Shikimate 3-Dehydroquinate Dehydratase and DAHP Synthase: Subcloning and Bioassay

Wong S.Y. and Yew W.S.

Department of Biochemistry, Faculty of Medicine, National University of Singapore
10 Kent Ridge Road, Singapore 117546

ABSTRACT

The shikimate pathway is the crucial aromatic biosynthesis pathway exclusive in microorganisms and plants, thus making it an attractive target for anti-microbial compounds and herbicides. Before designing anti-microbial compounds, understanding the structures and catalytic mechanisms of the shikimate enzymes is necessary. 3-Dehydroquinate dehydratase and DAHP synthase are selected as candidates for directed evolution to reveal the catalytic blueprints of shikimate barrel enzymes. A bioassay using an E. coli auxotroph, with the aroD gene coding for 3-dehydroquinate dehydratase deleted from the genome, was done to show that DAHP synthase will not be able to complement growth of the auxotroph before proceeding to subsequent directed evolution experiments. In an attempt to obtain soluble proteins for protein purification and crystallization purposes, the synthase from Bordetella parapertussis and the dehydratase from Archaeglobus fulgidus were subcloned from their respective pET-15b constructs to Tom-15b and pET-17b vectors to improve protein solubility. The synthase of Bordetella parapertussis remained insoluble but the dehydratase of Archaeglobus fulgidus subcloned to Tom-15b produced soluble protein upon expression at 37 °C.

INTRODUCTION

The shikimate pathway is conserved in prokaryotes, fungi, plants, algae and several apicomplexan parasites. It converts phosphoenolpyruvate and D-erythrose-4-phosphate to chorismate, a common precursor for aromatic amino acids, folic acid, entrochelin, para-aminobenzoate, and ubiquinone (Coggins et al., 2003; Webby et al., 2005). Since mammals lack the shikimate pathway, this pathway becomes a brilliant target for anti-microbial compounds and non-toxic herbicides (Coggins et al., 2003).

The long-term goal of this project is to obtain the catalytic blueprints of shikimate barrel enzymes by direct evolution of 3-dehydroquininate dehydratase and DAHP synthase. A better understanding of the structures and catalytic mechanisms of these shikimate enzymes will provide a detailed framework for the design of new anti-microbial compounds. Hence, producing a soluble protein that is able to be purified and crystallized is an essential first step in obtaining the structure of the enzyme. Eleven genes from archaebacteria, pathogenic and non-pathogenic prokaryotes were selected based on the availability of genomic DNA.
Experiments conducted previously in the lab have shown that DAHP synthase from *Bordetella parapertussis* (SBP) was found to be insoluble upon expression via the pET-15b vector. On the other hand, all six 3-dehydroquinate dehydratases were found to be soluble upon expression via the pET-15b vector, but dehydratase from *Archaeglobus fulgidus* (DAF) (only partially soluble under expression conditions at 37 °C). Hence, SBP and DAF were subcloned from their respective pET-15b constructs to Tom-15b and pET-17b vectors to obtain soluble expressed protein.

A bioassay was also done using an *E. coli* auxotroph with the *aroD* gene coding for 3-dehydroquinate dehydratase being inactivated via insertional deletion. Plasmids containing these eleven genes were transformed into the *E. coli* auxotroph and their growths were monitored. This bioassay was performed to ascertain that only plasmids bearing the 3-dehydroquinate dehydratase genes will complement growth of the auxotroph, whilst plasmids containing DAHP synthase genes will not be able to complement growth of the auxotroph. This is a crucial experiment to verify that the DAHP synthase will not be able to complement growth of the *aroD* auxotroph before directed evolution studies are carried out.

**MATERIALS AND METHODS**

**Subcloning of DAF and SBP**

For subcloning into Tom-15b and pET-17b, pET-15b plasmids containing DAF or SBP were first restricted with *NdeI* (NEB) and *BamHI* (NEB) and the reaction mixture was purified by electrophoresis gel. Vectors restricted with corresponding restriction enzymes were treated with calf intestinal alkaline phosphatase (NEB) to dephosphorylate the 5′-phosphate end to prevent self-religation. Ligation was carried out using T4 DNA ligase (NEB). The mixture was drop-dialyzed before electroporation into *E.coli* XL1Blue cells (Strategene) was performed and the transformed cells were resuspended in LB and plated onto LB agar plates supplemented with ampicillin. Then, colony-PCR was carried out to screen for the presence of insert-containing plasmid using T7Pro and T7Term primers and Taq DNA polymerase (NEB).

