A COLORIMETRIC METHOD FOR THE DETERMINATION
OF PEROXIDASE IN PLANT MATERIAL*

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In a previous paper (1) a colorimetric phenol oxidase method was presented which utilizes catechol as a mediator and in which the oxidation of a leucoindophenol is followed photometrically over a very short time interval. During the development of this method it was found that the same basic method and technique were applicable to the determination of peroxidase. In the latter case, oxidation of the leucoindophenol dye is catalyzed directly by peroxidase in the presence of hydrogen peroxide. Since it is often desirable to determine both phenol oxidase and peroxidase in a given plant material, a basically similar method for both enzymes was considered advantageous.

Most previous peroxidase methods have also been colorimetric and have utilized a variety of oxygen acceptors such as pyrogallol (2-4), leucomalachite green (5, 6), guaiacol (6), nadi reagent (7), and others. Lucas and Bailey (8) have also used an indophenol dye similar to that in the present work, but in a quite different way. Another method commonly used, especially in the assay of plant material, is that of Balls and Hale (9), in which pyrogallol is the oxygen acceptor, but the utilization of hydrogen peroxide is measured.

This paper includes (a) a brief discussion of factors involved in the proposed colorimetric reaction and in the enzyme preparation, (b) a description of the method adopted, (c) some results of its application to plant materials, and (d) some points of comparison with other methods.

EXPERIMENTAL

Colorimetric Reaction

The same general technique as that of the phenol oxidase method (1) was found suitable for peroxidase determination. A pH of 6.0 for the

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colorimetric reaction proved optimal for enzyme preparations from various sources, and at the same time permitted only a negligible rate of oxidation of the leuco dye by peroxide in the absence of enzyme. Buffer strength was not critical. Much less variation of peroxidase activity with different indophenol dyes was encountered than in the case of phenol oxidase. Although the common 2,6-dichlorobenzenoneindophenol was quite satisfactory, 2,6-dichlorobenzenoneindo-3'-chlorophenol afforded somewhat greater sensitivity, as shown in Table I. Since the latter dye had been adopted for the phenol oxidase method, some convenience as well as sensitivity was gained by also selecting this dye for the peroxidase determination. No evidence of enzyme inhibition by the dyes was observed, and small differences in the sensitivity of the method with the various dyes were due essentially to variations in their absorption coefficients.

The effects of leuco dye and peroxide concentrations on the rate of the enzyme reaction were necessarily interrelated. These relations are illustrated for an apple preparation in Figs. 1 and 2. It will be seen that the optimal peroxide concentration in the reaction mixture was at about 0.04 per cent for a leuco dye concentration of 3.5 \( \times 10^{-4} \) M. At this peroxide

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Acceptor*</th>
<th>Concentration</th>
<th>( \text{H}<em>{2}\text{O}</em>{2} )</th>
<th>Rate†</th>
<th>Michaelis constant</th>
<th>( \epsilon )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple (acetone ppt.)</td>
<td>3467</td>
<td>3.4 ( \times 10^{-4} )</td>
<td>0.04</td>
<td>100</td>
<td>1500</td>
<td>25</td>
</tr>
<tr>
<td>Same &quot;</td>
<td>3463</td>
<td>3.4 ( \times 10^{-4} )</td>
<td>0.04</td>
<td>100</td>
<td>1700</td>
<td>15</td>
</tr>
<tr>
<td>&quot;</td>
<td>Pyrogallol</td>
<td>0.033</td>
<td>0.04</td>
<td>0.3</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>&quot;</td>
<td>Leucomalachite green</td>
<td>1.2 ( \times 10^{-4} ) ca. 0.0004</td>
<td>0.04</td>
<td>0.3</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>Potato (acetone ppt.)</td>
<td>3467</td>
<td>3.4 ( \times 10^{-4} )</td>
<td>0.04</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same</td>
<td>3463</td>
<td>3.4 ( \times 10^{-4} )</td>
<td>0.04</td>
<td>61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horseradish (partially purified)</td>
<td>3467</td>
<td>3.4 ( \times 10^{-4} )</td>
<td>0.04</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same</td>
<td>3463</td>
<td>3.4 ( \times 10^{-4} )</td>
<td>0.04</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 3467 (Eastman Kodak Company), 2,6-dichlorobenzenoneindo-3'-chlorophenol; 3463 (Eastman Kodak Company), 2,6-dichlorobenzenoneindophenol; pyrogallol, 410 \( \mu \) filter used in measurement; leucomalachite green, in pH 4.0 buffer, 615 \( \mu \) filter used.

