DECLARATION

I declare that the work presented in this thesis is, to the best of my knowledge original, except as acknowledged in the text, and has not been submitted for a degree at any other university. Some parts of this research work have been published and a list of publications arising from this work is included in this thesis.

Gavin Marshall

March 2016
ABSTRACT

Haliotis Rubra hemocyanin is a promising antiviral candidate for treating Herpes Simplex Viruses, however this protein is not stable and forms irreversible aggregates, destroying any therapeutic activity. In this study we have analysed the stability of this protein, developing diagnostic techniques for measuring its stability, and have created a stable formulation which maintains its native structure even after long term storage. This work will allow us to properly test the antiviral activity of the protein by ensuring it reaches drug trials in a stable reactive form.

The extreme size of hemocyanins requires specialist techniques to study as they do not readily diffuse into electrophoresis gels and tend to aggregate easily. As the genome for Haliotis Rubra has not been investigated we have instead compared the primary structure of the hemocyanin to those for hemocyanin from other species using Mass Spectrometry, identifying that the Haliotis Rubra has high homology to Haliotis Tuberculata. Photon Correlation spectroscopy was then applied to identify a solubilised structure with a diameter of 44nm, indicating that the hemocyanin has a similar size shape and structure to the Haliotis Tuberculata didecamer with a molecular weight of 7.39MDa. This makes our hemocyanin one of the largest proteins to be studied.

Differential scanning calorimetry was then applied to the study of hemocyanin denaturation. The high resolution TA nanoDSC allowed calorimetric data to be obtained without the onset of aggregation during the denaturation transition. For the first time with Haliotis Rubra calorimetric traces have been obtained, with a denaturation temperature of 76.3°C showing that the stability of the pure protein was comparable to that from other species. Similarly to other hemocyanins which have been studied, the denaturation reaction was found to be entirely irreversible, necessitating the use of irreversible denaturation models for its study. The method of Sanchez-Ruiz was therefore applied, identifying an activation energy for the denaturation reaction of $528 \pm 63 \text{ kJ mol}^{-1}$.

It was determined that whilst the hemocyanin has a complex quaternary structure, this oligomeric form does not change during the denaturation. Aggregation, however, did occur alongside denaturation, with all samples showing visible aggregates upon removal from the calorimeter. Photon correlation spectroscopy was therefore applied, observing the onset of hemocyanin aggregation for the protein at 76°C, simultaneously to the denaturation. This method is novel to the study of proteins as extremely large molecular weights are required for testing, however this proved to be a simple and effective method for measuring the aggregation state of hemocyanin and may be widely applicable to the study of other large aggregating proteins.

Gavin Marshall PhD Thesis
The stability of this hemocyanin was then improved through formulation, with the effect of buffers, salts and sugars investigated as candidate stabilising adjuvants. All sugars promoted stable formulations, likely due to the excluded volume effect, with a maximum suitable concentration only limited by the solubility of the sugar. Buffers were found to have specific interactions with the protein, resulting in either a strongly destabilising effect or a promotion of stability and increase of denaturation temperature. Both TRIS and HEPES were strongly stabilising. Salts were found to stabilise the protein at certain concentrations, as long as divalent cations were not included, with an optimum concentration of 100mM observed.

In order to combine the stabilising adjuvants into a single formulation we identified the optimum mixture to be a 50mM HEPES at pH 7.4 with 100 mM NaCl and 100 mM sucrose solution which was formulated with 1 mg mL\(^{-1}\) *Haliotis Rubra* hemocyanin. This resulted in a stabilised formulation with a denaturation temperature of 78.9°C and a denaturation activation energy of 726 kJ mol\(^{-1}\), a stabilisation improvement by 38%.

This sample was then stored for nine months, with the sample retaining its thermal stability and oligomeric state. As we have formulated the protein into a solvent with FDA approved adjuvants and proven long term stability, the hemocyanin sample is now suitable for therapeutic testing. Creating a stable hemocyanin formulation will allow for viable drug testing and will show the true efficacy of native *Haliotis Rubra* hemocyanin.
ACKNOWLEDGEMENTS

I would like to thank my Supervisor, A/Prof Vincent Gomes for his long suffering support of this research, along with the rest of our research team, Prof. Fariba Dehghani, Dr Peter Valtchev, Fareed Saari and Negar Talaei Zanjani. To the research group at the UNSW BMSF who provided their excellent expert knowledge as well as use of their state of the art equipment I offer my great thanks. At UNSW I would especially like to thank Dr Anne Poljak and Dr Sohail Siddiqui who contributed so much wisdom.

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Thank you.

Gavin
PUBLICATIONS

Peer Reviewed Journal Articles


Conference Poster Presentations


G Marshall, V Gomes, P Valtchev, F Dehghani “Molluscan Hemocyanins: determining the structure and stability of living mega-molecules” Symposium of the Protein Society, Boston, USA 2013

Gavin Marshall PhD Thesis
# Table of Contents

CHAPTER 1. Introduction ........................................................................................................ 2

CHAPTER 2. Literature Review .......................................................................................... 6
  2.1. Protein Stability ......................................................................................................... 6
      2.1.1. Structure of proteins ....................................................................................... 6
      2.1.2. How to define stability of ideal protein systems ............................................ 7
      2.1.3. Irreversible denaturation of proteins ............................................................. 10
  2.2. Protein Formulation .................................................................................................. 12
      2.2.1. Stabilising and Destabilising Forces ............................................................... 12
      2.2.2. Effect of salt concentration on protein stability ............................................. 12
      2.2.3. Effect of sugars on protein stability ............................................................... 12
      2.2.4. Protein aggregation forces ............................................................................ 13
  2.3. Hemocyanin ............................................................................................................ 14
      2.3.1. Structure of hemocyanins ............................................................................ 14
      2.3.2. Hemocyanin Stability .................................................................................. 14
      2.3.3. Therapeutic Applications of hemocyanins ..................................................... 17

CHAPTER 3. Physical Characterisation of Hemocyanin ...................................................... 19
  3.1. Hemocyanin Sera .................................................................................................... 20
      3.1.1. Purification methods ...................................................................................... 20
      3.1.2. Protein concentration and purity ................................................................... 20
  3.2. Hemocyanin size .................................................................................................... 21
      3.2.1. Gel electrophoresis ...................................................................................... 22
      3.2.2. Photon Correlation Spectroscopy ................................................................ 24
  3.3. Hemocyanin Subunits ............................................................................................ 27
  3.4. Homology of Haliotis Rubra hemocyanin to hemocyanin of other species .......... 29
  3.5. Hemocyanin Charge ............................................................................................... 34
      3.5.1. Isoelectric Focussing .................................................................................... 34
      3.5.2. Titration Methods ....................................................................................... 35
      3.5.3. Zeta Potential Methods ............................................................................... 36
  3.6. Conclusions ............................................................................................................. 38

CHAPTER 4. Characterisation of hemocyanin stability ...................................................... 40
  4.1. Evaluation of DSC equipment for the study of hemocyanin denaturation ............ 41
  4.2. Analysis of irreversible denaturation ..................................................................... 44
Figure 2-1 A generalised protein stability curve, depicting a region of stability between the critical temperature (Tc) and the Melt temperature (Tm) for the protein. Temperatures beyond these points will lead to instability of the protein structure. [37] .................................................................................................................. 8

Figure 3-1 Gel electrophoresis of Haliotis Rubra hemocyanin showing the protein stuck in the stacking section. Lanes 1-5 and 6-11 all contain 20µg purified hemocyanin, run simultaneously to test resolution, L is a broad range ladder (6.5-200kDa) .......................................................................................................................... 22

Figure 3-2 BlueNative Gel showing Hemocyanin separated into two distinct protein bands. Lanes 1-3 are 20µg pure hemocyanin, L is a NativeMark Ladder, Lanes 5-6 are hemocyanin sera, Lane 7 is the purification filtrate and lane 8 is a sera sample stored for 3 months. ................................................................................................................ 23

Figure 3-3 BlueNative gel showing overloaded hemocyanin to check purity. Lanes 1, 4, are purified hemocyanin at 200µg, 100µg respectively, showing minor bands at lower molecular weight. ................................................................................ 23

Figure 3-4 Diameter of particles observed in Haliotis Rubra hemocyanin sera. The principal peak at 44nm is assigned to the hemocyanin quaternary structure ........................................................................................................... 25

Figure 3-5 Diameter of particles in hemocyanin sample after 10 min at room temperature or 10 min at 85°C ........................................................................................................................................................................ 26

Figure 3-6 Blue Native gel comparing purified hemocyanin in neutral buffer, with high pH separated hemocyanin subunits. Bands 1, 4, 7 show hemocyanin at pHs of 9, 10 and 11 respectively, with the two bands following each high pH sample being the initial sample at neutral pH and the protein after being returned to neutral pH. .................................................................................................................. 27

Figure 3-7 Gel used for the LCMS of Haliotis Rubra hemocyanin. Individual bands, as labelled, were cut and treated for LCMS. .................................................................................................................................. 31

Figure 3-8 IEF gels from Haliotis Rubra hemocyanin, showing no separation of hemocyanin through the gel ...................................................................................................................................................... 34

Figure 3-9 Titration of Haliotis Rubra hemocyanin with HCl from pH 7 to pH 2 .......................................................................................................................... 35

Figure 3-10 First derivative of the titration curve for Haliotis Rubra hemocyanin ........................................................................................................................................................................ 36

Figure 3-11 Zeta potential of Haliotis Rubra hemocyanin as a function of pH .......................................................................................................................... 37

Figure 4-1 Haliotis Rubra hemocyanin DSC trace in TA Q2000 .................................................................................................................................................. 41

Figure 4-2 DSC trace for hemocyanin in Setaram uDSC3 evo .......................................................................................................................... 42

Figure 4-3 DSC trace for hemocyanin in the TA nanoDSC .................................................................................................................................................. 43

Figure 4-4 (A) DSC Trace of native hemocyanin in TRIS buffer pH 7.6 at 1 K min⁻¹; (B) Rescan of denatured hemocyanin showing no endotherm upon reheating .............................................................................................................. 44

Figure 4-5 (A) DSC scan ramping to 75°C to measure onset of denaturation. (B) Rescan of hemocyanin sample after onset scan .................................................................................................................................................. 45

Figure 4-6 DSC traces for hemocyanin at scan rates of 0.2, 0.6, 1, 1.4 and 2 K min⁻¹ indicating that Tm is scan rate dependent .................................................................................................................................................. 46

Figure 4-7 DSC traces of hemocyanin at 0.19, 0.39, 0.64 mg mL⁻¹ .......................................................................................................................... 48

Figure 4-8 Method A to determine Ea for hemocyanin. Scan rates of 0.6, 1, 1.4 and 2 K min⁻¹ shown 50

Figure 4-9 Hemocyanin activation energy as a function of scan rate from 0.2 - 2.0 K min⁻¹ ................................. 51

Figure 4-10 Hemocyanin Ea as determined by method C: rate of energy evolution .................................................................................................................................................. 52

Figure 4-11 Aggregation profile as a function of temperature for hemocyanin in water .............................................................................................................. 54

Figure 5-1 DSC traces of Haliotis Rubra hemocyanin in buffers that reduce stability .................................................................................................................................................. 59

Figure 5-2 DSC traces of Haliotis Rubra hemocyanin in buffers which promote stability .................................................................................................................................................. 60
Figure 5-3 Aggregation of hemocyanin in high salt concentration TRIS buffer ........................................62
Figure 5-4 Changes in hemocyanin denaturation rates with change in ionic concentration...............63
Figure 5-5 Effect of changing both anion and cation valence on the stability of Haliotis Rubra hemocyanin ..................................................................................................................64
Figure 5-6 Effect of increasing sugar content on hemocyanin stability .................................................65
Figure 5-7 Effect of various monosaccharides on Haliotis Rubra hemocyanin ..................................67
Figure 5-8 Effect of changing polysaccharide size on Haliotis Rubra hemocyanin ...............................68
Figure 5-9 DSC curve for Haliotis Rubra hemocyanin in the most stable formulation identified, containing 50mM HEPES at pH 7.4 with 100mM NaCl and 100mM sucrose with 1mg mL-1 Haliotis Rubra hemocyanin ..................................................................................................................69
Figure 5-10 Aggregation behaviour of Haliotis Rubra hemocyanin in water compared with an optimised formulation ........................................................................................................70
Figure 5-11 DSC traces of Haliotis Rubra hemocyanin formulations after nine months of storage as either a liquid or a freeze dried solid .................................................................................71
Figure 5-12 Particle sizes of Haliotis Rubra hemocyanin formulation and its stored forms ...............72
Table List

