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Isolation and Purification of Recombinant EPSP Synthase from the Pathogenic Bacterium *Pseudomonas aeruginosa*

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ABSTRACT: The *Pseudomonas aeruginosa aroA* gene encodes an enzyme called 5-*enol*-pyruvylshikimate-3-phosphate (EPSP) synthase, which is the primary target of the herbicide glyphosate. We have amplified and cloned the *aroA* gene from the *P. aeruginosa* genomic DNA and subcloned it into a vector suitable for high level expression of a recombinant form of this enzyme in *Escherichia coli*. The *E. coli* transformed with the resulting plasmid, p*Trc*PA, produced the EPSP synthase in large quantities, which allowed it to be purified to homogeneity. Furthermore, site-directed mutants of *P. aeruginosa* ESPS synthase have been constructed in order to compare *in vitro* glyphosate sensitivity between the wild-type and the mutant enzymes. The k_{cat} and K_m values for substrates in both forward and reverse reactions were obtained from both wild-type and mutant EPSP synthases.

Abbreviations: IPTG, isopropyl-b-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; S3P, shikimate-3-phosphate; PEP, phosphoenolpyruvate.

KEYWORDS: EPSP synthase; *P. aeruginosa*; Isolation; Purification.

INTRODUCTION

Attention has been focused on the development of new antimicrobial agents due to the emergence of multi-drug resistant strains of pathogenic bacteria[1,2]. This consequently demands the identification of suitable drug targets. The suitability of the *aroA*-encoded EPSP synthase as a target for antimicrobial agents is supported, in part, by the finding that inactivation of *aroA* in a number of pathogens, such as *Salmonella typhimurium*[3], *S. typhi*[4], *Yersinia enterocolitica*[5] and *Shigella flexneri*[6] results in strains attenuated for virulence. However, *E. coli* EPSP synthase has been the prototype for most protein and enzymological studies[7], partly due to the availability of its crystal structure[8] and its use as the model for inhibition by the glyphosate[9]. For the development of new EPSP synthase antimicrobial agents, it would be advantageous to characterize the enzyme from the pathogen of interest, in terms of its activity and inhibition by lead compounds including glyphosate, which is known to demonstrate antimicrobial activity against *E. coli* and other pathogens[10,11]. It is also worth noting that some microorganisms, such as *P. aeruginosa*[10], *Pseudomonas* strains PG2982 and GLC11[12,13], *Arthrobacter* sp. GLP-1[14], the cyanobacteria *Anabaena varibilis* and *Synechocystis* sp. PCC6803[15] and *Burkholderia pseudomallei*[16], are naturally resistant to glyphosate.

P. aeruginosa is an opportunistic pathogen that infects immunocompromised individuals, including those suffering from severe burns or wounds, cystic fibrosis or AIDS. Treatment of this infection is rather difficult as the organism is resistant to a number of antibiotics and glyphosate[10]. Its EPSP synthase activity in crude cell extract was determined as a function of glyphosate concentration and showed an I_{50} value for glyphosate in the range of 2-6 mM, which was much higher than that of the *E. coli* enzyme (0.08-0.18 mM)[10]. It is thought that the ability of *P. aeruginosa* to demonstrate resistance against glyphosate may arise from its ability to utilize glyphosate as a phosphorus source[12,13]. As it is also possible that the *P. aeruginosa* EPSP synthase may be glyphosate resistant[10], the *aroA* encoding EPSP synthase from this organism was cloned as a prerequisite for detailed enzymological studies.

In this article, we report on the cloning of the *P. aeruginosa aroA* and construction of a system for the high level expression of a recombinant, native form of *P. aeruginosa* EPSP synthase in *E. coli*. We also described the purification and the initial characterization of enzyme activity for this recombinant form of *P. aeruginosa* EPSP synthase.

Materials and Methods

Cloning of *P. aeruginosa aroA* Gene

The genomic DNA from *P. aeruginosa* strain PAO1 was used for cloning. Using PCR primers designed to include the start and stop codons of the *aroA*, a 1.3 kb fragment was produced and used to probe a Southern blot which led to the subsequent isolation of a 1.5 kb SalI fragment. This fragment was cloned into the cloning vector pUC18, resulting in pUC18PA, and the transformed clone demonstrated complementation in *E. coli* strain AB2829, which is an *aroA* auxotroph.

