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Chorismate Mutase Isoenzymes from Selected Plants and Their Immunological Comparison with the Isoenzymes from *Sorghum bicolor*[1]

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ABSTRACT

The isoenzyme pattern of chorismate mutase (EC 5.4.99.5) was examined by diethylaminoethyl-cellulose chromatography in a wide variety of plants. All plants contained a regulated form of chorismate mutase (CM-1), and most contained an additional, unregulated form (CM-2). The regulatory properties of CM-1 differed significantly between plants. Antisera prepared against CM-1 and CM-2 from *Sorghum bicolor* were used to test immunological cross reaction of chorismate mutases from other plants. There was a high degree of similarity between chorismate mutase isoenzymes from *Sorghum bicolor* and *Zea mays* and some with *Hordeum vulgare*, but all other species studied were antigenically distinct from sorghum. No homology between the structure of CM-1 and CM-2 was detected within any species.

The shikimate pathway provides the precursors for the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in plants as well as in microorganisms (8, 12, 19). In plants, these amino acids are the precursors of a wide variety of secondary compounds such as alkaloids, lignin precursors, indole derivatives, flavonoids, and other phenolic compounds (24, 28). Chorismic acid occupies a pivotal position in this pathway and serves as a substrate for chorismate mutase and anthranilate synthase. Chorismate mutase gives rise to prephenic acid, the common precursor of phenylalanine and tyrosine, while anthranilate synthase catalyzes the first unique reaction in the pathway to tryptophan. Chorismate mutase, due to its regulatory properties, has been implicated to be a control point in the biosynthesis of phenylalanine and tyrosine (10, 14).

Chorismate mutase has been isolated in different forms from several plants (9, 13, 14, 22, 26). One form (designated CM-1) is activated by tryptophan and is inhibited by phenylalanine and tyrosine. The other form (designated CM-2) is not regulated by the aromatic amino acids. In tobacco (6) and sorghum (BK Singh, EE Conn, unpublished data), the two forms of chorismate mutase are compartmentalized within the cells: CM-1 in the chloroplast and CM-2 in the cytosol. On this basis, it has been postulated that separate and probably complete pathways of phenylalanine and tyrosine biosynthesis exist in both the chloroplast and the cytosol (17).

Even though all chorismate mutases are capable of catalyzing the same reaction, there is very little information available showing the molecular relationship of the two isoenzymes both within and between different species. Data available so far indicate that the size of chorismate mutase is different for the two isoenzymes and is species dependent. The mol wt of CM-1 has been found to range from 46,000 in alfalfa (26) to 56,000 in sorghum (22). The reported mol wt of CM-2 ranges even more widely from 36,000 for mungbean (11, 13) to 65,000 for tobacco (14). A simple and effective method of examining chemical similarity and structural homology of proteins is through the use of immunological cross reactions. Most of the evolutionary substitutions in proteins are immunologically detectable (25) and such studies have been carried out to examine the evolutionary divergence of proteins such as catalase, Cyt *c*, lysozyme and trypsin (1). This technique has also been used to identify structural similarities between isoenzymes (7, 23) as well as between different subunits of the same enzymes (15).

In spite of the importance of chorismate mutase in phenylalanine and tyrosine biosynthesis, the information available in the literature is limited to a very few species. In this report, we present the partial purification, the regulatory properties and antigenic similarity to the sorghum enzyme of chorismate mutases from several species, differing in morphology (monocots and dicots) and physiology (C_3, C_4, and CAM).

MATERIALS AND METHODS

Plant Material. The plants used for this study were selected on the basis of their morphological and physiological characteristics. Green seedlings of *Avena sativa* (C_3, monocot), *Brassica oleracea* (C_3, dicot), *Hordeum vulgare* (C_3, monocot), *Pennisetum typhoides* (C_4, monocot), and *Zea mays* (C_4, monocot) were grown by planting seeds in wet vermiculite in a growth chamber at 28°C under 12 h fluorescent illumination. Shoots from 6- to 7-d-old seedlings were used for the extraction of enzyme. Young, green leaves of *Amaranthus hypochondriacus* (C_4, dicot), *Hoya carnosa* (CAM, dicot), *Medicago sativa* (C_3, dicot), and *Xerosicyos danguyi* (CAM, dicot) were obtained from adult plants grown in a greenhouse and exposed to normal spring and summer daylengths. Young, green leaves from wild adult plants of *Eschscholtzia californica* (C_3, dicot) were used. *Spinacia oleracea* (C_3, dicot) leaves were obtained from a local supermarket.