Table 3-1 Comparison of measured diameters and volumes of whole and subunit hemocyanin......28
Table 3-2: MASCOT fits of individual bands separated from hemocyanin PAGE ......................32
Table 3-3 Key physical characteristics of Haliotis Rubra hemocyanin......................................38
Table 4-1 Comparison of resolution for three common DSC machines. ................................43
Table 4-2: Hemocyanin Tm variation with scan rate .................................................................47
Table 4-3 Hemocyanin Ea variation with scan rate ......................................................................52
Table 4-4: Denaturation temperatures for various hemocyanins..............................................53
Table 5-1: List of buffers which may be suitable for the formulation of Haliotis Rubra hemocyanin..59
Table 5-2 Comparative denaturation temperatures for Haliotis Rubra hemocyanin in various buffers prepared to 50mM........................................................................................................61
Table 5-3 Activation Energy parameters for Haliotis Rubra hemocyanin in solutions at various sucrose concentrations.............................................................................................................66
Table 5-4 Effect of Sugar identity on the stability of Haliotis Rubra hemocyanin.....................68
Table 5-5 Comparative stability of Haliotis Rubra hemocyanin in water and in an optimised formulation .............................................................................................................................70
6-1 Composition and properties of the final improved Haliotis Rubra hemocyanin formulation....78
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aciclovir</td>
<td>A family of drugs used to treat viral infections, most commonly HSV infections.</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>An added ingredient used in a formulation</td>
</tr>
<tr>
<td>AH</td>
<td>Abalone Hemocyanin</td>
</tr>
<tr>
<td>Alpha Helix (α-helix)</td>
<td>A common shape in protein secondary structures</td>
</tr>
<tr>
<td>apo form</td>
<td>Form of a protein with no bound oxygen</td>
</tr>
<tr>
<td>Beta Sheet (β-sheet)</td>
<td>A common shape in protein secondary structures</td>
</tr>
<tr>
<td>Bis-Tris</td>
<td>A buffer of Bis and TRIS</td>
</tr>
<tr>
<td>BLAST</td>
<td>A database of genomes used to match possible species for the source of a protein</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>chaotrope</td>
<td>An agent which disrupts the hydrogen bonding of water</td>
</tr>
<tr>
<td>Cp</td>
<td>Heat Capacity</td>
</tr>
<tr>
<td>Decamer</td>
<td>A quaternary structure composed of 10 subunits</td>
</tr>
<tr>
<td>Deconvolute</td>
<td>To separate multiple signals from a single curve.</td>
</tr>
<tr>
<td>Denaturation Temperature (Td)</td>
<td>The temperature at which the protein structure is disrupted</td>
</tr>
<tr>
<td>Didecamer</td>
<td>A quaternary Structure composed of 20 subunits</td>
</tr>
<tr>
<td>divalent</td>
<td>an ion with a charge of 2</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>DSC trace</td>
<td>The curve obtained from a DSC scan showing change in heat capacity as temperature is varied</td>
</tr>
<tr>
<td>Ea</td>
<td>Activation Energy</td>
</tr>
<tr>
<td>excipient</td>
<td>A non-active component in the drug formulation</td>
</tr>
<tr>
<td>FDA</td>
<td>The Food and Drug Administration of the USA</td>
</tr>
<tr>
<td>FS</td>
<td>Fluorescence Spectroscopy</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>Gibbs free energy (G)</td>
<td>A measure of total energy combining enthalpic and entropic contributions. Systems with lower total Gibbs free energy will be more stable than those with high Gibbs free energy.</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>The binding of glycans (sugars) to a protein</td>
</tr>
<tr>
<td>Hastelloy</td>
<td>A Low corrosion Metal</td>
</tr>
<tr>
<td>Heat Capacity (Cp)</td>
<td>The amount of heat required to increase the temperature of a sample.</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - A buffer</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric Focussing Gel Electrophoresis</td>
</tr>
<tr>
<td>kD</td>
<td>reaction rate constant</td>
</tr>
<tr>
<td>KLH</td>
<td>Key Hole Limpet Hemocyanin</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>lyophilisation</td>
<td>Freeze drying</td>
</tr>
<tr>
<td>MASCOT</td>
<td>A protein sequence database used for identifying the source of protein fragments</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>MDa</td>
<td>Megadalton, a unit of mass</td>
</tr>
<tr>
<td>mM</td>
<td>milliMolar - a measure of molar concentration</td>
</tr>
<tr>
<td>Monomer</td>
<td>A unit which can be bonded to other like units in a polymeric structure</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>Oligomer</td>
<td>A complex which contains a few monomeric units</td>
</tr>
<tr>
<td>osmolyte</td>
<td>Dissolved species which contribute to the ionic concentration of the sample.</td>
</tr>
<tr>
<td>oxy form</td>
<td>Oxygenated form of a protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphine buffered saline - A buffer</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon Correlation Spectroscopy</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of Hydrogen - A measure of relative acidity</td>
</tr>
<tr>
<td>phylum</td>
<td>A taxonomic category, ranking below kingdom</td>
</tr>
<tr>
<td>pi</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>pOH</td>
<td>Potential of Hydroxide - A measure of relative basicity</td>
</tr>
<tr>
<td>Primary Structure</td>
<td>The amino acid sequence of a protein</td>
</tr>
<tr>
<td>Q</td>
<td>Measured heat output at a point</td>
</tr>
<tr>
<td>Qt</td>
<td>Total heat evolved in a transition</td>
</tr>
<tr>
<td>Quaternary structure</td>
<td>The total structure formed when multiple polypeptide chains are included together in a protein</td>
</tr>
<tr>
<td>R</td>
<td>Universal Gas Constant</td>
</tr>
<tr>
<td>Recombinant</td>
<td>A technique for producing proteins artificially</td>
</tr>
<tr>
<td>Secondary Structure</td>
<td>The folding configuration of part of a protein sequence</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>Tertiary Structure</td>
<td>The complete folded structure of a single protein polypeptide chain</td>
</tr>
<tr>
<td>Tm</td>
<td>Melt temperature, the temperature of unfolding for a polymer (or protein)</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of Flight</td>
</tr>
<tr>
<td>TRIS</td>
<td>trisaminomethane - A common buffer used in protein formulations</td>
</tr>
<tr>
<td>Tris-Acetate</td>
<td>A buffer of Tris and Acetate</td>
</tr>
<tr>
<td>Z-average</td>
<td>The <strong>Z</strong> average is the intensity weighted mean hydrodynamic size of the ensemble collection of particles measured by dynamic light scattering (DLS).</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
CHAPTER 1. Introduction

Protein and peptide therapeutics are an increasingly important part of our pharmaceutical arsenal, with treatments from insulin replacement to novel antivirals becoming widespread [3]. Therapeutic proteins are not without their challenges, however, with initial candidates having low stability [4, 5] and therapeutic windows.

The efficacy of any protein pharmaceutical is limited by our ability to maintain the stability of the active protein. A protein which may have extremely strong drug action will not, if allowed to denature, aggregate or be removed from solution, show that activity in drug trials [6]. Before attempting such drug trials, therefore, it is crucial to create a stable protein formulation and to know under what conditions this stability will be maintained.

In order to create such a protein formulation we will need to understand the core properties of the protein, including what conditions will maintain or reduce this stability. Tools which can reliably measure if the formulation is stable must be validated, and such tools must be available to test the [7] formulation as it is used in drug trials.

Hemocyanins are a class of proteins which have been widely considered for their potential therapeutic responses to a wide range of conditions, from cancer [8] to viruses [9] with the effects varying dramatically based on the species of origin for the protein. Our lab has been studying the hemocyanin from Haliotis Rubra, specifically looking at its effect on the Herpes Simplex Virus family.

Herpes simplex viruses (HSV) affect nearly 75% of the Australian population (worldwide 55-90% with HSV-1) from which 20-25% develop painful, chronic and recurrent infections [10-12]. However to date no cure has been suggested for HSVs and current treatments only temporarily treat the symptoms of HSV infection. For this purpose the Aciclovir family of drugs being the only effective treatments currently available. These drugs treat the symptoms of herpes infections by inhibiting the viral DNA polymerase [13]. These treatments are therefore susceptible to drug resistance [10] and should be complemented with antiviral drugs which work via a different mechanism.

Whist hemocyanins have exciting therapeutic possibilities, their use is challenging due to the size and complexity of the protein [14], including a didecameric quaternary structure of up to 8 MDa [15], making them one of the largest protein families in existence. This means that normal formulation methods may not be suitable and as the solvation characteristics of the protein are vastly different from small peptides [16]. Further, whist most therapeutic proteins are now expressed recombinantly, hemocyanins are a poor candidate for this due to their size, complexity and glycosylation [17]. As it
may be possible that these factors are crucial to the drug action of the protein, we must investigate the protein by purifying it directly from the animal itself.

The aim of this study was, therefore, to create a verifiably stable formulation of *Haliotis Rubra* hemocyanin and a set of tools to verify its stability. In order to do this it is crucial to define the core properties of the protein, its stability, and to investigate all possible stabilising agents at our disposal. In order to arrive at a complete stable formulation, we have structured this research in a linear sequence first investigating tools to validate stability, then using those tools to define the stability of the unformulated protein. A list of possible stabilising adjuvants may then be compiled and investigated before creating a formulation which combined the most favourable conditions identified. It is hoped that a combination of these factors may allow for a formulation which would be suitable for drug testing.

Hemocyanins were first identified in 1900 and much research has been undertaken on their properties. Whilst this research has clearly shown that the properties of a hemocyanin are a factor of the species from which it is drawn, many possible avenues for investigating the stability of this protein exist in the literature. A review of this data, as well as the available methods for measuring and improving protein stability will be conducted in Chapter Two.

The physical properties of *Haliotis Rubra* hemocyanin will then be investigated in Chapter Three. As the genome for *Haliotis Rubra* has not been sequenced, and therefore the primary structure of its hemocyanin unknown, we will derive core information about the size, primary structure, aggregation and diffusion properties of the protein indirectly. These properties are key to establishing baseline properties of the protein so that we may have the core data required for models of the hemocyanin thermal stability, the properties of any aggregates formed and the possible quaternary arrangements of the protein.

Chapter Four will then investigate the thermal stability of *Haliotis Rubra* hemocyanin, investigating whether the protein may denature reversibly and what methods may be used to quantify the stability of the protein by analysing its denaturation behaviour. The previously derived information of the size and structure may allow us to calculate the activation energy of the hemocyanins denaturation reactions and to create baseline comparisons for data on the aggregation state of the hemocyanin before, during and after denaturation.

Further, in Chapter Five, we will formulate the hemocyanin into a variety of buffer and adjuvant solutions, comparing the stability of these formulations to that of the pure hemocyanin. Previous studies into the stabilising and destabilising effects of various adjuvants allow us to make predictions.
on what the effect of each of these additives may have on hemocyanin stability, however, due to the extreme size of the protein, as well as its unique properties, such as its high copper content, we anticipate that hemocyanin may not respond in the same manner as most small proteins would to this formulation. Indeed the extreme differences in stability properties measured for hemocyanins from other species suggests that a formulation tailored specifically to this hemocyanin may be needed in order to ensure its stability.

Finally we will combine and summarise the findings of this research in Chapter Six. Further lines of inquiry into the stability of other large proteins, and other experimental techniques which could be relevant to studying the stability of hemocyanins and indeed all large proteins will be investigated. As there are so many unusual variables involved in measuring the properties of such a large protein, we anticipate that many avenues for investigation will exist which may provide further insights. Study of the specific therapeutic effects of hemocyanin into herpes simplex viruses as well as other ailments will be considered,

It is hoped that by investigating the core stability issues of Haliotis Rubra hemocyanin we may facilitate the creation of viable and effective therapeutic Haliotis Rubra hemocyanin formulations and potentially give some frameworks into creating therapeutics from hemocyanins and other large proteins which may applied as broadly as possible.
Chapter 2: Literature Review
CHAPTER 2. Literature Review
In the following section we shall investigate the current literature covering protein structure, stability, the formulation of protein pharmaceuticals. This will include an evaluation of the relevant structural elements of hemocyanins, and proteins more generally.

2.1. Protein Stability

2.1.1. Structure of proteins
Proteins make up a large portion of the biomass within all living organisms, constituting the structure of cells, accounting for most of the cells mass and performing the bodily functions of the organism[18, 19] such as respiration, for which hemocyanins are responsible[20]. Information about both the covalent and folded structures of the protein are relevant to the relative stability of the various protein molecules, and hence shall be described here.

