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Note

Rapid chromatographic purification of glyphosate-sensitive 5-enolpyruvylshikimate 3-phosphate synthase from higher plant chloroplasts

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The plant and microbial enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (E.C. 2.5.1.19; alternative name 3-phosphoshikimate 1-carboxyvinyltransferase) is inhibited by the broad-spectrum herbicide glyphosate (N-phosphonomethylglycine) and is the principal site of the biological action of this xenobiotic[1–8]. EPSP synthase is present in plant tissues in relatively small quantities (0.03–0.06% of the total soluble protein[7,9]) but has been purified to homogeneity from pea seedlings[9] and a plant cell culture[7]. While the synthesis and cellular levels of the enzyme can be selectively increased in cell cultures by adaptation to the presence of glyphosate[2,7], the purification of EPSP synthase from plant tissues is in general a lengthy process and yields only microgram quantities of the pure enzyme.

EPSP synthase is predominantly a chloroplastic enzyme in pea seedling tissue[10] and is present in chloroplast lysates at higher specific activities than in crude tissue extracts[9,10]. We have utilized this compartmentation of the enzyme to develop a rapid small-scale purification of EPSP synthase from a range of higher plant species.

EXPERIMENTAL

Plant materials

Pea (*Pisum sativum* L. c.v. "Onward") seeds were germinated and grown in growth chambers as described previously[9]. Maize (*Zea mays* L.) plants were grown in similar growth chambers; leaf tissue was used from 10-day-old plants. Spinach (*Spinacia oleracea* L.) plants were greenhouse-grown; leaves were taken from approximately 6-week-old plants. Lettuce (*Lactuca sativa* L.) was obtained commercially.

Chloroplast preparation

Washed chloroplast preparations were made from 100–500 g fresh weight of leaf or shoot tissue by the method of Nakatani and Barber[11].

Fast protein liquid chromatography

Washed chloroplasts were lysed by dilution in 10–40 ml of 20 m*M* Tris (pH 7.5 with hydrochloric acid) containing 0.1 m*M* dithiothreitol (DTT) and 1 m*M* benzamidine hydrochloride (buffer A). The lysate was centrifuged at 80 000 *g* for 1 h at

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4°C. The supernatant was filtered through a 0.22-μm filter and applied to a HR5 Mono-Q column (Pharmacia, Milton Keynes, U.K.). The column was washed with 5 ml of buffer A and then eluted with a linear gradient of 0–20% 1 *M* sodium chloride in buffer A over a 20 min period using a Pharmacia Fast Protein Liquid Chromatography apparatus. Chromatography was performed at room temperature. The flow-rate was 1 ml/min and 0.5-ml fractions were collected. Fractions containing EPSP synthase activity were pooled and diluted tenfold with cold (4°C) 10 m*M* potassium citrate (pH 5.5 with hydrochloric acid) containing 0.1 m*M* DTT and 1 m*M* benzamidine hydrochloride for cellulose phosphate chromatography.

Cellulose phosphate chromatography

This was performed as described previously[9] at 4°C with a smaller column (1 ml volume) of cellulose phosphate. The enzyme was eluted with 1 m*M* phosphoenolpyruvate and 1 m*M* shikimate 3-phosphate and the active fractions were pooled and concentrated by dialysis against 1 l 50 m*M* Tris (pH 7.5 with hydrochloric acid) containing 0.1 m*M* DTT, 1 m*M* benzamidine hydrochloride, 1 m*M* EDTA and 50% (v/v) glycerol.

Analytical methods

EPSP synthase assays, protein determinations and polyacrylamide gel electrophoresis were as described previously[9]. Protein staining in gels was by the ammoniacal silver nitrate method of Wray *et al.*[12]. Analytical grade glyphosate was the gift of Dr. Stuart Ridley (I.C.I., Jeallott's Hill Research Station, Bracknell, U.K.).

RESULTS AND DISCUSSION

The chloroplast lysates contained EPSP synthase at specific activities of 0.12–0.24 nkat/mg protein (pea), 0.09–0.14 nkat/mg (spinach), 0.07–0.13 nkat/mg (lettuce) and 0.02–0.04 nkat/mg (maize). Substrate elution of EPSP synthase from cellulose phosphate, which is an important step in the purification of the enzyme from microbial[13] and plant[7,9] sources, gave only a partial purification (less than ten-fold) with the chloroplast lysates. A high-performance anion-exchange chromatographic step was therefore used to give an initial rapid (less than 40 min) purification of chloroplastic EPSP synthase prior to cellulose phosphate chromatography.