Enzyme Extraction. For the extraction of chorismate mutase, 20 to 25 g of shoots or leaf tissues were powdered in liquid N_2 and extracted at 0 to 4°C with one volume of buffer A (100 mM Tris-HCl, pH 8.0, containing 1 mM tryptophan and 0.1% 2-mercaptoethanol). The extract was filtered through two layers of cheesecloth (grade 60) and centrifuged at 60,000*g* for 90 min in a Beckman model L ultracentrifuge. All subsequent purification steps were carried out at 0 to 4°C.

Sephadex G-25 Desalting. The centrifuged supernatant was loaded at 7 ml/min onto a bed of Sephadex G-25 (2.5 × 29.0

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Table I. *Purification of Chorismate Mutase*

Plant	Fraction	Isoenzyme	Volume	Protein	Activity	Specific Activity
			ml	*mg*	*units*	*units/mg*
Amaranthus hypochondriacus	Sephadex G-25	CM-1 + CM-2	53.0	150.7	0.77	0.005
	DEAE-cellulose	CM-1	9.5	9.8	0.46	0.047
		CM-2	9.5	33.3	0.17	0.005
Avena sativa	Sephadex G-25	CM-1	43.0	299.9	2.86	0.009
	DEAE-cellulose	CM-1	8.3	65.1	1.63	0.025
Brassica oleracea	Sephadex G-25	CM-1	45.0	206.0	0.26	0.001
	DEAE-cellulose	CM-1	5.5	30.2	0.18	0.006
Eschscholtzia californica	Sephadex G-25	CM-1	40.0	162.0	0.81	0.005
	DEAE-cellulose	CM-1	10.0	75.0	0.75	0.010
Hordeum vulgare	Sephadex G-25	CM-1 + CM-2	43.0	181.5	0.86	0.005
	DEAE-cellulose	CM-1	10.5	37.1	0.49	0.013
		CM-2	6.0	1.4	0.11	0.075
Hoya carnosa	Sephadex G-25	CM-1 + CM-2	53.0	54.1	0.25	0.005
	DEAE-cellulose	CM-1	6.1	3.0	0.11	0.037
		CM-2	6.7	3.2	0.03	0.009
Medicago sativa	Sephadex G-25	CM-1 + CM-2	45.0	251.0	3.45	0.014
	DEAE-cellulose	CM-1	8.8	88.5	1.97	0.022
		CM-2	8.4	26.1	0.13	0.005
Pennisetum typhoides	Sephadex G-25	CM-1	40.0	86.9	1.96	0.023
	DEAE-cellulose	CM-1	15.0	10.4	1.40	0.135
Sorghum bicolor[a]	Sephadex G-25	CM-1 + CM-2	850.0	1443.0	59.8	0.041
	DEAE-cellulose	CM-1	25.0	84.0	22.3	0.265
		CM-2	34.0	230.0	28.9	0.126
Spinacia oleracea	Sephadex G-25	CM-1 + CM-2	43.0	74.7	1.20	0.016
	DEAE-cellulose	CM-1	7.5	37.9	0.86	0.023
		CM-2	4.5	6.4	0.26	0.041
Xerosicyos danguyi	Sephadex G-25	CM-1 + CM-2	53.0	62.5	0.75	0.012
	DEAE-cellulose	CM-1	7.2	4.4	0.21	0.048
		CM-2	19.1	12.7	0.21	0.016
Zea mays	Sephadex G-25	CM-1 + CM-2	52.0	153.9	6.89	0.045
	DEAE-cellulose	CM-1 + CM-2	16.5	61.2	6.00	0.098
	Amicon Blue A	CM-1	7.5	2.3	2.27	0.987
		CM-2	3.0	2.0	1.98	0.990

[a] Data from Singh *et al.* (22).

cm, coarse) equilibrated with buffer A. The column was eluted under the same conditions. Fractions (9 ml) were collected and assayed for chorismate mutase activity.