Expression Plasmid Construction

The pUC18PA was used as a template in the amplification of the *P. aeruginosa aroA* gene by PCR with two synthetic oligonucleotide primers. Primer 1 (5'-GAGTGAATTCCACAACAACGACCTGATTTATCTGG-3') incorporates a unique *Eco*RI site, whereas Primer 2 (5'-TGCTCAAAGCTTATCAATTGTTCTCCACGGCGACCCGG-3') introduces a unique *Hind*III site downstream of the TCA stop codon. PCR amplifications were performed in 25 ml solutions containing 2.5 ml of 10 X reaction buffer [100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-Cl (pH 8.75), 20 mM $MgSO_4$, 1% Tritonâ X-100, 1000 mg/ml BSA], 0.8 ml of *Pfu* polymerase (2.5 U/ml), 1.5 ml of DNA template (50 ng/ml DNA), 8 ml of mixed deoxyribonucleotide solution (400 mM of dNTPs), 5.2 ml of mixed oligonucleotide primers (1 mM of each primer), and 5 ml of 50 mM $MgCl_2$. A single cycle at 95 °C for 30 s followed by 30 cycles (95 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min), linked to a final cycle of 72 °C for 7 min was used to generate a 1320 bp fragment encoding the *P. aeruginosa aroA*. The fragment was gel-purified, digested with *Eco*RI and *Hind*III, and ligated into the *Eco*RI and *Hind*III sites of the IPTG-inducible expression vector p*Trc99*A to create plasmid pTrcPA (p*Trc99*A+*P. aeruginosa aroA*). Initial clones were obtained by transformation of the ligation mixture into *E. coli* AB2829. The PCR-generated fragment encoding PA (*P. aeruginosa aroA*), used for the construction of pTrcPA, was sequenced on an ABI/Perkin-Elmer 377 Automated Sequencer and analyzed with ABI Sequencing Analysis (v 3.3) and Factura (v 2.0).

Screening for IPTG-induced Overexpression

The expression was done by inoculating 10 ml LB containing 100 mg/ml ampicillin with a single colony of *E. coli* AB2829[pTrcPA] and then allowing the culture to grow overnight at 37 °C. The following day, 2 ml of culture was inoculated into 200 ml of fresh LB containing 0.1 mg/ml ampicillin and grown at 37 °C. At a culture A_{600} of 1.0, IPTG was added to a concentration of 0.2 mg/ml and the cultures were grown at 37 °C overnight. Cells were pelleted by centrifugation (4,000 *g* for 10 min) and sonicated (2 X 15 sec bursts at full power) in 50 mM K_2HPO_4/ KH_2PO_4 (pH 6.8). Insoluble material was removed by centrifugation (14,000 *g* for 20 min). Supernatant was assessed for the presence of over-produced EPSP synthase by SDS-PAGE and by assays for enzyme activity.

Gel Electrophoresis and Staining

Protein samples obtained from expression and purification were analyzed using polyacrylamide gradient Phast Gels in conjunction with a Phast electrophoresis system. SDS and native polyacrylamide gels were stained with Coomassie Brilliant Blue. Native gels were also stained for EPSP synthase activity. EPSP synthase activity was visualized after soaking in 50 mM glycine-KOH (pH 10) at 37 °C for 20-30 min. These gels were then transferred to a staining mixture [50 mM glycine-KOH (pH 10), 1 mM S3P, 2 mM PEP, 10 mM $CaCl_2$]. Staining was carried out at 37 °C until the white bands of the precipitated calcium salt were clearly visible.

