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EVIDENCE FOR A CYTOSOLIC NADP-MALIC ENZYME IN TOMATO

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; leaf; NADP-malic enzyme; chloroplast; antibodies; cDNA.

Abstract—The similarity and cellular location of NADP$^+$-malic enzyme (NADP-ME, EC 1.1.1.40) in developing fruit and other parts of the tomato (*Lycopersicon esculentum* Mill.) plant were investigated in order to clarify the role of the enzyme in metabolism. There appeared to be a single enzyme (native MW 260 kD, subunit MW 66 kD) in mature green tomato fruit; this was purified to a specific activity of 42.5 μmol mg^{-1} min^{-1} and apparent homogeneity on SDS-PAGE. Polyclonal antibodies raised against this protein achieved 90% precipitation of enzyme activity, but required purification in order to detect specifically the 66 kD protein on Western blots. The purified antibodies recognized a similar protein in tomato leaves, roots and stems. The specific activity of NADP-ME was at least nine times higher in supernatant than in chloroplast fractions in tomato fruit and leaf, consistent with the distribution of a cytoplasmic marker (alcohol dehydrogenase), but in contrast to a chloroplast marker (rubisco). The purified NADP-ME antibodies did not detect a 66 kD protein in chloroplast preparations. A partial NADP-ME cDNA isolated from a fruit library specifically hybridized with a 2.1 kb transcript in RNA preparations from tomato leaves and fruits at different stages of development. The existence of a minor, possibly chloroplastidic NADP-ME in tomato cannot be excluded; the major NADP-ME is a cytosolic protein which is present in all plant organs analysed.

INTRODUCTION

Tomatoes, like many fleshy fruits, accumulate malic and other organic acids during development. Such fruits frequently contain NADP$^+$-malic enzyme (NADP-ME; oxaloacetate-decarboxylating, EC 1.1.1.40) and this is assumed to be involved in the metabolism of malate, which occurs on ripening [1]. The ripening-related increase in activity of NADP-ME in certain fruits is consistent with this role [1], but in tomato (*Lycopersicon esculentum*) the specific activity of NADP-ME increased during fruit growth to a maximum at the mature green stage and then declined during ripening [2]. The detection of activity throughout the tomato plant raised further doubt about a specific role in fruit. El Shora and ap Rees [3] postulated that NADP-ME is involved in fatty acid biosynthesis or amino acid metabolism in plastids of C_3 plants; they showed that the enzyme was present in chloroplasts isolated from *Cucurbita* cotyledons and *Glycine* suspension cultures and argued that it is detected in the supernatant because of plastic breakage during homogenization. Chloroplastidic NADP-ME is well characterized from certain C_4 plants, notably *Zea mays* [4]. However, *Zea* is also known to contain a cytosolic NADP-ME, from which

understanding of the role of NADP-ME in tomato (and C_3 plants in general) and is a major focus of this paper. The possible occurrence of multiple forms of the enzyme needs to be considered in relation to this question and the hypothetical role of a specific enzyme in fruit or during fruit ripening. Assays of enzyme activity can be influenced by several factors and cannot completely resolve these questions. The presence of NADP-ME protein and the occurrence of multiple forms was tested immunologically. A cDNA clone for tomato NADP-ME was used in hybridization analyses to test for consistency between mRNA abundance and enzyme activity.

RESULTS AND DISCUSSION

Enzyme purification and antibody preparation

The purification scheme (Table 1) for tomato NADP-ME yielded 5.4% of the activity of the initial homogenate at a specific activity which was an order of magnitude higher than previously reported for tomato [6], but similar to that found for the potato enzyme [7]. Since NAD-ME also reduces NADP$^+$ [8], the possibility that this enzyme was present in the purified

Table 1. Purification of NADP-malic enzyme from mature green tomato fruit pericarp

