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NAD MALIC ENZYME FROM CITRUS FRUIT*

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Key Word Index—*Citrus sinensis*; *Citrus unshiu*; Rutaceae; NAD malic enzyme; purification; fruit development.

Abstract—NAD malic enzyme activity was found in the 15000 *g* precipitate of citrus leaf and fruit tissues. The enzyme activity in juice vesicle tissue did not change during the fruit growing period, but doubled following ripening. Partially purified enzyme was activated by CoA or FDP. Affinity for malate changed depending on enzyme concentration. The dependency was lost by addition of tricarboxylic acids but not dicarboxylic acids.

INTRODUCTION

Fruit development is a complex process and is divided into several stages distinct in metabolic properties and growth rate [1]. Among these metabolic properties, acid metabolism is the most interesting for the horticultural sciences. The amount of organic acids per fruit generally decreases accompanied by a decrease in juice acidity during fruit ripening. In climacteric fruit, this ripening process coincides with an increase in respiratory activity. A steep rise in activity of NADP malic enzyme (EC 1.1.1.40) was observed during ripening of apple, cherry and pear fruits [2–4]. Complete oxidation of organic acids to CO_2 and H_2O requires an additional supply of acetyl CoA. Pyruvate formation by malic enzyme is thought to provide acetyl CoA in some plant tissues [5–7].

Citrus fruits are thought to be a non-climacteric type. In these fruits increase in NADP malic enzyme activity was not so prominent during ripening. However, activities of malate dehydrogenase (MDH) (EC 1.1.1.37) and citrate synthetase (EC 4.1.3.7) increased prominently [1]. This suggests that enzymes other than NADP malic enzyme play an important role in decreasing acid during ripening of citrus fruits.

NAD malic enzyme (EC 1.1.1.39) is reported from cauliflower buds, tubers of Jerusalem artichoke, roots of sweet potato and leaves of C-4 photosynthetic plants (NAD malic enzyme type) [7–10]. From malate oxidation of avocado fruit, it is suggested that this enzyme also exists in the fruit tissue [11].

In this paper we report the presence of NAD malic enzyme in citrus fruit and its seasonal change in activity, and also some properties of the partially purified enzyme.

RESULTS AND DISCUSSION

Localization of NAD malic enzyme activity and its seasonal change

In both peel and juice vesicle tissues of satsuma mandarin, most activity of NAD malic enzyme was found in a particulate fraction (Table 1). It may be localized in mitochondria. In *Atriplex* and Jerusalem artichoke, the enzyme was reported in the mitochondrial fraction [7, 12, 13]. In leaf tissue, relatively high activity in the supernatant fraction will be due to damage of mitochondria during homogenization. Almost all of the NADP malic enzyme activity was in the supernatant

Table 1. Malic enzyme activity in mature fruit and leaf tissue of of satsuma mandarin (Sugiyama Unshiu)

Tissue	Fraction	Malic enzyme activity NAD-dependent	NADP-dependent
		(10^{-3} × units/g fr. wt)	
Juice vesicle	15000 *g* ppt	17.7	3.09
	supernatant	1.78	97.6
Peel	15000 *g* ppt	21.0	16.6
	supernatant	6.44	216
Leaf	15000 *g* ppt	145	24.0
	supernatant	353	1250

Both activities were assayed at pH 7.5. NAD dependent enzyme was assayed in the presence of 40 μM CoA.

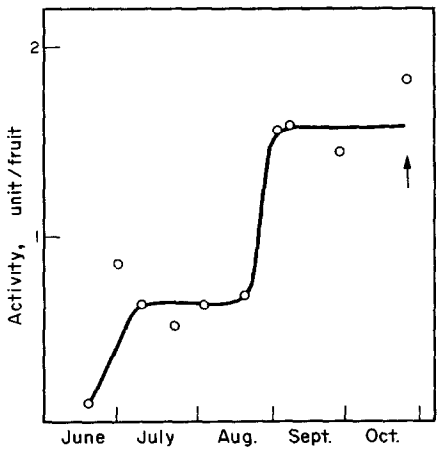

Fig. 1. Seasonal change in activity of NAD malic enzyme in juice vesicle tissue during development of satsuma mandarin (Miyagawa Wase). Arrow indicates ripening time.

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Table 2. Purification of NAD malic enzyme from citrus fruit (Fukuhara orange)

Fraction	Volume (ml)	Protein (mg)	Enzyme activity: NAD malic (10^{-3} × units)	NAD malic (units/g protein)	MDH (units)	NADP malic (10^{-3} × units)
Digitonin solubilized	192	187	25400	136	2610	2690
40–80% $(NH_4)_2SO_4$	16·2	36.7	8740	241	675	207
DE-52	16.0	1.14	3500	3070	2.31	15.1

fraction. Although intracellular localization of NADP malic enzyme from other fruit is not clear, the enzyme from other plant tissues was normally localized in the cytosol fraction [14].

