Isoperoxidases of (IAA oxidase) oxidase in oat coleoptiles

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Eight constitutive isoperoxidases were separated by the disc method of polyacrylamide gel electrophoresis from a lyophilized extract of 8-day-old oat (Avena sativa L., cv. Victory) coleoptiles. Both anodic and cathodic isoperoxidases were studied and differences in electrophoretic mobilities and hydrogen donor substrate specificities were revealed. In addition, by enzyme assay, cathodic and anodic isoenzymes were shown to possess differences in peroxidase and IAA (indole-3-acetic acid) oxidase activities.

Treatment of coleoptiles with 0.07 mM IAA for 24 h resulted in the repression of two slow-migrating anodic isoperoxidases; however, the same treatment also resulted in the induction of two slow-migrating cathodic isoenzymes which were shown to exhibit peroxidase and IAA oxidase activities.

Introduction

The literature is replete with studies on the multiple or isoenzymous nature of peroxidase in plant tissues (24). Studies comparing in vitro enzymic activities of extracted peroxidases with numerous hydrogen donor substrates suggest that these isoenzymes have much greater substrate specificity than had previously been discerned. This fact tends to buttress the many diverse metabolic and developmental functions in higher plants attributed to peroxidase (3, 4, 17, 22). Several recent investigations have provided even more evidence for the multifarious nature of peroxidase in plants. These studies have demonstrated differing physical, as well as chemical properties of isoperoxidases with varying distribution in plants (9, 27).

Concomitant with the investigation of the multiple nature of peroxidase in plants has been the quest to ultimately decipher the precise nature of the enzyme responsible for the destruction of endogenous auxin and thus modulation of growth in higher plants. Earlier studies by Stutz (30), using starch gel electrophoresis demonstrated that peroxidase and IAA3 oxidase activities were contained in the same macro-molecule. Yet, Sequeira and Mimeo (24), using ion-exchange filtration reported that in tobacco there were peroxidase isoenzymes with no perceivable IAA oxidase activity; this is a point of continuing controversy. Siegel and Galston (28) have provided evidence that IAA oxidase and peroxidase activities reside in the same macro-molecule since they demonstrated that apoperoxidase, provided with the proper cofactors, readily destroyed auxin without peroxidase activity, yet, the reconstituted peroxidase demonstrates vigorous peroxidative activity. More recent studies demonstrating the induction and repression of isoenzymes of peroxidase by natural and synthetic hormones also indicate a correlation of peroxidase and IAA oxidase activities (7, 11, 12, 13, 21, 29). The extraction and separation of isoperoxidases in oat coleoptiles and the investigation of their role in IAA oxidase activity are the subjects of this paper.

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ABBREVIATIONS: IAA, indole-3-acetic acid; IAA oxidase, indole-3-acetic acid oxidase; DCP, 2,4-dichlorophenol; TCP, 2,4,6-trichlorophenol; H2O2, hydrogen peroxide; benzidine-HCl, benzidine dihydrochloride.
Materials and Methods

Materials
All experiments used 8-day-old oat coleoptile tissue. Oat (Avena sativa L., cv. Victory) plants were grown in darkness on moist vermiculite at 25°C. After 72 h, the young seedlings were irradiated for 12 h with red light to suppress internodal growth (23). They were subsequently returned to darkness until the completion of 8 days of total growth. Coleoptiles were harvested and kept frozen until used for enzyme extraction.

Enzyme Preparation
Fifteen-gram samples of frozen coleoptiles were homogenized in 120 ml of cold 0.01 M phosphate buffer, pH 6.1, using a Waring semimicroblender. Homogenization was followed by two successive washes with buffer. To the total supernatant, an equal volume of acetone was added and the mixture was allowed to stand overnight (6). The acetone-insoluble fraction was collected by centrifugation at 10,000 g for 10 min. The precipitate was suspended in 15 ml of cold, distilled water, frozen, and freeze-dried for 24 h. The freeze-dried enzyme was stored in a desiccator for subsequent electrophoretic fractionations. All of the foregoing procedures were conducted at 4°C or below.