Purification step	Protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Yield (%)	Purification (fold)
Crude extract	195	103	0.53	100	1.0
$(NH_4)_2SO_4$ (30–55%)	40.0	43.1	1.08	41.9	2.0
DEAE-cellulose	6.54	22.3	3.40	21.6	6.4
Sephacryl S-300	4.54	18.9	4.17	18.4	7.9
Cibacron blue	0.13	5.53	42.5	5.4	80.3

isoenzymes in tomato and that other isoenzymes were lost during purification. There was some evidence of heterogeneity at the gel permeation and pseudo-affinity steps. Peak activity corresponded to a MW of 260 kD on the Sephacryl S-300 column, which agrees with earlier findings for this tetrameric protein [6]. This was followed by a shoulder at about 180 kD which could have resulted from partial dissociation of the protein. After elution of NADP-ME from the Cibacron blue column with NADP$^+$, further enzyme at lower specific activity could be eluted with 200 mM NaCl. On SDS electrophoresis followed by staining with Coomassie blue, both fractions were found to contain a protein with similar mobility to bovine serum albumin (66 kD), consistent with earlier findings [6]. No other bands were visible in the NADP$^+$ fraction whereas the NaCl fraction contained an additional protein.

Antibodies raised against the purified protein (NADP$^+$ fraction) were used to titrate the NADP-ME activity in a crude enzyme preparation. Approximately 50% of the initial activity remained in the supernatant after centrifugation of 0.5 μl of antiserum with 300 μl of enzyme. Further addition of antiserum caused nearly 90% loss of activity, but pre-immune antiserum had no effect (Fig. 1). This suggested that all of the NADP-ME in the crude extract was immunologically similar to the purified enzyme and is consistent with the presence of only one NADP-ME in the mature green fruit pericarp. However, Western blots of total fruit proteins with crude antiserum showed several bands, in addition to the 66 kD band, expected for the monomeric protein. The purified antibodies reacted with this band and weakly with a band at 74 kD, but did not cause immunoprecipitation of enzyme activity. It is thought that the purified antibodies recognized an epitope of the denatured protein (used in purification) which was not accessible in the native protein.

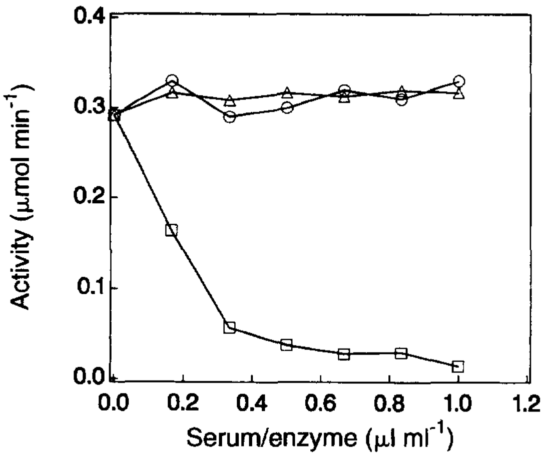


Fig. 1. Immunoprecipitation of NADP$^+$-malic enzyme in protein fraction from mature green tomato fruit pericarp. The enzyme extract was desalted on Sephadex G-25, incubated with serum at various dilutions for 12 hr at 2° and centrifuged

Localization of enzyme activity

The findings of El Shora and ap Rees [3] prompted us to consider the possibility that NADP-ME is a plastid enzyme in tomato. The experimental rationale required preparation of demonstrably intact chloroplasts and comparison of the partition of NADP-ME and chloroplast markers (GAPDH and rubisco) between chloroplast and supernatant. Since there is no evidence for association of NADP-ME with organelles other than chloroplasts, the absence of marker enzymes for other organelles (e.g. mitochondria) was not thought to be critical. The latency of NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity in osmotically buffered assay medium was used to assess intactness of chloroplasts [9]. All preparations showed some activity before addition of detergent, indicating that some lysis may have occurred during preparation; the increase of activity on addition of Triton X-100 indicates that intact chloroplasts constituted *ca* 60% of the preparation (Table 2). As expected, the specific activity of GAPDH associated with chloroplast preparations was at least 100 times higher than in the supernatant. Rubisco activities were generally lower than expected [10] and only three- to five-fold higher in the chloroplast than in the supernatant. It is likely that rubisco was assayed in its inactive state since the specific activity in tomato leaf chloroplasts was similar to that reported for rubisco-RUBP in the same material [11].

