Site-directed mutagenesis, expression and biological activity of *E. coli* 5- enolpyruvylshikimate 3-phosphate synthase gene

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Abstract
Site-directed mutagenesis (SDM) as a powerful technique was used to change two important and conserved amino acids in 5-enolpyruvylshikimate 3- phosphate synthase (EPSPS) gene of *E. coli*. The mutations changed glycine 96 to alanine and alanine 183 to threonine. These two amino acids are very important for intraction of the wide spectrum herbicide, glyphosate, to EPSP synthase enzymes. By designing mutagen primers and overlapping extension method, three kinds of altered bacterial EPSPS enzymes with first, second and both mutations were produced. These modified enzymes are expected to show decreased affinity for herbicide, with least alteration in their enzymatic activity. These altered genes were cloned under the control of chemically inducible T7 promoter and over expressed in *E. coli*. Biological activity analyses in the presence of glyphosate show that the bacteria containing the mutated enzymes, especially the enzyme with two mutations, were more tolerant to glyphosate.
Keywords: *E. coli*, 5- enolpyruvylshikimate 3-phosphate synthase, Glyphosate, Site-directed mutagenesis

INRTODUCTION

5- Enolpyruvylshikimate 3- phosphate synthase (EPSPS, EC 2.5.1.19) is a key enzyme in the shikimate pathway. This important pathway is responsible for the synthesis of aromatic amino acids in plants, bacteria and some fungi (Roberts *et al*., 1998). In plants, as much as 20% of all fixed carbon flows through the shikimate pathway leading to the formation of aromatic amino acids such as tyrosine, phenylalanine and tryptophan, as well as tetrahydrofolate, ubiquinon and vitamins K and E (Franz *et al*., 1997; Gruys *et al*., 1999).

The above enzyme catalyzes the reversible addition of the carboxyvinyl moiety of phosphoenolpyruvate (PEP) to shikimate 3- phosphate (S3P) (Eschenburg *et al*., 2002). Glyphosate [N-(phosphonomethyl) glycine] (Steinrucken *et al*., 1980) as a nonselective, broad-spectrum, post-emergence herbicide (Stallings *et al*., 1991) can interact with EPSPS-Shikimate-3-phosphate complex (Majumder *et al*., 1995).

The isolation of the *Escherichia coli* B variant, containing a highly glyphosate-tolerant EPSPS has been reported (Kishore *et al*., 1986). Isolation and sequencing of the *aroA* gene encoding this glyphosate-tolerant EPSPS revealed that the altered affinity for glyphosate was the result of a single amino acid substitution of alanine for glycine 96. Alignment of the amino acid sequences of EPSPS from certain plants, bacteria and also simple eukaryotes, revealed that glycine 96 is located in a highly conserved region of EPSPS, within a consensus sequence (Paul *et al*., 2005). There are several reports of amino acid substitution in the conserved regions of EPSPS conferring tolerance to

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glyphosate, for example changing alanine 183 to threonine and glycine 96 to alanine (Eichholtz *et al*., 2001) cause enzymatic tolerance to glyphosate. In these reports several methods such as *in vitro* mutagenesis were used to make amino acid substitutions in the conserved regions of the enzyme.

Site-directed mutagenesis (SDM) of the coding sequences of enzymes is a powerful technique for the investigation of structure-function relationships (Wagner *et al*., 1990) and has also been used to verify the roles of active site residues (Padgette *et al*., 1991). Furthermore, SDM promises to provide valuable information about the distribution of fitness effects of mutants (Taylor *et al*., 1996). In the present study we describe the use of *in vitro* PCR based SDM of the EPSPS gene in *E. coli* k12 in order to investigate the effect of amino acid alteration on enzyme activity in *E. coli* host cells in the presence of glyphosate. The changes in target amino acids were glycine at position 96 to alanine (Gly96Ala) and alanine at 183 to threonine (Ala183Thr). These changes were applied separately and also in combination form in one molecule. Wild-type and the mutated EPSPS genes were cloned and over expressed, under the control of chemically inducible T7 promoter. We also examined the tolerance of recombinant *E. coli* strains containing the mutated EPSPS genes against different concentrations of glyphosate.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions: *Escherichia coli* DH5α was used for cloning and mutation experiments. Bacterial cells were cultivated in Lauria-Bertani (LB) medium (10 g peptone, 5 g yeast extract and 10 g NaCl) at 37ºC. Plasmid pSK+ (Stratagene) was used in routine cloning and sequencing procedures.

