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5-*enol*-Pyruvyl-Shikimate-3-Phosphate Synthase from *Zea mays* Cultured Cells[1]

Purification and Properties

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The shikimate pathway enzyme 5-*enol*-pyruvyl-shikimate-3-phosphate (EPSP) synthase (3-phosphoshikimate-1-carboxyvinyl transferase, EC 2.5.1.19) was purified from cultured maize (*Zea mays* L. var Black Mexican Sweet) cells. Homogeneous enzyme preparations were obtained by a four-step procedure using ammonium sulfate fractionation, anion- and cation-exchange chromatography, and substrate elution from a cellulose phosphate column. The last step resulted in two well-separated activities of about the same molecular weight. A 2000- to 3000-fold purification, with an overall recovery of one-fourth of the initial activity, was achieved. Both EPSP synthase isoforms were characterized with respect to structural, kinetic, and biochemical properties. Only slight differences are seen in molecular mass, activation energy, and apparent affinities for the two substrates. A more pronounced difference was found between their thermal inactivation rates. Two EPSP synthase isoforms were also elucidated in crude homogenates by anion-exchange fast protein liquid chromatography. This allowed us to follow their expression during a culture growth cycle. One form was found at substantial levels throughout, whereas the other increased in exponentially growing cells and declined in late-logarithmic phase. The analysis of highly purified plastid preparations demonstrated a plastidial localization of both proteins. Possible functional roles for maize EPSP synthase isozymes, with regard to the dual-pathway hypothesis and to the recent findings on defense-related aromatic biosynthesis in higher plants, are discussed.

The sixth enzyme of the shikimate pathway leading to aromatic amino acid biosynthesis, EPSP synthase (3-phosphoshikimate-1-carboxyvinyl transferase, EC 2.5.1.19), catalyzes the reversible addition of the *enol*pyruvyl moiety of PEP to S3P. EPSP synthase activity has been detected and studied in a number of organisms, and the enzyme has been purified from bacteria, fungi, and dicotyledonous plants. Among monocots, studies have been reported only for *Sorghum bicolor* (Ream et al., 1988).

Interest in the characterization of plant EPSP synthase has been renewed since the enzyme has been identified as the target of the broad-spectrum postemergence herbicide glyphosate (Steinrücken and Amrhein, 1980). Glyphosate shows competitive inhibition for PEP and uncompetitive inhibition for S3P (Boocock and Coggins, 1983). Because EPSP synthase is absent in mammals, glyphosate has a very low toxicity for animals (Grossbard and Atkinson, 1985). The selection of mutant EPSP synthases or the isolation of naturally resistant enzymes and their transfer into the most widely cultivated crops should be of great agronomic value (Racchi, 1990).

An intact complement of aromatic-pathway enzymes leading to the production of amino acids needed for protein synthesis has been demonstrated within the plastids of higher plants (Bickel et al., 1978; Leuschner and Schultz, 1991). Aromatic amino acids are indeed required in the cytosol also, as precursors of a great variety of secondary metabolites (e.g. auxins, coumarins, flavonoids, lignins, folates, tannins, and alkaloids), so that the molecules from the shikimate pathway can account for up to 60% of the total plant dry weight (Haslam, 1974). In recent years, experimental evidence has been reported for an increasing number of plant species that supports the presence of distinct isozyme pairs with different subcellular localization (cytosolic and plastidial) for key branchpoint enzymes of aromatic biosynthesis (e.g. DAHP synthase [Ganson et al., 1986], shikimate dehydrogenase [Rothe et al., 1983], and anthranilate synthase and chorismate mutase [Poulsen and Veerporte, 1991]). On this basis, the universal existence in higher plants of analogous prechorismate pathways located simultaneously in both cell compartments has been hypothesized (dual-pathway hypothesis of aromatic biosynthesis; Jensen, 1986; Hrazdina and Jensen, 1992). The cytosolic enzymes, being unregulated, are assumed to provide precursors for secondary metabolism by a simple overflow mechanism. However, the presence of cytosol-localized isoforms has been well established only for the above-mentioned enzymes, and further experimental results are needed to confirm the existence of a complete extraplastidial shikimate pathway.

