The Coenzyme Requirement and Enzyme Inhibitors of Pineapple Indoleacetic Acid Oxidase*

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Extracts of pineapple stem tissue constitute the most potent known source of indoleacetic acid oxidase, despite the presence of an inhibitor which is difficult to remove without simultaneously removing some other factor necessary for maximal activity (1). Dialysis invariably lowers the enzyme activity as compared with photolysis, and even prolonged photolysis is never completely effective in removing the naturally occurring inhibitor from the enzyme extract.

Since the inhibitor was presumed to be a polyphenol, and since the enzyme extracts were rich in both peroxidase and catalase, the addition of hydrogen peroxide was tried as a means of destroying the inhibitor in pineapple indoleacetic acid oxidase preparations. The phenolic compounds were indeed oxidized, but the resulting enzyme preparation was devoid of indoleacetic acid oxidase activity despite the rapid and complete disappearance of peroxide from the solution. Addition of small amounts of boiled extract restored activity, demonstrating that the tissue contained an essential cofactor as well as the enzyme inhibitor for the indoleacetic acid oxidase. Subsequent work has shown that two different cinnamic acid derivatives were involved as modifiers of the enzyme (2). This paper will present evidence that one of these naturally occurring cinnamic acid derivatives, either p-coumaric acid or the more prevalent depside of p-coumaric acid and quinic acid found in the tissues (3), is a coenzyme for pineapple indoleacetic acid oxidase.

Knowledge of the coenzyme requirement has made possible the full activation of the enzyme system and thus the study of enzyme inhibitors. A previous report (2) has given evidence that a derivative of ferulic acid is the naturally occurring inhibitor of pineapple indoleacetic acid oxidase. This paper will present evidence on the inhibitory action of ferulic acid and related compounds.

METHODS

The technique for preparation of enzyme extracts of vegetative pineapple stems or leaf base tissue and the assay method for the indoleacetic acid oxidase were as previously described (1). One part of fresh tissue was blended with 1.5 parts of water, and the resulting homogenate was filtered to give the crude enzyme solution. Indoleacetic acid oxidase activity was measured by the Salkowsky reaction after a 10 minute reaction period with the acid. The solution was 0.005 M with respect to MnCl₂, and a 2 ml. volume of reaction mixture was used. Re-evaluation of the pH response curve has shown that the optimal activity of the enzyme occurs at approximately pH 4.0 rather than at pH 3.25 as used heretofore, with the manganese requirement the same at either pH. Most of the data in this paper were obtained at the higher pH for the oxidase assay.

Care to prevent trace contamination with copper was necessary, since even 0.25 p.p.m. of Cu⁺⁺ caused as much as 50 per cent inhibition of the indoleacetic acid oxidase.

To prepare the apoenzyme free from both inhibitor and coenzyme, 100 parts of the aqueous extract were treated twice with 5 parts of 0.1 M H₂O₂, with an interval of 15 minutes between the two additions of H₂O₂. The mixture was then held under refrigeration for at least an hour before use to insure complete disappearance of excess H₂O₂ by catalase activity. Such solutions invariably gave a negative test for peroxide.

In the study of enzyme inhibitors, near maximal activation of the enzyme was first achieved by addition of p-coumaric acid to the system.

RESULTS AND DISCUSSION

Effect of p-Coumaric Acid on Enzyme Activity—Enzyme preparations freed from cofactor have little or no activity, but with added p-coumaric acid, indoleacetic acid oxidation is very pronounced. As little as 3 ~l. of enzyme extract, equivalent to 1.2 mg. of fresh stem tissue and containing only 4.7 ~g. of protein, are able to oxidize 150 ~g. of indoleacetic acid in 10 minutes when 100 ~g. of p-coumaric acid are present along with 200 ~g. of substrate. It might be noted that this activity is far greater than that reported earlier (1) for photolyzed extracts. Approximately 100 ~l. of photolyzed (but not H₂O₂-treated) extract were required to oxidize as much indoleacetic acid under similar conditions.

