Malonic Acid Biosynthesis in Bush Bean Roots. II. Purification and Properties of Enzyme Catalyzing Oxidative Decarboxylation of Oxaloacetate [1, 2]

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In the preceding paper (4) evidence was presented that a soluble enzyme preparation from bush bean roots was capable of forming malonate from oxaloacetate (OAA). Carbons 3 and 4 of OAA were equally incorporated into malonate, suggesting that OAA undergoes an α-decarboxylation. In the presence of the crude enzyme preparation, Mn^{+2} appeared to be the only supplemental cofactor required for malonate formation.

The experiments reported in the present paper describe the partial purification and characterization of the enzyme(s) catalyzing the conversion of OAA to malonate.

Experimental Procedure

Materials. Plant materials and C^{14} labeled substrates were prepared as described in the previous paper (4).

Enzyme Assay. The basic procedure involved incubating OAA-4-C^{14} with enzyme and determination of the radioactive malonate formed. The complete incubation medium contained: 60 μmoles of acetate buffer, pH 5.4; 1 μmole of $MnCl_2$, 0.5 μmole of OAA-4-C^{14}, S.A. 140,000 cpm/μmole; 0.1 ml of boiled root extract; and enzyme to make a final volume of 1.0 ml. The reaction mixture was preincubated for 15 minutes in the absence of OAA-4-C^{14}, and incubated for 15 minutes in the presence of OAA-4-C^{14}. Preincubation and incubation temperatures were 30°. The reaction was stopped by addition of 0.1 ml of 12 N formic acid. The unreacted OAA-4-C^{14} was converted to unlabeled pyruvate and $C^{14}O_2$ by addition of 0.1 ml of freshly prepared 1:1 (V:V) mixture of 50 % citric acid and aniline (19). The reaction mixture was permitted to stand at room temperature for 30 minutes to effect complete decarboxylation. A 0.1 ml aliquot was then transferred to a glass planchet and the radioactivity measured using a Nuclear D-47 gas flow detector. Ascending chromatography of the reaction mixture using ether: acetic acid: H_2O (5:2:1) as solvent showed malonate to be the only radioactive compound present. The 1:1 citric acid-aniline solution was shown to have no destructive effect upon malonate-C^{14}. Control vessels with boiled enzyme (or enzyme omitted) showed negligible radioactivity after treatment with the 1:1 citric acid-aniline solution. The unit of activity is the amount of enzyme forming 1 μmole of malonate under the above conditions.

Purification of Enzyme. All operations were carried out at 0 to 4° unless otherwise stated. Crude homogenates from roots of bush bean (*Phaseolus vulgaris* L., var. Tendergreen) were prepared as described previously (4) and centrifuged 15 minutes at 30,000 × *g*. The 30,000 × *g* supernatant solution was made 0.005 M $MnCl_2$ and transferred to a hot water bath at 80°. When the enzyme solution reached 60° it was maintained at this temperature for 15 minutes, and then transferred to an ice bath. The solution was permitted to stand in ice for 30 minutes after reaching 4°. Denatured protein was removed by centrifugation at 30,000 × *g* for 15 minutes. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to make it 40 % saturated (24.3 g/100 ml heat-treated supernatant solution). The pH was maintained near 7.0 by addition of concentrated KOH. The solution was allowed to stand for 30 minutes and was centrifuged at 30,000 × *g* for 15 minutes; the residue was discarded. Additional solid $(NH_4)_2SO_4$ was added to the supernatant solution to make it 75 % saturated (24.5 g/100 ml heat-treated supernatant solution). The solution was allowed to stand for 30 minutes and was centrifuged at 30,000 × *g* for 15 minutes. The residue was taken up in 0.05 M tris at pH 7.5 in a volume 5 % that of the original heat-treated supernatant solution, and dialyzed 5 hours against 0.002 M tris at pH 7.5. Thirty ml of the dialyzed solution were transferred to a 1.7 × 14 cm chromatography column containing diethylaminoethyl-cellulose (DEAE-cellulose) equilibrated with 0.002 M potassium phosphate buffer at pH 7.0. The proteins were eluted stepwise with 70 ml volumes of potassium phosphate buffer, pH 7.0, at the following concentrations: 0.002, 0.005, 0.02, 0.04, 0.08, and 0.30 M. The effluent solutions were collected in 10 ml fractions and dialyzed against 0.002 M tris at pH 7.5 for 5 hours. Protein fractions eluting between 0.005 and 0.02 M buffer always showed the highest specific activity. Protein con-

