Plant Physiol. (1991) 97, 1381–1387
0032-0889/91/97/1381/07/$01.00/0

Received for publication March 5, 1991
Accepted July 24, 1991

Mitochondrial Malate Dehydrogenase from Corn[1]

Purification of Multiple Forms

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ABSTRACT

A method to fractionate corn (*Zea mays* L. B73) mitochondria into soluble proteins, high molecular weight soluble proteins, and membrane proteins was developed. These fractions were analyzed by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and assays of mitochondrial enzyme activities. The Krebs cycle enzymes were enriched in the soluble fraction. Malate dehydrogenase has been purified from the soluble fraction by a two-step fast protein liquid chromatography method. Six different malate dehydrogenase peaks were obtained from the Mono Q column. These peaks were individually purified using a Phenyl Superose column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified peaks showed that three of the isoenzymes consisted of different homodimers (I, III, VI) and three were different heterodimers (II, IV, V). Apparent molecular masses of the three different monomer subunits were 37, 38, and 39 kilodaltons. Nondenaturing gel analysis of the malate dehydrogenase peaks showed that each Mono Q peak contained a band of malate dehydrogenase activity with different mobility. These observations are consistent with three nuclear genes encoding corn mitochondrial malate dehydrogenase. Polyclonal antibodies raised against purified malate dehydrogenase were used to identify the gene products using Western blots of two-dimensional gels.

There is little information on the overall operation of the Krebs cycle in plant mitochondria, on its regulation, or its role during plant development (26). The information on most of the plant Krebs cycle enzymes is limited to the reports of activities in crude preparations with different enzymes being studied in different plants (3, 26). Little is known about their protein composition or genes. A few have been partially purified, and molecular studies have been carried out on malate dehydrogenase and citrate synthase with sequence data having been obtained (9, 23, 24).

It is our intention to look at the Krebs cycle in a single plant species. To achieve this, it is first necessary to undertake structural and functional characterization of the enzymes. We have developed a procedure to separate the mitochondrial proteins into physiologically relevant fractions. The soluble protein fraction of our procedure contains matrix proteins, including many of the Krebs cycle enzymes. We are developing a purification scheme that will make it possible to separate most of the Krebs cycle enzymes on a single column. We report the purification and characterization of one Krebs cycle enzyme, MDH.[2] Mitochondrial malate dehydrogenase has been previously purified from watermelon and the gene sequence obtained (9, 24, 25). However, in corn, genetic studies have indicated that there are three nuclear genes which code for the mitochondrial malate dehydrogenase (10, 11, 17, 18). The gene products and their interaction to produce the functional enzyme have not yet been characterized biochemically, and the genes have not been isolated or characterized. The purification and characterization of the different forms of MDH provides a starting point for these investigations and a linkage to the genetic information available in the literature (10, 11, 18).

MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemical Company. Seed corn, *Zea mays* inbred line B73, was obtained from the Nebraska Seed Foundation.

Mitochondrial Isolations

Mitochondria were isolated from 3-d-old, dark grown corn seedling shoots by the procedure of Day and Hanson (2), modified by using the centrifugation speeds of Schwitzguebel and Siegenthaler (20). The mitochondria were stored frozen at −80°C.

Fractionation of Mitochondria

Mitochondria were fractionated into membrane and soluble protein fractions. Mitochondria (50 mg) were diluted in Mops buffer (30 mM Mops, pH 8.0) to give a protein concentration of 1 mg/mL and then sonicated until the suspension cleared (about 20 s). The membranes were pelleted by centrifugation for 30 min at 100,000*g*. The supernatant containing the soluble protein fraction was centrifuged at 200,000*g* for 3 h to pellet large molecular weight complexes. The resulting supernatant which contained the soluble proteins

[1] This work was supported by the Center for Biotechnology, University of Nebraska-Lincoln.

[2] Abbreviations: MDH, NAD-malate dehydrogenase; FPLC, fast protein liquid chromatography.

was then concentrated to 4 mL using a Amicon Diaflo concentrator with a YM10 membrane, and stored at −80°C.