The response curve for added p-coumaric acid shows a linear relationship when the data are plotted as reciprocals of the reaction rate (μ of indoleacetic acid destroyed in 10 minutes) against μ of p-coumaric acid added (Fig. 1). The relationship holds for a wide range of enzyme levels.

The data thus suggest a mass action equilibrium involving 1 molecule of the enzyme apoprotein and 1 molecule of p-coumaric acid or its naturally occurring ester as a coenzyme,

\[ E + C \rightleftharpoons EC \]

for which one can formulate the dissociation constant,

\[ K = \frac{(E)(C)}{(EC)} \]

Thus, at constant reaction rate ((EC) constant), the free coen-
FIG. 1. Reciprocal plot of indoleacetic acid destruction as a function of p-coumaric acid content of the solution with different amounts of apoenzyme. The substrate level was constant at 200 γ of indoleacetic acid. There was no indoleacetic acid destruction by 10 µl of apoenzyme in the absence of p-coumaric acid.

FIG. 2. Logarithmic relationship of enzyme protein and p-coumaric acid at a constant rate of destruction of indoleacetic acid. The substrate level was constant at 200 γ of indoleacetic acid.

FIG. 3. Reciprocal plot to show competitive inhibition of indoleacetic acid substrate by high levels of p-coumaric acid (C).

Although the substrate and coenzyme obviously occupy different sites on the molecule for enzyme activity to be manifested, the p-coumaric acid also appears to be capable of attaching at the indoleacetic acid site and thus to be competitively inhibitory, although only at very high concentrations with respect to the enzyme. When minimal amounts of apoenzyme are present, very high levels of p-coumaric acid are inhibitory, and this inhibition can be reduced by raising the substrate level. A Lineweaver-Burk plot (Fig. 3) indicates this to be competitive inhibition at the substrate attachment site.

Coenzyme Activity of Related Compounds—The naturally occurring quinyl-p-coumarate in pineapple tissue also is effective as a cofactor for the indoleacetic acid oxidase. This was readily demonstrable by enzyme assays in the presence of cut-out areas of chromatograms representing the various spots detectable by paper chromatography of boiled extract. Strong stimulation of H₂O₂-treated enzyme was obtained only from the purple fluorescing area of the coumarate. The compound has not been isolated in pure form by means other than separation on chromatograms, and thus accurate assessment of the relative coenzyme potency of the oster and free acid has not been possible.

A number of compounds were tested for their ability to substitute for p-coumaric acid as a cofactor in the pineapple indoleacetic acid oxidase system. A comparison of activity could be made by determining the amount of the compound necessary to effect destruction of 100 γ of indoleacetic acid under a standard set of conditions. Data on active compounds are summarized in Table I. (Several of the compounds listed stimulated the destruction of as much as 50 γ of indoleacetic acid but failed to stimulate the destruction of 100 γ at any level.)

The high potency of p-coumaric acid as a cofactor is shared by α-naphthol and β-naphthol; all other compounds tested showed appreciably less activity. It is evident that the enzyme does not have absolute specificity in its coenzyme requirement.

A few generalizations on structures can be made. All of the compounds which show appreciable coenzyme activity are characterized by having a single free hydroxyl group on an aromatic ring. Most frequently, there is additional substitution para to the phenolic group. None of the compounds which show coenzyme activity sufficient to stimulate destruction of indoleacetic
acid by 100 \( \gamma \) has appreciable inhibitory activity when the enzyme is activated with \( p \)-coumaric acid (Table II). Most of the large variety of compounds tested had little or no coenzyme activity (Tables II and III) despite a close structural relationship to some of the active materials, further illustrating the structural specificity of the cofactor. For example, \( \alpha \)-coumaric acid is without activity on pineapple indoleacetic acid oxidase.

For the indoleacetic acid oxidases of other plants, these phenolic compounds have varied activities (Table I). Thus \( \alpha \)-naphthol and \( \beta \)-naphthol, with powerful coenzyme activity for the pineapple enzyme, are inhibitors for the analogous enzyme in peas.