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Table I

Summary of Purification of Enzyme Catalyzing the Conversion of Oxaloacetate to Malonate

Fraction	Protein mg/fraction	Activity* units/fraction	Specific activity	Recovery %
30,000 × *g* supernatant	862.0	6888	8	...
Heat and $MnCl_2$	374.0	6358	17	92
$(NH_4)_2SO_4$ (40–75 %)	99.4	3168	32	46
DEAE (0.005–0.02 M)	5.1	1657	325	24

* A unit of activity is the amount of enzyme forming 1 μmole of malonate under assay conditions described in text.

tents were measured by the methods of Warburg and Christian (24) and Lowry et al. (11).

A summary of the purification data appears in table I. A purification of approximately 40-fold was obtained. All data presented in the rest of this paper were obtained with enzyme purified through the $(NH_4)_2SO_4$ stage. Most experiments were verified with enzyme purified through the DEAE-cellulose chromatography stage.

Results

Figure 1 illustrates the rate of malonate formation as a function of time and enzyme concentration.

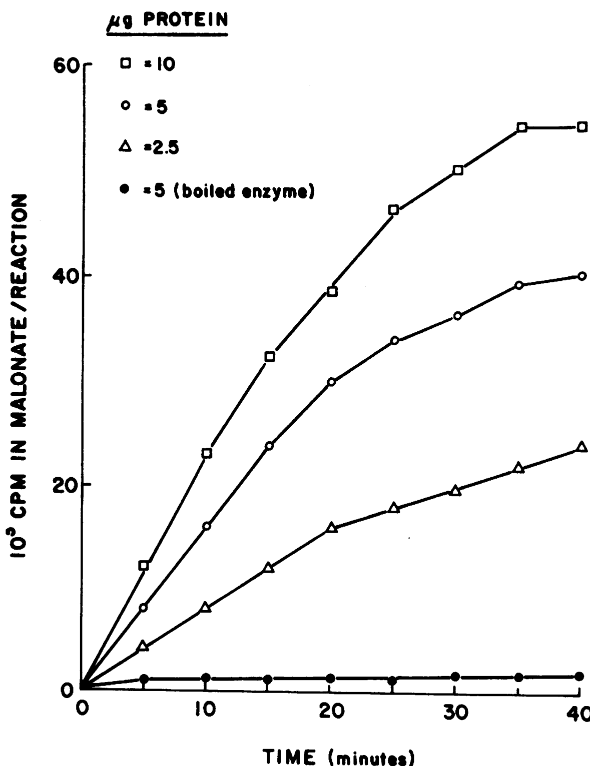


Fig. 1. Rate of malonate formation as a function of time and enzyme concentration. Conditions of reaction as defined under Enzyme Assay, except: 2 μmoles OAA-4-C^{14}; protein concentration as indicated; incubation time as indicated. Enzyme purified through the $(NH_4)_2SO_4$ step.

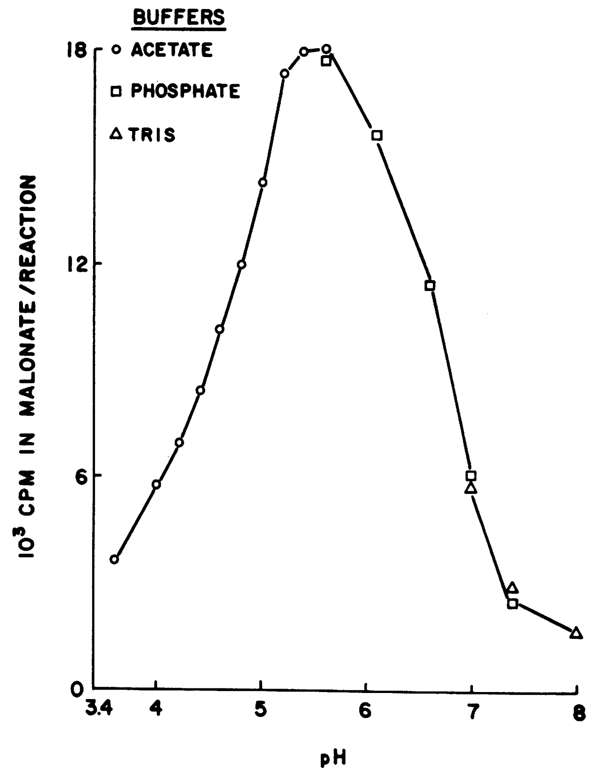


Fig. 2. pH activity curve of enzyme. Conditions of reaction as defined under Enzyme Assay, except: buffers as indicated.

