The purification of 5-enolpyruvylshikimate 3-phosphate synthase from an overproducing strain of *Escherichia coli*

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Received 4 November 1983

The *Escherichia coli aroA* gene which codes for the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase) has been cloned from the λ-transducing bacteriophage λ*pserC*. The gene has been located on a 4.7 kilobase pair *Pst*I DNA fragment which has been inserted into the multiple copy plasmid pAT153. *E. coli* cells transformed with this recombinant plasmid overproduce EPSP synthase 100-fold. A simple method for the purification of homogeneous enzyme in milligram quantities has been devised. The resulting enzyme is indistinguishable from enzyme isolated from untransformed *E. coli*.

5-Enolpyruvylshikimate 3-phosphate synthase *3-Phosphoshikimate 1-carboxyvinyltransferase*
aroA *gene* Escherichia coli *Shikimate pathway* *Glyphosate*

1. INTRODUCTION

The discovery that glyphosate (*N*-phosphonomethylglycine, 'Roundup'), a successful, broad-spectrum, post-emergence herbicide, acts by blocking aromatic amino acid biosynthesis at the level of EPSP synthase (EC 2.5.1.19, alternative name 3-phosphoshikimate 1-carboxyvinyltransferase) has focussed a great deal of attention on this enzyme and its mechanism of action [1–6]. Until recently the only reported purifications to homogeneity of EPSP synthases were those of the multifunctional enzymes containing this activity found in *Neurospora crassa [7–9]* and *Euglena gracilis* [10]. Monofunctional enzymes have now been purified to homogeneity in our laboratory from both *Escherichia coli* [11] and pea seedlings [12]. In no case however has the enzyme been purified on a sufficient scale to allow detailed mechanistic and protein chemical studies to be performed. To assist our studies in this area we have cloned the *E. coli* gene *(aroA)* coding for EPSP synthase and inserted it into the multicopy plasmid pAT153 [13]. We report here on the use of this plasmid to transform *E. coli* cells so that they overproduce EPSP synthase about 100-fold. A simple method for purifying milligram quantities of the enzyme is presented together with evidence that the overproduced enzyme is identical with the enzyme previously isolated from *E. coli* K12 cells [11].

Abbreviations: Bis-tris, bis(2-hydroxyethyl) iminotris-(hydroxymethyl)methane; EPSP, 5-enolpyruvylshikimate 3-phosphate; PEP, phospho*enol*pyruvate; SDS, sodium dodecyl sulphate; shik 3-*P*, shikimate 3-phosphate

2. MATERIALS AND METHODS

2.1. *Reagents*

All the coupling enzymes and other reagents for the enzyme assays and the enzyme purification were obtained as in [3,11]. Restriction enzymes were purchased from Bethesda Research Laboratories (Cambridge, England) and digestions carried out under their recommended conditions. Calf intestinal phosphatase was from Boehringer (Lewes,

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00145793/84/$3.00

England and *Staphylococcus aureus* V8 protease from Miles (Slough, England).

2.2. *Enzyme assays*

Enzyme activity in crude extracts and at each stage of the purification was determined by assay in the reverse direction by coupling the formation of PEP to the pyruvate kinase and lactate dehydrogenase reactions as in [11].

For kinetic studies EPSP synthase was assayed in the backward direction as above except that the assay buffer was 50 mM Bis-tris-KOH (pH 7.0), 50 mM KCl, 2.5 mM $MgCl_2$. In the forward direction it was assayed by coupling to the chorismate synthase reaction and continuously measuring the formation of chorismate at 275nm [11]. The assay mixture contained 50 mM Bis-tris-KOH (pH 7.0), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM FMN, 20 μM NADPH, 10 m units partially purified *N. crassa* chorismate synthase [3], PEP, shik 3-*P* and, where appropriate, glyphosate.

All assays were conducted at 25°C. One unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol substrate/min.

2.3. *Construction of pKD501 and pKD502*

Digestion of λp*serC* DNA [14] was carried out with *Pst*I and the fragments separated by electrophoresis on a low-melting-point agarose gel [15]. The region of the gel containing the 4–5 kilobase pair fragments was excised and the DNA extracted [15]. This DNA was ligated to pAT153, which had been treated with *Pst*I and calf intestinal phosphatase, and used to transform $CaCl_2$-treated *E. coli* strain AB2829 (CGSC2829) [16]. Cells were plated onto L agar containing tetracycline (20 μg/ml). After overnight growth colonies were replica plated onto minimal medium. Colonies growing on minimal medium were picked and checked for ampicillin sensitivity. Plasmid DNA was extracted from 10 tet^R, amp^S colonies [17], and characterised by restriction enzyme digestion.