Electrophoresis
Before electrophoretic separation, lyophilized protein was resuspended into 2 ml of 0.01 M phosphate buffer, pH 6.1. This was equally divided into each of 10 columns, with each column receiving 0.2 ml of the resuspended protein, which in most cases was a total amount of 2 mg for the anodic separation. Isoenzymes were separated by polyacrylamide gel electrophoresis. Anodic gel columns were composed of 7.5% (w/v) polyacrylamide in the separation section and 2.5% (w/v) polyacrylamide in the spacer and sample sections. Cathodic gel columns were composed of 5% (w/v) polyacrylamide in the separation section and 3.5% (w/v) polyacrylamide in the sample section. A Buchler Polyanalyst polyacrylamide gel electrophoresis apparatus was used with a Buchler Model 3-1009 power supply. Anodic separation was conducted according to the procedures of Davis (2), with the exception that the order of the gel layers was reversed. Cathodic separation was conducted according to the procedures of Reisfeld et al. (20). Electrophoresis was conducted at 4°C.

Detection of Isoenzymes
After electrophoresis, gels were removed from the tubes and stained to reveal the locations of either peroxidase activity or IAA oxidase activity. Staining to reveal peroxidase activity was achieved by utilization of two different hydrogen donor substrates. When benzidine-HCl was used, modification of the procedures by Novacky and Hampton (17) was used. The staining medium consisted of 30 ml of 0.05 M benzidine-HCl, 30 ml of 3 mM H2O2, and 70 ml of 0.01 M phosphate buffer, pH 6.1. When guaiacol was used as the substrate, modification of the procedure by Stuber and Leavings (29) was used. The staining medium consisted of 50 ml of 0.015 M guaiacol, 20 ml of 5 mM H2O2, and 20 ml of 0.01 M phosphate buffer, pH 6.1. Modification of the standard procedures was necessary to effect greater resolution in polyacrylamide gels. IAA oxidase activity was revealed by the procedure of Endo (5) with fast blue BB, modified by substitution of DCP for TCP as the phenolic cofactor. Fast blue BB salt (Cl 37175) was purchased from Sigma Chemical Co., St. Louis. Staining was conducted for 1 to 2 h with guaiacol, 3 to 5 h with benzidine-HCl, and 7 to 8 h with fast blue BB; staining was conducted at 37°C.

Peroxidase and IAA Oxidase Assay
Bands of enzymic activity were sliced from the gels and eluted by maceration of the gel segments into 5 ml of 0.01 M phosphate buffer, pH 6.1, and shaking in a Dubnoff metabolic shaking incubator for 2 h at 30 rpm. The concentration of eluted isoenzymes was determined by a modification of the method of Lowry et al. (14) in which horseradish peroxidase was used as the standard instead of the usual bovine serum albumin. IAA oxidase activity was determined by the method of Goldacre et al. (8) with the amount of residual IAA estimated with the modified Saikowski reagent (10). Peroxidase assay was conducted according to the method of McCune (15).

Results

Migration and Staining Properties
Eight isoperoxidases comprise the peroxidase and IAA oxidase activities of oat coleoptiles in this investigation, a property which corroborates the findings of Stuber and Leavings, who studied the isoenzymatic nature of peroxidase in coleoptiles from several varieties of oat (29). After electrophoretic separation for 2 h, anodic isoperoxidases demonstrated greater electrophoretic mobility (Table 1) with more uniform distribution from top to bottom in the acrylamide gels than cathodic fractions (Fig. 1). Cathodic fractions separated for an equal length of time tended to be rather slow-moving except for a dense peroxidase zone (Fig. 1E), C1, revealed when benzidine-HCl was used as the substrate. This irregular zone of peroxidase activity seems not to be unique to oat tissues since it has also been reported in the investigations of Gordon and Aldridge (9) on tomato stem tissues. From cytochemical studies, they found that this fraction was unlike soluble cytoplasmic peroxidase in that it was tightly bound to cell wall fragments and required the addition of salts or lowering of pH for resolution. An in vitro study of tea leaf peroxidase by Takeo and Kato (31) provided similar results. Also evident were the differences in detection of isoperoxidases by the two substrates used. Localization with guaiacol was characterized by a single hue with maximum visualization within 2 h. Peroxidase activity was revealed much more...
slowly when benzidine-HCl was used and maximum visualization was preceded by several oxidative color changes as previously reported by DeJong (3). Benzidine-HCl was apparently more effective as a substrate in that it revealed the greater number of bands (Fig. 1A). But guaiacol seemed to be more effective in revealing slower moving fractions (Figs. 1B and 1F), although if absolute mobility is considered, benzidine-HCl was shown to reveal the two slowest isoperoxidases. Guaiacol proved to be the more specific in detecting isoperoxidases, as evidenced by the fact that an isoperoxidase, A3, revealed as one band by benzidine-HCl (Fig. 1A).