Construction of the *P. aeruginosa aroA* Mutants

In order to make the site-directed *P. aeruginosa aroA* mutant, the PCR-based mutagenesis protocol adapted in our laboratory was applied as follows. The protocol requires two separate PCR, with pTrcPA plasmid as template and four primers. Primers 1 and 2 (5'-GAGTGAATTCCACAACAACGACCTGATTTATCTGG-3'and5'-CAGACCGCTGAGCAGACGCATGGAAGTAGCGGAGTT-3', respectively, for plasmid pTrcPAG99A, and 5'-GAGTGAATTCCACAACAACGACCTGATTTATCTGG-3'and5'-CGGCTGGGCCGCCAGCAGACCGCTGAGCGGACGCAT-3', respectively, for plasmid pTrcPAL104P) produce a fragment containing the site-directed mutation. Primers 3 and 4 (5'-ACTTCCATGCGTCTGCTCAGCGGTCTGCTG-3'and 5'-TGCTCAAAGCTTATCAATTGTTCTCCACGGCGACCCGG-3', respectively, for plasmid pTrcPAG99A, and 5'-CTCAGCGGTCTGCTGGCGGCCCAGCCGTTC-3' and 5'-TGCTCAAAGCTTATCAATTGTTCTCCACGGCGACCCGG-3', respectively, for plasmid pTrcPAL104P) create another fragment, which has a region overlapping to the first fragment but does not include the mutation site. Primers 1 and 4 also incorporate the restriction sites at the 5' end of the first fragment and 3' end of the second fragment, respectively. The two resulting fragments are then used as templates in a second PCR with primers 1 and 4. This generates the entire gene containing the mutation of choice. This fragment was ligated into p*Trc99*A to create plasmids pTrcPAG99A (p*Trc99*A + *P. aeruginosa aroA* mutated at G99 to A) and pTrcPAL104P (p*Trc99*A + *P. aeruginosa aroA* mutated at L104 to P). Initial clones were obtained by transformation of the ligation mixtures into *E. coli* strain AB2829. The screening for IPTG-induced overexpression of the two mutants was performed as previously described.

Purification of EPSP Synthase from PA, PAG99A and PAL104P

Crude Cell Extract (Step i)-The *E. coli* strain AB2829[pTrcPA] was grown and harvested as previously described. Cells were resuspended in 50 mM K_2HPO_4/KH_2PO_4 phosphate (pH 6.8) containing 0.4 mM DTT (buffer A) and lysed by sonication. Insoluble material was removed by centrifugation. The resulting supernatant was treated with 100 mg/ml DNAse and 100 mg/ml RNAse for 30 min at 4 °C and then re-centrifuged.

DEAE-Sepharose Anion-exchange Chromatography (Step ii)- A Pharmacia XK-16/40 column containing DEAE-Sepharose attached to the BioCAD 700E Workstation was equilibrated with buffer A at a flow rate of 3 ml/min. The final supernatant from step i was loaded onto the column. The column was washed with buffer A and column bound proteins were eluted with a linear gradient of 0-1.0 M NaCl in buffer A, followed by 1 M NaCl in buffer A, while 4-ml fractions were collected. The protein peak fractions were assessed for purity using SDS-PAGE, pooled and concentrated by centrifugation in a Macrocep 30 K at 4000 *g*, 4 °C. The concentrate was then assayed for EPSP synthase activity.

Superdex 200 Gel Filtration Chromatography (Step iii)- A Pharmacia Superdex 200 HR 10/30 gel filtration column attached to a Pharmacia FPLC system was first equilibrated at a flow rate of 0.2 ml/min with buffer B [25 mM K_2HPO_4/KH_2PO_4 (pH 6.8) containing 0.4 mM DTT] and the concentrated pooled fractions from step ii were loaded onto the column. 1-ml fractions eluted by buffer B were collected, assessed for protein composition and purity by SDS-PAGE, pooled, and assayed for the enzyme activity as previously described.

Resource Q Anion-exchange Chromatography (Step iv)- A Pharmacia Resource™ Q column attached to the BioCAD 700E Workstation was equilibrated with buffer C [10 mM K_2HPO_4/KH_2PO_4 (pH 6.8) containing 0.4 mM DTT] at the flow rate of 5 ml/min. The pooled fractions from step iii were loaded onto the column. The column was washed with buffer C and eluted with a linear gradient of 0-1.0 M NaCl in buffer C. 1.5-ml fractions were collected, assessed for purity by SDS-PAGE, pooled, and assayed for enzyme activity as previously described.

Protein Analysis

Protein concentrations were determined as described by Bradford using the Bio-Rad protein assay. N-terminal amino acid sequencing was performed on the Resource Q-purified PA after gel electrophoresis and transfer to ProBlott PVDF membrane. Samples were sequenced with an ABI 477A Protein Sequencer. The final purified PA was also analysed for the amino acid composition. 10 nmoles of the Resource Q™-purified EPSP synthase was used for the determination of amino acid compositions using an Alpha Plus II Amino Acid Analyzer.