The primary structure of the protein is defined by the polypeptide sequence of bound amino acid residues which are synthesised within cells by the condensation reaction between amino acids to form peptide bonds between the carboxyl group and the amino group of two amino acids. Twenty different amino acids, defined by the structure of their side chain, are utilised to create polypeptides with a variety of different properties. As some amino acid residues are polar and others are non-polar, the protein will fold into a structure where the non-polar residues are shielded from the surrounding water molecules, whilst the polar residues are free to interact with water and other polar residues through a number of intermolecular forces[21]. This creates a situation where the folded structure is thermodynamically favourable to the unfolded structure, as the free energy of the system with a folded protein in water is lower than that of an unfolded protein[22]. The total molecular weight, solubility and ionic charge will be determined by the protein's primary structure.

The secondary structure of the protein consists of the folded shapes which are formed in the hiding of non-polar residues, within segments of a protein's primary structure. The two most common folding patterns are the α-helix and the β-sheet. They are predominantly stabilised by the hydrogen bonding between amide and carboxylic acid groups on the protein's side chains. An α-helix is formed when the polypeptide chain coils around itself, hydrogen bonding between every four amino acid residues. A β-sheet, however, forms when hydrogen bonding occurs between segments of the polypeptide chain which lie parallel to each other[18]. Most proteins form some combination of α-helices and β-sheets, connected by unstructured linking sections. The final shape, function and stability will be determined by the relative prevalence of secondary structure elements in the final structure[23].
The tertiary structure of the protein is comprised of the complete folded structure of an entire polypeptide chain. It is determined by the arrangement of all the secondary structure elements present in the polypeptide and is stabilised by a range of intermolecular forces, including hydrogen bonding, hydrophilic and hydrophobic interactions and Van der Waals forces [24]. These interactions exist particularly between the various side chains of the amino acid residues and between the side chains and the solvent in the system [25].

For complex proteins, in which multiple polypeptide chains assemble to form the complete protein, a further level of organisation, called the quaternary structure, exists. In this structure each individual polypeptide is referred to as subunit. The structure may consist of repeating identical subunits, such as in keratin [26], or contain different subunits, such as haemoglobins [27]. Hemocyanins have been shown to form a series of different complex quaternary structures, depending on the phylum from which they are extracted [20, 28, 29].

Upon denaturation, proteins unfold and refold into many different conformations. This changes are most pronounced in the secondary and tertiary structures, however separation of the quaternary structure prior to denaturation is often observed. The primary structure of the protein, however, is not disturbed during denaturation. It has been suggested that during reversible denaturations the structure of the protein does not change dramatically and there is more likely an expansion of the original shape and a loss of intramolecular bonding [30]. Separation of secondary structures from α-helices into β-sheets or random coils, is also observed [31, 32]. This leads to a final denatured structure most often referred to as a molten globule [33].

### 2.1.2. How to define stability of ideal protein systems

A stable protein may be defined as one which is in its natural functional state. This requires that the protein has preserved its primary structure and is correctly folded state [32]. Naturally, this is the state which a protein will be found when functioning within an organism. This correctly folded state is one maintained as a thermodynamic local energy minimum of the entire protein and solvent system, which we describe with the Gibbs free energy function. The stable state will therefore have a Gibbs free energy lower than other folding possibilities [34].

\[
\Delta g(T) = \Delta h(T) - T\Delta s(T)
\]

This Gibbs free energy function is relevant to all thermodynamically controlled reactions, however as the entropy of the system cannot be directly measured, it must be expressed in another manner.
Privalov [35, 36] shows a method for expressing the Gibbs free energy stability function in terms of the denaturation temperature and heat capacity of a protein as follows.

\[
\Delta g(T) = \Delta h(T_d) \left( \frac{T_d - T}{T_d} \right) - (T_d - T) \Delta C_p \ln \left( \frac{T_d}{T} \right)
\]

If this function is then plotted with Gibbs free energy as a function of temperature, we get the specific protein stability curve [33] for that protein system (Figure 2-1).

![Figure 2-1 A generalised protein stability curve, depicting a region of stability between the critical temperature (Tc) and the Melt temperature (Tm) for the protein. Temperatures beyond these points will lead to instability of the protein structure. [37]

As the protein is solubilised into solution, the Gibbs free energy for the system will depend on the character of the solution as well as the protein, therefore the stable conformation observed within that solution and its characteristic heat capacity and denaturation temperatures will also be a function of that formulation.

Unlike monomeric proteins, the stability of multimeric proteins is closely linked to concentration as, by Le Chatelier’s principle, any dissociation which increases overall concentration will push the reaction to mitigate this change. Thus when analysing the stability of a multimer it is crucial to develop a series of thermodynamic state functions which take concentration into account[38]. The equilibrium constant for the folding/unfolding reaction must be characterised and this is quantified by the Gibbs free energy, as the equilibrium position will occur at the free energy minimum[16, 39].
\[ K = e^{-\frac{\Delta G^o}{RT}} \]

As usual the temperature dependence of the enthalpy change for the equilibrium is calculated using the van’t Hoff equation.

\[ \frac{\partial R \ln K}{\partial \left(\frac{1}{T}\right)} = -\Delta H_{vh} \]

Note that the calorimetric enthalpy does not equal that of the van’t Hoff enthalpy

\[ \Delta H_{cal} \neq \Delta H_{vh} \]

The heat capacity of the system is also temperature dependant and this must be taken into account.

\[ \Delta C_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p \]

This may be integrated to yield

\[ \Delta H(T) = \Delta H(T_m) + C_p(T - T_m) \]

Note that the melt temperature \( (T_m) \) is defined as the temperature where exactly half of the protein is denatured.

It follows that the entropy change for this reaction is

\[ \Delta S(T) = \Delta S(T_m) + \Delta C_p \ln \left(\frac{T_m}{T}\right) \]

Once again yielding the protein stability curve.

A molecular partition function may be used to quantify the equilibrium position. This is defined as:

\[ Q = \frac{[N] + [U]}{[N]} = 1 + \frac{[U]}{[N]} = 1 + K \]

Where:

- \( Q \) = The molecular partition function
- \( K \) = Equilibrium constant
- \([N], [U]\) = The concentrations of the native and unfolded states respectively

It follows that a fractional population can be constructed for each species as
\[
\alpha_N = \frac{1}{Q} = \frac{1}{1 + K} \quad \alpha_U = \frac{K}{Q} = \frac{K}{1 + K}
\]

The fractional population can then be experimentally measured by quantifying the change in spectroscopic signal (S) as the reaction progresses.

\[S_{obs} = \alpha_N S_N + \alpha_U S_U\]

A spectroscopic signal gives different information to a DSC measurement of the unfolding process. As K (and \(\alpha_n\)) is a function of \(T_m\), \(\Delta H(T_m)\), \(\Delta C_p\) both of these methods can be used to provide this information.

### 2.1.3. Irreversible denaturation of proteins

A method for the analysis of irreversible denaturation occurring under a two state model was derived by Sanchez-Ruiz [40]. The model assumes that an irreversible first order transition occurs for which the denaturation temperature is scan rate dependant. These are conditions required under the two state model kinetic model of protein denaturation. It is also assumed that the rate constant [22, 41] of the denaturation follows the Arrhenius equation

\[k = A \exp\left(-\frac{E}{RT}\right)\]

as is expected.

The applicability of the two state model was tested through analysis of the activation energy for the denaturation. This was done by the mathematical extrapolation of the above conditions when applied to the denaturation rate constant, the heat capacity at various scan rates, the heat evolved as a function of temperature and the heat capacity at the denaturation temperature. If the two state model applies to the measured denaturation these plots will show good agreement to their models and the activation energy calculated will agree.

Firstly the rate constant of the denaturation, assumed to follow the Arrhenius equation, was calculated as a function of temperature:

\[k = \frac{vC_p}{Q - Q_t}\]

Where \(C_p\) and \(Q_t\) are a function of temperature.
The data is then plotted on an Arrhenius plot where the slope of the resulting line is equal to the activation energy.

Secondly the heat evolved at any set temperature follows

\[
\ln \left( \ln \left( \frac{Q_t}{Q_t - Q} \right) \right) = \frac{E}{R} \left( \frac{1}{T_m} - \frac{1}{T} \right)
\]

Thus the slope obtained from plotting the Left hand side of this equation vs. 1/T will be equal to E/R allowing for calculation of the activation energy.

Thirdly the denaturation temperature will be dependent on scan rate as follows

\[
\frac{v}{T_m^2} = \frac{A E}{R} e \left( \frac{-E}{RT_m} \right)
\]

Allowing for the calculation of activation energy from the slope of the plot of ln(v/Tm) vs. 1/Tm.

Finally the activation energy was calculated directly from the heat capacity at the denaturation temperature as follows

\[
E = \frac{eRT_m^2C_p^m}{Q_t}
\]

The initial paper, as well as subsequent publications[42, 43], shows that for any irreversible denaturation the two state model, as tested by the above conditions, correlates well with experimental data. This allows for the calculation of useful thermodynamic data from irreversible transitions. Prior to this approaches to calculating the energy associated with denaturation required the assumption of reversibility, as per Anfinsen’s experiment, to satisfy the reversible thermodynamic model. As no current study has found a hemocyanin denaturation to be reversible, this method will allow for the calculation of thermodynamic data from DSC experiments.
2.2. Protein Formulation

2.2.1. Stabilising and Destabilising Forces
The stability of a native protein is a function of the Gibbs free energy for the entire system in which the protein is solvated. Thus in order to increase the stability, we may either promote interactions which increase the strength of bonding within the protein, or increase the energy contribution of the solvent towards the native state[3]. A number of approaches have been considered to stabilise proteins, generally with the addition of excipients, covalent modification or lyophilisation techniques [44, 45]. However, experience has shown that no one approach is effective for all proteins, and a separate formulation must be considered for any new protein pharmaceutical[4, 46].

2.2.2. Effect of salt concentration on protein stability
Ionic salts have long been added to protein formulations in order to change their stability. Whilst the relative effect of each of the salts changes depending on the structure of the protein in question, the general order of stabilising effect remains the same. This was first observed by Hofmeister in 1888, and has been widely studied since.[47-50] Whilst the Hofmeister series lays a specific order of destabilising to stabilising ions, other contributing effects have been observed such as a specific interaction of multiply charged ions with the protein secondary structure [51, 52]. Further, some ions including Calcium and Magnesium have been seen to induce aggregation, particularly in unstable proteins. [53]

2.2.3. Effect of sugars on protein stability
The addition of sugars into a protein formulation has long been considered as a method for stabilising the protein with early studies indicating that the denaturation temperature of the protein may be increased by as much as 18.5°C [54], yet the exact mechanism by which the stability is increased is yet to be determined.

It was suggested by Arakawa [55] that all stability gains were due to the preferential hydration of the protein, however the earlier study by Back [54] suggests that the major contributor to the stability increase was through changing the free energy of the water in solution, rather than the energy of the protein itself. Benton[56], however, suggests that the stabilisation effect may have an enthalpic contribution, instead of a entropic crowding only effect. Likely, both of these energetic contributions are at play in the increased stability of the protein as they can both contribute to the free energy of the entire system.

As these theories all involve stabilising interactions, there are no reports of sugar additions destabilising protein formulations, however the relative effect of sugars have been reported to vary.
Furlan [57] reported that the stability increase for bovine plasma was correlated with the molecular weight of the sugar addition, with sucrose performing better than glucose. This would suggest that the excluded volume theory was dominant, as the larger sugar was excluded to a higher extent. Arakawa [55], however, found that the difference between the stabilising effect of Lactose and Glucose were minor, suggesting that this gave evidence that the relative interaction of the sugar with water, and not the excluded volume was the dominant force.

Whilst these theories all suggest that sugars should promote stability proportional to their concentration with no limit [58], this has not been observed. A decrease in the protective effect of the sugar was identified at above 10 % w/v by Furlan [57] and Arakawa [55] reporting a low dependence of sugar concentration on the stabilising effect. Back [54] had earlier suggested this was due to the sugar structures formed in solution. If structures such as Micelles were created by the sugars, then their relative effect on the free energy of the water would be minimised [59, 60].

These studies suggest that whilst sugar additions to a protein formulation will certainly have a stabilising effect, the effect of sugar identity, and concentration may not be so pronounced. Further if the stabilising effect of the sugar was dependant on sugar size, it would give evidence that the excluded volume effect was more pronounced than the free energy change of the water system due created by the sugar.

2.2.4. Protein aggregation forces
Proteins are often observed to aggregate whenever non-polar sections of protein meet, forming a more stable structure as an aggregate than an individual protein. This effect may be measured directly by circular dichroism [61] due to the induced changes in folding. Whilst this process may be either kinetically or thermodynamically controlled [62], the process is always irreversible, and hence no thermodynamic character can be directly measured.

