Testing 'Prometheus' – Investigating predictions about protein folding made by an analytical computer program.

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Abstract

In this article I reflect on my experience of taking on an Undergraduate Research Opportunities Scheme (UROS) summer project in a biochemical research laboratory. I expanded my technical skills working with both DNA and protein, as my project focused on the enzyme T4 lysozyme and how changing single amino acids in the gene that encodes it also changes the stability of the overall protein. This was done to assist a larger group of researchers, a collaboration between the University of Lincoln and Rutgers University, USA. We want to prove that the analytical computer program 'Prometheus', which is said to be able to predict how proteins fold and how likely each amino acid is to accept new interactions with other compounds, is accurate and can aid in the discovery and improvement of new and existing drug molecules. The UROS experience also helped prepare me for my upcoming dissertation project and made me more educated on what to expect during a career in scientific research.

Keywords: Protein stability, T4 lysozyme, Prometheus, research experience

Introduction

The Undergraduate Research Opportunities Scheme (UROS) is a competitive scheme that enables students to engage with the material they have learned at university in a different, more hands-on way. They can experience what it would be like to be part of a research team and take on independent tasks as a researcher. To take part in UROS, the student must apply with an academic supervisor and a project proposal. Upon successful application, the student receives a bursary to fund their project, almost simulating a professional grant application.
My project centred around the protein folding and expression of the protein lysozyme, and how its stability may change when introducing specific point mutations. The newly developed computer program 'Prometheus' has made predictions on how the amino acids change which surrounding amino acids they interact with. Our goal was to prove this theoretical basis using thermal denaturation trials with the wild-type protein and the mutated proteins.

Project Background

Proteins are fundamental biological molecules responsible for running the processes in our body that keep us alive. They are made up of a linear chain of amino acids that fold up to give each protein its unique structure.
Understanding protein structure and protein stability are crucial factors in developing new drug targets and improving already existing ones. The interaction between the target protein and target amino acids and the drug determines how well the drug works (Abbasi Mesrabadi et al., 2023). The computer program “Prometheus” reduces the structure of a protein to a heat map, where it is easy to identify the most interaction-happy amino acids. This could be revolutionary for drug discovery, as it would be much easier to identify where the best binding sites in a drug target are. Professor Lawrence Williams, the developer of “Prometheus”, has made predictions about how single amino acid mutations in the bacteriophage T4 protein lysozyme can increase the protein’s stability. To validate this theoretical basis, I measured the thermal stability of the wildtype protein against mutated versions of the protein to identify possible changes in their structure.

Literature Review

The protein lysozyme is an important part of the innate immune system, due to its ability to kill bacteria by attacking and destroying their cell wall. They are part of the organelles called lysosomes, which essentially act as the digestive system of the cell. After lysing open the bacterial cell wall, they degrade and digest the waste material (Cooper & Hausman, 2000).
It was discovered by Alexander Fleming due to the ability of nasal secretions (which are rich in lysozyme) to prevent bacterial growth, even before he discovered penicillin (Ferraboschi et al., 2021).
Drug-target interactions are a crucial part of developing new drugs and improving medical treatment for many diseases. Identifying or predicting how strong the binding affinity of a particular drug to its target is, is one of the biggest modern research areas in the biomedical field (Shim et al., 2021). It is much quicker and easier to examine already known compounds for new uses, than try to find completely new ones. Due to its ability to act against gram-positive bacteria, new applications of lysozyme in medicine have been considered, especially since its nature as a recombinant protein means it is at a lesser risk of bacteria developing resistance against them (Ferraboschi et al., 2021).
The analytical computer program ‘Prometheus’, developed by Professor Lawrence Williams at Rutgers University, can reduce the structure of a protein to a heat map (Williams et al., 2019). The redder the amino acids, the more willing they are to form more interactions with other amino acids and other compounds. The bluer the amino acids, the less likely they are to accept new interactions (Williams et al., 2019).
With this data, it is then possible to make predictions about surface interactions and how a compound may fit into a binding pocket.

Lysozyme has been found to be very tolerant of mutations like amino acid replacements, due to its already high stability. This makes it a very good subject for

protein folding experiments and how single amino acid changes might affect the folded structure of a protein.
A previous study of the protein T4 lysozyme by Brian Matthews identified a single amino acid change that switched the 117th amino acid from a serine to a valine (Baase et al., 2010). This mutation increased the stability of the protein, such that the protein was five degrees Celsius more stable when heated. Prometheus predicts that serine 117 actually affects the 121st amino acid leucine, increasing its interaction with neighboring amino acids which affects the stability.

Methodology

As part of this greater project, I was given data produced by Prometheus in form of a heat map of specific amino acids that may affect its stability. I used this data to decide which amino acids to mutate and examine.
To start with, we attempted to use the Q5 Site-Directed Mutagenesis Kit to insert our mutations into the T4 wild-type (WT) template DNA using PCR, a polymerase chain reaction to denature the DNA, insert the sequence with our mutation and re-extend the new DNA strand. Unfortunately, the Q5 kit was unable to insert the mutation successfully, so we switched to the KAPA Taq PCR kit. After each PCR the product was examined using an agarose gel. The PCR product is inserted into the gel along with a dye that attaches to the DNA as it slowly runs through the gel. The clearer the band when examined under UV light, the better the mutation has bound to the DNA and inserted itself.
To insert the DNA into bacterial cells and express the protein with its new mutations, we used the pET22b vector and inserted them into competent cells. The cells were then grown on ampicillin-resistant agar plates.