Native Gel Analysis

This method is the general procedure for staining for activity of enzymes that catalyze the release of phosphate by utilizing the insolubility of calcium phosphate. Prior to staining for activity, approximately 1 μg protein samples were separated by electrophoresis on native polyacrylamide gels. Gels to be stained for activity were soaked in 50 mM glycine-KOH (pH 10) at 37 °C for 20-30 min. These gels were then transferred to a staining mixture [50 mM glycine-KOH (pH 10), 1 mM S3P, 2 mM PEP, 10 mM $CaCl_2$]. Staining was carried out at 37 °C until the white bands of the precipitated calcium salt were clearly visible. The effect of glyphosate on EPSP synthase activity in this assay was also determined. The protocol was performed in the same way as described above but glyphosate was added to the staining mixture at one of the following concentrations: 20 mM, 200 mM, 2 mM or 20 mM.

Kinetic Characterization

Spectrophotometer measurements were made on a Perkin-Elmer Lambda 3 UV-visible spectrophotometer interfaced to a personal computer.

EPSP synthase Assay-Enzyme assay was carried on in the reverse direction at 25 °C by coupling the formation of PEP to the pyruvate kinase and lactate dehydrogenase reactions[17]. The assay solution (1.0 ml) contained 0.5 ml of 2 X assay buffer [1 mM phosphate buffer (pH 7.0), 25 mM ADP, 1 mM $MgCl_2$], 50 ml of 2 mM NADH, 50 ml of 0.7 mg/ml EPSP and 5 ml of mixed 3.5 U pyruvate kinase/ 5.0 U lactate dehydrogenase. The progress of NADH oxidation was monitored spectrophotometrically at 340 nm (e_{340} = 6200 M^{-1} cm^{-1}). One unit of EPSP synthase activity is defined as the amount of EPSP synthase catalyzing the conversion of 1 mmol of substrate per minute.

For determination of steady-state parameters, rate measurements were made as described above except that the assay buffer was 50 mM Bis-Tris-propane (pH 7.0), 50 mM KCl, 2.5 mM $MgCl_2$, 25 mM ADP, 10 mM NADH and 5 ml of mixed 3.5 U pyruvate kinase/ 5.0 U lactate dehydrogenase. A 5-s delay was allowed after initiation of the reaction. The apparent K_m for EPSP was determined by varying its concentrations while maintaining a constant concentration of PO_4 (50 mM). The K_m for PO_4 was determined by maintaining EPSP concentration constant (50 mM) while varying PO_4 concentrations. The rate associated with each individual assay solution was determined using an enzyme concentration in the range between 0.01-0.02 mM. The K_m values for both EPSP and PO_4 were estimated by fitting the appropriate experimental rates to the Michaelis-Menten equation using the weighted non-

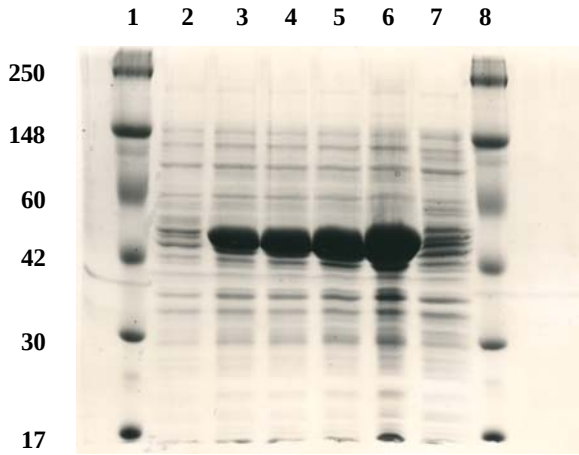

Fig 1. Expression of the cloned *P. aeruginosa* EPSP synthase gene in *E. coli* AB2829. Gel electrophoresis of the crude extracts from cells carrying non-induced pTrcPA (2, 7) and pTrcPA (3-6) cultured in the presence of IPTG at 37 °C for 2, 4, 6 h and overnight, respectively. Lanes 1 and 8 contain MW standards with sizes given in kDa to the left of the picture.

linear regression data analysis package Grafit. The k_{cat} value or catalytic constant was calculated using the equation $k_{cat} = V_{max}$/[enzyme concentration].

Results

Mutant construction and expression- Using PCR based mutagenesis, the following mutants were obtained: G99A and L104P *P. aeruginosa* EPSP synthases. Curiously, only the mutants cloned into the pTrc99A vector system were able to complement the *E. coli aroA* auxotroph. The mutants cloned into the pUC18 system failed to complement the *E. coli aroA* auxotroph, unlike their analogous wild-type construct. It is known that the p*Trc99*A system is leaky and therefore may produce higher levels of proteins compared to pUC18 constructs. In addition, as native gel analysis and kinetic data did indicate that all mutants were active (although with lower activity levels compared to wild-type enzymes), it is plausible that increased levels of protein production (in p*Trc99*A constructs) were necessary to achieve complementation.

