

**Communication**

# Evidence from *Solanum tuberosum* in Support of the Dual-Pathway Hypothesis of Aromatic Biosynthesis<sup>1</sup>

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## ABSTRACT

Key branchpoint enzymes of aromatic amino acid biosynthesis, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS) and chorismate mutase (CM), have previously been shown to exist as separate compartmentalized isozymes in the chloroplasts and cytosol of tobacco, sorghum and spinach. Although additional examples of plants containing these isozyme pairs are accumulating, some studies in the literature report the presence of only the single plastidic DS or CM enzyme. Such apparent exceptions contradict the universality of pathway organization existing in higher plants that is implied by the dual-pathway hypothesis of aromatic biosynthesis. Since potato (*Solanum tuberosum*) exemplifies a case where only a single species of both DS and CM have been reported, we selected this system for further analysis. The DS-Mn and DS-Co isozyme pair, exhibiting all of the differential properties described in *Nicotiana glauca*, have now been identified in *S. tuberosum*. Likewise, partial purification via DEAE-cellulose chromatography revealed two isozymes of CM in disks excised from tubers of *S. tuberosum*. The differential regulatory properties of these isozymes were comparable to the CM-1 and CM-2 isozymes of *N. glauca*.

An intact complement of aromatic-pathway enzymes exists within the plastid compartment of higher plants (11). This pathway is subject to tight allosteric control at early-, mid-, and late-pathway branchpoints (11). Aromatic amino acids are additionally required in the cytosol, not only for protein synthesis, but as precursors for an extensive metabolic network responsible for the output of secondary metabolites that include phenylpropanoids, lignins, flavonoids, and tannins (12). Distinct isozyme pairs, one plastidic and the other cytosolic, have been identified for DAHP<sup>2</sup> synthase (5), chorismate mutase (4, 26), and anthranilate synthase (2). In each case, the cytosolic isozyme species lacks the allosteric control that is characteristic of its plastidic isozyme counterpart. One can reasonably assume that the isozyme compartmentation rigorously demonstrated in tobacco, sorghum, and spinach

applies to other systems where isozyme pairs exhibiting the same distinctive properties have been characterized, e.g. mung bean (25).

The existence of isozyme species corresponding to most of the steps between DAHP synthase and chorismate mutase has been reported (19, 20, 22, 24). While cytosolic location of such isozymes have been indicated (19, 20, 24), clear characterizations have not yet been accomplished. We assume that the presence of cytosolic DAHP synthase (DS-Co) and cytosolic chorismate mutase (CM-2) indicates that the intervening succession of enzymes is also present, thus providing a second intact pathway in the cytosol. The hypothesis that dual biosynthetic pathways of aromatic amino acid biosynthesis existing in spatially separated compartments is a universal characteristic of higher plants is mainly based upon the large and growing list of higher plant species that possess the isozyme pairs of DAHP synthase (DS-Mn and DS-Co) and chorismate mutase (CM-1 and CM-2).

On occasion, the apparent absence of one isozyme species has been reported. For chorismate mutase, the undetected isozyme has always been the unregulated, cytosolic species: CM-2. For DAHP synthase, the vastly differing properties of plastidic and cytosolic isozymes has commonly resulted in the description of one isozyme or the other, depending upon the arbitrary choice of assay conditions (see discussion by Ganson *et al.* [5]). The absence of the isozyme pairs in even a single plant species would contradict the universal existence in higher plants of a regulated plastidic pathway and an unregulated cytosolic pathway of aromatic biosynthesis (dual-pathway hypothesis).

An important apparent exception to the proposed universality of separately compartmented isozyme pairs has been *Solanum tuberosum* where the existence of only a single chorismate mutase (CM-1) (15) and a single DAHP synthase (probably DS-Mn) have been reported (21). In order to resolve this, we have, therefore, extended our studies to *S. tuberosum*, and report our findings of the existence of isozyme pairs of both DAHP synthase and chorismate mutase. These isozyme pairs are virtually identical to the model isozyme pairs characterized in *Nicotiana glauca* (4, 5) and in *Sorghum bicolor* (26).