DEAE-Cellulose Chromatography. The pooled fractions from the G-25 column were loaded onto a Whatman DE 52 column (2.2 × 15.0 cm) equilibrated with buffer A. The column was then washed with two bed volumes of equilibration buffer and eluted with a linear salt gradient (500 ml, 0–0.5 M KCl in buffer A). Fractions (9 ml) were collected and assayed for chorismate mutase activity. The fractions with peak activity were pooled separately and concentrated by ultrafiltration in an ultrafiltration cell (Amicon, PM 10 membrane) at a pressure of 25 p.s.i.

Chorismate Mutase Assay. Chorismate mutase activity was measured by estimation of the product, prephenate, after its conversion by acid to phenylpyruvate (4). The phenylpyruvate concentrations were determined using its A at 320 nm (E = 17,500 M^{-1} cm^{-1}) in alkali. Standard reaction mixtures (0.5 ml) contained 50 mM Tris-HCl (pH 8.0) and 1 mM chorismate. The reaction was started by the addition of enzyme, incubated at 25°C for 10 min and was stopped with 0.1 ml of 6 N HCl. Acid conversion of prephenate to phenylpyruvate was complete after 10 min at room temperature at which point 0.4 ml of 4 N NaOH was added. The A of 320 nm was measured immediately against a 1 N NaOH blank. One unit of enzyme activity is defined as 1 μmol of prephenate produced in 1 min. To perform activation and inhibition experiments, enzyme preparation was passed

Table II. *Summary of the Total Enzyme Activity, the Activation of CM-1 by Tryptophan and the Neutralization of CM-1 and CM-2 by Antiserum*

Details of the experiments are described in "Materials and Methods."

Plant	Type	No. of Isoenzyme	Total Enzyme Activity	Activation of CM-1 by 1 mM Tryptophan	Antiserum for 50% Inhibition	
					CM-1	CM-2
			unit/g fresh wt	*-fold*	*μl/unit*	
Amaranthus hypochondriacus	C_4, Dicot	2	0.031	17.1	NR[a]	NR
Avena sativa	C_3, Monocot	1	0.114	1.4	NR	IA[b]
Brassica oleracea	C_3, Dicot	1	0.013	2.1	NR	IA
Eschscholtzia californica	C_3, Dicot	1	0.032	2.4	NR	IA
Hordeum vulgare	C_3, Monocot	2	0.040	9.6	1040	>7000
Hoya carnosa	CAM, Dicot	2	0.010	6.0	NR	NR
Medicago sativa	C_3, Dicot	2	0.138	2.2	NR	NR
Pennisetum typhoides	C_4, Monocot	1	0.078	11.0	NR	IA
Sorghum bicolor[c]	C_4, Monocot	2	0.199	TD[d]	55	35
Spinacia oleracea	C_3, Dicot	2	0.048	TD	NR	NR
Xerosicyos danguyi	CAM, Dicot	2	0.030	4.0	NR	NR
Zea mays	C_4, Monocot	2	0.276	16.4	325	205

[a] No reaction. [b] Isoenzyme absent. [c] Data from Singh and Conn (21) and Singh *et al.* (22). [d] CM-1 activity was totally dependent upon the presence of tryptophan.

through a Sephadex G-25 (PD 10) column to remove tryptophan. The column was equilibrated with buffer A, omitting tryptophan.

Protein Determination. Protein concentrations were determined by the method of Bradford (2) using crystalline BSA as the protein standard.

