Purification and Properties of Apple Fruit Malic Enzyme[1]

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Summary. Malic enzyme was isolated and purified from mature apple fruits (*Malus sylvestris*, Miller) by utilizing procedures probably applicable to other soluble enzymes in this and similar tissues.

The physical properties of apple fruit malic enzyme are similar to those reported for malic enzyme from other plant and animal sources. It is specific for L-malate, TPN and requires a divalent cation for activity. In contrast to the pigeon liver enzyme, supplemental TPN is not required for oxalacetic decarboxylase activity of the fruit enzyme. The pH optimum of the malic enzyme varied with the L-malate concentration and the nature of the divalent cation present. D-Malate activated the oxidation of L-malate at rate-limiting concentrations.

Apple fruits probably contain most, if not all of the photosynthetic and respiratory enzymes considered ubiquitous in higher plants. This has been indicated from studies with intact apple fruits, tissue slices and mitochondrial preparations. However, there are only a few reported instances where the specific enzymes associated with interconversions of major metabolites have been identified, notably those of Harley et al. (4) for amylase and Dilley (2, 3) and Hulme and Wooltorton (6) for malic enzyme. The combination of high acidity, low protein and high polyphenol content of apples which has caused difficulty in the isolation of active mitochondria from apple fruit, has also hindered enzyme studies. A procedure for the isolation and purification of malic enzyme from apple fruit is described here. This procedure circumvents many of the problems inherent in the use of apple, and probably other fruit tissue, for enzyme studies. Consequently this procedure may prove generally useful in the study of enzyme changes during fruit ontogeny.

Materials and Methods

Plant Materials and Reagents. Mature apple fruits, *Malus sylvestris*, (Miller), of several cultivars used in this investigation were obtained from the University orchards at East Lansing and maintained in refrigerated storage at 0 to 1°. TPN was obtained from Nutritional Biochemical Corporation; L-malic acid from California Biochemical Corporation; diethyldithiocarbamic acid (DIECA) from Eastman Organic Chemicals and polyethylene glycol-4000 (PEG-4000) from Union Carbide Chemical Company. Other reagents employed were of analytical grade.

Tissue Preparation. Apple fruits were peeled, cored, and sliced on an apple peeling apparatus and the cortical tissue was diced into 1 cm wedges. The diced tissue was rinsed briefly with 1×10^{-3} M $NaHSO_3$ to retard oxidation of the cut surface. The tissue was submerged in 2 times its weight of a 0.2 M K_2CO_3 solution containing 5×10^{-3} M DIECA and vacuum infiltrated. All steps in the purification procedure were performed at 0 to 2°.

Enzyme Extraction and Purification. The infiltrated tissue (0.1–1 kg) was rinsed briefly and disintegrated by blending at 0° for 2 minutes in the presence of an equal weight of a 5×10^{-3} M solution of DIECA, pH 7. The suspension was filtered with suction or pressed through a milk filter and the residue was discarded. The pH of the filtrate was generally between 9.5 and 10.5.

Mg^{++} Treatment. When the enzyme was obtained from ripe fruits, it was necessary to precipitate gel forming substances, presumably pectic acids, prior to further purification. The filtrate was adjusted to pH 9.5 and sufficient magnesium acetate was added to obtain a concentration of 0.01 M. The solution was centrifuged at $10,000 \times g$ for 15 minutes and the resulting pellet discarded. When the Mg^{++} treatment was not employed, the original filtrate was adjusted to pH 9.5 and centrifuged.

PEG-4000 Fractionation. The supernatant solution was adjusted to pH 5.5 with 5 N acetic acid and PEG-4000 was added at the rate of 0.4 g per ml of solution. The pH was readjusted to 5.5 and the solution was stirred for 15 minutes. Following centrifugation at $10,000 \times g$ for 15 minutes, the pellet was suspended in 0.05 M glycylglycine, pH 7, (2 ml

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per 10 g initial weight of tissue) and clarified by centrifugation at 15,000 × *g* for 15 minutes. The resulting solution contained a high percentage of initial malic enzyme activity and could be stored at 0° or frozen at minus 15° for several weeks with no appreciable loss in activity.

