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ENZYMATIC OXALATE DECARBOXYLATION IN *ASPERGILLUS NIGER*

II. HYDROGEN PEROXIDE FORMATION AND OTHER CHARACTERISTICS OF THE OXALATE DECARBOXYLASE

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SUMMARY

O_2-dependent oxalate decarboxylase (oxalate carboxy-lyase, EC 4.1.1.2) is isolated of a higher degree of purity. During the decarboxylation reaction, in addition to formate and CO_2, traces of H_2O_2 and oxidation products of aromatic amines and phenols are formed; these aromatic compounds are added with the purpose of stimulating and protecting the enzyme during its catalytic action. When the partial pressure of O_2 is increased the enzyme is denatured at an increasing rate; the amount of H_2O_2 and oxidation products formed are also increased. The results suggest that the oxalate decarboxylation proceeds with a single electron transfer.

INTRODUCTION

Oxalate decarboxylase (oxalate carboxy-lyase, EC 4.1.1.2) has been discovered by SHIMAZONO[1] in *Collybia velutipes*. SHIMAZONO AND HAYAISHI[2] cultivated this xylophagous fungus during 27 days adding oxalic acid to the medium to induce the formation of decarboxylase. EMILIANI AND BEKES[3] obtained the same enzyme from *Aspergillus niger* strains which produce a considerable amount of citric acid. Aspergillus grows so rapidly, that 3 days after inoculation it is possible to extract the enzyme from the mycelium. When the pH of the medium is very low (pH 1–1.5) the mold cells contain the oxalate decarboxylase and the liquid medium citric acid only; but when the pH is higher (pH $>$ 2), the enzyme is not produced and besides citric acid, oxalic acid is present in the medium. More recently LILLEHOJ AND SMITH[4] extracted a decarboxylase of the same type from *Myrothecium verrucaria*, which is a wood-destroying mold as *C. velutipes*.

The enzymes obtained from all 3 molds have the same fundamental feature, *i.e.* they decarboxylate the oxalate to produce formate and CO_2 and are inactive in absence of O_2, but they differ in optimal pH, sensitivity to oxygen, K_m, *etc.*

The present work deals with some new properties of *Aspergillus niger* decarboxylase which has now been obtained in a higher degree of purity.

MATERIALS AND METHODS

Organism used and culture conditions

The Aspergillus strain and the culture conditions have been described previously[3].

Manometric method

Enzyme activity was determined by measuring the amount of CO_2 produced in 30–60 min in the Warburg apparatus[6]. This analysis was carried out in an atmosphere of air or a mixture of N_2 and O_2. The main cavity of the flask contained: 0.6 ml (120 μmoles) of 0.2 M sodium oxalate (pH 5.2); 0.1 ml (1 mg) of Tween 80; 0.2 ml (4 μmoles) of *o*-phenylenediamine and 2.1 ml of 0.2 M acetate buffer (pH 5.2). Side arm: 0.4 ml of acetate buffer and 0.1 ml of the enzyme diluted so as to yield 150–300 μl of CO_2 in 1 h. The temperature was 30°.

Determination of H_2O_2

To determine the amount of H_2O_2 produced during oxalate decarboxylation, the property of peroxidase of oxidizing the *o*-phenylenediamine was used. The intensity

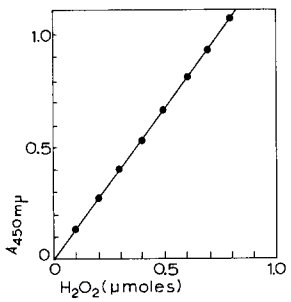

Fig. 1. Determination of H_2O_2. 0.6 ml (120 μmoles) of oxalate (pH 5.2); 0.2 ml (4 μmoles) of *o*-phenylenediamine; 0.1 ml (1 mg) of Tween 80; different amounts of H_2O_2 as indicated in the figure; acetate buffer 0.2 M (pH 5.2) to complete 3 ml. Temperature, 30°. To this mixture 0.1 ml (10 μg) of peroxidase and, after 1 min, 4 ml of 0.2 M HCl were added. Readings at 450 mμ with a Coleman Model 14 spectrophotometer.

of the yellow color formed was proportional to the amount of H_2O_2. Fig. 1 shows the results obtained by addition of H_2O_2 to the mixture used in the manometric method for determination of decarboxylase activity, but without this enzyme.