Activity of NAD malic enzyme in juice vesicle tissue per fruit remained almost unchanged from the end of June to mid-August (Fig. 1). Since the cell number of the tissue was constant during this period [1], the results suggest that the activity per cell is constant. The activity of the enzyme per fruit doubled during ripening (Fig. 1). This period coincided with the time of decreasing citrate content [1].

Purification of NAD malic enzyme

Juice vesicle tissue (0.7–1.2 kg) of mature Fukuhara orange was homogenized with the same weight of medium containing 0.2 M Tris–HCl buffer (pH 7.5), 0.5 M sucrose, 3 mM $MnCl_2$, 1 mM dithiothreitol (DTT) and the calculated amount of pH-adjusting medium. After straining through nylon cloth, the filtrate was centrifuged at 15000 *g* for 20 min, the resulting pellet was suspended in a medium composed of 0.2 M Tris–HCl buffer (pH 7.5), 1 mM $MnCl_2$ and 1 mM DTT, then mixed with digitonin solution in a final concentration of 0.5%. The mixture was centrifuged at 15000 *g* for 15 min. The extraction from pellet was repeated 3 times and the combined supernatant was designated as digitonin solubilized fraction. This fraction was adjusted to pH 6.5 and the protein fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 40–80% saturation was dissolved in 10 to 20 ml of medium containing 0.02 M Tris–HCl buffer (pH 7.3), 1 mM DTT, 1 mM $MnCl_2$ and 1 mM malate (column medium). The dissolved protein was passed through a Sephadex G-25 column equilibrated with column medium. The resulting eluate was loaded on a DE-52 column (170 × 14 mm) which had been equilibrated with column medium. The enzyme was eluted with a 100 ml linear gradient of 0.05 to 0.2 M KCl in column medium; flow rate was 16.8 ml per hr. The active fraction of the eluate showed a single peak and the fraction was concentrated in a collodion bag (designated as DE-52 fraction). All these procedures were carried out at 4°. The procedure is summarized in Table 2.

Properties of partially purified NAD malic enzyme

Stoichiometry of the enzyme reaction was checked using the DE-52 fraction as enzyme. Pyruvate was assayed enzymatically after 4 min [15]. The ratio of the products (pyruvate/NADH) was from 1 to 0.99. When malic enzyme activity was measured at pH 7.5 with NADP and Mg, it was very low in the DE-52 fraction (Table 2), so contamination of NADP malic enzyme was assumed to be negligible. MDH activity measured by NADH oxidation was relatively high, but activity toward the reverse reaction was almost negligible in our assay system. Our system was therefore considered to be applicable to the assay of NAD malic enzyme.

In the presence of 40 μM of CoA and 4 mM of $MnCl_2$, the optimum pH of the NAD malic enzyme was 6.9 using MES–KOH and HEPES–KOH buffers. Optima for the enzymes from cauliflower and Jerusalem artichoke were reported to be between 6.7 and 6.9 in the absence of CoA [6, 7]. For the enzyme from C-4 photosynthetic plant, the optimum was higher than that of citrus enzyme in the presence of CoA [16].

In the absence of effectors, such as FDP or CoA, the saturation curve for malate was sigmoidal and the sigmoidicity was dependent on the enzyme concentration. Higher enzyme concentrations gave higher affinity for malate and lowered interaction coefficient ($n = 3$ for 10 μl enzyme per reaction mixture, $n = 1.3$ for 40 μl enzyme) (Fig. 2a). Even in the presence of FDP or CoA, the affinity was dependent on enzyme concentration. When 40 μl of enzyme were used, the saturation curve for malate was normalized by addition of these activators, and the $s_{(0.5)}$ value for malate was 0.83 or 1.86 mM in the presence of FDP or CoA respectively (Fig. 2b and 2c). On the other hand, the effect of citrate on the reaction was different from the effects of FDP and CoA. In the presence of citrate, malate concentration giving half-maximum velocity was 6.6 mM and the value appeared to be independent of enzyme concentration. Dependency of *n*-value on enzyme concentration was rather small