## MATERIALS AND METHODS

### Plant Material

Tubers of Idaho potatoes (*Solanum tuberosum*) were purchased from a local supermarket, washed with deionized

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<sup>2</sup> Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; KDOP, 3-deoxy-D-manno-octulosonate 8-phosphate; CM, chorismate mutase; Pipes, 1,4-piperazinediethanesulfonic acid; Epps, N-[2-hydroxyethyl]-piperazine-N'-3-propanesulfonic acid; DTT, dithiothreitol; PEP, phosphoenolpyruvate.

water, and soaked in water containing 25  $\mu\text{g}/\text{mL}$  streptomycin sulfate. Disks 12 mm in diameter and 13 mm thick were excised from the center tissue of the tubers and placed in Petri dishes (15 mm diameter) containing streptomycin-saturated filter paper (Whatman No. 3). The Petri dishes were wrapped in aluminum foil and stored in a cabinet at room temperature. Disks were harvested after 0 to 2 d, frozen with liquid nitrogen, and ground to a fine powder in a Waring blender. Powder was stored at  $-80^{\circ}\text{C}$ .

#### Extract Preparation (DAHP Synthase)

Frozen powder of tuber tissue (3 g/2 mL buffer) was thawed in buffer A: 50 mM K phosphate (pH 7.2) containing 0.5% (v/v)  $\beta$ -mercaptoethanol. The extract was clarified by centrifugation at 29,000g for 30 min and filtered through Miracloth to remove a lipid pellicle. A one-tenth volume of 2% protamine sulfate in buffer A was slowly added to the extract. The resulting suspension was stirred for 10 min and centrifuged at 29,000g for 20 min. The pellet was discarded and finely ground ammonium sulfate was added to the supernatant to give 60% of saturation. After being stirred for 10 min, the suspension was centrifuged at 29,000g for 20 min. The pellet was resuspended in a minimal volume of buffer B: 10 mM K Epps, (pH 7.5), 50 mM KCl, and 0.5 mM PEP. This was passed through a Sephadex G-25 (PD-10) column equilibrated with buffer B.

#### DEAE-Cellulose Chromatography (DAHP Synthase)

Extract containing approximately 80 mg protein was loaded onto a DE-52 (Whatman column (1.5  $\times$  18 cm)) equilibrated in buffer B. The column was then washed with two bed volumes of buffer B before application of a 400-mL gradient (50 to 500 mM KCl). The flow rate was 30 mL/h and fractions of 100 drops (2.9 mL) were collected and assayed for enzyme activity as described below.

#### Assay of DAHP Synthase

DS-Mn activity was assayed in a 200- $\mu\text{L}$  reaction mixture containing 50 mM K Epps (pH 8.0), 3 mM PEP, 0.6 mM erythrose-4-P, 0.5 mM DTT, and 0.5 mM  $\text{MnCl}_2$ . DS-Co activity was assayed in a 200- $\mu\text{L}$  reaction mixture containing 50 mM K Epps (pH 8.6), 3 mM PEP, 3 mM erythrose-4-P, and 10 mM  $\text{MgCl}_2$ . KDOP synthase was assayed in a 200- $\mu\text{L}$  reaction mixture containing 50 mM bis-tris-propane (pH 6.5), 3 mM PEP, and 3 mM arabinose-5-P.

Elapsed reaction times of 30, 20, and 60 min at  $37^{\circ}\text{C}$  were used in assays for DS-Mn, DS-Co, and KDOP synthase, respectively. The reaction was stopped by addition of 50  $\mu\text{L}$  of 20% trichloroacetic acid. The appearance of the products, DAHP and KDOP, were assayed at  $A_{549}$  as described by Jensen and Nester (13) using the chemical method of Weissbach and Hurwitz (29) as adapted by Srinivasan and Sprinson (28).

#### CM Assay, Extract Preparation, and Anion-Exchange Chromatography

Frozen potato powder was thawed in 1 volume of extraction buffer C (100 mM Pipes [pH 7.2], 150 mM KCl, 1 mM EDTA,

1 mM DTT, and 10% [w/v] polyvinylpyrrolidone, insoluble) squeezing through four layers of cheesecloth and 1 layer of Miracloth, and centrifuged at 15,000g for 25 min. The supernatant was desalted by passage through a Sephadex G-25 column (2.5  $\times$  50) equilibrated in buffer D (10 mM Pipes (pH 7.2), 20 mM KCl, 1 mM EDTA, and 0.5 mM DTT). Fractions containing CM activity were then applied to a DE-52 column (2.5  $\times$  15 cm) previously equilibrated with buffer D. The column was washed with two bed volumes of the same buffer, and a 500-mL gradient (50 to 350 mM KCl) was applied. The column flow rate was approximately 3 mL/min and 2.9-mL fractions were collected. CM activity was assayed as previously described (8, 9) using a concentration of 1.22 mM chorismate.