In the case of EPSP synthase, studies on *Petunia hybrida* cultured cells showed that the enzyme is located predominantly in the chloroplast and is synthesized as a precursor in

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Abbreviations: BMS, Black Mexican Sweet; DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; EPSP, 5-*enol*-pyruvyl-shikimate-3-phosphate; FPLC, fast protein liquid chromatography; S3P, shikimate-3-phosphate.

the cytoplasm (della-Cioppa et al., 1986). The presence of EPSP synthase isozymes has been described in only a few plant species (*Pisum sativum* [Mousdale and Coggins, 1985b], *Nicotiana sylvestris* [Ream and Porter, 1984; Rubin et al., 1984], *S. bicolor* [Ream et al., 1988]), but neither detailed kinetic nor molecular characterization has been reported, nor has the subcellular localization been investigated. A minor portion of EPSP synthase activity in *P. sativum* has indeed been associated with the cytosol, being absent in density-gradient-purified chloroplasts (Mousdale and Coggins, 1985b).

Here we describe the detection and purification to electrophoretic homogeneity of two EPSP synthase isoforms from maize cultured cells. A comparison of their physical and functional properties failed to point out any remarkable differences between the two presumptive isozymes. Subcellular fractionation studies also suggested that both of these proteins are functionally located in the plastid. However, whereas one form was constitutively expressed throughout the culture cycle of growth, the other was detectable only in actively proliferating cells.

MATERIALS AND METHODS

Chemicals

The ammonium salt of S3P was prepared according to Coggins et al. (1987); the purity of the preparations and the concentration of the compound were determined by the HPLC method described by Mousdale and Coggins (1985a).

Biochemicals were purchased from Sigma. DEAE-Sephacel, Mono-Q 5.5, and mol wt markers were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). Bio-Gel P6DG was from Bio-Rad. Cellulose phosphate (P11) was obtained from Whatman Biochemicals (Maidstone, UK). Analytical grade glyphosate (acid form) was from Riedel-de Haën (Seelze, Germany).

Cell Culture

Zea mays L. var Black Mexican Sweet (BMS) cells, originally obtained from the laboratory of Dr. V. Walbot (Stanford, CA), were cultured in liquid suspension on a rotary shaker (100 rpm) in the dark at 26 ± 1°C. Every 2 weeks cells were subcultured by transferring 10-mL aliquots into 100 mL of fresh medium containing Murashige and Skoog salts (Murashige and Skoog, 1962) with 0.3% (w/v) Suc and 1 mg L^{-1} 2,4-D.

Enzyme Assay

EPSP synthase activity was measured at 35°C in the forward direction. The activity of the purified enzyme was measured by determining the inorganic phosphate release using the malachite green dye assay method (Lanzetta et al., 1979) with minor modifications. The reaction mixture contained, in a final volume of 0.1 mL, 100 mM Hepes-NaOH, pH 7.4, 1 mM S3P, 1 mM PEP, and a limiting amount of enzyme (10–25 pkat). After an appropriate incubation period (up to 20 min), the reaction was stopped by the addition of 1 mL of colorimetric solution [9.2 mM malachite green and 8.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 1 M HCl, with 2 g L^{-1} 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate added to stabilize color development] followed, after exactly 1 min, by 0.1 mL of 34% (w/v) Na citrate solution. After 15 min at room temperature, samples were read at 660 nm against unincubated blanks. Under these conditions, the molar absorption coefficient was found to be 75,000 M^{-1} cm^{-1}.

This method was also routinely used during purification by adding to the assay mixture 0.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ to inhibit phosphatases. EPSP synthase activity was determined reading sample A_{660} against blanks in which S3P had been omitted or, alternatively, in which 10 mM glyphosate had been added. Phosphatase activity was measured with *p*-nitrophenyl phosphate as substrate as described (Malamy and Horecker, 1964).

Enzyme Purification

Step 1. Preparation of Crude Extract

Cells grown to mid-linear phase were harvested, weighed, powdered in liquid nitrogen, and resuspended in 2 mL g^{-1} buffer A [50 mM Hepes-NaOH, 10% glycerol (v/v), 5 mM GSH, 0.1 mM EDTA, 0.01 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, pH 7.0] with 1% polyvinylpolypyrrolidone (w/v) added to prevent oxidation of phenolic compounds. All subsequent operations were carried out at 0 to 4°C. The homogenate was clarified by centrifugation at 5,000*g* for 30 min, and the resulting supernatant was further centrifuged at 20,000*g* for 30 min.

Step 2. Ammonium Sulfate Fractionation

The crude extract was fractionated with ammonium sulfate and the 55 to 70% saturated fraction was collected by centrifugation at 12,000*g* for 20 min; pellets were resuspended in half-strength buffer A and dialyzed three times for 2 h each time against 10 volumes of the same buffer.