Photon correlation spectroscopy is the most direct method for observing protein aggregation [63, 64], however the resolution of the technique limits its applicability to all but the largest proteins or aggregates. Protein aggregates are a problem for protein formulation due to the destructive and irreversible nature of their interaction. [65] Protein aggregation may be measured in a number of ways, including [63, 66]
2.3. Hemocyanin

Hemocyanin is the name given to a class of respiratory proteins which uses a dicopper centre to bind dioxygen and transport it through the body. These proteins are found in the blood of all Arthropods and Molluscs. Whilst the primary structure of all hemocyanins shows a high homology, the quaternary structure of hemocyanins varies between species and varies dramatically between Arthropods and Molluscs[15]. Hemocyanins were first discovered in 1900 [67, 68] however analytical research on the structure and properties of hemocyanins began in the 1980s [69].

2.3.1. Structure of Hemocyanins

Molluscan hemocyanins are characteristically composed of subunits of 340-400kDa which are further organised into a quaternary structure formed of decamers, or multiples thereof, of identical subunits[69, 70]. The individual subunits are single polypeptide chains containing 8 copper containing functional units, of which 8 different functional unit structures have been isolated. In the case of hemocyanin from the key hole limpet (the most studied species) this leads to an overall quaternary structure of 8MDa. Extensive articles and reviews exist defining molluscan hemocyanin structures [14, 15, 70-72]; however these structures will not be considered in detail here.

The hemocyanin of the Arthropod must also be considered as the quaternary structure of arthropoda hemocyanin is vastly different to that of molluscan hemocyanin, which may be manifest in the thermal properties of the protein[73]. Arthropoda hemocyanin, whilst containing similar functional and subunit conformations to molluscan hemocyanin, creates hexameric quaternary structures. Various species form multiples of this with the most common being the hexamer and the dodecamer.

2.3.2. Hemocyanin Stability

Previous studies have been conducted on the stability of hemocyanin using a variety of techniques, namely DSC on the hemocyanin of the Gastropod Rapana thomasiana [74] and Arthropod Pulinurus vulgaris [43]. Circular dichroism for a variety of arthropoda and mollusca [73], Fluorescence spectroscopy for a variety of arthropoda [75] and FTIR for a variety of species[76]. These articles have all found the denaturation of hemocyanins to be an irreversible process and have further analysed various features of the hemocyanin stability.
The earliest study on the thermal denaturation of hemocyanin [43] gives evidence that the denaturation of hexameric hemocyanin follows the two state kinetic model of protein denaturation implying that the unfolding of the protein at the denaturation temperature is cooperative and happens as a single step, as opposed to a denaturation in which several partially unfolded states may occur. Later research[74] confirms this finding using similar methods on dissimilar species. The implication of this is that the vant Hoff equation cannot be applied directly to calculate the protein stability curve for hemocyanins.

The two state model was tested following the procedure of Sanchez-Ruiz [40]. It was observed by Guzman-Casado and Idakieva that the denaturation data for hemocyanin closely fitted the above mathematical models for the two state model. Within each paper the calculated activation energies were comparable for Guzman-Casado [43] an $E_a = 383 \pm 30 \text{ kJ/mol}$ was recorded and for Idakieva [74] activation energies of $E_a = 597 \pm 20 \text{ kJ/mol}$. The far higher activation energies in the later paper are attributed to the use of molluscan hemocyanin compared with the arthropod hemocyanin considered earlier. This is however in contrast to the following studies which consider the separated subunits of both arthropod and molluscan hemocyanin finding no correlation of stability with Phylum.

It should also be noted that the uncertainty in the activation energy found in these papers is higher than expected based on the study which created this method ($E_a \pm 8 \text{ kJ mol}^{-1}$). One possible explanation for the lower certainty found in this paper was proposed to be a second denaturation peak existing near the end of the normal thermal denaturation. Such a peak was observed when heating the hemocyanin to its denaturation temperature, cooling it and heating through the denaturation. This method should allow the previously observed denaturation to occur on the first heating and any secondary denaturation to follow. Such a peak was observed 3°C higher than the normal denaturation and must be tested for in further thermal denaturation studies.

It has also been found that the two state model did not accurately describe the behaviour of hemocyanin monomers. A possible explanation for this was suggested to be differences in the denaturation parameters for the different subunits found in hemocyanin macrostructures[43].

This paper also compares the same calculations for samples of different hemocyanin concentration between 0.2-3 mg/mL. It was found that the denaturation temperature was not correlated with this concentration. This leads to the conclusion that the oligomerization state of the protein does not change during the denaturation. Le'Chateliers principle requires that changes in oligomerization state create a concentration dependence of denaturation temperature.
Fluorescence spectroscopy has also been utilised in the study of hemocyanin stability [75], however in this case no calculation of thermodynamic properties was conducted. The study instead focused on denaturation due to pH and chemical denaturants. It was discovered that the chemical denaturation of many hemocyanins followed a multistage denaturation and subsequent aggregation of the hemocyanin monomers, a further hypothesis that three chemical denaturation peaks were observed due to the existence of three distinct domains in the subunit structure. Whilst this is a logical conclusion, it is in contrast to thermal stability studies which show a two state denaturation [43, 74]. It is expected that both chemical and thermal denaturation will follow the same kinetics.

Circular dichroism was utilised to measure the denaturation temperature of a series of hemocyanins [73]. This study confirmed that the thermal denaturation of hemocyanin was irreversible as expected. A series of hemocyanins from both the mollusca and arthropoda phylum’s were then compared showing a range of melt temperatures from 65-92°C (covering the range found before [43, 74]), the melt temperature was not, however, correlated to phylum, suggesting that the quaternary organisation of the hemocyanin does not play a major role in the denaturation. It was also found that the denaturation temperature was dependant on oxygen binding with the oxy form having a denaturation temperature on average 11°C higher than the Apo form [73]. This implies that for reproducibility of results samples must be either homogeneously oxygenated or deoxygenated.

Finally one study has been performed measuring thermal stability with temperature controlled FTIR measurements [76]. This study focussed on the possible effects of glycosylation on the denaturation temperature. By studying the hemocyanin functional units separately they were also able to conclude that the a range of denaturation temperatures exist for the various functional units (as proposed earlier [43]). It was also discovered that the effect of oxygenation had a stabilising effect of up to 20°C supporting the measurements of [73]. The study, however, did not find any correlation between the glycosylation of the functional units and the denaturation temperature. This was unexpected as a correlation between glycosylation and stability had been previously established for other glycoproteins.

It can be concluded, therefore, that studies on the thermal denaturation of hemocyanins have found the denaturation to be irreversible and to follow the two state model. Various hemocyanins display a wide range of denaturation temperatures; however there appears to be no correlation between quaternary structure or glycosylation on the denaturation temperature.
2.3.3. Therapeutic Applications of hemocyanins

Hemocyanins are considered to be promising candidates for a number of different pharmaceutical applications including as antiviral agents, antimicrobial agents and as carriers for anticancer agents. These wide ranging applications of hemocyanins make them a prime candidate for therapeutic testing, with drug trials well established in some fields. As each of these applications are theorised to utilise hemocyanin by different mechanisms, they will be outlined separately.

One common method for creating cancer vaccines is to conjugate their sugar moieties onto a protein in order to induce an immune response to them[77]. Hemocyanin from the Keyhole Limpet (KLH) has frequently been used as a carrier protein for this purpose. KLH was found to elicit one of the strongest immune responses of the possible carriers tested, making it a viable conjugation option[78, 79].

Hemocyanins from three different species have been considered for their antiviral activity, Penaeus Monodon [9], Rapana Thomasiana [80] and Haliotis Laevigata [81]. Whilst Zhang[9] and Genova-Kalou[80] both provide evidence that whole hemocyanin protein has strong antiviral activity against white spot syndrome virus and herpes simplex 1 and 2 viruses respectively, Dang [81] instead suggests that it is the sugars and small peptides in the hemocyanin hemolymph sample instead which provide the antiviral activity they measured (towards herpes simplex 1 virus).

There is also some evidence that hemocyanins perform an antiviral function within their organism [82], possibly exhibiting enzymatic activity and conversion to phenoloxidase to fight infection [83]. This may explain the antiviral effects observed from hemocyanins in vivo.

Arancibia [84] has also suggested that the immunostimularory effects of hemocyanins were limited by a lack of stability, highlighting the importance of creating a stable hemocyanin for pharmacological testing. Whilst the current studies into hemocyanins therapeutic value paint an incomplete picture of the medicinal potential for hemocyanins, it is clear that hemocyanins possess some drug activity and as such are worth studying in greater depth.
Chapter 3: 
Physical Characterisation Of Hemocyanin
CHAPTER 3. Physical Characterisation of Hemocyanin

Hemocyanin from *Haliotis Rubra* was obtained as uncharacterised blood sera from *Haliotis Rubra* abalone. Whilst previous research and anecdotal evidence had identified some potential therapeutic activity of the sera, particularly towards the mouth borne herpes simplex two virus, no evidence was yet available on what components of the blood sera had this activity. It was also entirely unknown whether any active compounds could be separated from the sera whilst retaining their stable active states.

Previously published research on the blood of Molluscs suggested that the major protein component of the sera would be a hemocyanin, and whilst it was certain that the sera contained hemocyanin, the concentration of that hemocyanin could not be assumed. Further, any research into the properties and stability of the hemocyanin content would require an investigation into the physical properties of the protein as it is already established that these properties vary greatly depending on the species from which the hemocyanin was derived. *Haliotis Rubra* hemocyanin has not previously been investigated in the literature, and as such this will be the first work to assess it. In this chapter the core physical properties of *Haliotis Rubra* sera, and its contained hemocyanin are investigated.

As few contemporary protein samples have been obtained by purification from animal sources and as the hemocyanin protein was expected to have an extremely high molecular weight, we have investigated various purification and identification methods which are not commonly used in current molecular biology[85]. The large size (high molecular weight) of the presents many challenges, including low permeability of the protein into PAGE or IEF gels, diffusion properties more resembling suspended particles than dissolved proteins, rapid aggregation of large protein quaternary structures and the use of computer models which assume that all proteins have a lower molecular weight than hemocyanin.

This chapter will discuss the challenges associated with first purifying *Haliotis Rubra* hemocyanin from blood sera, how to measure purity when standard gels are not designed for proteins over 500 kDa, identifying the closest available homologous hemocyanins using mass spectrometry and how to coax methods usually used for suspended particles into the analysis of protein size and charge.
3.1. Hemocyanin Sera
Sera containing the blood extracted directly from live adult *Haliotis Rubra* abalone was delivered to our labs in 1 L bottles from Marine Biotechnology Australia in Hobart. The first challenge with this sera was identifying suitable sterilisation and purification methods for a protein of exceptional size. The deep blue colouring of the sera indicated that a large quantity of hemocyanin existed in solution, owing to the dicopper centres used for oxygen transport in hemocyanins.

The sterilisation and purification of small proteins is a relatively simple task, however the expected size of hemocyanin protein required careful planning, particularly to avoid filtering out or denaturing the protein during this task.

3.1.1. Purification methods
Hemocyanin sera samples were first sterilised through a 0.22 µm filter cartridge and separated into 3 mL aliquots for storage at 4°C. Individual samples of purified hemocyanin were then prepared by isolation over a 100 kDa Amicon filter tube.

The buffering solutions to be used for the hemocyanin samples were prepared, by dissolving the given concentration in MilliQ water, and chilled to 4°C. For initial testing 100mM TRIS buffer was applied. The 3 mL of hemocyanin sera was placed into the Amicon tube and centrifuged at 4000 rpm at 4°C for 20 minutes resulting in a retentate of ~0.5 mL expected to contain the hemocyanin protein. The 2.5mL of filtrate, expected to contain no hemocyanin, was either retained for analysis or discarded. The retentate was then diluted to the original 3 mL with cold buffer and centrifuged again. This was repeated five times to increase the purity of the retained proteins. The final product was expected to contain material between 0.22µm and approximately 100 kDa within the chosen buffer solution. This was then analysed for purity, protein concentration, final pH and visually inspected for large aggregates before storage at 4°C.

3.1.2. Protein concentration and purity
The protein concentration and the purity of any proteins within the sample were required for validation of any subsequent analysis. Protein concentration was calculated using the Qubit 2.0 Fluorometer system, from ThermoFisher Scientific, according to its specified instructions and reagents. This method had previously been validated against the Bradford Assay within our research group by Dr Yan Er.

