LEADER Feasibility Study Technical Data Report

Activities undertaken during the project

- Harvested limpets and set up tanks.
- Investigated influences of abiotic and biotic factors on amount of hemocyanin produced by individual limpets.
- Developed hemocyanin extraction and purification methods.
- Harvesting and processing of slipper limpets in the lab.
- Early buffer concentration experiments.
- Scale up hemocyanin extraction trial.
- Development of freezing trial methodology and protocol.
- Successful scale-up of harvesting and processing at Pembroke Dock facility.
- Investigation of buffer formulations for purified hemocyanin.
- Dialogue initiated for stakeholder engagement.
- Ongoing structural analysis at Cardiff University.
- Analysis of protein degradation issues.
- Freezing trial.
- Partial V8 proteolysis of hemocyanin.
- Temperature dependant autocleavage of purified hemocyanin.
- Replication of proteolysis experiments.
- Sequence analysis of slipper limpet hemocyanin.
- EM and Cryo-EM analysis with partial 3D reconstruction.
- Meetings and presentations regarding the project.
- Completed V8 proteolysis of hemocyanin.
- Replication of proteolysis experiments.
- Ongoing analysis of slipper limpet hemocyanin.
- EM and Cryo-EM analysis with full 3D reconstruction.

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Activities

Harvesting

Slipper limpets were harvested at low tide and stored in holding tanks. They were then processed in the lab for volume extraction and refinement per harvest period. Regular low tide days allowed for increased harvesting resulting in increased volume processing in the lab.

Haemocyanin Extraction, Purification

The slipper limpets were processed by hand and the gut was removed allowing for the dissection of the haemolymph production site. Once the gut had been dissected the haemolymph was harvested using a pipette.

The harvested haemolymph was then centrifuged for 30 minutes to remove large impurities. The supernatant was then transferred to another tube and then centrifuged for 4 hours, after which the supernatant was removed, leaving a haemocyanin pellet at the base of the tube.

Concentration Analysis

The haemocyanin pellets were diluted to a 1:1000 ratio to allow for a Bradford assay to be conducted on the sample. Approximately 200 µl of hemocyanin was harvested per limpet with an average overall protein concentration of 92.2 mg/ml.

Sample	Volume (µl	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbanc	Concentration 1:1000 (mg/	Concentration (mg/m
1	15	0.361	0.369	0.377	0.369	0.073	73
2	15	0.309	0.389	0.331	0.343	0.048	48
3	15	0.383	0.401	0.385	0.389666667	0.093	93
4	15	0.386	0.4	0.399	0.395	0.098	98
5	15	0.366	0.371	0.377	0.371333333	0.075	75
6	15	0.473	0.448	0.461	0.460666667	0.161	161
7	15	0.445	0.441	0.436	0.440666667	0.142	142
8	15	0.376	0.391	0.406	0.391	0.094	94
9	15	0.311	0.315	0.34	0.322	0.028	28
10	15	0.407	0.411	0.407	0.408333333	0.11	110

Sample	Volume (µl)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbanc	Concentration (mg/ml)
1	15	0.776	0.814	0.778	0.789333333	0.5
2	15	0.589	0.599	0.594	0.594	0.25
3	15	0.462	0.453	0.457	0.457333333	0.125
4	15	0.375	0.363	0.361	0.366333333	0.075
5	15	0.329	0.322	0.323	0.324666667	0.05
6	15	0.298	0.293	0.295	0.295333333	0.025

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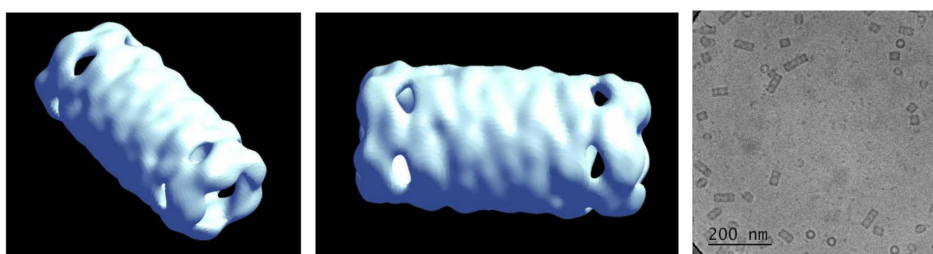
Structural Analysis

EM and Cryo-EM was used to analyse the haemocyanin to gain more information about its structure and possibly create a 3D reconstruction of the protein. The haemocyanin was reconstructed to 30Å from EM data sets. The successful preparation of hemocyanin on Cryo-EM grids with good preliminary low-resolution data allows for use of other facilities, like Bristol University, to obtain better data sets and reconstruct the protein at a much better Å.



Freezing Trials

Triplicate batches of slipper limpets were frozen with the plan to defrost and process a batch of triplicates every month for six months. Haemolymph will be extracted and the haemocyanin purified to investigate the effects of freezing on the final product.

The first batch was defrosted and the slipper limpets displayed physical destruction of the gut and internal organs which created a mush on defrosting rather than organs and blood as expected.

