ENZYMATIC DECARBOXYLATION OF OXALIC ACID

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Large quantities of oxalic acid accumulate in the cell sap of various plants and as a major product of carbohydrate metabolism by many molds. A recent investigation in our laboratory revealed that oxalic acid was produced from oxalacetic acid by an enzyme system which was isolated from the mycelia of Aspergillus niger (1). It was also demonstrated that oxalic acid was converted to formic acid and CO₂ by an enzyme isolated from a soil bacterium (2). The latter reaction proceeded anaerobically and required ATP, CoA, Mg²⁺, ThPP, and acetate.

The present communication is concerned with the purification and properties of an enzyme which is obtained from the mycelia of a wood-destroying fungus, Collyvia veltipes. This enzyme catalyzes the decarboxylation of oxalic acid with the formation of stoichiometric quantities of formic acid and carbon dioxide as follows:

\[
\text{COOH} \quad \rightarrow \quad \text{HCOOH} + \text{CO}_2
\]

In contrast to the oxalic decarboxylase of a soil bacterium, however, the enzyme from C. veltipes does not show a requirement for any of the cofactors mentioned above. Instead, it requires a small amount of oxygen, although the over-all reaction does not stoichiometrically utilize oxygen. Preliminary reports of this work have been published (3, 4).

EXPERIMENTAL

Manometric Method—CO₂ was determined by a conventional Warburg technique at 37°C. Unless otherwise specified, the reaction was measured in an atmosphere of air with a 2 ml. reaction mixture containing 400 µmoles of potassium citrate and 10 µmoles of potassium oxalate at pH 3.0. The reaction was started by the addition of the enzyme. 1 unit of activity was expressed as the amount of enzyme which causes the evolution of

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The following abbreviations are used throughout this report: ATP, adenosine triphosphate; CoA, coenzyme A; ThPP, thiamine pyrophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.
1 μmole of CO₂ in a 1 hour period, and the specific activity was expressed in units per mg. of protein. Protein was determined by the phenol method (5).

Anaerobic experiments were carried out as follows: 9 ml. of a water solution, pH 3.0, containing 50 μmoles of potassium oxalate, and 2 mmoles of potassium citrate were saturated with helium gas which had been passed through chromous chloride solution. A 1.8 ml. aliquot of this solution containing 10 μmoles of oxalate and 400 μmoles of citrate was placed in the main compartment of stoppered manometer flasks in which air had previously been replaced by helium. The enzyme solution and the other chemicals were added to the side arms of the manometer flask while it was being flushed with helium. Then the flasks were attached to the manometer and helium was passed through the entire manometer space for 10 minutes with shaking.

Spectrophotometric Assay—In order to determine the rate of the reaction with minute quantities of the substrate, the disappearance of oxalic acid was followed by determining at 25° the absorption at 220 or 230 μm in a Beckman model DU spectrophotometer with quartz cells having a 1 cm. light path. 50 μmoles of phosphate buffer, pH 3.0, were used in a total volume of 1.0 ml. with 1 μmole of oxalate and enzyme. Fig. 1 shows the ultraviolet absorption spectrum of oxalic acid at pH 3.0.

Determinations—Formic acid was determined by an unpublished method of J. C. Rabinowitz. In the presence of ATP, formic acid, and an enzyme from Clostridium cylindrosporum, tetrahydrofolic acid is stoichiometrically converted to N-formyltetrahydrofolic acid. The reaction can be determined by the increase in absorption at 356 μm. The incubation mixture (1.0 ml.) contained the following components, expressed in micromoles: maleate buffer, pH 7.0, 25; FeSO₄, 0.5; MgCl₂, 20; mercaptoethanol, 2.5; tetrahydrofolic acid, 1; ATP, 2. The mixture was incubated at 37° for 3 minutes; 0.1 ml. of a partially purified enzyme preparation of C. cylindrosporum was then added and incubation continued for 10 more minutes. 1 ml. of 5 per cent perchloric acid was then added, and the supernatant solution was used for the spectrophotometric determination.

Growth Conditions and Preparation of Crude Extract—C. veltipes (strain S) was grown on the surface of a medium containing 5 per cent dextrose, 1 per cent peptone, 0.1 per cent KH₂PO₄, 0.05 per cent MgSO₄·7H₂O, and 1 per cent Difco malt extract at pH 5.2. The medium (200 ml.) was

Grade A helium gas was obtained from the Bureau of Mines, Department of Interior, and was reported to contain less than 0.002 per cent (by volume) oxygen.