Preparation of crude extract

Crude extract preparation was performed using 3-day-old mycelium, according to the method mentioned before[3] with the difference that the washing of mycelium was carried out with running tap water for 30 min.

Purification of the enzyme

All operations were performed at 2–3°. 2 l of cold methanol were added to 2 l of crude extract with continuous stirring, and then were allowed to stand for 30–60 min. The precipitate was separated by centrifugation (10 000 × *g*, 10 min) and suspended

in 200 ml of 0.2 M acetate buffer (pH 5.6). After 5–6 h the suspension was clarified by centrifugation at 30 000 × *g* for 15 min and the supernatant filtered through a glass filter with the purpose of eliminating some floating lipids. The precipitation with methanol, redissolution in the acetate buffer and clarification were repeated again 3 times, each time reducing the volumes (see Table I). After this procedure, most of the enzyme was in the fraction precipitated between 15 and 30% of methanol (v/v). The precipitate was dissolved in 4 ml of 0.2 M acetate buffer (pH 5.6) and the solution was centrifuged at 30 000 × *g* for 15 min; the clear supernatant had a light yellow color and under ultraviolet light gave an intense purple fluorescence.

The final purification was performed on a DEAE-cellulose column (2 cm × 6 cm) prepared according to PETERSON AND SOBER[7] and equilibrated with 0.02 M acetate buffer (pH 5.6). The enzyme solution was diluted with water to lower the buffer

TABLE I

PURIFICATION OF OXALATE DECARBOXYLASE

Fraction	*Total vol. (ml)*	*Total units**	*Protein (mg/ml)*	*Specific activity* (units/mg protein)*
Crude extract	2000	1250	2.5	0.2
1st precipitation (50% methanol)	200	1190	3.0	1.9
2nd precipitation (50% methanol)	40	1110	3.0	9.1
3rd precipitation (50% methanol)	16	992	4.3	14.4
Fractional precipitation (15–35% methanol)	4	880	4.8	45.4
DEAE-cellulose	10	400	0.5	80.0

* A unit is the amount of enzyme that catalyzes the decarboxylation of 1 mole of oxalate per min.

concentration to 0.02 M, applied to the column and washed successively with 50 ml of 0.04, 0.10 and 0.13 M acetate buffer (pH 5.6). The 0.1 M effuent contained a purple fluorescent substance. The decarboxylase was eluted with 0.2 M acetate (pH 5.6). The flow rate was 1 ml/min and the volume collected in each tube was 5 ml. Fractions 3 and 4 had the highest specific activity. The enzyme solution purified in this manner was colorless and did not fluorescence under ultraviolet light. All determinations performed to establish the presence of catalase[8], peroxidase[9] or polyphenol-oxidase[10] gave negative results. Negative results were also obtained for the analysis of iron with α,α'-dipyridyl[11] and copper with diphenyl-dithiocarbazone[12].

Reagents

Catalase was obtained from Boehringer; peroxidase, bovine albumin and Tween 80 from Sigma: *p*-phenylenediamine, hydroxyquinone, pyrogallol and Triton X from British Drug Houses Ltd.; *o*-phenylenediamine and catechol from Aldrich; DEAE-cellulose from Eastman. All other chemicals used were of reagent quality.

RESULTS

Stability of the enzyme

Decarboxylase dissolved in acetate 0.2 M buffer (pH 5.6) did not lose its activity for several months when kept at 0–3° (freezing of this solution caused a partial inactivation of the enzyme).