The rate of the reaction was linear with time to 15 minutes for enzyme concentrations up to 5 μg of protein per reaction mixture. The enzyme concentrations used in this study were always within the linear range. It may also be noted in figure 1 that when the enzyme preparation was boiled, malonate formation was negligible, indicating the reaction was enzymatic.

Properties of Enzyme. Figure 2 illustrates the pH activity curve of the enzyme. Acetate buffer was used over the pH range 3.6 to 5.6; phosphate buffer over the pH range 5.6 to 7.7; and tris buffer over the pH range 7.0 to 8.0. A boiled enzyme control was included with each treatment. The activity curve in figure 2 represents the net enzymatic synthesis of malonate at each pH level. The data indicate an optimum activity about pH 5.4.

Table II

Mn^{+2} Requirement for Enzyme

Metal	Concentration	10^3 cpm in malonate
Experiment 1		
None	...	0
$MnCl_2$	2.5×10^{-4} M	12.0
$MnCl_2$	5.0×10^{-4} M	20.0
$MnCl_2$	20.0×10^{-4} M	20.0
Experiment 2		
None	...	0
$MnCl_2$	20.0×10^{-4} M	20.0
$MgSO_4$	"	2.6
$FeSO_4$	"	2.2
$CdSO_4$	"	1.3
$Co(NO_3)_2$	"	0.1
$Al_2(SO_4)_3$	"	0.1
$NiSO_4$	"	0
$ZnCl_2$	"	0

The enzyme shows an absolute dependence upon added Mn^{+2}. The Mn^{+2} stimulation was maximal at a final concentration of 5×10^{-4} M, table II, experiment 1. The Mn^{+2} requirement could not be satisfied by a variety of other divalent cations, table II, experiment 2. A boiled enzyme control was included with each metal tested. Values presented in table II represent the net enzymic synthesis of malonate.

Table III illustrates the effect of compounds known to inhibit certain types of enzyme-catalyzed reactions. Azide decreased malonate formation by 60 % at 10^{-4} M and by 90 % at 10^{-3} M. Parachloro-mercuribenzoate (PCMB) and arsenite had no effect on the enzyme, indicating that sulfhydryl groups probably do not participate in the reaction.

The requirement for O_2 in the conversion of OAA to malonate is illustrated in figure 3. Two series of reaction mixtures were placed in Warburg vessels. One series was gassed 10 minutes with N_2 and stoppered; the other series was gassed 10 minutes with air and stoppered. Following a 15-minute preincubation, OAA-4-C^{14} was tipped into the reaction chamber. At the indicated times the reactions were stopped and malonate formation assayed in the usual manner. In the N_2 atmosphere malonate formation was inhibited 80 %. It would appear O_2 is the terminal electron acceptor in the oxidation of OAA to malonate. The requirement for O_2 in the conversion of OAA to malonate thus provides an alternate assay to measure the rate of the reaction.

Table III

Effect of Inhibitors on Enzyme

Inhibitor	Concentration	10^3 cpm in malonate
None	...	18.0
Azide	10^{-4} M	7.0
Azide	10^{-3} M	1.8
Arsenite	10^{-3} M	18.0
Arsenite	10^{-2} M	18.0
PCMB	10^{-6} M	18.0
PCMB	10^{-4} M	17.0