Oxosorbent, chromous chloride solution, Burrell Corporation, Pittsburgh, Pennsylvania.

We wish to express our gratitude for the kind collaboration of Dr. J. C. Rabinowitz in carrying out the formic acid assay with us.
placed in Blake bottles of 1 liter capacity and incubated at 25°. About 25 days after inoculation, the reaction of the medium was brought to about pH 3.0 by the addition of about 2.5 mmoles of oxalic acid to each culture flask. The mycelia were harvested 2 to 3 days after the addition of oxalic acid. The culture medium was removed by filtration through a double layer of cheesecloth, and the mycelial pad was washed twice with cold distilled water and stored at -20°.

50 gm. of frozen mycelia were ground in a Waring blender for 3 minutes with about 10 gm. of crushed dry ice, and the fine powder was extracted with approximately 150 ml. of 0.1 M potassium citrate buffer, pH 3.2, for 10 minutes at 4° with constant mechanical shaking. The suspension was centrifuged at 10,000 X g for 20 minutes at 0–2°. The supernatant solution thus obtained (crude extract) usually contained about 1.5 to 2.0 mg. of protein per ml.

Material—Crystalline catalase was obtained from the Worthington Biochemical Corporation. α-Ketomalonate is a product of the H. M. Chemical Company, Ltd.

Results

Purification of Enzyme—All the manipulations were carried out at approximately 3°. Cold acetone (500 ml.) was added dropwise to 1000 ml. of crude extract with constant mechanical stirring. The precipitate was removed by centrifugation, and the precipitate formed upon further addition of 500 ml. of acetone was dissolved in about 30 ml. of 0.1 M potassium acetate buffer at pH 4.5 (first acetone fraction).
The first acetone fraction was dialyzed for 12 hours against 100 volumes of 0.02 M potassium acetate buffer at pH 4.5, and a small amount of precipitate formed during the dialysis was removed by centrifugation. To 10 ml. of the supernatant solution were added 6.8 ml. of cold acetone dropwise with constant mechanical stirring. The precipitate was removed by centrifugation, and 3.2 ml. of cold acetone were added to the supernatant fluid in the same manner. The precipitate thus formed was collected by centrifugation and was dissolved in 2 ml. of 0.1 M potassium acetate buffer at pH 4.5 (second acetone fraction).

The second step was repeated with the second acetone fraction, and the final preparation was dissolved in the same buffer (third acetone fraction).

The result of a typical purification is shown in Table I. Approximately 400-fold purification was achieved with an over-all yield of about 35 per cent. The specific activity of crude extracts varied from 8 to 73 among the six different batches, but the specific activities of the final preparations were almost identical and ranged from 3166 to 4040.

<table>
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<th>Steps</th>
<th>Total volume</th>
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<th>Total protein</th>
<th>Specific activity</th>
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<td>100</td>
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<td>10,900</td>
<td>3.4</td>
<td>3166</td>
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</table>

**Table I**

Purification of Oxalic Acid Decarboxylase

20 liters of culture (500 gm. of wet mycelium).

Stoichiometry and Cofactor Requirement—When 10 μmoles of oxalic acid were incubated under standard assay conditions in the Warburg manometer flask, 9.8 μmoles of CO₂ were produced and no measurable oxygen consumption was observed. After the reaction was over, the incubation mixture was removed from the flask and an aliquot was used for determination of formic acid; 10.3 μmoles of formic acid were produced. This result indicated that the over-all reaction was a stoichiometric decarboxylation of oxalic acid to form formic acid and CO₂, and therefore it appeared to be identical with the enzymatic reaction previously described by Jakoby, Ohmura, and Hayashi (2).

However, when the purified enzyme was dialyzed against cold distilled water for several days, there was little loss of activity, and the addition of CoA, ATP, Mg²⁺, acetate, or cocarboxylase, alone or in combination,
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did not show any stimulation. When the enzyme was treated with Dowex 1 formate, the rate of the reaction was reduced to about 20 per cent of the original. However, the addition of either boiled crude extracts or ATP, CoA, Mg++, acetate, and cocarboxylase did not increase activity.

These observations suggested to us that either the cofactors were bound extremely tightly to the enzyme protein or that the properties of the Collybia enzyme were different from those of the one previously described from a soil bacterium.