Antiserum Preparation. Details of the immunization protocol and antiserum preparation have been previously described (21). Separate sets of mice were immunized with either CM-1 (5 mice) or CM-2 (4 mice) from *Sorghum bicolor.* Four injections of chorismate mutase emulsified with an equal volume of Freund's adjuvant into the backs of 4- to 6-week-old BALB/c mice were made (10 μg protein per mouse per injection). Two weeks after the final injection, serum was collected from these mice separately and tested for the antibody of chorismate mutase. High titer sera were pooled and used as a source of polyclonal serum without further purification. Serum collected from the same mice before immunization was used as the control.

Neutralization of Chorismate Mutase Activity with Antiserum. The effect of antisera on chorismate mutase activity was determined in reaction mixtures containing 0.2 mg BSA, 0.05% 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0), antiserum, and chorismate mutase in a total volume of 50 μl. Controls for each reaction series contained either no serum or the preimmune serum. After incubating at room temperature for 30 min, the mixtures were further incubated on ice for 2 h before centrifugation at 10,000*g* for 5 min. An aliquot of the supernatant in each reaction mixture was assayed for chorismate mutase activity. Prephenate formed in the tube containing no serum or preimmune serum was the same and was designated as 100% chorismate mutase activity. The activity data from other reactions were expressed as a percent of this activity. The enzyme activity in the control tubes was also used to calculate the units of chorismate mutase activity present in each tube.

RESULTS

Isolation and Separation of Chorismate Mutase Isoenzyme. Crude extracts prepared from all species exhibited very high background due to strong absorbance by phenolics under the assay conditions described. Chorismate mutase activity could, therefore, be estimated accurately only after Sephadex G-25 chromatography which separated the proteins from the phenolic compounds. The specific activity of chorismate mutase at this step was as low as 0.001 unit/mg protein in *B. oleracea* to as high as 0.045 unit/mg protein in *Z. mays* (Table I). Total activity varied greatly between different species ranging from 0.010 unit/g fresh weight of *H. carnosa* to 0.276 unit/g fresh weight of *Z. mays* (Table II). These values varied by 10 to 50% between different preparations depending upon the age and source of the tissue. This variation does not affect the conclusion that there are large differences between species in the chorismate mutase activity per unit weight of tissue. In all species, the activity was partially inhibited by phenylalanine and tyrosine, and activated by tryptophan (data not presented).

Chromatography on DEAE cellulose of the extract from the previous step yielded a single peak of chorismate mutase activity in *A. sativa, B. oleracea, E. californica, P. typhoides,* and *Z. mays* (Figs. 1 and 2). Chorismate mutase activity from the remaining group of plants was separated into two peaks (Fig. 1). The enzyme activity in the second peak (CM-1) as well as in the group of plants with a single peak was activated by tryptophan and inhibited by phenylalanine and tyrosine. In the group of plants with two peaks of chorismate mutase activity, the first peak (CM-2) was insensitive to these amino acids.

Recovery of a single peak of chorismate mutase activity from some plants may be due to the lack of separation of isoenzymes by DEAE cellulose chromatography in these species. Therefore, attempts were made to separate isoenzymes of chorismate mutases by chromatography of the activity obtained after elution from DEAE-cellulose column. Indeed, two peaks of activity were resolved after chromatography of the enzyme from *Z. mays* on Amicon Matrex Blue A (Fig. 2B). The first peak of activity did not bind to the resin and was inhibited by phenylalanine and tyrosine and activated by tryptophan. The second peak of activity (CM-2) was recovered by salt gradient elution and was found to be unaffected by the aromatic amino acids. Separation of isoenzymes by Amicon Matrex Blue A chromatography was not achieved in other species.