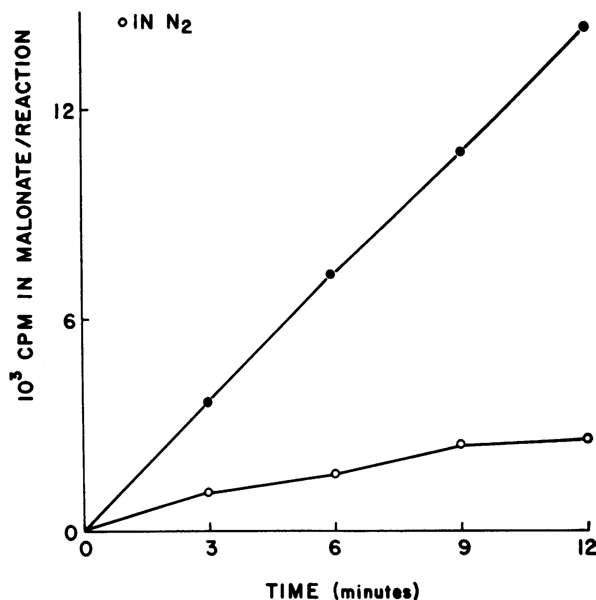


FIG. 3. Requirement for O_2 in the conversion of OAA to malonate.

The stoichiometry of the reaction was measured in the following manner: 0.3 mg protein from the 40 to 75 % $(NH_4)_2SO_4$ fraction was preincubated with 1 μmole of Mn^{2+} and 120 μmoles of acetate buffer, pH 5.4, in a Warburg vessel. Following a 15 minute preincubation period, 27.0 μmoles unlabeled OAA were tipped into the reaction chamber and O_2 uptake and CO_2 evolution measured on a Warburg respirometer periodically over a 3-hour period. The stock solution of OAA employed in this experiment was freshly prepared and assayed for pyruvate and OAA spectrophotometrically by DPNH oxidation with lactic dehydrogenase and malic dehydrogenase respectively. After the 3-hour incubation period, the reactions were stopped by adding dry Dowex 50-H^+ cation exchange resins. The reaction mixtures were again assayed for OAA and pyruvate. Malonic acid was isolated from the reaction mixture by chromatographing an aliquot of the reaction mixture in ether: acetic acid: water (5:2:1) and eluting with water. The eluted malonic acid was then determined by alkali titration. Since there is a simultaneous nonenzymic decarboxylation of OAA to pyruvate and CO_2, a control vessel was included in which OAA was incubated under the same conditions as the complete system, but in the absence of the enzyme. Table IV illustrates results obtained from the enzymic and nonenzymic decarboxylation of OAA.

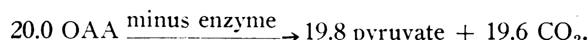
It may be noted from the nonenzymic data that 20.0 μmoles of OAA disappeared with the simultaneous

Table IV

Stoichiometry of OAA Decarboxylation in the Presence and in the Absence of Enzyme

	µmoles					
	Complete			Minus enzyme		
	Initial	Final	Δ	Initial	Final	Δ
OAA	27.0	1.1	25.9	27.0	7.0	20.0
Pyruvate	1.2	10.0	8.8	1.2	21.0	19.8
Malonate	0.0	15.2	15.2	0.0	0.0	0.0
O_2 consumed (µl)			7.7			0.0
CO_2 evolved (µl)			24.5			19.6

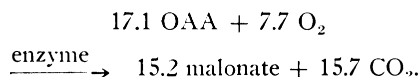
formation of 19.8 µmoles of pyruvate and 19.6 µmoles of CO_2, yielding the following equation:



$$20.0 \text{ OAA} \xrightarrow{\text{minus enzyme}} 19.8 \text{ pyruvate} + 19.6 \text{ } CO_2.$$

These data approach the theoretical calculated values of 1 mole OAA yielding 1 mole of pyruvate and 1 mole of CO_2.

During the enzymic conversion of OAA to malonate 8.8 µmoles of pyruvate were produced, therefore, 8.8 µmoles of CO_2 must be subtracted from the total CO_2 evolution in order to determine how much CO_2 was formed during the enzymic decarboxylation of OAA to malonate. Similarly, a value of 8.8 µmoles must be subtracted from the OAA metabolized in order to determine how much OAA was converted to malonate. With these corrections the following stoichiometric equation may be written for the enzymic conversion of OAA to malonate:



$$17.1 \text{ OAA} + 7.7 \text{ } O_2 \xrightarrow{\text{enzyme}} 15.2 \text{ malonate} + 15.7 \text{ } CO_2.$$

These results approach the theoretical calculated values of 1 mole OAA yielding 1 mole of malonate and 1 mole of CO_2 with the simultaneous consumption of ½ mole O_2. All efforts to show the reverse reaction were unsuccessful.