### TABLE 1
Electrophoretic migration of isoperoxidase and IAA isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme activity</th>
<th>Migration interval, mm</th>
<th>Electrophoretic mobility, mm/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benidine-HCl, hydrogen donor</td>
<td>A1</td>
<td>35-52</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>18-22</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>7-14</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>3-4</td>
</tr>
<tr>
<td></td>
<td>A5</td>
<td>0-1</td>
</tr>
<tr>
<td>Guaiacol, hydrogen donor</td>
<td>A1</td>
<td>7-8</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>13-14</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>9-11</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>18-21</td>
</tr>
<tr>
<td>Oxidative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benidine-HCl, hydrogen donor</td>
<td>C1</td>
<td>22-37</td>
</tr>
<tr>
<td>Guaiacol, hydrogen donor</td>
<td>C1</td>
<td>4-5</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>7-8</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>4-5</td>
</tr>
</tbody>
</table>

Note: Migration interval and electrophoretic mobility values represent averages of 18 estimations. All estimations were made from electrophoretic separation of the same lyophilized preparation. Anodic separation was conducted in 7.5% gels and cathodic in 5% gels.

*Electrophoretic mobility = (migration interval) / 2 (ref. 25).

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**Fig. 1.** Diagram of peroxidase and IAA oxidase isoenzymes from oat coleoptiles separated by polyacrylamide gel electrophoresis of lyophilized enzyme preparations. Columns represent detection of isoenzymes from staining and treatment as follows: (A and E) benzidine-HCl + H2O2 without IAA pretreatment of coleoptiles; (B and F) guaiacol + H2O2 without IAA pretreatment; (C and G) fast blue BB + IAA without IAA pretreatment; (D) benzidine + H2O2 with 24 h 0.07 mM pretreatment of coleoptiles; and (H) fast blue BB + IAA with 24 h 0.07 mM IAA pretreatment of coleoptiles.
was subsequently revealed as two bands by guaiacol (Fig. 1B) and even possibly as three bands when IAA oxidase activity was determined (Fig. 1C).

The IAA oxidase activity revealed by staining with fast blue BB suggested that not all isoperoxidases were capable of IAA destruction (Figs. 1C and 1G). Anodic and cathodic oxidase fractions exhibited differences in color densities as described by Endo (5) but in reverse order, that is, anodal IAA oxidase isoenzymes developed as transparent yellow bands and cathodic isoenzymes were revealed as rather opaque yellow bands. In agreement with these findings, we found that IAA oxidase bands always corresponded in part or completely with a previously revealed peroxidase band (Fig. 1).

Peroxidase and IAA Oxidase Assays

From the resolution of isoperoxidases and IAA oxidases by the staining techniques used (Fig. 1), it became apparent that not only were there qualitative differences in various isoenzymes, as evidenced by visualization with guaiacol and benzidine, but that there were, also, quantitative differences as well, as shown by band size and density. It was of interest to determine whether there was any correlation between the relative size and density of the bands and their activity in peroxidative and oxidative catalysis. It was found that there was no correlation between size of bands and peroxidase activity (Table 2) or IAA oxidase activity. In fact, it is noteworthy that at least one fraction, A2 (Fig. 1C), exhibited IAA oxidase activity in staining, yet the same fraction demonstrated no measurable in vitro auxin destruction. Two additionally interesting phenomena were also apparent. All cathodic isoenzymes possessed both peroxidase and IAA oxidase activities, a point which could not be supported by results from anodic studies (Table 2). In general, cathodic isoenzymes demonstrated greater enzymic activity, both peroxidase and IAA oxidase, than anodic isoenzymes (Table 2).

Induction and Repression of Isoenzymes by IAA

Induction and repression of isoperoxidases has been widely reported in the literature (7, 11, 21, 29) and a few studies have reported the effect of hormone treatment on IAA oxidase activity (12, 32). Since the above experiments had provided significant data on untreated tissue, it was quite opportune to conduct comparative study on auxin-treated tissue to determine the effect of supraoptimal auxin concentration on peroxidase and IAA oxidase constitution and activity. After treatment of tissues for 24 h with 0.07 mM IAA, the two slowest moving peroxidase bands previously resolved by benzidine-HCl in staining (Fig. 1A) were shown to have been repressed when detection was conducted with the same substrate (Fig. 1D). In contrast to the repression by IAA on the anodic peroxidases, induction of two oxidative bands was revealed by detection with fast blue BB (Fig. 1G and H).