Recombinant DNA technologies: Standard DNA technologies was used (Sambrook *et al*., 2001). Oligonucleotide synthesis and DNA sequencing reactions were carried out at MWG-Biotech (Ebensburg, Germany).

EPSPS gene amplification and cloning: Isolation of genomic DNA from *E. coli* K12 was carried out according to Sambrook *et al.,* 2001. The EPSPS gene was amplified using specific primers P1 forward 5′-CGGGATC*CATGG*AATCCCTGACG TTACAA-3′ and P2 reverse 5′-GCGGATCCTCAGGCTGCCTGGC-TAATC-3′ with *Bam*HI site at the 5′ end of each primer. By this strategy and considering the 5′ sequence of the amplified fragment, the *Nco*I site (CCATGG) was also formed at this end.

EPSPS amplification was performed in a 50 µl total volume containing 500 ng of template DNA, 10 pM of each primer, 3 mM of Mg^{2+}, 0.2 mM of each dNTP and 2.5 units of *pfu* DNA polymerase. The amplification cycles included: hot start at 94°C for five minute, followed by thirty cycles of denaturation at 94ºC for 60 seconds, annealing at 63ºC for 60 seconds and extension at 72ºC for 90 seconds. The extension time was given increment of five seconds between the tenth and the last cycle. The final extension was carried out at 72ºC for ten minutes. The PCR product was purified from the agarose gel by high pure PCR product purification kit (Roche Diagnostic, Germany). Restriction enzyme analysis was carried out using *Bgl*II, *Hinc*II and *Taq*I. The PCR product was cloned in the pSK+ phagemid (pSK-ECEPS) and sequenced in both directions with standard T7 and T3 primers by the dideoxy chain termination method.

Site-directed mutagenesis: According to designed primers and PCR-based SDM procedures which were described by Kahrizi *et al*. (2007), the required mutations (Gly96Ala, Ala183Thr and both mutation) were introduced into the *E.coli* EPSPS gene.

Cloning and sequencing of the genes: Wild-type and the mutated EPSPS genes were cloned into the *Bam*HI site of the pSK+ phagemid. The recombinant plasmids were analyzed and confirmed by restriction enzyme digestion and PCR using specific primers. The plasmids with desired inserts were sequenced by the dideoxy chain termination methods, using standard T7 and T3 primers. The sequences result were compared with other sequences stored in the Gene bank and aligned by using the Blast software (www.ncbi.nlm.nih.gov) to confirm the mutations.

Expression of wild type and mutated EPSPS genes: The genes encoding the wild-type and mutated EPSPS from *Escherichia coli* were cloned into an expression plasmid from the pET system, pET24d, which was under the control of the T7 promoter. For construction of the expression vectors, the plasmid was digested with *Nco*I and *Bam*HI restriction endonucleases and the ligation was carried out using genes which were digested with the same enzymes. Recombinant plas-

mids were introduced into competent *E. coli* cells and were verified on the basis of restriction enzyme analyses and PCR with specific primers.

For protein expression, the pET-derived plasmids were transformed in to the competent *E.coli* strains BL21 (DE3) plysS host cells. A single colony of transformed *E. coli* BL21 (DE3) plysS was used to inoculate 5 ml of LB medium containing kanamycin (30 µg/ml) and chloramphenicol (34 µg/ml). For recombinant protein expression 1 ml of overnight culture was inoculated into 50 ml of 2x LB medium containing the same concentration of kanamycin and cultivation was continued at 37ºC, with vigorous shaking at 300 rpm, for a further two hours until the optical density (OD) at 600 nm reached to 0.4. Expression was induced by adding different concentrations of isopropyl-β-D-galactopyranoside (IPTG), 0.2-1 mM. One milliliter samples were taken at 2, 4 and 18 h after induction. The samples were centrifuged immediately at 8000 rpm, 4ºC, for 8 min and pellets were stored at -20ºC for primary analyses on 12% polyacrylamid gel electrophoresis.

