# The Purification and Substrate Specificity of a Soluble Squalene-Hopene Cyclase Enzyme.

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## Introduction

This project was carried out within the Exeter Biocatalysis Centre to investigate the substrate specificity of a squalene-hopene cyclase (SHC) enzyme. SHCs are a family of enzymes that catalyse the production of hopene from a linear precursor, squalene (figure **1**). This single-step biotransformation results in the formation of five ring structures, thirteen covalent bonds and nine stereocentres, making it one of the most complex enzymatic reactions in nature[1]. The product of the reaction, hopene, is an important precursor in the synthesis of several fragrance and flavour molecules.

Typically, SHCs exist as membrane-bound proteins, however, the Littlechild group have been able to identify a novel bacterial variant that lacks a membrane binding region and can be cloned and overexpressed in *Escherichia coli* in soluble form (see figure **2**). The crystallographic structure of this new SHC has been determined (PDB code: 8PAK[2]). Characterising its activity towards squalene as a substrate is of great interest, as it has the potential to be used for the sustainable synthesis of hopene in the health care and fragrance industries.

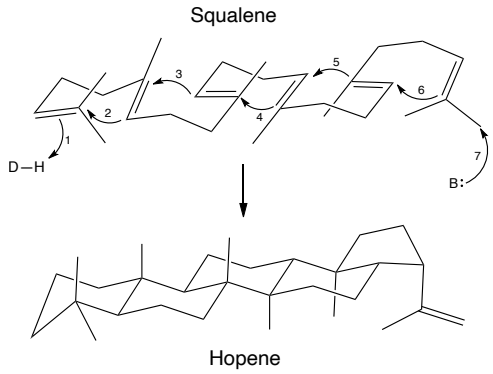


**Figure 1**: Adapted from [3]: Schematic showing the conversion of squalene into hopene with the numbered arrows indicating the sequence of intermediate electron transfers. Within the active site of SHC enzymes, a highly-conserved aspartic acid residue (labelled here as "D") carries out the proton transfer that induces the polycyclisation of squalene. Once this has occured, a non-specific basic residue (labelled as "B") will accept a proton to form the final product, hopene.

## Aims of the project

- Overexpress the enzyme in *E. coli* cells that have been transformed with an appropriate recombinant plasmid
- Purify the enzyme using fast protein liquid chromatography on gel filtration and anion-exchange columns.
- Use sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the level of protein over-expression and final purity of the enzyme.
- Assay the enzyme's activity towards squalene as a substrate
- Crystallise the enzyme with a known inhibitor. The crystals will be used to solve the structure of the enzyme-inhibitor complex which will provide further information regarding the binding of substrates within the active site.

## Methods used

### Overexpression and purification

A pET11a+ plasmid containing the gene encoding for the SHC enzyme was transformed into *E. coli* BL21 (DE3) cells via heat shock. These cells were then grown in LB broth at 37°C up to an optical density at 600nm of 0.6. Expression of the gene was induced by adding 1mM isopropyl $\beta$-D-thiogalactopyranoside (IPTG). After incubating overnight at 20°C, the cells were harvested by centrifugation and resuspended in 50mM MOPS, pH 7.0, 100mM NaCl. Sonication was then used to disrupt the cells and the resulting lysate was clarified by centrifugation. Size-exclusion chromatography (SEC) and anion-exchange chromatography columns were then used to purify the protein from the crude supernatant. Samples from the cell lysate and the subsequent purification steps were run on SDS-PAGE to confirm the level overexpression and purity of the SHC enzyme.

### Assay

The enzyme's activity towards squalene was assayed using a recently published method[4]. Here, 20mM squalene was added to 0.284mg/mL enzyme with 0.5%w/v Tween 80 or Triton X-100 detergent being used to solubilise the squalene. The reaction was then incubated at 30°C for 17 hours. All reactions were carried out in triplicate with negative controls that contained 20mM squalene and 0.5%w/v detergent, without any enzyme present. This procedure was repeated using a temperature of 40°C. The samples were then analysed by HPLC to confirm the presence of hopene.

### Crystallography

SHC enzyme was concentrated to 40mg/mL and mixed with 45$\mu$M of a known inhibitor that was dissolved in dimethylsulfoxide (DMSO) and methyl acetate. Microbatch crystallisation trials were then set up with Morpheus®Fusion protein crystallisation screens using an Oryx8 crystallization robot. Crystals appeared within one week in several conditions, these were harvested and sent to the Diamond Light Source Synchrotron to obtain high resolution diffraction data.