**Effect of Ferulic Acid on Enzyme Activity**—The pronounced inhibitory effect of ferulic acid on the active pineapple indoleacetic acid oxidase system is seen in Fig. 4. It is also evident that where inadequate cofactor (\( p \)-coumaric acid) is present, low levels of ferulic acid may actually be stimulatory. However, in no case does ferulic acid activate the indoleacetic acid oxidase to anywhere near the activity effected by \( p \)-coumaric acid. Higher levels of ferulic acid are invariably inhibitory.

The ability of ferulic acid to suppress indoleacetic acid oxidation by the enzyme is not only dependent on the amount of \( p \)-coumaric acid present but also on the indoleacetic acid substrate level. There is evidence that the ferulic acid inhibition may occur because of competition with either the substrate (indoleacetic acid) or the coenzyme (\( p \)-coumaric acid). An attempt to isolate these two effects for kinetic analysis is shown in Figs. 5 and 6. Other attempts have been less clear-cut.

Where apoenzyme is limited but coenzyme is present in appreciable quantities, the ferulic acid may be expected to compete primarily with the substrate. This is indeed observed (Fig. 5), and suggests that the ferulic acid and indoleacetic acid may compete for the same site on the enzyme molecule. At lower levels of substrate, moderate amounts of ferulic acid inhibited

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Millimicromoles required* in presence of pineapple enzyme</th>
<th>Other indoleacetic acid oxidase systems†</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p )-Coumaric acid</td>
<td>20</td>
<td>Inhibits peas (4)</td>
</tr>
<tr>
<td>( \alpha )-Naphthol</td>
<td>20</td>
<td>Inhibits peas (4)</td>
</tr>
<tr>
<td>( \beta )-Naphthol</td>
<td>20</td>
<td>Inhibits peas (4)</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
| \( p \)-Hydroxyhydrocin-
                             namic acid         | 100                                                      |                                        |
| 2,4-Dichlorophenol        | 300                                                      |                                        |
| Scopoletin                | 400‡                                                     |                                        |
| \( p \)-Hydroxyphenylacetic acid | 500                                                    | Stimulates rice (10), peas (4)          |
| Phenol                    | 700                                                      |                                        |
| \( p \)-Hydroxyphenylpyru-
                             vic acid         | 700‡                                                     |                                        |
| 7-Hydroxycoumarin         | 900                                                      |                                        |
| Resorcinol                | 2000                                                     | Stimulates wheat (9)                    |
| Ferulic acid              | 10‡                                                      | Inhibits rice (10), lupine (6)          |
| Guaiacol                  | 40‡                                                      |                                        |

* Expressed as millimicromoles to effect destruction of 100 \( \gamma \) and 50 \( \gamma \) of indoleacetic acid in 10 minutes by 40 µl. of \( \text{H}_2\text{O}_2 \)-treated indoleacetic acid oxidase solution in the presence of 200 \( \gamma \) of indoleacetic acid.

† Reference citation in parentheses.

‡ Inhibitory activity also manifested by this compound, but not by the others.

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Millimicromoles required* in presence of pineapple enzyme</th>
<th>Other indoleacetic acid oxidase systems†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>0.6</td>
<td>Inhibits peas (11)</td>
</tr>
<tr>
<td>Daphnetin</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Hydrocaffeic acid</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
| 3,4-Dihydroxyphenyl-
                             alamine        | 2                                                        |                                        |
| Catechol                  | 3                                                        |                                        |
| Pyrogallol                | 3                                                        |                                        |
| Gallic acid               | 5                                                        |                                        |
| Hydroquinone              | 5                                                        |                                        |
| 5-Hydroxytryptophan       | 5                                                        |                                        |
| \( p \)-Quinone            | 6                                                        |                                        |
| Esculetin                 | 6                                                        |                                        |
| 5-Hydroxytryptamine       | 10                                                       |                                        |
| (serotonin)               |                                                          |                                        |
| 5-Hydroxy IAA             | 20                                                       |                                        |
| Sinapic acid              | 30                                                       |                                        |
| Ferulic acid              | 40‡                                                      |                                        |
| Homovanillic acid         | 50                                                       |                                        |
| Guaiacol                  | 80‡                                                      |                                        |
| 2,4-Dinitrophenol         | 100                                                      |                                        |
| Scopoletin                | 2000‡                                                     |                                        |
| \( p \)-Hydroxyphenylpyru-
                             vic acid         | 4000‡                                                     |                                        |
| \( \beta \)-Hydroxyethylhydra-
                             zine         | 5000                                                      |                                        |
| Maleic hydrazide          | 6000                                                      |                                        |
| Tryptophan                | 8000                                                      |                                        |
| 4-Hydroxycoumarin         | 90                                                        |                                        |

