Differential Activities of Chorismate Mutase Isozymes in Tubers and Leaves of Solanum tuberosum L. \(^1\)

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ABSTRACT

Chromatography on DEAE cellulose equilibrated with Pipes buffer resolved three forms of chorismate mutase (CM) in tubers and leaves of Solanum tuberosum: CM-1A and CM-1B were activated by tryptophan and inhibited by phenylalanine and tyrosine; CM-2 was unaffected by these aromatic amino acids. When compared to freshly excised discs, day-old tuber discs demonstrated a 4.5-fold increase in CM-1 activity following wounding. By contrast, CM-2 activity levels were not affected by this treatment. In aged tuber discs the CM-1:CM-2 activity ratio was 9:1. However, in green leaves the CM-1:CM-2 activity ratio was 1:4 suggesting organ specific regulation for the expression of these isozymes. The CM-1 isozymes isolated from both tubers and leaves shared similar native molecular weight values of 55,000, \(K_w\) values of 40 to 56 micromolar, and inhibition by phenylalanine (110–145 micromolar concentrations required for 50\% inhibition) and tyrosine (50–70 micromolar concentrations required for 50\% inhibition). The resolution of CM-1 into two forms occurred only in the presence of Pipes buffer. When this buffer was replaced with Aces, Bes, imidazole or Tris, only a single peak of CM-1 activity was observed. In these buffers CM-2 eluted as a shoulder on the CM-1 peak. Analytical isoelectric focusing of the CM-1 fraction followed by assay of the gel yielded only one form of CM-1 with an isoelectric point of 5.0. Gel filtration studies with Pipes buffer yielded molecular weights of 60,000 for both CM-1A and CM-1B indicating these forms are not the result of aggregation. The two forms of CM-1 may be artifacts generated by Pipes buffer.

Chorismate mutase (EC 5.4.99.5) isozymes have been well resolved by chromatography on DEAE cellulose in a number of plants (5, 6, 14, 15). Isozyme CM\(^2\)-1 has been shown to be activated by tryptophan and inhibited by phenylalanine and tyrosine, whereas CM-2 is not regulated by these aromatic amino acids. However, in other studies, only CM-1 activity could be detected following anion exchange chromatography (9, 15). In a previous investigation we detected only CM-1 activity in tubers of Solanum tuberosum following DEAE cellulose chromatography in citrate-phosphate buffer (9). Recent studies with parsley cell cultures (KF McCue, EE Conn, unpublished data) indicated that CM-1 and CM-2 may coclute on DEAE cellulose. This suggested that a similar phenom might be occurring in the potato system. Utilizing DEAE cellulose equilibrated with Pipes buffer, Morris et al. (12) have recently detected CM-2 in potato tubers. We also report the resolution of CM-1 and CM-2 isozymes from tubers and green leaves of S. tuberosum. The data presented in this study revealed that of the five buffers employed, complete resolution of tuber CM isozymes on DEAE cellulose occurred only with Pipes buffer.

The separation of CM-1 and CM-2 by chromatography on DEAE cellulose allowed us to observe the effects of wounding and organogenesis on the expression of these shikimate pathway enzymes. Similar studies have been reported for the differential expression of DAHP synthase isozymes in Nicotiana silvestris cell cultures (3) as well as for CM isozymes in cell cultures and green leaves of N. silvestris (6). However, it is difficult to extrapolate the physiological significance of cell culture data to intact plants. The results presented in this paper constitute the first report of differences in the expression of CM-1 and CM-2 isozymes from an intact plant.

MATERIALS AND METHODS

Plant Material

Solanum tuberosum L. cv White Rose tubers were purchased at a local store, washed with distilled water, blotted dry, and stored in the dark at 4°C. Tubers stored for up to 4 weeks were employed in all studies. Potato plants were propagated by excision of sprouts from tubers stored at room temperature, followed by planting in vermiculite. The plants were maintained in a growth chamber at 25°C, with a light-dark cycle of 9 h:15 h.

Disc Incubation Conditions

Discs were excised and incubated for 3 d as previously described (9).