Various organic acids were tested as substrates for the enzyme by measuring their rate of oxidation on the Warburg respirometer, table V. When OAA was replaced by pyruvate, indoleacetate, α-ketoglutarate, malate, or malonate, oxygen uptake was very low.

Table V

Substrate Specificity of Enzyme

Reaction mixture contained 20 µmoles substrate as listed, 120 µmoles acetate buffer pH 5.4; 1 µmole $MnCl_2$; 0.1 ml boiled root extract; and 0.3 mg protein from 40 to 75 % $(NH_4)_2SO_4$ fraction in a volume of 4.0 ml. Reaction mixtures incubated 1 hour at 30° on a Warburg respirometer.

Substrate	O_2 uptake µl/hour
None	5
OAA	120
Pyruvate	6
Indoleacetate	7
α-ketoglutarate	4
Malate	4
Malonate	5

The 30,000 × *g* supernatant solution accounted for essentially all the enzyme activity observed in the 1000 × *g* homogenate. The 30,000 × *g* precipitate was resuspended in 0.5 M sucrose, centrifuged, and resuspended in 0.25 M sucrose. This mitochondrial preparation was unable to catalyze malonate formation; the mitochondria were functional, however, as attested by their ability to oxidise succinate. It would appear, therefore, that the enzyme under study is essentially soluble.

Cofactor in Boiled Root Extract. The boiled root extract was prepared by boiling the 30,000 × *g* supernatant solution for 20 minutes and removing the denatured proteins by centrifugation. Omitting

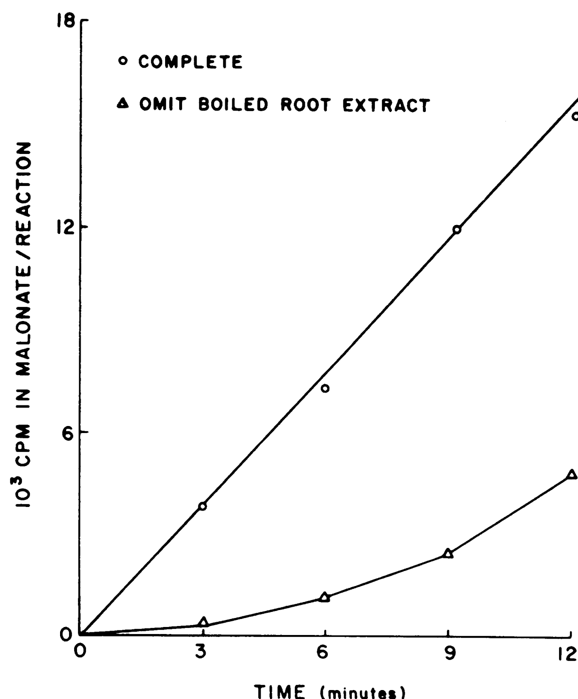


FIG. 4. Requirement for cofactor in boiled root extract.

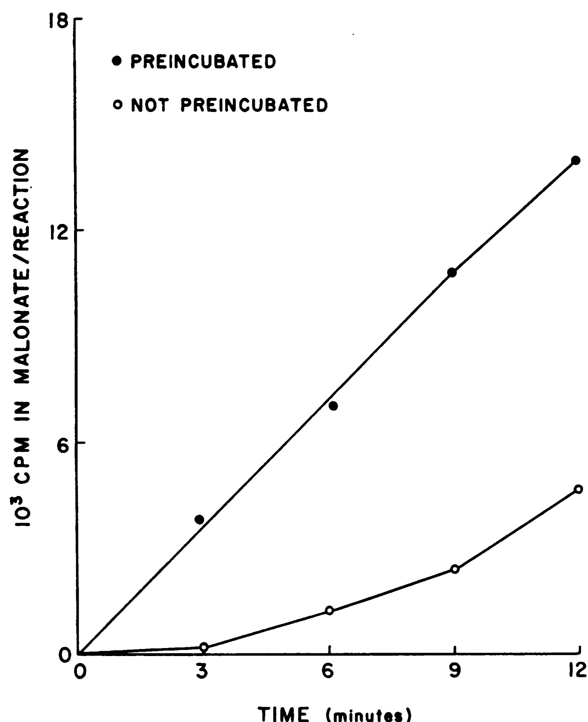


FIG. 5. Effect of preincubation on time lag in malonate formation.