#### Protein Assays

Protein determinations were carried out using the method of Bradford (1) with bovine serum albumin as a reference standard.

#### Biochemicals

PEP (trisodium salt), erythrose-4-phosphate (sodium salt),  $\beta$ -mercaptoethanol, Epps, bovine serum albumin, and enzyme-grade ammonium sulfate were purchased from Sigma. DTT and Pipes were from Research Organics, and Bradford reagent was from BioRad. Chorismate (80% pure) was prepared (6) in our laboratory.

## RESULTS

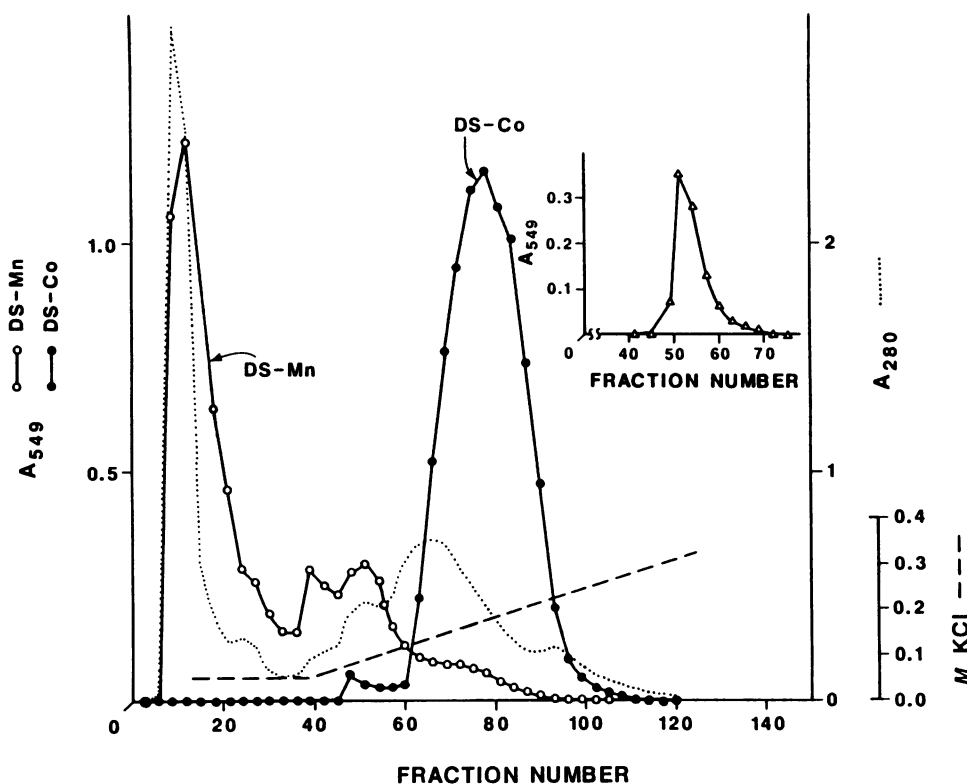
#### Resolution of DAHP Synthase Isozymes

The DS-Mn and DS-Co pair of isozymes were completely separated following ammonium sulfate fractionation and anion-exchange chromatography. Figure 1 shows the profiles obtained with the selective assays for DS-Mn and for DS-Co. The DS-Mn isozyme eluted in the wash eluate fractions, while the DS-Co isozyme eluted in the gradient at about 0.18 M KCl.

Unexpectedly, a third isozyme eluted at about fraction 50. Although this enzyme catalyzes the reaction of DAHP synthase, a distinct preference for arabinose-5-P over erythrose-4-P was noted. This KDOP synthase requires neither DTT (as does DS-Mn) nor a divalent metal (as does DS-Co). Hence, under assay conditions carried out at low pH with arabinose-5-P (or erythrose-4-P) and PEP, but lacking DTT and a divalent metal, a single peak of activity at about fraction 50 was apparent (Fig. 1, inset). The apparent peak at about fraction 40 under DS-Mn assay conditions is an artifact caused by the overlapping elution of DS-Mn and 'KDOP synthase.'