Step 3. Anion-Exchange Chromatography

The ammonium sulfate fraction was loaded at a constant flow of 60 mL h^{-1} onto a DEAE-Sephacel column (2.5 × 10 cm) equilibrated with half-strength buffer A. After extensive washing with the same buffer, enzyme activity was eluted with a linear gradient from 0 to 250 mM NaCl (500 mL), collecting 5-mL fractions. Active fractions were pooled and proteins were concentrated in a centrifugal Ultrafree filter unit (Millipore) by centrifugation for 10 to 30 min at 2500*g*. The concentrate was buffer-exchanged by passage through a Bio-Gel P6DG column equilibrated with 50 mM Mes-NaOH buffer, pH 5.5, containing 5% glycerol (v/v), 2.5 mM GSH, 0.1 mM EDTA, and 0.1 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (buffer B).

Step 4. Cation-Exchange Chromatography

The sample was applied at a flow rate of 40 mL h^{-1} to a cellulose phosphate column (1.6 × 10 cm) equilibrated with buffer B. The column was washed with 50 mL of buffer B supplemented with 0.5 mM PEP and then eluted with a linear gradient from 0 to 500 mM NaCl in buffer B (250 mL), collecting 5-mL fractions. Active fractions were concentrated and desalted as above.

Step 5. Substrate Elution Chromatography

After the pH was adjusted to 6.0, the sample was applied at a constant flow rate of 10 mL h^{-1} to a cellulose phosphate column (1.6 × 5 cm) equilibrated with buffer C (as buffer B, but pH 6.0). The column was washed until no protein was detected in the eluate; EPSP synthase was then eluted with a linear gradient of 0 to 0.5 mM PEP and S3P in buffer C (200 mL), collecting 2.5-mL fractions. Active fractions of the two resulting activities were pooled, concentrated, and dialyzed against buffer D (20 mM Hepes-NaOH, pH 7.0, containing 20% [v/v] glycerol and 2.5 mM GSH). Enzyme preparations were stored at −20°C. Under these conditions, EPSP synthase activity was found to be stable for at least 3 months.

Protein Determination

Protein concentration was determined by the method of Bradford (1976), using BSA as standard.

Electrophoresis

Discontinuous SDS-PAGE was performed at 20°C by the method of Laemmli (1970) with a 4% stacking and a 10% separating gel. Gels were silver stained for proteins over a previous Coomassie blue R250 stain by using the method described by Heukeshoven and Dernick (1985) with slight modifications.

Nondenaturing electrophoresis of purified proteins was run at 4°C with separating gels at various acrylamide concentrations; gels were silver stained or stained for EPSP synthase activity as described (Nimmo and Nimmo, 1982).

Isoform Detection in Crude Extracts

Cells harvested at various stages during the growth cycle of the culture were resuspended in 2 mL g^{-1} buffer A and homogenized in a Teflon-in-glass Potter homogenizer with 15 strokes. After centrifugation at 20,000*g* for 20 min, solid ammonium sulfate was added to the crude extract to give 70% saturation. Proteins were pelleted by centrifugation, resuspended in buffer E (50 mM Tris-HCl, pH 7.4, containing 5% [v/v] glycerol, 1 mM DTT, and 0.1 mM EDTA), and column-desalted against the same buffer. Then, extracts were further centrifuged at 100,000*g* for 60 min and 1-mL aliquots of the resulting supernatant were injected onto a Mono-Q 5.5 FPLC column equilibrated with buffer E. Proteins were eluted at a flow rate of 0.5 mL min^{-1} using a computer-controlled (Kontron 450) linear gradient from 0 to 80 mM NaCl (30 mL), and 0.5-mL fractions were collected.

Subcellular Fractionation and Markers' Assay

Plastids from cells grown to mid-log phase were isolated by rate-zonal sedimentation on discontinuous Percoll gradients as described (Miernyk, 1985). Plastidial stroma preparations were made by diluting washed plastids with buffer E and centrifuging at 100,000*g* for 60 min. The supernatant was analyzed for EPSP synthase isoform content as above. The activity of marker enzymes (alcohol dehydrogenase, catalase, Cyt *c* oxidase, Mn-dependent DAHP synthase, and ribulose-1,5-bisphosphate carboxylase) in crude and in density-gradient-purified plastidial extracts was determined as described previously (Kerster and Deley, 1966; Lück, 1962; Wharton and Tzagoloff, 1967; Muday and Herrmann, 1992; Lilley and Walker, 1974, respectively).

