Phytochemistry, 1971, Vol. 10, pp. 1811 to 1815. Pergamon Press. Printed in England.

PROPERTIES OF MALIC ENZYME (DECARBOXYLATING) FROM PULP OF MANGO FRUIT (*MANGIFERA INDICA*)

SHANTHA KRISHNAMURTHY and M. V. PATWARDHAN
Central Food Technological Research Institute, Mysore-2A, India

(*Received* 8 *September* 1970, *in revised form* 16 *November* 1970)

Abstract—Malic enzyme (decarboxylating) has been purified 200-fold from pulp of the fruit *Mangifera indica*. Some of its properties are described. Reversibility of the reaction is demonstrated. The enzyme has pH optimum around 7·2 and Km for NADP 16·6 μM.

INTRODUCTION

HULME and co-workers[1] have reported that in the apple fruit the onset of the climacteric is accompanied by an increase in the activities of malic enzyme (E.C. 1.1.1.40) and pyruvate decarboxylase (E.C. 4.1.1.1). The malic acid content was found to decrease at the same time. It was suggested that this increase in the activities of the two decarboxylating enzymes may be one of the factors responsible for the increased liberation of carbon dioxide that accompanies the climacteric phase of the fruit. Modi and Reddy[2] showed that NADP-dependent malic enzyme is present in ripe mangoes.

A similar increase in malic enzyme activity was noticed in this laboratory while studying the climacteric behaviour of the mango fruit. In this paper are reported some properties of this enzyme isolated from the pulp of the mango fruit at the time of climacteric peak.

RESULTS

Table 1 gives the steps in the purification of the enzyme extracted from freeze dried, cell-free extract of the mango pulp. In the absence of pectinol treatment, viscous solutions

TABLE 1. PURIFICATION OF MANGO FRUIT (PULP) MALIC ENZYME

Procedure	Volume (ml)	Total units	Protein (mg/ml)	Specific activity* (units/mg protein)	% yield	Purification
1. Freeze dried powder	80·0	78	9·8	0·009	100	1
2. Supernatant after pectinol treatment	78·0	60	7·8	0·033	80	3·7
3. 60% $(NH_4)_2SO_4$ fraction	10·0	45	4·5	0·37	56	40·1
4. DEAE-Cellulose 0·2 M Tris HCl buffer eluate	5·5	11	0·2	2·0	14	220·0

The reaction mixture consisted of 16 mM Tris HCl buffer pH 7·4, 3·3 mM $MnCl_2$, 3·3 mM potassium malate and enzyme to a total volume of 3·0 ml at 28°. Reaction was started by addition of 0·2 μmole NADP and followed at 340 nm in Beckman DU-2 Spectrophotometer.

* Specific activity is expressed as Absorptivity change at 340 nm/min/mg protein.

Abbreviations: NAD: Nicotinamide adenine dinucleotide; NADP: Nicotinamide adenine dinucleotide phosphate; NADPH: Reduced Nicotinamide adenine dinucleotide phosphate.

[1] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. Lond.* **B 158,** 514 (1963).
[2] V. V. MODI and V. V R. REDDY, *Ind. J. Exp. Biol.* **5,** 233 (1967).

were obtained and interfered with ammonium sulfate fractionation. The enzyme has been purified about 200-fold and all work reported here is carried out at this stage of purity. The pH optimum for the pure enzyme is 7·2 when assayed by the reduction of NADP.

As most of the work described in the present investigation is based on NADP reduction for assay of malic enzyme activity, it was necessary to rule out the presence of malate dehydrogenase (E.C. 1.1.1.37) in our enzyme preparation. This later enzyme, although generally NAD-specific, may, according to Ochoa[3] have some small activity with NADP. Results in Table 2 indicate the absence of dehydrogenase activity in mango malic enzyme, since activity is dependent on the presence of Mn^{2+}. For comparison, studies with malate dehydrogenase of rat liver mitochondria are also included. Based on this, interference due to the dehydrogenase activity in our malic enzyme assay, was ruled out.

TABLE 2. EFFECT OF MANGANESE AND EDTA ON MALIC ENZYME

Additions	Malic enzyme NADP (mango pulp)	Malate dehydrogenase NAD (Rat liver mitochondria)
Nil	0	4·0
Mn^{2+}	12·8	4·0
Mn^{2+} + EDTA	0	4·0

Assay of malic enzyme was done as in Table 1.

For malate dehydrogenase assay reaction mixture contained: 15 mM Tris HCl buffer pH 9·0; 1 mM NAD; 3·5 mM potassium malate and enzyme.

Activity expressed as nm mole NADPH or NADH formed/min/mg protein.

