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ACTIVITY OF PEAR FRUIT MALIC ENZYME; ITS REGULATION BY METABOLITES

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Key Word Index—*Pyrus communis*; Rosaceae; pear fruit; malic enzyme; allosteric properties; maturation.

Abstract—Sigmoid kinetics are reported for the pear malic enzyme (L-malate NADP oxidoreductase, EC 1.1.1.40). Responses have been obtained from possible allosteric effectors. The physiological significance of these responses to metabolites is discussed in relation to a regulatory role of this enzyme in maturation.

INTRODUCTION

NADP malic enzyme probably plays an important role in the maturation of pear fruit. Its activity rises to a maximum at the climacteric maximum and this increase is accompanied by a synthesis *de novo* of the enzyme [1–3]. Allosteric properties of malic enzyme have been described with enzyme extracted from various plants [4–6] and Davies and Patil [7] studied metabolite regulation with extracts from potato tuber. Our purpose, here, was to study the kinetic properties of pear fruit malic enzyme and to see if a metabolite regulation may have a function in maturation.

RESULTS

Requirement for a bivalent cation

The enzyme does not show any activity in the absence of a divalent cation. At pH 7.5, with saturating concentrations of Mg^{2+} and Mn^{2+}, the activity with Mn^{2+} is 3 times that with Mg^{2+}. The activity decreases with higher concentrations of Mg^{2+}, while it stays at a maximum rate with the same concentration of Mn^{2+}. The affinity of the enzyme for Mn^{2+} is much greater than for Mg^{2+}; the concentrations producing half maximum velocity are 16 μM and 1 mM respectively and the saturating concentrations of Mn^{2+} and Mg^{2+} are 1 mM and 5 mM respectively.

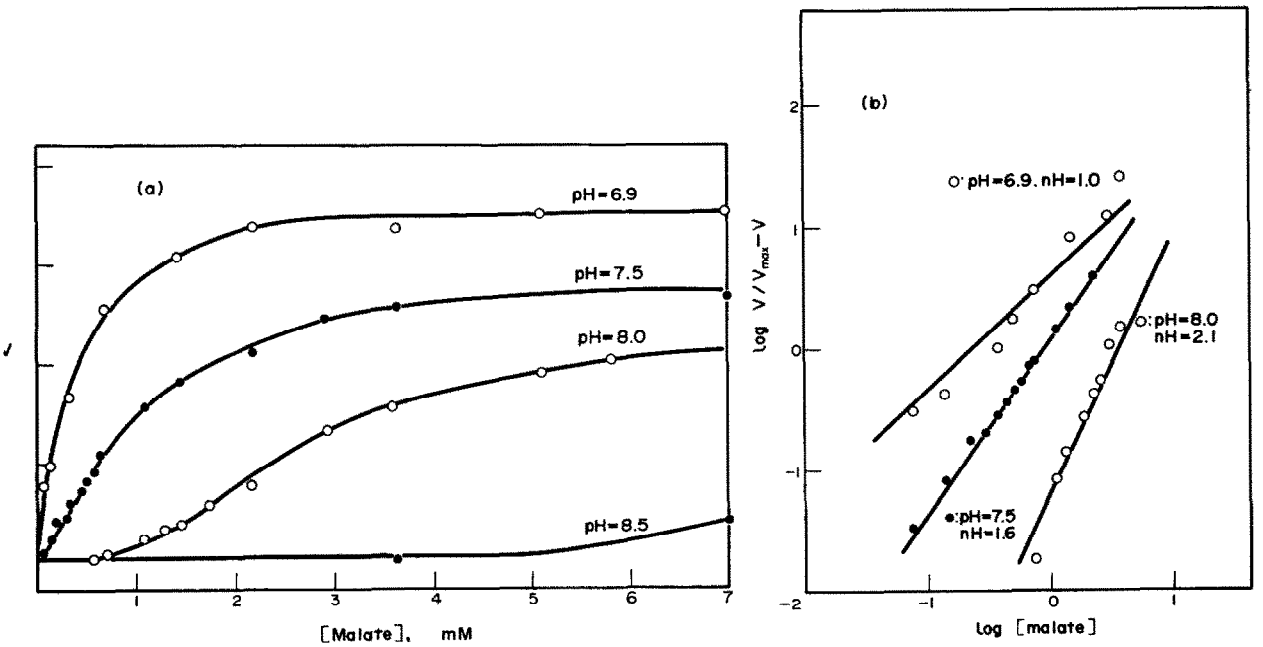

Fig. 1. Effect of pH and malate concentration. (a) Direct plot of rate versus malate concentration. (b) Hill plot. Enzyme activity is determined as described in the text except that malate concentration and pH vary as indicated.

