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The Isoperoxidases of Pisum sativum

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Summary. The heterogeneity of the peroxidases in peas was examined by starch gel electrophoresis. Comparisons were made between tall and dwarf cultivars and among organ systems developed in light and darkness. Isoperoxidase bands could be grouped as cathodic, anodic and near-neutral (at pH 9.0) types. The cathodic set stained well with guaiacol oxidation products whereas some anodic bands reacted preferentially with 2,6-dimethoxyphenol. Some near-neutral bands were aceto-carmine positive and may have been organellar.

Each organ had a characteristic isozyme pattern, and the band patterns in corresponding organs from different varieties were far more alike than were the patterns in the different organs within each variety. Ontogenetic changes were marked in all 3 organ systems, principally in the cathodic bands. The effect of light on isozymal patterns was quantitative rather than qualitative, possibly influencing the isoperoxidases secondarily via its effect upon organ physiology and development.

Peroxidase may have a significant role in the regulation of cell growth and differentiation (3, 8, 23), though its precise function in the cell is as yet poorly understood. The molecular heterogeneity of this enzyme (9, 10, 11, 20) has been appreciated for many years. While the significance of molecular heterogeneity is unknown, the existence of isozymes would appear to increase the biochemical versatility of organisms, and to protect the organism against loss of function occasioned by mutation or environmental stresses. It has been shown that several peroxidase isozymes are quantitatively altered by gibberellic acid application to dwarf corn plants (13, 14, 23). Although it is possible to increase the activity of root tissue peroxidase by incubation of tissue in IAA (4, 8, 17), it has also been possible to repress 1 specific isoperoxidase by addition of IAA to young pea stem sections (16).

Since ontogenetic changes in the patterns of growth and differentiation at the cellular level must be preceded by quantitative or qualitative changes at the molecular level, it is pertinent to examine the kinds and quantities of the various peroxidase isozymes which plants may contain during the various stages of their ontogeny. In the present study, existing methods of electrophoresis have been adapted to plant peroxidases in order to conduct an analysis of changing enzyme patterns during development.

Peroxidase has been given the designation 1.11.1.7 by the Committee on Nomenclature of the International Union of Biochemists (18). Some peroxidases have been assigned different numbers on the basis of their substrates, but generally, these enzymes do not exhibit absolute substrate specificity (12). Kinetic studies have further detailed the host of responses which can be elicited from these enzymes (7, 19, 22).

As a simple expedient the following nomenclature will be used throughout this paper: A) A (anodic) and C (cathodic) will designate the charge the enzyme carried under given electrophoretic conditions. It is realized that the net charge of the molecule will vary with the pH of the solutions, as well as with infolding or exposure of charged groups which may alter the electrophoretic mobility or isoelectric point. Therefore, the symbols A and C are entirely operational. B) Since there are different patterns for the major plant parts, a subscript will be selected to indicate organ source. Only 3 will be used, $_r$ = root, $_s$ = shoot, which includes stem, leaves, apex, etc. and $_c$ = cotyledon. C) A numeral will be used to indicate the enzyme's position relative to the origin, that is, isozymes closest to the origin will be called "1" with the more rapidly moving components being given higher numbers.

Unfortunately, from such a system no relationship may necessarily be drawn between peroxidases from different organs, since C_r3 may in no way resemble C_c3. Also A_r4 from pea may be very different from A_r4 from corn. But until some interrelationship between the isoperoxidases is found, such descriptive labelling of these enzymes should suffice.

Material and Methods

Plant Material. The 2 major varieties used during the course of this investigation were a tall pea (*Pisum sativum* L. cultivar Alaska) and a dwarf pea (cultivar Progress No. 9) supplied by the Asgrow Seed Company of Orange, Connecticut. All seeds were initially soaked in dilute (12 %) Clorox (a commercial preparation of hypochlorite) for 10 to 15 minutes. They were then thoroughly rinsed in running tap water, and were allowed to imbibe water in either the dark or the light for 6 to 8 hours before planting in vermiculite. The light-grown plants were kept on a 24 hour photoperiod under a bank of Daylight and White fluorescent tubes (ca 1200 ft-c) supplemented with about 10 % of the total energy as incandescent light. The dark grown plants were maintained in total darkness except for minimal handling under a safe light (Sylvania green fluorescent tube wrapped in 3 layers each of duPont amber and green cellophane). Temperatures were 23° (light) and 26° (dark).

