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Purification of 5-enolpyruvylshikimate 3-phosphate synthase from *Escherichia coli*

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A procedure for the purification of 5-enolpyruvylshikimate 3-phosphate synthase from *Escherichia coli* is described. Homogeneous enzyme of specific activity 17.7 units/mg was obtained in 22% yield. The key purification step involves substrate elution of the enzyme from a cellulose phosphate column. The subunit M_r was estimated to be 49000 by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The native M_r was estimated to be 55000 by gel filtration, indicating that the enzyme is monomeric.

5-Enolpyruvylshikimate 3-phosphate synthase (EC 2.5.1.19) catalyses the reversible transfer of the enolpyruvate group of phosphoenolpyruvate to the 5-hydroxy group of shikimate 3-phosphate. The product, 5-enolpyruvylshikimate 3-phosphate (EPSP), is an intermediate on the biosynthetic pathway leading to chorismate and thus to aromatic compounds. This enzyme has been reported to be the site of action of the commercially important herbicidal compound glyphosate (Steinrucken & Amrhein, 1980; Boocock & Coggins, 1983).

Previous studies on EPSP synthases have centred on attempts to elucidate the mechanism of this unusual reaction. Investigators have used partially purified bacterial EPSP synthases and have examined equilibrium and kinetic isotope-exchange effects (Bondinell *et al.*, 1971; Ife *et al.*, 1976; Grimshaw *et al.*, 1982). The results are in agreement with the addition–elimination mechanism proposed by Levin & Sprinson (1964), but do not exclude other possible mechanisms, for example that involving a substituted enzyme intermediate. Only one other enzyme catalyses a similar reaction, the enolpyruvyl transferase (EC 2.5.1.7) of peptidoglycan biosynthesis (Gunetileke & Anwar, 1968; Cassidy & Kahan, 1973; Kahan *et al.*, 1974; Zemell & Anwar, 1975).

The only reported purifications to homogeneity of EPSP synthases are those of the multifunctional enzymes containing this activity found in *Neurospora crassa* (Lumsden & Coggins, 1977; Gaertner & Cole, 1977) and *Euglena gracilis* (Patel & Giles, 1979). Multifunctional enzymes containing EPSP synthase also occur in other species of fungi (Ahmed & Giles, 1969; Bode & Birnbaum, 1981), although their purification has not been reported. The EPSP synthase activity in prokaryotes (Berlyn & Giles, 1969) and in photosynthetic organisms (Berlyn *et al.*, 1970; Koshiba, 1979) appears to occur as a separate enzyme by the criteria of sucrose-density-gradient centrifugation and ion-exchange chromatography. None of these monofunctional EPSP synthases has previously been purified. We now describe here the purification to homogeneity of EPSP synthase from *E. coli*.

Abbreviation used: EPSP, 5-enolpyruvylshikimate 3-phosphate.

Materials and methods

Reagents

All the reagents and coupling enzymes except those specified below were obtained from Boehringer Corp., Lewes, East Sussex, U.K., or Sigma Chemical Co., Poole, Dorset, U.K. DEAE-Sephacel, Sephacryl S200 (superfine grade) and phenyl-Sepharose CL-4B were obtained from Pharmacia, London W.5, U.K., and cellulose phosphate (P11) was from Whatman Biochemicals, Maidstone, Kent, U.K. Shikimate 3-phosphate was a gift from Dr. G. A. Nimmo, and partially purified *N. crassa* chorismate synthase was a gift from Mr. M. R. Boocock.

The barium salt of EPSP was prepared essentially as described by Knowles *et al.* (1970) except that 6 forward units of *N. crassa arom* EPSP synthase activity (Boocock & Coggins, 1983) were used for the enzymic conversion. Before use, EPSP was converted into its potassium salt by the addition of a 5-fold excess of K_2SO_4.

Purification of EPSP synthase

All steps after the breaking of the cells were performed at 4°C.

Step 1: extraction and centrifugation. *E. coli* (strain A.T.C.C. 14948) cells (20g), broken by two passages through a French pressure cell, were extracted with a total of 60ml of 100mM-Tris/HCl buffer, pH7.5, containing 1mM-EDTA and 0.4mM-dithiothreitol. Deoxyribonuclease I (0.5mg) was added and the extract was stirred for 1h, then centrifuged at 38000*g* for 30min. The resulting supernatant is termed the crude extract.

Step 2: fractionation with $(NH_4)_2SO_4$. Powdered $(NH_4)_2SO_4$ was slowly added with stirring to the crude extract to give a final concentration of 291g/l (50% saturation). The solution was stirred for 30min, and the precipitate was removed by centrifugation at 23000*g* for 30min. More $(NH_4)_2SO_4$ was added to the supernatant to give a final concentration of 416g/l (70% saturation), and the resulting precipitate was collected by centrifugation at 23000*g* for 30min. This precipitate was dissolved in 100mM-Tris/HCl buffer, pH7.5, containing 0.4mM-dithiothreitol and dialysed overnight against 1000ml of 50mM-Tris/HCl buffer, pH7.5, containing 50mM-KCl and 0.4mM-dithiothreitol (buffer A).