Protein purity was determined by both native and denaturing gel electrophoresis methods in precast Invitrogen gels. For denaturing PAGE, a 3-8% Tris-Acetate gel was used as it was the largest porosity gel available. For native gels, the blue native Bis-Tris kit was applied.
3.2. Hemocyanin size
Hemocyanin, a protein family containing some of the largest proteins ever discovered, poses many difficulties for the application of standard protein analysis methods, including but not limited to electrophoresis, FTIR and Circular Dichroism methods, which rely on the diffusion, solubility and mobility of the protein and have been optimised for the analysis of much smaller proteins such as albumin. Thus, before utilising any standard methods for the analysis of hemocyanin, we must first validate their applicability to this protein.

We are presented, however, with an opportunity to apply techniques usually inaccessible to proteins, particularly those based on the diffraction of light through soluble samples, as the sheer size of the protein may give diffraction patterns more similar to suspended particles than those of mobile dissolved proteins. In particular the quaternary structure of a protein as large as *Haliotis Rubra* hemocyanin will become large enough to be detected by their scattering patterns.

This chapter discusses the methods we have discovered for the investigation of the size of hemocyanin and the related challenges posed to conventional technologies.
3.2.1. Gel electrophoresis

Conventional methodologies require the application of gel electrophoresis to determine the size and purity of any protein solution. This includes the use of standardised protein solutions to calibrate the distance of travel to the size of the protein, however as no commercial standards include a protein as large as hemocyanin, the data had to be interpolated to the size of the protein.

![Gel electrophoresis of Haliotis Rubra hemocyanin showing the protein stuck in the stacking section. Lanes 1-5 and 6-11 all contain 20µg purified hemocyanin, run simultaneously to test resolution, L is a broad range ladder (6.5-200kDa)](image)

**Figure 3-1** Gel electrophoresis of Haliotis Rubra hemocyanin showing the protein stuck in the stacking section. Lanes 1-5 and 6-11 all contain 20µg purified hemocyanin, run simultaneously to test resolution, L is a broad range ladder (6.5-200kDa)

**Figure 3-1** shows a standard BIS-TRIS SDS PAGE gel, of the largest available porosity, containing hemocyanin and a BioRad broad range molecular weight ladder (6.5-200kDa). The bunching of the hemocyanin at the top of the gel indicates that the protein was too large to enter the pores of the gel and has instead stuck into the stacking section of the gel. Other gel systems, such as the Blue Native system, utilising larger porosity gels and different staining systems were then investigated.
Figure 3-2 BlueNative Gel showing Hemocyanin separated into two distinct protein bands. Lanes 1-3 are 20µg pure hemocyanin, L is a NativeMark Ladder, Lanes 5-6 are hemocyanin sera, Lane 7 is the purification filtrate and lane 8 is a sera sample stored for 3 months.

The Invitrogen BlueNative system gel in Figure 3-2 allowed for a penetration of hemocyanin into the gel and a separation of two distinct bands from the protein sample. Both of these are of larger molecular weight than the NativeMark protein ladder, with a largest band of 1236 kDa, indicating that these bands must both be multimers of hemocyanin, potentially the decamer and didecamer.

Figure 3-3 BlueNative gel showing overloaded hemocyanin to check purity. Lanes 1, 4, are purified hemocyanin at 200µg, 100µg respectively, showing minor bands at lower molecular weight.
Figure 3-3 shows a gel then run with a heavily overloaded sample of hemocyanin in order to determine if impurities exist in the sample. This leads to a primary band which is thick and has poor resolution, however if any impurities exist in low concentration they will be magnified by the increased sample size. Whilst it is not possible to discern impurity bands in the normally loaded sample, the overloaded sample shows many distinct bands, indicating some impurities. Of more interest, however, are two strong bands which form on either side of the 720 kDa ladder band, as these may be the individual hemocyanin subunits.

Gel electrophoresis, whilst not able to determine any quantitative data about the hemocyanin, owing to its large molecular weight, has shown us that the filtered hemocyanin product predominantly contains an extremely large protein, almost certainly a hemocyanin, which may be separable into smaller units. The purity of this sample was found to be high, considering its source as a blood sample. We now have a starting point with which to study the hemocyanin directly.

3.2.2. Photon Correlation Spectroscopy
In order to assess what conformational structures exist within a formulated hemocyanin sample we wished to analyse the size of the suspended protein directly. If the sample was large enough to investigate by optical methods, as we anticipate, then this would allow a direct, simple and powerful way to directly assess whether the hemocyanin in a sample existed as subunits, larger quaternary structures or as aggregates in the formulation we prepared.

Photon correlation spectroscopy was utilised, through a Malvern Zetasizer 2000 to measure the physical size of suspended molecules within Haliotis Rubra hemocyanin sera. As the complete hemocyanin itself was expected to have a diameter above 10 nm, it was expected that the protein would be observable by PCS.

Immediately prior to testing, hemocyanin sera, of previously determined concentration (tested by the Q-Bit method), was diluted into water in concentrations between 0.01–1 mg mL⁻¹ and placed in PCS cuvettes. As a measure of true sample diameter, the Z-average measure was used as per manufacturers instruction as it corrects for over representation of large particles. The diameter of particles was tested with samples prepared in triplicate, and the composition of each sample also measured in triplicate to allow for identification of erroneous results (often observed due to dust contamination).

Whilst samples of each concentration within the above range gave similar results, samples of 0.1 mg mL⁻¹ showed the lowest polydispersity and highest repeatability of the sample. As it is standard
practice to tune the output of the zetasizer to the best performing concentration, all further samples were prepared at 0.1mg mL\(^{-1}\).

**Figure 3-4** Diameter of particles observed in *Haliotis Rubra* hemocyanin sera. The principal peak at 44nm is assigned to the hemocyanin quaternary structure

Samples of sera filtered into MilliQ water (pH7.0), Figure 3-4, show that a Z-average diameter of 44 nm. This is similar to the expected size of hemocyanins from other abalone species, and as such it is assigned to the whole quaternary structure of the hemocyanin. Experiments were performed using the default Malvern settings, with distribution calculated using the Z-average calculation. This size was confirmed for samples of hemocyanin filtered into water and various buffers and concentrations with a Z-average diameter of 43 ± 3 nm.

Samples were then retested after sitting at room temperature and at 85°C for 10 min to determine what aggregation states existed.
It was found that after only 10 min in pure water, hemocyanin had begun to aggregate noticeably, with a prominent peak forming at approximately 1000 nm. After treatment at 85°C aggregates at 500 nm and over 2000 nm were observed. Determining the accurate size of the aggregates proved difficult as repeat testing yielded constantly changing values. This was expected due to the continuing aggregation process and hence constantly changing particle size. Further, due to the large mass and low count of large aggregates, they are poorly measured by dynamic light scattering. Whilst this makes determining exact aggregate size impossible, the identification of aggregation behaviour was none the less determined.

It was found therefore, that testing of hemocyanin particle size via the zetasizer was a reliable method for determining if a hemocyanin sample had aggregated, or if it was in its native state. This method, whilst very effective, is not commonly used to study protein conformations as it can only pick up proteins above 10 nm in diameter. Thus dynamic light scattering methods have allowed an adapted method for studying hemocyanin usually reserved for suspended particles.

This PCS method for monitoring hemocyanin aggregation is a rapid and effective way to study the size of aggregates within the sample. Whilst it is not widely applicable to protein aggregation studies, due to the required minimum particle size, we have found it to be a viable method for proteins as large as hemocyanin.

Figure 3-5 Diameter of particles in hemocyanin sample after 10 min at room temperature or 10 min at 85°C
3.3. Hemocyanin Subunits

Whilst hemocyanin naturally forms into large didecameric quaternary structures, these structures are composed of individual single chain subunits which themselves have folded structures. Revealing the properties of these individual subunits would yield insights into the degree of stability afforded by the tertiary and quaternary structures as well as allowing us to probe the stability and potential therapeutic effects of individual subunits as well as the whole hemocyanin.

Whilst many possible candidate methods may be able to separate hemocyanin into its subunits, most of these methods, including chemical denaturants, temperature and enzymatic lysis would be destructive of the folded structure of the hemocyanin. One candidate method, however, presents as potentially non-destructive, treatment in alkaline pH.

To test if alkaline pHs could separate hemocyanin subunits, samples of hemocyanin were dialysed into glycine buffers with pHs ranging from 8-10. The first and fourth and seventh bands of Figure 3- show hemocyanin in pHs of 9 10 and 11 respectively, with the two bands following each high pH sample being the initial sample and the protein after being returned to neutral pH.

![Figure 3-6](image)

**Figure 3-6** Blue Native gel comparing purified hemocyanin in neutral buffer, with high pH separated hemocyanin subunits. Bands 1, 4, 7 show hemocyanin at pHs of 9, 10 and 11 respectively, with the two bands following each high pH sample being the initial sample at neutral pH and the protein after being returned to neutral pH.
As it can be seen the high pH samples have dissociated from their large molecular weight band. The marker in lane five shows a band of 720kDa between the two separated subunit bands, showing that the molecular weights of the two subunits must be on either side of this size.

In order to verify the size of the proteins in the high pH samples, we tested them with the zetasizer procedure first used to determine whole hemocyanin diameter.

Table 3-1 Comparison of measured diameters and volumes of whole and subunit hemocyanin

<table>
<thead>
<tr>
<th></th>
<th>Whole</th>
<th>Subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-average diameter (nm)</td>
<td>43</td>
<td>20</td>
</tr>
<tr>
<td>Volume (nm$^3$)</td>
<td>41600</td>
<td>4200</td>
</tr>
<tr>
<td>Ratio of volumes</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>

As the ratio of volumes observed for the hemocyanin and its subunits are almost perfectly 10, there is a high probability that we have observed whole hemocyanin separating into dimers in the formulated sample.
3.4. Homology of Haliotis Rubra hemocyanin to hemocyanin of other species

Blood sera from the black lipped abalone (*Haliotis Rubra*) has been studied via mass spectrometry to determine the composition of the proteins it contains. Electrophoresis was first performed on the sera to isolate the proteins from the sample. The high molecular weight bands were then treated via a tryptic digest and tested via mass spectrometry to compare their structures to published genome sequences.

It was found that each of the bands tested were composed of hemocyanin or hemocyanin fragments. Whilst it was expected that the largest proteins in the sera must be hemocyanins, this data has provided the first confirmation of this hypothesis. The data also suggests that the other bands separated by electrophoresis are the result of hemocyanin degradation as opposed to sample impurities. Hemocyanin from the European green ormer abalone (*Haliotis Tuberculata*) has been identified as the most homologous protein to those measured, with further similarity to other abalone species found. Only a low homology to that of the more widely studied keyhole limpet was identified.

Whilst it is simple to collect indirect evidence that we are working with hemocyanin, such as an extremely large size, copper content and resulting blue appearance, proving protein identity requires identification of the primary structure. This is traditionally performed either by a direct Edman sequencing of the amino acid residues or matching of a protein to a part of the complete genome for the species. As the genetic sequences for a number of molluscs have been published, we have chosen to look for genetic matches for this protein through the use of mass spectrometry.

Whilst the genome sequence for *Haliotis Rubra* has not been studied, the sequence for coding hemocyanin in a number of other abalone species has been recorded. As the shape and size of the protein are similar to those for the hemocyanin from other mollusca, it is assumed that a highly conserved sequence exists and the homology between the proteins will be high. This will be tested by the level of overlap measured for our hemocyanin and that from these other species.

A series of sera samples from the abalone were prepared, including a purified hemocyanin sample, hemocyanin subunits, prepared in a pH 8.5 glycine buffer and raw hemocyanin sera samples. These samples were then purified by blue native gel electrophoresis to yield distinct pure bands. After fixing of the gel, the intact protein was immobilised in the gel and the individual bands were cut. Each band sample was then further purified to remove any unwanted salts or impurities. The bands were then individually treated with trypsin to fragment the protein. The resulting small peptides were released from the gel due to their low molecular weight. The individual peptide mixtures from each band were then taken to mass spectrometry. An LCMS used to separate and identify the individual peptides...
within each sample. These were then charged and accelerated within an electric field. The time of flight was measured to determine an accurate mass of each observed peptide.

For each band, dozens of small peptide fragments were observed. As these fragments often overlapped, possible larger sequences could be derived. The protein database “MASCOT” was used to identify the protein primary structures which could yield the observed peptides. Each peptide mass was compared to the database individually. Many of these peptides would be common to a series of proteins, however some will be unique. The potential protein matches which could yield a significant number of the observed peptides are then tabulated and presented to the user as an identification tool.

