The Promotion of Indole-3-acetic Acid Oxidation in Pea Buds by Gibberellic Acid and Treatment

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ABSTRACT

Terminal buds of dark-grown pea (Pisum sativum) seedlings have an indole-3-acetic acid oxidase which does not require Mn$^{2+}$ and 2,4-dichlorophenol as cofactors. Oxidase activity is at least 50 times higher in buds of tall peas than in dwarf seedlings. Administration of gibberellic acid to dwarf peas stimulates both growth and indoleacetic acid oxidase activity to the same levels as in tall seedlings. By contrast, indoleacetic acid oxidation assayed in the presence of Mn$^{2+}$ and 2,4-dichlorophenol proceeds at similar rates regardless of gibberellin application. Treatment of tall peas with the growth retardant AMO-1618 reduces growth and oxidase activity. Such treated seedlings are indistinguishably dwarf. The enzyme does not appear to be polyphenol oxidase, nor do the results suggest that reduced activity in dwarf buds is due to higher levels of a dialyzable inhibitor. The peroxidative nature of the oxidase is probable.

The presence of IAA oxidase in plant tissues has been firmly established (23). The loss of added IAA from tissue extracts has been attributed in part to this enzyme. There is evidence that IAA oxidase is a peroxidase-based enzyme (5, 24) and studies in vitro with horseradish peroxidase revealed that IAA is oxidized via a multiplicity of intermediates to oxindole-3-carbinol, which is converted to methyleneoxindole in a postenzymatic step (8, 15). In view of the inverse correlation between the levels of this enzyme and ability of pea tissue to respond to IAA, Galston and Dalberg (6) proposed that IAA oxidase is a growth-regulatory system. Thus, the enzyme is thought to inactivate IAA, thereby controlling the endogenous auxin level.

In the course of our studies on IAA biosynthesis, we observed that cell-free extracts of etiolated pea buds possess considerable IAA oxidase activity when assayed without cofactors. This paper presents some new information concerning this enzyme in relation to GA treatment and the dwarf-tall growth habit of peas along with experiments bearing on the question of IAA oxidation.

MATERIALS AND METHODS

Seeds of Pisum sativum L., cv. Progress No. 9 or Alaska, were soaked in tap water with or without 0.2 mM GA (a gift from Abbott Laboratories) for about 7 hr and sown in moist vermiculite in polyethylene trays. These were placed in a darkened cabinet in a dark room maintained at 25°C. The seedlings were handled in dim green light (a fluorescent tube wrapped in three layers each of amber and green DuPont cellophane).

Terminal buds were harvested from 4-day-old seedlings, weighed, and placed immediately in cold 0.1 M phosphate buffer, pH 6.1. All steps in the preparation of enzyme for IAA oxidase assays were carried out at 1 to 3°C in green light. The tissue was homogenized in a Sorvall Omnimixer, and the homogenate was centrifuged at 30,000 g for 30 min. The supernatant fraction was adjusted to volume and used as the crude enzyme. Normally, extract of 75 apices with a fresh weight of about 600 mg for Progress peas and about 400 mg for Alaska or GA-treated Progress peas was adjusted to a final volume of 10 ml.

IAA oxidase activity was determined in an assay medium consisting of 9 ml of crude enzyme and 1 ml of IAA (Mann Research Laboratories) at a starting concentration of 30 μg/ml. Each reaction mixture was incubated in a water bath shaker at 30°C. Initial and residual IAA concentrations at various time periods were determined by the Salkowski method as described by Tang and Bonner (25) except that a development time of 20 min was used. Absorbance at 530 nm was determined in a Bausch and Lomb Spectronic 20 colorimeter. All operations during the assay were conducted in green light. In some cases, the kinetics of IAA oxidation were followed in a Coleman Hitachi model 124 recording spectrophotometer by determining the increase in absorbance at 247 nm (22).

Peroxidase activity was assayed by following the linear increase in absorbance at 15-sec intervals at 470 nm in the colorimeter. The reaction mixture contained 0.1 ml of enzyme previously diluted 1:100; 1.9 ml of 0.05 M phosphate buffer, pH 6.1; and 1 ml of a solution of 5 mM guaiacol and 5 mM H$_2$O$_2$.

Protein content was measured according to the method of Lowry et al. (13).