During analysis in the Warburg apparatus the enzyme underwent a denaturation. However, when the procedure was carried out at a temperature not higher than 30° and in the presence of air, it was possible to protect the enzyme by adding albumin and *o*-phenylenediamine[3]; under these conditions the reaction was practically of zero order for 30–60 min.

Albumin could be replaced by gelatin or by surface-active non-ionic substances like Tween 80, Tween 20 or Triton X. On the contrary, surface-active ionic substances, either anionic (sulfonated alcohols) or cationic (quaternary bases) inactivated the

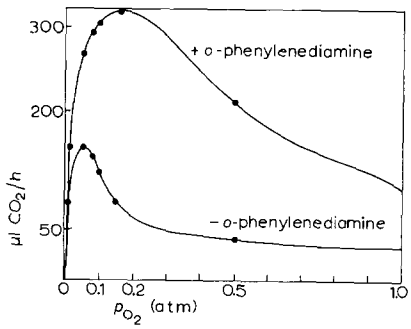

Fig. 2. Influence of the partial pressure of oxygen on the enzyme activity. Gas phase, mixture of N_2 and O_2. Other conditions see *Manometric method.*

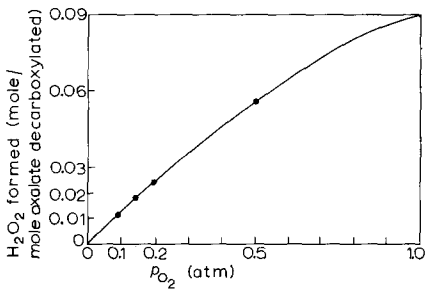

Fig. 3. Influence of the partial pressure of O_2 on the H_2O_2 formation during the decarboxylation. Determination of the enzyme activity as in Fig. 2 and H_2O_2 as in Fig. 1.

enzyme very rapidly. *o*-Phenylenediamine could be replaced, though with less efficiency, by reducing reagents such as *o*- and *p*-diphenols, *o*- and *p*-aromatic diamines, *etc.* Strongly reducing substances (hydroxylamine, sulfite, dithionite, etc.) in $5 \cdot 10^{-4}$ M concentration inhibited the decarboxylase immediately and completely.

Influence of the partial pressure of oxygen

The influence of the partial pressure of O_2 on the activity of the enzyme was studied by replacing the air by mixtures of O_2 and N_2 in the Warburg apparatus. As shown in Fig. 2, in the absence of *o*-phenylenediamine the activity was maximal with a partial pressure of 0.04 atm of O_2. With *o*-phenylenediamine the maximum was obtained at 0.2 atm. When the oxygen pressure was lower than the optimal, the enzyme showed a decreased activity and it became totally inactive in anaerobiosis. On the other hand, O_2 pressures higher than optimal accelerated the denaturation of the decarboxylase even in the presence of *o*-phenylenediamine.

Oxidation of the o-phenylenediamine and other reducing substances during decarboxylation

When the analysis was carried out in presence of air (p_{O_2}, 0.21 atm) using

o-phenylenediamine and sufficient enzyme to produce more than 300 μl of CO_2 in 1 h, the color of the liquid in the Warburg flask turned faintly yellow because of a slight *o*-phenylenediamine oxidation. Other substances (such as benzidine, tetramethyl-*p*-phenylenediamine, NADI reagent, *etc.*) which had the property of protecting the enzyme during oxalate decarboxylation were also oxidized under these conditions. The degree of oxidation of *o*-phenylenediamine and of the other compounds increased with the activity of the enzyme.

Formation of hydrogen peroxide

The formation of H_2O_2 during decarboxylation was proved by the following experiments.

(a) The decarboxylation reaction was carried out under standard conditions, but without the addition of *o*-phenylenediamine. After 1 h, *o*-phenylenediamine or one of the above mentioned substances, that had the property of stimulating and protecting decarboxylase (and that at the same time were substrate for peroxidase), was added. Color did not appear, but on addition of peroxidase an immediate positive reaction was obtained; the color developed depended on the oxidable substance aggregated, with *o*-phenylenediamine it is deep yellow-orange.