Large differences between species were found in the activation of CM-1 by 1 mM tryptophan. CM-1 showed as little activation as 1.4-fold in *A. sativa* while at the other extreme, it showed complete dependence of activity on the presence of tryptophan

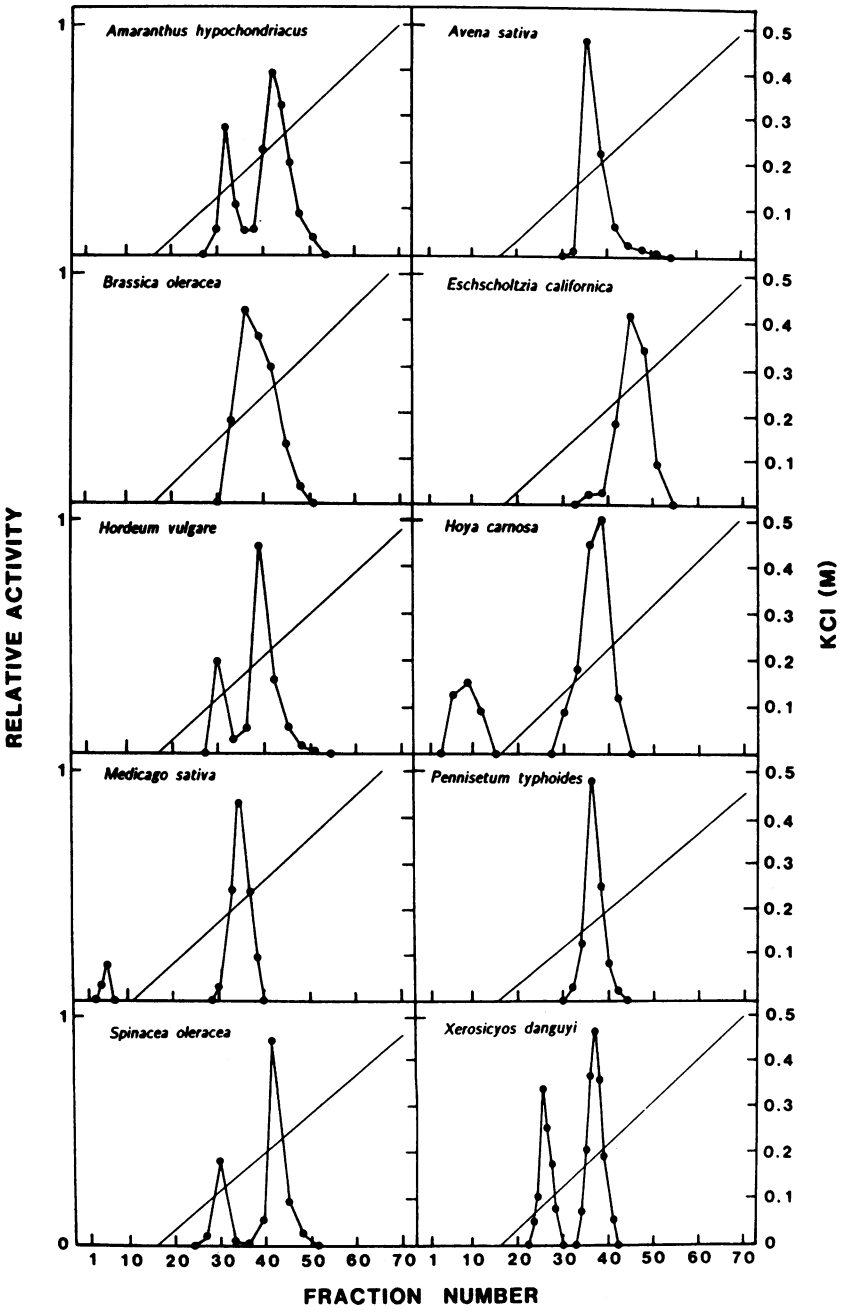

FIG. 1. Chromatography of chorismate mutase isoenzymes from different plants on DEAE-cellulose column.

in *S. oleracea* (Table II). Lack of CM-1 activity in the absence of tryptophan was also observed in sorghum (11). The degree of inhibition of CM-1 activity by phenylalanine and tyrosine also varied greatly between species (data not shown).