RESULTS

Kinetics of IAA Oxidation. The oxidation of IAA by crude enzyme preparations from etiolated Alaska and Progress No. 9 pea buds without added cofactors is shown in Figure 1. Alaska apices have considerable IAA oxidase activity. By contrast, activity is low in Progress No. 9 pea buds. Treatment of peas with GA gave another unexpected result (Fig. 1). Added GA produces at least a 35-fold (50-fold on a per unit protein basis) increase in enzyme activity in dwarf pea buds. Only a small promotion in activity is attained in buds of the tall pea. The IAA oxidase activities of tall and GA-treated dwarf peas are similar. Subsequent work revealed that oxidase activity is distributed evenly over the plumule and adjoining 3-mm apical crook segment but rapidly decreases basipetally in the shoot (Ockerse and Mumford, unpublished).

The velocity of the reaction increases with IAA concentrations between 12 and 300 μg/ml of IAA. No inhibition could be detected at the highest concentration used.
late growth of etiolated dwarf peas, the effect of the hormone on etiolated tissue is often thought not to be of any significance. Ockerse and Galston (18) reported earlier that GA produces a 21⁄2-fold increase in length of the third internode of Progress No. 9 cuttings. The results reported here (Fig. 2) support the conclusion that GA produces a marked growth response in dwarf but not in tall pea plants.

It is noteworthy that etiolated Alaska and GA-treated Progress No. 9 peas form recurved terminal buds. Without GA, recurrature has never been observed in dwarf seedlings.

**Peroxidase and Polyphenol Oxidase Activity.** In the following experiment the correspondence between increased activities of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IAA Oxidase Activity</th>
<th>Peroxidase Activity</th>
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<tr>
<td></td>
<td>- Cofactors</td>
<td>+ Cofactors</td>
</tr>
<tr>
<td>Progress</td>
<td>0.8</td>
<td>43.4</td>
</tr>
<tr>
<td>Progress + GA</td>
<td>48.0</td>
<td>53.0</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>3.9</td>
</tr>
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**Fig. 1.** Kinetics of IAA oxidation by etiolated Alaska and Progress No. 9 pea buds. Effect of treatment with GA or AMO-1618. A 10-ml reaction mixture contained 300 μg of IAA, 0.1 M phosphate buffer (pH 6.1), and enzyme from 75 buds.

**Table I. Influence of Cofactors on IAA Oxidase Activity of Terminal Buds from Etiolated Progress No. 9 Peas Treated with or without GA**

For the assay without cofactors, a 10-ml reaction mixture contained 350 μg of IAA at the start, 0.1 M phosphate buffer (pH 6.1), and extract from 75 buds. A 10-ml reaction mixture with cofactors contained, in addition to the above components, 0.1 mM MnCl₂ and 0.1 mM dichlorophenol.

<table>
<thead>
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<th>Treatment</th>
<th>IAA Oxidase Activity</th>
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The quaternary ammonium derivative, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride (AMO-1618), known to inhibit gibberellin biosynthesis (1), has a pronounced effect on IAA oxidase activity. Treatment of Alaska pea seeds with 0.2 mM AMO-1618 reduces activity of the enzyme in the buds to a level characteristic of the dwarf pea (Fig. 1). Such treated seedlings are indistinguishably dwarf in their growth habit.

By contrast, IAA oxidation assayed in the presence of cofactors appears not to be affected by prior treatment of tissue with GA. For the experiment summarized in Table I, the buds of Progress No. 9 seedlings (untreated and GA-treated) were assayed at 75 buds per 10 ml of reaction mixture with or without 0.1 mM dichlorophenol and 0.1 mM MnCl₂. It is evident that, with cofactors, activity is about the same regardless of hormone application.

**Gibberellin and Epicotyl Elongation in Dark-grown Peas.** Although Lockhart (12) demonstrated that gibberellin can stimu-
Galston (14) showed that gibberellic acid (GA) increases plumule size (i.e., leaf size), this may explain the increase in oxidase activity in the plumules. GA appears to enhance activity to that found in Alaska (Table II). Activity in light-growth Progress No. 9 pea buds is about 50% less than that present in the dark-grown dwarf pea. However, if GA oxidase activity is expressed on a bud basis, activity is about the same, regardless of cultivar difference, GA treatment, or growth of dwarf seedlings in the light.

A comparison of the change in ultraviolet absorption spectrum of IAA in the presence of horseradish peroxidase (Worthington, RZ 1.12) without cofactors and an enzyme preparation from pea buds reveals that oxidation probably proceeds via a similar series of intermediates. In both cases 3-methyloxindole appears to be the end product. The enzyme preparation was a 40 to 90% (w/v) saturated (NH₄)₂SO₄ fraction of etiolated Alaska pea buds.

Further purification studies revealed that the oxidase activity is associated with the supernatant of the 40 to 70% fraction from which it cannot be recovered with (NH₄)₂SO₄ (Ockerse and Mumford, unpublished). Addition of 0.5 mM KCN to the enzyme preparation prevents the oxidation of IAA completely. The results are consistent with the suggestion that the oxidase is a peroxidase-based enzyme.