Further to this, the database “BLAST” was then used to determine homology between proteins. In this case it is used to determine the level of homology between each of the hemocyanins which have previously been published.

Gel electrophoresis was conducted on a series of hemolymph samples. This included samples which were purified and unpurified as well as samples which had been treated at pH 2 and pH 10.5 before purification. As can be seen in Figure 3-7 below, all bar the base treated samples (lanes 5 + 6) are extremely similar.

The base treated samples, however, show a series of new bands and a disappearance of the extremely high molecular weight bands. Similar observations have been made for other hemocyanins, in which base treatment leads to a separation of the quaternary structure into subunits. Two distinct bands form at approximately 400 kDa and it is hypothesised that these may belong to two different isoforms of the hemocyanin subunit, as is the case for some hemocyanins.
From the gel electrophoresis a total of nineteen individual bands were chosen to be cut and treated, the bands which were then taken to mass spectrometry are labelled. It was expected that the larger molecular weight bands may be hemocyanins and the lower molecular weight bands may be impurities or fragments. The chosen bands were chosen to provide a wide variety of molecular weight bands.

The following table illustrates that a MASCOT search identified a hemocyanin as the most likely parent for the measured peptides in every sample. The first isoform of hemocyanin from *Haliotis Tuberculata* [86, 87] was measured as the most homologous protein to the sample in every case, where results are ranked on their matching score. A score above 100 is considered a positive result and due to the exceptional size of hemocyanins, very high scores could be attained. It is also useful to look at the number of protein sequences which were directly matched which in most cases is over twenty distinct peptides. The number in brackets indicates how many of these sequences are unique to the protein. The total coverage, also documented for comprehensiveness, is less significant as a high coverage would never be achieved for such a large protein and in particular one where the species is not directly matched from the database.

**Figure 3-7** Gel used for the LCMS of *Haliotis Rubra* hemocyanin. Individual bands, as labelled, were cut and treated for LCMS.
It is notable that whilst a strong homology is found to various species of abalone, a much weaker homology is identified for other molluscan hemocyanins such as that of the keyhole limpet. It was also found that for the highly homologous species, the *Haliotis Tuberculata* and the *Haliotis Diversicolor*, two different isoforms were documented. Whilst most of the measured bands were positive for isoform one band nine (corresponding to a lower molecular weight band of base treated sample) was much stronger in its identification of isoform two. Whilst isoform one was still present in this sample, it is highly likely that the lower molecular weight bands have been concentrated in a second isoform of the hemocyanin.

As we are searching for hemocyanin matches to a similar but not identical species to *Haliotis Rubra* it is expected that some amino acid residues will have been modified through the evolutionary processes since the divergence of the two species. MASCOT is equipped to search for such modifications by specifying only one protein match to the sample and researching for specific masses associated with possible modifications. This method has allowed us to propose a series of potential amino acid substitutions based on observed residue masses which would match a divergent search.

In one case, utilising divergent search results suggested 27 single amino acid substitutions, increasing the score and coverage by more than double. Similar results were indicated for other bands and these results indicate a high probability that substitutions have occurred. A mapping of the *Haliotis Rubra* genome would be necessary in order to confirm this hypothesis.
In order to support the hypothesis that amino acid substitutions were possible a BLAST search was performed on the most homologous hemocyanin, *Haliotis Tuberculata*. In this case the known sequence for *Haliotis Tuberculata* hemocyanin was compared only with other published structures. This revealed that over 60% of the protein sequence was conserved within the abalone family. Sections of the structure were also observed for other hemocyanins; however the similarity was much lower. This explains why the overlap between our hemocyanin and other abalone hemocyanin is high, whilst the overlap with other mollusca is low.

This experiment has given the first hard evidence that we are indeed studying a hemocyanin and that the primary structure of it is highly similar to that of *Haliotis Tuberculata*. The increased homology between our sample and other abalone, and the low homology to other families indicates that the properties and structure of the protein may be conserved within the abalone family; however the properties may be vastly different to those observed for other species such as the keyhole limpet.
3.5. Hemocyanin Charge

The charge on the protein determines its aggregation behaviour, and is based primarily on the charge of the amino acid residues which make up the protein. This can be estimated from the primary structure or measured directly. Proteins will readily form aggregates when in a solution with a pH near their isoelectric point (pI) as the net charge on their surface will be close to zero and hence there will be no charge repulsion. At pHs away from the proteins pI the protein molecules will all have like charge, and hence will repel, reducing the aggregation driving forces.

Standard protein protocols call for measurement of the isoelectric point using an isoelectric focussing gel (IEF) often accompanied by a theoretical determination of the pI calculated from the sequence of the protein. However for Haliotis Rubra hemocyanin these methods will prove problematic as the size of the protein may inhibit migration of the protein within the IEF gel, as has occurred for PAGE gels, and the sequence of the protein is unknown. We therefore have considered techniques usually used for larger charged particles in order to determine the net charge on the hemocyanin and hence approximating its isoelectric point. The two most promising options were an auto titration of the protein and the measurement of its zeta potential in solutions of changing pH.

3.5.1. Isoelectric Focussing

The charge on small proteins is generally measured using an isoelectric focussing gel electrophoresis technique in which a gel is cast in which has a pH gradient. The protein will then migrate through the gel to the point where the pH of the gel is equal to the pI of the protein.

IEF gels were run on the whole protein, denatured protein and pH separated subunits of the protein to investigate if it were possible to get hemocyanin to travel through any IEF gels. Figure 3-8 shows the gel images obtained from IEF gels of the hemocyanin in each of its states.

![Image](image.png)

**Figure 3-8** IEF gels from Haliotis Rubra hemocyanin, showing no separation of hemocyanin through the gel

Whilst IEF gels have been specifically cast for large proteins such as hemocyanin [88] it can be seen in Figure 3-8, unfortunately, that no states of the protein could enter the IEF gels and as such a clean pI
for the protein could not be found from this method. Whilst this means we had to rely on less commonly used titration and zeta potential methods for the determination of hemocyanin isoelectric point.

### 3.5.2. Titration Methods

An alternative method for establishing the pI of the protein is to titrate the protein solution across its possible pI range to identify titration end points which would correspond to protein pIs. Extremely sensitive titration equipment has been developed for such titrations including the Metrohm Titrando, which was used for this purpose.

![Titration of Haliotis Rubra hemocyanin with HCl from pH 7 to pH 2](image)

**Figure 3-9** Titration of *Haliotis Rubra* hemocyanin with HCl from pH 7 to pH 2

Whilst the raw data shows no obvious titration end points, the first derivative of the titration curve can be used to determine if any hidden points of inflexion are present. In this case we see two prominent inflexion points, at pH 3.05 and pH 5.97.
The two separate inflexion points in this titration indicate that there were two different pHs at which the protein could be ionised. Either may be the pI for the protein; however both may be problematic for the stability of the hemocyanin.

### 3.5.3. Zeta Potential Methods

If a protein, or any other suspended material in a solution, is charged it will migrate when placed within an electric field and by measuring the rate of this migration the net charge can be calculated. For proteins, the surface charge will depend on the pH of the containing solution, such that when the pH of the solution is equal to the pI of the protein there will be no charge on the protein. A Malvern Zetasizer was therefore used to measure this effect, as was analysed by the zeta potential measured by the machine. The pI point (at which no surface charge exists) is therefore equal to the solution pH where there is a zeta potential of zero.

Hemocyanin solutions were created in dialysed buffers at pHs from 3-7 at the optimal concentration of 1mg/mL and their zeta potential was measured in triplicate. Whilst the data required that multiple buffers be used to cover the entire pH range, the overlaps of these different buffer systems showed that their measured zeta potentials were comparable.
Figure 3-11 Zeta potential of Haliotis Rubra hemocyanin as a function of pH

Figure 3-11 shows the aggregated zeta potential data for Haliotis Rubra hemocyanin from pH 3-7 over which the isoelectric point of the protein was crossed. The highly positive zeta potential of the hemocyanin in acidic conditions indicating a positive charge on the protein. Correspondingly a negative charge was observed at pHs close to neutral. The pI of the protein was determined to be between 4.8 and 5.0. Other hemocyanins previously been reported to have a pI range (4.4-5.5 [89]) which is consistent with this data.

This is consistent with titration data suggesting possible pI points at pH 3.05 and 5.97. Considering the much more extensive literature supporting the use of zeta potential to measure protein isoelectric points, the zetasizer data is preferred over the Metrohm titration data. This suggests a Haliotis Rubra hemocyanin isoelectric point of 4.9.

We have determined the Haliotis Rubra isoelectric point as 4.9. This is the pH at which there will be no net charge on the protein. At this pH the protein molecules will not electrostatically repel each other and therefore protein aggregation will be rapid. As there is also some evidence, from the Metrohm data, that other titration points of no charge exist at 3.05 and 5.97, these pHs may also result in increased aggregation and as such any acidic conditions with a pH 6 or below should be avoided when formulating this protein.
3.6. Conclusions

Chapter three has investigated the physical properties of *Haliotis Rubra* hemocyanin. Whist these properties are simple to derive for most proteins, the extreme size of hemocyanins has made the measurement of any physical properties challenging. However, in order to further study the stability of this complex protein, we must have an accurate understanding of its size, composition and charge.

When the protein was purified from a blood sample, over a 100 kDa filter, the remaining sera was found to contain almost exclusively hemocyanin. By use of electrophoresis and dynamic light scattering we identified that this protein forms a single folded structure of 43 nm in diameter. By comparing the primary structure in LCMS we then determined that the composition of the protein was most similar to that of *Haliotis Tuberculata*, indicating that a subunit size of 370 kDa and a quaternary structure of 7.39 MDa. The hemocyanin was also found to have an isoelectric point of 4.69.

The key characteristics of Haliotis Rubra hemocyanin, as determined in Chapter 3, are summarised in Table 3-3 below.

<table>
<thead>
<tr>
<th>Table 3-3 Key physical characteristics of Haliotis Rubra hemocyanin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diameter of native protein</strong></td>
</tr>
<tr>
<td><strong>Molecular Weight</strong></td>
</tr>
<tr>
<td><strong>Isoelectric Point</strong></td>
</tr>
<tr>
<td><strong>Diameter of Subunit</strong></td>
</tr>
</tbody>
</table>

In this chapter we have investigated properties and techniques which are rarely applied to modern protein science. This was necessitated by the sheer size of the hemocyanin and also the source, as it is rare in modern protein science to purify proteins from blood sera (with recombinant methods now preferred). Whilst this is less convenient than expressing the protein recombinantly, it does allow us to investigate a protein sample which may not be possible to obtain any other way. The size of this protein, however, has allowed us to use photon correlation spectroscopy methods to assess aggregation which are not generally available to protein science and these may be widely applicable to the study of extremely large proteins.
Chapter 4: 
Characterisation of 
Hemocyanin Stability
CHAPTER 4. Characterisation of hemocyanin stability

In order to create a formulation of *Haliotis Rubra* hemocyanin with improved stability, we must first define how the stability of our hemocyanin formulation will be assessed and then create a baseline stability of the protein with which to compare the formulated samples to. As the application of the hemocyanin will be as a liquid pharmaceutical product, we have defined the stability against those required for the pharmaceutical. Namely, that it must remain in its active form during storage at room or fridge temperature for a period of up to two years.

Whilst other researchers in our group will be able to assess the efficacy of the protein as a pharmaceutical, directly relating these results to the stability of the formulation may introduce unexpected variables into the measurement. It is worthwhile, therefore, to first measure the stability of the protein against more conventional biochemical measures, particularly the retention of its normally folded, non-aggregated state.

Measuring the size of a hemocyanin molecule, as well as any aggregated protein, was examined in Chapter Three utilising dynamic light scattering methods. However, in order to determine under what conditions aggregation occurs we will have to extend this method to measuring the onset and progress of the aggregation process.

Assessing the folded state of the protein, however, is possible by a number of techniques including differential scanning calorimetry (DSC), circular dichroism (CD) and Fourier Transform Infrared Spectroscopy (FTIR). Of these, we have focussed on utilising DSC due to its extensive use in the measurement of protein folding. Further, whilst we have made many attempts to apply CD and FTIR to create comparative results during our study, the rapid aggregation of the hemocyanin during the experimental procedure has overshadowed any data we may have obtained from these techniques. The thermal stability of hemocyanins from some other organisms has already been studied with DSC, providing an approximate indication of the expected thermal stability of *Haliotis Rubra* hemocyanin. Crucially these hemocyanins were all found to denature irreversibly, which would indicate that not only the unfolding temperature, but also the rate of that unfolding reaction were measures of its stability.