* Expressed as millimicromoles to lower indoleacetic acid destruction by 100 \( \gamma \) and 50 \( \gamma \) with 10 µl. of \( \text{H}_2\text{O}_2 \)-treated indoleacetic acid oxidase solution, 12 \( \gamma \) of \( p \)-coumaric acid, 200 \( \gamma \) of indoleacetic acid, and a 10 minute reaction period.

† Reference citation in parentheses.

‡ Coenzyme (stimulator) activity also manifested by this compound, but not by the others.
TABLE III

Compounds showing essentially no inhibitor or coenzyme activity

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Coenzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl umbelliferone</td>
<td>Cinnamic acid</td>
</tr>
<tr>
<td>Coumarin</td>
<td>o-Coumaric acid</td>
</tr>
<tr>
<td>6-Methyl coumarin</td>
<td>3,4-Methylenedioxybenzaldehyde</td>
</tr>
<tr>
<td>3-Methyl coumarin</td>
<td>3,4-Dimethoxybenzaldehyde</td>
</tr>
<tr>
<td>Coumarin-3-carboxylic acid</td>
<td>2,3-Dimethoxybenzaldehyde</td>
</tr>
<tr>
<td>5,7-Dihydroxy-4-methyl coumarin</td>
<td>2,4-Dimethoxybenzaldehyde</td>
</tr>
<tr>
<td>Other phenol derivatives</td>
<td>Miscellaneous</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>Salicylaldehyde</td>
<td>a-Naphthylecetic acid</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>a-Naphthylecarbinol</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>Gibberellic acid</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Kinetin</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>Tryptamine</td>
</tr>
<tr>
<td>o-Coumaric acid</td>
<td>Benzoic acid</td>
</tr>
<tr>
<td>3,4-Methylenedioxybenzaldehyde</td>
<td>8-Hydroxyquinoline</td>
</tr>
<tr>
<td>3,4-Dimethoxybenzaldehyde</td>
<td>Quinic acid</td>
</tr>
<tr>
<td>2,3-Dimethoxybenzaldehyde</td>
<td>Shikimic acid</td>
</tr>
<tr>
<td>2,4-Dimethoxybenzaldehyde</td>
<td>Malic acid</td>
</tr>
<tr>
<td>3,4,5-Trimethoxycinnamic acid</td>
<td></td>
</tr>
<tr>
<td>2,4-Dimethoxycinnamic acid</td>
<td></td>
</tr>
<tr>
<td>2,4-Dimethylhydroxycinnamic acid</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Effect of added ferulic acid on activity of indoleacetic acid oxidase before and after activation with p-coumaric acid.

the enzyme more than would be expected from the data in Fig. 5. At lower levels of p-coumaric acid, inhibition by ferulic acid at different substrate levels did not conform to simple competitive inhibition kinetics, perhaps owing to an ability of the inhibitor to compete simultaneously with the substrate and with the coenzyme.

Where the substrate is present in appreciable quantities and the coenzyme is limited, the ferulic acid may be expected to compete primarily with the coenzyme. This situation is illustrated in Fig. 6. Evidence for competitive inhibition was lacking if the concentration of p-coumaric acid was outside of a relatively narrow range or if the concentration of ferulic acid was too high (16 γ, Fig. 6).

