THIOOXIDASE, A NEW SULFHYDRYL-OXIDIZING ENZYME FROM PIRICULARIA ORYZAE AND POLYPORUS VERSICOLOR

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In the course of a study of the enzymes of Piricularia oryzae Cav., the fungus causing the blast disease of rice, it was observed that activity of crude polyphenoloxidase preparations from culture filtrates was not inhibited by sodium diethyldithiocarbamate (DEDTC), a widely used inhibitor of copper-containing enzymes. Further investigation revealed that such preparations contained an enzyme which catalyzes the oxidation of DEDTC and related compounds. This enzymatic reaction appears not to have been described in the literature, and the enzyme seems to be distinct from other enzymes operating on sulfhydryl-containing substrates. The enzyme, which was later demonstrated also in culture filtrates from the wood-rotting fungus Polyporus versicolor, is referred to in this paper as thiooxidase.

Methods

Cell-free culture filtrates of P. oryzae (strain No. 775) and of P. versicolor, prepared as described elsewhere (1), were used as the source of the enzyme. Activity was assayed manometrically in a Warburg apparatus at 30.4°. In a typical experiment, 0.5 ml. of 0.1 M DEDTC was placed in the side arm, 0.1 ml. each of di-n-hexylamine and 10 per cent potassium hydroxide together with a filter paper wick was placed in the center well, and, in the main compartment, 1.0 ml. of buffer (McIlvaine, pH 6.0 or 7.0), together with an enzyme solution and water sufficient to make a total volume of 3.0 ml.

Because DEDTC tends to decompose into carbon disulfide and diethylamine at even slightly acid pH, it was necessary to provide a trapping reagent for the volatile carbon disulfide produced. The alkaline di-n-hexylamine solution proved adequate at pH near 7, but in some experiments at pH 6 the evolution of carbon disulfide was so rapid that positive pressures resulted. To correct for this, blanks containing all the components except enzyme were always included.

Sodium dimethyldithiocarbamate was prepared from dimethylamine hydrochloride, carbon disulfide, and sodium hydroxide. Potassium dithio-
acetate was synthesized by treating methyl magnesium iodide with carbon disulfide, followed by acidification, steam distillation, and addition of potassium hydroxide. Potassium methyl-, ethyl-, propyl-, isopropyl-, butyl-, and amylxanthates were prepared by treating solutions of the corresponding alcohols with potassium hydroxide and carbon disulfide. All other reagents were obtained from commercial sources and were used without further purification.

Results

Identification of Product of DEDTC Oxidation—The most likely oxidation product of DEDTC appeared to be tetraethylthiuram disulfide (TETDS), according to the following reaction:

$$2(C_2H_5)_2N\text{-}[C-SH + \frac{1}{2}O_2 \rightarrow (C_2H_5)_2N\text{-}[C-S-S-C-N(C_2H_5) + H_2O}$$

The oxidation product, which has very low solubility in water, soon causes the reaction mixture to become turbid and later separates in crystalline form. After several hours, the crystalline yellow precipitate was separated by centrifugation and washed thoroughly with water. The product was recrystallized three times from 95 per cent ethanol. The infrared spectra of the purified material and of a similarly recrystallized authentic specimen of TETDS were identical, as were the melting points and mixed melting point (71–72° uncorrected; literature value, 70°).

The oxidation product of sodium dimethyldithiocarbamate was shown in the same way to be tetramethylthiuram disulfide.

There was no evidence of formation of thiuram disulfides from the dithiocarbamates in the absence of enzyme or in the presence of boiled enzyme.

The product from the enzymatic oxidation of thiophenol was obtained as white needles which, after recrystallization from aqueous ethanol, melted at 59.5° (uncorrected). According to the literature, diphenyl disulfide is a white compound, m.p. 61°.

Kinetics and Stoichiometry—As shown in Fig. 1, the rate of enzymatic DEDTC oxidation remains constant so long as the substrate concentration is non-limiting. In Fig. 2 is seen the direct proportionality between the rate of DEDTC oxidation and enzyme concentration and in Fig. 3 the effect of substrate concentration on the rate of the reaction is shown.