Fig. 2. Effect of oxygen on the rate of enzymatic decarboxylation of oxalate. The incubation mixture contained, in 2.0 ml., 0.024 mg. of enzyme protein (second acetone fraction), 10 µmoles of potassium oxalate, and 400 µmoles of potassium citrate buffer, pH 3.2. The branched side arm contained 10 units of catalase in one end and 2, 0.5, or 0.2 µmole of H₂O₂ and 3 µmoles of potassium phosphate buffer, pH 7.0, in a total volume of 0.3 ml. in the other end; temperature, 37°. The values were corrected for oxygen evolution.

Fig. 3. Effect of quinone and hydroquinone on the rate of enzymatic decarboxylation of oxalate. The incubation mixture contained 0.024 mg. of enzyme protein (second acetone fraction), 10 µmoles of potassium oxalate, and 400 µmoles of potassium citrate buffer at pH 3.2. Air was introduced at 90 minutes and exact readings were started at 105 minutes.

**Effect of Oxygen on Rate of Reaction**—It was previously observed that this reaction did not proceed under strictly anaerobic conditions and that introduction of air into the manometer flasks restored the activity to the original level (3). As shown in Fig. 2, when the atmosphere was completely replaced by the helium gas (see under “Methods”), the gas production was almost negligible. In confirmation of the previously reported results, when air was introduced into the gaseous phase, the reaction proceeded immediately. In order to test whether oxygen is needed for the reaction, and how much oxygen is needed to restore 100 per cent activity, various amounts of oxygen were evolved in the manometer flasks as follows: A double side arm manometer flask was employed, one of the side
arms of which had two reservoirs (American Instrument Company, Inc., catalogue No. 5-224). In one of these, 10 units of catalase were placed, and various amounts of hydrogen peroxide were placed in the other reservoir. Usually the reaction was started by tipping in the oxalate solution from one side arm, and gas production was followed for about 30 minutes to make sure that the rate of the reaction was almost negligible. Then the catalase and \( \text{H}_2\text{O}_2 \) were mixed in the branched side arm so that a known amount of oxygen was liberated in the manometer gas phase. As shown in Fig. 2, about 1 \( \mu \)mole of oxygen was necessary to restore the complete activity. With 0.25 \( \mu \)mole of oxygen the effect was about one-fourth, and with 0.1 \( \mu \)mole of oxygen stimulation was about 10 per cent, of that observed with 1 \( \mu \)mole of oxygen. In the absence of oxygen the enzyme appears to be completely stable, and even after 5 hours incubation the enzyme showed the original level of activity, once air was introduced.

The requirement for oxygen was strictly specific and could not be replaced by any of the following compounds at concentrations of 0.01, 0.001, or 0.0001 M: \( \text{H}_2\text{O}_2 \), paraquinone, 2-methyl-1,4-naphthoquinone, flavin adenine dinucleotide, flavin mononucleotide, DPN, TPN, \( \text{KNO}_3 \), and cytochrome c. Hydroquinone and quinone exhibited no effect under anaerobic conditions, but when the air was introduced quinone exhibited a marked inhibition, as shown in Fig. 3, whereas hydroquinone stimulated the reaction by 60 to 100 per cent. Certain other reducing agents, such as catechol, ascorbic acid, and \( \text{FeSO}_4 \) showed some stimulating effect, but glutathione, cysteine, or cystine had no effect at \( 10^{-3} \) and \( 10^{-4} \) M. Chelating agents such as ethylenediaminetetraacetic acid, \( \text{KCN} \), and \( \alpha,\alpha' \)-dipyridyl (\( 10^{-3} \) or \( 10^{-4} \) M) had no effect, nor did \( p \)-chloromercuribenzoate (\( 10^{-4} \) M) have any inhibitory action on the most purified enzyme preparation.

![Fig. 4. Heat stability of oxalic decarboxylase at various pH levels. The enzyme solution (first acetone fraction) containing approximately 2 mg. of protein per ml. was heated at 78° for 10 minutes, cooled, and assayed with the standard assay system. The pH was adjusted by the cautious addition of \( \text{N KOH} \) and \( \text{H}_2\text{SO}_4 \).](http://www.jbc.org/)


Stability—Crude enzyme solutions at various pH values were prepared by the cautious addition of HCl or KOH and heated at 78° for 10 minutes. The activity of these solutions was tested by the manometric technique. The enzyme lost only about 5 per cent of the activity at pH 4.0, but lost almost 90 per cent at pH 2.0 and 7.0 (Fig. 4). The crude enzyme preparation of pH 4.3 did not show any decrease in activity after storage at 0° for 45 days.