EPSP synthase in chloroplast lysates was purified 30–100 fold by chromatography on Mono-Q anion-exchange columns (Figs. 1 and 2) with a recovery of greater than 95% of the applied enzyme activity. The resulting specific activity lay within the range (1–8 nkat/mg) routinely obtained before cellulose phosphate chromatography in the multi-step purification procedure from tissue homogenates previously described[9]. Subsequent substrate elution of the adsorbed enzyme from cellulose phosphate resulted in enzyme preparations containing only trace protein contaminants as revealed by silver-staining of polyacrylamide gels (Fig. 3). The entire purification process from the beginning of chloroplast preparation to the completion of cellulose phosphate chromatography could be accomplished in 6–8 h. Overall recovery of the EPSP synthase was 10–20% from an initial 1–3 nkat of the enzyme. Because of the low protein concentration in the purified enzyme preparations the final specific activities were difficult to determine accurately. However, the specific

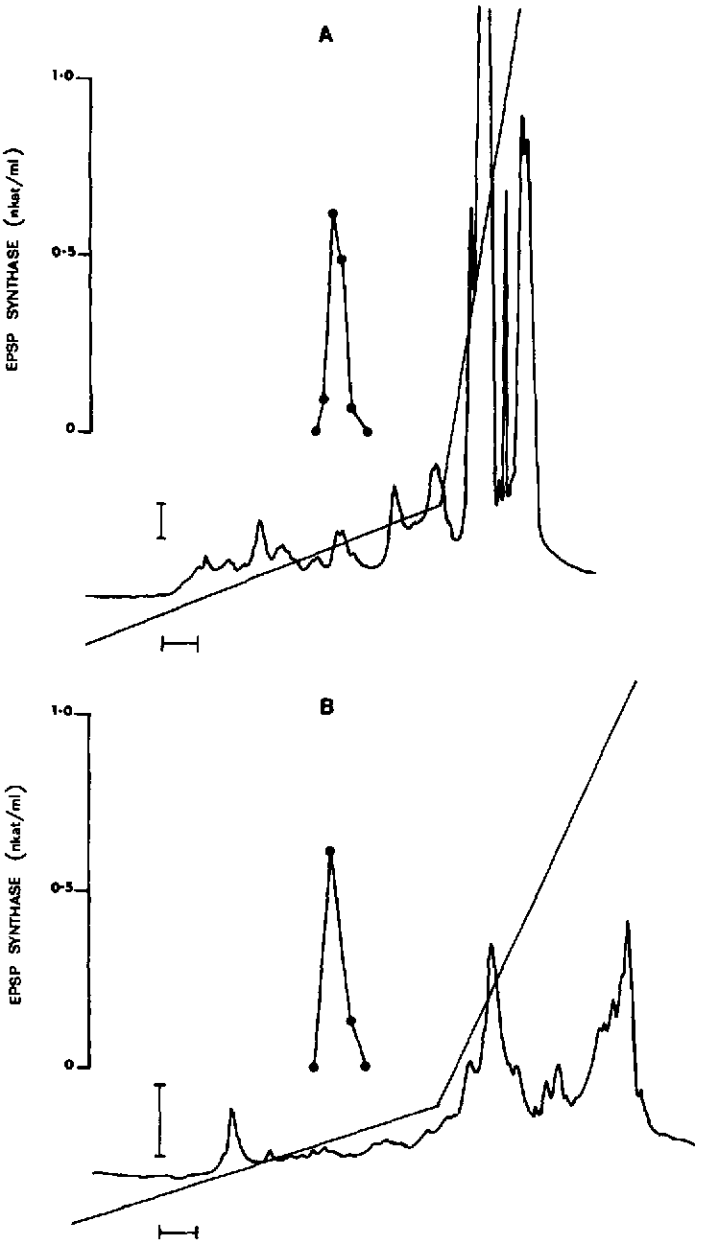

Fig. 1. Chromatography of spinach (A) and maize (B) chloroplast lysates containing EPSP synthase on Mono-Q anion-exchanger. Gradient profiles: 0–200 m*M* sodium chloride in 20 min, 200–1000 m*M* sodium chloride in 5 min (A) or 15 min (B). Vertical bar: 0.1 $A_{280\ nm}$; horizontal bar: 2 min. Flow-rate: 1 ml/min.