2.4. *Growth of EPSP synthase overproducing strain*

E. coli strain AB2829/pKD501 was grown in 500-ml batches on minimal medium (M9 salts plus 0.2% (w/v) glucose [15]) in 2-l shaking flasks at 37°C. Cells were grown into stationary phase (A_{650} 1.3–1.5) and then harvested by centrifugation and stored at −20°C.

2.5. *Purification of EPSP synthase*

E. coli (strain AB2829/pKD501) cells (~20 g) were broken in a French pressure cell and extracted into 100 mM Tris-HCl (pH 7.5), 0.4 mM DTT (buffer A) containing 1 mM EDTA as in [11]. This crude extract was fractionated with $(NH_4)_2SO_4$ and the enzyme isolated in the 50–70% precipitate [11] which was collected by centrifugation and dialysed against 1 l 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.4 mM DTT (buffer B).

The dialysed fraction was loaded onto a column of DEAE-Sephacel (100 ml bed volume) previously equilibrated with buffer B. The column was then washed with 4–5 column volumes of buffer B and EPSP synthase activity was eluted with a linear gradient of 50–250 mM KCl in 50 mM Tris-HCl (pH 7.5), 0.4 mM DTT (gradient volume 1 l, flow rate 100 ml/h). Fractions containing EPSP synthase activity were pooled and dialysed overnight against 1 l buffer A.

The dialysed enzyme solution was made 30% saturated in $(NH_4)_2SO_4$ and loaded onto a column of phenyl-Sepharose (40 ml bed volume) previously equilibrated with buffer A containing 0.8 M $(NH_4)_2SO_4$. The column was washed with 3 column volumes of this buffer and then the enzyme was eluted with a decreasing gradient of 0.8–0.0 M $(NH_4)_2SO_4$ in buffer A (gradient volume 300 ml, flow rate 60 ml/h). Fractions containing EPSP synthase activity were pooled and dialysed against 3 changes of 2 l 10 mM potassium citrate (pH 6.0), 0.4 mM DTT (buffer C) for a total period of 24 h.

After dialysis the enzyme solution was loaded onto a cellulose phosphate column (P11, 30 ml bed volume) previously equilibrated with buffer C. The column was then washed with this buffer until the A_{280} of the eluate was about 0.01. EPSP synthase was eluted with buffer C containing 1 mM PEP and 1 mM shik 3-*P*. The eluted enzyme was concentrated by vacuum dialysis, then dialysed against buffer B containing 50% glycerol (v/v) for long-term storage at −20°C.

2.6. *Determination of protein*

Protein was determined as in the method of Bradford [18].

2.7. *Polyacrylamide gel electrophoresis*

Electrophoresis was carried out as in [19] in 7% polyacrylamide gels at 4°C. Gels were either stained for protein using Coomassie brilliant blue [7] or for EPSP synthase activity [20]. Electrophoresis in the presence of SDS was performed as in [21] with a 3% stacking gel and a 10% running gel.

2.8. *Limited proteolysis and one-dimensional peptide mapping [22]*

Samples of purified EPSP synthase (2.5 μg overproduced enzyme, 1 μg 'wild-type' enzyme)) were boiled for 2 min in 125 mM Tris–HCl (pH 6.8), 0.5% (w/v) SDS, 10% (v/v) glycerol, 0.0001% (w/v) bromophenol blue in a total volume of 50 μl. Then 20 μl of a solution of *Staphylococcus aureus* V8 protease (0.5 mg/ml in the same buffer) was added. After 75 min at 37°C digestion was stopped by adding SDS and 2-mercaptoethanol to give final concentrations of 2% (w/v) and 10% (v/v), respectively, and then boiling for 2 min. Samples were then subjected to polyacrylamide-gel electrophoresis in the presence of SDS and the gels stained for protein using the silver method [23].

2.9. *Gel permeation chromatography*

Gel permeation chromatography was performed on a 60 × 0.75 cm TSK G2000 SW column (L.K.B., South Croydon, England) at room temperature with 0.067 M potassium phosphate (pH 6.8) as the mobile phase. Injections (2 μg each of calibration proteins, 0.8 μg purified EPSP synthase) were made in 25 μl. The flow rate of 0.5 ml/min was provided by a model 303 pump (Gilson, Villiers-le-Bel, France). The column eluate was monitored at 215 nm with a model M300 variable wavelength UV detector (Michrom, Crowborough, England) fitted with an 8 μl flow cell. The M_r calibration proteins employed were sperm whale myoglobin (17 000); bovine erythrocyte carbonic anhydrase (29 000); chicken ovalbumin (45 000) and bovine serum albumin (68 000).