Step 3: DEAE-Sephacel chromatography. The dialysed material was loaded on to a column of DEAE-Sephacel (bed volume 60ml) equilibrated in buffer A. The column was washed with this buffer until the A_{280} of the eluate reached a constant value of approx. 0.4. The column was then eluted with a linear gradient of 50–250mM-KCl in 50mM-Tris/HCl buffer, pH7.5, containing 0.4mM-dithiothreitol (total volume 400ml; flow rate 70ml/h). Fractions (6ml) containing EPSP synthase activity were pooled and dialysed overnight against 1000ml of 100mM-Tris/HCl buffer, pH7.5, containing 0.4mM-dithiothreitol (buffer B).

Step 4: phenyl-Sepharose chromatography. Solid $(NH_4)_2SO_4$ was added to the dialysed enzyme to give a final concentration of 164g/l (30% saturation). This solution was stirred for 15min, and then loaded on to a column of phenyl-Sepharose (bed volume 40ml) equilibrated in 0.8M-$(NH_4)_2SO_4$ in buffer B until the A_{280} of the eluate was less than 0.1. The enzyme was eluted with a decreasing gradient of $(NH_4)_2SO_4$ (0.8–0.0M) in buffer B (total volume 300ml). Fractions containing EPSP synthase activity were pooled (Fig. 1) and dialysed overnight against 2×2000ml of 10mM-potassium citrate buffer, pH6.0, containing 0.4mM-dithiothreitol (buffer C).

Step 5: cellulose phosphate (P11) chromatography. The dialysed material was loaded on to a column (bed volume 5ml) of cellulose phosphate equilibrated in buffer C and washed with the same buffer until the A_{280} of the eluate was less than 0.01. EPSP synthase was eluted with buffer C containing 1mM-phosphoenolpyruvate and 1mM-shikimate 3-phosphate.

The fractions containing EPSP synthase activity were pooled (Fig. 2) and the substrates were removed on a column (27cm×2.5cm) of Sephadex G-25 (medium grade) equilibrated in buffer A. Solid $(NH_4)_2SO_4$ was added to give a final concentration of 164g/l (30% saturation), and the enzyme was bound to a column of phenyl-Sepharose (bed volume 2ml) equilibrated in buffer B containing 0.8M-$(NH_4)_2SO_4$ and eluted in buffer B. Fractions containing EPSP synthase activity were pooled and dialysed against 500ml of buffer A containing 50% (v/v) glycerol and stored at −20°C.

Assay of EPSP synthase activity

EPSP synthase activity was assayed at 25°C by two methods.

Assay method 1. The enzyme was as a routine assayed in the reverse direction by coupling the release of phosphoenolpyruvate to the pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) reactions. Oxidation of NADH was monitored at 340nm in a total volume of 1ml. The assay mixture contained (final concentrations) 100mM-potassium phosphate buffer, pH7.0, 2.5mM-ADP, 2.5mM-$MgCl_2$, 0.1mM-NADH, 50μM-potassium EPSP (to initiate assay) and 3 units of pyruvate kinase and 2.5 units of lactate dehydrogenase.

Assay method 2. The purified EPSP synthase was assayed in the forwards direction by coupling to chorismate synthase. Chorismate ($\varepsilon_{275} = 2630 M^{-1} \cdot cm^{-1}$; Gibson, 1970) formation was monitored at 275nm in a total volume of 1ml. The assay mixture contained (final concentrations) 50mM-triethanolamine hydrochloride/KOH buffer, pH7.0, 50mM-KCl, 2.5mM-$MgCl_2$, 10μM-NADPH, 10μM-FMN, 0.5mM-phosphoenolpyruvate (to initiate the assay), 0.5mM-shikimate 3-phosphate and 0.05 unit of partially purified *N. crassa* chorismate synthase (Boocock & Coggins, 1983).

One unit of enzyme activity is defined as the amount of enzyme catalysing the conversion of 1μmol of substrate/min.

Determination of protein

Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed by the method of Davis (1964) in 7%-polyacrylamide gels at 4°C. After electrophoresis, gels were stained either for protein or for EPSP synthase activity as described by Nimmo & Nimmo (1982). Electrophoresis in the presence of sodium dodecyl sulphate was performed

by the method of Laemmli (1970), with a 3% stacking gel and a 10% running gel.