(b) The decarboxylation reaction was carried out as in (a). After 1 h the reaction mixture was dialyzed. When one of the oxidizable compounds and peroxidase were added to the diffused fraction, a positive reaction was obtained.

(c) In every case, if catalase was added to destroy H_2O_2 before the addition of peroxidase, the reaction was negative.

The formation of traces of H_2O_2 could involve a consumption of O_2. We attempted to determine these trace amounts with the respirometer of Warburg; however with our apparatus the data obtained were not significant.

Amount of hydrogen peroxide produced during the decarboxylation

Influence of some factors. Determination of the H_2O_2 formed during the oxalate decarboxylation was accomplished in the following manner: at the end of the enzyme activity determination, in the presence of *o*-phenylenediamine, the side arm of the Warburg flask was opened to introduce 0.1 ml (10 μg) of peroxidase and after 1 min, 4 ml of 0.2 M HCl. The mixture (which turned yellow after the addition of peroxidase) was transferred to a spectrophotometric tube for absorbance determination (see Fig. 1). Under standard conditions the amount of H_2O_2 produced, fluctuated between 1.5 and 3.0% of the decarboxylated oxalate (mole/mole); for example, with a production of 270 μl of CO_2 (12.3 μmoles), an average of 0.2 μmole of H_2O_2 was obtained.

In order to be sure that the amount of H_2O_2 produced was related to the amount of decarboxylated oxalate, several assays were performed: in one case the amount of enzyme was varied from 2.5 to 0.4 μg of protein; in another case the reaction time was varied (from 10 to 60 min), so that a production of CO_2 of 50–300 μl/h was obtained. In every case the amount of H_2O_2 formed was 1.5 to 3% of the CO_2 produced (mole/mole). These assays indicated that the formation of H_2O_2 was related to the decarboxylation of oxalate, although it could not definitively be ascertained that it was not due to a contaminant. Experiments were performed, therefore, in which the amount of enzyme was maintained constant and its activity varied by modifying the pH (from 4.6 to 5.8) and the concentration of oxalate (from 10 to 120 μmoles). Under these

conditions the amount of H_2O_2 fluctuated also between 1.5 and 3.0% of the decarboxylated oxalate. Thus, it was concluded that the formation of H_2O_2 was a consequence of the decarboxylation.

Since O_2 was necessary and, at the same time, injurious for decarboxylase, it was likely that the partial pressure of O_2 would be important for H_2O_2 formation*. In fact, it can be seen in Fig. 3 that the amount of peroxide formed (referred to CO_2, *i.e.* to decarboxylated oxalate), increases on raising the p_{O_2}. It does not increase in an absolute manner, because, as shown before (Fig. 2), an increase in the percentage of O_2 in the mixture N_2–O_2 produces a more rapid loss of enzymatic activity during the analysis.

In conclusion it can be affirmed that the amount of H_2O_2 formed depends on the activity of the decarboxylase and the partial pressure of oxygen.

Influence of the hydrogen peroxide. It was indicated before that when the amount of CO_2 produced in the standard analytical conditions was more than 300 μl/h, the liquid became faintly yellow due to oxidation of the *o*-phenylenediamine. With 300 μl of CO_2, 0.20 μmole of H_2O_2 accumulated during 1 h in the flask of the Warburg apparatus. In order to investigate whether this quantity of H_2O_2 was important, as far as the oxidation of *o*-phenylenediamine was concerned, 1 μmole of H_2O_2 was added to the standard mixture but without decarboxylase. Under these conditions no color appeared during a 1-h period at 30° in the presence of air (p_{O_2} = 0.21 atm).

Another possibility was that H_2O_2 could be harmful to the enzyme. To clarify this, the activity of the decarboxylase was determined with addition of 0.2–0.3 μmole of H_2O_2 to the mixture. There was no difference to the control. However higher amounts were inhibitory; *e.g.* by adding 1 μmole of H_2O_2 the enzyme showed an activity about 10% less than the control.

