Comparison of Chorismate Mutase Isozyme Patterns in Selected Plants

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ABSTRACT

A wide variety of plants have been assayed to determine if they contain three isozymes of chorismate mutase (EC 5.4.99.5) as does alfalfa (Medicago sativa L.) or two isozymes, as does mung bean (Phaseolus aureus). The isozymes were separated by disc electrophoresis. All anthophyta with the exception of some closely related Leguminosae contained three isozymes of chorismate mutase. The one coniferophyta (a pine), and pterophyta (a fern) and one microphylophyta (a Selaginella) assayed contained two isozymes of chorismate mutase. All plants assayed contained measurable chorismate mutase levels and at least two isozymes of chorismate mutase.

In an effort to determine if most plants contain two or three isozymes of chorismate mutase, we surveyed a wide variety of anthophyta and a more limited variety of more primitive plants.

MATERIALS AND METHODS

Plant Material. Unless otherwise indicated all plant material was 6- to 7-day-old seedlings grown on cheesecloth wicks. Green plants were grown in a greenhouse and exposed to normal spring day lengths. Etiolated plants were grown in sealed drawers. Unless otherwise indicated the entire seedling was used for making extracts.

Pine needles were gathered on April 25 from a pine growing in the wooded area near the laboratory. All other mature plant material was taken from greenhouse-grown plants just before or after flowering.

Extracts. Nitrogen powders were prepared by dropping pieces of plant material into a Waring Blender containing liquid N2 and grinding until well powdered. Acetone powders were prepared by grinding plant material in a Waring Blender containing dry ice and acetone, collecting the powders on a Büchner funnel, and washing them with cold acetone until the washings were colorless. Powders were extracted twice with 0.10 mol K-phosphate (pH 6.5) at a ratio of 2 g of nitrogen powder to 1 ml of buffer or 1 g of acetone powder to 1 ml of buffer. The extracts were clarified by centrifugation for 20 min at 30,000g in the SS34 head of a Sorvall model RC2-B refrigerated centrifuge. All operations including electrophoresis and elution were performed at 4 C.

Electrophoresis. Electrophoresis was performed on 6% (w/v) polyacrylamide gels prepared according to Davis and Ornstein (5) with the exception that no spacer or sample gels were employed. Bromphenol blue was employed as the tracking dye. Fifty μl of the first mycelial extraction made 10% (w/v) in sucrose was applied gently under buffer to each of six gels, each approximately 9 cm long. The gels were subjected to a current of 3 to 4 mamp/tube. Immediately at the end of each run five gels of each set were frozen in liquid N2 and stored overnight at 20 C. One gel of each set was stained for protein by immersion for 12 hr in 2.5% (w/v) Amido Schwarz. These were destained by repeated flushing with 7% (w/v) acetic acid.

Gel Extraction and Assay. The frozen gels were lined up with dye fronts matching and cut into sections 3 mm long. Corresponding sections from each gel were pooled and put into a test tube (1.2 x 7.5 cm) to which was added 1 ml of 0.10 mol K-phosphate (pH 6.5). Gels and buffer were incubated for 4 to 6 hr with occasional hand shaking. When the dye band was evenly distributed between gel and buffer, we assumed that protein elution was completed. Elution of chorismate mutase activity ranged from 80 to 100% of that originally applied, with the exception of that from beans, 67% (Table III). Appropriate aliquots were used for assays of chorismate mutase, using a slight variation of a method developed by Nishioka and Woodin (24). Assay mixtures consisting of 0.60 ml of 0.10 mol K-phosphate (pH 6.5), containing 1 μmol of

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3 Abbreviations: DAHP: deoxyarabino-heptulosonic-acid-phosphate; SK: shikimate kinase.
chorismic acid and an appropriate aliquot of gel eluate, were incubated at 37°C for 30 min. The reaction was stopped by adding 0.20 ml of 28% (w/v) trichloroacetic acid. After a 15-min incubation at room temperature, 2.4 ml of 1.25 M borate-2.5 M K-phosphate (pH 8.5), was added. At 300 nm was read after 30 min, using a Coleman-Hitachi model III single beam spectrophotometer. One unit of enzyme activity represents 1 μmol of product produced in 1 min. Protein concentration was determined by the biuret method (11).

