An Electrophoretic Analysis of the Isozymes of Malate Dehydrogenase in Several Different Plants

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Abstract. The particulate and soluble fractions of cell-free extracts from seeds, roots, and leaves of 10 different plants were examined electrophoretically for isozymes of malate dehydrogenase. Distinct isozyme patterns were observed for each plant and even for the individual tissues of each species. There were some isozymes in several different plant extracts with equal electrophoretic mobilities, but there was no isozyme band that was common to all tissues or to all plants.

Malate dehydrogenase (EC 1.1.1.37) is an ubiquitous enzyme, easily extracted, and stable in cell-free preparations. Therefore it is one of the more thoroughly studied enzymes in regard to its properties as a catalyst and as a protein. Studies with malate dehydrogenase obtained from animal tissue have shown that this enzyme exists in 2 forms: a soluble form (i.e., present in the supernatant of tissue homogenates that have been centrifuged at 100,000 × g or greater), and a particulate form that is associated with mitochondria (1, 4, 8, 12). The evidence is clear that the soluble and particulate enzymes are 2 different proteins. More recently, it was found that both the soluble and particulate malate dehydrogenases may be further subdivided into isozymes which are separable by means of electrophoresis (5, 6, 11). Kitto et al. (5) have studied the isozymes present in chicken mitochondria and have found no differences among them in their catalytic properties or amino acid composition. This group suggested that the word “conformers” would be a better term than “isozymes” for their catalytically-active fractions.

Isozymes of malate dehydrogenase have been shown to be present in plant tissues also, such as corn scutellum (13), the imbibed pea seed (14), legume root nodules (3), and bean leaves (9, 10). This report will be concerned with the demonstration of various isozymes of malate dehydrogenase in seeds, root, and leaf (or shoot) of several other plant species.

Methods

The same general procedures for growth and enzyme extraction were followed for all plants studied. Plants used were: pea (Pisum sativum); mung bean (Phaseolus aureus); soybean (Glycine max); cotton (Gossypium hirsutum); pumpkin (Cucurbita pepo); radish (Raphanus sativus); cabbage (Brassica oleracea); corn (Zea mays); oat (Avena sativa); and wheat (Triticum vulgare). Extracts were made from both seeds and seedlings. The tissues were homogenized in a small amount of a solution composed of 2 M KCl, 0.2 M tris (pH 8), and 0.02 M EDTA (pH 8) so that the tissue juices would contribute the major share of extract volume. This process was used to be certain that the concentration of enzyme in the extract would be sufficiently high that isozymes would be detectable on starch gel slices after the extracts had been subjected to electrophoresis. One of the purposes of the above salt-buffer solution was to add a buffer to the reaction mixture to neutralize any acids that might be present in the tissues. Another function of the salt-buffer solution was to introduce an adequate quantity of KCl into the extract to protect cellular organelles from osmotic rupture. It was determined empirically that an initial concentration of 2 M KCl was needed to achieve this result since the final volume of the extracts could be as much as 3 times larger than the volume of salt-buffer solution originally added.

Homogenates of Seeds. Seeds were immersed in aerated water at room temperature for 4 to 6 hours. The imbibed seeds were ground in a Waring Blender in the presence of 0.5 ml of cold salt-buffer solution per g of dry seed. The slurry was squeezed through 2 layers of cheesecloth and the resulting residue discarded.

1 Contribution from the United States Salinity Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, United States Department of Agriculture, Riverside, California, in cooperation with the 17 Western States and Hawaii.
Fig. 1. Photograph of a starch gel slice in which the soluble fraction of leaf extracts from 10 different plants have undergone vertical electrophoresis at 300 volts for 18 hours at 2 to 5° in a buffer solvent composed of 2% tris, 0.2% EDTA, and 0.15% boric acid. The pH of this mixture was 8.6. The top of the gel slab was connected to the cathode of the power supply, and the bottom was in a solvent reservoir connected to the anode. The gel slice was stained for the enzyme, malate dehydrogenase. The extracts were placed in the slots of the gel in the following order beginning at the left edge: Slot No. 1, pea; No. 2, mung bean; No. 3, soybean; No. 4, cotton; No. 5, pumpkin; No. 6, radish; No. 7, cabbage; No. 8, corn; No. 9, oat; No. 10, wheat.
Growth and Homogenization of Seedlings. Seeds were germinated by placing them in moist paper towels and incubating them at 25°. After 4 days, the resulting seedlings were removed from the towels and transferred to aerated one-half strength Hoagland's solution. The plants were allowed to grow at 28° with a 12-hour photoperiod. Light was supplied by a bank of fluorescent and tungsten lamps with an intensity of 3.88 × 10^3 lux at plant height. After another 6 to 16 days (depending on the species), the plants were harvested.