Purification of Malate Dehydrogenase

The concentrated soluble protein fraction was subject to ion exchange chromatography using a Mono Q column on a Pharmacia FPLC system. The soluble protein fraction (2 mL) was loaded onto a Mono Q column (anion exchange) that had been previously equilibrated with 50 mM NaCl in Mops buffer. The column was washed with 15 mL of this buffer, and then proteins were eluted with a linear gradient of 50 mM to 250 mM NaCl in Mops buffer. The malate dehydrogenase peaks were then made up to 3 M NaCl with solid NaCl and run separately over a Phenyl Superose (hydrophobic interaction) column. The column was washed with 10 mL of 3 M NaCl in Mops buffer. Malate dehydrogenase activities were eluted with a linear gradient of 3 M to 1 M NaCl in Mops buffer. Peaks of malate dehydrogenase activity were concentrated with an Amicon Diaflo concentrator with a YM10 membrane.

Enzyme Assays

All enzyme activities were measured at 25°C and the initial linear rates were measured. Unless indicated otherwise, the assay buffer consisted of 100 mM KH_2PO_4, pH 8.0. Malate dehydrogenase activity was assayed by measuring the change in absorbance at 340 nm. For oxaloacetate reduction the assay buffer contained 0.2 mM oxaloacetate and 0.2 mM NADH, and for malate oxidation the assay buffer contained 25 mM malate and 2 mM NAD. Cytochrome oxidase activity was measured as oxygen uptake in a Rank Brothers (Cambridge, UK) electrode using approximately 125 μg of mitochondrial protein in assay buffer containing 1 mM ascorbate, 250 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine and 50 μM cytochrome *c*. NADH dehydrogenase activity was measured at 600 nm using 200 μM NADH and 60 μM 2,6-dichlorophenolindophenol in assay buffer. Other enzyme activities were measured using the following methods: citrate synthase (22), fumarase (12), aconitase (6), pyruvate dehydrogenase (19), and succinate dehydrogenase (15).

Gel Electrophoresis

Native gels were done by the method of Ferl *et al.* (7). SDS-PAGE was carried out in the buffer system of Laemmli (13) with resolving gels formed from a 13 to 16% (w/v) acrylamide gradient. Silver staining was done by the procedure of Merril *et al.* (16). MDH activity staining was done by the method of Siciliano and Shaw (21). Two-dimensional PAGE was performed in a Bio-Rad 2D-Mini-Gel apparatus, according to the manufacturer's instructions with the following modification. The isoelectric focusing gels were prepared for the first dimension by adding the protein samples to the gel solution before the gels were poured, and no overlay was used.

Antibody production and Western blotting were carried out as described previously (5).

Protein Quantitation

Protein quantitation was by the Lowry method as modified by Larson *et al.* (14). For the column fractions, protein estimates were obtained by densitometric scanning of SDS-PAGE gels. Protein standards of 50 ng to 1 μg of BSA were run on the gel alongside the column fractions. A standard curve was obtained and the amount of protein in the column fractions was estimated from that curve.

RESULTS

Fractionation of Mitochondria

To facilitate our attempts to purify individual mitochondrial proteins it was first necessary to develop a method to fractionate mitochondria to yield physiologically relevant protein fractions. Details of the fractionation procedure are given in "Materials and Methods." Diluted mitochondria were disrupted by sonication and the membranes were then pelleted by ultracentrifugation. The supernatant was centrifuged again to pellet high mol wt protein complexes. The resultant supernatant was concentrated. This procedure yielded three protein fractions, a membrane fraction, a soluble high mol wt protein fraction, and a soluble protein fraction which should contain the matrix proteins. When the fractions were run on SDS-PAGE gels (Fig. 1) major differences in the protein composition of these fractions were seen.