† All \( \text{H}_{2}\text{O}_{2} \) concentrations were optimal.

‡ Rates for each enzyme preparation relative to those in bold-faced type.

§ \( \epsilon \) = absorption coefficient = \((\log I_0/I)/(L(\text{cm.}) \times C \text{ (mM per liter)})\).

∥ This value is for purpurogallin in alcohol.
concentration the leuco dye was not at an optimal concentration. With lower peroxide concentrations an optimal dye concentration could be reached, but this was at such a level that there was danger of precipitation of dye in the stock solutions. Thus the best practical choice for maximum sensitivity seemed to be about 0.04 per cent peroxide and 3 to 4 \( 10^{-4} \text{M} \) leuco dye. Under these conditions good proportionality between rate of oxidation of the leuco dye and enzyme concentration was obtained, as illustrated in Fig. 3.

![Graph showing rates of peroxidase reactions with apple and sweet potato preparations.](http://example.com/graph.png)

**Fig. 1.** The effect of peroxide concentration on the peroxidase reaction with apple and sweet potato preparations. Leuco dye concentration, \( 3.5 \times 10^{-4} \text{M} \).

It may be pointed out that enzyme preparations vary in their sensitivity to peroxide, as previously reported (10). As may be noted in Fig. 1, the sweet potato preparation was markedly inhibited by peroxide concentrations above the optimum, while an apple preparation showed little or no inhibition at such levels. This variation in sensitivity was considered in the design of a routine method. Inhibitory peroxide concentrations were usually indicated by non-linear rate curves and were generally avoided.

**Enzyme Preparation**

Much of the present work was carried out simultaneously with studies on the phenol oxidase method (1) and included investigation of the effects
DETERMINATION OF PEROXIDASE

of sampling and grinding techniques, pH, ascorbic acid addition, acetone precipitation, temperature, and time. Peroxidase was similar in its behavior to phenol oxidase in many respects but showed generally greater stability. A pH of 7 to 8 was found to be optimal for the stability of peroxidase during preparation for apple and potato tissue. There was no evidence that the use of ascorbic acid during grinding was beneficial. Re-

![Image](http://www.jbc.org/)

**Fig. 2.** The relation of leuco dye concentration and rate of the peroxidase reaction with an apple preparation. Peroxide concentration, 0.04 per cent.

coveries of enzyme activity after 80 per cent acetone precipitation were usually better than 90 per cent, but this procedure was not required, since simple homogenates were found to be as stable as acetone precipitates. Peroxidase differed from phenol oxidase in that diluted homogenates were more stable in peroxidase activity in the presence of gelatin. It was generally possible to use the same enzyme preparation for the determination of both peroxidase and phenol oxidase activities, except that the aliquot of homogenate for peroxidase determination was diluted with gelatin solution.
Standard Procedure

Reagents—

Leuco dye. An approximately 0.001 M solution of 2,6-dichlorobenzenone-endo-3'-chlorophenol (35 mg. per 100 ml.) is prepared. To obtain the leuco dye reagent, the above stock is first diluted, after ascorbic acid standardization, to 5.5 × 10⁻⁴ M. The dye is reduced by first adding 2 ml. of a freshly prepared 0.2 per cent suspension of 5 per cent palladized asbestos for each 200 ml. of solution and then bubbling hydrogen through the solution until colorless. 3 to 4 ml. of 0.1 M phosphate-citrate buffer (pH 6.0) per 200 ml. of solution are added to reduce autoxidizability, the solution is quickly filtered through retentive paper in a Büchner funnel,
and the hydrogen bubbling is resumed during the use of the leuco dye reagent. Further detail on the leuco dye preparation is given in the description of the phenol oxidase method (1).