Immunological Comparison of Chorismate Mutase. Antisera produced against highly purified CM-1 and CM-2 from sorghum were used to test cross-reaction with the chorismate mutases isolated from species described above. Neutralization of enzyme activity was chosen as a means to detect the antigen-antibody interaction primarily due to the ability of this method to demonstrate the binding of antibody with the specific enzyme under investigation. Enzymes isolated from species other than *H. vulgare* and *Z. mays* did not show any inhibition when incubated with the sorghum antisera. Increasing amounts of antiserum 1 (against CM-1) inhibited the activity of CM-1 alone in both *H. vulgare* and *Z. mays*; CM-2 activity was not inhibited in either case (Figs. 3A and 4A). Similarly, increasing amounts of antiserum 2 (against CM-2) inhibited the activity of only CM-2, CM-1 not being affected (Figs. 3B and 4B). This suggests the presence of common antigenic determinants within each isoenzyme from *S. bicolor*, *H. vulgare*, and *Z. mays*. Even though these isoenzymes contain common antigenic determinants, the amount of antiserum (μl/unit) required for 50% inhibition of chorismate mutase activity was much higher in *Z. mays* and *H. vulgare* as compared to that in *S. bicolor* (Figs. 3 and 4; Table II). The degree of inhibition of both isoenzymes by antisera from *S. bicolor* was higher in *Z. mays* than in *H. vulgare*.

DISCUSSION

Our results clearly demonstate that there is great diversity in the isoenzyme patterns of chorismate mutase in higher plants. All species examined in the present study exhibited the presence of the regulated form of chorismate mutase (CM-1). On the other hand, the unregulated form of chorismate mutase (CM-2) could

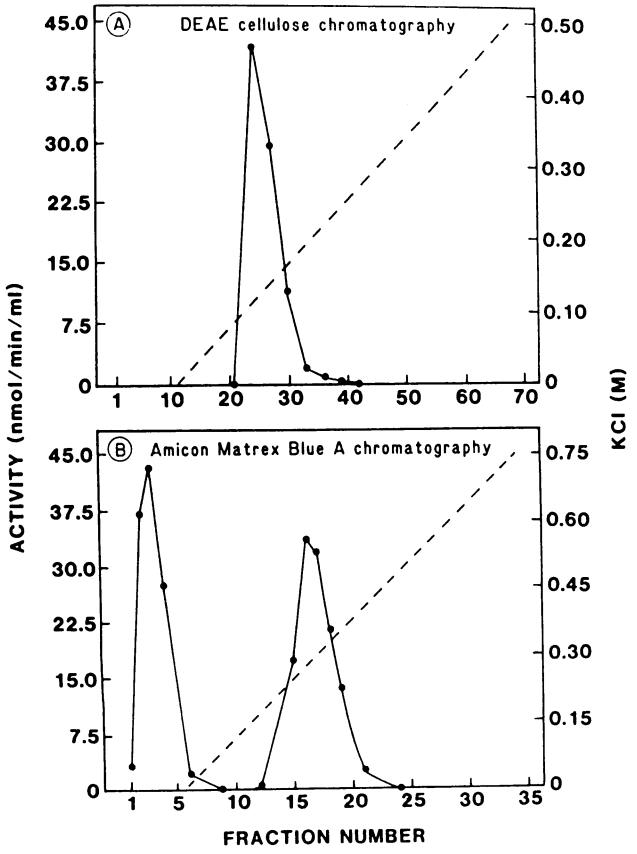

FIG. 2. Chromatography of chorismate mutases from *Z. mays* on DEAE-cellulose (A) and Amicon Matrex Blue A (B).