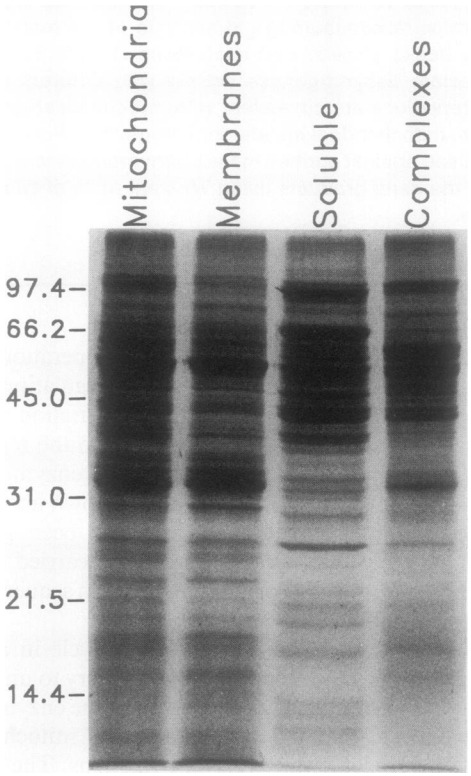

Figure 1. SDS-PAGE of mitochondrial fractions. Mitochondria were fractionated as described in "Materials and Methods." Fractions containing 30 μg of protein each were separated on 13 to 16% SDS-PAGE gels and proteins were visualized by Coomassie blue staining.

Table I. *Distribution of Enzyme Activities in Mitochondrial Fractions*

Enzyme	Specific Activity	Complex	Soluble	Membrane
	μmol/min/mg protein		*% of total*	
Matrix proteins				
MDH	28.3	9.1	75.3	15.6
Citrate synthase	0.597	19.3	55.3	25.4
Aconitase	0.528	6.9	56.9	36.2
Fumarase	0.300	ND[a]	ND	ND
Pyruvate dehydrogenase	0.030	100	0	0
Membrane proteins				
Succinate dehydrogenase	0.029	2.5	7.0	90.5
NADH dehydrogenase	1.82	7.3	41.3	51.4
Cyt oxidase	0.488	3.3	0	96.7
Total protein		8.1	18.8	73.1

[a] ND = not detectable.

The three fractions were assayed for matrix and membrane enzyme activities (Table I). Most of the activity of the matrix Krebs cycle enzymes was found in the soluble fraction. Pyruvate dehydrogenase was only detected in the high molecular weight complex fraction as anticipated. More than 90% of the activities of integral membrane proteins succinate dehydrogenase and cytochrome oxidase were found in the membrane fraction.

A relatively large proportion of the activity of matrix enzymes, MDH, citrate synthase, and aconitase was found associated with the membrane fraction. This may reflect the association of these enzymes with each other and the inner mitochondrial membrane. More harsh treatments, such as a low salt wash, could reduce the levels of these enzymes in the membrane fraction, but were not used because of the potential for removing extrinsic membrane proteins. NADH dehydrogenase activities were found almost evenly distributed between the membrane and the soluble protein fractions. This supports the view that some of these membrane associated dehydrogenases are easily removed as previously described (1, 3, 4).

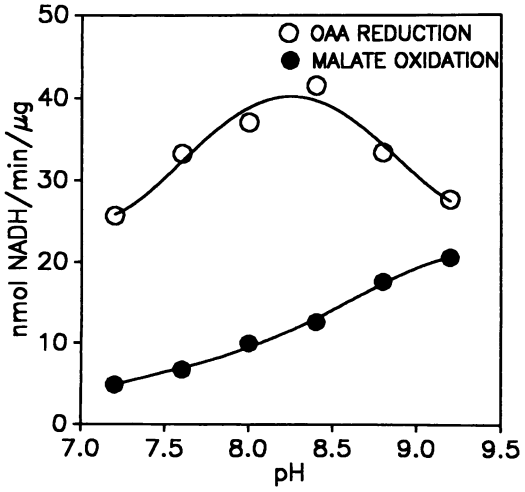

Figure 2. Effect of pH on the reactions of mitochondrial malate dehydrogenase. pH optima were obtained using a buffer consisting of 20 mM Mes, 20 mM Tes, and 20 mM Tricine.