*Hydrogen peroxide.* 0.48 per cent hydrogen peroxide is prepared fresh from 30 per cent "superoxol."

**Method**

The following procedure was adopted for routine determination in plant materials: (1) A weighed sample of tissue, fresh or frozen, is ground first in a Waring blender and then a portion in the Potter-Elvehjem homogenizer (11), or in the homogenizer alone if the sample is small, until the cells are ruptured. Sufficient 0.2 M K$_2$HPO$_4$, depending on the acidity of the tissue, is included to maintain a pH of 7 to 8. Dilution of tissue in the homogenate is 1:10. The homogenate is diluted to the appropriate concentration for assay with 0.5 per cent gelatin solution and assayed as soon as possible or stored for short periods in an ice bath. (2) To a colorimeter tube (20 X 150 mm. test-tube) are added 2.5 ml. of 0.2 M phosphate-citrate buffer (pH 6.0), 7.5 ml. of 5.5 X 10^{-4} M leuco dye, 1.0 ml. of 0.48 per cent hydrogen peroxide, and finally 1.0 ml. of the enzyme preparation. The tube is quickly swirled for mixing and inserted in the colorimeter with a 645 nm filter. After adjusting the lamp rheostat to give a transmission of 80 to 100 per cent, galvanometer readings are taken at 5 second intervals, usually in the interval from 15 to 45 seconds after mixing. An interval timer with a flashing light signal is convenient.

Transmission values are plotted against time on semilog paper, and the rate was determined from the straight line and expressed as $\Delta \log I$ per minute. These relative rates can be converted to an absolute basis by the following expression:

$$\frac{\Delta \log I \text{ per min.} \times \text{dilution factor}}{3.50 \times \text{volume}} = \text{rate} \left( \frac{\text{micromoles}}{\text{ml.} \times \text{min.} \times \text{gm.}} \right)$$

where "dilution factor" is the volume of enzyme preparation divided by the fresh weight of tissue, "volume" that of the reaction mixture, usually 12 ml., and 3.50 is the optical density at 12 ml. volume in the standard tube of 1 $\mu$m of oxidized dye. A more detailed explanation of the expression "$\Delta \log I$ per minute" as a measure of rate is given in the report on phenol oxidase (1).

**Some Applications and Properties of Method**

Possible interference in the proposed method by other oxidative enzymes was evaluated. Direct oxidation of the leuco dye (without peroxide) occurred in some plant tissues, the so called "dye oxidase" reaction previ-
It is conceivable that phenol oxidase and cytochrome oxidase in the presence of their natural substrates could interfere, though this is unlikely because of the high dilution of the tissue. Catalase might interfere by competing for the peroxide substrate. Studies with varying proportions of peroxidase and catalase\(^1\) showed that relatively high catalase concentrations were necessary for appreciable interference which was evidenced by falling off in the rate curves. No such interference was encountered in the plant materials investigated.

### Table II

**Peroxidase Activity of Various Plant Tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dilution</th>
<th>Activity, ( \mu \text{m} \times \text{min.}^{-1} \times \text{ml.}^{-1} \times \text{gm.}^{-1} ) fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple fruit, fresh</td>
<td>1:400</td>
<td>120</td>
</tr>
<tr>
<td>Potato tuber, fresh</td>
<td>1:1,000</td>
<td>7030</td>
</tr>
<tr>
<td>Horseradish root, frozen</td>
<td>1:30,000</td>
<td>128</td>
</tr>
<tr>
<td>Carrot root, fresh</td>
<td>1:200</td>
<td>490</td>
</tr>
<tr>
<td>Beet root, frozen</td>
<td>1:2,000</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Sweet potato tuber, frozen</td>
<td>1:2,000</td>
<td>153</td>
</tr>
<tr>
<td>Pea, frozen</td>
<td>1:1,000</td>
<td>336</td>
</tr>
<tr>
<td>Spinach, frozen</td>
<td>1:1,000</td>
<td>63</td>
</tr>
<tr>
<td>Mushroom, frozen</td>
<td>1:10</td>
<td>22</td>
</tr>
<tr>
<td>Cabbage, fresh</td>
<td>1:5,000</td>
<td>1905</td>
</tr>
<tr>
<td>Asparagus stems, fresh</td>
<td>1:2,000</td>
<td>336</td>
</tr>
<tr>
<td>Radish roots, fresh</td>
<td>1:2,000</td>
<td>423</td>
</tr>
<tr>
<td>Celery tops</td>
<td>1:100</td>
<td>31</td>
</tr>
</tbody>
</table>