Table 3. Effect of acids in TCA cycle on NAD malic enzyme

Addition (10 mM)	V_{max} [10^{-3} × units (% of control)]		K_m [$s_{(0.5)}$] (mM)	Remarks	
None	5.40	(100)	[8.5]	A	S
Citrate	3.70	(68)	[6.15]	B	S
Isocitrate	6.24	(115)	5.88	B	M
cis-Aconitate	7.40	(137)	4.16	B	M
Oxalacetate	3.34	(62)	[5.7]	A	S
2-Oxoglutarate	4.54	(84)	6.06	A	M
Succinate	4.76	(88)	3.17	A	M
Fumarate	5.88	(109)	2.02	A	M

Reaction mixture was same as the standard mixture except for addition of malate and acids. Each mixture contained 5.24 μg protein of DE-52 fraction. Both V_{max} and K_m were determined from double reciprocal plots. A: K_m value was variable depending on enzyme concentration. B: K_m value was independent on enzyme concentration. S: Malate saturation curve showed sigmoidal. M: The curve was a Michaelis–Menten type.

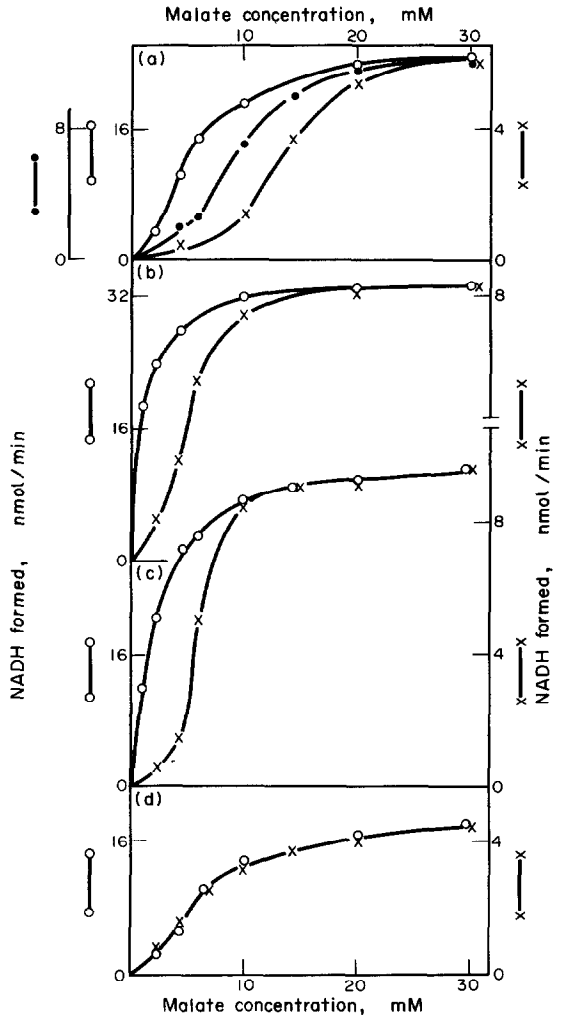

Fig. 2. Effect of activators and enzyme concentration on malate saturation curve of NAD malic enzyme. Reaction mixtures were the same as the standard mixture except for malate concentrations varied and existence of activators. DE-52 fraction (115 μg protein/ml) was used as enzyme. A, no addition; B, FDP 500 μM; C, CoA 40 μM; D, citrate 10 mM. —○— activity with 40 μl of enzyme; —●— activity with 20 μl of enzyme; —×— activity with 10 μl of enzyme. To compare saturation curves, activities with 20 and 40 μl of enzyme were plotted on the scales reduced twice and 4 times respectively.

(n = 1.9 for 10 μl enzyme, 1.4 for 40 μl enzyme) (Fig. 2d).

These findings led us to examine further the effect of acids of the TCA cycle (Table 3). In the presence of aconitate or isocitrate, K_m values were independent of enzyme concentration, as in the case of citrate. Aconitate was the most effective activator in lowering K_m value and increasing V_{max}. However, in the presence of dicarboxylic acids such as succinate, oxalacctate, fumarate of 2-oxoglutarate, the K_m value for malate changed, depending on enzyme concentration.

NAD malic enzyme from higher plant tissues

The role of NAD malic enzyme in C-4 photosynthetic leaves is thought to be the release of CO_2 for refixation via the Calvin cycle in bundle sheath cells. On the other hand, in C-3 plants this enzyme is assumed to control mitochondrial pH or to oxidize malate for energy or carbon source [7, 17]. In fruit tissue, NAD malic enzyme has not been reported.