E. coli expression of PA, PAGA and PALP- The levels of IPTG-induced expression of PA, PAGA and PALP were analyzed by SDS-PAGE. The prominent new protein bands having an apparent mass of 50 kDa appeared 2 h after induction and increased to a maximum level within 15 h (Fig.1). Analysis of these lysates using native gel showed the presence of a white band demonstrating significant levels of EPSP synthase activity when appropriately stained.

Purification of P. aeruginosa EPSP synthase- The

Table 1. Purification of expressed *P. aeruginosa* EPSP synthase from 1 L of *E. coli* cell culture.

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	202.0	277.0	1.4	100
DEAE-Sepharose	65.5	111.0	1.7	40
Superdex 200	49.9	101.0	2.0	36
ResourceQ	10.2	87.2	8.5	31

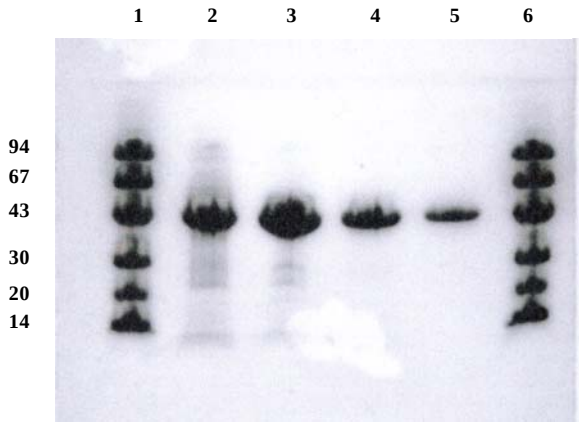

Fig 2. Purification of wild-type *P. aeruginosa* EPSP synthase as monitored by SDS-PAGE. Lanes 1 and 6, standard molecular weight markers with numbers on the left in kDa; Lane 2, crude cell extract; Lane 3, pooled active fractions after DEAE Sepharose; Lane 4, pooled active fractions after Superdex 200; Lane 5, pooled active fractions after Resource Q.

Table 2. Purification of expressed *P. aeruginosa* G99A EPSP synthase from 1 L of *E. coli* cell culture.

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	231.0	131.0	0.57	100
DEAE-Sepharose	110.0	44.6	0.40	34
Superdex 200	50.0	43.1	0.86	33
ResourceQ	29.2	39.2	1.34	30

Table 3. Purification of expressed *P. aeruginosa* L104P EPSP synthase from 1 L of *E. coli* cell culture.

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	139.0	85.7	0.62	100
DEAE-Sepharose	58.7	36.9	0.63	43
Superdex 200	18.9	34.5	1.83	40
ResourceQ	15.5	29.4	1.91	34

purification of both wild-type (Table 1, Fig.2) and mutant (Table 2 and 3) enzymes was accomplished by a four-step protocol. The first purification step, DEAE ion exchange chromatography, was very efficient and able to remove most of the contaminating proteins. The EPSP synthase eluted in 0.25 M NaCl. The active fractions were pooled and further purified by Superdex 200 gel filtration chromatography. The final purification step, a Resource™ Q column, gave a pure protein corresponding to about 50 kDa band on SDS gels for both wild-type and mutant enzymes (Figure 2).

Protein Analysis of wild-type P. aeruginosa EPSP synthase- The N-terminal sequence for the first 5 amino acids confirms the presence of the new N-terminal sequence, Met-Glu-Phe, predicted by the clone insert, followed by His and Asn corresponding to the predicted N-terminal sequence of the EPSP synthase arising from the pTrcPA construct. The extinction coefficient of enzyme was calculated as follows. The calculated EPSP synthase concentration was 28.84 nmoles/ml (0.288 X 10^{-4} M). Using the molecular mass (54,040 Daltons), this corresponds to a concentration of 1.56 mg/ml in the original sample. The absorbance of this sample at 280 nm was 0.593 in a 1-ml quartz cuvette. Using the Beer-Lambert Law which relates absorbance (A) to c (concentration): A = e.c.l where e_{280} is the extinction coefficient and l is the pathlength of the cuvette, it can be shown that e for *P. aeruginosa* EPSP synthase at 280 nm is 20590 $M^{-1}cm^{-1}$. Using a cuvette with a pathlength of 1 cm it follows that 1.0 of A_{280} units is equivalent to 2.62 mg/ml of EPSP synthase.