Calcium Phosphate Gel Adsorption. The glycylglycine extract of the PEG-4000 precipitate was adjusted to pH 5.5 with 2 M acetic acid and calcium phosphate gel was added at the rate of 0.3 mg of gel solids per mg protein present and stirred for 15 minutes. The suspension was centrifuged at 10,000 × *g* for 5 minutes. The pellet was rinsed by suspending in 0.1 M KCl (0.5 ml per 10 g initial weight of tissue) and centrifuged at 10,000 × *g* for 5 minutes. The enzyme was eluted from the gel with 0.1 M potassium phosphate, pH 7 (0.25 ml per g initial weight of tissue).

Polyacrylamide Gel Electrophoresis. The calcium phosphate gel eluate was concentrated by dialysis against a saturated solution of PEG-4000 to an approximate concentration of 1 mg of protein per ml. One-tenth ml of the concentrate was applied per gel column (7.5% acrylamide) prepared according to Ornstein and Davis (12). Electrophoresis was conducted at 2 milliamps per column (0.5 cm diameter) at room temperature. The gel columns were stained with Amido Schwarz (Naphthol Blue Black) and subsequently destained electrolytically in 7.5% acetic acid. Companion gel columns (not stained) were sectioned immediately into 1 mm discs and the proteins eluted with 0.05 M glycylglycine and assayed to ascertain the location of malic enzyme on the stained gel columns. An alternate procedure of slicing the gel column lengthwise and staining one-half while assaying the corresponding half was used to verify the location of the enzyme.

Enzyme Assay. Malic enzyme activity was determined by the spectrophotometric procedure employed by Ochoa (11). The assay conditions are contained in the legends for the appropriate figures. A change of 0.01 OD unit per minute at 340 mμ was considered 1 malic enzyme unit. OD values were converted to μmoles TPNH formed per minute employing 6.22×10^6 as the extinction coefficient. Oxalacetic decarboxylase activity was determined at 25° according to Ochoa (11) with a unit of activity being that producing 1.0 μl of CO_2 in 10 minutes.

Protein Determination. Protein content was determined by Kjeldahl digestion of trichloroacetic acid precipitates at purification stages prior to calcium phosphate. Protein content of the calcium phosphate gel eluate was estimated spectrophotometrically (8) by the procedure of Warburg and Christian. The method of Lowry et al. (9) or UV absorption procedures (8) were not applicable prior to elution of protein from calcium phosphate because of interference from phenolic contaminants present. Protein solutions containing PEG-4000 could not be determined by methods based on UV absorption, Folin-Ciceaulteau, or biuret reagents.

Results

Enzyme Purification. A summary of the purification of apple fruit malic enzyme is given in table I. Total malic enzyme activity recovered from a given lot of fruit tissue was dependent upon the extracting medium (table II). The pH of the extracting medium was less critical than the composition of the medium. Thus, extracts obtained when the tissue was blended in distilled water resulted in a homogenate pH of 3.5 yet the enzyme recovery was only slightly less than that obtained with K_2CO_3, NH_4OH and Tris that yielded homogenate pH values of 10.2, 9.1 and 8.7 respectively. The lowest enzyme recoveries were obtained with phosphate in the extracting medium which gave intermediate pH values. The recoveries were essentially the same in phosphate extracts of pH 7.2 and 6.4 suggesting phosphate rather than pH as the factor responsible for the low recoveries.

Table I. *Summary of Purification of Apple Fruit Malic Enzyme*

The enzyme was prepared from one kg of cortical tissue of Northern Spy apples after 6 months air storage at 0°. The enzyme assay consisted of Tris-glycylglycinate, pH 7.3, 300 μmoles; $MnSO_4$, 3.6 μmoles; TPN, 0.24 μmole; malate, 9 μmoles, and enzyme in a total volume of 3.0 ml at 20°.

Stage	Yield of protein mg	Specific activity Units/mg protein	Total enzyme yield Units
Crude extract*	496	141	70,000
Mg^{++} treatment*	287	230	66,000
PEG fractionation	130	524	68,000
$Ca_3(PO_4)_2$ eluate	12.8	3140	40,000

* Enzyme activity and protein estimation was obtained following dialysis.

Table II. *Influence of pH and Extracting Medium on the Recovery of Malic Enzyme from Apple Fruit*

Extracting medium	pH of Medium	pH of Homogenate	Relative activity*
0.2 M Potassium carbonate	11.8	10.2	100
0.2 M Ammonium hydroxide	10.6	9.1	96
0.2 M Tris	10.2	8.7	89
0.2 M Potassium phosphate	9.1	7.2	23
0.2 M Potassium phosphate	6.8	6.4	22
H_2O	7.0	3.5	84

* Activity relative to that obtained with the 0.2 M K_2CO_3 extraction. Activity determinations were made after fractionation with PEG-4000 (see text) with assay stated in table I.