The sigmoidal nature of the malate saturation curve of NAD malic enzyme is common to the enzymes from both C-3 and C-4 photosynthetic plants. Activation effects of CoA in lowering K_m and increasing V_{max} are also observed on the enzymes from plant tissues [16, 17]. K_m values for Mn and NAD reported here (Table 4) were similar to those of enzymes from cauliflower and Jerusalem artichoke. But K_m values for Mg and NADP were several times higher than those of enzymes from these plants [6, 7, 17]. For the enzymes from C-4 plants, Mg was not effective and the activity was lost rapidly in air [9]. These phenomena were, however, not observed in the case of the citrus enzyme. We found that affinity for malate depended on enzyme concentration, but this dependency was not found in NAD malic enzyme previously reported. These comparisons suggest that the NAD malic enzyme reported here is a different type of enzyme from either Jerusalem artichoke, cauliflower or C-4 photosynthetic plants.

During ripening of pear, cherry and apple, the activity of NADP malic enzyme increased considerably [2–4]. Therefore, this enzyme is assumed to be necessary in ripening and climacteric respiration of these fruits. On the other hand, Lance *et al.* reported pyruvate formation during malate oxidation in avocado mitochondria, which was dependent on NAD [11]. It suggests that NAD malic enzyme acts as a respiratory enzyme in ripening of the fruit. Ripening behaviour of citrus fruits is quite different from that of apple, pear and cherry fruit. The main acid in citrus fruits is citrate and decrease of acid during ripening is slow and continues for a prolonged period. NADP malic enzyme activity increased only 20% during ripening of citrus fruit [1], but activity of NAD malic enzyme increased *ca* two fold during the period. Since this enzyme activity was dependent on enzyme concentration, as shown in Fig. 2, it is probable that the increase in enzyme levels observed during the ripening results in an increase in malate decarboxylation *in vivo*. Sugar accumulation during maturation of citrus fruit suggests inactivation of glycolysis and in consequence the accumulation of free CoA. It seems likely that the CoA accelerates malate decarboxylation *in vivo*. Our results therefore

Table 4. Kinetic constants of NAD malic enzyme for Fukuhara orange fruit

Requirement	V_{max} (10^{-3} × units)	K_m (mM)
Mg^{2+}	4.40	5.00
Mn^{2+}	4.00	0.065
NAD	5.00	0.67
NADP	2.86	3.45

The reaction mixture was same as the standard mixture except for the addition of the requirements indicated in the table. Each reaction mixture contained 11.4 μg of protein of DE-52 fraction. Both V_{max} and K_m were determined from double reciprocal plots.

suggest that the NAD malic enzyme acts as a pyruvate supplier in decreasing citrate of citrus fruits during ripening.

EXPERIMENTAL

Fukuhara orange, a variety of sweet orange (*Citrus sinensis* Osbeck) and Sugiyama Unshiu and Miyagawa Wase, 2 varieties of satsuma mandarin (*Citrus unshiu* Marcovich) were used. In 1977, fruit was harvested from leaf shoots of mature trees for the study of seasonal change in enzyme activity. In the fruit, anthesis was from May 11 to 13 and ripening time was the end of October.

Homogenization and fractionation of tissues. Tissues were ground with 2–4 ml per g tissue wt of homogenizing medium composed of 0.05 M HEPES–KOH buffer (pH 7.5), 0.5 M sucrose, 3 mM $MnCl_2$ and 1 mM DTT using a mortar and pestle with sea sand. In the case of juice vesicle tissue, the pH of the homogenate was adjusted to 7.5 by adding N NaOH containing 0.5 M sucrose (pH adjusting medium). The homogenate was centrifuged at 15000 *g* for 15 min. Enzyme in the pellet was solubilized by addition of Triton X-100 at a final concn of 0.1%.

Assay of enzymes and protein. Before the assay, low MW substances were removed from each enzyme prepn by passing through a column of Sephadex G-25. For NAD malic enzyme, a standard reaction mixture contained 40 mM HEPES–KOH buffer (pH 6.9), 20 mM malate, 2 mM NAD, 4 mM $MnCl_2$, 1 mM DTT and enzyme in a total vol of 0.5 ml. The mixture was incubated at 30°, and the increase in A at 340 nm was recorded. In the case of crude extracts, A increased rapidly in the first 1 or 2 min from endogeneous MDH. Then MDH activity was inhibited by the oxalacetate produced. The remaining linear increase rate was measured as activity of NAD malic enzyme. Using the purified enzyme after DE-52 column chromatography, the initial rate was used as the enzyme activity. MDH activity was measured as described in ref. [18]. For NADP malic enzyme, the reaction mixture was composed of 40 mM HEPES–KOH buffer (pH 7.5), 10 mM malate, 2 mM NADP, 4 mM $MgCl_2$, 1 mM DTT and enzyme in a total vol of 0.5 ml. Increase in A at 340 nm was measured. For each enzyme, one unit of activity is defined as the activity that catalyses 1 μmol of substrate per min. Protein content was assayed by the method of ref. [19].

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