On the other hand it was possible that the presence of H_2O_2 was necessary for decarboxylase activity. An experiment was performed in which peroxidase (10 μg) was added to the mixture, at the beginning, with the purpose of consuming the H_2O_2 simultaneously with its formation by *o*-phenylenediamine oxidation. Under these conditions the liquid turned yellow, although decarboxylase activity was not different from that of a control without peroxidase. There was no difference in the activity of the enzyme, either when the analysis was performed in the presence of an excess of catalase (10 μg).

In conclusion, these experiments could not prove any effect of the H_2O_2 formed under standard conditions of analysis.

DISCUSSION

By bubbling air, or even pure O_2, through the solution of decarboxylase for 1 h, no inactivation was observed; but in the presence of oxalate the enzyme was denatured. Evidently, in the presence of oxalate the decarboxylase is more susceptible to attack by O_2. Antioxidants, which have an E° between 0.7 and 0.8 V, protect and stimulate

* It had previously been investigated whether, in standard analytical conditions, the *o*-phenylenediamine was oxidized under higher than normal p_{O_2} without addition of enzyme. After an hour of shaking in the Warburg apparatus, the $A_{450\ m\mu}$ was determined as indicated in Fig. 1. Up to p_{O_2} = 0.5 atm no yellow color appeared. By increasing O_2 concentration a faint color could be observed; *e.g.*, with pure O_2 the $A_{450\ m\mu}$ was 0.05. The values obtained without adding decarboxylase were used to correct the values of Fig. 3 obtained with enzyme.

the enzyme. On the contrary more reducing substances inhibit the enzyme probably by interfering with or destroying a complex enzyme–O_2–oxalate. Likewise the low activity of enzyme at low p_{O_2} (see Fig. 2) is possibly due to a difficulty in the formation of this complex; in anaerobiosis the enzyme is completely inactive.

During decarboxylation traces of H_2O_2 are formed in amounts approximately proportional to the enzyme activity and to the p_{O_2}. On the other hand, the H_2O_2 is not necessary for the decarboxylation reaction and cannot replace O_2. Therefore we can conclude that these trace amounts of H_2O_2 originate from a secondary reaction.

At present, further studies are required to elucidate the mechanism of oxalate decarboxylation. However, the O_2-dependence, the formation of traces of H_2O_2 and the slight oxidation of phenols and aromatic amines indicate that this reaction proceeds with a single electron transfer. An hypothetical model for the reaction mechanism can be formulated such as that shown in Fig. 4; this model is in harmony with our results and is consistent with all other information mentioned in the literature.

We suppose that the decarboxylase forms a complex with the O_2, indicated in reaction A of Fig. 4. In this complex the O_2 is activated, although the decarboxylase

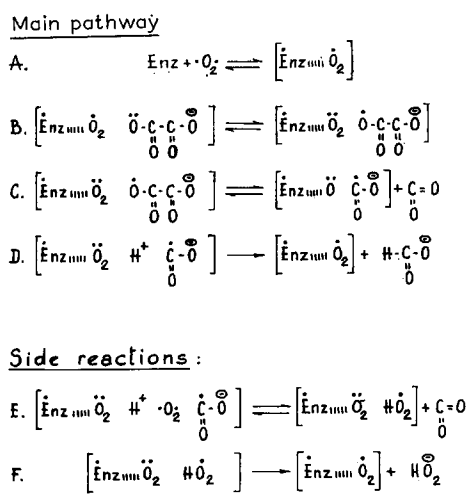

Fig. 4. Tentative mechanism for oxalate decarboxylation.