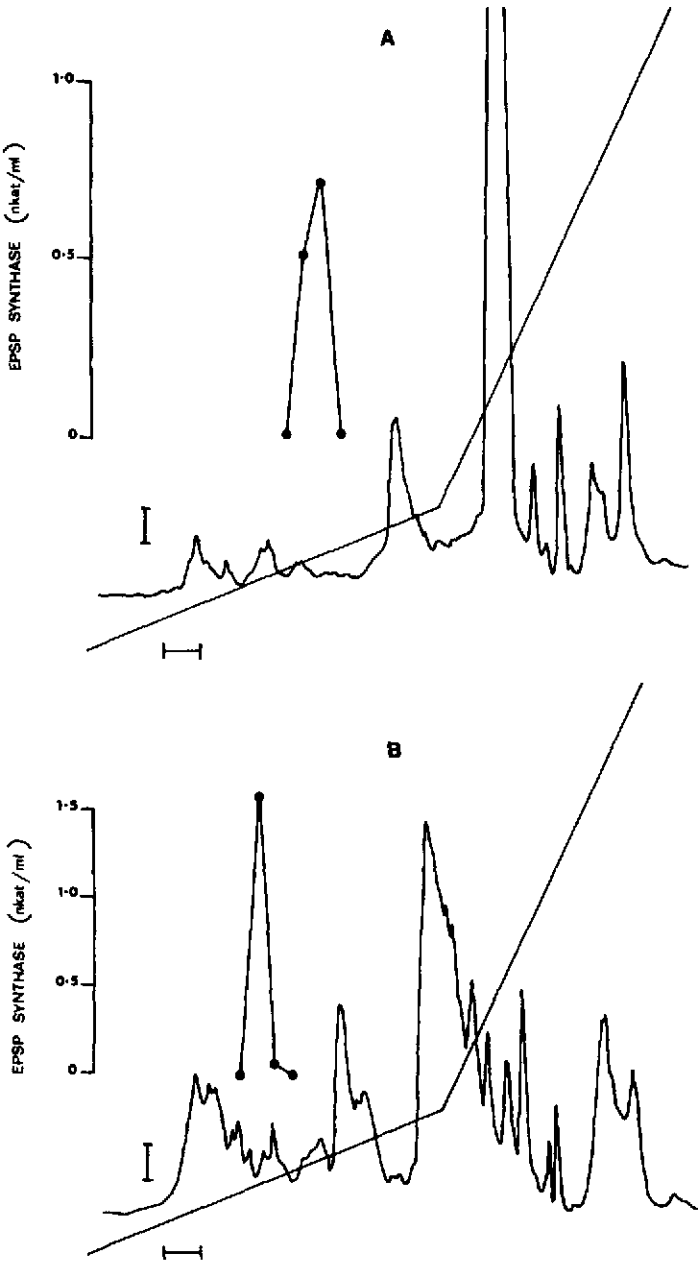

Fig. 2. Chromatography of lettuce (A) and pea (B) chloroplast lysates containing EPSP synthase on Mono-Q anion exchanger. Gradient profiles: 0–200 m*M* sodium chloride in 20 min, 200–1000 m*M* sodium chloride in 15 min. Vertical bar: 0.1 $A_{280\ nm}$; horizontal bar: 2 min. Flow-rate: 1 ml/min.