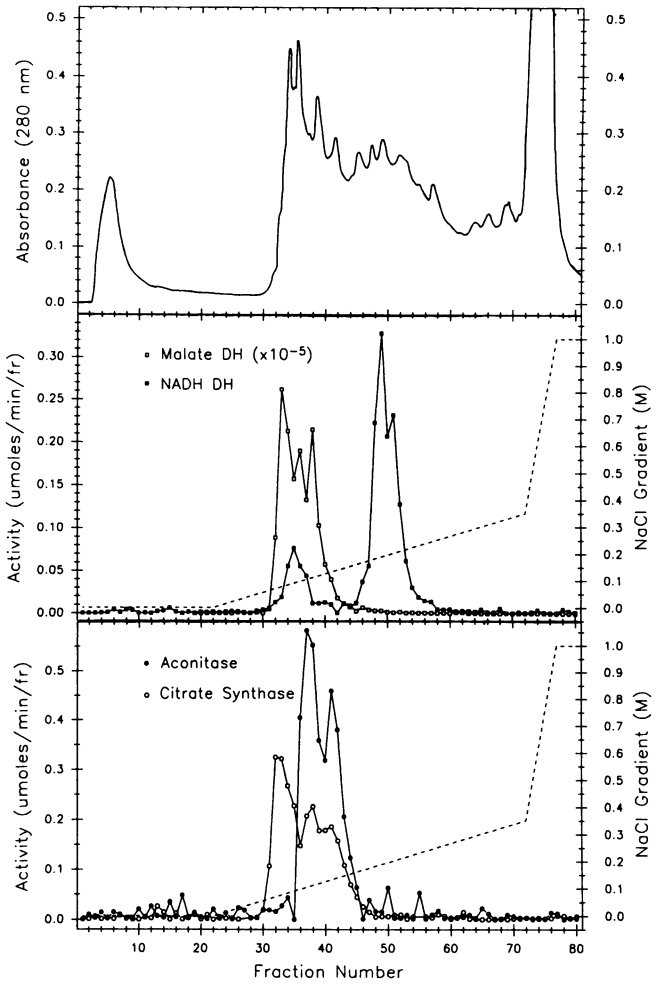

Figure 3. Separation of soluble proteins on Mono Q. Details are given in "Materials and Methods." Upper panel, protein elution profile (absorbance at 280 nm). Lower panel, separation of mitochondrial enzyme activities found in the soluble fraction. Two milliliters of the concentrated soluble fraction were loaded on the Mono Q, and 500 μL fractions were collected.

pH Optima

Initial characterization of the corn malate dehydrogenase was carried out using sonicated mitochondria. pH optima were determined for both malate oxidation and oxaloacetate reduction by malate dehydrogenase over the range 6.4 to 9.2. This range was chosen because it would include the physiologically relevant pH range. The pH curves are shown in Figure 2. The optimum pH for oxaloacetate was 8.4. The optimum pH for malate oxidation was greater than 9.2. There

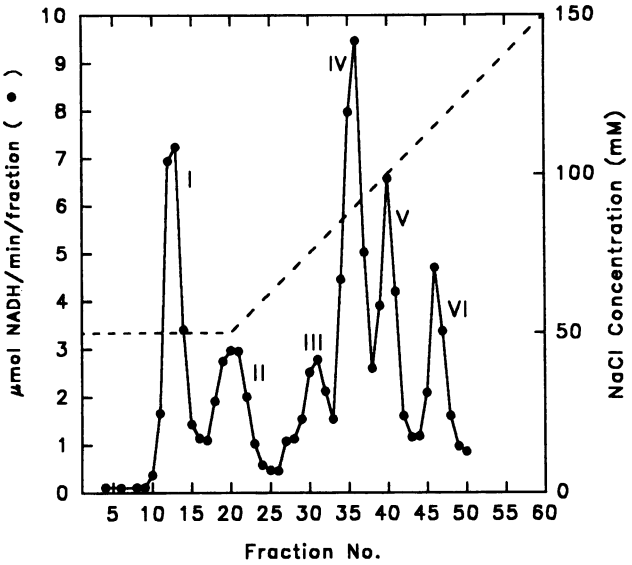

Figure 4. Separation of MDH isoenzymes on Mono Q. Distinct peaks of activity are labeled with roman numerals. Two millliters of the concentrated soluble fraction were loaded onto the Mono Q, and 500 μL fractions were collected.

was a greater level of activity in the reduction of oxaloacetate as noted previously (25, 27).

Purification of MDH

In an initial purification procedure the soluble protein fraction was loaded onto an FPLC Mono Q column preequilibrated with 30 mM Mops, pH 8.0, and after washing, the protein was eluted with a 0 to 350 mM NaCl gradient. Under these conditions major peaks of four soluble enzyme activities were detected and partially separated (Fig. 3). Further refinement of this column should yield partial resolution of other soluble mitochondrial enzymes.