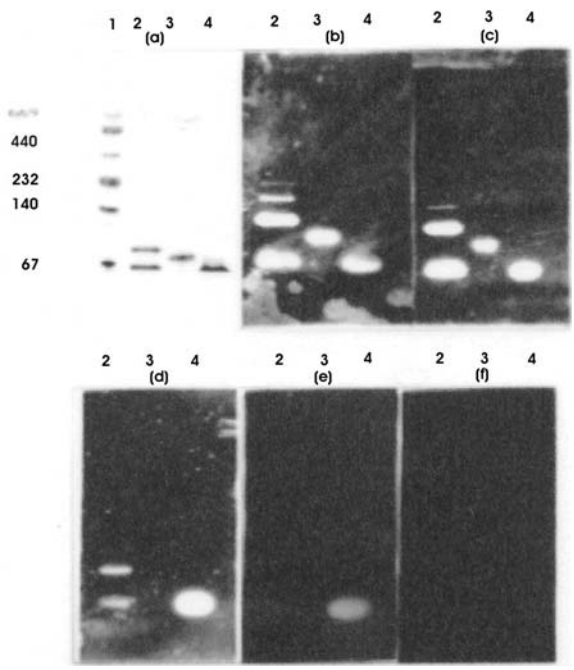

Fig 3. The effect of glyphosate concentration on EPSP synthase activity demonstrated by native gel analysis. Lane 1, standard molecular weight markers with sizes on the left in kDa; Lane 2, 1.5 g of purified *E. coli* EPSP synthase; Lane 3, 1.0 g of purified *H. influenzae* EPSP synthase; Lane 4, 1.0 g of purified *P. aeruginosa* EPSP synthase; (A), coomassie staining; (B), activity staining with no glyphosate; (C), with 20 mM glyphosate; (D), with 200 mM glyphosate; (E), with 2 mM glyphosate; and (F), with 20 mM glyphosate.

Native gel analysis- The activities of EPSP synthases were observed from the appearance of diffuse white bands which demonstrated that recombinant EPSP synthases from *P. aeruginosa* are active and capable of carrying out the enzyme reaction in the forward direction. Comparison of the Coomassie staining native gels to the activity gels of the EPSP synthases from *P. aeruginosa* demonstrates that the activity band stains co-migrate on the gels at the same positions of the purified enzymes. The glyphosate inhibition on EPSP synthases was also tested. For comparison, the activity of *E. coli* EPSP synthase to be inhibited in this assay was also tested. Although the aggregation of *E. coli* EPSP synthase was observed as shown from several higher molecular mass bands on the Coomassie stained gel, the presence of glyphosate still affected this enzyme's activity. It was shown that a glyphosate concentration of 200 mM was able to substantially inhibit the activity of *E. coli* EPSP synthase but only weakly inhibited the *P. aeruginosa* enzyme. However, at 20 mM glyphosate the *P. aeruginosa* enzyme activity was substantially inhibited, as shown by the disappearance of the white band shown in Fig. 3.

Kinetic Characterization- The observed K_m values (EPSP, PO_4) and k_{cat} values of wild-type and mutant *P. aeruginosa* enzymes are given in Table 4. The K_m (EPSP, PO_4) observed for the G99A and L104P mutants are very similar to those for the wild-type, except that an order of magnitude higher K_m (PO_4) was observed for the L104P mutant. Lower k_{cat} values (EPSP, PO_4) were

Table 4. Kinetic parameters for selected enzymes with EPSP synthase activity.

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Wild-type *P. aeruginosa*	EPSP	2.2 ± 0.5	7.4 ± 0.4	3.4 x 10^6
G99A *P. aeruginosa*	EPSP	1.9 ± 0.3	1.2 ± 0.04	6.6 x 10^5
L104P *P. aeruginosa*	EPSP	2.5 ± 0.4	2.7 ± 0.1	11.0 x 10^5
		(mM)		
Wild-type *P. aeruginosa*	PO_4	5.6 ± 1.6	5.4 ± 0.4	9.6 x 10^2
G99A *P. aeruginosa*	PO_4	7.3 ± 0.7	1.0 ± 0.03	1.3 x 10^2
L104P *P. aeruginosa*	PO_4	24.8 ± 4.8	2.8 ± 0.2	1.1 x 10^2

also observed for the mutants giving the k_{cat}/K_m for the mutants nearly an order of magnitude lower compared to those for the wild-type.