Electrophoresis. Starch gel zone electrophoresis was performed on tissue macerates (16) essentially according to the methods of Smithies (24). The method is summarized in Fisher Scientific Company Technical Data Bulletin S-676. The buffer systems and apparatus utilized have been described previously (16).

Enzyme Assays after Electrophoresis. On starch gel, peroxidases were usually assayed by pouring over the freshly cut inner surface a suitable substrate and H_2O_2 in 0.2 M pH 5.8 phosphate buffer. Peroxidase moves well in an electric field only at pH 8 or higher, but shows no activity in alkaline media. The high molarity of the pH 5.8 buffer was required to overcome the buffering capacity of the gel (pH 9.0) and allow for the appearance of the active enzymes. The substrate which proved most versatile was 5 mM guaiacol together with equimolar H_2O_2. However, certain peroxidases were resolved by using other substrates such as orthodianisidine (Nutritional Biochemicals Co.) and 2,6-dimethoxyphenol (Eastman Organic Chemicals) applied at a concentration of 5 mM with hydrogen peroxide. Pyrogallol was also employed, but its oxidation product is too water soluble for critical use. The oxidation products of mesidine are exceedingly pH sensitive, hence unstable. In addition to phenolic substrates, benzidine was used according to the technique of van Duijn (5).

It was noted that during starch gel electrophoresis of plant extracts, a sharp white band migrating toward the anode appeared after the pH had been lowered. If the gels were then placed in a refrigerator overnight, the starch in the immediate vicinity of this line became liquefied. A similar phenomenon, reported from cattle serum (2), has been attributed to amylase. No further assays were made on this enzyme, but since it was found in most pea tissues, it has been used in this study as an internal standard in ascertaining uniformity of electrophoretic conditions.

Gels were sliced transversely after completion of the electrophoresis, yielding duplicate half gels. This allowed direct comparisons between peroxidase substrates, the determination of proteins (by nigrosin staining), nucleoproteins (by acetocarmine), or other enzymes such as catalase.

Results

Roots. Roots from both Alaska and Progress peas have only 3 cathodic peroxidases (fig 1). Root tips from embryos removed before germination, which occurs between the second and third day, generally possess equal amount of C_r1 and C_r2 and variable amounts, though usually less, of the C_r3 component. From these ungerminated roots, the isozyme pattern is less sharp than that of growing tissues. In root tips from older plants (10–14 days) and in the non-meristematic tissues from even quite young plants (3 days old) the majority of the peroxidase is of the slower moving form, C_r1.

Secondary roots show patterns which are indistinguishable from those of the primary root, irrespective of their maturity. Separations made before and after the secondary roots have manifested a geotropic curvature produced no detectable differences. Adventitious roots from the first node of dark grown corn plants (*Zea mays* L. cv. Golden Bantam) show a peroxidase pattern identical with that found in the primary root system. Stem cuttings from light grown Progress peas placed in water form callus tissue around the injured area from which roots may subsequently arise. These callus roots possess peroxidases not normally asso-

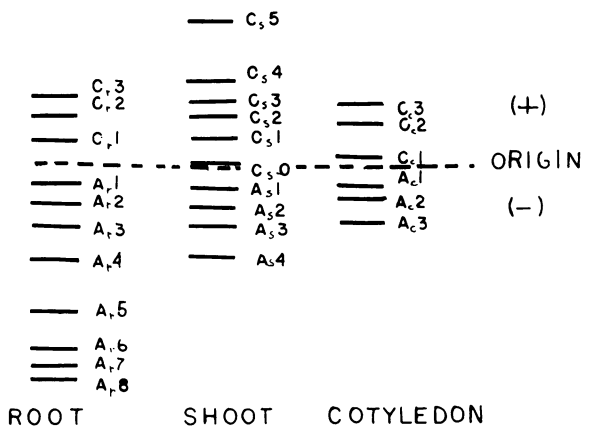

FIG. 1. Isoperoxidases in principle organs of the pea. Band C_r2 migrates approximately 1 cm during the 90 minute separation, and all other bands are drawn relative to this scale.

ciated with root tissue. As might be expected, their electrophoretic patterns, though very root-like, include some shoot peroxidases which migrate more rapidly.