The consistent observation of several peaks of MDH activity on Mono Q indicated that there may be more than one form of MDH separating by ion exchange chromatography. Further refinement of the Mono Q separation by using a 50 to 150 mM NaCl gradient has resulted in the separation of six peaks of MDH activity (Fig. 4). Four peaks eluted in the region of 80 to 120 mM NaCl (III, IV, V, VI). Additionally two peaks of MDH activity (I, II) were eluted during the washing of the column with 50 mM NaCl following loading. However, these peaks eluted after the major peak of unbound protein which indicated that there had been a weak interaction with the column. A summary of the characteristics of the MDH peaks from the Mono Q is presented in Table II. The majority of the activity is found in only three of the peaks, namely I, II, and IV.

All the peaks were individually purified on a Phenyl Superose (hydrophobic interaction) FPLC column. The fractions were loaded onto the column in 3 M NaCl. The protein was eluted with a linear gradient from 3 to 1 M NaCl. This step resulted in the purification to homogeneity of the different forms of malate dehydrogenase. A typical purification is shown for peak IV in Table III. Peak III was the only peak which eluted from the phenyl superose column under 2 M NaCl (Table II). The remaining five eluted between 2.5 and 2 M NaCl. Despite their very different elution from the Mono Q column, peaks I and IV eluted at the same salt concentration from the Phenyl Superose. Establishment of the specific activities and kinetic properties of all 6 isozymes will require a considerable effort because of the low yields obtained (Table III).

SDS and Native Gel Analysis of MDH Isozymes

The subunit composition of the purified MDH peaks was investigated with both one- and two-dimensional SDS-PAGE. With one-dimensional PAGE three peptides of 37, 38, and 39 kD are observed. The six peaks of activity from the Mono Q column correspond to all possible dimers of these three peptides (Fig. 5). This observation was confirmed by two-dimensional SDS-PAGE (data not shown). Western analysis of two-dimensional gels of corn mitochondrial proteins, using antisera raised in mice against purified MDH, recognized three major and two minor protein spots (Fig. 6). An antiserum raised in rabbits by Newton (8, 17) recognized a similar pattern (data not shown). Two-dimensional gel analysis of the individual purified MDH activities has shown that the three major protein spots correspond to the three different gene products. The minor protein spots recognized by the antibodies do not copurify with any of the Mono Q peaks. At this

Table II. *Summary of the Six MDH Activities Purified from Corn Mitochondria*

Mono Q Peak	Elution	Total Activity	Phenyl Superose Elution	Peptides[a]	Native Gel Band[b]	Genes[c]
	mM NaCl	*μmol/min*	*M NaCl*	*kD*		
I	50	40.8	2.30	39	1	*MDH-2*
II	50	27.9	2.05	39, 38	2	*MDH-2, MDH-1*
III	75	7.77	1.80	38	3	*MDH-1*
IV	90	34.2	2.30	39, 37	4	*MDH-2, MDH-3*
V	100	14.8	2.25	38, 37	5	*MDH-1, MDH-3*
VI	115	8.24	2.40	37	6	*MDH-3*

[a] Determined by SDS-PAGE. [b] Determined by MDH activity staining of nondenaturing gels. [c] Determined by comparison with the MDH activity staining of native gels of corn in Newton and Schwartz (18).

Table III. *Purification of Peak IV Mitochondrial Malate Dehydrogenase*

Purification Step	Total Activity	Total Protein	Specific Activity
	μmol/min	*mg*	*μmol/min/mg protein*
Whole mitochondria	1780	48.7	36.6
Soluble protein	1490	3.9	382
Mono Q peak IV	8.65	0.005	1730
Phenyl Superose	4.56	0.0016	2850

time we can only speculate on the minor spots, but they likely represent some modification or degradation of the primary gene products. The observation of the three major peptides is consistent with previous genetic studies which indicated that there are three genes for mitochondrial malate dehydrogenase (10, 11, 17, 18).