3. RESULTS AND DISCUSSION

The λ-transducing bacteriophage λp*serC* carries the region of the *E. coli* chromosome around 20 min. It had been shown previously that the *aroA* gene is carried by this bacteriophage [14]. Since the *aroA* gene occurs as a single copy in lysogens of λp*serC* it was decided to sub-clone the gene into the multicopy vector pAT153 [13] in order to facilitate overexpression of the enzyme. Data obtained from restriction endonuclease mapping and sequencing p*serC* [24] suggested that the *aroA* gene was probably located on a 4.6 kilobase pair *PstI* fragment within the chromosomal insert. This DNA fragment was isolated by preparative electrophoresis and ligated into pAT153. Ligation mixtures were used to transform *E. coli* AB2829 (an *aroA* strain [16]) and the transformed cells were plated onto L agar containing tetracycline. After overnight growth colonies were replicate plated onto minimal medium. Because of the auxotrophic requirements of AB2829, only colonies carrying recombinant plasmids containing the *aroA* gene would grow under these conditions. From the hundreds of colonies which grew on minimal medium 100 were picked and checked for ampicillin resistance and plasmid DNA was extracted from tet^R, amp^S colonies. Restriction endonuclease mapping established that all the clones carried the anticipated 4.6 kilobase pair *Pst*I fragment. Two clones (pKD501 and pKD502) were retained for further examination; each carries the 4.6 kilobase pair DNA fragment but in opposite orientations.

Measurement of the EPSP synthase activity in crude extracts of *E. coli* AB2829/pKD501 established that this strain was an excellent source of the enzyme. The specific activity was 0.42 units/mg compared with about 0.004 units/mg for *E. coli* K12 (strain ATCC14948).

Details of the purification of EPSP synthase from *E. coli* AB2829/pKD501 are given in table 1. The purification scheme is similar to that employed for the purification of the enzyme from *E. coli* K12 [11]. The key steps were ion exchange chromatography on DEAE-Sephacel, hydrophobic chromatography on phenyl-Sepharose and combined substrate elution from cellulose phosphate. A polyacrylamide gel showing the protein components at each stage of the purification is shown in fig. 1. Homogeneous enzyme, as judged by polyacrylamide gel electrophoresis under native conditions (not shown) and in the presence of SDS (fig. 1), was obtained in 30% overall yield. The purification required was only 50-fold and the purification required from the ammonium sulphate fraction was 26-fold compared with the

Table 1

Purification scheme for EPSP synthase from pKD501 transformed *E. coli* AB2829

Step	Stage	Volume (mg/ml)	Protein (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (-fold)
1	Crude extract	77	10.4	4.35	335	100	0.42	1
2	50–70% $(NH_4)_2SO_4$	40	6.8	5.55	222	66	0.82	1.9
3	DEAE-Sephacel	81	0.65	2.12	171	51	3.2	4.0
4	Phenyl-Sepharose	60	0.14	2.25	135	40	16.1	38
5	Cellulose phosphate	23	0.21	4.43	102	30	21.1	50

The results presented are for a purification from 18 g of cells. Enzyme activity was assayed in the reverse direction (see section 2)