Some purification with very little loss in total enzyme activity was obtained by treating the initial extract with 0.01 M Mg^{++} at pH 9.5. However, the primary benefit derived from the addition of Mg^{++} was the precipitation of an interferring substance (presumably pectic acid) that coprecipitated with the enzyme forming an unmanageable gel during PEG-4000 fractionation. Enzyme activity was sharply reduced at a Mg^{++} concentration of 0.01 M when the precipitation was performed at pH values below 9.0 (table III). Enzyme activity was also greatly reduced at pH 9.0 with a Mg^{++} concentration of 0.05 M. The slight reduction in enzyme recovery at pH 9.0 with 0.02 M Mg^{++} indicates the critical nature of Mg^{++} concentration. Extracts obtained from fruit late in the storage season required 0.02 M Mg^{++} to effectively precipitate the gel forming substance. This may be a consequence of the increase in soluble pectic substances which occurs during fruit ripening. The necessity of including the Mg^{++} step can be judged from the quantity of gel formed during the PEG-4000 fractionation step.

Table III. *Influence of pH and Mg^{++} Molarity on the Recovery of Malic Enzyme from Apple Fruit*

Magnesium acetate was added to aliquots of the crude filtrate of a McIntosh apple fruit homogenate to obtain the Mg^{++} concentrations at the pH indicated. The pH of the filtrate was adjusted before and readjusted following adding the magnesium acetate.

pH	Mg^{++} molarity*	Relative activity*
9.5	0.01	100
9.0	0.01	100
7.0	0.01	25
5.0	0.01	5
9.0	0.02	90
9.0	0.05	0

* Enzyme activity was determined after PEG-4000 fractionation and is expressed relative to that obtained with 0.01 M Mg^{++} at pH 9.5. Assay conditions as stated in table I.

Malic enzyme was precipitated with 40 % (w/v) PEG-4000 over a wide pH range (table IV). Enzyme recovery was improved at progressively lower pH values between 9.0 and 6.0. Precipitation at pH 6.0 yielded the most enzyme, however, more inert protein accompanied it than at higher pH levels as evidenced by a lower specific activity. Fractional precipitation of malic enzyme with PEG-4000 at pH 8.0 in 10 % (w/v) increments through 40 % (w/v) yielded fractions with higher specific activities than was obtained with a single 40 % (w/v) fraction. However, enzyme recoveries in the individual fractions were not sufficiently high to warrant the use of increment fractionation.

Adsorption of malic enzyme on calcium phosphate gel at pH 5.5 and elution at pH 7.0 resulted in approximately a 6-fold purification with respect to the PEG-4000 step and recovery of 60 to 80 % of the activity. Fractional adsorption on calcium phosphate gel resulted in fractions with a higher purification but lower recoveries. Attempts to employ calcium phosphate purification prior to PEG-4000 fractionation were not successful.

Table IV. *Influence of pH During PEG-4000 Precipitation on Malic Enzyme Recovery from Apple Fruit*

PEG-4000 was added at the rate of 0.4 g/ml solution to an aliquot of McIntosh apple extract after Mg^{++} treatment. The pH was adjusted to the indicated value before and after the addition of PEG-4000. Assay conditions as stated in table I.

pH	Relative enzyme activity	Relative specific activity
9.0	100	100
8.0	108	98
7.0	120	88
6.0	132	78