Enzyme Purification

All of the following steps were carried out at 4°C. Fresh or 3 d old discs (11 g fresh weight) were homogenized in a mortar containing 1.0 g quartz sand, 7.0 mL of 0.1 mM Pipes buffer (pH 7.2) containing 5 mM EDTA, 150 mM KCl, 1.0 mM DTT, and 50 mM ascorbate. Potato leaves (9 g fresh weight) were ground to a fine powder in liquid nitrogen, and the enzyme extracted with 10 mL of the above buffer system. The homogenate was filtered through two layers of cheesecloth and centrifuged for 20 min at 10,000 g. The supernatant was

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\(^2\) Abbreviations: CM, chorismate mutase; DAHP, 3-deoxy-d-arabino-heptulosonate 7-phosphate; Pipes, 1,4-piperazinediethanesulfonic acid; Aces, N-[2-acetamido]-2-aminoethanesulfonic acid; Bes, 2-[bis(2-hydroxy)amino]ethanesulfonic acid; DTT, dithiothreitol.

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decanted and chromatographed on a Sephadex G-25 column (2.5 × 48 cm) equilibrated with 10 mM Pipes buffer (pH 7.2) containing 20 mM KCl and 0.5 mM DTT. Fractions (6.5 mL) were collected and assayed for protein content by measuring the absorbance at 280 nm. Fractions containing protein were pooled and applied onto a DEAE cellulose column (21 × 1.5 cm) equilibrated with the above buffer. The column was washed with this buffer until unbound proteins were eluted as determined by absorbance at 280 nm. Bound proteins were eluted with a linear 20 to 350 mM KCl gradient (450 mL, total volume) in the above buffer. Fractions (5.7 mL) were collected and assayed for CM activity. Studies with protease inhibitors were identical to the above procedure with the inclusion of 10⁻⁶ M leupeptin, 10⁻⁶ M pepstatin A, and 1.0 mM PMSF. Further studies with other buffers were performed as described above except Pipes was replaced by Aces, Bes, imidazole or Tris. All buffers had a final pH of 7.2. For the Tris buffer system, final concentrations employed during homogenization and purification were 0.2 mM and 25 mM, respectively. Additionally, chromatography was performed at a higher ionic strength with imidazole containing 40 mM KCl.

Molecular Weight Determination

Peak activity fractions obtained from DEAE cellulose were chromatographed on a Sephadex G-25 column (Pharmacia PD-10) equilibrated with one of the buffers described below. Native mol wt were determined by chromatography on Sephadex G-150 (2.5 × 85 cm) equilibrated with either 50 mM imidazole (pH 7.2) containing 0.1 mM NaCl, or 50 mM Pipes (pH 7.2) containing 0.1 mM NaCl. Fractions (5.7 mL) were collected and assayed for CM activity as described below. Calibration standards employed were alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, and ribonuclease.

Isoelectric Focusing

Protein samples were obtained by concentrating DEAE cellulose fractions by ultrafiltration, followed by desalting on Sephadex G-25 (Pharmacia PD-10) equilibrated with 10 mM potassium phosphate (pH 7.0). IEF was carried out at 10°C on LKB PAGplates (pH range 4.0–6.5). The gel was preequilibrated for 30 min prior to sample application. Electrofocusing was then performed according to manufacturer’s specifications. CM activity was localized in the gel by incubating gel slices (2.5 × 5.0 mm) in the assay mixture described below (0.2 mL, total volume). After incubation at 25°C for 30 min, aliquots (0.163 mL) were removed and added to 0.1 mL of 2 N HCl to stop the reaction. Product determination was the same as described below. The pH gradient was determined by incubating gel slices (5.0 × 5.0 mm) in deionized water (0.7 mL, total volume) for 30 min at room temperature, followed by measurement of the pH.