RESULTS

Purification of Maize EPSP Synthase

When cells were harvested in middle exponential growth phase, crude extracts from BMS suspension cultures contained EPSP synthase at a specific activity of 0.43 ± 0.08 nkat mg^{-1} protein. The results of the purification procedure are outlined in Table I. Ammonium sulfate fractionation and ion-exchange chromatography succeeded in both a preliminary enrichment of EPSP synthase and the removal of unspecific phosphatases (data not shown), which can interfere with the enzyme assay and substrate elution on phosphocellulose. However, the addition of 0.1 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, which selectively inhibits hydrolases without affecting EPSP synthase catalytic rate, to the column buffer allowed us to perform a separation on a cellulose phosphate column even in the presence of residual contamination by phosphatases. The use of a first phosphocellulose column as a simple cation exchanger, equilibrated at low pH values and washed with buffer supplemented with 0.5 mM PEP before eluting the enzyme, greatly enhanced the subsequent, high-resolution purification step. When the former was omitted, several other proteins co-eluted with EPSP synthase during substrate elution chromatography (not shown). The maximum specific activity observed after the second cellulose phosphate column reflects a purification of over 2000-fold, with an overall yield

Table I. *Purification of Z. mays EPSP synthase*

The results presented are for a typical purification starting from 200 g (fresh weight) of cultured maize cells.

Step	Total Activity	Protein	Specific Activity	Purification	Yield
	nkat	*mg*	*nkat mg^{-1}*	*fold*	%
Crude extract	512	1032	0.5	1	100
55–70% ammonium sulfate	482	282	1.7	3.4	94.1
DEAE-Sephacel	446	44.2	7.8	20.2	87.1
Cellulose phosphate, first column	346	7.8	44.3	88.6	67.6
Cellulose phosphate, second column					
Enzyme I	63	0.062	1016	2032	12.3
Enzyme II	66	0.042	1578	3155	12.9

of more than 25%. Purified enzyme preparations were substantially stable: between 75 and 85% of the activity was retained during 30 d of storage at 0°C or 3 months of storage at −20°C.

Chromatographic Evidence for Two Enzyme Forms

The first purification steps gave no evidence supporting the presence of EPSP synthase isoforms. Only a single activity was evident after both anion- and cation-exchange chromatography. Even during the last step, if the cellulose phosphate column was eluted by directly adding high concentrations of the two substrates, only one activity could be detected (data not shown). In contrast, when substrate elution was carried out by a linear gradient from 0 to 0.5 mM S3P and PEP at a very low flow rate, two well-separated EPSP synthase activities were recovered in the eluate (Fig. 1). This result was highly reproducible, and the same pattern was obtained when purification was performed in the presence of protease inhibitors (1 mM PMSF and 1 μM pepstatin A). The relative proportion of the two activities varied. The pools obtained by collecting the most active fractions, designated I and II according to their order of elution, were used for the subsequent characterization.

Homogeneity of Purified EPSP Synthases

The purified enzymes were electrophoretically homogeneous: when silver stained, denaturing polyacrylamide gels showed only a single band in both cases (Fig. 2, lanes B and C). The mobilities of the two proteins, although very similar, were not identical. Figure 2 also shows the electrophoretic pattern obtained when the same preparations were analyzed in the absence of detergents (lanes D and E). A second, somewhat diffuse band in lane E probably reflects a tendency of protein II to aggregate under native conditions. A similar behavior was also seen during gel-permeation chromatography of the purified enzyme at low ionic strength (data not shown). When the native gel was stained for EPSP synthase activity, the same bands were observed (lanes F and G).

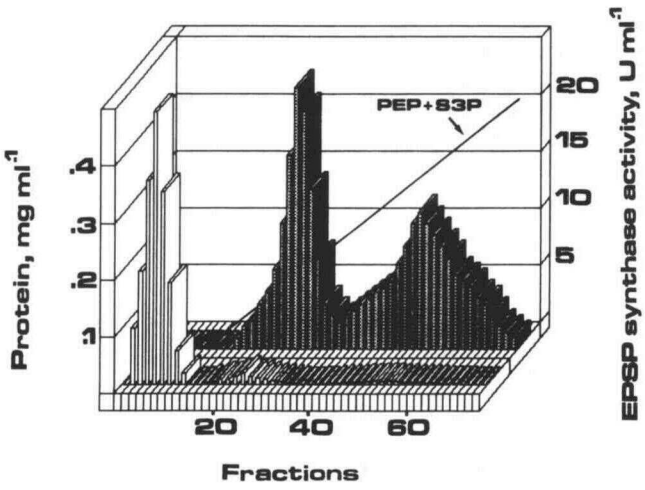

Figure 1. Purification of EPSP synthase from the BMS cell line. Substrate-elution chromatography on a phosphocellulose column that was equilibrated at pH 6.0. Fraction volume = 2.5 mL. Sample elution with a linear gradient of both the substrates of EPSP synthase (PEP and S3P) resulted in two well-separated enzyme activities.