According to the equation above, 0.25 mole of oxygen is consumed per mole of substrate oxidized. Experimentally, only 70 to 80 per cent of the theoretical oxygen absorption was observed (Fig. 1), owing, presumably, to the concurrent non-enzymatic non-oxidative decomposition of the substrate. Hydrogen peroxide is not produced during the oxidation of DEDTC, as evidenced by the fact that added catalase did not influence
the oxygen consumption in the presence of enzyme preparations demonstrated to be catalase-free.

_Properties of Thiooxidase_—The crude enzyme is quite stable at low temperature and may be stored for weeks at 4° without appreciable loss of activity. It is inactivated completely after 5 minutes at 100°. 50 per cent acetone produces a precipitate containing most of the activity.

Exhaustive dialysis against distilled water did not diminish activity of the preparations, indicating that no soluble cofactors are involved in the reaction.

Because of the increasingly rapid non-enzymatic decomposition of DEDTC with increasing acidity, only fragmentary data were obtained as

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![Graph 1](image1.png)

**Fig. 1.** Rate of enzymatic oxidation of diethyldithiocarbamate as a function of time; pH 6.0. The flask contents were as described in the text. All measurements were corrected for non-enzymatic evolution of carbon disulfide.

![Graph 2](image2.png)

**Fig. 2.** Rate of oxidation of diethyldithiocarbamate as a function of enzyme concentration; pH 6.0.
to the pH dependence of the enzymatic reaction. The rate of the enzyme-catalyzed oxidation was considerably less at pH 7.0 than at pH 6.0 and too slow to be measured at pH 8.0.

Activity is inhibited by azide, cyanide, glutathione, and cysteine, as shown in Table I.

Occurrence of Thiooxidase—The enzyme has been demonstrated in the cell-free filtrates from cultures of the rice-blast fungus, *P. oryzae*, and of the wood-rotting fungus, *P. versicolor*. It occurs also in the mycelium of *Piricularia*; the *Polyporus* mycelium has not been investigated.

Relation of Thiooxidase to Polyphenoloxidases—Although the phenoloxidases and thiooxidase usually occur together in the culture medium, the sulfhydryl-oxidizing and phenol-oxidizing activities appear to belong to distinct enzymes. The thiooxidase is demonstrable as early as 65 hours (Table II), whereas the laccase and metapolyphenoloxidase do not appear until considerably later. As the cultures age, the ratios thiooxidase-laccase and thiooxidase-metapolyphenoloxidase change markedly.

![Fig. 3. Rate of oxidation of diethyldithiocarbamate as a function of substrate concentration; pH 7.0.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.7 x 10⁻³ M</td>
</tr>
<tr>
<td>Azide</td>
<td>100</td>
</tr>
<tr>
<td>Cyanide</td>
<td>96</td>
</tr>
<tr>
<td>Cysteine</td>
<td>90</td>
</tr>
<tr>
<td>Glutathione</td>
<td>96</td>
</tr>
</tbody>
</table>
Furthermore, the several enzymes are not equally sensitive to inhibitors. Thus, $10^{-6}$ M azide inhibited both laccase and metapolyphenoloxidase 50 per cent but decreased the activity of thiooxidase by only 25 per cent.

Thiooxidase activity has been eliminated completely from some preparations by fractional precipitation of the phenoloxidases with acetone and ammonium sulfate.

Substrate Specificity—Besides the dialkyl dithiocarbamates, the following substances were oxidized by thiooxidase: alkylxanthates (R = methyl, ethyl, n-propyl, isopropyl, n-butyl, or n-amyl), thioacetate, dithioacetate, dithiooxalate, thioglycolate, thiophenol, p-bromophenylthiopseudourea, 2-mercaptobenzothiazole, 5-amino-2-benzimidazolethiol, thiohistidine, and ergothioneine. The following were not oxidized: n-butyl mercaptan, mercaptoethylamine, thioglycerol, mercaptosuccinic acid, cysteine, and glutathione. Obviously the structural requisite for oxidizability is not merely the possession of a sulfhydryl group. With the exception of thioglycolic acid, which is oxidized rather slowly, all the compounds which are oxidized by the enzyme possess a sulfhydryl group attached to a carbon atom which is linked by a double bond to another carbon, nitrogen, oxygen, or sulfur atom:

$$\begin{align*}
\text{C} = \\
\uparrow \\
\text{SH}
\end{align*}$$

<table>
<thead>
<tr>
<th>Age of culture (hrs.)</th>
<th>Activity, μl. of O₂ per hr. per ml.</th>
<th>Ratio, laccase/thiooxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thiooxidase*</td>
<td>Laccase†</td>
</tr>
<tr>
<td>65</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>139</td>
<td>320</td>
<td>198</td>
</tr>
<tr>
<td>168</td>
<td>470</td>
<td>390</td>
</tr>
<tr>
<td>240</td>
<td>603</td>
<td>605</td>
</tr>
<tr>
<td>288</td>
<td>690</td>
<td>680</td>
</tr>
</tbody>
</table>

* Substrate 0.0167 M sodium diethyldithiocarbamate, pH 6.0.
† Substrate 0.043 M hydroquinone, pH 4.0.
‡ Substrate 0.086 M resorcinol, pH 7.0.
DISCUSSION

Apart from the claim of Bertrand and Gavard (2, 3), which has been refuted by Tissières (4), that cysteine is oxidized to cystine by laccase, the only report of direct enzymatic oxidation of sulfhydryl compounds to disulfides is that of Mandels (5). He described the occurrence in spores of Myrothecium verrucaria of an enzyme capable of oxidizing cysteine, glutathione, homocysteine, and thiophenol to the corresponding disulfides. This enzyme, moreover, was unaffected by cyanide, azide, and DEDTC, and did not catalyze the oxidation of thioacetic acid. Hence it appears entirely unrelated to thiooxidase.

The role of thiooxidase is at present entirely conjectural; it is not inconceivable that it may function as a terminal oxidase. According to Ward (6), the slime mold Physarum polycephalum contains an atypical ascorbic acid oxidase which is stimulated by DEDTC and TETDS but not by cysteine or glutathione. He suggested that these compounds may act as models of some unknown carrier which functions by shuttling between the —SH and S—S forms. Thiooxidase might be presumed to participate in the oxidation and reduction of such a carrier.

In this connection it is of considerable interest that TETDS has been shown recently to occur in the mushroom Coprinus atramentarius (7), suggesting the possibility that diethyldithiocarbamate itself may also be of natural occurrence. Conceivably DEDTC and TETDS might be not merely models in Ward’s system but the actual carriers. Enzymatic reduction of thiuram disulfides both by fungi and by animal tissue has been reported (8–11).

The oxidizability of ergothioneine by thiooxidase suggests the possibility also that this compound, which is of widespread occurrence in plant and animal tissue but of presently unknown role, may function in such an oxidation system.

SUMMARY

Cell-free culture filtrates of Piricularia oryzae and Polyporus versicolor contain an enzyme which catalyzes the direct oxidation by atmospheric oxygen of compounds containing the structure >C—SH. Oxidizable substrates include dithiocarbamates, alkylxanthates, thioacetate, dithioacetate, dithiooxalate, thiophenol, thioglycolate, thiohistidine, and ergothioneine, among others. The dithiocarbamates are oxidized to the corresponding disulfides. Butyl mercaptan, thioglycerol, cysteine, glutathione, and mercaptosuccinic acid are not oxidized.

The new enzyme, which has been designated “thiooxidase,” appears to be distinct from previously described enzymes that effect oxidation of sulfhydryl compounds.
We are indebted to Mr. George Svarnas and Mr. Freeman Young for synthesizing the sodium dimethyldithiocarbamate and potassium dithioacetate, to Mr. David Stefanye for determining the infrared spectra, and to Dr. Robert C. Baldridge for generously supplying the ergothioneine. We wish also to acknowledge the assistance of Mr. Jules J. Weisler, Mr. Bertram W. Fuhr, and Mr. Richard A. Pamplin with some of the experiments.

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