The material was processed, SDS-PAGE and Bradford Assay were used to determine protein degradation. The results showed that extracting haemocyanin from the frozen samples was not viable and the trial was stopped. Snap-freezing was investigated but was not trialled due to the cost.

Proteolysis

An enzyme (Endoproteinase Glu-C from *Staphylococcus aureus* strain V8) was used to cleave proteins at specific points in their structure to help determine their overall structures. The use of the V8 protease resulted in cleavage of two functional units achieved. It is suspected that these are FU-g and FU-h. V8 protease treatment can clearly cleave at specific points but is not successful in cleaving all 7-8 FU's. Experimental repeats showed the same full cleavage of the two functional units alone.

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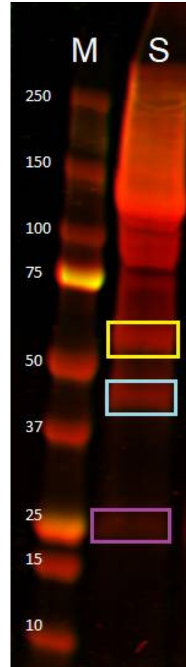
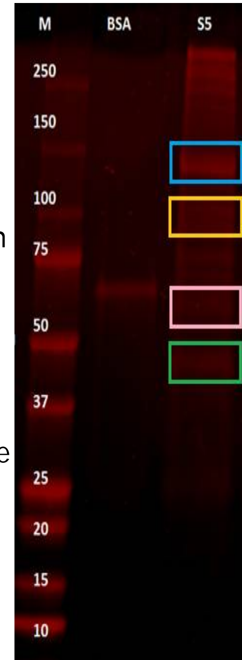
Proteolysis Results

- 4-20% TrisGlycine Gradient Gel.
- Blue area shows FU-g banding pattern
- Yellow area shows FU-h banding pattern
- Purple banding pattern is representative of the enzyme used during the experiment.



- 4-20% TrisGlycine Gradient Gel.
- Green shows FU-g banding pattern also present in first experiment.
- Pink area shows FU-h banding pattern present in earlier experiment.
- Blue and orange banding patterns are higher order cleavage fragment.

Repeat gel image



SDS-PAGEs displaying the sample broken down by the V8 protease (S) as well as protein markers (M). FU-g/FU-h are the 7th and 8th functional unit on a haemocyanin subunit that have been cleaved off by the protease (displayed on the images above as well as smaller fragments that have been cleaved off).

Autocleaving

Furthermore, an experiment was carried out to determine if an external factor was breaking down the protein or if it was autocleaving itself. Autocleavage of hemocyanin has been observed within molluscan Concholepas concholepas (CCH). CCH shares certain characteristics with slipper limpet hemocyanin (SLH) including the two subunits.

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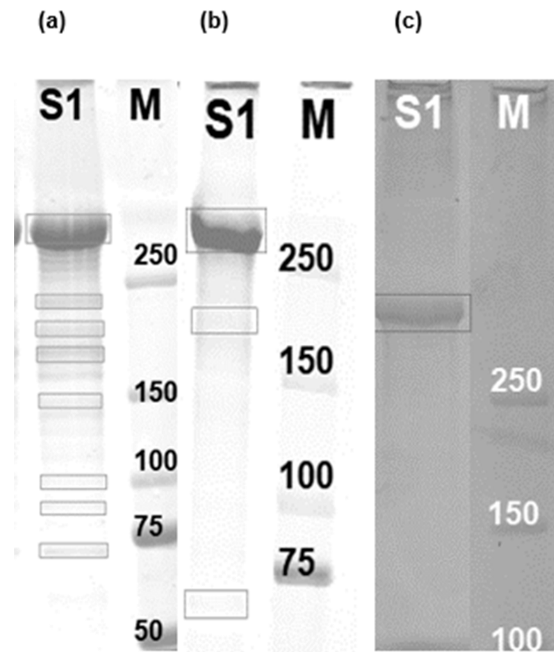
Autocleaving Results

The fragment patterns displayed in each case were consistent throughout all 5 samples at differing temperatures.

The range of molecular weights observed could be a result of having a range of cleaving positions along the chain.

EM imaging of the protein suggested no degradation occurred, so autocleaving is a possibility.



SDS-PAGE gel of sample (S1) and protein marker (M) with each sample being exposed to different temperatures to test for the spontaneous cleavage of the haemocyanin subunits.

Sequence Analysis

Previously deposited DNA and RNA sequencing data can be analysed to assemble and deduce the amino-acid sequences of proteins. Sequence comparisons allow points of similarity or dissimilarity between old and new sequences to be analysed, allowing prediction of a potential sequence for a protein of interest. There is plenty of data regarding keyhole limpet hemocyanin sequences for us to compare to.

Several individual transcripts were discovered, these require further analysis. This hints at potentially unique characteristics of slipper limpet hemocyanin. Sequence for possible FU's within SLH_1 were theorised and mapped out in comparison to KLH, displaying an almost perfect fit.