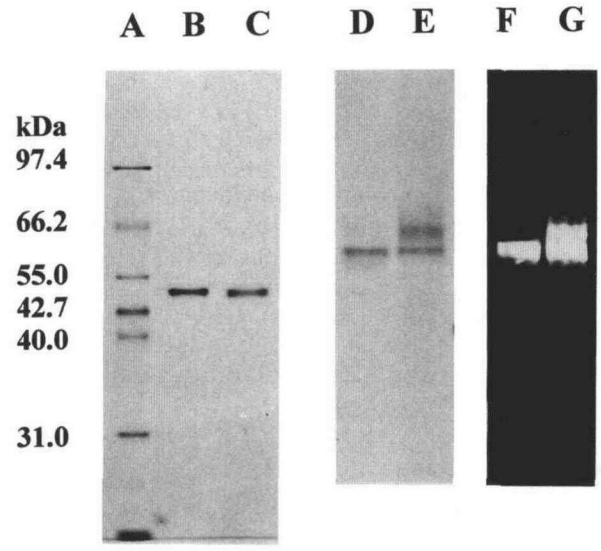

Figure 2. Electrophoresis of purified maize EPSP synthase under native and denaturing conditions. Aliquots (approximately 100 ng of protein, except for lanes F and G, in which a 10-fold higher quantity was loaded) of the two proteins obtained following substrate elution chromatography were analyzed by both SDS-PAGE (lanes A–C; 10% acrylamide) and native PAGE (lanes D–G; 6% acrylamide). Lanes B, D, and F, Enzyme I. Lanes C, E, and G, Enzyme II. Gels were silver stained (lanes A–E) or stained for EPSP synthase activity (lanes F and G). The molecular masses of protein standards run on the same denaturing gel (lane A) are also indicated.

Structural and Functional Characterization of the Purified Proteins

To ascertain whether possible differences between the two isoforms occurred that could account for a distinct functional role in cellular metabolism, the purified proteins were thoroughly characterized with regard to structural, kinetic, and biochemical properties. Data obtained, summarized in Table II, failed to demonstrate any striking difference between the two isoforms.

Gel-filtration experiments confirmed the minor difference in molecular mass that was detected by SDS-PAGE. Results obtained with respect to temperature and pH optima were very similar. The activities of both proteins showed only a single broad maximum of activity between pH 7.0 and 8.0 (not shown) and were rapidly inactivated at temperatures exceeding 60°C. Slight differences were found for affinity constants and temperature dependence of enzyme activity. As reported for the enzyme from other sources, the broad-spectrum herbicide glyphosate strongly inhibited the activity of both maize EPSP synthase isoforms. At a concentration of 1 mM glyphosate, enzyme activity was completely suppressed, and 50% inhibition was achieved with 6 to 8 μM glyphosate. The resulting K_i values were almost identical with PEP as the variable substrate, but were slightly different with

Table II. *Properties of EPSP synthase isoforms from BMS cultured cells*

Data from Forlani (1992).

Property	Pool I	Pool II
Molecular mass (kD)		
By SDS-PAGE	54.9	53.6
By native-PAGE	58.7	58.1
Stokes radius (Å)	31.7	31.4
Temperature optimum (°C)	55	55
Activation energy (kJ mol^{-1})	59.1	55.7
pH optimum	7.4	7.5
Isoelectric point	5.9	5.9
$K_{m(app)}$ for PEP (μM)[a,b]	33.1	27.9
$K_{m(app)}$ for S3P (μM)[a,b]	32.6	28.7
K_i for glyphosate, with respect		
to PEP (μM)[b,c]	0.13	0.12
to S3P (μM)[c,d]	9.4	12.0

[a] The second nonvariable substrate was fixed at a concentration of 1 mM. [b] Mean of the values obtained on two separate enzyme preparations. [c] Competitive inhibition, slope replots. [d] Uncompetitive inhibition, intercept replots.

respect to S3P. As expected, because EPSP synthase is not a regulated branchpoint enzyme, the activities of both purified proteins were unaffected by aromatic amino acids, chorismic acid, or anthranilic acid (data not shown).