Biological activity of mutated and wild type enzymes: In order to calculate the inhibitory concentration of glyphosate for *E. coli* (BL21, DE3, plysS), the bacteria were grown overnight at 37ºC, 300 rpm, in liquid M9 minimal media (Sambrook *et al*., 2001). One hundred microliters of a 10^{-6} dilution of culture sample was added to solidified M9 media containing increasing amounts of glyphosate. The concentration of glyphosate, which inhibited the growth of empty *E. coli* host cells, represented the basal inhibitory concentration. For assessing the effect of glyphosate on *E. coli* host cells containing the wild-type and mutated EPSPS genes, the recombinant bacteria were cultured separately in 20 ml of M9 minimal media at 37ºC with shaking at 250 rpm. When the OD_{600} of each culture reached 0.4, expression was induced by the addition of 0.2 mM IPTG. After 30 minutes several quantities (from 0.0 to 20 mM with 2 mM increments) of glyphosate were added to each bacterial culture and the cultivations were continued for a further two hours. One hundred microliters of the 10^{-6} dilution of bacterial culture from each experiment were cultivated in solidified M9 minimal media containing the same concentration of inhibitor for the porpose of colony counting and appointment of the LD50 factor. The experiments were repeated three times.

RESULTS

Site directed mutagenesis: Total genomic DNA was isolated from *E. coli* and the EPSPS gene was amplified using specific primers. The gene was cloned and its nucleotide content was verified by sequencing (data not shown). SDM was carried out using designed primers and four intermediate fragments, A (300 bp), B (1000 bp), C (500 bp) and D (800 bp) were amplified (Figs. 1A-D).

The intermediate fragments (A+B and C+D) were attached to each other to make two different EPSPS genes carrying single mutations (Fig. 2A). Similarly, the EPSPS gene with two mutations (Gly96Ala and Ala183Thr) was constructed by using the EPSPS gene with Gly96Ala as a template and P5 and P6 primers. For further analysis the 1.3 kb fragment was subjected to restriction endonuclease analysis using *Bgl*II, *Hinc*II and *Taq*I enzymes. The *Bgl*II gave three fragments (690, 330 and 300 bp) while *Hinc*II gave two fragments (800 and 500 bp) and the *Taq*I resulted in four fragments (580, 530, 120 and 40 bp). In the case of *Taq* I digestion the two small bands could not be observed (Fig. 2B).

The mutated genes were cloned in to the pSK+ plasmid. The positive clones were confirmed by PCR with specific primers and restriction enzyme (*Bam*HI). By comparing the sequencing and their alignments it with other sequences in the gene bank, the insertion of the necessary mutations and the absence of undefined mutations were verified.

Expression of the EPSPS genes: Four target genes were cloned into the pET24d vector at the *Nco*I and *Bam*HI sites.The best conditions for induction of pro-

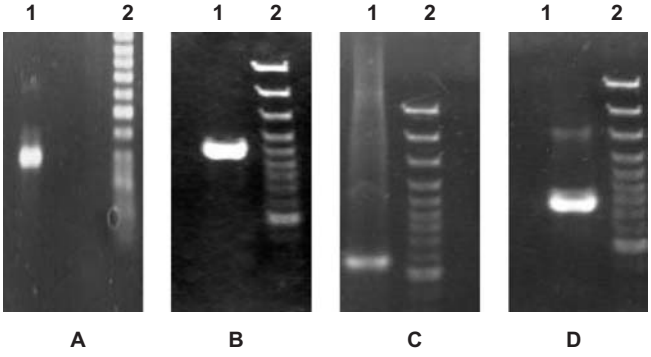

Figure1. Amplification of four intermediate fragments. A: Amplification of 300 bp fragment. B: Amplification of 1000bp fragment. C: Amplification of 500 bp fragment. D: Amplification of 800 bp fragment. In all figures lane 2 is the 100 bp ladder.

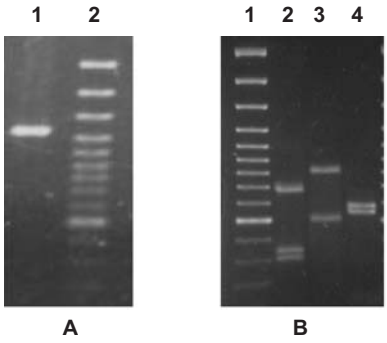

Figure 2. Amplification and restriction enzyme analyses of mutated *E. coli* EPSP synthase gene, A: lane 1: Amplified mutated gene. Lane 2: 100 bp ladder B: Restriction enzyme analyses. Lane 1: 100 bp ladder. Lane 2: Fragments produced by *Bgl*II digestion. Lane 3: Fragments produced by *Hinc*II digestion. Lane 4: Fragments produced by *Taq*I digestion (For detail see the text).

teins were obtained two hours after addition of 0.2 mM IPTG at an OD_{600} = 0.4. The results of recombinant enzyme production by one of the *E .coli* strains containing the mutated gene is shown on 12% SDS-PAGE, before and two and four hours after induction (Fig. 3).