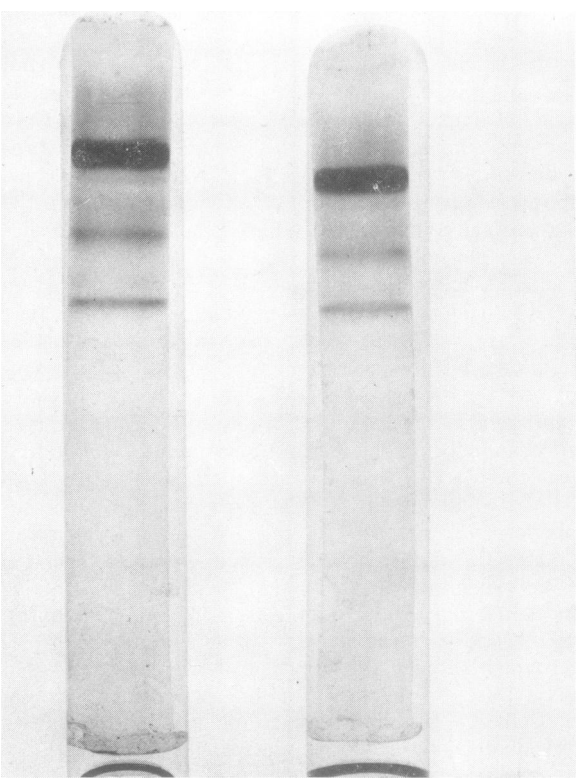


FIG. 1. Polyacrylamide gel electrophoretic pattern of proteins present in the $Ca_3(PO_4)_2$ gel eluate employed to characterize the properties of apple fruit malic enzyme. Approximately 40 μg of protein (specific activity of malic enzyme = 3100) was applied at the cathode end (top of photograph) of each column. Malic enzyme activity was confined to the dark band at the upper end of the column (see text).

Acrylamide gel electrophoresis of the enzyme preparation after elution from calcium phosphate revealed the presence of 6 distinct protein bands (fig 1). Malic enzyme was found to be the dominant protein. Electrophoresis of the soluble proteins present in a crude extract from the fruit after concentration by gel infiltration with Sephadex G-25 also revealed a dominant band with mobility similar to that of the purified malic enzyme.

Optimum pH. The effect of pH upon the activity of apple fruit malic enzyme is illustrated in figure 2. Maximum activity was observed between