The anodic peroxidases from the root are composed of several broad bands close to the origin. These slow moving bands may be intact cellular organelles, since A_r1 and A_s1 can be stained with acetocarmine. In addition, roots possess A_r4, which appears equivalent to an enzyme found in all parts of the plant except the cotyledons, but most abundantly in meristematic regions. A_r5 is a peroxidase which is unique to root tissue. Under certain conditions even more rapidly migrating anodic root peroxidases designated A_r6, A_r7 and A_r8 may be discerned. The latter 3 are very electrophoretically mobile, indicative of a greater negative charge per unit mass, and are found in the main root in the area associated with secondary root initials. These anodic peroxidases, A_r4 to 8, are frequently not visible with guaiacol and tend to fade rapidly; however, 2,6-dimethoxyphenol or o-dianisidine alone or in combination with guaiacol bring out these regions of peroxidative activity more easily.

Cotyledons. Cotyledons possess 3 cationic peroxidases. These enzymes are distinct from any of the cationic peroxidases associated either with the root or shoot tissues. On the other hand, anionic peroxidases A_c1 and A_c2 tend to be similar in nature to the comparable enzymes from other pea tissue. Cotyledons possess no peroxidase equivalent to A_r or $_s4$, i.e. the meristematic peroxidase.

Although pea cotyledons are senescent organs, they are nevertheless important during germination and are critical in dark grown, vermiculite cultured plants. Nevertheless we could find no evidence for the direct transport, similar to that of small molecules, of any cotyledonary peroxidase to stem or root.

Cotyledons ground in the dry state or at any time up to about 18 hours after the start of imbibition showed no electrophoretically mobile peroxidases, although there was some activity in the immediate vicinity of the origin. Virtually no activity was detected when the homogenate was assayed colorimetrically for peroxidase. It can therefore be concluded that there was little or no active peroxidase present at this phase, since one would expect electrophoresis to separate the enzyme from any soluble inhibitors that might be present. By 24 hours after imbibition, the electrophoretically mobile peroxidases had appeared, and peroxidase-positive material at the origin had diminished. Two bands (C_c2 and C_c3) were seen. Forty-eight hours after the start of the experiment, these 2 bands have intensified and a third (C_c1) has appeared. Between a week and 10 days in the dark, the C_c3 band of the cotyledon diminishes, and C_c1 and C_c2 become the major active bands (fig 2).

It should be noted that in light and darkness, the cotyledonary peroxidases show identical patterns for the first 3 days. In light, however, even after a week, no C_c1 has appeared. Even after several weeks, only a small amount of C_c1 is seen in light grown plants, but at no time attains the intensity of a major band as it does in dark-grown material.

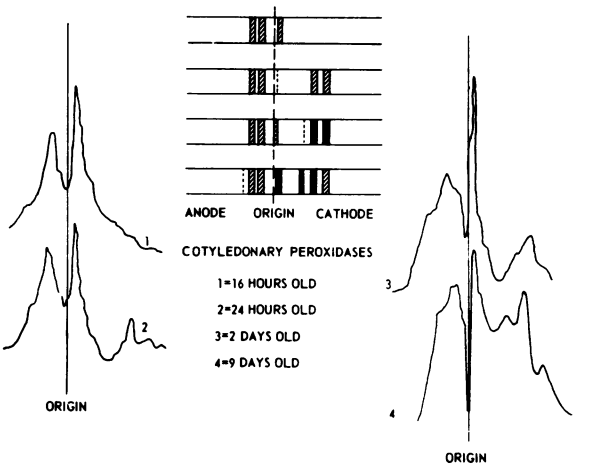

FIG. 2. Time course of cotyledonary peroxidase development as shown by gel densitometer traces at critical time intervals.

Shoots. Although shoots are composed of many organs, they nevertheless have been grouped together here because relative isoperoxidase activities vary little among organs in the shoot; some of the minor peroxidases may not be found in some organs at certain times, but their patterns are similar.