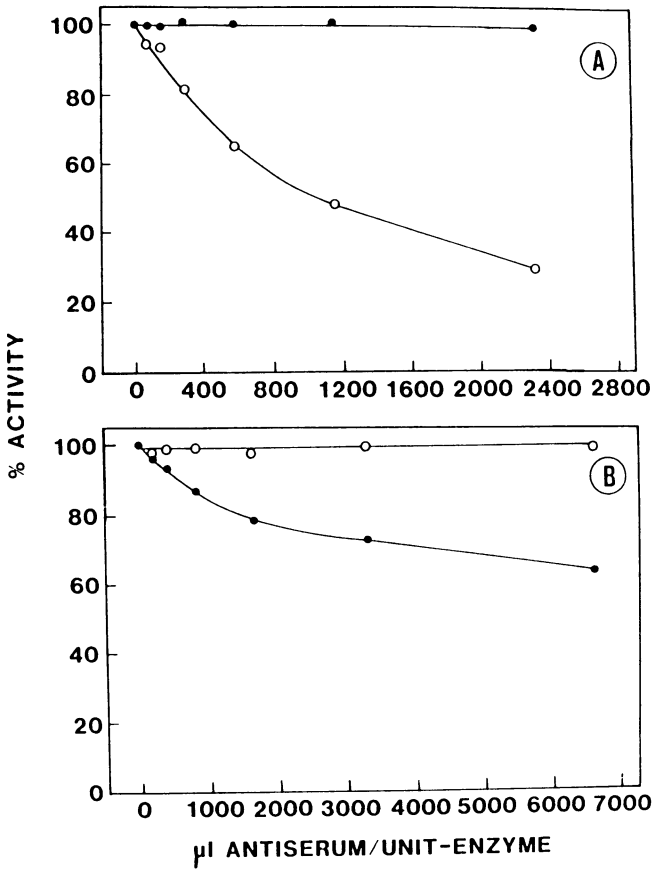

FIG. 3. Neutralization of chorismate mutase activity from *H. vulgare* with increasing amounts of antiserum 1 (○——○) or antiserum 2 (●——●). (A) CM-1; (B) CM-2.

only be isolated from selected species (Fig. 1). Our inability to detect CM-2 in some species suggests the presence of only one form of chorismate mutase in such plants; however, lack of separation of the isoenzymes with the chromatographic technique employed cannot be ruled out. Although CM-2 has been found to be highly stable in other species examined previously (11, 13, 14, 22), we also cannot eliminate the possibility of the loss of this isoenzyme during purification.

Reports from another laboratory (26, 27) have indicated presence of three isoenzymes in a large number of plant species including three species examined in this work (*A. sativa*, *M. sativa*, and *Z. mays*). However, these findings have not been confirmed by any other laboratory. In the present work, only one peak of chorismate mutase activity was observed after DEAE cellulose chromatography of the extracts from *Z. mays* (Fig. 2A) which is in agreement with a previous report (3). Similar contradictory results were obtained for *Nicotiana tabacum* by Goers and Jensen (14) who argued that the third isoenzyme reported earlier (27) may prove to be an electrophoretic variant of one of the four allelic CM-1 cistrons of *N. tabacum*. Authors (26, 27) presenting the evidence for three isoenzymes of chorismate mutase argued that the lack of a third chorismate mutase isozyme in some plants was due to CM-3 (the third isoenzyme) being highly unstable. However, lack of support for their findings from any other laboratory makes the existence of CM-3 questionable.

Several enzymes of glycolysis and the pentose phosphate pathway are present in higher plants as isoenzymes that are compartmentalized, most frequently in the plastids and the cytosol. Similar findings have been made for some of the shikimate pathway enzymes (17) including chorismate mutase (6) and on this basis arguments are being made that separate biochemical pathways of aromatic amino acid biosynthesis may exist in the cytosolic and the plastid compartments of a plant cell (17). However, our recovery of only one form of chorismate mutase from several plants would argue against such a hypothesis. A detailed study of isoenzyme patterns, their properties and subcellular localization, is required before a generalization of this sort can be made.