However, a pronounced difference between the two isoforms was found in heat-inactivation experiments. Incubation of the purified proteins for increasing times at 45°C resulted in a faster disruption of EPSP synthase activity I. As shown in Figure 3, the time required to reduce its catalytic rate by 50% was about 20 min, whereas 50% of the initial activity of the enzyme II was still retained after a 60-min treatment.

Detection of EPSP Synthase Isoforms in Crude Extracts

Since the lack of any apparent difference in their functional properties could suggest that the appearance of two activities might be ascribed to an experimental artifact arising during the purification procedure (e.g. by means of a partial proteolysis of a single protein), crude extracts were analyzed for EPSP synthase activity following anion-exchange FPLC. The use of a very narrow NaCl gradient allowed us also to detect the presence of two enzyme forms in the desalted 0 to 70% ammonium sulfate fraction (Fig. 4). Rechromatography of the two isoforms resulted in each case in a single activity at the same ionic strength. The purified proteins were then buffer-exchanged and eluted under the same experimental conditions. In both cases the analysis of the eluate showed a single activity corresponding to one of the two activities present in crude extracts, eluting in the reverse order than from the cellulose phosphate column. Moreover, the first, less-abundant isoform detectable in crude extracts showed a more pronounced thermal stability, a result consistent with those previously obtained with the purified proteins.

Subcellular Localization and Expression Pattern of the Two Isoforms

The availability of a method to detect EPSP synthase isoforms in crude preparations made subcellular localization experiments feasible. Plastids were purified from BMS cells by rate-zonal sedimentation on discontinuous Percoll gradients. Plastidial stroma preparations were analyzed for EPSP synthase isoforms and marker enzyme content, and data were compared to the corresponding specific activities measured in crude extracts. Results (Table III) clearly demonstrated a plastidial localization of both EPSP synthase isoforms. Finally, since the ratio between the two isoforms in crude extracts varied slightly among experiments and was quite

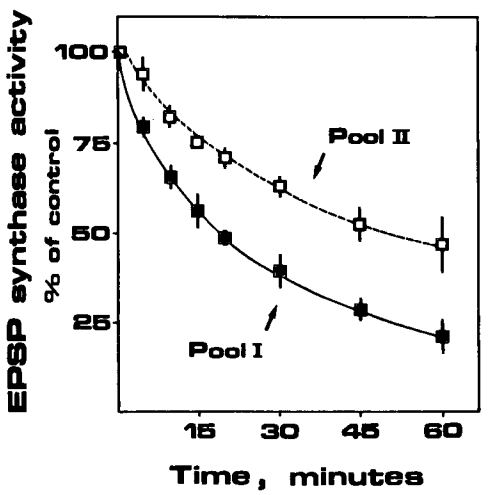

Figure 3. Heat sensitivity of the two EPSP synthase isoforms. The activity of the purified proteins, buffer-exchanged with 10 mM Hepes-NaOH, pH 7.4, and diluted to a concentration of 0.4 nkat mL^{-1}, was measured at 35°C after incubation of up to 60 min at 45°C. Data, expressed as percentage of untreated controls, are averages ± SD of four independent determinations.

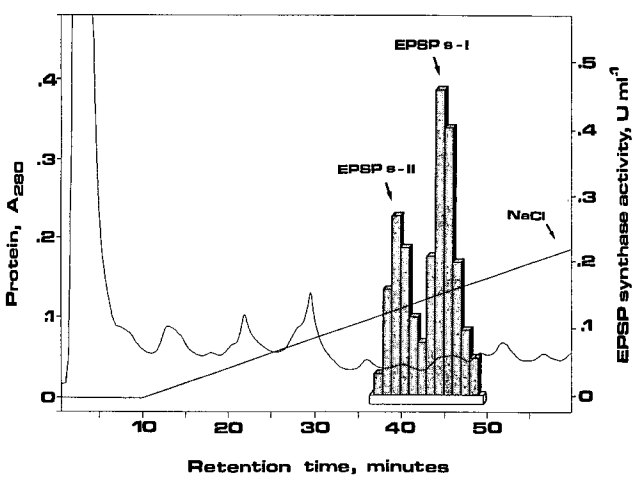

Figure 4. FPLC detection of maize EPSP synthase isoforms in crude extracts. The desalted 0 to 70% ammonium sulfate fraction from cells grown to mid-log phase was centrifuged for 60 min at 100,000*g*; a 1-mL aliquot of the resulting supernatant was injected onto a Mono-Q 5.5 column. Sample elution: 0–80 mM NaCl gradient. Fraction volume = 0.5 mL.