No attempt was made to separate bundle sheath and mesophyll tissues in corn and it is likely that chloroplasts from mesophyll tissue outnumbered bundle sheath chloroplasts (containing NADP-ME) in the final preparation. Nevertheless, the distribution of NADP-

Table 2. Specific activities (in μmol min^{-1} mg^{-1}) of NADP-malic enzyme, rubisco and NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in chloroplast (lysed with 0.1% Triton X-100 prior to protein and enzyme assays) and supernatant fractions of immature green tomato fruit pericarp, tomato leaves and corn leaves

	GAPDH		NADP-ME		Rubisco	
Tissue	Chloroplast	Supernatant	Chloroplast	Supernatant	Chloroplast	Supernatant
Tomato fruit	169 (68)*	0.49	16.6	156	2.5	1.1
Tomato leaf	97 (40)	0.54	0	17.5	27.0	4.9
Corn leaf	170 (65)	1.36	120	23.5	13.9	4.2
CV† (%)	11.2	43.1	32.0	18.7	29.6	20.0

*Assay before addition of detergent.

†Coefficient of variation for three independent preparations.

leaves, the specific activity of NADP-ME was at least nine times higher in the supernatant than in the chloroplasts (Table 2). Alcohol dehydrogenase was detected only in tomato fruit; activity in the supernatant was 212 nmol mg^{-1} min^{-1} and no activity was detected in chloroplasts. These data do not exclude the presence of NADP-ME in tomato chloroplasts, but it is unlikely that the enzyme detected in the supernatant was entirely derived from broken chloroplasts.

Antigenic localization of ME protein

The purified antibodies to tomato fruit NADP-ME detected a protein at similar MW (*ca* 66 kD) in total protein preparations from tomato fruit and leaf and in corn leaf (Fig. 2). A similar protein was also detected in tomato stem and root and in corn root (results not shown). This protein was not detected in any chloroplast preparation although a higher MW protein (*ca* 75 kD) was detected in tomato fruit chloroplasts (Fig. 2). The cross reaction with a component of total protein in corn, but not in corn chloroplasts, is consistent with the presence of another NADP-ME which is distinct from the chloroplastidic form and is thought to be cytosolic [12, 13]. If a chloroplastidic form exists in tomato it is distinct from the NADP-ME, which was purified from fruit; this enzyme appears to be cytosolic and a similar protein exists in other plant organs.

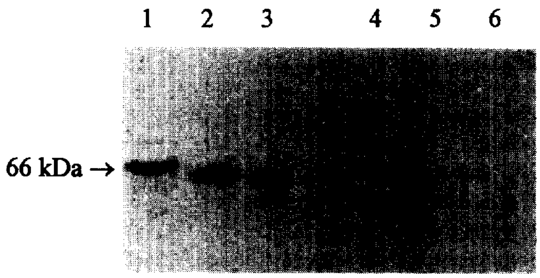


Fig. 2. Western blot analysis of NADP^{+}-malic enzyme in whole protein and chloroplast preparations (50 μg protein).

Expression of ME mRNA

Exhaustive screening of two libraries did not yield a full-length cDNA for NADP-ME. The 1.95 kb clone (Fig. 3) isolated from the λZAPII library contains a 1707 bp open reading frame which is 60 bp shorter than the full-length sequences from other C_3 plants, bean and poplar [14, 15]. The tomato sequence contains the expected dinucleotide binding site and is 75–80% homologous with corresponding regions of cDNAs from corn, *Flaveria trinerva*, *F. linearis* and poplar [4, 14, 16, 17]. The homology with bean is only 72% [15]. The 1.5 kb cDNA isolated from the λgy11 library represented the end of the larger cDNA 3′ from the first of two internal *Eco* RI sites.

Northern hybridization analysis with the tomato cDNA probe detected a transcript of similar size (*ca* 2.1 kb) in fruit at four stages of development and in tomato leaves (Fig. 4). Although a corn NADP-ME cDNA [4] was used in the isolation of the tomato cDNA, no transcript was detected in corn leaves. It is possible that the stringency of hybridization precluded detection. While it is possible that a distinct transcript for chloroplastidic NADP-ME in tomato could have escaped detection, the data are consistent with production of a similar mRNA in tomato fruit at different stages of development and in tomato leaf.