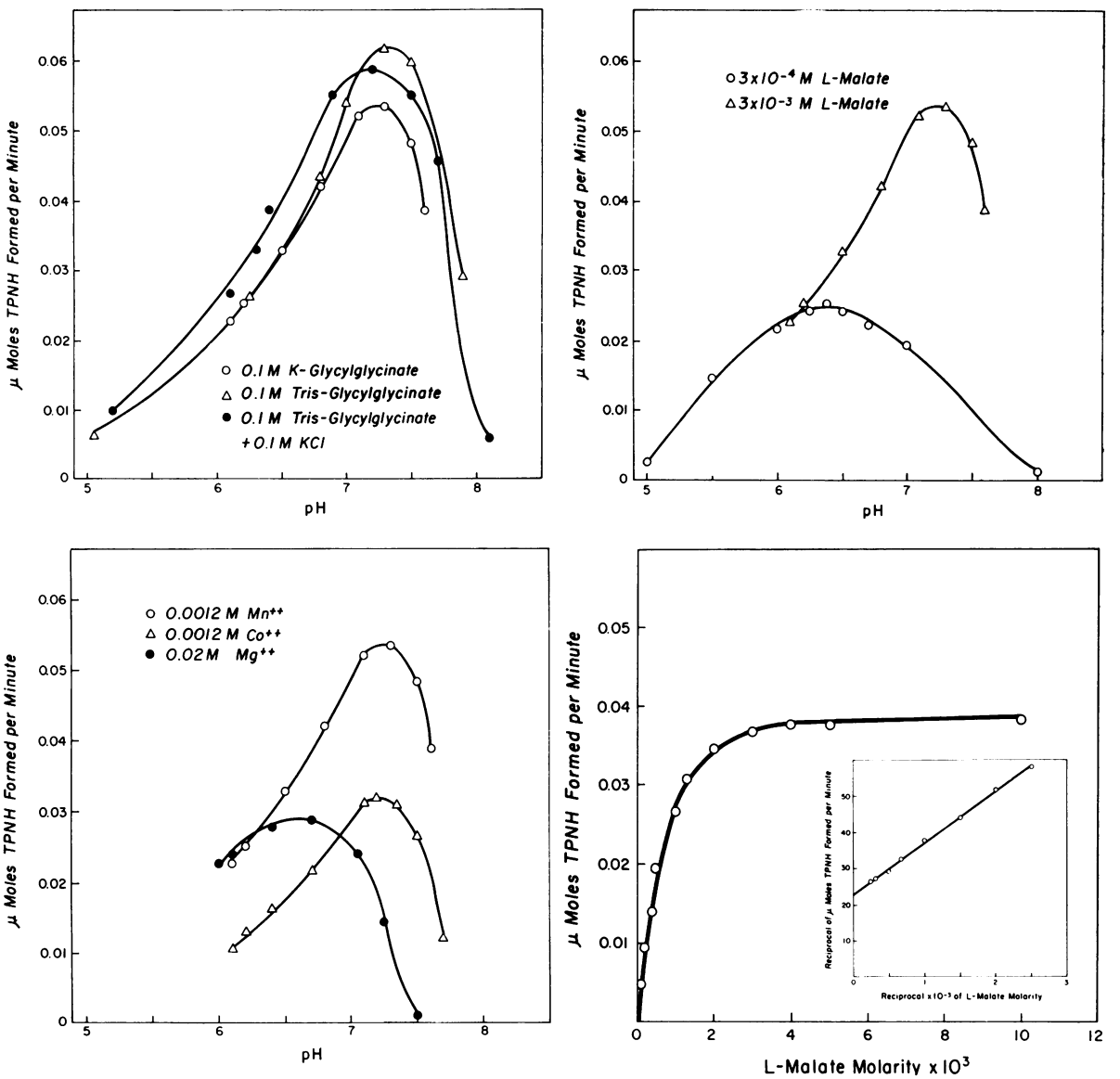


FIG. 2. pH Optimum of apple fruit malic enzyme in buffers of differing ionic strength. The assay consisted of L-malate, 9 μmoles; $MnSO_4$, 3.6 μmoles; TPN, 0.48 μmole; buffer, 300 μmoles at pH indicated; and enzyme in a total volume of 3.0 ml at 20°. (*Upper left.*)

FIG. 3. Influence of L-malate concentration on pH optimum of apple fruit malic enzyme. The assay conditions as in figure 2 employing Tris-glycylglycinate buffer with the indicated final L-malate concentration. (*Upper right.*)

FIG. 4. Influence of pH on the efficacy of divalent cation activation of apple fruit malic enzyme. The assay conditions as in figure 2 employing Tris-glycylglycinate buffer with indicated final concentration of Mn^{++}, Co^{++} and Mg^{++}.

FIG. 5. Effect of L-malate concentration on the activity of apple fruit malic enzyme. The assay consisted of K-glycylglycinate, pH 6.4, 75 μmoles; $MnSO_4$, 0.18 μmole; TPN, 0.24 μmole; enzyme, and the indicated concentration of L-malate in a final volume of 3.0 ml. (Inset) Reciprocal plot of reaction velocity versus malate concentration.

pH 7.1 and 7.3. The pH optimum was not affected by the ionic strength of the assay medium as indicated by the similarity in activity among the 3 buffer systems employed. Slightly higher activities were obtained with Tris-glycylglycinate in the presence or absence of 0.1 M KCl as compared to K-glycylglycinate in the region of the optimum pH.

The pH optimum was dependent upon the concentration of L-malate in the assay medium (fig 3) as reported by Rutter and Lardy (13). At 3×10^{-4} M L-malate the pH optimum was 6.3 to 6.5 and rather broad while at 3×10^{-3} M L-malate maximum activity was observed at pH 7.2 and activity fell off sharply on either side of the optimum. The optimum pH was 7.2 when Mn^{++} or Co^{++} was the activating cation but was shifted downward approximately 0.6 of a pH unit when Mg^{++} was employed to satisfy the divalent cation requirement (fig 4). The cation comparisons were performed in the presence of 3×10^{-3} M L-malate. The pH-activity curve obtained with Mg^{++} had a less clearly defined optimum than that obtained with Mn^{++} or Co^{++}. The broad pH optimum with the Mg^{++} assay may be related to the similar and broad pH optimum observed for Mn^{++} with the low malate concentration (10^{-4} M) assay (fig 3).