Discussion

Cloning and sequencing of the P. aeruginosa aroA gene- A BLASTX search using the translated sequence from the ORF in the resulting fragment indicated that it was an EPSP synthase homologue. Alignment of the protein sequence from *P. aeruginosa* EPSP synthase to other homologues demonstrated the highest identity of 88% with *P. stutzeri*. Interestingly, EPSP synthase from the Gram-negative *P. aeruginosa* shows marked amino acid identities with homologues from the Gram-positive *Bacillus subtilis* and *Staphylococcus aureus* (46% and 42%, respectively), compared to 31% with the homologue from the Gram-negative *E. coli*. Based upon the sequence alignments, Maskell (1993)[18] reported two conserved regions in EPSP synthase. The first region (^{92}LGNAGTA according to *E. coli* numbering) is conserved in all known EPSP synthase sequences except those from *B. subtilis* and *Mycobacterium tuberculosis*. The other region (^{384}DHRMAMCF according to *E. coli* numbering) is conserved between all the EPSP sequences from Gram-negative bacteria. Although these two regions in the *P. aeruginosa* enzyme share some identity to those from Gram-negative bacteria, it is important to point out that some of the amino acid residues in these regions share more identity to those of Gram-positive bacteria. The sequence of the *P. aeruginosa* EPSP synthase shows a similar pattern of gaps and insertions with the homologues from Gram-positive bacteria. In addition, based on the available three dimensional structures of EPSP synthase and the related enzyme UDP-*N*-acetylglucosamine enolpyruvyltransferase (EPT), Schonbrunn et al. (1996)[19] identified a repetitive structural motif $LX_3G(A)$, which is part of a loop connecting the a helix with the b strand in each of the six folding units of EPT, and found in four of the folding units of EPSP synthase (folding units 1, 2, 4 and 5). These "conserved" sequences generally show high conservation amongst the EPSP synthase sequences from Gram-negative, with the *P. aeruginosa* sequence sharing higher similarity to those from the Gram-positive bacteria. However, at this level, it is difficult to analyse how sequence differences in EPSP synthases might relate to differences in function.

Expression of Recombinant P. aeruginosa EPSP Synthase in E. coli- In the case of *P. aeruginosa*, production of substantial quantities of soluble active protein eliminates the need to use large-scale preparations of virulent pathogen. The use of the *E. coli* host strain AB2829, which lacks endogenous EPSP synthase activity, eliminates the possibility of background contamination, which can affect both protein isolation and activity assessments.

In this study, we have constructed a recombinant *P. aeruginosa* EPSP synthase that has three residues, Met-Glu-Phe, prior to the start codon. Construction of the pTrcPA plasmid required the additional of these residues to create an *Eco*RI restriction site without substantially altering the sequence of the N-terminal residues of the *aroA*-encoded gene product. PCR amplification of the *aroA* from *P. aeruginosa* with the appropriately placed *Eco*RI (5') and *Hind*III (3') restriction sites allowed insertion of the *aroA* into the *pTrc*99A plasmid to take advantage of the highly efficient IPTG-inducible *trc* promoter. The construct pTrcPA, after induction with IPTG, overproduced the *aroA* product as a soluble protein. However, two extra amino acid residues, Glu and Phe, are present between the ATG start codon (encoding Met) and His (the second translated amino acid of *P. aeruginosa* EPSP synthase). The structural effects of adding two amino acids to this particular enzyme may not be as detrimental as might be predicted from the structure of the *E. coli* enzyme. In particular, the topological diagram shows that the N-terminus of the *E. coli* enzyme forms part of a b strand which is contained within folding unit 1 of the structure[8]. It is conceivable that insertion of extra amino acids near the N-terminus could disrupt the proper folding of the protein. Comparison of the sequence alignment between the *E. coli* and *P. aeruginosa* enzymes shows that the *P. aeruginosa aroA* sequence has a four amino acid extension at the N-terminus, which suggests that the N-terminus may be exposed in this particular enzyme and that it could possibly tolerate a dipeptide insertion. It followed that *in vitro* enzyme activity and native gel assays of crude extracts, and *in vivo* complementation indicated that the protein overproduced by this system is highly stable and active.

