Indole-3-acetic Acid Oxidase from Peas

I. OCCURRENCE AND DISTRIBUTION OF PEROXIDATIVE AND NONPEROXIDATIVE FORMS

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ABSTRACT

Tissues of etiolated pea seedlings variety Alaska were examined for the presence of peroxidative and nonperoxidative forms of indoleacetic acid (IAA) oxidase. Enzymes were extracted in a sequence involving acetone powder preparation from pea tissues, buffer extraction of the powder, ammonium sulfate precipitation, dialysis, lyophilization, and acrylamide gel electrophoresis. Electrophoretically separable proteins were assayed for IAA oxidase activity with the Salkowsk test, and peroxidase activity was based on the color reaction with benzidine and H₂O₂. Each tissue examined contained several nonperoxidative IAA oxidases. No tissue contained more than three peroxidative IAA oxidases, whereas the plumule hooks (a tissue with a high IAA oxidase activity) contained no detectable peroxidases. The results indicate that nonperoxidative IAA oxidases might play a major role in the regulation of IAA content in pea seedlings.

IAA is the best known naturally occurring auxin, and it participates in controlling many phases of growth and differentiation. Levels of free IAA are, in turn, regulated via synthesis, binding, esterification, and enzymatic degradation catalyzed by IAAO. There have been many reports of IAAO in a wide variety of plants (9, 18) with the destruction capabilities increasing in a tip to base manner (5, 17) or increasing with physiological age (5) and inversely correlated with the IAA concentration (4, 5, 12). The physical nature of IAAO has remained controversial. Galston et al. (6) and others (7, 9, 18) have proposed that IAAO and peroxidase activities are shown by the same enzyme. This concept rests on the frequent demonstration that many IAAO preparations contain peroxidases and that purified HRP can degrade IAA in vitro. Most researchers have studied either crude or only partially purified enzyme preparations, or focused only on peroxidases that could be detected (14). Previous research (21, 26) has succeeded in isolating nonperoxidative IAAO fractions. Since multiple peroxidases in pea seedlings have been shown (14, 22, 25), the same condition may exist for nonperoxidative IAAO. In evaluating this possibility, our preliminary research demonstrated that nonperoxidative IAAO enzymes could be isolated from pea seedlings. The scope of the research in this report was to utilize a set of standard biochemical techniques to screen electrophoretically separable proteins for both peroxidative and IAAO activities. This paper reports the results of these studies as applied to several tissues of pea seedlings.

MATERIALS AND METHODS

Plant Tissue. Seeds of Pisum sativum L. var. Alaska were soaked in distilled H₂O for 4 h and then grown in the dark on wet paper towels in plastic trays for 8 days at 18 C. The seedlings were harvested under dim green light and were sectioned into roots, first internodes, second internodes, and plumule hooks. The fresh tissue sections were packaged separately in convenient amounts (25–50 g) in plastic bags and frozen until required for further analysis.

Preparation and Extraction of Acetone Powders. Acetone powders were prepared from the frozen tissue by blender homogenizing 25 g tissue in two successive 100-ml aliquots of cold acetone. The homogenate was collected by Büchner filtration through Whatman No. 1 filter paper following both grindings. The homogenate was air-dried until free of acetone odor, and the resulting dry powder was weighed and freezer-stored in closed containers.

One g of acetone powder was ground in two successive 20-ml aliquots of 25 mM phosphate buffer (pH 6.2) in a mortar chilled in an ice bath. The extract was collected by Büchner filtration through Whatman No. 1 paper after each grinding. The combined filtrates, comprising the protein extract, were diluted to 50 ml with phosphate buffer.

IAAO Assay. IAAO activity was determined on an aliquot of the extract by measuring residual IAA following dark incubation with shaking at 30 C. The enzyme incubation mixtures contained: 142 μmol phosphate buffer (pH 6.2), 3.05 μmol PCA, 5.7 mmol IAA, 30 μmol MnCl₂, and 2.0 ml enzyme preparation giving a total volume of 10 ml. Reaction was initiated by addition of IAA. Residual IAA was determined colorimetrically according to Gordon and Weber's (8) modification of the Salkowski test, except that Fe(NO₃)₃ replaced FeCl₃ (16). Aliquots of 2.0 ml were withdrawn from the incubation mixtures after 0 and 50 min incubation and placed in flasks containing 5.2 ml of 5 μM CH₃CO₂ and 0.5 ml of 0.1 μM Fe(NO₃)₃, and then diluted to 10 ml with distilled H₂O. After incubating the Salkowski mixtures in the dark for 60 min, A was measured at 535 nm.