In this Chapter we will define and assess the stability of unformulated hemocyanin and establish methods for the investigation of hemocyanin stability which may be applied to other formulations and potentially other large proteins for which measuring stability may also be challenging.
4.1. Evaluation of DSC equipment for the study of hemocyanin denaturation

Selection of an appropriate DSC is an essential step in obtaining accurate and reliable experimental results, particularly with a protein prone to aggregation. Many potential DSC machines are available options for measuring calorimetry traces and three (the TA Q2000, Setaram µDSC3 and the TA nanoDSC) were tested for use with hemocyanin. *As Haliotis Rubra* hemocyanin is anticipated to undergo a complex denaturation high resolution may be needed to isolate individual transitions. Further, as the denaturation is likely accompanied by aggregation, we must be able to distinguish the energy due to aggregation from that due to denaturation.

These three DSC machines cover the range of equipment available in Australia. A trace of the typical data from each machine is included as well as some key data from it. Figure 4-1 shows the trace from a TA Q2000 series coin cell DSC. A constant ramp rate of 2°C was applied to the liquid hemocyanin sample in the heating direction only, delivering the trace in Figure 4-1. These machines are among the most common DSC instruments available and are most commonly used to investigate synthetic polymers.

![Figure 4-1 Haliotis Rubra hemocyanin DSC trace in TA Q2000](image)

This equipment is based at the University of Sydney and is primarily designed for testing solid materials packed into disposable cells. Special high pressure hermetic pans were used to test a 70% by mass sample of hemocyanin in water, the lowest concentration detectable in the trace. As this setup is not
designed for aqueous samples resolution is poor and one large peak is observed from 65-85°C covering multiple possible transitions.

A 2010 uDSC3 evo machine from Setaram instruments was then tested. This machine is based at LaTrobe University and utilises removable 1 mL hastelloy pressure cells for sample holding. Sample concentrations from 1-10 mg/mL were used to identify the sharpest signal. It can be seen in Figure 4-2, that three transitions are being recorded, two fine peaks and one broad trough. In this case the peak position of the 71.3°C peak was found to be heavily scan rate dependant, and the peak at 76.5°C corresponds best to peaks observed in other apparatus. Recent research[53, 65] has suggested that using various adjuvants may reduce aggregation effects and hence may reduce the broad trough and change the temperature dependence of the small peak. The sharpness of the peaks obtained, however, shows the high resolution of the machine.

![DSC trace for hemocyanin in Setaram uDSC3 evo](image)

**Figure 4-2** DSC trace for hemocyanin in Setaram uDSC3 evo
Finally the TA nanoDSC has been tested extensively. This is a relatively new machine (it was produced in 2012) with inbuilt capillary cells designed specifically to reduce aggregation effects in denaturing proteins. This machine is based at the University of New South Wales.

![Figure 4-3 DSC trace for hemocyanin in the TA nanoDSC](image)

It can be seen from these three spectra that whilst the earlier Qseries does not yield suitable data for protein analysis, both the uDSC3 and the nanoDSC yield good calorimetric traces, containing obvious peaks which are easily deconvoluted. Whilst the resolution on the Setaram µDSC 3 was the sharpest, the ability of the nanoDSC to avoid aggregation during the denaturation reaction may allow more viable results. The core data from these is summarised in Table 4.

**Table 4-1 Comparison of resolution for three common DSC machines.**

<table>
<thead>
<tr>
<th>Equipment used</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Width of peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA Qseries 2000</td>
<td>73°C</td>
<td>-</td>
<td>11°C</td>
</tr>
<tr>
<td>Setaram uDSC3 evo</td>
<td>76.5°C</td>
<td>71.3°C</td>
<td>5°C</td>
</tr>
<tr>
<td>TA nDSC</td>
<td>75.8°C</td>
<td>81.2°C</td>
<td>7°C</td>
</tr>
</tbody>
</table>

As the TA Q-series DSC provided poor resolution and the SETARAM µDSC showed protein aggregation during denaturation, the TA nanoDSC was found to be the most suitable DSC for measuring the denaturation of hemocyanin and will therefore be used for future investigations.
4.2. Analysis of irreversible denaturation

With the TA nanoDSC we then attempted to optimise the conditions, testing procedure and data analysis for *Haliotis Rubra* hemocyanin. As this was one of the first experiments done with the machine, and the first utilising large proteins, this experiment served as a validation of the equipment as well as the methods.

As analysis of denaturation data depends on the reversibility and order of the denaturation reaction, we first had to establish the irreversibility and concentration dependence of the transition in order to choose a suitable model.

4.2.1. Irreversibility of thermal denaturation

The thermal denaturation of this hemocyanin has been determined to be irreversible due to a lack of endotherm upon rescanning of the denatured protein (as shown in Figure 4-4). Samples were heated from 40°C to 95°C at 1 K min⁻¹ before cooling and reheating.

![Figure 4-4](image)

**Figure 4-4 (A)** DSC Trace of native hemocyanin in TRIS buffer pH 7.6 at 1 K min⁻¹; **(B)** Rescan of denatured hemocyanin showing no endotherm upon reheating

In no case was an endotherm observed after heating the sample past its denaturation transition. To investigate if secondary processes such as aggregation were occurring during the onset of denaturation, samples were then heated to the denaturation onset before cooling and rescanning through the transition.
Two distinct denaturation peaks were observed for the unfolding of this hemocyanin. A previous study noted the possible existence of a second shoulder on the hemocyanin denaturation endotherm [74] indicating a complex unfolding without complete deconvolution. The improved separation of these peaks in this study may be attributed to the higher resolution of the TA nanoDSC instrument we used. The separation of these peaks (Figure 4-4) indicates that the two denaturations are distinct and the removal of only the first peak in the onset scan (Figure 4-5) confirms that their progression is independent of one another.

Deconvolution techniques may be used to model the separate peaks determine the activation energy of the second peak [74], however this would require inferring an unknown slope to the onset of the partially covered second peak. As the Sanchez Ruiz model is highly sensitive to the slope of the denaturation onset [40], the deconvoluted peak was considered too unreliable to be evaluated by this method.

![Graph](image)

**Figure 4-5 (A)** DSC scan ramping to 75°C to measure onset of denaturation. (B) Rescan of hemocyanin sample after onset scan

When the hemocyanin samples were heated to the onset of denaturation, then cooled and rescanned, a smaller denaturation peak was observed indicating that a partial unfolding had occurred (Figure 4-5). The presence of an endotherm on the rescan indicates that the denaturation did not then proceed
through aggregation. It was therefore concluded that the denaturation is entirely irreversible and this is not the result of destructive high temperature processes.

In order to determine if the transition was kinetically controlled, the dependence of Tm on scan rate was established, as shown in Figure 4-6.

![DSC traces for hemocyanin at scan rates of 0.2, 0.6, 1, 1.4 and 2 K min⁻¹ indicating that Tm is scan rate dependent](image)

**Figure 4-6** DSC traces for hemocyanin at scan rates of 0.2, 0.6, 1, 1.4 and 2 K min⁻¹ indicating that Tm is scan rate dependent

The denaturation temperature varied from 75.5-80.5 °C, increasing with increase in scan rate. This was expected as the kinetic theory of irreversible denaturation requires a dependence of peak position on scan rate. This happens due to the heating rate and denaturation rate being of a similar scale. The transition is therefore under kinetic control.
Irreversibility of hemocyanin denaturation has been observed for other species such as the Gastropod *Rapana Thomasiana* [74] and the Arthropod *Pulinurus Vulgaris* [43]. Reversible denaturation has not been observed for any hemocyanin to date. The presence of visible aggregates in denatured samples suggests that aggregation effects occur simultaneously to the denaturation. As this reaction leads to irreversibly unfolded proteins, it is expected that the exposed internal residues will be drawn together to lower unfavourable interactions and leading to aggregation.
4.2.2. Concentration dependence

As hemocyanin forms an oligomeric structure, separation of the individual subunits may occur during the denaturation process. In order to determine if this is occurring, DSC traces were collected at varying protein concentrations (Figure 4) from 0.19 - 0.64 mg mL\(^{-1}\) at a constant scan rate of 1 K min\(^{-1}\).

The initial denaturation peak for each of these samples was measured to be 79.0 °C, indicating that the denaturation temperature is independent of concentration over the range of 0.19-0.64 mg/mL. This leads to the conclusion that the rate determining step of the transition does not involve a change of oligomeric state. Whilst a change in oligomeric state may occur in concentrations outside this range, this data shows that a change of oligomeric state is not inherent to the denaturation reaction. Changes in oligomeric state by definition result in a change in concentration, and if this was to occur, Le Chateliers Principle would require a resultant reaction, altering the kinetics of the reaction. As this was not observed, no change in oligomeric state could have occurred. It should be noted, however, that this does not preclude changes in oligomeric state following the rate determining denaturation.

Figure 4-7 DSC traces of hemocyanin at 0.19, 0.39, 0.64 mg mL\(^{-1}\)

The initial denaturation peak for each of these samples was measured to be 79.0 °C, indicating that the denaturation temperature is independent of concentration over the range of 0.19-0.64 mg/mL. This leads to the conclusion that the rate determining step of the transition does not involve a change of oligomeric state. Whilst a change in oligomeric state may occur in concentrations outside this range, this data shows that a change of oligomeric state is not inherent to the denaturation reaction. Changes in oligomeric state by definition result in a change in concentration, and if this was to occur, Le Chateliers Principle would require a resultant reaction, altering the kinetics of the reaction. As this was not observed, no change in oligomeric state could have occurred. It should be noted, however, that this does not preclude changes in oligomeric state following the rate determining denaturation.
4.2.3. Activation Energy parameters

The Sanchez-Ruiz method [40] allows for the calculation of the activation energy for a particular transition assuming that it follows the two state mechanism

\[ N^k_D U \]

where the unfolding occurs at a reaction rate (kD) and follows the Arrhenius relation:

\[ k_D = A \exp(-E_a/RT) \]

To demonstrate whether the reaction follows the above mechanism, four different simplifications of the Arrhenius equation are then derived based on the four different assumptions below which must each be true, and will yield the same activation energy (E_a), if the reaction is irreversible and under kinetic control. If each of these relations hold, then as shown by Sanchez-Ruiz [12], the mechanism determined is an accurate one for the denaturation process and the activation energy of the transition is given by the average value obtained from the four methods.

Whilst the peak position of an irreversible denaturation may change with approach rate, the activation energy for a particular transition is theoretically consistent and the Arrhenius plots are linear. If the transitions were reversible, this would not hold true.

**Method A**

Method A is based on the premise that the rate of heat evolution is proportional to the activation energy[40]. This rate may be calculated as follows:

\[ k_D = \nu C_p/(Q_t - Q) \]

Where the rate constant for the denaturation reaction k_D is dependent on the scan rate (\( \nu \)) the total heat of the transition (Q_t) and the heat capacity (C_p) and heat evolved up to a particular temperature. If the transition follows the behaviour of an irreversible transition, then the rate constant can be equated to the Arrhenius equation. Plotting 1/T vs. ln(k_D) (Figure 4-8) helps to linearise this relation with the slope equal to \( E_a/R \).
The slope of each data set, and hence the activation energy, was determined independently yielding an average activation energy of $588 \pm 29 \text{ kJ mol}^{-1}$ across the samples.

**Method B**

The second method relates the change in melting temperature to the scan rate at which the melt temperature is recorded. This scan rate dependence is proportional to the activation energy of the protein:

$$\frac{v}{T_m^2} = AR \frac{E_a}{E_a} e^{-\frac{E_a}{RT_m}}$$

A plot of $\ln(v/T_m^2)$ vs $1/T_m$, with a slope of $E_a/R$, was derived and is shown in Figure 4-9.
This method was applied to five scan rates from 0.2 – 2.0 K min\(^{-1}\) (Figure 4-9). The analysis yielded an activation energy of 464 kJ mol\(^{-1}\). This is significantly lower than that calculated via Method A; however method B may have higher uncertainty as it uses only peak position to determine stability.

**Method C**

For method C, the rate of energy evolution within the system is proportional to the activation energy barrier preventing energy evolution, through the following relationship:

\[
\ln \left( \ln \left( \frac{Q_t}{Q_t - Q} \right) \right) = \frac{E}{R} \left( \frac{1}{T_m} - \frac{1}{T} \right)
\]

When the left hand side of the expression is plotted against 1/T (Figure 4-10) then the slope will be equal to \(E_a/R\).
The slope of each data set was derived independently yielding an average activation energy of $E_a = 591 \pm 120$ kJ mol$^{-1}$ which is in close agreement with the value obtained by Method A.