**Interactions in Activation and Inhibition of Enzyme**—The data reported above on the multiple effects of p-coumaric acid, ferulic acid, and indoleacetic acid in combination with the apoprotein of pineapple indoleacetic acid oxidase indicate that a complicated series of equilibria are involved in the naturally occurring mixture of these several components. The observed interactions are brought together into a hypothetical scheme in Fig. 7.

It is inferred that there are two adsorption sites on the enzyme apoprotein, a particular one of which must be occupied by the indoleacetic acid substrate and the other by the phenolic cofactor for oxidation to take place. It is also suggested that the p-coumaric acid cofactor as well as the natural ferulic acid inhibitor...
may attach at either site on the protein, though with differing affinities. Such attachments lead to inactive combinations.

In brief, the postulated scheme has had to include the observations that: p-coumaric acid is a very efficient cofactor for the enzyme (Fig. 1); at very high levels, p-coumaric acid inhibits, with higher indoleacetic acid levels reducing this inhibition (Fig. 3); ferulic acid can inefficiently serve as a cofactor for the enzyme in the absence of p-coumaric acid (Fig. 4); ferulic acid soon starts to inhibit, and is immediately inhibitory in the presence of adequate p-coumaric acid (Fig. 4); raising the indoleacetic acid level reduces this ferulic acid inhibition (Fig. 5); raising the p-coumaric acid level within a certain range also lowers the inhibition from a given amount of ferulic acid (Fig. 6).

**Inhibitory Activity of Related Compounds**—In pineapple tissue, ferulic acid occurs largely in combination as an ester. That the ester is a strong inhibitor of indoleacetic acid oxidase was evident by paper chromatography of extracts followed by enzyme assays in the presence of cut-out areas of chromatograms where the ferulic ester was detected. However, since this natural compound has not been completely identified or otherwise obtained in pure form, the relative inhibitory activity cannot be accurately compared with that of free ferulic acid.

Compounds which show some ability to inhibit the activated pineapple indoleacetic acid oxidase system are listed in Table II. Inhibitory activity is expressed as the amount of the compound which diminished the quantity of indoleacetic acid destroyed by 100 μg under a standard set of conditions. (One compound listed lowered indoleacetic acid destruction by 50 μg, but failed to lower it by 100 μg at any level.)

Many compounds are much more potent than ferulic acid in inhibiting the indoleacetic acid oxidase system. Highest inhibitory activity is associated with compounds having an o-dihydroxy group. All of the compounds with appreciable inhibitory activity have at least one free hydroxyl group on an aromatic hydroxyl group. None of the compounds which show high inhibitory activity has appreciable coenzyme activity for the unactivated indoleacetic acid oxidase (Table I).

Most of these phenolic compounds tested by other workers show similar inhibitory activity for the indoleacetic acid oxidases of other plants (Table II). An exception is 2,4-dinitrophenol, which inhibits the enzyme in pineapple, stimulates that in Lens culinaris, and is without effect in peas.

A large number of compounds tested had little or no inhibitory activity (Tables I and III) despite a close structural relationship to some of the active materials.

**SUMMARY**

p-Coumaric acid or a quinyl-p-coumarate present in pineapple leaf and stem tissue serves as a coenzyme for pineapple indoleacetic acid oxidase. Solutions of apoenzyme may be prepared from crude enzyme extracts by peroxidative destruction of the accompanying polyphenolic substances. Evidence indicates that the p-coumaric acid is in mass action equilibrium with the enzyme protein.

Ferulic acid, either free or as an ester occurring in pineapple tissue, has weak cofactor activity in the absence of p-coumaric acid but is a strong enzyme inhibitor of the activated indoleacetic acid oxidase system.

Many phenolic compounds exhibit some degree of activity in substituting for p-coumaric acid as a cofactor for indoleacetic acid oxidase or in simulating ferulic acid as an inhibitor. However, they do not commonly accompany the enzyme in pineapple tissue.

A scheme reconciling the several activating and competitive interactions of indoleacetic acid, p-coumaric acid, and ferulic acid in the indoleacetic acid oxidase system is proposed.

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**REFERENCES**

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