Conclusions

The evidence presented in this paper is for a single NADP-ME gene in tomato, which is expressed in fruit throughout development, and in leaves. Because the cDNA is incomplete at the 5′ end it is not known whether the sequence includes a region coding for a transit peptide, which would be required for chloroplast processing. However, such sequences do not occur in the NADP-ME cDNAs of other C_3 species [14, 15], contradicting the hypothesis that the enzyme is generally located in the chloroplast [3]. The enzymological and immunological data in this paper support a cytosolic location for the enzyme and the presence of a similar protein throughout the plant. This in turn suggests that

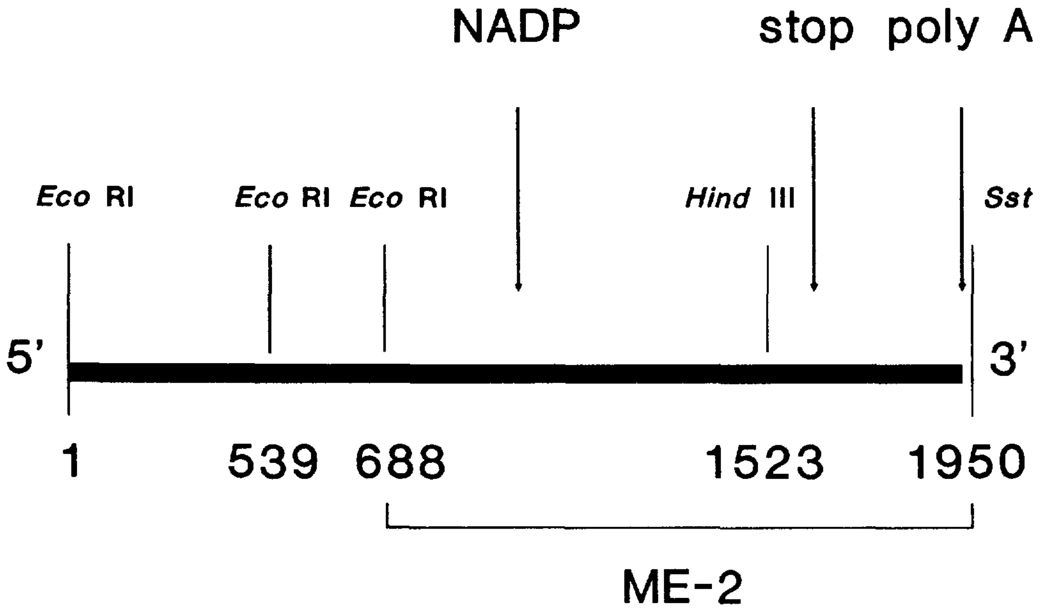


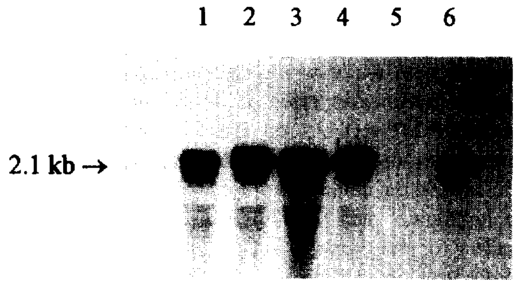
Fig. 3. Structure of cDNA clone for tomato fruit NADP$^+$-malic enzyme isolated from λZAPII library showing position of restriction sites, region coding for dinucleotide binding site (NADP), stop codon, region coding for poly A tail and the overlap with the clone isolated from a λgt11 library (ME-2).

in biosynthesis or metabolic shunts have been suggested [5]. The increase in specific activity during fruit growth could be related to a role in CO_2 generation for fruit photosynthesis [18]. None of these alternate roles precludes the possibility that the NADP-ME is responsible for the consumption of malate, which is associated with ripening [2].

EXPERIMENTAL

Plant material. Tomato (*L. esculentum* Mill., cv. Ohio 7814 [19]) and corn (*Zea mays* L. cv. Golden Bantam) were grown in a greenhouse at 20° (day) and 15° (night).

NADP-ME extraction and purification. NADP-ME extraction and purification was carried out at 0–2° according to published procedures [2, 6] except for the inclusion of a final pseudo-affinity purification step. Frs from the gel filtration column containing NADP-ME activity were pooled, concd and applied to a Cibacron-Blue 3GA agarose (Sigma) column (2 × 0.5 cm) equilibrated with 25 mM Tris–HCl, 1 mM mercaptoethanol and 1 mM $MnSO_4$ (pH 7.3). After elution of the unbound proteins in the same buffer, NADP-ME was eluted with buffer containing 5 mM NADP. Throughout the procedure, elution was monitored by absorbance at 280 nm and protein was assayed by dye binding [20].