the boiled root extract from the reaction mixture caused a pronounced time lag in malonate formation, figure 4. Following the lag period, the rate of malonate synthesis increased rapidly. Since the conversion of OAA to malonate is an oxidative decarboxylation, it was suspected the boiled extract may be supplying a necessary cofactor for a dehydrogenase type of enzyme. Lipoic acid, cytochrome c, DPN, TPN, CoA, TPP, and each of the six mono-, di-, and tri-phosphonucleotides were unable to eliminate the lag period in malonate formation. The nature of the cofactor present in boiled root extract is under investigation.

Preincubation. When OAA-4-C^{14} was added directly to the reaction mixture containing buffer, enzyme, boiled root extract, and Mn^{2+}, a pronounced time lag in malonate formation resulted, figure 5 (lower curve). When a similar reaction mixture was preincubated at 30° for 15 minutes prior to addition of OAA-4-C^{14} the rate of malonate formation was immediately linear, figure 5 (upper curve). In a separate experiment it was shown that omitting the enzyme, Mn^{2+}, or boiled extract during preincubation also caused a time lag in malonate formation. This observation indicates that all 3 components are required during the preincubation period to overcome the time lag in malonate formation.

During the conversion of OAA to malonate by metmyoglobin, Vennesland et al. (20, 21) were confronted with a similar lag in malonate formation. They reported catalytic amounts of H_2O_2 shortened the lag period. The following experiment was undertaken to ascertain the effect of H_2O_2 upon the system under study. In this experiment the reaction mixtures were not preincubated. Figure 6 (upper curve) illustrates that when 0.01 µmole H_2O_2 was added to the complete reaction mixture, the rate of malonate formation was immediately linear. The addition of H_2O_2 in the absence of boiled extract was ineffective in eliminating the lag in malonate formation, thus indicating that H_2O_2 cannot replace the requirement for boiled crude extract. It may be calculated that approximately 0.1 µmole of malonate was formed during the reaction, figure 6 (upper curve). This value exceeds by 10-fold the amount of H_2O_2 added, thus indicating a catalytic and perhaps cyclic role for H_2O_2.

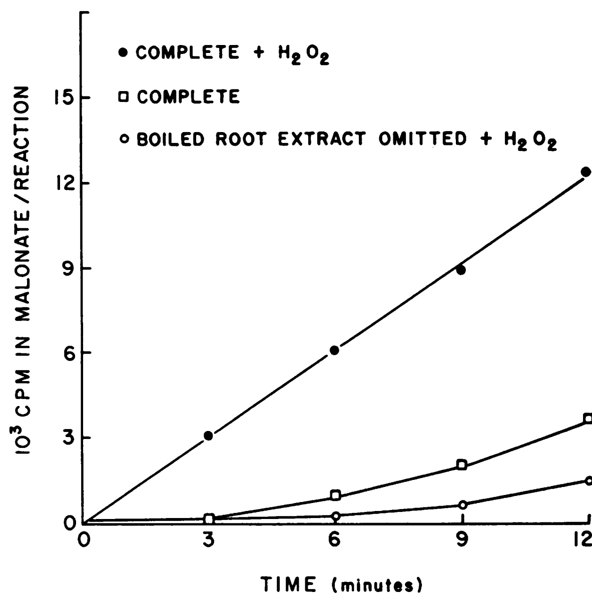


FIG. 6. Effect of H_2O_2 on the rate of malonate formation.

Discussion

The present paper describes the partial purification and characterization of an enzyme(s) from bush bean roots that catalyzes the oxidative decarboxylation of OAA to malonate. At least 10 different metabolic pathways leading to the formation of malonate have been reported. The enzymes participating in these pathways include: acetylCoA carboxylase, transcarboxylase, thiokinase and thioesterase, barbiturase, malonic semialdehyde dehydrogenase, acyl-CoA-CO_2 exchange, and metmyoglobin. The properties of the enzyme from bean roots are quite distinct from each of the enzymes listed, except metmyoglobin.