Enzyme Assays

CM activity was determined essentially as previously described (9). Column fractions were assayed at a final chorismate concentration of 66 μM. Recovery and characterization studies for CM-1 (A and B) and CM-2 were performed with 0.3 mM and 1.1 mM final chorismate concentrations, respectively. All CM-1 (A and B) assays were performed in the presence of 0.1 mM tryptophan (except where noted). Assays were linear with the addition of up to 0.130 mL of enzyme, and for up to 30 min incubation time.

Chemicals

Chorismic acid was purchased from Sigma. The chorismate concentration was determined by measuring the absorbance at 274 nm (ε=2630). Gel filtration media were purchased from Pharmacia, Inc. DEAE cellulose (DE-52) was obtained from Whatman, Ltd., England. All other chemicals were purchased from Sigma.

RESULTS AND DISCUSSION

DEAE cellulose chromatography in the Pipes buffer system described here resulted in excellent separation of CM-1 and CM-2 activities in both tubers and green leaves (Fig. 1, A and B). Additionally, CM-1 activity was resolved into two peaks, CM-1A and CM-1B. Both of these fractions were activated by tryptophan and inhibited by phenylalanine and tyrosine.

![Figure 1](https://www.plantphysiol.org)
Table I. Properties of CM Isozymes Isolated from Tubers and Leaves of S. tuberosum

<table>
<thead>
<tr>
<th>Assays were performed as described under &quot;Materials and Methods.&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubers</td>
</tr>
<tr>
<td>CM-1A</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>Tryptophan activation</td>
</tr>
<tr>
<td>$I_{50}$ phenylalanine*</td>
</tr>
<tr>
<td>$I_{50}$ tyrosine</td>
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<tr>
<td>$M_0$ (imidazole buffer)</td>
</tr>
<tr>
<td>$M_0$ (Pipes buffer)</td>
</tr>
<tr>
<td>pH optimum</td>
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* Amount of inhibitor required for 50% inhibition when assayed in the absence of tryptophan.  
* No inhibition up to 1.0 mM inhibitor concentration.  
* Not determined.

Table I. Properties of CM Isozymes Isolated from Tubers and Leaves of S. tuberosum

However, these CM-1 isozymes may be artifacts of the Pipes buffer system as discussed later. CM-2 activity was characterized by insensitivity to the aromatic amino acids that are end products of the shikimate pathway (Table I).

We have reported an increase in the activity of a tryptophan sensitive form of CM following wounding of Solanum tuberosum tubers (9). Studies with transcriptional and translational inhibitors suggested this increase was a result of de novo synthesis of CM-1. The apparent absence of CM-2 reported earlier (9) was based, in part, on the observation that, in a crude extract, the tryptophan activation ratio remained constant during aging of wounded discs. The resolution of CM-2 in potato tubers indicates that the tryptophan activation ratio is not a reliable measure for the presence of CM isozymes if the fractional amount of CM-2 is small. Since CM-2 activity was not detected in the previous study the effect of wounding on CM-2 activity was not determined. In the present study, comparison of elution profiles from fresh and aged tuber discs illustrates that CM-2 activity does not increase in response to wounding (Fig. 1A). By contrast, CM-1 activity in aged discs increased 4.5-fold over the activity in fresh discs (Fig. 1A). CM-1 has been proposed to participate in a plastidial shikimate pathway that exclusively supplies aromatic amino acids for protein biosynthesis (8). This increase in CM-1 activity may be due to an increased demand for phenylalanine and tyrosine required for protein synthesis following wounding.