Native gels of the soluble protein fraction and the purified MDH isozymes were stained for MDH activity (Fig. 7). Several bands of MDH activity were seen in the soluble protein fraction which were not completely resolved in the gel. The purified MDH isozymes, however, all migrated to different positions, clearly showing the presence of six different isozymes. Comparison of this pattern of staining with the work of Newton and Schwartz (17, 18) has led us to tentatively assign the peptides as products of the *MDH-1*, *-2*, and *-3* genes as shown in Table II.

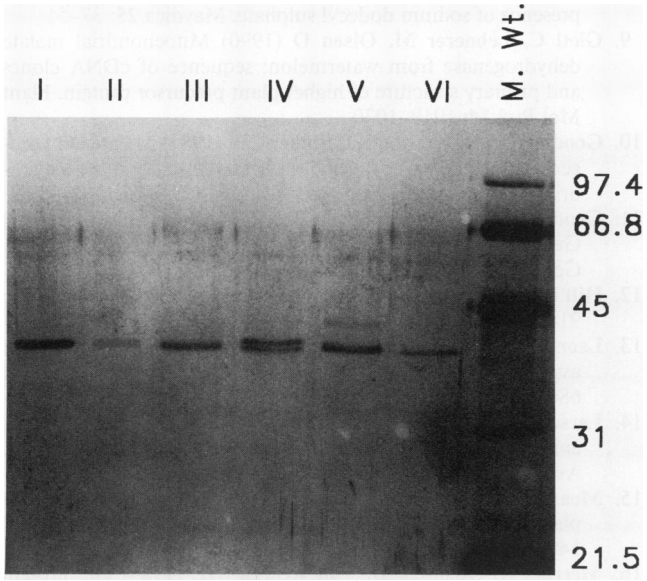

Figure 5. SDS-PAGE of purified MDH isoenzymes. Peaks of MDH activity fom the Mono Q were individually purified using a Phenyl Superose column. The most active fraction for each isozyme was concentrated and run on SDS-PAGE. The proteins were visualized by silver staining. Standard mol wt markers are shown in the left lane. Refer to Table II for subunit composition of each malate dehydrogenase isoform.

DISCUSSION

We have developed a useful fractionation of mitochondria that greatly facilitates the purification of matrix enzymes. Initial isolation of the mitochondria already yields a substantial improvement over purification schemes which begin with plant tissue. The ability to remove the membrane fraction which accounted for the majority of the mitochondrial protein, while retaining most of the matrix enzyme activity, provided an invaluable start to the purification of soluble matrix enzymes. Initial separation of a few of the soluble mitochondrial activities has been achieved.

Malate dehydrogenase was purified to homogeneity by using a two-step FPLC procedure after mitochondrial fractionation. We successfully purified six forms of malate dehydrogenase and characterized their subunit peptide composition using one- and two-dimensional PAGE. The six different forms of MDH represent the hetero- and homodimers of three peptides. The three peptides are most likely gene products of the three mitochondrial MDH genes that were predicted from previous genetic studies.