Vennesland (20, 21) reported metmyoglobin catalyzed an oxidative decarboxylation of OAA to yield

malonate. The metmyoglobin catalyzed reaction showed a Mn^{+2} requirement, O_2 requirement, azide inhibition, and a time lag in substrate oxidation which could be shortened by small quantities of H_2O_2. The similarity in properties between the metmyoglobin and the bean root enzyme catalyzed reactions is readily apparent.

The properties cited above are nearly identical to the properties of various peroxidase catalyzed oxidations. Peroxidase catalyzed IAA oxidation has been reported to possess Mn^{+2} requirement (22), O_2 requirement (5), aromatic cofactor requirement, azide inhibition, and lag in substrate oxidation (25). Similar properties have been reported for the peroxidase catalyzed oxidation of DPNH (1, 10), dihydroxy fumarate (2), *B*-3-indolyl propionate and 3-indolyl-*n*-butyrate (8), 2-nitropropane (13) and miscellaneous dicarboxylic acids (9). The similarity in properties between the reaction under study and the above peroxidase catalyzed oxidations suggests that the bean root enzyme may catalyze the oxidation of OAA by peroxidase activity. This latter suggestion is supported by the following observations.

Kenten and Mann (9) reported that horse-radish peroxidase could oxidase OAA in the presence of Mn^{+2} and *p*-cresol; they did not report the products of the reaction. Shannon and Lew (16) confirmed that horse-radish peroxidase catalyzed the oxidation of OAA and showed that malonate was the product of the reaction. The requirement for Mn^{+2} was absolute; the boiled extract from bean roots was a more active cofactor than *p*-cresol. Horse-radish peroxidase and the bean root enzyme exhibited nearly identical properties in catalyzing the conversion of OAA to malonate. The 2 enzymes showed the same inhibitor response, O_2 requirement, and approximately the same pH activity curve. The time lag in malonate formation could be reduced in each case by preincubation treatments and by the addition of catalytic quantities of H_2O_2 (16).

de Vellis (3) demonstrated that the bean root enzyme preparation under study possessed peroxidase activity at each stage of purification. Peroxidase activity was assayed spectrophotometrically by measuring the rate of *o*-dianisidine oxidation (26). Upon assaying the enzyme preparation at each stage of purification, de Vellis showed a concomitant and parallel increase in specific activity for *o*-dianisidine oxidation and malonate-C^{14} formation.

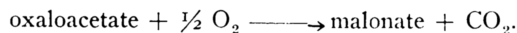
On the basis of the above observations, it is suggested that the bean root enzyme(s) under study catalyzes the oxidative decarboxylation of OAA by peroxidase activity.

Although various mechanisms have been proposed to explain peroxidase catalyzed oxidations (1, 2, 5, 12, 17, 18, 23), the precise functions of Mn^{+2}, aromatic cofactor, and H_2O_2 are not well established. The most recent evidence suggests that these constituents participate in a complex sequence of enzymic and nonenzymic reactions in which cyclic free radical formation is involved (14, 15).

In view of the abundance of malonic acid in bean roots (4) and in view of the apparent absence or low activity of acetylCoA carboxylase in root tissues (4, 6, 7), the enzyme under study is most probably a major participant in malonate biosynthesis in bean roots and perhaps other tissues.

Summary

An enzyme preparation from bush bean roots that catalyzes the oxidative decarboxylation of oxaloacetate to yield malonate was partially purified by heat and $MnCl_2$ treatment, $(NH_4)_2SO_4$ fractionation, and chromatography on a diethylaminoethyl-cellulose column. The enzyme requires manganous ions and an unidentified constituent present in boiled root extract. The enzyme is inhibited by azide. The pH optimum is approximately 5.4 and the reaction is essentially irreversible. Prior incubation of the enzyme with manganous ions and boiled root extract was shown to eliminate a time lag in malonate synthesis. Including a catalytic quantity of H_2O_2 in the reaction mixture substituted for the preincubation treatment. Stoichiometry studies showed the enzyme catalyzed the following reaction:



$$\text{oxaloacetate} + \tfrac{1}{2}\,O_2 \longrightarrow \text{malonate} + CO_2.$$

It is suggested that the bean root enzyme understudy is a peroxidase enzyme that catalyzes the oxidation of oxaloacetate in a manner analogous to other peroxidase catalyzed oxidations. It is probable that the enzyme under study constitutes a major pathway in malonate biosynthesis in bush bean roots and perhaps other tissues.

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