**Protein expression**

Proteins were expressed in *E.coli* strain BL21 (DE3) (Strategene). Transformed cells were grown at 37 °C and 25°C overnight in LB supplemented with ampicillin. Half of the culture was induced with IPTG while the other half was left uninduced. The cells were harvested by centrifugation, and lysed by sonication (Heat Systems). Subsequently, the lysate was centrifuged, the supernatant was removed and the pellet was resuspended in binding buffer. Samples were run on SDS PAGE to analyze protein solubility.

**Bioassay**

*E. coli aroD* auxotrophs (KanR, CamR) transformed with pET-15b constructs containing the eleven genes were cultured overnight in LB with ampicillin, kanamycin, and chloramphenicol. Cell cultures were centrifuged, resuspended in M9 salts and washed three times before inoculating into M9 minimal media with and without glucose and supplemented with ampicillin, kanamycin, and chloramphenicol. The cultures were incubated at 37 °C with shaking at 200 rpm. Bacterial growth
curves were obtained by monitoring the growths using a UV/Vis spectrophotometer (Beckman) at 600 nm, and plotting the average of duplicates.

RESULTS AND DISCUSSION

<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>Pellet/Supernatant</th>
<th>IPTG Induction</th>
<th>Vector</th>
<th>Pellet/Supernatant</th>
<th>IPTG Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tom-15b</td>
<td>Pellet</td>
<td>Induced</td>
<td>1</td>
<td>pET-17b</td>
<td>Pellet</td>
</tr>
<tr>
<td>B</td>
<td>Tom-15b</td>
<td>Supernatant</td>
<td>Induced</td>
<td>2</td>
<td>pET-17b</td>
<td>Supernatant</td>
</tr>
<tr>
<td>C</td>
<td>Tom-15b</td>
<td>Pellet</td>
<td>Uninduced</td>
<td>3</td>
<td>pET-17b</td>
<td>Pellet</td>
</tr>
<tr>
<td>D</td>
<td>Tom-15b</td>
<td>Supernatant</td>
<td>Uninduced</td>
<td>4</td>
<td>pET-17b</td>
<td>Supernatant</td>
</tr>
</tbody>
</table>

As shown in Figure 1, SBP expressed in Tom-15b and pET-17b with overnight incubation at both 25 °C and 37 °C showed no soluble protein. SDS PAGE clearly illustrated that the proteins were still largely present in the insoluble pellet fractions. Changes in incubation temperature and IPTG induction brought no significant improvements to protein solubility.

DAF showed no expression at all when incubated overnight at 25 °C as demonstrated in Figure 2 but soluble proteins were obtained for both Tom-15b and pET-17b vectors when incubated at 37 °C. DAF was slightly soluble when expressed in pET-17b but was considerably soluble when expressed in Tom-15b. Addition of IPTG had brought no significant different to the DAF expression.

His tag is a frequently used fusion tag able to increase the solubility of recombinant proteins and is commonly used for affinity purification. Hence, vectors like pET-15b and Tom-15b that add His-tag to the protein end terminus were used here. pET-15b construct will add 6X His tag to protein N-terminus and it has a thrombin cleavage site after the His tag and before Nde I recognition site. As the genes were cloned in between Nde I and BamH I restriction sites, this His tag could be easily cleaved off using thrombin protease after purification using nickel ions chromatography. Tom-15b is modified from pET-15b created by Dr. Toomas Haller (Dr.J.A. Gerlt’s lab, University of Illinois) which adds an N-terminus 10X His tag instead of 6X His tag to the protein. Increasing the number
of histidine residues and thus positive charges on the protein, the solubility of the protein could be enhanced. pET-17b is a construct that fuses a T7 tag to protein N-terminus. Since the cloning was done between Nde I and BamH I sites, the T7 tag was lost and thus allow native protein expression without any fusion. As a result, the protein product could not be purified simply by nickel ion chromatography and other purification techniques or combination of several techniques could be applied. For instance, ion exchange, hydrophobic interaction, gel filtration, reversed phase, and so forth.

In this bioassay, *E. coli* aroD auxotrophs transformed with DAHP synthases showed no growth complementation in M9 minimal media supplemented with glucose. In contrast, auxotrophs transformed with 3-dehydroquinate dehydratases showed significant growth within a day. This bioassay is important to verify that DAHP Synthases have no promiscuous activity and would not be able to complement auxotrophs lacking 3-dehydroquinate dehydratase activity. This would provide a crude foundation for this project before proceeding to the directed evolution studies of DAHP synthase to 3-dehydroquinate dehydratase.

REFERENCES