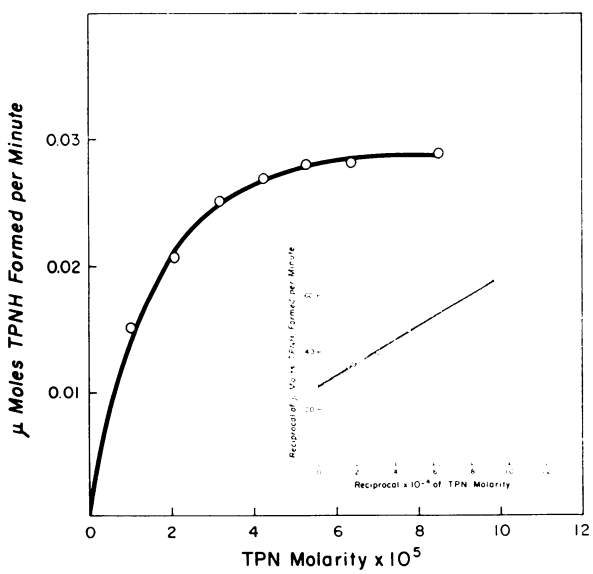
Effect of Substrate Concentration. The effect of L-malate concentration on enzyme activity is shown in figure 5. The Michealis constant (K_m) (see insert in fig 5) for L-malate was 5.5×10^{-4} at pH 6.4 and 20°. The K_m values reported for L-malate for the enzyme from wheat germ was 7×10^{-4} M at pH 7.3 (11), while for the pigeon liver enzyme the K_m at pH 7.5 was 3.0×10^{-4} M (13). The K_m for L-malate varied with pH of the assay medium in the same manner as reported by Rutter and Lardy (13). The effect of TPN and Mn^{++} concentration on enzyme activity is shown in figures 6 and 7. The concentration of TPN and Mn^{++} at one-half maximal reaction velocity under the assay conditions employed was 1.6×10^{-5} M and 9.2×10^{-5} M, respectively, which are of the same order of magnitude as those reported for the enzyme from other sources (11).

Co-factor Requirements. The enzyme required the presence of a divalent metallic cation for oxidation of L-malate. The relative effectiveness of the cations employed was $Mn^{++} > Co^{++} >> Mg^{++}$. The pH of the assay medium had a pronounced effect on the efficacy of Mg^{++} to activate the reaction as shown

Table V. *Oxalacetic Decarboxylase Activity of Apple Fruit Malic Enzyme*

Three separate malic enzyme preparations were assayed manometrically for oxalacetic decarboxylase activity. The enzyme was obtained from Delicious fruits and was assayed following PEG-4000 fractionation. The complete reaction mixture contained: 0.1 M acetate, 10^{-3} M $MnSO_4$, 5×10^{-5} M TPN, 0.33 mg per ml bovine serum albumin, 10^{-2} M oxalacetate and 100 units of malic enzyme. The pH and temperature were 5.1 and 25°, respectively.

Reaction mixture	Preparation I	II	III	Avg
		μl CO_2/10 min		
Complete	53	43	44	47
− TPN	54	49	46	50
− Enzyme	10	3	4	6
− Mn^{++}	9	2	3	4



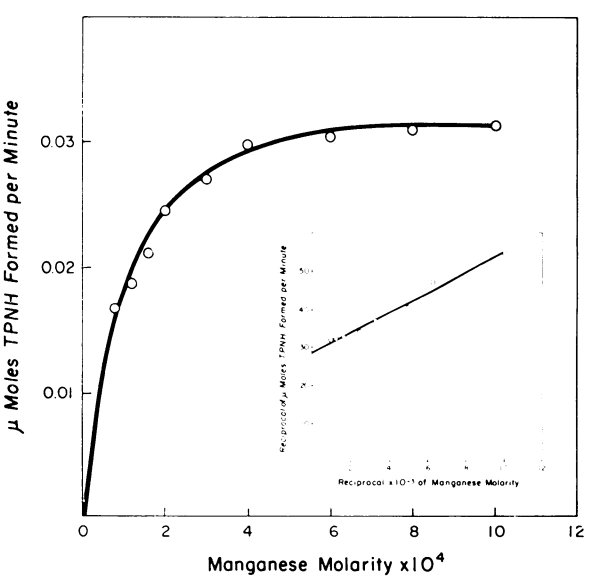


FIG. 6. Effect of TPN concentration of apple fruit malic enzyme activity. The assay conditions as in figure 5 employing 3×10^{-3} M L-malate and varying the TPN concentration.

FIG. 7. Effect of Mn^{++} concentration on the activity of apple fruit malic enzyme. The assay conditions as in figure 5 varying Mn^{++} concentration.

in figure 4. At pH 7.5, 0.02 M Mg^{++} was completely ineffective whereas Mn^{++} and Co^{++} at a much lower concentration (0.0012 M) adequately satisfied the cation requirement.