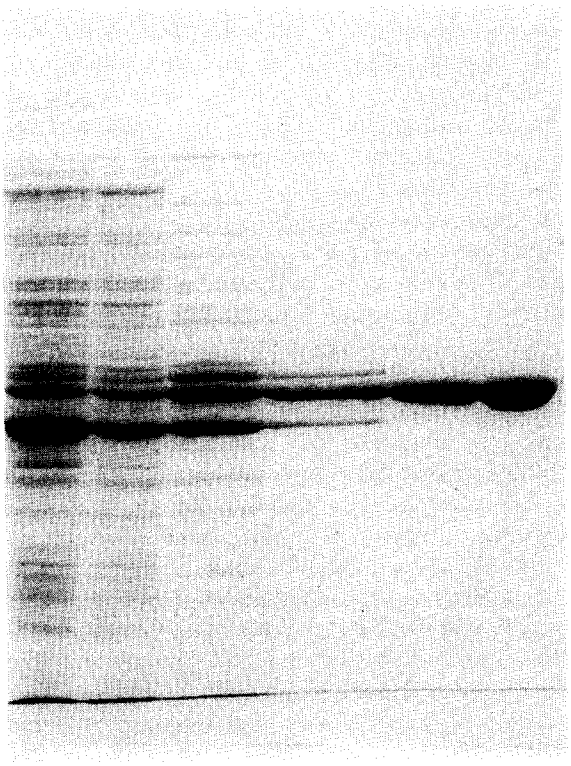

Fig. 1. Purification of EPSP synthase from *E. coli* strain AB2829/pKD501. This 10% polyacrylamide gel run in the presence of SDS monitors the purification of EPSP synthase. Track A, 50–70% ammonium sulphate fraction (68 μg protein); track B, enzyme eluted from DEAE-Sephacel column (32 μg); track C, enzyme eluted from phenyl-Sepharose column (7 μg); tracks D and E, enzyme eluted from cellulose phosphate column (4 μg and 7.5 μg).

846-fold required from *E. coli* K12 [11]. Since the overproduced enzyme was required for protein chemistry and enzymology it was essential to demonstrate that it was identical to that purified from the wild type K12 strain ATCC14948.

Firstly, it was found that the overproduced enzyme and the wild type enzyme were indistinguishable on polyacrylamide gel electrophoresis under native conditions and in the presence of SDS. The subunit M_r estimated by polyacrylamide gel electrophoresis in the presence of SDS was 49 000 [11]. As shown in fig. 2 identical fragmentation patterns were obtained when the two enzymes were partially digested with *S. aureus* V8 protease. This confirmed that the two enzymes had the same peptide composition and in particular that the overproduced enzyme contained the complete EPSP synthase amino acid sequence.

Both enzymes eluted in the same fraction from a TSK G2000 SW gel permeation column. This established that enzyme isolated from the two sources had the same native M_r of 42 000 and confirmed that *E. coli* EPSP synthase is monomeric. This estimate of the native M_r value is lower than the value of 55 000 obtained previously for the K12 enzyme by gel permeation chromatography on Sephacryl S200 [11]. The native and the subunit M_r values reported here for the K12 enzyme and the overproduced enzyme are very similar to those obtained by the same methods for the pea seedling enzyme (subunit M_r 50 000 and native M_r 44 000 [12]). The discrepancy between the native M_r values for the K12 enzyme obtained by gel permeation chromatography on Sephacryl S200 and on TSK G2000 SW is consistent with other observa-

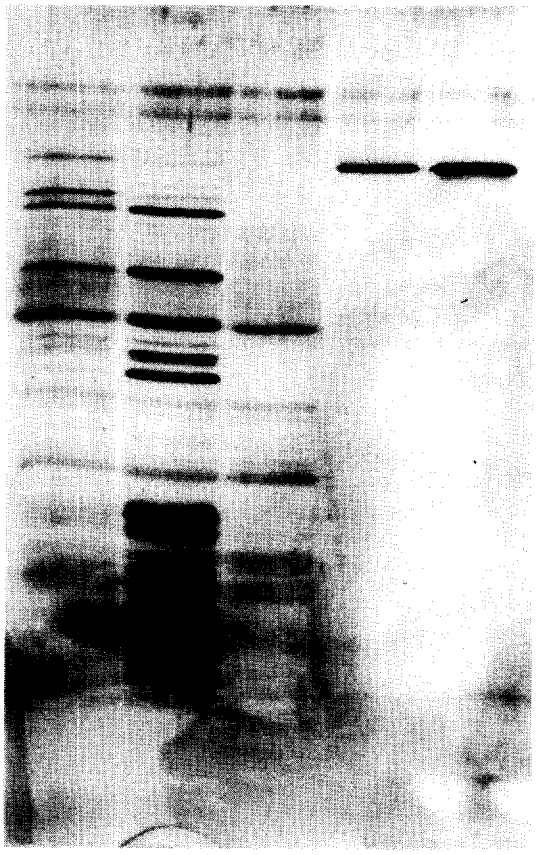

Fig. 2. One-dimensional peptide maps of wild type *E. coli* EPSP synthase and the overproduced EPSP synthase. Samples of EPSP synthase isolated from the overproducing strain AB2829/pKD501 and from the wild type strain ATCC14948 were subjected to limited proteolysis with *S. aureus* V8 protease as described in section 2. Track A, a digest of 1 μg wild type enzyme; track B, a digest of 2.5 μg overproduced enzyme; track C, V8 protease control; track D, 0.3 μg wild type enzyme; track E, 0.7 μg overproduced enzyme. The 15% polyacrylamide gel run in the presence of SDS was stained for protein by the silver method.

tions made in our laboratory. We have noted several examples of enzymes which give significantly different M_r values on these two types of column (Chaudhuri, S. and J.R. Coggins, unpublished).