The plants were separated into roots, stems, and leaves (or roots and shoots for the monocotyledons). Each tissue was weighed and placed in a chilled mortar along with 1 ml of the cold salt-buffer solution per 3 g (fr wt) of tissue. The tissue was macerated with a pestle at a relatively smooth suspension was obtained. The suspension was squeezed through 2 layers of cheesecloth and the residue discarded without further washing.

Preparation of Particulate and Soluble Enzyme Fractions. The seed and tissue homogenates were centrifuged at 750 × g for 15 minutes to remove starch grains and debris. This step and all subsequent centrifugations were performed at 0 to 5°. The supernatant was centrifuged a second time at 20,000 × g for 30 minutes and, after being decanted from the pellet, centrifuged once more at 140,000 × g for 60 minutes. The pellet formed by centrifuging at 20,000 × g is labeled the particulate fraction, and the supernatant from the centrifugation at 140,000 × g is the soluble fraction. The pellet sedimenting at 140,000 × g was discarded because experiments (not described here) showed that this material had little, if any, malate dehydrogenase activity.

The particulate fraction was washed 2 times with 0.5 m KCl in 0.05 m tris (pH 8) and finally suspended in 5 ml or less of this buffered solution. The soluble fraction was passed through a Sephadex G-50 column equilibrated with 0.01 m tris (pH 8). This process removed not only the large amount of KCl in the extract but also a major portion of pigmented material. Approximately 80 to 105% of the protein and essentially all malate dehydrogenase activity placed on columns were recovered in the "excluded volume" eluate.

All fractions were stored in the frozen state until they were to be used. This treatment, in the case of the particulate fraction, released all the malate dehydrogenase activity from the particles with recovery of the enzyme in a soluble form.

Protein was measured colorimetrically by the Folin-Ciocalteau phenol method (7). However, since plant extracts contain a lot of material that also reacts with this reagent, the following procedure was used to remove such interfering compounds. The protein in an aliquot of each extract was precipitated in 7% perchloric acid. The precipitate was washed twice in water, twice in 95% ethanol, and once more in water. The washed precipitate was dissolved in approximately 3 ml of 0.5 × NaOH and then was accurately diluted to a known volume with water. A portion of this alkaline solution was used for the protein assay.

Malate dehydrogenase was assayed for activity and for the effect of NaCl on activity, as previously described (14). Vertical starch gel electrophoresis of tissue extracts and the detection of malate dehydrogenase in such gels were performed by the method of Fine and Costello (2).

Results

Malate dehydrogenase activity was present in all tissues of all plants (table 1). The specific activities of the enzyme in crude homogenates varied somewhat, but none was notably larger or smaller than any of the others. As was found in pea seed extracts (14), upon separation of the various extracts into particulate and soluble fractions, a major portion of the enzymatic activity was in the soluble fraction.

The soluble and particulate fractions were examined electrophoretically for the presence of isozymes of malate dehydrogenase. The results obtained with stem tissue extracts are omitted because in the few extracts examined, the isozyme

Table 1. Activity of Malate Dehydrogenase in Crude Plant Extracts

<table>
<thead>
<tr>
<th>Plant</th>
<th>Activity of extract</th>
<th>Specific activity (mg protein)</th>
<th>Activity of extract</th>
<th>Specific activity (mg protein)</th>
<th>Activity of extract</th>
<th>Specific activity (mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>160</td>
<td>4.6</td>
<td>40.0</td>
<td>8.9</td>
<td>20.0</td>
<td>15.4</td>
</tr>
<tr>
<td>Mung bean</td>
<td>300</td>
<td>5.7</td>
<td>2.1</td>
<td>1.8</td>
<td>2.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Soybean</td>
<td>155</td>
<td>3.1</td>
<td>52.0</td>
<td>2.5</td>
<td>15.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Cotton</td>
<td>180</td>
<td>4.2</td>
<td>12.4</td>
<td>4.4</td>
<td>10.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>75</td>
<td>15.6</td>
<td>45.0</td>
<td>6.5</td>
<td>12.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Radish</td>
<td>90</td>
<td>4.1</td>
<td>3.3</td>
<td>1.2</td>
<td>1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Cabbage</td>
<td>105</td>
<td>3.3</td>
<td>10.0</td>
<td>3.8</td>
<td>13.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Corn</td>
<td>60</td>
<td>12.5</td>
<td>9.3</td>
<td>2.8</td>
<td>6.2</td>
<td>3.3</td>
</tr>
<tr>
<td>Oat</td>
<td>36</td>
<td>5.2</td>
<td>29.0</td>
<td>4.8</td>
<td>11.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Wheat</td>
<td>60</td>
<td>10.2</td>
<td>14.5</td>
<td>3.2</td>
<td>18.0</td>
<td>18.0</td>
</tr>
</tbody>
</table>