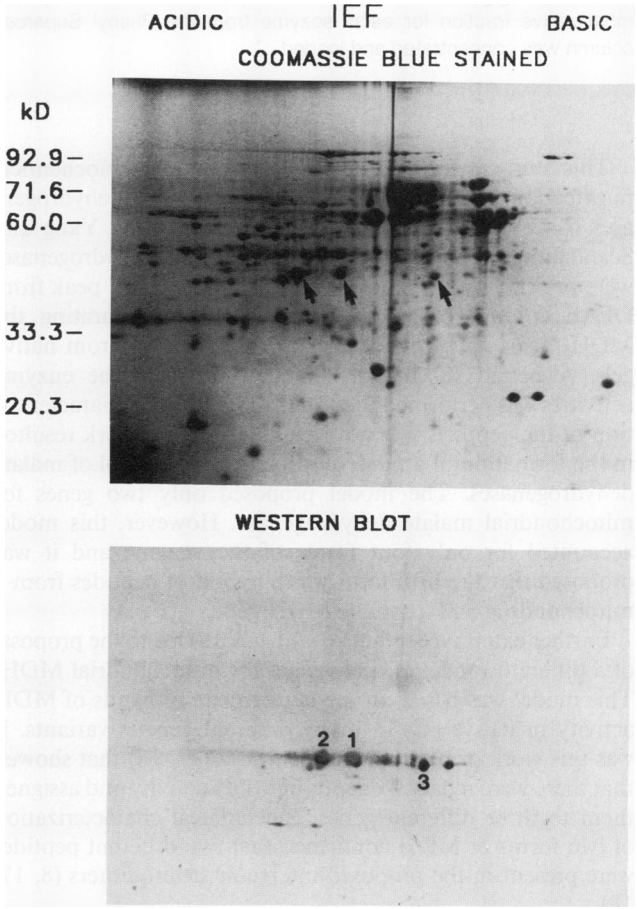

Figure 6. Two-dimensional SDS-PAGE of total corn mitochondrial proteins. Top panel, visualization of the corn mitochondrial proteins with Coomassie blue staining. MDH peptides are indicated. Bottom panel, Western blot of a two-dimensional gel which was probed with the mouse anti-MDH antibody. MDH gene products are labeled as follows: *MDH-1*, 1; *MDH-2*, 2; and *MDH-3*, 3 (refer to Table II).

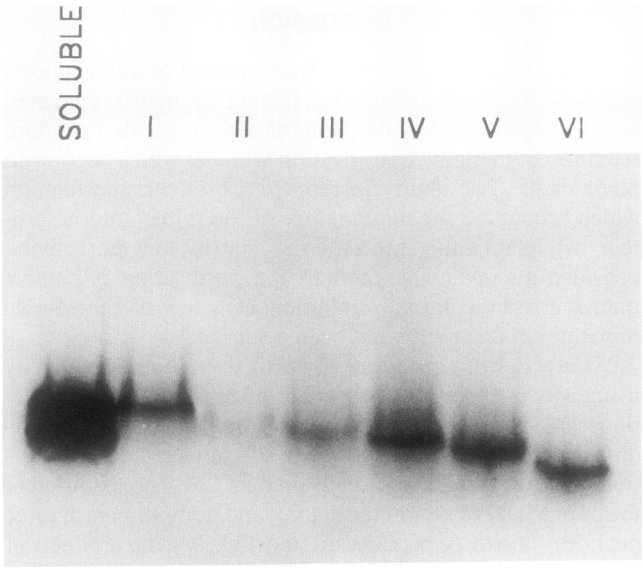

Figure 7. Separation of native MDH isoenzymes by nondenaturing PAGE. Purified MDH isoenzymes were run on 7.5% acrylamide nondenaturing gels and the gels were stained for MDH activity. The most active fraction for each isozyme from the Phenyl Superose column was concentrated and loaded.

This study is the first time that a complete biochemical purification of the six corn mitochondrial malate dehydrogenases has been undertaken. In previous work by Yang and Scandalios (27), five mitochondrial malate dehydrogenases were purified by obtaining a mitochondrial MDH peak from DEAE column chromatography and then separating the MDH forms by cutting bands of MDH activity from native gels. Although substantial characterization of the enzyme activities was performed the subunit structure or characterization of the peptides was not investigated. This work resulted in the formation of a model for the genetic control of malate dehydrogenases. The model proposed only two genes for mitochondrial malate dehydrogenase. However, this model accounted for only four forms of the enzyme and it was proposed that the fifth form was a hybrid of peptides from a mitochondrial and a soluble MDH gene.

Further extensive studies (10, 11, 17, 18) led to the proposal of a different model of three genes for mitochondrial MDH. This model was based on the comparison of bands of MDH activity in native gels in many different genetic variants. It was this work of Newton and Schwartz (17, 18) that showed that there were in fact six bands of MDH activity and assigned them to three different genes. Biochemical characterization of two forms of MDH confirmed that two different peptides were present in the proposed intergenic heterodimers (8, 17, 18).

In this study, we have observed striking differences in the total activities of each isozyme isolated and purified. Those forms containing the 39-kD peptide, which we have proposed to be the product of *MDH-2*, accounts for 75% of the activity purified. It has been difficult to establish the specific activities and kinetic properties of each isozyme because of low yields. Further research in this direction, along with the molecular characterization of the three different malate dehydrogenase genes, will yield insight into the reasons for the observed differences in isozyme activity.

ACKNOWLEDGMENT

The authors wish to thank Dr. Kathleen Newton for the gift of the rabbit anti-MDH antibody.

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