Determination of M_r by gel filtration

A column (1.1 cm × 56 cm) of Sephacryl S200 (superfine grade) equilibrated in buffer A was calibrated with the following markers: horse heart cytochrome *c* (M_r 12400) Margoliash *et al.*, 1961); yeast phosphoglycerate kinase (M_r 47000) (Krietsch & Bücher, 1970); pig heart malate dehydrogenase (M_r 70000) (Thorne & Kaplan, 1963); calf intestine alkaline phosphatase (M_r 100000) (Engström, 1961); pig heart lactate dehydrogenase (M_r 144000) (Castellino & Barker, 1968); rabbit muscle pyruvate kinase (M_r 237000) (Steinmetz & Deal, 1966).

Results and discussion

The purification of *E. coli* EPSP synthase is summarized in Table 1. With the use of our coupled assay it proved to be very difficult to estimate the amount of enzyme in crude extracts because the blank rate of NADH oxidation was very high. After $(NH_4)_2SO_4$ fractionation EPSP synthase activity was located in the 50–70%-saturation fraction. The enzyme was eventually purified 843-fold and in 22% yield from this fraction.

To obtain electrophoretically homogeneous enzyme, three chromatographic steps were required. The first step (elution profile not shown) involved ion-exchange chromatography on DEAE Sephacel, and the second step hydrophobic chromatography (Shaltiel, 1974) on phenyl-Sepharose (Fig. 1). These two steps resulted in a 143-fold purification in 57% overall yield.

The final purification step involved substrate elution of the enzyme from a cellulose phosphate column. The enzyme was bound to the column at low pH (6.0) and eluted with a mixture of shikimate 3-phosphate and phosphoenolpyruvate. Enzyme activity was not eluted with phosphoenolpyruvate alone, and elution with shikimate 3-phosphate alone resulted in a broad peak of enzyme activity. Under the column elution conditions employed the bound enzyme undoubtedly catalyses the reaction of the two substrates to give EPSP. It seems very likely that it is this EPSP that leads to the elution of a narrow peak of enzyme activity (Fig. 2). Our very limited supply of EPSP precluded its use as an eluent.

The failure of phosphoenolpyruvate to elute the enzyme is consistent with our evidence that *E. coli* EPSP synthase, like the *N. crassa* enzyme (Boocock & Coggins, 1983), has an ordered mechanism in which shikimate 3-phosphate binds to the enzyme first (A. Lewendon & J. R. Coggins, unpublished work). It is interesting to compare this purification of *E. coli* EPSP synthase with the purification of another phosphoenolpyruvate-utilizing enzyme, the tryptophan-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (EC 4.1.2.15) of *N. crassa* (Nimmo & Coggins, 1981*a*). This latter enzyme has a rapid-equilibrium ordered mechanism in which phosphoenolpyruvate is the first substrate to bind (Nimmo & Coggins, 1981*b*), and it can also be eluted from a cellulose phosphate column with its first binding substrate, in this case phosphoenolpyruvate (Nimmo & Coggins, 1981*a*).

E. coli EPSP synthase appeared to be completely stable throughout the purification provided that dithiothreitol (0.4 mM) was present. The final purified EPSP synthase was freed of the eluting substrates by gel filtration on Sephadex G-25 and was dialysed against buffer A containing 50% (v/v) glycerol for long-term storage. The pure enzyme was stable for at least 4 months when stored in this solvent at −20°C.

The specific activity of the purified EPSP synthase was 62 units/mg when assayed in the forward direction and 17.7 units/mg when assayed in the reverse direction. The purified enzyme exhibited only one protein band (R_F 0.48) on simple polyacrylamide-gel electrophoresis, and this coincided with the band of EPSP synthase activity. The purified enzyme also exhibited only one protein band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

Table 1. *Purification scheme for E. coli EPSP synthase*

The results presented are for a typical purification starting from 20 g of *E. coli* cells. Enzyme activity was assayed in the reverse direction (assay method 1; see the Materials and methods section). Abbreviation: N.D., not determined.

Step	Stage	Vol. (ml)	Concn. of protein (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
1	Crude extract	74	5.5	N.D.	N.D.	—	—	—
2	50–70%-satn. $(NH_4)_2SO_4$	33	6.4	0.135	4.4	100	0.021	1
3	DEAE-Sephacel	52	0.22	0.058	3.0	68	0.264	12.6
4	Phenyl-Sepharose	38	0.022	0.066	2.5	57	3.0	143
5	Cellulose phosphate*	1.2	0.044	0.780	0.96	22	17.7	843

* Data for enzyme eluted from cellulose phosphate are for gel-filtered and glycerol-dialysed enzyme.

The subunit M_r of *E. coli* EPSP synthase was estimated to be 49000 by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Molecular-exclusion chromatography on Sephacryl S200 demonstrated that the native enzyme had M_r 55000. The same M_r value was obtained both for the homogeneous enzyme and for the EPSP synthase activity present in 100000*g* supernatants of *E. coli* cell extracts. *E. coli* EPSP synthase thus appears to be a monomeric enzyme.