Discussion

The results of investigations reported here show that peroxidase in Avena coleoptiles is isoenzymous and abides in two major groups, anodic and cathodic. Separation of the two groups of isoenzymes required use of two different gel concentrations, a fact which was previously reported by Shannon et al. (26) while studying horseradish peroxidase. But the larger difference in gel concentration required in this investigation suggests that a greater difference in molecular weights exists between the two groups studied here than those previously reported. While anodic separation resulted in a rather

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Protein conc., µg/ml</th>
<th>Peroxidase activitya</th>
<th>IAA oxidase activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anodic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>555</td>
<td>2.5 × 10⁻²</td>
<td>3.1 × 10⁻⁵</td>
</tr>
<tr>
<td>A2</td>
<td>340</td>
<td>1.1 × 10⁻²</td>
<td>0</td>
</tr>
<tr>
<td>A3</td>
<td>370</td>
<td>6.7 × 10⁻³</td>
<td>2.2 × 10⁻⁴</td>
</tr>
<tr>
<td>A4</td>
<td>220</td>
<td>3.9 × 10⁻²</td>
<td>0</td>
</tr>
<tr>
<td>A5</td>
<td>120</td>
<td>6.2 × 10⁻⁵</td>
<td>1.4 × 10⁻⁴</td>
</tr>
<tr>
<td>Cathodic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>30</td>
<td>2.5 × 10⁻¹</td>
<td>3.2 × 10⁻³</td>
</tr>
<tr>
<td>C2</td>
<td>280</td>
<td>4.0 × 10⁻²</td>
<td>8.7 × 10⁻⁴</td>
</tr>
<tr>
<td>C1</td>
<td>1400</td>
<td>1.6 × 10⁻¹</td>
<td>1.8 × 10⁻⁴</td>
</tr>
</tbody>
</table>

NOTE: Bands of peroxidase and IAA oxidase activity were revealed by staining two tubes from each separation. The corresponding sections were removed from the remaining gels columns with a single-edge razor blade and macerated in a 5-ml disposable syringe. The macerated gels were taken up in 5 ml of 0.01 M phosphate buffer pH 6.1 and eluted for 2 h by shaking at 50 rpm. Extractions were made from pooled elutions of 20 gels.

aActivity measured in nanomoles of guaiacol destroyed per minute per microgram of protein.

bActivity measured in ngoles of IAA destroyed per hour per microgram of protein.
uniform distribution in electrophoretic migration, cathodic separation resulted in comparatively slower and more localized migration (Table 1). Such findings support suggestions of greater structural similarities among cathodic than anodic isoenzymes (18, 26).

Substrate specificity is exhibited by the individual isoperoxidases, both anodic and cathodic (Table 1). This fact is quite well known for plant peroxidase (1, 18), but unique in this study is the fact that visualization of peroxidase by staining with guaiacol indicated that only a few slow-migrating isoperoxidases could utilize it as a substrate (Figs. 1B and 1F) yet all eluted isoenzymes effected its catalysis. The answer to this may be revealed in the findings of Paul and Stigbrand (18), who report that isoperoxidases containing larger amounts of arginine and methionine possessed much greater capacity for catalysis of guaiacol than those with a smaller amount. It can thus be perceived that isoenzymes with smaller amounts of these two amino acids when restricted to a more compact configuration by the limiting pore size of the polyacrylamide gels may not be able to exhibit sufficient affinity for guaiacol to provide a color reaction. Similarly, conformational change due to pore size restriction may effect sufficient separation of reactive groups to provide a fraction such as A3 (Fig. 1B), which appears to be two different isoenzymes when revealed by staining with guaiacol.

Enzymic activity was consistently higher in cathodic isoenzymes than in anodic isoenzymes (Table 2), but no isoenzyme from either group exhibited exclusive IAA oxidase activity. However, two anodic isoperoxidases demonstrated no measurable in vitro auxin destruction. A similar finding has been reported by Wetmore (32). The correlation of higher peroxidase and IAA oxidase activities in cathodic isoenzymes suggests that the same structural properties thought to favor guaiacol destruction may also favor auxin destruction. More important is the fact that supraoptimal auxin treatment causes repression of two anodic peroxidases (Fig. 1D) but induction of two cathodic peroxidases with considerable IAA oxidase activity (Fig. 1H). Lavee and Galston (11) and Macnicol (16) have reported the same kind of results from studies with tobacco pith tissues and green pea seedlings, respectively. In the investigations reported here, it appears that supraoptimal auxin levels elicit physiological responses which require the specificity of cathodic isoperoxidases. We feel that our results and the report by Ridge and Osborne (19) that cathodic isoperoxidases located in plant cell walls modulate cell wall expansion and cellular growth through maintenance of certain auxin and hydroxyproline levels warrant further study on the role of cathodic isoperoxidases in plant growth and development.

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14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and