#### Differential Properties of DS-Mn and DS-Co

Further characterization of the DS-Mn and DS-Co isozymes resolved were done to compare the extent to which individual characteristics resemble those of the isozyme pair in *Nicotiana glauca*. The characteristics, upon which the differential assay procedures developed for *N. glauca* depend, were virtually identical. Thus, 0.6 mM erythrose-4-P



**Figure 1.** Chromatographic separation on a DEAE-cellulose matrix of DAHP synthase isozymes, DS-Mn and DS-Co, from potato disks that had been incubated 2 d after slicing (wounding). DS-Mn, recognized under assay conditions optimal for DS-Mn, washed through the column without retardation. DS-Co, recognized under assay conditions optimal for DS-Co, eluted in the KCl gradient (dashed line). The inset shows a third peak of activity assayed with arabinose-5-P and PEP as KDOP synthase.

was saturating for DS-Mn, while at least 6.0 mM was required to saturate DS-Co. DS-Mn exhibited a residual activity of about 9% without  $Mn^{2+}$ .  $Mn^{2+}$  was the sole divalent metal capable of stimulating DS-Mn. DS-Co exhibited an absolute requirement for a divalent cation, the order of preference being 10 mM  $Mg^{2+}$ , 0.5 mM  $Co^{2+}$ , and 0.5 mM  $Mn^{2+}$ . The activity of DS-Co was optimal in the vicinity of pH 8.6, compared to pH 8.0 for DS-Mn. The DS-Mn isozyme required DTT, and the relatively slow activation as a function of time (hysteresis) demonstrated by Ganson *et al.* (5) was observed with the potato isozyme as well. The DS-Co isozyme, on the other hand, was slightly inhibited (14%) by 0.5 mM DTT. The combined effects of these differential properties are apparent by inspection of the profiles obtained in Figure 1 by use of the procedures for differential assay of DS-Mn and DS-Co.

#### Resolution of Chorismate Mutase Isozymes

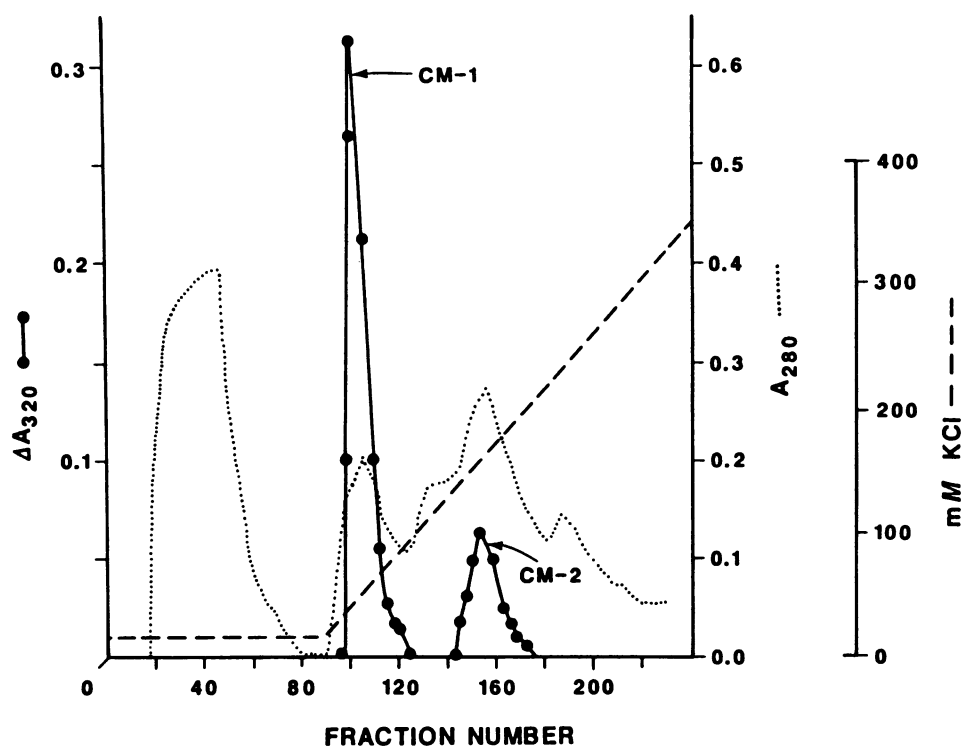
Total chorismate mutase activity was stable in desalted crude extracts, as well as in fractions obtained following DEAE-cellulose chromatography. However, the recovery of chorismate mutase was improved by the addition of L-tryptophan which stabilizes CM-1. Figure 2 shows that two peaks of chorismate mutase were cleanly resolved by use of DEAE-cellulose chromatography. The leading peak exhibited all of the properties of the plastidic CM-1 isozyme. Under standard assay conditions, it was activated 50% by 0.5 mM L-tryptophan and inhibited 40 and 55% by 0.5 mM L-phenylalanine or L-tyrosine, respectively. Chromatofocusing of the pooled CM-1 fractions on a Mono HR 5/20 column (data not shown) separated three peaks of activity. The significance of these isoforms, if any, is unknown.