Analyses of biological activity : The tolerances of the bacteria containing the mutated EPSPS were analyzed against different concentrations of herbicide (0-20 mM, with two mM increments). A level below 2 mM glyphosate completely inhibits the growth of *E. coli* - BL21 (DE3) plysS as a negative control. To analyse the biological activity of the target enzymes in recombinant *E. coli* cells, the bacteria were counted and a decrease in their viability were measured (Fig. 4).

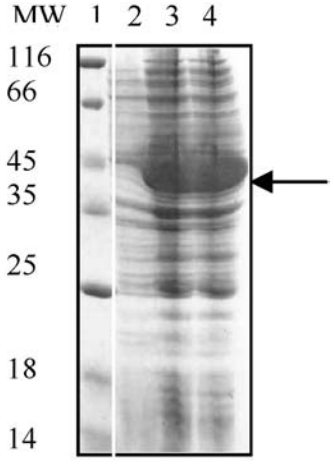

Figure 3. Induction of recombinant *E. coli* Bl21 DE3 plysS contains the mutated EPSPS enzyme. Lane1- Molecular weight Marker (KD), Lane 2- Bacterial colony before induction, Lane 3 – Bacterial colony Two hours after induction, 4- Bacterial colony four hours after induction.

DISCUSSION

In modern farming, chemicals have special roles in the control of biotic stresses, and among these chemicals, herbicides are very important. The herbicides glyphosate play a critical role in weed control. Despite widespread and long-term use of glyphosate, weeds have evolved limited resistance to this herbicide (Baerson *et al*., 2002). Glyphosate inhibits the shikimic acid pathway which provides a precursor for the synthesis of aromatic amino acids (Eichholtz *et al*., 2001).

The pervious study of the EPSPS and attachment of glyphosate to this enzyme, showed that the over expression of the EPSPS gene and amino acid substitutions in the conserved regions of target molecule are two important strategies for engineering glyphosate resistance. For the first time, Padgett and his coworkers (1991) reported the isolation of an *E. coli* B variant, containing a highly glyphosate-tolerant EPSPS. The further analysis of glyphosate-tolerant EPSPS revealed that the altered affinity for glyphosate was the result of a single amino acid substitution of alanine for glycine at residue 96. Glyphosate-tolerant *Salmonella typhimurium* strain has also been reported wherein the tolerance to glyphosate results from the same amino acid substitution to serine (Stalker, 1985). Alignment of the amino acid sequences of EPSPS from different organismes show that these two amino acids (Gly 96 and Ala 183 in *E. coli*) are located in a highly conserved region. Glycine at this position produces a very strong hydrogen bond between its nitrogen and the oxygen from the phosphate group of glyphosate and alanin 183 is an important amino acid for EPSPS and glyphosate interaction (Schonbrunn, 2001). But these two amino acids residue are not involved in the active site of EPSPS. Therefore, substitution for these amino acids can alter the affinity of glyphosate for the enzyme.

Due to the knowledge about the interaction between glyphosate and its target enzyme, many scientists have tried to introduce certain changes in EPSPS in order to make it resistant to glyphosate. Previously, Eichholtz and his coworkers (2001) have described the modified gene encoding glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate synthase. By using the M13 mutagenesis method they introduced mutations in the first 660 bp fragment of the wild type gene coding for the enzyme. Studies have shown that such changes could also increase glyphosate tolerance in transgenic plants (Kahrizi *et al.,* 2007). By using such an approach, we have attempted to use PCR based