These experiments confirmed that the overproduced EPSP synthase was chemically identical with the wild type *E. coli* K12 enzyme. It now remained to establish that the kinetic properties of the overproduced enzyme were also the same as those of the wild type enzyme. The specific activity of the overproduced enzyme was 21.1 units/mg which is very similar to the value of 17.7 units/mg previously reported for the enzyme from *E. coli* strain ATCC14948 [11]. The kinetic parameters of the two enzymes are given in table 2. The K_m and the K_i values for glyphosate, which is a competitive inhibitor with respect to PEP [3, 12], are essentially identical as one would expect for two separate preparations of the same enzyme. Preliminary kinetic parameters are now available for both the *E. coli* and pea seedling enzymes. These can be compared with the limiting K_m values previously reported for the *N. crassa* enzyme [3]. As can be seen in table 3 the K_m values for shik 3-*P*, PEP and EPSP are very low. It should be noted that EPSP is a very effective product inhibitor of the forward reaction (Boocock, M.R. and J.R. Coggins, unpublished) and it was therefore essential to use a sensitive coupled assay system so that reliable estimates of the K_m values could be made. The second interesting point is that the pea seedling enzyme has a very much lower K_i for glyphosate than the *E. coli* and the *N. crassa* enzymes. This means that glyphosate is a far more effective inhibitor of the pea seedling enzyme than of the *E. coli* or *N. crassa* enzymes. This is most clearly shown by the ratio K_i for glyphosate/K_m for PEP which for the pea seedling enzyme is 0.015 while for the *E. coli* and *N. crassa* enzymes the ratios are 0.56 and 0.41, respectively.

One further property of the overproducing strain is worthy of comment. The growth of *E. coli* [4] and other bacteria and plant cell cultures [5, 6] is normally severely inhibited by glyphosate. Mutant strains that overproduce EPSP synthase have been shown to be highly tolerant to glyphosate [4, 5]. The EPSP synthase overproducing strain described here (AB2829/pKD501) was found to grow normally on plates containing 50 mM glyphosate as was another transformed strain ATCC14948/pKD501. In comparison the wild type strain ATCC14948 failed to grow on plates containing 3 mM glyphosate.

The data presented here establish that EPSP synthase purified in milligram quantities from the overproducing *E. coli* strain AB2829/pKD501 is chemically and enzymatically indistinguishable

Table 2

Comparison of the kinetic parameters of EPSP synthase purified from wild type *E. coli* K12 (ATCC14948) and from the overproducing strain AB2829/pKD501

Kinetic parameter	*E. coli* ATCC14948 enzyme	*E. coli* AB2829/pKD501 enzyme
K_m for PEP	15 μM (200 μM shik 3-*P*)	16 μM (200 μM shik 3-*P*)
K_m for shik 3-*P*	3.5 μM (500 μM PEP)	2.5 μM (200 μM PEP)
K_m for phosphate	2.5 mM (50 μM EPSP)	2.5 mM (50 μM EPSP)
K_m for EPSP	not determined	3.0 μM (50 mM phosphate)
K_i for glyphosate	1.0 μM (200 μM shik 3-*P*)	0.9 μM (200 μM shik 3-*P*)

Details of the assay conditions are given in section 2. The co-substrate concentrations are given in brackets

Table 3

Comparison of the kinetic parameters of three purified EPSP synthases

Kinetic parameter	*E. coli* AB2829/pKD501 enzyme	*N. crassa* enzyme	*P. sativum* enzyme
K_m for PEP	16 μM	2.7 μM	5.2 μM
K_m for shik 3-*P*	2.5 μM	0.36 μM	7.7 μM
K_m for EPSP	3.0 μM	0.25 μM	5.2 μM
K_m for phosphate	2.5 mM	1.8 μM	4.0 mM
K_i for glyphosate	0.9 μM	1.1 μM	0.08 μM

The data for the *E. coli* enzyme (this paper) and the *Pisum sativum* enzyme [12] were obtained at fixed co-substrate concentrations as specified elsewhere. The data for the *N. crassa* enzyme

from enzyme purified from the wild type strain ATCC14948. The availability of large quantities of pure, active protein will greatly facilitate further mechanistic and structural studies on this enzyme.

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