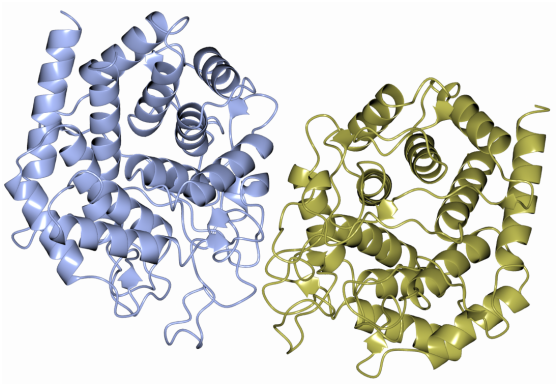

**Figure 2**: Cartoon representation of the SHC enzyme under investigation.

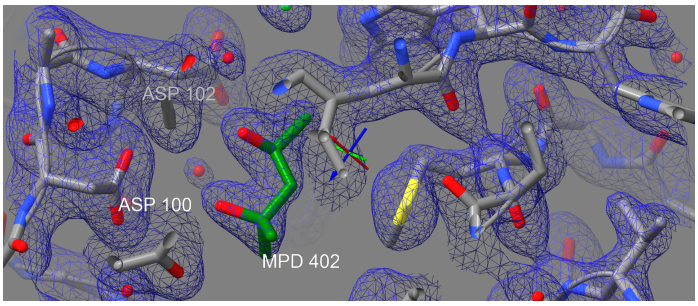


**Figure 3**: Diagram showing the structure of the enzyme's active site solved from the crystal diffraction data. The molecule shown in green and red is 2-methyl-2,4-pentanediol (MPD) which is a chemical additive in the Morpheus®Fusion crystallisation screen. Presence of MPD in the active site confirms that binding did not occur between the enzyme and inhibitor during crystallisation.

## Results and discussion

### Assay

Analysis of the assay samples is still in progress. This is due to an unexpected delay in the purchase of the specific column required for HPLC, as per the method in [4].

### Crystallography

X-ray diffraction datasets were collected and processed through an automatic pipeline based on a software called XDS[5]. Using these data, the structure of the enzyme was determined to a resolution of 1.4$\mathring{A}$. This was achieved through molecular replacement, using another recently-determined SHC structure as a search model. This structural resolution is the highest that the Exeter Biocatalysis Centre have been able to obtain for this enzyme and will therefore be useful for modelling in future crystallographic studies.

Upon inspection of the structure, it is clear that a molecule is present within the active site (see figure **3**). The contours of this molecule's electron density match the structure of 2-methyl-2,4-pentanediol (MPD), a chemical component of the Morpheus®Fusion crystallisation screen. The presence of this molecule within the enzyme's active site indicates that enzyme-inhibitor binding did not occur during crystallisation.

## Future directions in which the project could be taken

The assay samples will be stored at 4°C until they can be analysed by HPLC. The results from this analysis will largely determine the future directions of the project. If conversion from squalene to hopene is observed, the next objectives will focus on optimising the reaction conditions (e.g. pH, temperature) and scaling up the reaction for hopene production. If there is no reaction, different parameters of the assay method, such as reaction time and detergent concentration will be altered to try and elicit enzyme activity towards the substrate.

It is also important that more crystallisation trials are set up to gain insight into the binding of substrates within the enzyme's active site. Cocrystallising the enzyme and inhibitor will be repeated, with detergents being added to promote binding between the two species. The method of soaking native enzyme crystals in a solution of the inhibitor will also be carried out to try and successfully capture complexation.

## Departures from original project plan

There were no departures from the original project plan.

## Value of the studentship

*Research group*: Soren been hands-on with all the techniques described and worked alongside other members of the Littlechild group. He has obtained new results that have contributed to the SHC project that was originally funded by Innovate UK Grant No. 104457.

*Student*: This studentship has allowed me to gain excellent experience in the field of biocatalysis. I have enjoyed gaining valuable insight into the importance of chemistry within biological systems and how this can be used to improve the sustainability of industrial processes. I have been able to improve my adaptability and flexibility through proactive learning whilst also enhancing my communication proficiency and ability to work as part of a team. Alongside these transferable skills, I have developed competence in numerous biochemical techniques which has helped to increase my lab confidence immensely. Ultimately, the experience has intensified my passion for research and my desire to pursue PhD programmes relating to biotechnology and sustainable chemistry.

## Acknowledgements

Funding of the studentship by the Biochemical Society is gratefully acknowledged. The success of this programme has contributed to achieving their strategic objectives which focus on promoting the importance of molecular bioscience as well as providing career and lifelong development to all its members. Thank you for your generosity.

Thanks must also be given to the Exeter Biocatalysis Centre for hosting the studentship; they consistently provided exceptional supervision and fascinating insight into their work throughout this enriching experience.

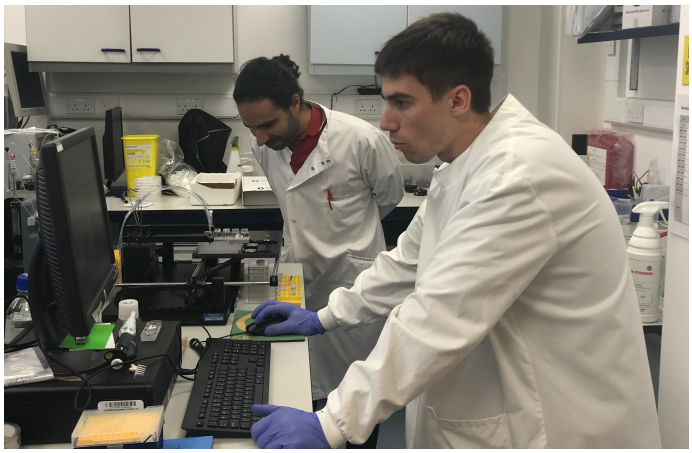

**Figure 4**: Image of Soren and Simone setting up crystallisation trials using the Oryx8 robot.

## References


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