Protein concentration was determined on an aliquot of the enzyme preparation according to Lowry et al. (13).

Salt Fractionation. The protein extract was then subjected to stepwise ammonium sulfate precipitation, adding solid ammonium sulfate to the extract to achieve a desired level of saturation, based on the nomogram of Dixon (3). Precipitation was performed at ice bath temperatures, proceeding in units of 10% increase in saturation. Dissolution of salt was followed by waiting 20 min for a precipitate to appear. Precipitates were collected by centrifugation for 5 min at 5,000g. The precipitates were dissolved in 15 ml phosphate buffer, and dialyzed against distilled H₂O at 10 C for 48 h. These protein samples were then lyophilized and freezer-stored in closed containers until examined by polycrylamide gel electrophoresis.

Gel Electrophoresis. Electrophoresis was performed by the methods of Davis (2), with the following modifications. (a) Ly-
Oxidized preparations were dissolved in 4.0 ml large pore gel and 0.3 ml was applied per tube. (b) Spacer gel consisted of 0.15 ml large pore gel per tube. Initially, we experienced the problem of variation among the 12 tubes with respect to migration distances of the dye fronts. We resolved this problem by carefully measuring the volume of unpolymerized separating gel placed in each tube. By holding the volume constant among the tubes, rather than height of solution per tube, the difference in migration distance of the dye between the fastest and slowest tubes was no more than 2 to 3 mm. We suspect that the initial problem was due to slight variations in the inside diameter of the glass tubing, as well as lengths of the tubes. (c) Electrophoretic separation was performed at cold temperature maintained by circulating ice water around the lower buffer reservoir, and the applied current was 2 mamp/tube for 10 min, followed by 4 mamp/tube until the dye marker had reached the ends of the gels. Twelve gel tubes were run simultaneously.

Gels were removed immediately from the tubes and stained for peroxidase bands by placing one gel in a solution composed of H2O2 and benzidine, according to Brewer (1). We conducted a preliminary survey of chromogenic agents by screening with protein fractions from the first internodes of the pea seedlings. Benzidine, o-dianisidine, and pyrogallol gave the same staining pattern. We elected to use only benzidine, because it rapidly yielded an intensely blue product. Protein bands were located by placing a second gel in 1% (w/v) Amido Schwarz in 7% (v/v) acetic acid and stained overnight. Destaining was electrophoretic at 15 mamp using 7% acetic acid as the electrolyte. To prevent diffusioning of the separated bands, the 10 remaining gels were immediately sliced transversely into 2-mm-thick discs using a Plexiglas slicing template. Homologous gel sections were collected and stored in glass vials at 10 C until results of the stains had been obtained.

By stacking the gel sections into running tubes and applying a 2- to 3-mamp current for 6 to 8 h, the appropriate gel sections were electrophoretically eluted into a small dialysis bag in the Tris-glycine buffer of Davis (3). These samples were then dialyzed, and aliquots were assayed for protein concentration and IAAO activity.

In summary, each ammonium sulfate protein fraction was subjected to electrophoresis. One gel was stained for peroxidase activity, one for protein, and the remaining 10 gels were cut into 2-mm-thick discs. Discs corresponding to visible protein bands were eluted and assayed for IAAO activity. A nonperoxidative IAAO is defined as an enzyme from a disc whose position did not correspond to a peroxidase positive band, but did correspond to a visible protein band which exhibited IAAO activity of at least 1 μg IAA destroyed/μg protein in 50 min at 30 C.

**Effect of Pea Extraction Scheme on Loss of Heme from HRP.**

HRP, used as a heme-containing model system, was subjected to the entire pea enzyme extraction sequence to monitor the potential for dissociating heme from peroxidases. The heme content of proteins was measured by determining Reinheitzahl (RZ) values. RZ is defined as the ratio of A at 402 nm to the A at 275 nm (19).