Table 3 gives the cation requirement of the enzyme. Although manganese gave maximum activity both cobalt and magnesium were also effective. Similar observation has been made for malic enzyme from *Lactobacillus arabinosus*.[4] As shown in Table 4, the presence of NADP is not necessary for the decarboxylation of oxaloacetate which is also performed by malic enzyme, although the presence of cation is necessary for both activities.

TABLE 3. EFFECT OF METAL ION ON MALIC ENZYME ACTIVITY

Cation (3×10^{-3} M)	nm μmole $NADPH_2$ formed/min
Mn^{2+}	7·2
Co^{2+}	5·6
Mg^{2+}	4·6
Nil	0·02

Conditions of assay same as in Table 1.

[3] S. OCHOA, *Methods in Enzymology*, Vol. 1 (edited by S. P. COLOWICK and N. O. KAPLAN), p. 739, Academic Press, New York (1955).

[4] P. M. NOSSAL, *Biochem. J.* **49**, 407 (1951).

TABLE 4. OXALOACETATE DECARBOXYLASE ACTIVITY OF PURIFIED ENZYME (μl CO_2 EVOLVED/10 min/mg PROTEIN)

Enzyme	Complete	−NADP	−Enzyme	$-Mn^{2+}$
1. 60% Ammonium sulfate fraction	40	40	4	3
2. DEAE-cellulose eluate	107	108	33	6

Complete reaction mixture contained: 40 mM acetate buffer pH 5, 0·4 mM manganese chloride; 8 mM oxaloacetate; 0·2 μmole NADP and enzyme protein 100 μg. Each value represents mean of three determinations. The reaction was carried out in Warburg apparatus at 30°.

In another experiment, the reversibility of the enzyme reaction was studied. The enzyme was incubated in presence of labelled bicarbonate, NADPH, pyruvate and $MnCl_2$ at pH 6. At the end of the incubation period, the reaction mixture was deproteinized and malic acid formed was purified and isolated as described under experimental section. Malic acid isolated showed incorporation of radioactivity although in small amount; out of 10^5 counts/min added mixture, 5230 counts/min were incorporated in malic acid, whereas when pyruvate was omitted from the reaction mixture only 1045 counts/min were incorporated. This low incorporation is expected, as two reactions with different pH optima are involved and thermodynamically conditions are more favourable in the forward direction. In another experiment with unlabelled bicarbonate the product formed was located in a single spot of R_f 0·60 and authentic malic acid has R_f 0·64 in *n*-butanol–formic acid–water (10:12:15) system. Figure 1 shows the activity of the enzyme during the oxidation of $NADPH_2$ in the absence (curve 1), in the presence (curve 2) of $NAHCO_3$ and during the reduction of NADP (curve 3).

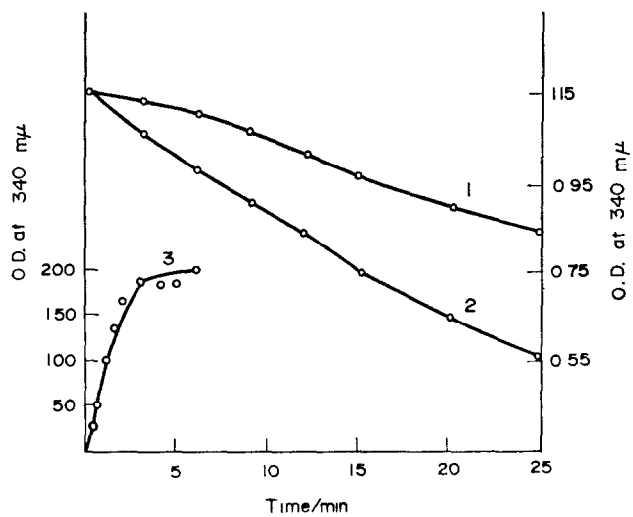

FIG. 1. REVERSIBILITY OF MALIC ENZYME REACTION

Curves 1 and 2 denote $NADPH_2$ oxidation and Curve 3 denotes NADP reduction. Reaction mixture for curves 1 and 2 consisted of: 60 mM acetate buffer pH 6·0; 3 mM $MnCl_2$; 0·2 μmoles NADPH; 8 mM $NaHCO_3$; 16 mM sodium pyruvate and enzyme in a final volume 3 ml. Reaction was started by addition of NADPH. In curve 1, $NaHCO_3$ was omitted. For curve 3 experimental conditions are same as in Table 1.

DISCUSSION

Malic enzyme (decarboxylating) has been purified about 200-fold from the pulp of the mango fruit. The fruits were near the climacteric maximum stage which is attained on 9th day after harvest. Attempts to further purify the enzyme resulted in loss of activity of the enzyme. The general properties of this enzyme appear to be similar to those from other sources.[5]

The enzyme is NADP specific with a pH optimum 7·2. The Km with NADP is 16·6 μM. Manganese was a more effective co-factor than cobalt. This is in agreement with the results of Dilley[5] for malic enzyme from apple at pH 7·2.