Because it has been shown that polyphenol oxidase can oxidize IAA (11), this possibility was also examined. The reaction mixture contained 4.5 ml of enzyme and 0.5 ml of 0.02 m catechol. Assay of enzyme activity at 405 nm showed no increase in absorbance over 2-hr period, and the experiment was terminated. This suggests that the enzyme is not a polyphenol oxidase.

Extent of Inhibitor and Cofactor Contribution. To determine if the difference in IAA oxidase activities between dwarf and tall peas is due to a difference in concentration of a dialyzable inhibitor or cofactor of the enzyme, the following experiment was conducted. A 10-ml enzyme preparation from 100 dark-grown Progress No. 9 or Alaska pea buds in 0.1 m phosphate buffer, pH 6.1, was dialyzed against 200 ml of water at 2 °C for 24 hr with continuous stirring. The dialysate was reduced in volume to 4 ml, and 3 ml were added to 7 ml of an enzyme preparation from 75 Alaska or Progress No. 9 pea buds together with IAA. It is evident from the results in Table III that enzyme activity in tall peas is not due to higher levels of a dialyzable cofactor nor is the low activity in dwarf pea buds the result of high levels of a dialyzable inhibitor.

**DISCUSSION**

In the present work it has been shown that an enzyme exists in terminal pea buds which oxidizes IAA in the absence of added cofactors. This enzyme has been referred to in the text as IAA oxidase with the realization that it may be different from the IAA oxidase described by Galston (14). Further purification studies revealed that the oxidase activity is associated with the supernatant of the 40 to 70% fraction from which it cannot be recovered with (NH₄)₂SO₄ (Ockerse and Mumford, unpublished).

Table III. Influence of Dialysates from Etiolated Progress No. 9 and Alaska Pea Buds on the IAA Oxidase Activity of Etiolated Alaska Pea Buds

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Addenda</th>
<th>IAA Oxidase Activity (μg IAA oxidised per 25 min per 10 mg protein)</th>
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</thead>
<tbody>
<tr>
<td>Alaska</td>
<td>None</td>
<td>63.6</td>
</tr>
<tr>
<td>Alaska</td>
<td>Progress dialysate</td>
<td>63.6</td>
</tr>
<tr>
<td>Alaska</td>
<td>Alaska dialysate</td>
<td>61.4</td>
</tr>
<tr>
<td>Progress</td>
<td>None</td>
<td>1.2</td>
</tr>
<tr>
<td>Progress</td>
<td>Alaska dialysate</td>
<td>1.2</td>
</tr>
</tbody>
</table>

The IAA oxidase reported in the present paper is a peroxidase, the different results may be explained as follows. In the above cited study (14), pyrogallol was used as a substrate for peroxidase. Using guaiacol as substrate, Ockerse (17) reported that GA stimulates the activity of one isoperoxidase in green pea stem segments. In the present investigation GA also appears to enhance total peroxidase activity in terminal buds of dwarf peas. Since isoperoxidases do not oxidize guaiacol and pyrogallol with the same degree of effectiveness, it is conceivable that different results are obtained with different substrates. Alternatively, our data show that IAA oxidase activity is highest in the youngest part of the stem and rapidly falls as the tissue matures. Since in their work McCune and Galston (14) measured peroxidase activity in relatively mature internodes, their data would be consistent with ours.

The synergistic interaction between gibberellin and auxin is well documented (3, 18). To explain the interaction, it has been suggested that gibberellin-induced growth proceeds through an auxin-sparing mechanism (20) via a decrease in IAA oxidase activity, thereby producing higher auxin levels in the tissue. In support of this concept, several investigators have reported an increase in auxin concentration (16, 19) and the level of a phenolic inhibitor of IAA oxidase (4) and a decrease in IAA oxidase activity (21, 27) following application of gibberellin to intact plants. Subsequent work with rice seedlings (10) did not support the thesis that interaction between the hormones proceeds via an auxin-sparing system, albeit that IAA oxidase appears to control coleoptile elongation in this tissue. Other investigators have reported no effect of GA on IAA oxidase (3, 26), and recently GA has been shown to increase the oxidase activity (2, 7). Since the evidence presented here indicates that CA increases IAA oxidase activity, our work does not support a hypothesis based on an auxin-sparing system.

The findings presented here are difficult to interpret in view of current ideas on the relation of GA, IAA, and IAA oxidase to stem growth. Detailed studies of this relationship are now in progress.

**LITERATURE CITED**