Ten mg of HRP was dissolved in 1 ml of phosphate buffer, and then hand-agitated with 20 ml of cold acetone. The mixture was centrifuged at 4,600g for 5 min, then the acetone supernatant was scanned in the Soret region for free heme. The protein pellet was dissolved in 50 ml of phosphate buffer and brought to ammonium sulfate saturation under ice bath conditions. The precipitated proteins were collected by centrifugation. Pellet and supernatant were individually dialyzed, and the dialyzed proteins were combined and lyophilized. RZ values were determined for the lyophilized sample and for an untreated sample of HRP, type I using 25 mm phosphate buffer (pH 6.2).

**RESULTS**

The data in Table I report the distribution of peroxidative and nonperoxidative IAAO in salt fractions of extracts of etiolated Alaska pea tissues. Only those proteins corresponding to visible bands following electrophoretic destaining were assayed for IAAO activity. In addition, all peroxidases correspond to a protein-staining band, while some proteins exhibited neither peroxidative

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nor IAAO activities. Because no further physical or chemical characterization was conducted to distinguish enzymes, a given IAAO may be represented more than once in these tissues. In all of the tissues analyzed, there was a greater number of nonperoxidative IAAO protein bands than there were peroxidative IAAO proteins. With one exception (second internodes, 70%), the IAAO with the highest specific activity in each salt fraction was nonperoxidative. There appeared to be no orderly trend in either the number or activities of IAAO proteins when progressing toward the apex of the pea seedling. However, the number of peroxidases and peroxidative IAAO (Table I) decreased when approaching the apex. It is noteworthy that the plume hooks (a tissue with a high IAAO activity) yielded no detectable peroxidases. In agreement with previous findings (15), IAAO activity was not always salt-precipitated, and the IAAO with the highest specific activity appeared in the salt-saturated supernatant of the first internode extract.

The possibility that the scheme used for extracting proteins from pea internodes could cleave peroxidase to heme plus apoperoxidase was examined. Curve A (Fig. 1) shows that there is no detectable heme in a concentrated acetone extract of fresh pea internode tissue. When HRP was agitated with cold acetone and collected by centrifugation, a small amount of protein-bound heme remained in the acetone supernatant (curve B) as shown by Soret absorption at 405 nm. There was no evidence for any absorption peak at 385 nm that would indicate any free heme. The presence of protein-bound heme in the acetone-precipitated pellet is evident from curve C. The peroxidase solution from curve C then was treated by ammonium sulfate precipitation, dialysis, and lyophilization. Following all of the extraction steps, heme remained attached to the protein moiety (curve D) giving an RZ value of 0.69 as compared to an RZ value of 0.72 for untreated HRP, type I.

DISCUSSION

This study has shown that proteins separated by electrophoresis from pea seedlings may be grouped into four categories: (a) those having both IAAO and peroxidative activities; (b) nonperoxidative enzymes with IAAO activity; (c) peroxidases with no IAAO activity; (d) nonperoxidative proteins with no IAAO activity. The classical assumption has been that IAAO is a peroxidative enzyme, and crystalline HRP has been used for a model system in enzyme studies. The data contained in this research clearly show that peroxidative action is most often not a requirement for in vitro enzymic destruction of IAA. Siegel and Galston (24) demonstrated that full IAAO activity was retained by the apoenzyme of commercial HRP after removal of the heme group. They concluded that peroxidase and IAAO activities were due to two distinct sites on the enzyme. From this it is apparent that the action of horseradish peroxidase as an IAAO is independent of its action as a peroxidase.