Glyphosate inhibition- Glyphosate has been shown to disrupt the shikimate pathway, which is crucial for aromatic amino acid synthesis in bacteria, plants and microbial eukaryotes, by inhibiting EPSP synthase in the sixth step of the pathway[20]. It is known that a number of bacteria are sensitive to glyphosate, including *E. coli*, *S. typhimurium* and *K. pneumoniae*, with K_i values for glyphosate for their EPSP synthases of approximately 1 mM[21,22,23]. In contrast, natural glyphosate tolerance is observed in other microorganisms, including *P. aeruginosa*[10]. The origin of this resistance in *P. aeruginosa* is assumed to be related to its ability to utilize glyphosate as a sole phosphorus source by degrading it with a C-P lyase into sarcosine, which is further cleaved by a sarcosine oxidase-dehydrogenase enzyme to glycine. Glycine is incorporated into proteins and nucleic acids in their biosynthesis. It was also suggested that the origin of its glyphosate tolerance could be because its

EPSP synthase is actually resistant to glyphosate, as observed from its reported I_{50} value (2-6 mM) compared to that of *E. coli* (0.08-0.18 mM)[10]. The native gel analysis demonstrated that a glyphosate concentration of 20 mM (two orders of magnitude higher compared to that for the *E. coli* enzyme) was required, in order to obtain full inhibition of recombinant *P. aeruginosa* EPSP synthase. This result suggested that the *P. aeruginosa* enzyme shows resistance to glyphosate. The molecular basis by which glyphosate resistance is acquired in this enzyme is still unclear at this point and further characterization of this enzyme needs to be done.

Mutants of P. aeruginosa EPSP synthase- In order to extend the studies undertaken here on glyphosate inhibition, mutants of the *P. aeruginosa* EPSP synthase were constructed. The first amino acid selected for mutation was G96 (*E. coli*), equivalent to G99 in the enzyme from *P. aeruginosa*, which has been the subject of numerous studies. This position is located in the proposed glyphosate binding site (^{93}GXX^{96}GTXXR according to *E. coli*). Although G96 is conserved among EPSP synthases of both glyphosate-sensitive organisms, such as *E. coli*, *H. influenzae*, *S. typhimurium*, *K. pneumoniae* and *B. subtilis*, and glyphosate-tolerant *P. aeruginosa* EPSP synthases, mutation of G96 to A can result in increased glyphosate tolerance, as demonstrated for *K. pneumoniae*[24], *E. coli, P. hybrida*, soybean, and maize[25]. For the mutant created in this study, the G99A *P. aeruginosa* mutant shows a significant decrease in specific activity and an order of magnitude higher K_m value for PO_4, although the K_m value for EPSP was relatively similar to the wild-type enzyme. Its k_{cat}/K_m value is decreased by an order of magnitude compared to the wild-type enzyme indicating that this site is likely to interact with substrates and/or products.

The second position selected for mutation was P101 (*E. coli*), equivalent to L104 in the *P. aeruginosa* enzyme. It was noted that replacement of the P101 residue with S conferred glyphosate tolerance to EPSP synthase enzymes of *S. typhimurium* and *P. hybrida*[21,25,26]. It was shown that P101 is one turn away from the N-terminus of the second helix in folding unit 3, which is located near the domain cross-over point of *E. coli* EPSP synthase[8]. However, it was also suggested by Stallings et al. (1991)[8] that this proline residue might not be directly involved in substrate or inhibitor binding but affect the orientation of nearby residues (such as R100), which would in fact interact with ligands. The R100K mutation in *E. coli* EPSP synthase was made, but the K_i (glyphosate) was found to be very similar to that of the wild-type enzyme (0.24 for R100K and 0.13 mM for wild-type)[27]. Unlike the R100K *E. coli* mutant, the R100K *B. subtilis* enzyme showed more sensitivity to glyphosate compared to the wild-type enzyme (K_i = 0.06 for wild-type and 0.005 mM for mutant enzyme)[28]. Alignment of conserved regions of the translated amino acid sequences of EPSP synthases shows that P101 (*E. coli*) is not conserved in EPSP synthases. Interestingly, the EPSP synthase of *P. aeruginosa* reported to be tolerant to glyphosate, contains L101 instead of P. The "reversion" mutant L104P was constructed in this study and surprisingly showed a significant increase in the K_m for PO_4, suggesting that this mutation may indeed alter glyphosate binding and may result in a glyphosate-resistant mutant.

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