Chemicals. Chorismic acid was prepared according to Gibson (6). All other chemicals were purchased from Baker (inorganic salts and acids, solvents) or Sigma (organic compounds) as the purest grade available and used without further purification. Chorismic, caffeic, fericulic, and 3,4-dimethoxyxycinnamic acids were chromatographed on Brinkmann thin layer plates (cellulose NM300 UV-254) in methanol-butanol-benzene-water (2:1:1:1, v/v). Each exhibited only one UV absorbing spot.

**RESULTS**

As can be seen from Table III, there are two distinct patterns of chorismate mutase isozyme distribution in higher plants. The more primitive plants assayed, such as the pine, fern, and Selaginella, each contain two isozymes of chorismate mutase. Except for the mung bean and a few closely related Leguminosae (peas, French beans), all anthophyta surveyed contain the three isozymes of chorismate mutase (CM1, CM2, and CM3) previously reported in extracts of alfalfa (27).

The isozyme patterns reported in Table III were based on relative mobility in the electrophoresis system used. The relative mobilities of the three chorismate mutase isozymes in alfalfa have...
already been well established (27) and were used as a standard. In all plants assayed chorismate mutase peaks appeared in essentially the same segments of gel. In those plants containing three isozymes of chorismate mutase, peaks of activity were found in segment 17 (varied from 14 to 19) for CM₁, segment 23 (varied from 22 to 25) for CM₂, and segment 28 (varied from 26 to 29) for CM₃. The peaks of activity in those plants containing two isozymes of chorismate mutase appeared in segments 17 to 19 for CM₁ and segments 22 to 26 for CM₂.

Since each isozyme of chorismate mutase in mung bean (CM₁, CM₂ and alfalfa (CM₁, CM₂, CM₃) is distinguished by its effector sensitivity (8, 27) as well as by its electrophoretic mobility (this paper and ref. 27), knowledge of metabolite sensitivity of mutase isozymes is necessary to confirm isozyme type. Aliquots from appropriate gel eluates having high chorismate mutase activity were assayed for sensitivity to both aromatic amino acids and to selected lignin precursors (Table IV). Other plants surveyed for chorismate mutase isozyme metabolite sensitivity patterns were etiolated corn seedlings, green and etiolated pea seedlings, green and etiolated cucumber seedlings, fern leaves, and mature squash. Results for these plants were substantially the same as reported here for green corn seedlings. Thus, the metabolite sensitivity of the mutase isozymes for all plants investigated parallels that previously reported for CM₁, CM₂, and CM₃ in alfalfa (27) or CM₁ and CM₂ in mung bean (8).

Previous assays using mung bean extracts had been performed at 25 C (8). Since all assays reported in Tables III and IV had been performed at 37 C, we explored metabolite sensitivity of mung bean CM₁ and CM₂ at both 25 C and alfalfa CM₁, CM₂, and CM₃ at a variety of temperatures. For all forms of chorismate mutase examined, metabolite sensitivity at all temperatures investigated was essentially the same as reported in Table IV and had been previously reported (27).

Plant age or plant part had little effect on the number of isozymes found. We had previously shown this to be the case with alfalfa (27) where the number and relative amount of each chorismate isozyme were relatively constant whether green seedlings, etiolated seedlings, mature stems, flowers, or leaves were surveyed.

Extracts from seedlings and more mature plants of corn and tobacco contained three isozymes of chorismate mutase. Similarly, extracts of both mature and seedling (green and etiolated) peas and mung beans exhibited only two mutase isozymes. However, the ratios of CM₁ and CM₂ in mung bean seedlings were different in green and etiolated tissues (Woodin and Nishioka, unpublished).