The data reported here do not negate the possible importance of peroxidases in IAA metabolism, since several peroxidases in various tissue sections are active in oxidizing IAA in vitro. These results show that two enzyme types exist which may act in the control of auxin-related phenomena: nonperoxidative IAAO and IAAO which may function independently as peroxidases. Sequeira and Mineo (21) have reported a nonperoxidative IAAO in crystalline HRP that they believed to be responsible for the oxidation of IAA by the commercial enzyme. Siegel and Galston (24) suggested that the nonperoxidative IAAO reported by Sequeira and Mineo (21) was an artifact resulting from accidental removal of heme during extraction. Siegel and Galston reported that they had been unable to detect free apoperoxidase from peas showing IAAO activity. The results reported from this research show that nonperoxidative IAAO can be isolated from peas. Although it is possible that these nonperoxidative IAAO enzymes may be the apoperoxidase sought by Siegel and Galston (24), the isolation procedure is not considered sufficiently harsh to dissociate heme from the peroxidases present. Utilizing our extraction protocol, we were unable to achieve significant dissociation of heme from a purified sample of HRP (Fig. 1). This is highly suggestive that the nonperoxidative property of some of the IAAO enzymes cannot be attributed to heme removal during extraction.

Our results may require some qualification as to the general applicability of other published results, inasmuch as it has been felt that peroxidase activity is a good indicator of IAAO activity, and that the active site of IAAO contains a heme group. Sequeira and Mineo (21) clearly demonstrated that some peroxidase and IAAO activities were separable when fractionating tobacco root extracts on SE-Sephadex C-50 with a high molarity buffer. At least six peaks of IAAO activity were generated, one major peak of which had no peroxidative activity. Hoyle (10) was unable to verify the separability of peroxidase and IAAO activities based on gel filtration of extracts of birch leaves. The one peak of IAAO activity had the same elution volume as that of the peroxidative activity. More recently, Hoyle (11) has demonstrated the high resolving power of isoelectric focusing by fractionating commercial HRP samples into numerous isoperoxidases. When commercial HRP was subjected to gel filtration only one peak of peroxidase activity was observed (10). Gel filtration has limited resolving power, and activity profiles should indeed be interpreted cautiously (27). We are simply acknowledging the possibility that a single peak from gel filtration is not rigorous proof of enzyme homogeneity, and that the coincidence of two enzyme activities in a single elution peak also could be interpreted as being due to two different enzymes with similar mol wt. In addition, others (23) have proposed to stain for IAAO activity by reacting p-dimethylaminoazinomethyldiehyde with an enzyme reaction product. However, because the true identity of the IAAO reaction product is speculative (20), the IAAO stain as an indicator may not be highly accurate. Another IAAO stain (11) has included IAA as well as H2O2 and a peroxidase substrate. We can envision a color reaction taking place without IAA actually being a reactant. Consequently, such a stain conceivably could generate too high a correlation being drawn between IAAO and peroxidative activities.

These comments emphasize the necessity of reevaluating many papers dealing with the IAAO/peroxidase dilemma. In so doing, it is likely that alternate interpretations of results could be forthcoming, and this would underscore our contention that there is room for an alternate concept regarding the physical nature of IAAO.

In summary, we have reported research which has demonstrated...
that some proteins which can be isolated from pea seedlings exhibit IAAO activity, but without peroxidative activity. This does not prove that these activities are the primary function of these enzymes in vivo. If IAAO indeed does function in regulating levels of IAA in vivo, then this role is most precisely defined by studying physical properties of a rigorously purified IAAO. A well characterized IAAO protein may then be studied with respect to those factors which regulate its synthesis, activity, and degradation. Presently, much of this information with respect to an IAAO does not exist. We have conducted preliminary studies on some properties of high purity IAAO, and a report of these results is in preparation.

**LITERATURE CITED**

7. GOLDACKI PL 1961 The indole-3-acetic acid oxidase-peroxidase of peas. In RM KLEIN, ed. Plant Growth Regulation. Iowa State Univ Press, Ames, pp 143-147
CORRECTIONS

Bryant, Stephen D., and Forrest E. Lane. Indole-3-acetic Acid Oxidase from Peas. I. Occurrence and Distribution of Peroxidative and Nonperoxidative Forms.
Page 696, column 2, paragraph 4, line 4 should be corrected to read: ... 5.7 \mu mol IAA.

Page 1188, Figure 1, vertical legend should be corrected to read: Potassium-stimulated ATPase Activity (\mu moles Pi/mg protein/h).
Page 1188, column 1, line 9 should be corrected to read: may have contributed to the apparent sharp inflections in activity between segments.