of C 6/8 was significantly reduced, and at pH 7 avoided altogether. This observation suggests that earlier failures to isolate basic IPER may have been due to *in vitro* degradation of these IPER. It has been reported that basic horseradish peroxidases are gradually converted to more acidic forms by prolonged incubation at high pH (6).

Certain steps in the purification procedure apparently cause the *in vitro* degradation of the most basic IPER into a series of increasingly more acidic IPER, which appear to be in part identical with IPER normally formed in wounded tissue.

DISCUSSION

The analysis of IPER by means of the described thin layer acrylamide slab gel used in conjunction with adequate internal standards offers the following methodological advantages as compared to starch or disc gel electrophoresis: (*a*) large sample numbers (up to 30) containing IPER ranging from pI 3.5 to 11 can be separated simultaneously; (*b*) individual IPER or groups of IPER can be compared both to each other and to well known reference molecules with respect to their electrophoretic behavior, facilitating comparisons within and between gels; (*c*) use of the stain 3-amino-9-ethyl carbazole, which yields a stable, purple-brown precipitate, makes recording of gels immediately after staining unnecessary. The thin, clear gels can be stored indefinitely and are thus available for scoring, photographing, or densitometric scanning when convenient; (*d*) duplicate samples of the same extract may be stained for protein, peroxidase, or other enzyme activity in different sections of the same gel. Staining for peroxidase can be followed by staining for proteins in the same gel section—the product of peroxidase staining persists through the protein-staining procedure.

Separation and characterization of IPER by IEF appear to be less suitable, because the range of pI represented by acidic and basic IPER cannot be accommodated by a single pH gradient (see also ref. 5). The preliminary observation of *in vitro* degradation of basic IPER into more acidic forms which comprise IPER normally present in wounded potato tissue has important implications for work aimed at the analysis of the genetic control and function of IPER. The results show that minor, stepwise alterations of the basic IPER molecule may change its electrophoretic behavior substantially, apparently by affecting its charge, without inactivating the catalytic site. Whereas the basic IPER C 6/8 is the molecular species normally present in the cytoplasm and cell walls of dividing, nonsuberizing cells, all other, more acidic IPER occur only in the suberizing cells. If during wound-healing these IPER should arise by degradation or posttranslational modification of C 6/8, this would mean that only this latter IPER, but none of the

others, would be under genetic control. Inasmuch as IPER found *in vivo* and IPER resulting from *in vitro* degradation of IPER C 6/8 are similar, it is likely that during degradation both *in vivo* and *in vitro* well defined fragments containing positive charges are eliminated from the surface of the enzyme molecule and thus alter its charge in a regular, stepwise manner. If all multiple forms of IPER should be demonstrated to arise by such stepwise modification of a single molecular species, the search for differential physiological functions of different IPER might be in vain. Differences in substrate affinity, pH optimum, and other biochemical properties, as recently demonstrated for IPER in horseradish and tobacco (6, 8), might be the consequence of secondary modifications of the basic IPER—whatever its "functional purpose" may be—rather than the result of selection for certain functional properties. The demonstration, by IEF, of as many as 40 different bands of IPER in commercial preparations of horseradish peroxidase (5) makes the search for their physiological function questionable. On the other hand, control of IPER by multiple gene loci has been demonstrated in barley, maize, and cucurbits (9).

Evidence for the probable *in vitro* degradation of IPER in materials other than potato is presented in several recent studies. For example, Hoyle (5) examined three commercial preparations of horseradish peroxidase by IEF. All three products were crude powders but contained substantially different distributions of IPER. The product which totally lacked the most basic IPER contained the highest activity of the most acidic IPER, suggesting that methods used in the preparation of this product enhanced the degradation of the basic IPER into the more acidic forms. Tobacco pith tissue, like potato, contains four major groups of IPER, which were shown to range in pI from 4 to 8.3 for the most basic group (7). However, in other work, two groups of IPER migrated to the cathode in a pH 8.3 starch gel (1), *i.e.* these basic IPER must possess a pI of at least 9, as observed in potato. As mentioned earlier, during IEF with wide range Ampholines (pH 3.5–9.5) the most acidic and basic IPER in potato and horseradish tend to migrate out of the gel and may thus escape attention (5).

In potato as well as tobacco it appears as if the most basic IPER are present in significant quantities only in growing cells and tissues, like tobacco callus, wounded pith of tobacco or potato, and tips of shoots and roots in potato, but not in stationary ones like those of leaves (2, 7). Detection of basic IPER may therefore also depend on the choice of experimental material.

In agreement with Scandalios' (9) recent plea for methodological improvements in the study of IPER and the need for caution in the interpretation of observed changes in IPER related to plant development, our work illustrates both the possibilities of and necessity for methodological improvements in the analysis of IPER, which must be achieved before we can hope to gain an understanding of the role of IPER in plant development.

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