Extensive kinetic characterization of CM-1 and CM-2 from these plants was not carried out. However, a wide range of activation of CM-1 by tryptophan between different species was found (Table II). Neither the isoenzyme pattern nor the activation by tryptophan could be correlated with the morphology or the physiology of the species. Even though these data provide information regarding the diversity in the kinetic properties of the enzyme from various sources, they are not useful in identifying homology in the structure of the protein. However, immunological comparison helps in this regard and also examines evolutionary divergence. The presence of common antigenic determinants in the enzymes from a group of plants indicates that these plants are derived from a common ancestor (see introductory remarks). In the present study, inhibition of chorismate mutase activity by the antiserum was used as a basis to measure the amount of antigen-antibody complex. This method can be used to screen for the cross-reactivity of antigens with a single antiserum (16). Only the enzymes isolated from *H. vulgare* and *Z. mays* reacted with sorghum antisera. Enzymes from several other monocots and all dicot species failed to show any cross-reaction. Chorismate mutases from *Z. mays* reacted very strongly with the antisera and therefore appear to be most closely related to sorghum chorismate mutases. The absence of a high degree of immunological relatedness between chorismate mutases from the species examined in the present study suggests that chorismate mutase is a highly divergent protein.

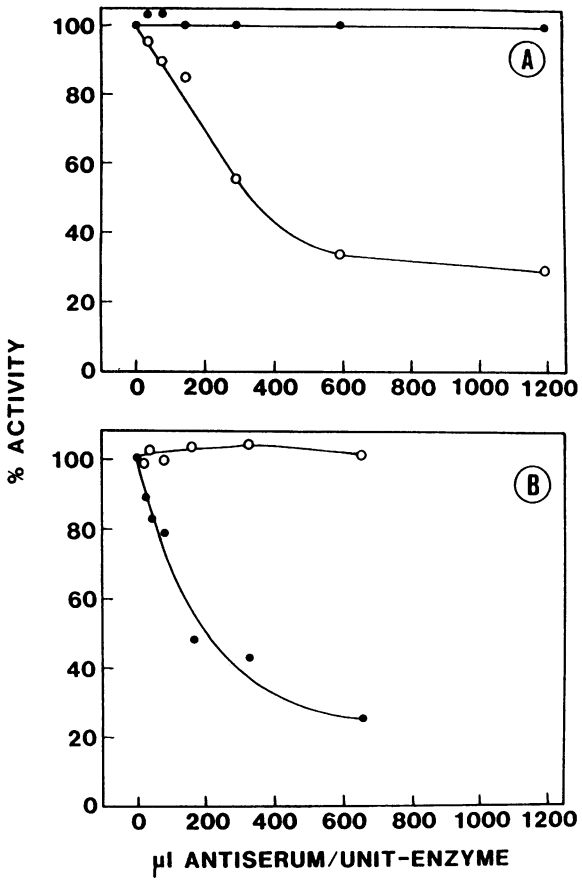

FIG. 4. Neutralization of chorismate mutase activity from *Z. mays* with increasing amounts of antiserum 1 (○——○) or antiserum 2 (●——●). (A) CM-1; (B) CM-2.

Lack of cross-reaction between antiserum 1 and CM-2 or antiserum 2 and CM-1 from either *H. vulgare* or *Z. mays* supports our previous findings with sorghum isoenzymes (21) that the two isoenzymes are immunologically distinct. On this basis, it is reasonable to speculate that the two isoenzymes are the products of separate genes. Similar results have been reported for tryptophan synthase from bacteria (5, 18, 20). In this case, no antigenic determinants were shared by the two *trp* A and *trp* B gene products. However, a high degree of cross-reactivity was found within each gene product in the enteric bacteria.

Plants used in this study were chosen from different morphological (monocot and dicot) and photosynthetic (C_3, C_4, and CAM) types. No relationship between these plant types and the chorismate mutase isoenzyme pattern or their kinetic properties was observed. Immunological cross-reaction was found betweeen some plants from monocots (*H. vulgare*, *S. bicolor*, and *Z. mays*) alone suggesting homology in the structure of this protein within this group. However, lack of cross-reaction with the enzyme from other plants (*A. sativa* and *P. typhoides*) indicates the homology observed within this group is restricted to a limited number of plants.

In summary, chorismate mutase is a highly divergent enzyme. The differences between species are found in the isoenzyme pattern of chorismate mutase as well as in the kinetic, physical and immunological properties of these isoenzymes. Results presented here also demonstrate that there was little relation between these properties and the morphology or the physiology of the species.

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