does not possess any metallic cation. It has been shown by CILENTO AND ZINNER[13–15], however, that organic compounds can transfer or accept one electron, thus activating the O_2. We can formulate the association of enzyme with O_2 as a transfer of one O_2-electron to an electron reservoir (such as tryptophan or tyrosine, present in the decarboxylase) rather like a charge transfer complex; this is shown in Fig. 4 as $\dot{E}nz_{\cdots\cdots}\dot{O}_2$; thus, a peroxide and an oxalate radical can be formed (reaction B) and the latter can decompose into CO_2 and the radical $\overset{\dot{C}-O}{\underset{O}{\|}}$ shown in (C). DRUMMOND AND WATERS[17] proved the formation of these two radicals by oxidation of oxalate with manganic ions. The last radical can now receive an electron from the peroxide complex and, in the presence of H^+, rearrange to formate ion (reaction D).

On the other hand the trace amounts of H_2O_2 formed as byproduct may arise

from a competition between complex radicals and O_2 as electron acceptors. An example is shown in (E) and (F). This phenomenon increases with the increase of O_2 partial pressure.

Interpretation of the mechanism by which the *o*-phenylenediamine stimulates and protects the enzyme during its catalytic action is more difficult. It is known that this and other analogous compounds give and receive electrons easily; therefore it is possible that they collaborate with the enzyme in the activation of the O_2.

We have not been able to verify the formation of free radicals by electron paramagnetic resonance spectroscopy; however, it can be foreseen that it will not be easy to obtain this direct physical evidence. According to our hypothesis, the radicals would form complexes with the enzyme and would not be free in the solution as has been shown in the case of peroxidase[18]. On the other hand, in order to detect radicals joined to the decarboxylase it would be necessary to use an enzyme concentration 300–500 times higher than those used in the present work. In such a case it would be necessary to overcome difficulties due to pH buffering, rapidity of operation, *etc.*

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REFERENCES

1 H. SHIMAZONO, *J. Biochem. Tokyo*, 42 (1955) 321.
2 H. SHIMAZONO AND O. HAYAISHI, *J. Biol. Chem.*, 227 (1957) 151.
3 E. EMILIANI AND P. BEKES, *Arch. Biochem. Biophys.*, 105 (1964) 488.
4 E. B. LILLEHOJ AND F. G. SMITH, *Arch. Biochem. Biophys.*, 109 (1965) 216.
5 E. EMILIANI, F. FALCO AND A. I. RICCIARDI, *Rev. Fac. Ing. Quim. Univ. Nacl. Litoral. Sta. Fe. Arg.*, 24 (1955) 27.
6 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques*, Burgess, Minneapolis, Minn., 1959.
7 A. PETERSON AND H. A. SOBER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. V, Academic Press, New York, 1963, p. 3.
8 D. HEBERT, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, 1962, p. 784.
9 B. CHANCE AND A. C. MAEHLY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, 1962, p. 773.
10 C. R. DAWSON AND R. J. MAGEE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, 1962, p. 817.
11 F. D. SNELL AND C. T. SNELL, *Colorimetric Methods of Analysis*, Vol. II, Van Nostrand, New York, 1949, p. 316.
12 F. D. SNELL AND C. T. SNELL, *Colorimetric Methods of Analysis*, Vol. II ,Van Nostrand, New York, 1949, p. 112.
13 G. CILENTO AND K. ZINNER, *Biochim. Biophys. Acta*, 120 (1967) 84.
14 G. CILENTO AND K. ZINNER, *Biochim. Biophys. Acta*, 143 (1967) 88.
15 G. CILENTO AND K. ZINNER, *Biochim. Biophys. Acta*, 143 (1967) 93.
16 P. GEORGE, *International Symposium on Oxidase and Related Oxidation Reduction System*, Vol. I, Wiley, Amherst, Mass., New York, 1964, p. 3.
17 A. Y. DRUMMOND AND W. A. WATERS, in W. A. WATERS, *Mechanism of Oxidations of Organic Compounds*, Methuen, London, 1964, p. 104.
18 I. YAMAZAKI, H. S. MASON AND L. PIETTE, *J. Biol. Chem.*, 235 (1960) 2444.