Table I shows that the leuco dye used in the present method affords much greater sensitivity of measurement than either pyrogallol or leucomalachite green. With pyrogallol, with which the Michaelis constant was found to be similar to that when the indophenol dye served as acceptor, the difference seemed to be due to the relative absorption coefficients and possibly to some inhibitory effect of pyrogallol on the enzyme. With malachite green, however, which has a higher absorption coefficient than the indophenol dye, the difference in sensitivity would seem to result from differences in the specific reaction rates of the intermediate compound, peroxidase-H\(_2\)O\(_2\), with the two oxygen acceptors (see Chance \(5\)). Guaiacol and nadi reagent were found to be much less satisfactory in the present method than any of the above acceptors. The high sensitivity of the

\(^1\) We are indebted to Professor J. B. Sumner of the Biochemistry Department of Cornell University for a sample of crystalline beef liver catalase.
Determination of Peroxidase

The method was also demonstrated by comparison with the Balls-Hale method (9) in blanching studies on peas and beans. Accurate determinations of fractional per cents of residual peroxidase activity after blanching were possible only with the present method.

Much of the work reported in this paper was done with apple, potato, and horseradish preparations, but a survey of other available plant materials was made to test the method further. The results in Table II show the wide range of peroxidase activity encountered and the magnitude of the control rate (without peroxide). Most of this may be due to "dye oxidase," but since the preparations were all unpurified homogenates, some may have been due to other oxidases with their natural substrates, at least when the homogenate could not be greatly diluted.

**DISCUSSION**

The indophenol method has been readily adapted for peroxidase determination with the same technical advantages of speed, convenience, and sensitivity as that described previously for the phenol oxidase method (1). The leuco dye selected afforded higher sensitivity than either the pyrogallol or leucomalachite green acceptors which have been most commonly used in the past. The method has one disadvantage in comparison with the Balls-Hale method (9) that applies, however, to any method which employs an acceptor that may be an oxidase substrate and which measures substrate change. With materials of relatively low peroxidase activity and high "dye oxidase" activity, control determinations without peroxide are necessary and peroxidase activity must be calculated by difference.

As has been observed with pyrogallol and malachite green (6, 10, 12), excessive peroxide concentrations may cause suboptimal rates with some enzyme preparations. Although this was only rarely observed in the present work, it may complicate the use of fixed peroxide concentrations for the assay of a wide variety of tissues. A single peroxide concentration, therefore, may not serve to compare the peroxidase activities of different tissues accurately, but would be valid for comparing changes in activity in the same or similar tissues.

Preparation of the tissue for peroxidase analysis was similar to that for phenol oxidase (1); hence with most materials the same preparation served for both determinations.

**SUMMARY**

A new method of increased speed and sensitivity for determining peroxidase in plant tissue is described. Leuco-2,6-dichlorobenzenoneindo-
3'-chlorophenol is oxidized by peroxidase in the presence of hydrogen peroxide, and the rate of color formation is found to be linear and proportional to enzyme concentration under the conditions described. The influence of peroxide and leuco dye concentration and of the method of enzyme preparation was investigated and a standard procedure adopted. Assay results from representative plant tissues illustrate the flexibility of the method and comparative data with other peroxidase methods emphasize its sensitivity.

BIBLIOGRAPHY

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