Influence of pH and Substrate Concentration—Maximal activity for the decarboxylation was observed in the range of pH 2.5 to 4.0 (Fig. 5). The influence of varying concentrations of the substrate is illustrated in Fig. 6. The apparent dissociation constant ($K_m$) of the enzyme and substrate is approximately $2.05 \times 10^{-3}$ M.

Substrate Specificity—Oxalic acid was the only substrate found to be decarboxylated by the purified enzyme preparation. Pyruvic, malonic, succinic, glycolic, citric, malic, α-ketomalonic, oxalacetic, and formic acids were all inactive. The rigid specificity of this enzyme makes it an excellent tool for the specific and quantitative determination of oxalic acid.

DISCUSSION

It appears that there are at least three different enzymes which decarboxylate oxalic acid in nature. In higher green plants (6) and mosses...
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(7), oxalate is metabolized by an oxidative process yielding CO₂ and hydrogen peroxide. Flavin derivatives appeared to be involved in this reaction (8). In a soil bacterium (2), oxalate is decarboxylated under anaerobic conditions in the presence of ATP, CoA, Mg⁺⁺, cocarboxylase, and acetate. This type of reaction appears to be commonly found in nature, since enzymes from Neurospora crassa⁶ and Torula utilis⁶ were found to be stimulated by the addition of CoA and ATP. In the so called white rot fungi (3), which do not accumulate oxalate in the acidic culture media, the third, oxygen-dependent type of oxalic decarboxylase was found.

The evidence presented in this communication indicates that oxalic acid decarboxylase from C. veltipes is different from the enzyme isolated previously from a soil bacterium in its cofactor requirements, pH optimum, and other properties. The peculiar requirement for oxygen has not been explained. Under anaerobic conditions, none of the oxidizing agents so far tested satisfied the requirement for oxygen. However, under aerobic conditions, these oxidizing agents, such as H₂O₂ or paraquinone, usually inhibited the enzyme. Certain reducing agents, such as ascorbic acid, hydroquinone, FeSO₄, and catechol, stimulated the reaction in the presence of oxygen.

Since the oxidative decarboxylation of oxalic acid in higher green plants and mosses was shown to yield H₂O₂ (Equation 1), it was postulated that H₂O₂ might be reducing oxalic acid to form formic acid and oxygen in the Collyvia enzyme system (Equation 2). The net reaction (Equation 3) is the anaerobic decarboxylation of oxalate to produce stoichiometric quantities of formate and CO₂, but oxygen was needed only in a catalytic quantity.

\[
\begin{align*}
(1) & \quad \text{COOH} + O_2 \rightarrow 2\text{CO}_2 + \text{H}_2\text{O}_2 \\
(2) & \quad \text{COOH} + \text{H}_2\text{O}_2 \rightarrow 2\text{HCOOH} + O_2 \\
(3) & \quad 2\text{COOH} \rightarrow 2\text{CO}_2 + 2\text{HCOOH}
\end{align*}
\]

However, when H₂O₂ was added directly or generated in situ by glucose and notatin under anaerobic conditions, it did not replace oxygen. The addition of catalase or catalase with ethanol did not inhibit the enzyme activity under aerobic conditions. Therefore, involvement of hydrogen

⁶ Unpublished observation by Yuichi Yamamura.
⁶ Unpublished observation by Yoshitaka Saito.
peroxide did not appear likely, although this hypothesis could not be ruled out completely.

SUMMARY

Oxalic decarboxylase from *Collyvia veltipes* was purified about 400-fold with an overall yield of about 35 per cent. The enzyme is most active at pH 3.0 and most stable at pH 4.5. It acts specifically on oxalic acid and produces stoichiometric quantities of CO₂ and formic acid. In contrast to the previously reported oxalate decarboxylase from a soil bacterium, this enzyme does not show any requirement for adenosine triphosphate, coenzyme A, cocarboxylase, acetate, or Mg ion. However, it is active only in the presence of small amounts of oxygen, although the over-all reaction does not involve net oxidation.

BIBLIOGRAPHY

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