The trailing peaks of chorismate mutase activity recovered from the DEAE-cellulose column was easily recognized as the CM-2 isozyme by its total insensitivity to allosteric effects mediated by aromatic amino acids. Chromatofocusing of the CM-2 isozyme band resulted in the elution of a single peak of activity at pH 4.17.

#### DISCUSSION

The location of the regulated isozymes of DAHP synthase and chorismate mutase (DS-Mn and CM-1) in plastids and the location of the unregulated isozymes (DS-Co and CM-2) in the cytosol of plants as unrelated as tobacco (4, 5), spinach (11, 14), and sorghum (26) suggested that this dual-pathway arrangement might be a generalized property of higher plants. The existence of strikingly similar isozymes of DAHP synthase and chorismate mutase in other organisms where sub-cellular localization was not established, *e.g.* mung bean (25), leaves little doubt that the compartmentation would be the same. Isozymes corresponding to CM-1 and CM-2 have been described in numerous plant species (7, 27, 30). We have described differential assay procedures that allow the selective assay of DS-Mn or DS-Co in mixtures (5). Application of this procedure to crude extracts prepared from cauliflower, broccoli, soybean, alfalfa, wheat, rye, and potato indicated the presence of both DS-Mn and DS-Co in all of these. Only one of these was isolated from cauliflower in a previous study (10). We have pointed out (6) that the diametrically opposite properties of DS-Mn and DS-Co inevitably means that optimization to one isozyme will mask the presence of the other.

Potato tuber was also reported to possess a single species of DAHP synthase (21). This was contrary to results obtained by Ganson *et al.* (5) using the differential assay procedure



**Figure 2.** Chromatographic separation on a DEAE-cellulose matrix of chorismate mutase isozymes from potato disks that had been incubated 1 d after slicing (wounding). The isozyme bands were identified by assessment of sensitivity (CM-1) or lack of sensitivity (CM-2) to allosteric effects of aromatic amino acids.

with crude extracts. Since potato tuber tissue also was reported to possess a single chorismate mutase (15), it was deemed important to study potato in more detail. Even a single exception would create a basis for rethinking the dual-pathway hypothesis.

Both isozyme CM-1 and isozyme CM-2 were separated and identified by the multiple allosteric properties of CM-1 and the absence of allosteric sensitivities in CM-2. Kuroki and Conn (16) have recently confirmed the presence of both CM species in potato. We also found both isozymes of DAHP synthase (18). One cannot be sure which of these was purified from potato (21) since the purified enzyme was not characterized; the properties attributed to the purified enzyme were in fact determined at various stages of partial purification, where unknown mixtures of isozyme may have been present.

We have shown (17) that most or all enzymes of primary aromatic biosynthesis (including DS-Mn, DS-Co, CM-1, and CM-2) are induced by mechanical wounding. The experiments described here utilize tissue harvested relatively early in the time course of enzyme elevation triggered by wounding. Streptomycin, an inhibitor of plastidic protein synthesis, was present after wounding to minimize microbial contamination. Assuming, in the absence of a positive control, that plastidic protein synthesis was indeed blocked, the results indicate that DS-Mn and CM-1 are nuclear encoded. Additionally, they must either require no posttranslational processing for activity or are not dependent upon plastid protein synthesis for posttranslational processing.

Not only were the two isozymes of DAHP synthase, DS-Mn and DS-Co, found, but a third isozyme was resolved. Although this isozyme can utilize erythrose-4-P, its substrate preference is for arabinose-5-P. Hence, it catalyzes the reaction of KDOP synthase, an enzyme of lipopolysaccharide

synthesis in gram-negative bacteria. No function is currently known for this enzyme in higher plants. The three isozymes of DAHP synthase in *Pisum sativum* reported by Rothe *et al.* (23) could have been DS-Mn, DS-Co, and the KDOP synthase.

In conclusion, potato tuber tissue has been shown to possess the isozyme pairs, DS-Mn/DS-Co and CM-1/CM-2, these isozymes having all of the salient properties characterized in detail in other plants. It would appear that potato also possesses the differently compartmented pair of anthranilate synthase isozymes (3) which was demonstrated in tobacco (2).

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