Substrate Specificity. L-Malate was the only dicarboxylic acid tested which was oxidized by the enzyme. However, the enzyme decarboxylated oxalacetate (table V) in the presence of a divalent cation as has been reported previously (11). Whereas, TPN greatly stimulated oxalacetic decarboxylase of the pigeon liver enzyme (13) it did not significantly affect the fruit enzyme activity. Dihydroxymaleate and dihydroxytartarate (tetrahydroxysuccinate) at 1×10^{-3} M were potent inhibitors of the malic enzyme while malonate, maleate and meso-tartarate were less inhibitory at the same concentration. Dihydroxymaleate was a competitive inhibitor. The nature of the inhibition by the other acids was not determined. D-Malate was not a substrate for the enzyme; however, at 1×10^{-3} M in the presence of rate-limiting concentrations of L-malate, it activated the oxidation of L-malate (fig 8). The activation decreased as the

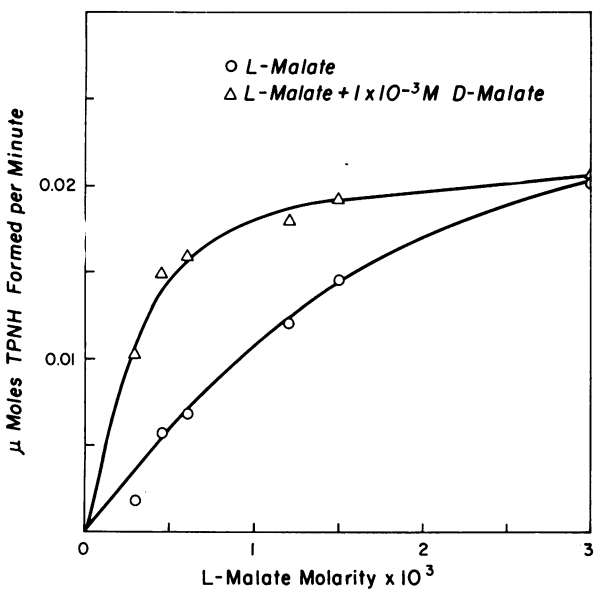


FIG. 8. Effect of D-malate on the activation of apple fruit malic enzyme. The assay consisted of K-glycylglycinate, pH 6.4, 75 μmoles; $MnSO_4$, 0.18 μmole; TPN, 0.24 μmole; enzyme, and the indicated concentration of L-malate in the presence and absence of 3 μmoles of D-malate in a total volume of 3.0 ml at 20°.

concentration of L-malate reached the saturation level. Activation by D-malate was similar at pH 6.4 and pH 7.3 and required the presence of a divalent cation. Activation of this reaction by D-malate differs from that recently reported by Billiar, et al. (1) wherein D-malate was found to promote the synthesis or activation of L-malate dehydrogenase while it inhibited its activity in vitro. Studies of the D-malate enhancement of L-malate oxidation are being continued.

Discussion

The procedure described for the isolation and purification of malic enzyme from apple fruit tissue overcomes difficulties encountered with this and similar tissue. High cell sap acidity per se is not a major obstacle in the extraction of active malic enzyme but functions indirectly by favoring oxidation of polyphenols which in turn inactivate the enzyme (5). Amylase inactivation was encountered by Harley et al. (4) in apple fruits. They suggested tannins as a possible factor responsible for the inactivation. Incorporation of a polyphenolase inhibitor during tissue extraction and its presence in crude extracts is essential to prevent enzyme inactivation. An alternative procedure developed by Jones and Hulme (7) and Hulme and Jones (5) for preparing mitochondria from apple fruits is based on the complexing of polyphenols with polyvinylpyrrolidone and thus prevent phenol oxidation.

Attempts to purify apple fruit malic enzyme by ammonium sulfate or ethanol fractionation were inferior to the PEG-4000 and $Ca_3(PO_4)_2$ procedure employed. The residual PEG-4000 present in the suspended pellet following PEG-4000 precipitation formed an aqueous polymer 2-phase system with ammonium sulfate which resulted in the accumulation of inactive protein at the interface.

Quantitative studies of malic enzyme are currently under way to determine the relationship between the synthesis of this enzyme and the marked increase in malic acid decarboxylation capacity of apple fruit observed during the respiratory climacteric (2, 10).

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