Shoots contain C_s1, C_s2 and C_s4 in most of their organs, with C_s2 being most abundant. In maturing stem tissue, C_s3 appears progressively with age, but its appearance can be repressed by application of IAA (16) or related auxins (15). Ockerse (15) has reported a C_s5 peroxidase in stem sections of Progress peas which appears to increase as a direct function of gibberellic acid concentration. C_s5 or one with similar electrophoretic mobility is one of the major components from light grown leaf, stipule or tendril material, though it is not found in stem, unexpanded leaf or apical meristem from Alaska peas.

The ontogeny of shoot material is complex because of the number of organs and cell types involved. Therefore, dark grown material has been most studied, because little growth other than cell elongation of the stem occurs. Nodal regions were initially selected, since they contain the complex organizational potential for forming stipules, leaflets, petioles and tendrils in the light. From figure 3, one can see a shift in the peroxidases of the nodal regions as one progresses up the plant.

In the oldest node, most of the peroxidase is C_s1 and C_s2, with relatively less of C_s3. By the time one has reached the apex, C_s1 has become a minor band and C_s3 predominates. A given node showed

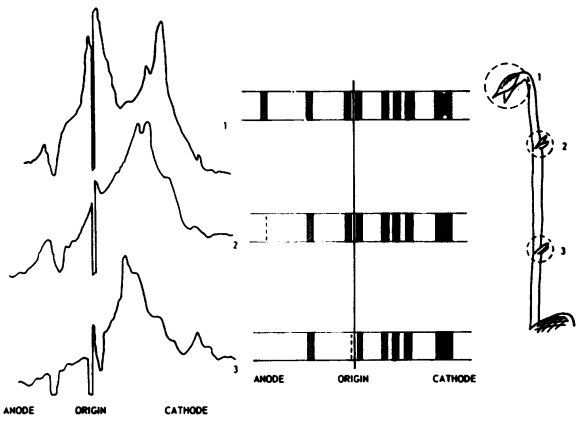

FIG. 3. Topographic distribution of isoperoxidases in etiolated pea stem nodes as shown by gel densitometer traces and diagramatic gel drawings.

similar changes in pattern as a function of time. It should be noted that the internodal regions where the tissue has not yet elongated did not contain C_s3 (16), yet it was detected in the more mature and fully differentiated stem. Progress peas, which are not dwarf when grown in the dark, showed a pattern which was very similar. Alaska peas almost always have a peroxidase (C_sO) which moves only slightly from the origin toward the cathode, and this peroxidase was never observed in Progress peas regardless of the conditions under which they were grown. Since this peroxidase represents only a small fraction of the total peroxidases, it has not been studied further.

The anionic peroxidases were always few in dark grown plumular material; A_s1 was generally present, but A_s2 and A_s3 could not always be detected, except as broad smears in the general region where they would be expected to migrate. It may be that these peroxidases represent enzymes which in the light may be packaged or incorporated into the developing chloroplast or other organelle whose development is enhanced by light. Accordingly, perhaps they are analogous to the peroxisomes found by deDuve (6). The A_s4 band, corresponding directly to the A_r4 of roots, was again found most abundantly in the active meristematic regions and showed a decreased amount as one analyzed progressively lower portions of the plant.

In light grown material, leaflets, stipules and tendrils (all parts of the leaf) possess C_s1, C_s2, perhaps C_s3 and abundance of C_s4 and C_s5. It is the C_s5 peroxidase which can be found rarely and then only in trace amount in stem sections unless they have been treated for a number of hours with gibberellic acid. It is premature to say whether this peroxidase from GA treated stems is identical to that found in green leaf tissue.

The Effect of Light on Isoperoxidase Patterns. When electrophoresis was carried out in starch gel using borate-NaOH pH 9 buffer, with direct maceration of the plant material immediately before insertion into the gel, no obvious effects, such as others had reported (11, 22), of light and dark treatments of the plants was seen. Roots placed in light lost some of their peroxidase activity; IAA raised the peroxidase levels both in dark and light treated roots (table I), but their isozymal patterns remained qualitatively the same. When 3 day old dark and light grown Alaska seedlings (at this stage, the plants are just beginning to break ground and both groups are still arched) were compared electrophoretically, only 1 difference could be found, i.e. there were higher levels of C_s5 in the apex from light grown plants. As the plants got older, it was progressively more difficult to demonstrate C_s5 in dark grown material, and easier to find this isoperoxidase in light grown plant material, especially in leaf tissue. Stem sections from unexpanded internodes from dark- and light-grown plants show the same phenomenon with respect to the appearance of band C_s3 with maturity, and its repression by IAA.