Our results indicate no effect of NADP on the decarboxylating activity of the enzyme towards oxaloacetate as is the case of apple enzyme.[5] This is in contrast with pigeon liver enzyme where NADP addition is known to stimulate the decarboxylating activity 6-fold;[6] NADP inhibits the oxaloacetate decarboxylating activity of malic enzyme from flax rust uredospores enzyme.[7]

EXPERIMENTAL

Material

Mature mango fruits were collected from a local garden and allowed to ripen at room temp. (28°). Fruits in the region of the climacteric maximum were taken for purification of enzyme. Previous work based on the pattern of carbon dioxide evolution by whole fruit has indicated that mango fruit reaches the climacteric peak around the 9th day after harvest.

Extract

80 g mango pulp was homogenized for 3 min with 30 ml of 0·25 M sucrose containing 0·1% polyvinylpyrrolidone (PVP 40) the pH being maintained at 7·0 by addition of 0·1 N NaOH in 0·25 M sucrose. The extract was filtered through cloth and centrifuged at 10,000 *g* for 15 min. At this stage, the supernatant was freeze-dried and stored at 0°. This preparation retained its activity for one year. For further purification, the freeze-dried powder was reconstituted with 5 vol. of 0·02 M Tris HCl buffer, pH 7·0.

Purification

To 50 ml of the (reconstituted) extract were added 200 mg pectinol-R_{10} (Rohm-Haas) and allowed to stand for 20 min with stirring at room temp. The solution was then centrifuged and the precipitate discarded. From the supernatant the fraction precipitated by 60% $(NH_4)_2SO_4$ fraction was collected. This fraction was suspended in 5 ml 0·02 M Tris HCl buffer pH 7·0, dialysed for 4 hr, centrifuged and the clear supernatant was collected. This solution was placed on a DEAE cellulose column (6 × 1·5 cm) from which the enzyme was eluted with a step-wise gradient of Tris HCl buffer (0·02 → 0·05 → 0·1 → 0·2 M) at pH 7·4. The major part of the activity, located in 0·2 M buffer fraction, was collected and used in all the work described in this paper.

Assay

The enzyme was assayed by NADP reduction by the method of Ochoa *et al.*[8] Oxaloacetate decarboxylase activity was assayed manometrically as described by Viega Salles and Ochoa.[9]

Reversibility of the Enzyme Reaction

This was studied by (i) NADPH oxidation in presence of $NaHCO_3$ (labelled and unlabelled) and pyruvate and (ii) identification of malic acid as the reaction product. Assay conditions are given in Fig. 1.

Identification of Malic Acid

Using ^{14}C-labelled Na_2CO_3, pyruvate and NADPH as substrates, the reaction at the end of an incubation period was stopped by the addition of 1 ml of 6 N HCl and the precipitate removed by centrifugation. To

[5] D. R. DILLEY, *Plant Physiol.* **41,** 214 (1966).
[6] W. J. RUTTER and H. A. LARDY, *J. Biol. Chem.* **233,** 374 (1958).
[7] M. A. JOHNSON and S. S. FREAR, *Phytochem.* **2,** 75 (1963).
[8] S. OCHOA, A. MEHLER and A. KORNBERG, *J. Biol. Chem.* **174,** 979 (1948)
[9] J. B. VIEGA SALLES and S. OCHOA, *J. Biol. Chem.* **187,** 849 (1950).

the supernatant, 50 mg of unlabelled malic acid was added and a few drops of H_2O_2 were added to remove unreacted pyruvate and any trace of oxaloacetate. The solution was boiled for 1 min to remove H_2O_2 and then concentrated to a small volume. This was placed on Dowex-2-acetate column (8 × 2 cm). The column was washed with H_2O and eluted with 1 N HCl. The eluate was evaporated to dryness and the residue taken up in Et_2O, filtered and precipitated with benzene. The malic acid thus obtained was weighed and counted for radioactivity in a windowless gas flow counter. The ether–benzene treatment was repeated twice till the counts in malic acid were constant.

For chromatography of the malic acid formed, no carrier malic acid was added and unlabelled $NaHCO_3$ was used. The H_2O_2 treated reaction mixture prepared as described in the previous paragraph was concentrated and spotted on Whatman No. 3 paper. The chromatograms were run for 16 hr with *n*-butanol–acetic acid–water (5:1·5:5) and *n*-butanol–formic acid–water (10:2:15) respectively as solvents. Authentic malic acid was co-chromatographed with the sample.

Protein was estimated by the method of Lowry *et al.*[10]

[10] O. H. Lowry, N. J. Rosenbrough, A. C. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).