To make and then purify the WT protein, we once again had to adapt. The method originally used by Tsugita and Inouye (1968) and Matthews and Remington (1974) was unfit for our version of the protein, so we adapted and combined their method with that of Sloane et al. (1996).
First, we transformed some of the T4 WT in competent cells, continued to grow the protein in lysogeny broth, a nutritionally rich medium, with the help of IPTG. It was then purified using a CM Sepharose column. The resin forms a matrix that the denatured cell broth can flow through, with the proteins attaching to the matrix while the waste liquid with the rest of the cells is expelled as flowthrough. We then used a NaCl step gradient, which is made of different buffers at increasing concentrations of NaCl, to slowly elute the protein from the resin column. We planned to identify where exactly the protein sits using an SDS-PAGE gel and examining all the buffers next to each other.
Then we wanted to examine the thermal stability using thermal denaturation trials, comparing the WT protein to the proteins containing the different mutations. This was achieved through adding a fluorescent dye to the protein and heating it, until it unfolds, and the dye can attach to it. A difference in their fluorescence curves would

show if their melting temperature changed. An increased melting temperature shows an increase in protein stability.

Results

Due to the possibility that the results I achieved during my summer project may be used as part of a bigger article to be published in a more subject-specific journal, I am unable to go into a lot of detail.
What I can share is that during our experiments we had to keep adjusting our methods due to the results we got, like changing the denaturation temperature of our PCR rounds to optimise the attachment of the mutation strand to the rest of the template DNA. This meant that our process had to be adjusted significantly, extending our work by at least two weeks, since we had to perform multiple PCRs and clean-up steps in-between to get clean DNA at the end.
We were able to fully insert four mutations into competent cells, R125A, R125F, R95H and R96H. While we managed to express and produce a lot of viable wild type protein, extracting it from the filter column proved to be more difficult than expected. Therefore, for the thermal denaturation curve we used hen egg white lysozyme (HEWL) instead (Fig. 1).

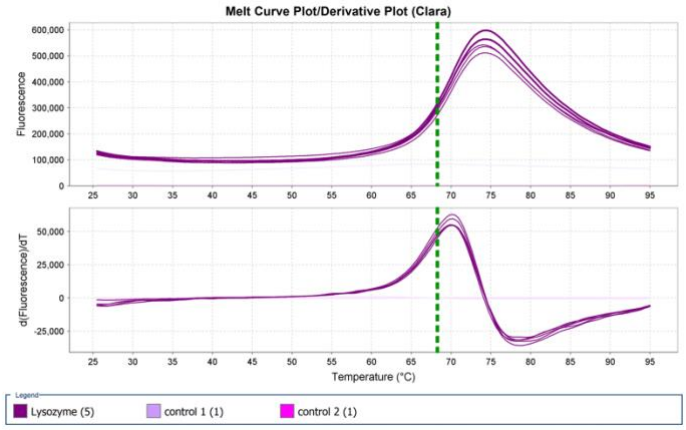

Figure 1.
A thermal denaturation curve depicting the fluorescence of HEWL (upper graph) and the first derivative of that data (lower graph). The Tm over 5 repeats is 68.33 +/- 0.04.

UROS Experience

This UROS project gave me a lot of valuable insights into the world of scientific research. It showed me that, unlike in movies where everything seems to work on the first try, real projects require a lot of trial and error. But you learn and improve with every failed attempt. During my time of working on this project, we had to adapt and re-strategize frequently, to be able to work with and improve on the results we were getting.
In a lab that deals almost exclusively with small, fickle things like proteins and DNA, you never quite know how an experiment is going to turn out or what results you are going to end up with, or even how long it is going to take you. This was an important realisation for me, and it is something I will have to consider when continuing my

education in a master's programme. Even when it comes to choosing careers, it made it clear to me that with academia there is a lot of uncertainty sometimes, not just when it comes to grants and project allowances, but also getting results in a timely manner.
Overall, this UROS project was a very enlightening experience. Being able to use the skills and knowledge I learned during my lectures at university in an actual lab and research environment felt incredibly rewarding. The results I achieved at the end of my project were maybe not exactly what I was hoping for, but the technical skills I learned made the experience more than worth it.

Conclusion

To conclude, Prometheus is a very promising computer program that, once proven accurate, should vastly improve drug discovery research. I learned how to work effectively in a professional laboratory, how to work with both DNA and protein. Having to adapt our methods multiple times and even make up our own really forced me to think on my feet and think outside the box. Switching from Q5 to KAPA elongated our project by about 2 weeks, so we had to cut out some other experiments we wanted to do, helping me prioritise what techniques I really wanted to learn and what I wanted to get out of my project at the end of the summer. It also helped me understand the project better, as you can only adapt something successfully if you really understand why every chemical is used the way it is in the original method.
The new skills I acquired will be incredibly useful in my career and further academic study, like during a master's program or PhD.

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