The M_r of unpurified *E. coli* EPSP synthase was previously estimated to be 38000 by sucrose-density-gradient centrifugation (Berlyn & Giles, 1969). The M_r values for several other unpurified bacterial EPSP synthases have also been estimated by sucrose-density-gradient centrifugation and, with the exception of the *Bacillus subtilis* enzyme (for which a range of values were reported), all were found to be in the range 33000–45000 (Berlyn & Giles, 1969). The M_r values of partially purified

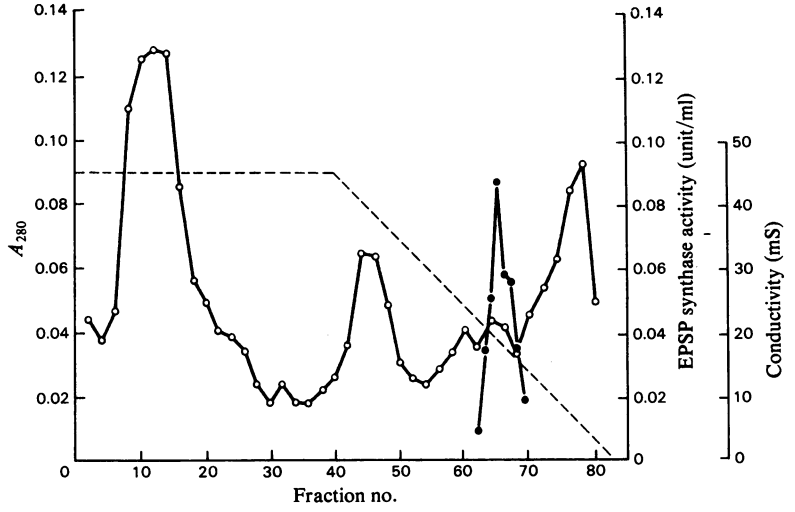

Fig. 1. *Chromatography of E. coli EPSP synthase on phenyl-Sepharose (step 4 of the purification scheme)*
Enzyme from step 3 (3 units in 52ml) was treated with $(NH_4)_2SO_4$ and loaded on to a 40ml phenyl-Sepharose column as described in the Materials and methods section. The column was eluted with a 300ml decreasing linear gradient of $(NH_4)_2SO_4$ (0.8–0.0M) in buffer B. The flow rate was 70ml/h, and 6ml fractions were collected. O, A_{280}; ●, EPSP synthase activity; ——, conductivity.

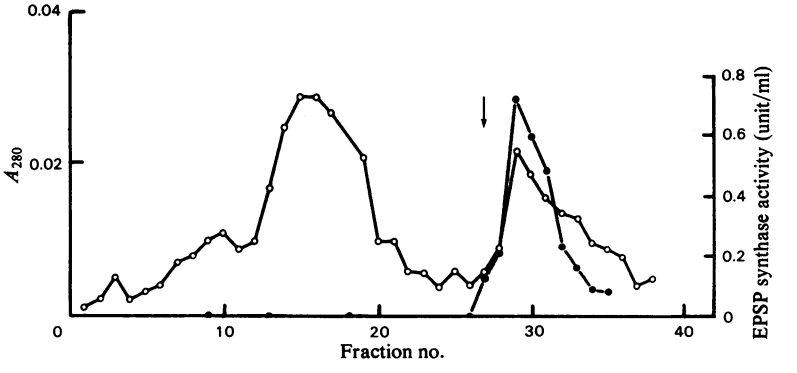

Fig. 2. *Chromatography of E. coli EPSP synthase on cellulose phosphate (step 5 of purification scheme)*
Enzyme from step 4 (2.5 units in 38ml) was loaded on to a 5ml cellulose phosphate column as described in the Materials and methods section. The column was washed with buffer C, and at the arrow buffer containing 1mM-shikimate 3-phosphate and 1mM-phosphoenolpyruvate was applied. The flow rate was 7.2ml/h, and 2.4ml fractions were collected. O, A_{280}; ●, EPSP synthase activity.

EPSP synthase isolated from mung beans, *Phaseolus mungo* (Koshiba, 1979), and the fungus *Hansenula henricii* (Bode & Birnbaum, 1981) have been estimated by molecular-exclusion chromatography to be 44000. All these estimates of M_r are in the same range as our value for the *E. coli* enzyme.

Note added in proof (received 29 March 1983)

In their 1983 Newsletter, the Nomenclature Committee of IUB (NC-IUB) and the IUB–IUPAC Joint Commission on Biochemical Nomenclature (JCBN) have suggested that EPSP synthase should be given the systematic name 3-phosphoshikimate 1-carboxyvinyltransferase.

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