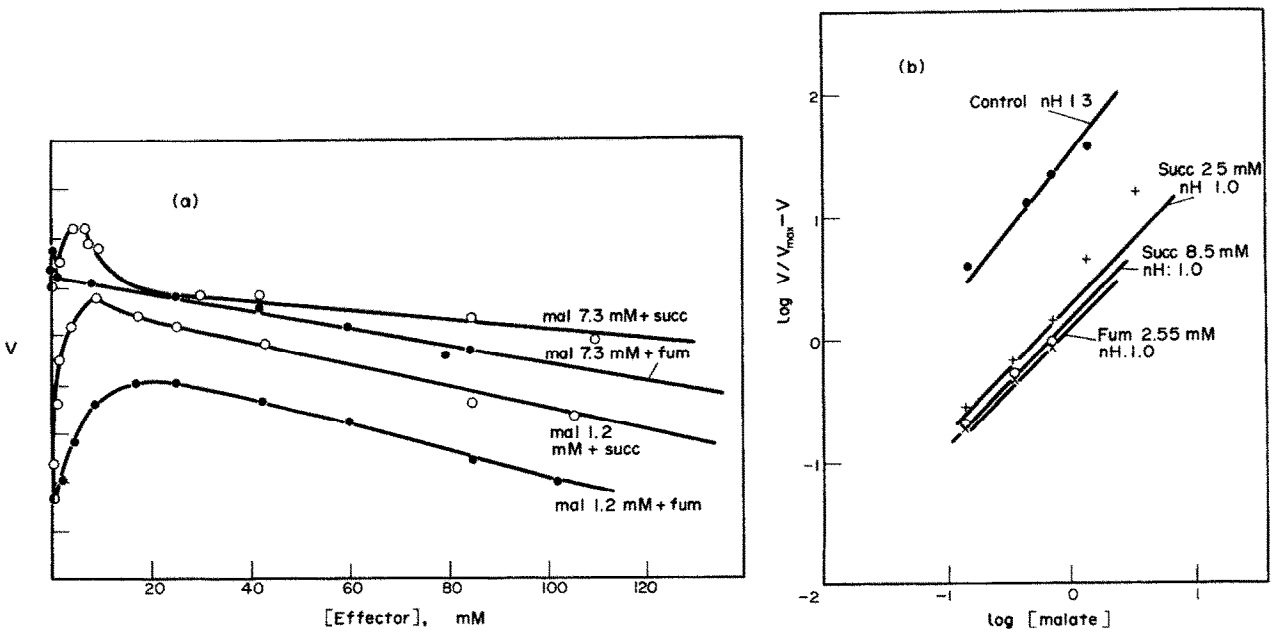

Fig. 2. Effect of succinate and fumarate. (a) Enzyme activity is assayed as described in the text except that the malate concentration is 7.3 mM or 1.2 mM. ○: sucinnate; ●: fumarate. (b) Hill plot. ●: control (no succinate); +: succinate 2.5 mM. ○: succinate 8.5 mM; ×: fumarate 2.55 mM.

Effect of NADP concentration

The plot of rate versus NADP concentration shows normal Michaelis–Menten kinetics. Under the conditions of the standard assay the apparent K_m is 16 μM for NADP.

Effect of malate concentration

The plot of rate versus malate concentration approximates to normal Michaelis–Menten kinetics at pH values below 7. Under the conditions of the standard assay at pH 6.9, the apparent K_m for malate is 0.45 mM. At pH values above 7 the enzyme exhibits sigmoid kinetics. When the results are plotted in the form of the Hill equation, the coefficient (*nH*) is 1 at pH 6.9 but is greater than 2 at pH values above 7.5.

Effect of NADPH concentration

The rate of decarboxylation up to $E_{340} = 0.8$ is linear. When the assay mixture contains NADPH (0.13 mM), there is no detectable inhibition.

Activators of malate decarboxylation

(a) *dicarboxylic acids* (Fig. 2). Two dicarboxylic acids, succinate and fumarate, which gave no reaction in the absence of malate, activated the enzyme when assayed at pH 7.5. There is an optimum concentration at which these dicarboxylic acids stimulate malate decarboxylation. In both cases, high concentrations produce a pronounced inhibition. These dicarboxylic acids eliminate the sigmoidicity in the plot of rate versus malate concentration. The Hill coefficient (*nH*) is now 1 instead of 1.3. The percentage stimulation produced by the dicarboxylic acids is greatest when the malate concentration is low and when the acid concentration is high. At high concentrations of malate the dicarboxylic acids behave as inhibitors. (b) *Ethyl alcohol.* Alcohol stimulates malate decarboxylation at low malate concentrations but gives no reaction at high malate concentrations.