Phenylalanine is also a precursor for chlorogenic acid which is a secondary metabolite produced in response to wounding (11). CM-2 has been proposed to be part of a complete cytosolic shikimate pathway that, in addition to supplying aromatic amino acids for protein synthesis, also provides precursors for secondary metabolism (8). This suggested that an increase in secondary metabolism might be correlated with an increase in CM-2 activity, particularly since isozymic forms of DAHP synthase demonstrated differential timing of their expression in Nicotiana silvestris suspension cultures (2, 3). In these cultures, the plastid associated DAHP synthase demonstrated maximum activity during early exponential growth, whereas the cytosolic isoenzyme activity was greatest during late exponential growth. This was suggested to reflect a shift from primary to secondary metabolism during the growth cycle. In S. tuberosum tubers, however, the differential responses to wounding by CM-1 and CM-2 suggest that CM-1, rather than CM-2, is critical to this wound response. In addition to the absence of wound induced activation, CM-2 represented only 10 to 15% of the total CM activity in aged discs. This does not exclude an association between CM-2 and secondary metabolism and may simply represent a priority for channeling phenylalanine and/or tyrosine into protein synthesis rather than secondary metabolism following wounding. It is apparent that the putative roles for CM-1 and CM-2 in partitioning carbon flow between primary and secondary metabolism are not easy to define at this time. The wound response in S. tuberosum tubers may provide a good model to study the relationship between CM isozymes and these metabolic pathways.

In contrast to tubers, CM-2 predominates in green leaves (compare A and B in Figs. 1 and 2). In this organ, CM-2 represented 77% of the total activity. The elution profile for CM-2 in leaves does not accurately reflect the true CM-1:CM-2 ratio. This is due to the difference in $K_m$ values between CM-1 (A and B) and CM-2 (Table I). Since column fractions were assayed at a final chorismic concentration of 66 µM, CM-2 was not saturated. However, total recovery studies were performed at substrate concentrations six times the $K_m$ value for each isozyme.

Whereas the ratio of CM-1:CM-2 activity in fresh tubers was 2:1, the activity ratio in leaves was 1:4, suggesting organ specific regulation for the expression of CM-2. This data indicates a physiologically significant role for CM-2 in green potato leaves. A similar change in the expression of CM isozymes was observed in cell suspension cultures and green leaves of N. silvestris where CM-1 comprised most of the activity in cell cultures and CM-2 was the predominant isozyme in green leaves (1, 6). This shift in the ratio of isozyme activities was suggested to reflect increased secondary metabolism in leaves versus cell cultures in exponential growth (6). However, the participation of CM-2 in a unique shikimate pathway associated with secondary metabolism has not been rigorously demonstrated.

The CM-2 isozymes from tubers and green leaves also had different affinities for chorismic acid. CM-2 from tubers had a $K_m$ of 55 µM, almost four times lower than the $K_m$ for the leaf enzyme. This difference in $K_m$ values might be artifactual due to side reactions that are unique to leaf homogenates and further purification of these CM-2 isozymes is necessary be-
amino acids. Subsequent studies by other researchers failed
to reproduce this pattern and these tryptophan sensitive iso-
yzmes have been suggested to be artifacts (6, 15). Although
kinetic studies with CM-1A and CM-1B from S. tuberosum
tubers indicated virtually identical pH optima, $K_m$ values, and
similar inhibition by phenylalanine and tyrosine (Table I),
additional results from a variety of chromatographic methods
suggested these forms may be artifacts.

Pipes was very effective in resolving CM-1 and CM-2 in
both tubers and leaves. However, it is an anionic buffer and
due to possible interactions with the gel, its use with anion
exchange columns is discouraged (13). Further studies were
initiated to determine the possible effect that Pipes buffer
might have on the appearance of CM-1 isozymes.

Homogenization and purification of aged tuber discs were
carried out in four other buffer systems. Two anionic buffers,
Aces and Bes, and two cationic buffers, imidazole and Tris,
were employed. In all cases, only a single peak of CM-1
activity could be resolved on DEAE cellulose (Fig. 2A). Since
the elution profiles for all buffers were essentially identical,
only the results with imidazole are shown. In contrast to the
elution profile in Pipes buffer, CM-1 eluted well into the
gradient and CM-2 appeared as a shoulder on the trailing
edge of the peak. The failure of Aces and Bes to resolve CM-
1 isozymes indicated a specific effect by Pipes buffer and not
a general effect of anionic buffers. Furthermore, when CM-1
isolated from chromatography in imidazole buffer was re-
chromatographed on DEAE cellulose equilibrated with Pipes,
CM-1A and CM-1B isozymes were resolved (Fig. 3). The
small peak of activity at fraction 83 was probably due to
contamination of CM-1 with CM-2 since these activities were
not fully resolved in the preceding step.