Table III. *Specific activity of EPSP synthase isoforms and marker enzymes in crude extracts and in density-gradient-purified plastids*

Crude extracts and plastidal lysates were subjected to centrifugation at 100,000*g* for 60 min prior to analysis. EPSP synthase isoforms were quantified after anion-exchange FPLC, and activities refer to the overall activities measured in the extracts just before the injection. The results, expressed in nkat mg^{-1}, are means of two independent experiments. In density-purified plastids, the relative activity of the two EPSP synthase forms could not be quantified; the overall activity is therefore reported. n.d., Not detected.

Enzyme	Crude Extracts	Density-Purified Plastids		Plastidial Stroma
		Before lysis	After lysis	
EPSP synthase I	0.426	0.27	4.96	6.32
EPSP synthase II	0.164			1.79
Alcohol dehydrogenase	8.85	0.88	0.92	0.67
Catalase	1,045	295	351	188
Cyt c oxidase	8.09	3.21	3.30	4.21
Mn-dependent DAHP synthase	0.007	0.003	0.061	0.082
Ribulose-1,5-bisphosphate carboxylase	0.045	n.d.	n.d.	0.496

different from that obtained through the four purification steps, the specific activity of maize EPSP synthase isoforms was quantified in extracts from cells harvested at different stages during the growth cycle of the culture. The results, outlined in Table IV, indicated that although one isoform persisted throughout the cycle at substantial levels, the other, significantly less abundant, increased with the onset of exponential growth and declined in stationary phase.

DISCUSSION

In this paper we describe the purification of maize EPSP synthase from BMS cultured cells. The cell line employed was initially selected as the source of the enzyme because it produced EPSP synthase with higher specific activity than a few other tested lines of the same cultivar (Forlani et al., 1992). To obtain electrophoretically homogeneous enzyme, three chromatographic steps were required. The final purification step involved substrate elution from cellulose phosphate. The enzyme bound to the phosphocellulose column only at low pH values. Above pH 6.2, the resin did not retain EPSP synthase activity, the elution of which was simply slowed down. The enzyme could be selectively eluted with a mixture of the two substrates (S3P and PEP) only if the column had been previously equilibrated at a pH between 5.7 and 6.1. Below pH 5.6, the enzyme bound tightly to cellulose phosphate and could be eluted only by high ionic strength. This behavior is consistent with that observed upon IEF of the purified EPSP synthase: substrate elution can be achieved only at a pH near its isoelectric point, at which the enzyme is retained only by its affinity to the phosphate groups and not by other ionic interactions. Enzyme activity could also be eluted from the cellulose phosphate column with either PEP or S3P alone, but elution resulted in a broad peak.

The last step in the purification procedure allowed us to separate two EPSP synthase isoforms. Two activities were

Table IV. *Specific activity of maize EPSP synthase isoforms during the growth cycle of BMS cultured cells*

Days from Subculture	Fresh Weight	Overall Activity[a]	Relative Activity[b]	
			EPSPs-II	EPSPs-I
	$mg\ mL^{-1}$	$pkat\ mg^{-1}$	%	
2	26 ± 3	754 ± 54	36.7	63.3
4	33 ± 7	656 ± 91	44.1	56.9
7	65 ± 15	552 ± 72	40.7	59.3
10	90 ± 18	559 ± 62	36.8	63.2
12	106 ± 10	623 ± 53	30.1	69.9
14	116 ± 4	747 ± 70	20.3	79.7
17	113 ± 6	760 ± 84	12.2	87.8
20	104 ± 7	488 ± 93	8.5	91.5

[a] Values ± SD refer to the activity measured in the desalted 0–70% ammonium sulfate fraction after centrifugation at 100,000*g* for 60 min, and are means of three independent experiments. [b] Activities estimated following anion-exchange FPLC as described in "Materials and Methods."

recovered in the eluate after substrate elution chromatography when it was performed using a linear gradient of both S3P and PEP. The relative ratio of these enzyme forms varied somewhat from experiment to experiment. The presence of EPSP synthase isoforms is consistent with previous reports. A minor proportion of extraplastidial EPSP synthase activity was described in *Pisum sativum* (Mousdale and Coggins, 1985b). A second peak of activity was also reported in extracts from *Nicotiana sylvestris* cultured cells (Ream and Porter, 1984; Rubin et al., 1984). Three isoforms were separated by anion-exchange HPLC from dark-grown seedlings of *Sorghum bicolor* (Ream et al., 1988). A partial characterization of these putative isozymes has been described only in the latter study (Ream et al., 1988): in this case the authors failed to find any significant difference between the two major forms.