Inhibitors of malate decarboxylation

(c) *Na oxalate* (Fig. 3). At pH values below 7, oxalate inhibits the enzyme, changing the approximately Michaelis–Menten kinetics into sigmoid kinetics. The Hill coefficient varies from 1.2 to 1.8. At pH values above 7.2 where the plot of rate versus malate concentration is clearly sigmoid, oxalate inhibits the enzyme and increases sigmoidicity. The Hill coefficient does not vary significantly: its value is near 1.6. (d) *Other organic acids* (Fig. 4). As previously noted a number of organic acids activate the enzyme when present in low concentration. However, a number of compounds (pyruvate, α-ketoglutarate, citrate, Na-oxaloacetate) inhibit the enzyme at all concentrations tested (0 to 120 mM). These dicarboxylic acids increase the sigmoidicity in the plot of rate versus malate concentration. The same concentration of citrate and oxaloacetate involves an equal percentage inhibition of the enzyme activity. The addition of these inhibitors changes the Hill coefficient significantly from 1.4 to 1.2 for α-ketoglutarate, 2 for pyruvate and 2.1 for citrate.

DISCUSSION

A prerequisite for the study of the detailed kinetics of

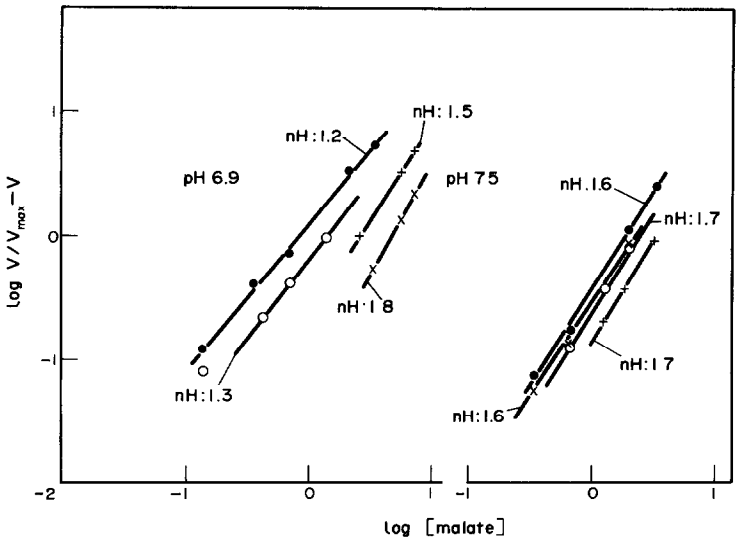

Fig. 3. Effect of Na oxalate; Hill plot. ●: Na oxalate 0 mM; ○: Na oxalate 0.08 mM. ×: Na oxalate 0.4 mM; +: Na oxalate 0.8 mM. Enzyme activity assay as described in the text except that the malate concentration varies and that different oxalate concentrations are tested. The pH is 6.9 or 7.5.

the activity of plant enzymes is the development of suitable extraction procedures which retain the full allosteric properties of the protein. We have developed methods for the isolation of malic enzyme in crude extracts from climacteric pears which are stable for up to 7 days at 0° with the full retention of the allosteric properties. We have similarly prepared extracts from pre-climacteric fruit which although having lower catalytic activity have similar allosteric properties to those of preparations from climacteric fruit.

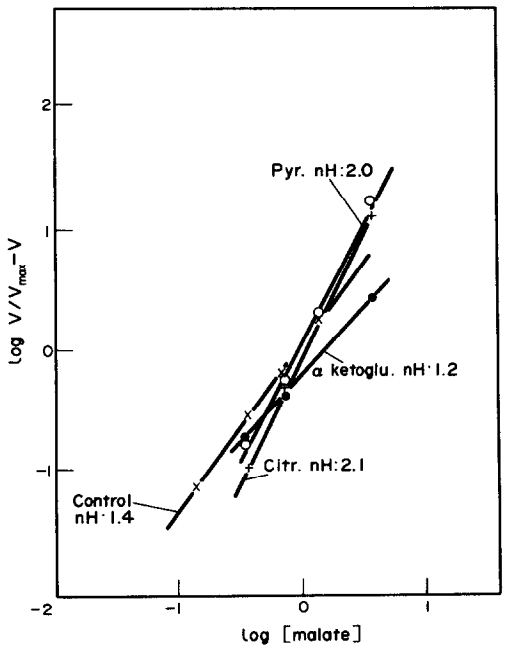

Fig. 4. Effect of some effectors; Hill plot. ×: control; ○: pyruvate; ●: α-ketoglutarate. +: citrate and Na oxaloacetate. Enzyme activity assay as described in the text with and without inhibitors and varying malate concentration. The pH is 7.5.