Antibody production and purification. A male New Zealand White rabbit was immunized at 3-week intervals, twice with 50 μg purified NADP-ME in Freund's complete adjuvant and finally with 100 μg NADP-ME in Freund's incomplete adjuvant at the University Immunology lab. Serum was collected from the final bleed by centrifugation at 1500 *g* for 10 min after clotting for 24 hr at 0°. Antibodies were purified by their affinity for purified and denatured NADP-ME [21].

Chloroplast isolation. Leaf samples were homogenized in 10 vols 50 mM Tricine–KOH (pH 7.9), 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and 1% PVP-40T, and green fruit pericarp samples were homogenized in 5 vols of this 'grinding buffer' for 4 sec with a Polytron (Brinkman Instruments, Westbury, NY). Homogenates were filtered through 8 layers of muslin and the filtrate centrifuged for 3 min at 1500 *g*. Tomato leaf and fruit pellets were resuspended in resuspension buffer (grinding buffer without PVP) and applied to a centrifuged for 3 min at 1500 *g* and resuspended once more. Corn leaves were not sepd into bundle sheath and mesophyll prior to chloroplast prepn, but the crude chloroplasts were processed as described for bundle sheath chloroplasts [23]. The first supernatant from all prepns was centrifuged for 20 min at 20 000 *g* and applied to a Sephadex G-25 column (18 × 1.5 cm) equilibrated and eluted with 10 mM MOPS–KOH (pH 7.0), 0.5 mM $MnSO_4$, and 1 mM mercaptoethanol. The void fr. was collected for assay of cytosolic enzymes.

Enzyme assays. NADP-ME, NAD-malic enzyme (EC 1.1.1.39), alcohol dehydrogenase (EC 1.1.1.1) and rubisco (EC 4.1.1.39) were all assayed spectrophotometrically by published procedures [6, 24–26]. Chloroplast prepns were lysed with 0.1% Triton X-100 prior to these assays. The intactness of the original chloroplast prepns was assayed by the increase in activity of $NADP^+$-GAPDH (EC 1.1.1.8) on addition of 0.1% Triton X-100 to an assay mixt. containing 0.33 M sorbitol [9]. The protein content of enzyme prepns was estimated by dye binding [20].

Protein electrophoresis and immunological detection. 'Total proteins' were extracted from tissue samples with Me_2CO at −20° and resuspended in sample buffer at 100° [10]. An estimate of protein content was obtained [20] on parallel samples extracted in buffer without SDS or mercaptoethanol, so that constant amounts of protein could be loaded on to gels. Total protein and protein frs were sepd on 8% polyacrylamide gels [27]. Proteins were visualized by staining with Coomassie blue and by Western blotting. This involved transfer to nitrocellulose in a semi-dry transfer unit (Hoefer Instruments, San Francisco, CA) and excess sites were blocked with 1% BSA in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20. The filter was incubated with malic enzyme antibodies, purified as described above, for 1 hr. The bound antibodies were visualized with goat anti-rabbit IgG-alkaline phosphatase conjugate as directed by Promega (Madison, WI).

NADP-ME cDNA. A 1.3 kb *Eco* RI fragment of corn NADP-ME cDNA [4] was cloned in pBS(+) and used for random primer labelling with [^{32}P]dCTP. The product was used to screen a λgt11 cDNA library prepd from mature green 'VFN8' tomato (Clonetech, Palo Alto, CA), following standard procedures [28]. A 1.5 kb cDNA was isolated and sequenced by the dideoxy chain termination procedure [29]. This cDNA was used for Northern and Southern hybridization analysis and to screen a λZAPII cDNA library from ripe tomato (cv. Rutgers). This library yielded a 1.95 kb cDNA, which was sequenced and given the Genbank accession number L27509.

RNA hybridization. Various tomato tissues and corn leaves were frozen and ground in liquid N_2 prior to extraction of RNA with PhOH–$CHCl_3$ [30]. Samples of total RNA (20 μg, based on absorbance at 260 nm)

radiography [31, 32]. The probe was a fragment of *ca* 1.5 kb from the 3′ end of the tomato NADP-ME cDNA.

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