1 Δ OD min^-1 ml^-1
2 Δ OD min^-1 mg^-1 protein
patterns in stems were the same as those of leaf extracts. Figure 1 is a photograph of a starch gel slice in which the soluble fraction of leaf extracts of 10 plants has been subjected to electrophoresis, and the gel stained for malate dehydrogenase. However, photographs do not reproduce the actual gel stain too clearly. Therefore, all results are presented as drawn facsimiles; the edges of bands in the gel are actually not as distinct as shown in the facsimiles.

The results of this survey are obvious from an examination of the gel patterns (figs 2 and 3) and therefore need be discussed only in general terms. Both soluble and particulate fractions of all plant extracts contained isozymes of malate dehydrogenase. Resolution was variable from very poor in the case of cotton to reasonably distinct banding for pumpkin extracts. Even though only 1 or 2 bands can be observed in tissue extracts of cotton, the bands are so broad that it suggests that either there are several enzymes whose rates of migration are so close that they overlap, or the isozymes of this plant do not migrate uniformly under these conditions.

The electrophoretic patterns (the number of bands and the mobility of each band) differed widely in the separate tissues. The patterns may be compared by separating the isozymes of each extract of the same anatomical structure on the same gel slab (fig 2). However, in the absence of an independent marker against which the mobilities of the bands could be directly measured, the electrophoretic separation experiments were repeated several times but each time, the order in which the extracts were applied to the sample slots was varied. The results in each experiment were consistent with those shown in figure 2. The most striking feature of isozyme patterns of the same tissue from different plants is the lack of a common pattern. Wheat and oat extracts showed some degree of similarity to one another (and even identity in particulate fractions), as did radish and cabbage extracts. However, patterns of the fractions and extracts of the other plants were different from one another. In addition, there was no single isozyme band that was common to all 10 plants, although there were some isozymes in extracts of several plants that had similar electrophoretic mobilities.

While 1 or more isozymes distinctive for a certain morphological structure were not found, it is still possible that isozymes occur which are typical of the whole plant rather than of just 1 tissue. Therefore, the extracts were re-examined by electrophoresis of the extract fractions of each plant species on 1 gel slab (fig 3). Stained gel slices in which the extracts of pea, mung bean, soybean, radish, cabbage, and corn had been subjected to electrophoresis showed 1 isozyme band that existed in all 6 extract fractions of the individual plant. Usually, it was the fastest or next to fastest migrating band. However, no such common band was observed in the stained gel slices of the other plants examined.

Other generalizations concerning the isozyme patterns of these tissues do not seem possible. Although there may be 1 or more isozymes with the same mobility in the various tissue fractions of 1 plant, in comparing the 3 soluble fractions or the 3 particulate fractions to one another, the over-all patterns were not the same. Consequently, it must be concluded that each plant synthesizes a complement of isozymes of malate dehydrogenase that is distinctive not only for the plant but also for the tissue of the plant.

Because of the large variation in the number and electrophoretic mobility of isozymes in plant tissue, it seemed possible that there might be a variation in other properties of this enzyme as well. One
property that may be easily assayed in crude extracts and is pertinent to the mission of this Laboratory is the response of enzyme activity to changes in ionic strength. It was shown previously (14) that an isolated isozyme of malate dehydrogenase from pea seeds was stimulated by NaCl if the concentration of salt did not exceed 0.02 M. Higher concentrations of salt were inhibiting. Both the soluble and particulate fractions of the extracts of the 10 plants studied here responded to NaCl in the same qualitative manner and to approximately the same degree. The rate of oxalacetate reduction at pH 7.5 increased 50 to 100% with a concentration of NaCl of approximately 0.01 to 0.02 M, while higher concentrations inhibited the reaction. Thus, it appears that, at least for this 1 property, the 1 or several isozymes responsible for the major portion of activity in each extract are similarly affected by NaCl.

Literature Cited


Fig. 3. Facsimiles of electrophoretic patterns in starch gel of 10 different plants compared on a species basis. Tissue extracts are in the same sequence in all gels beginning on the left side in the following order: Slot No. 1, particulate fraction of seed extract; No. 2, soluble fraction of seed extract; No. 3, particulate fraction of leaf (or shoot) extract; No. 4, soluble fraction of leaf (or shoot) extract; No. 5, particulate fraction of root extract; No. 6, soluble fraction of root extract. Conditions for electrophoresis are the same as in figure 1.