Since the Pipes buffer system had a slightly higher ionic
strength than the imidazole buffer, the ionic strength of the
latter was doubled from 20 mm to 40 mm KCl to determine
if the resolution of CM-1 isozymes was a function of this
parameter. This resulted in CM-1 eluting slightly earlier in

![Figure 2](https://example.com/fig2.png)

**Figure 2.** Elution profiles of CM chromatographed on DEAE cellulose
equilibrated with imidazole buffer. A, CM elution profile for aged discs
(●); B, CM elution profile for green leaves (●). Fractions (5.7 mL)
were collected and assayed as described under "Materials and Meth-
ods." No CM activity was detected in the load and wash fractions
and only gradient fractions are shown.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** DEAE cellulose chromatography of CM-1. CM-1 obtained
from DEAE cellulose chromatography in the presence of imidazole
buffer was rechromatographed in the presence of Pipes buffer.
Fractions (5.7 mL) were collected and assayed for CM activity (●) as
described under "Materials and Methods." No CM activity was de-
tected in the load and wash fractions and only gradient fractions are
shown.
the gradient, but again, only a single activity peak was observed (data not shown). When this fraction was subjected to IEF, subsequent assay of the gel yielded only one CM-1 peak, with a pl of 5.0 (data not shown).

It is unlikely that Pipes uniquely protects one of the iso-
zymes from degradation or denaturation since the total amount of CM-I activity (CM-1A + CM-1B) in the Pipes buffer system is 95% of the total CM-1 activity isolated in the imidazole system. Likewise, CM-1A and/or CM-1B do not appear to be the result of proteolysis since homogenization and purification in Pipes buffer containing four general protease inhibitors (EDTA, PMSF, leupeptin, pepstatin A) yielded an isozyme pattern identical to that observed in the absence of protease inhibitors (data not shown).

Gel filtration of CM-1A and CM-1B on Sephadex G-150 equilibrated with imidazole buffer yielded individual native \( M_r \) values of 55,000 (Table 1). These values are almost identical to the \( M_r \) reported for CM-1 earlier (10). Additional studies were performed on the same column equilibrated with Pipes to determine if aggregation might be occurring in the presence of this buffer. Native \( M_r \) values of 60,000 were obtained for both CM-1A and CM-1B (Table 1) indicating that aggregation is not responsible for the existence of these tryptophan sensitive isozymes. Although the exact nature of this heterogeneity is not known, the isolation of a single form of CM-1 in five different buffers (9; this study) and the detection of a single CM-1 activity following IEF suggest that CM-1A and CM-1B are artifacts generated by Pipes buffer. However, of all the buffers tested, Pipes was the only one that successfully resolved CM-1 and CM-2 activities. It appears this buffer has specific properties that facilitate this separation, and a similar role in the resolution of CM-1A and CM-1B cannot be ruled out.

In conclusion, chromatography on DEAE cellulose in the presence of Pipes buffer separated three forms of CM. The nature and origin of the two tryptophan sensitive isozymes is unknown, and they may be artifacts. The resolution of CM-1 and CM-2 in tubers revealed different responses to wounding, the CM-1:CM-2 ratio being 2:1 in fresh discs and increasing to 9:1 in aged discs. In contrast to the relatively high activity of CM-1 in aged discs, CM-2 comprised the majority of total CM activity in green leaves with a CM-1:CM-2 ratio of 1:4. This dramatic shift in the ratio of isozyme activities suggests organ specific regulation for the expression of CM-1 and CM-2 isozymes. Unfortunately, the underlying regulatory mechanisms are unknown. Although the CM-1:CM-2 ratios in various organs and tissues from *Sorghum bicolor* have been reported (14), no significant differences were observed. *S. tuberosum* tubers and leaves provide an ideal system to study the regulation of CM isozyme expression in response to wounding and organogenesis. Further studies will hopefully provide insight into the specific role for CM-2 in potato leaves.

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LITERATURE CITED