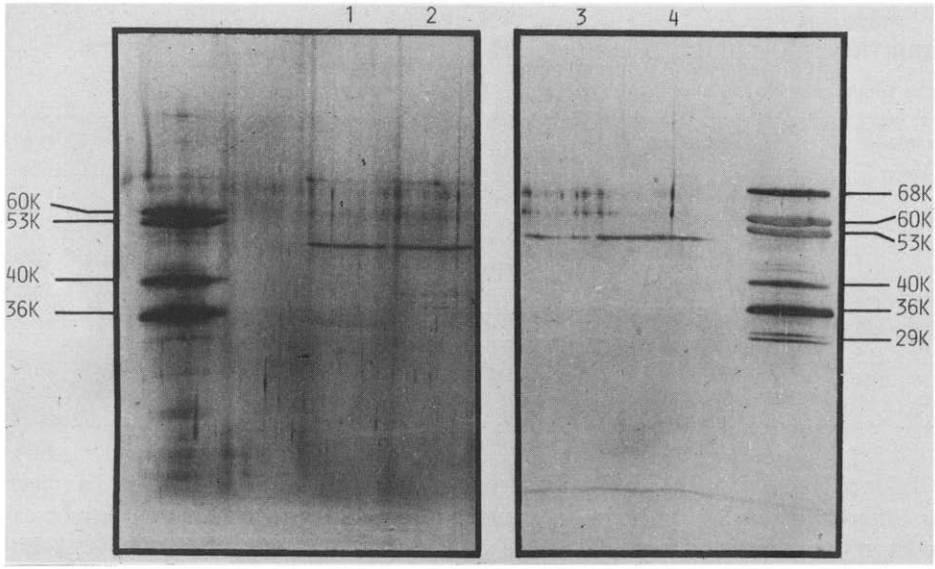

Fig. 3. Polyacrylamide gels of purified EPSP synthases in the presence of 0.1% sodium dodecyl sulphate. Lane 1: enzyme from spinach chloroplasts; lane 2: enzyme from spinach leaf extract (method of ref. 9); lane 3: enzyme from pea chloroplasts; lane 4: enzyme from pea shoot extract. Molecular weight markers: bovine serum albumin (68 000), catalase (60 000), glutamate dehydrogenase (53 000), aldolase (40 000), glyceraldehyde 3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000).

activities obtained (approximately 200 nkat/mg protein measured in the forward enzyme direction) were at least as high as those obtained when larger scale purifications were carried out from pea[9] and spinach tissue homogenates. The subunit molecular weight of EPSP synthase from pea, spinach, lettuce and maize chloroplasts was 51 000 ± 500. The native molecular weight of the enzyme purified from pea seedling and spinach and wheat (*Triticum vulgare* L.) leaf extracts is approximately 45 000 (ref. 9 and unpublished results); this suggests that the higher-plant chloroplastic EPSP synthases are monomeric and of similar subunit size.

The rapid small-scale purification is particularly suitable for the assessment of any variation among plant EPSP synthases in sensitivity towards inhibition by glyphosate. Few reports have appeared of natural tolerance to the herbicide; the major proposed mechanisms have been low uptake or translocation of the herbicide[14,15] and high plant regenerative capabilities[16,17]. Plant cell cultures grown in the presence of glyphosate contained increased tissue levels of EPSP synthases which were, however, no less sensitive to the herbicide *in vitro*[7,18]. Some *Pseudomonas* species contain a relatively insensitive EPSP synthase and the enzyme from a range of microbial sources has been found to be less inhibited by glyphosate than is the case with higher plant EPSP synthases[19]. The chloroplastic EPSP synthases purified from the three dicotyledonous species (pea, spinach and lettuce) and the monocotyledonous species maize showed similar inhibition by glyphosate (Table I).

Another application of this rapid purification strategy is with species from which the enzyme cannot be purified by traditional enzymological procedures because

TABLE I

INHIBITION OF PURIFIED CHLOROPLASTIC EPSP SYNTHASES BY GLYPHOSATE

Assay conditions: 500 μM shikimate 3-phosphate, 100 μM phosphoenolpyruvate; 25°C.

Glyphosate (μM)	*EPSP synthase (nkat/ml)*			
	Pea	*Spinach*	*Lettuce*	*Maize*
0	0.28 (100)	0.20 (100)	0.12 (100)	0.24 (100)
0.5	0.19 (68)	0.11 (55)	0.09 (75)	0.17 (61)
5.0	0.08 (29)	0.06 (30)	0.05 (42)	0.065 (27)
50.0	0	0	0	0

of the low stability of the enzyme in crude tissue extracts. This problem has been encountered in this laboratory with lettuce and maize leaf extracts and may be expected in those species whose tissues accumulate phenolic and other reactive secondary products.

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