Progress in cloning, expression and purification of 5-enolpyruvylshikimate-3-phosphate synthase from pathogens causing meningitis

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Bacterial meningitis is a major health problem worldwide. Even in the developed world, one child in every thousand may contract meningitis before the age of ten [1]. The majority of meningococcal infections occur in children under five, and the mortality is about 5-10% [1]. In these cases, death occurs soon after the symptoms become apparent. The major causes are Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae. The increasing resistance to ampicillin and chloramphenicol among Hib isolates, and penicillin resistance in pneumococci complicates the choice of antibiotic for therapy [2,3]. Active immunization of children offers the only real long-term solution to reducing the incidence of these infections. Meningococci cause most of the disease in developed countries, but an effective vaccine remains elusive [1].

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme from the prechorismate or "shikimate" pathway, has been identified as a target for antibiotic development. This is because this biosynthesis pathway for the essential aromatic amino acids is present in bacteria and fungi (as well as plants) but is absent in vertebrates; thus, compounds inhibiting any one of the enzymes in this pathway would be expected to have minimal side effects in animals or man.

EPSPS catalyses the reversible condensation of phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) with the transfer of the carbovinyl group from PEP to S3P to form 5-enolpyruvylshikimate-3-phosphate [4]. EPSPS has been shown to be the target of the herbicide glyphosate [5], a competitive inhibitor with respect to PEP. It is therefore conceivable that compounds similar to glyphosate might be exploited as antimicrobial drugs, inhibiting EPSPS and thus starving the organism of essential aromatic compounds. In addition, pathogenic bacteria unable to express active EPSPS, i.e. which are araA-, are attenuated for virulence: live attenuated vaccines from these mutants provide immune protection from virulent challenge [6].

In order to assess the pharmacological suitability of EPSPS inhibitors, we are interested in studying the structure and function of this enzyme. To this end, we are aiming to clone, express and purify this enzyme from the pathogenic bacteria N. meningitidis and H. influenzae. The cloning of the araA gene from N. meningitidis is of particular interest as it represents an ideal target for the development of an effective live vaccine.

To clone the araA gene from N. meningitidis, oligonucleotide primers were designed based on back translation of two highly conserved regions from reported araA sequences and these were used in a PCR on N. meningitidis chromosomal DNA as template. The PCR product was a 900 bp fragment of the araA gene, which was cloned in E. coli and sequenced. This probe was effective in identifying a homologue in a library of N. meningitidis chromosomal DNA in Lambda ZAP II (Stratagene, La Jolla, CA, USA). However, sequencing revealed that although the 3' end of the araA gene was present, approx. 250 bp at the 5' end were missing from this clone; no clones carrying the entire araA gene were found. In an alternative approach, the probe was used to identify by Southern hybridization a variety of restriction fragments of genomic N. meningitidis DNA that carry the araA gene, but for unknown reasons none of these could be cloned in E. coli. In order to circumvent this problem, an anchor PCR approach was undertaken in which the various restriction fragments ligated into the cloning vector pUC19, were used as templates. Oligonucleotide primers were designed to bind to regions near the 5' and 3' ends of the determined 900 bp sequence, and these were used in combination with primers that bind to the vector in order to selectively amplify the two ends of the araA gene. A PCR product was obtained that could be shown to contain the 3' end of the araA gene, confirming the validity of this approach. Another PCR product obtained from the 5' end has also been obtained in low yield, but has yet to be cloned and sequenced for its identity to be confirmed. The sequence determined so far resembles all other known araA gene products over a wide range of species.

The H. influenzae araA gene, which has been cloned and sequenced previously [7], was subcloned into the inducible expression vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) and over-expressed in an E. coli araA- strain (BRD048), yielding soluble and active protein in great abundance. EPSPS activity was assayed as described previously [8]. EPSPS was purified by modification of the original procedure for purification of E. coli EPSPS [8,9]. All steps were carried out at 4°C unless otherwise indicated. Cell cultures were grown in shaken flasks (225 rpm, 37°C) in LB medium with ampicillin. Expression was induced at 37°C for 24 h with IPTG (0.2 mg/ml). Cells were grown up overnight and harvested by centrifugation, yielding approx. 8 g of cell pellet (wet weight) per litre culture. After storage at -70°C, the cell paste was thawed and resuspended in three volumes of extraction buffer (50 mM NaCl in stock buffer: 50 mM potassium phosphate buffer pH 7.0, 1 mM EDTA, 0.4 mM DTT) and then sonicated for 90 s in total at max. 8°C. The whole cell lysate was further diluted with two volumes of extraction buffer, stirred with approx. 0.5 mg DNAse for 1 h, and centrifuged at 40000 g for 30 min to remove cell debris. The crude extract contained approx. 70 mg protein per gram cells (wet weight). In preparation for hydrophobic interaction chromatography, the crude extract was further diluted with two volumes of extraction buffer, stirred with approx. 0.5 mg DNAse for 1 h, and centrifuged at 40000 g for 30 min to remove cell debris. The crude extract contained approx. 70 mg protein per gram cells (wet weight). In preparation for hydrophobic interaction chromatography, the crude extract was further diluted with two volumes of extraction buffer, stirred with approx. 0.5 mg DNAse for 1 h, and centrifuged at 40000 g for 30 min to remove cell debris. The crude extract contained approx. 70 mg protein per gram cells (wet weight).

Table 1. Purification of EPSPS from Haemophilus influenzae

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Activity (U/ml)</th>
<th>Spec. Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>(NH)2SO4</td>
<td>81</td>
<td>1.0</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>Mono Q</td>
<td>75</td>
<td>0.32</td>
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<td></td>
<td></td>
<td>77</td>
<td>0.24</td>
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