The possible physiological significance of the effects of metabolites on malic enzyme activity are summarised in Fig. 5. Since the concentration of malate decreases during maturation in the pear, the effects of Kreb's cycle intermediates on malic enzyme activity were studied at a range of malate concentrations. OAA, citrate, isocitrate and α-ketoglutarate, which are products of malate in the Kreb's cycle, inhibit malic enzyme at all the malate and effector concentrations used. Succinate and fumarate, precursors of malate activate the enzyme at low concentrations of substrate and effector while at high concentrations of substrate succinate and fumarate act as inhibitors. Pyruvate, the precursor of oxalacetate, and oxalate inhibit malic enzyme at all concentrations tested. In general, it is clear that high concentrations of Kreb's

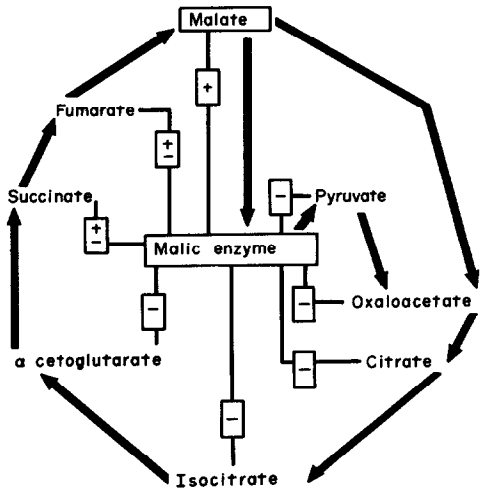

Fig. 5. Effect of various effectors on the activity of malic enzyme. +: activator; −: inhibitor; ±: activator or inhibitor as a function of malate or effector concentrations.

cycle intermediates inhibit malic enzyme with the exception of succinate and fumarate which activate the enzyme at low concentrations.

The physiological role of malic enzyme in fruit ripening is unclear although it has been implicated as a component of the malate decarboxylating system [8] which develops during the ripening of some fruits. It has been suggested that malic enzyme may provide an important source of NADPH for synthetic processes during ripening when other sources of NADPH such as photosynthesis and the pentose phosphate pathway are not fully active.

EXPERIMENTAL

Pears (*Pyrus malus*, cv Passe-Crassane) harvested at the beginning of November, stored at 0° during 12 weeks, ripened subsequently at 15° and extracted at the climacteric maximum.

Extraction. Peel tissues were frozen in liquid N_2 and lyophilised. Extraction was performed on 2 g of pulverized material by homogenizing in 12.5 ml of cold extraction soln (Tris 0.5 M pH 7.5; Dieca 0.01 M; mercaptoethanol 0.011 M. PEG 3%. 1 g of Polyclar and $CaCl_2$ 0.1 M were added just before use). The extract was obtained after a centrifugation (30 min, 15 000 *g*).

Enzyme assay. The standard assay was carried out at pH 7.5 by measuring the increase in A_{340} associated with $NADP^+$ reduction. The assay mixture contained Tris buffer (pH 7.5 0.08 M) $MnSO_4$ (1 mM); $NADP^+$ (0.33 mM) and Na malate (7.3 mM) in a vol. of 3 ml. The reaction was started by the addition of 0.02 ml of enzyme. Under these conditions, the relation between reaction rate and enzyme concentration was linear. Assays were carried out at 25° in a recording spectrophotometer.

1. Hartmann, C. J. R. (1975) *Colloques Internationaux C.N.R.S.* **238**, 235.
2. Hartmann, C. J. R., Boulay, M. and Moulet, A. M. (1975) *C.R. Acad. Sci. Paris* **281**, 135.
3. Boulay, M. (1975) *Thèse de 3ème Cycle*, Université d'Orléans.
4. Frenkel, R. (1972) *Biochem. Biophys. Res. Commun.* **47**, 931.
5. Dilley, D. R. (1966) *Plant Physiol.* **41**, 214.
6. Davies, D. P., Nascimento, K. M. and Patil, K. D. (1974) *Phytochemistry* **13**, 2417.
7. Davies, D. P. and Patil, K. D. (1974) *Biochem. J.* **137**, 45.
8. Hulme, A. C. and Rhodes, M. J. C. (1971) *Pome Fruits* in *The Biochemistry of Fruits and the Products* (Hulme, A. C. ed.) **2**, p. 303. Academic Press, London.