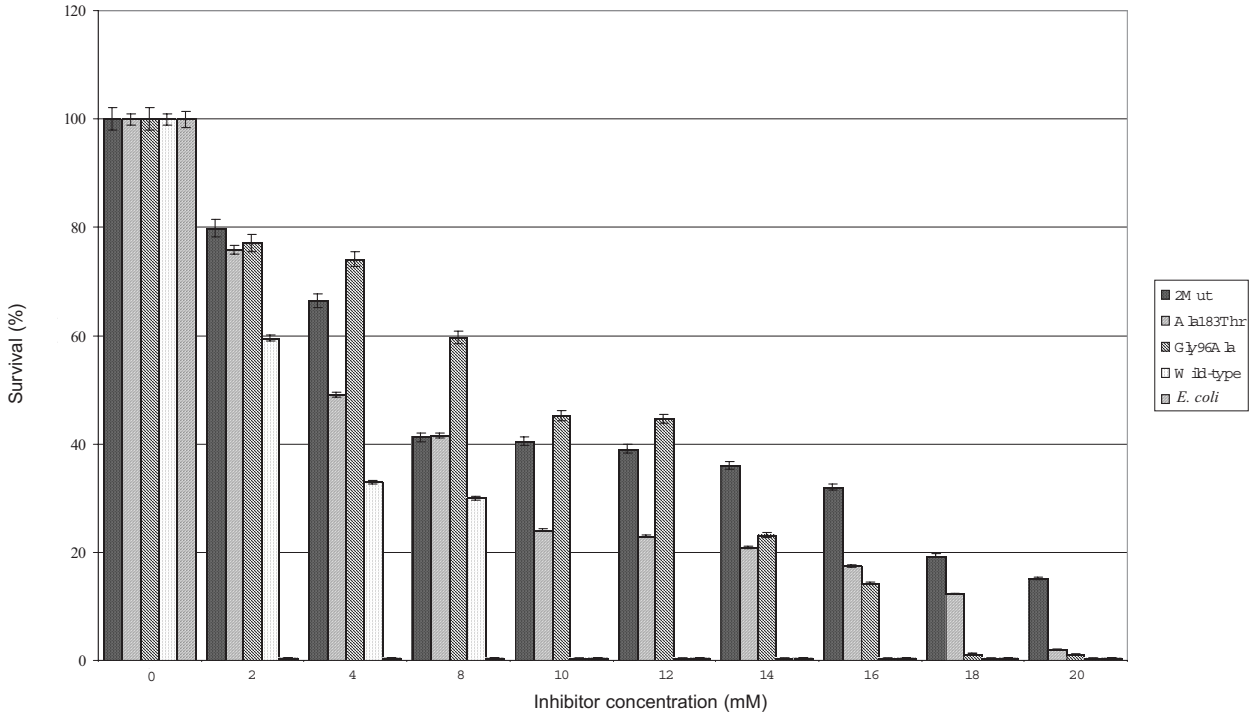

Figure 4. Survival of *E. coli* cells containing wild-type and the mutated genes (2MUT, Ala 183 Thr, Gly 96 Ala) and empty *E. coli* host cell (*E. coli*) in the presence of different concentrations of glyphosate.

SDM, as a simple, reliable and reproducible technique, to design three forms of EPSPS mutations (Gly96Ala and Ala183Thr and both of them) and compare their biological activities with the wild-type enzyme.

The biological activity analyses of recombinant bacteria containing the four forms of the EPSPS gene (wild-type and three mutated forms) show different levels of tolerance to glyphosate. Comparison of these recombinant bacteria show that the *E. coli* cells with extra copy of wild type EPSPS are more sensitive to the glyphosate herbicide. Principally the tolerance in these cells only occured by increasing the natural enzyme production. The bacteria with an additional copy of the un-modified EPSPS gene can continue growing in the presence of 10 mM glyphosate. But the levels of the tolerance in bacteria harboring the mutated form of the EPSPS gene are much higher. On the other hand the extensive synergistic effects of mutation in the right residue and gene amplification are outstanding.

The results indicate that in the presence of 4,8,10 and 12 mM of inhibitor, the colonies containing the Gly96Ala mutation are obviously more resistant than the wild-type and other mutated forms. However from 14 mM up to 18 mM, Ala183Thr shows higher tolerance in comparison with other mutants. The bacteria with the EPSPS enzyme containing two mutations show more tolerance in the presence of higher concentrations of glyphosate (Up to 20 mM). This finding may indicate that the binding of glyphosate to its target molecule occurs through several amino acid residues involving different kinds of interactions and furthermore, it seems that the affinity of these residues for interaction with glyphosate depends on different concentrations of the inhibitor.

The comparison of several bacterial colonies show that 10 mM represents the critical concentration of the glyphosate inhibitor. At this concentration the growth of colonies containing an extra copy of the wild type gene were completely inhibited.

In conclusion, the rapid and reliable PCR based SDM method and biological activity assay of the modified enzyme by simple procedure, allowed us to introduce new mutations and analyze the effect of such manipulations on tolerance to the glyphosate herbicide precisely, prior to using this altered gene for genetic transformation of higher organisms.

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