### DISCUSSION

Chorismate mutase patterns in higher plants vary somewhat with taxonomic type. Vascular but primitive plants, such as the fern and Selanginella (cryptograms) and pine (coniferophyta), contained two isozymes of chorismate mutase (CM₁ and CM₂) while the more complex anthophyta studied here, with the minor exception of some members of the Lotoideae subfamily of the Leguminosae, all contained three isozymes of chorismate mutase.

Our sampling of nonflowering plants was extremely limited. This reflects the fact that we found extracts of most ferns, conifers, and mosses very viscous and difficult to assay for chorismate mutase activity both before and after electrophoresis. Our aim was to manipulate the extracts as little as possible and so the only alterations in general procedure were to concentrate the Selanginella and fern extracts 2-fold and the Eichornia extract 2.5-fold before electrophoresis. It is still entirely possible that had we exploited the nonvascular and/or nonseedbearing plants more completely we would have found among them some plants containing more or less than two isozymes of chorismate mutase or had we explored the angiosperms more completely some plants would have been found which are not in the Leguminosae family but do contain only two chorismate mutase isozymes.

It is also possible, considering prior work on plants containing three chorismate mutase isozymes (27), that the lack of a third chorismate mutase isozyme in some plants is due to CM₂ being highly unstable.

There are two points of interest in this study: (a) the functional significance of two versus three isozymes of chorismate mutase; and (b) the relation of chorismate mutase isozyme patterns to taxonomic types.

The functional significance of three or even two isozymes of chorismate mutase to an organism is difficult to assess without...
detailed knowledge of the metabolites which are formed from chorismate and/or prephenate and the ensuing metabolic pathways to these metabolites. In plants the conversion of chorismate to prephenate is an important event necessary for the ultimate productions of the lignin precursors cinnamic, caffeic, ferulic, and sinapic acids all of which are also either phytoalexins or precursors of such (13). Thus, it may well be that there are periods in a plant's development, such as a disease state or when a rapid rate of lignification occurs, that the presence of a chorismate mutase such as CM2 which is sensitive to changes in concentration of lignin precursors, but not to the aromatic amino acids, would be advantageous. During rapid growth spurts protein synthesis predominates, and CM1- and CM4-type chorismate mutase activity might help ensure a balanced supply of aromatic amino acids. An answer to the question of the advantages to an organism for three versus two chorismate mutase isoforms would require detailed studies of chorismate mutase isozone levels during growth and development and during stress situations of various plants.

Crawford (4), in a detailed review of the enzymes catalyzing synthesis of tryptophan from chorismate, cites the notable uniformity presented by the reactions of the pathway and the great variety of regulatory patterns and associations in the enzymes catalyzing the pathway. The same can be said of those enzymes in the pretryptophan pathway catalyzing synthesis of chorismate from erythrose-4-P and P-enolpyruvate. With the possible exception of T. rubrum the pathway for synthesis of chorismate and for prephenate is the same in all organisms investigated. The regulatory patterns in the pathway vary strikingly with the type of organism investigated, varying from no apparent regulation in Streptomyces sp. (9) to the complex enzyme aggregations reported for Bacillus subtilis (15) and several fungi (13, 17).

Jensen and Pierson (18) have found evidence for some divergence of the enzymes for the synthesis of L-tyrosine in blue-green algae (Table II). They report that in these organisms the transamination step precedes the reduction step so that prephenate is transaminated and the product of that reaction (pretyrosine) is then oxidized to tyrosine via an NAD-dependent reaction. They speculate that such a unique pathway confined to one set of organisms may reflect the sequence of evolutionary events leading to their divergence. The same may be true of varying patterns of isozyme distribution.

The result of the study presented here provides preliminary evidence that in higher plants the isozyme pattern of at least one enzyme (chorismate mutase) in the shikimate pathway is related to taxonomic type and warrants more definite comparative studies of chorismate mutase isoforms and of other enzymes in the shikimate pathway in higher plants.

LITERATURE CITED

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