Also, in the case of maize a high degree of similarity exists between the enzyme forms. We compared the physical and kinetic properties of the two purified proteins, but characterization experiments did not succeed in finding any difference that could account for a distinct role in cellular metabolism. However, some factors do suggest the occurrence of true isozymes versus the possibility of an experimental artifact. The addition of protease inhibitors did not change the relative ratio of the two isoforms. The same EPSP synthase isoforms could also be detected and separated in crude extracts. In this case their ratio varied significantly (2:1 in crude extracts, about 1:1 in purified preparations), but according to their differential stability, so that the final yield of the less abundant isoform was expected to be higher than that of the more abundant isoform. The two isoforms showed a different pattern of expression during the culture growth cycle. One was expressed throughout the process, the other was detected preferentially during the exponential phase of growth. Such a result seems to rule out the possibility that the BMS cells might not be homozygous for EPSP synthase. Moreover, two EPSP synthase activities have been detected in extracts from another BMS cell line, which showed a natural tolerance to the herbicide glyphosate (Forlani et al., 1992). Enzyme I closely resembled one of those isoforms in that it is glyphosate sensitive and expressed throughout the culture growth cycle.

Distinct isozyme pairs, one plastidial and the other cytosolic, have so far been identified for key branchpoint enzymes of aromatic amino acid biosynthesis such as DAHP synthase (Ganson et al., 1986) and chorismate mutase and anthranilate synthase (Poulsen and Veerporte, 1991). Only the isoforms located in the chloroplast were shown to be subjected to strong allosteric control (Goers and Jensen, 1984). Because cytosolic location of enzymes catalyzing other steps in the prechorismate pathway has been reported (Rothe et al., 1983; Mousdale et al., 1987), dual biosynthetic pathways of aromatic biosynthesis in spatially separated compartments have been hypothesized to exist as a universal characteristic of higher plants (Jensen, 1986).

However, data obtained with density-gradient-purified plastidial preparations clearly demonstrated a plastidial localization of both maize EPSP synthase isoforms. Moreover, several reports have been published in recent years describing gene pairs encoding DAHP synthase (Dyer et al., 1990; Zhao and Herrmann, 1992) and also the anthranilate synthase α subunit (Niyogi and Fink, 1992) and the Trp synthase β subunit (Berlyn et al., 1989; Last et al., 1991), both of which show putative chloroplast transit peptides at their amino termini. The molecular data now available point to an exclusively plastidial localization of the shikimate pathway. Cytosolic isozymes have been interpreted as the result of aberrant processing of a plastidial protein in cultured cells (Singh et al., 1991). The occurrence of more distantly related genes that encode cytosolic isozymes could not be ruled out. In the case of EPSP synthase, at least four genes have been detected in rape (Gasser and Klee, 1990) and six have been detected in tobacco (Goldsbrough et al., 1990). On the other hand, cytosolic isoforms devoted to the production of aromatic amino acids for the synthesis of secondary metabolites might not be expressed in cultured cells.

At least for some control points in the aromatic amino acid pathway, regulation in plants seems to involve differential expression of duplicated genes, both of which express plastid enzymes. Our results on maize EPSP synthase suggest the possibility that two distinct whole prechorismate pathways could exist in the plastid, even if they are differentially expressed (in distinct cell types or at different times). Biochemical evidence suggests that one EPSP synthase isoform is constitutively expressed, whereas the other seems to be regulated, possibly by increased demand of aromatic amino acids, since it can be detected only in actively proliferating cells. This would be consistent with data previously described for both DAHP synthase and anthranilate synthase. Steady-state mRNA levels and enzyme specific activities of anthranilate synthase-1 and Mn-dependent DAHP synthase increased dramatically following both wounding and bacterial pathogen infiltration, whereas the other isoforms were unaffected (Dyer et al., 1989; McCue and Conn, 1989; Niyogi and Fink, 1990; Muday and Herrmann, 1992). This has been interpreted as being due to the synthesis ex novo of aromatic metabolites as indole-glucosinolates and phytoalexins required in the plant defense response. But an augmented need for aromatic amino acids for protein synthesis in rapidly proliferating maize cells might also determine the transcription of an inducible EPSP synthase isoform. Work is now under way to elucidate this point.

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