Cotyledons of light-grown plants show ontogenetic peroxidase patterns similar to the dark grown ones, but the sequence develops more slowly. Thus, the cotyledonary peroxidases from a 10 day old light grown plants appear quanitatively more like a chronologically younger version from dark grown plants.

Discussion

Enzymes isolated from a single source, such as the sixth internode of peas, can vary in isozymic composition as a function of hormone treatment and age of the plant. The source and time of harvest as well as the methods of purification and

Table I. *Peroxidase Induction in Roots by 0.1 mM IAA*

Treatment	Mg fr wt/ 5mm root tip	Relative peroxidase activity* per root tip	per mg fr wt
Dark —IAA	3.0	0.2	0.067
Dark +IAA	3.2	0.35	0.109
Light —IAA	3.0	0.06	0.020
Light +IAA	2.5	0.15	0.060

* Activity is defined as Δ OD (470 mμ)/t (min) for a first order reaction. The reaction mixture is composed of 5 mM guaiacol and 5 mM H_2O_2 in 0.1 M phosphate buffer pH 5.8

handling are relevant to the kinds and rates of reactions which the extracted peroxidases may subsequently carry out.

It is of interest that peroxidases from a single plant vary both quantitatively and qualitatively, when different organs are compared. In fact, the differences are more profound when root and shoot of a single plant are compared, than when 1 organ is studied from 2 different varieties.

Organ specific differences found in peroxidase of peas is neither unique for peas nor for the enzyme. Preliminary experiments with a number of higher plants, both monocots and dicots, all showed organ differences; furthermore, phosphorylase and amylase demonstrate differences in electrophoretic mobility as a function of both the organism and plant tissue utilized. The recent experimental demonstration (21) that 5 of the 6 cationic isoperoxidases from winter rye are synthesized de novo during seed imbibition and germination, lends credence to a hypothesis involving differential gene action. However, it is also not unlikely that the differences may reflect different patterns of development in terms or organellar synthesis, since chloroplast, myeloblasts, etc., are not uniformly distributed throughout the plant. Possibly the divergent patterns of development are initially reflected in changes in such components, so that changes in isozyme pattern may be a shadow of many subsequent events leading to a differentiated organism.

The effects of light may reflect only relative differences in turnover of storage materials and the enzymes required to utilize them. These metabolic processes are necessarily more rapid in dark grown plants than in those which are able to photosynthesize. It is reasonable to inquire as to whether differences seen in a specific molecule are in fact a result of variations in the molecule per se, or in its environment.

It should be noted that we have found 2 very basic proteins, C_s3 and C_s5 (basic since they still possess negative charges at pH 9.0) which may be regulated by hormones associated with growth and differentiation. We have also demonstrated (21) using winter rye that the most rapidly migrating basic peroxidase appears to be carried over in the dry seed, whereas the 5 other basic peroxidases are synthesized de novo during imbibition and germination. These observations might be of interest in view of the emphasis recently placed on basic proteins as modifiers of gene action (1).

With the exception of the minor group A_r4 to 8, which is fast moving, negatively charged and guaiacol negative, the isoperoxidases are not differentiated by substrate specificity. Neither are there any regular patterns of kinetic differences among them. Distinction, therefore rest solely upon the intrinsic electrohydrodynamic differences which electrophoresis reveals. Conceivably other differences may exist as well and should be reviewed briefly. A) Among the vast array of natural and laboratory chemicals which are known to be peroxidized, greater or lesser differences may exist in substrate specificity; B) The isoperoxidases may differ either qualitatively or quantitatively in the phenolic and/or metallic cofactors required for IAA oxidase or NADH oxidase function; and C) A full physico-chemical characterization of the isoperoxidases may reveal differences that suggest differentiation of the isozymes in environmental or ultrastructural terms rather than as catalysts, per se.

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