**Method D**

The fourth method directly calculates the activation energy based on the total energy released at the denaturation peak. This is related as follows:

$$E_a = eR C_p^m T_m^2 / Q_t$$

The activation energy was estimated for each of the samples as shown in Table 3.

Table 4-3 Hemocyanin Ea variation with scan rate

<table>
<thead>
<tr>
<th>Scan rate /K min$^{-1}$</th>
<th>$E_a$/kJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>434</td>
</tr>
<tr>
<td>1.0</td>
<td>512</td>
</tr>
<tr>
<td>1.2</td>
<td>549</td>
</tr>
<tr>
<td>1.4</td>
<td>545</td>
</tr>
<tr>
<td>2.0</td>
<td>451</td>
</tr>
</tbody>
</table>
Using method D, an average activation energy of $498 \pm 50$ kJ mol$^{-1}$ was computed which is in agreement with the value obtained via method A.

Thus it was established that the denaturation of *Haliotis Rubra* hemocyanin could be reliably modelled as an irreversible denaturation through the method of Sanchez-Ruiz. Whilst methods A through D should theoretically provide the same result for the activation energy, this rarely seems to occur in literature and the agreement found here (464-591 kJ mol$^{-1}$) is within what has been previously published (Table 3-1).

Table 4-4: Denaturation temperatures for various hemocyanins

<table>
<thead>
<tr>
<th>Hemocyanin Species</th>
<th>Technique</th>
<th>Tm /°C</th>
<th>Ea /kJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haliotis Rubra</em> (This work)</td>
<td>DSC</td>
<td>76.3</td>
<td>528 ± 63</td>
</tr>
<tr>
<td><em>Palinurus Vulgaris</em> [43]</td>
<td>DSC</td>
<td>63.1</td>
<td>383 ± 30</td>
</tr>
<tr>
<td><em>Rapana Thomasiana</em> [74]</td>
<td>DSC</td>
<td>83.0</td>
<td>597 ± 20</td>
</tr>
<tr>
<td><em>Helix Pomatia</em> [90]</td>
<td>DSC</td>
<td>85.7</td>
<td>517 ± 27</td>
</tr>
<tr>
<td><em>Megathura Crenulata</em> [73]</td>
<td>CD</td>
<td>68.0</td>
<td>-</td>
</tr>
<tr>
<td><em>Haliotis Tuberculata</em> [73]</td>
<td>CD</td>
<td>76.0</td>
<td>-</td>
</tr>
<tr>
<td><em>Rapana Thomasiana</em> [73]</td>
<td>CD</td>
<td>79.0</td>
<td>-</td>
</tr>
<tr>
<td><em>Helix Pomatia</em> [76]</td>
<td>FTIR</td>
<td>81.0</td>
<td>-</td>
</tr>
<tr>
<td><em>Eurypelma Californicum</em> [91]</td>
<td>DSC</td>
<td>89.9</td>
<td>-</td>
</tr>
</tbody>
</table>

This data is also similar to that found for hemocyanins from other species, such as Gastropod *Rapana thomasiana* ($E_a = 597 \pm 20$ kJ mol$^{-1}$) [74] and Arthropod *Pulinurus vulgaris* ($E_a = 383 \pm 30$ kJ mol$^{-1}$) [43] using DSC. Analysis of the stability of various formulations of *Haliotis Rubra* hemocyanin will therefore be analysed by this procedure.
4.3. Aggregation behaviour

4.3.1. Change in particle size with temperature

One key factor in the stability of a protein in solution is its ability to resist aggregation, as protein aggregates are generally biologically inactive and therefore not in their stable native state. As was established in Chapter Three, native hemocyanin is an extremely large protein (approximately 40nm) and it is possible to measure the particle size of its native conformation directly through use of photon correlation spectroscopy. It was also determined that aggregates can be identified via this method. In order to test the stability of the sample, we will now investigate the temperature at which aggregation begins and the rate at which it progresses.

As the Malvern Zetasizer does not have an accurate differentially controlled heating apparatus, discrete measurements every 1°C have been made during increasing temperature and as such no differential action can be displayed in this data. It does, however, give some insight into the point of aggregation onset. In Figure 4-11 the onset of aggregation is shown for a system of hemocyanin dialysed into deionised water. Samples of hemocyanin, dialysed into MilliQ water, with no adjuvants, were filtered through a 22μm filter into the sample tubes. Following the method outlined in Chapter 3, Z average distributions of the particle size were then measured every 1°C. The diameter of the most prominent peak is shown here for each measurement.

![Figure 4-11 Aggregation profile as a function of temperature for hemocyanin in water](image)
As shown in Figure 4-11 aggregation is seen to begin at approximately 75°C in a pure water environment and shows how the hemocyanin rapidly aggregates as the temperatures rises above this. The aggregation onset appears to coincide with the denaturation temperature of the hemocyanin as may be expected due to the exposure of normally hydrophobic residues to the solution.

4.4. Conclusions

In this chapter we have discussed the thermal stability of *Haliotis Rubra* hemocyanin, with a focus on the application of differential scanning calorimetry to the study of hemocyanin denaturation. The study of calorimetry equipment discovered that the TA nanoDSC can provide high resolution DSC traces, without the onset of aggregation during the denaturation transition. Other equipment, however, could not avoid this aggregation, making the choice of calorimeter important in obtaining viable DSC data.

Calorimetric traces have been obtained for the first time for the hemocyanin from *Haliotis Rubra*, with a denaturation temperature of 76.3°C showing that the stability of the pure protein was comparable to that from other species. Like all hemocyanins which have been studied, the denaturation reaction was found to be entirely irreversible, necessitating the use of irreversible denaturation models for its study. The method of Sanchez-Ruiz was therefore applied, identifying an activation energy for the denaturation reaction of \(528 \pm 63 \text{ kJ mol}^{-1}\).

It was determined that whilst the hemocyanin has a complex quaternary structure, this oligomeric form does not change during the denaturation. Aggregation, however, does occur alongside denaturation, with all samples showing visible aggregates upon removal from the calorimeter. We therefore investigated the aggregation behaviour of the protein using photon correlation spectroscopy in a Malvern zetasizer. This method is not generally applied to the study of proteins as the minimum size of particle for monitoring is above that of most proteins. By placing the protein sample into the zetasizer and scanning up in temperature we observed the onset of aggregation for the protein at 76°C, simultaneously to the denaturation. This is a simple and effective method for measuring the aggregation state of large proteins and may be widely applicable to the study of other large aggregating proteins.

By defining the stability of pure *Haliotis Rubra* hemocyanin, we have been able to construct a picture of the thermal stability of this protein. This will allow a benchmark of stability which we can then improve upon with formulation studies in the following chapter.
Chapter 5: Improved Hemocyanin Stability Through Formulation
CHAPTER 5. Improved hemocyanin stability through formulation

Previous chapters have established the inherent properties of *Haliotis Rubra* hemocyanin and found that without improvements to the stability of our formulations, the hemocyanin rapidly denatures and aggregates when stored in water solutions. In order to create viable pharmaceuticals from the hemocyanin, we must create formulations which allow the protein to remain stable through testing and administering of the therapeutic.

To this end, *Haliotis Rubra* hemocyanin will be formulated with a series of excipients which are often used in protein formulations. Many additives, such as buffers, salts and sugars are often added into protein formulations to increase stability, however the relative effect of each of these additives may be specific for to the protein chosen and as such their effects must be studied individually if we wish to create an optimal formulation.

Whilst no other studies have been aimed directly at formulating the hemocyanin from any organism, studies into the thermal characteristics of other hemocyanins do provide a range of initial formulations which we will investigate. Further, studies into the formulation of other, usually much smaller and conformationally simpler proteins, give some clues as to which formulating agents are worth considering in combination with hemocyanin. Sugars are found to have generally stabilising effects, while the choice of salts, pH and buffer may have various results.

In order to quantify the relative stability of a series of formulations we will primarily compare the denaturation temperature \( T_m \) and the activation energy \( E_a \) for the first unfolding peak, as was established in Chapter Four, where increased \( T_m \) or \( E_a \) indicate increased stability. Whilst it is tempting to consider the maximum apparent heat capacity for the peak as a measure of stability, this must be avoided as the prominence of this peak incorporates both the activation energy of the transition and the quantity of protein involved in the transition, complicating interpretation of the data. Measurements of the aggregation rate for the sample will be used to corroborate changes in stability.

We intend to identify a number of excipients which promote stability and, combining them in their most beneficial concentrations, come to a final optimal hemocyanin formulation. If successful, this formulation may provide much longer term stability for the hemocyanin sample than we currently have, facilitating the testing of the pharmaceutical properties of *Haliotis Rubra* hemocyanin without the threat of sample degradation compromising results.
5.1. Formulation Method

All hemocyanin samples were initially purified into a solution containing TRIS buffer at 100 mM NaCl as described in Chapter Three. Samples were then further formulated into the desired environment by the following method. The desired buffer was mixed from dry ingredients into MilliQ water. A 2 mL sample containing 10 mM Hemocyanin in TRIS was placed into a 100 kDa filter tube and spun down to approximately 200 mL in a centrifuge at 4000 rpm. This tube was refilled to 2 mL and agitated to remove any protein from the membrane filter. This process was repeated five times as per the method in Chapter 3 leaving a final sample of 2 mL of approximately 1 mM hemocyanin in the new buffer solution. This exact concentration of this sample was then tested via the Qubit method (established in Chapter 3) and recorded for use in data analysis. The formulated samples were then tested via DSC and PCS to determine their stability properties using the methodology developed in Chapter Four.

In order to facilitate a complementary range of excipients to be combined in formulation, commonly applied excipients were surveyed and found to fall into the following categories; buffering agents, ionic salts and solution modifiers (frequently sugars). These categories of excipients shall therefore each be investigated as means of altering hemocyanin stability.

5.1. Effect of excipients on hemocyanin stability

5.1.1. Effect of buffer identity

Buffer identity plays a role in the stability of a protein formulation as it not only maintains the sample pH, but may interact with the protein directly through intermolecular forces or chaotropic effects. The buffering range and ionic charge will each play a role in the formulation stability, however as no general rule for the stabilising or destabilising effect of buffers exist, they will have to be tested specifically for the protein to be formulated [46].

Buffers were chosen to cover the representative sample of commonly used protein buffers as well as covering a range of possible buffer pH ranges as shown in Table 5-1. As was determined in Chapter 3 formulations in pure water as well as the commonly used phosphine buffered saline (PBS) buffer results in immediate aggregation of *Haliotis Rubra* hemocyanin and as such TRIS, the first identified buffer which could retain stability, has been used as our baseline buffer. A buffer concentration of 50mM was used in all buffer tests to maintain ionic strength.
Table 5-1: List of buffers which may be suitable for the formulation of *Haliotis Rubra* hemocyanin

<table>
<thead>
<tr>
<th>Buffer species</th>
<th>Buffering range (pH)</th>
<th>Concentration used (mM)</th>
<th>pH of buffer used (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>3.6 – 5.6</td>
<td>50</td>
<td>4.5</td>
</tr>
<tr>
<td>Phosphate</td>
<td>5.8 - 8</td>
<td>50</td>
<td>7.4</td>
</tr>
<tr>
<td>HEPES</td>
<td>6.8 – 8.2</td>
<td>50</td>
<td>7.4</td>
</tr>
<tr>
<td>TRIS</td>
<td>7.3 – 9.0</td>
<td>50</td>
<td>7.4</td>
</tr>
<tr>
<td>PBS</td>
<td>7.4</td>
<td>50</td>
<td>7.4</td>
</tr>
<tr>
<td>Glycine/NaOH</td>
<td>8.4-10.6</td>
<td>50</td>
<td>8.5</td>
</tr>
</tbody>
</table>

The buffers have been divided here into those which provided a more stable hemocyanin sample compared to water and those which destabilised hemocyanin compared to a water formulation. This was determined by the shape of the calorimetric traces collected, where destabilising buffers tended to create multiple poorly resolved denaturation peaks. This is attributed to the protein having already partially unfolded in the buffer environment, creating unexpected transitions. Figure 5-1 shows the destabilising buffers.

![Figure 5-1 DSC traces of Haliotis Rubra hemocyanin in buffers that reduce stability](image-url)
Initially we attempted to formulate hemocyanin into PBS buffer as it was the reported buffer used for formulations of hemocyanin from *Megathura Crenulata*, however we discovered that this resulted in denaturation and aggregation of *Haliotis Rubra* hemocyanin as can be seen by the lack of distinct peaks in Figure 5-1. Buffers which could be used at a neutral pH were formulated to pH 7.4 and the others were formulated within their buffering range as stated below.

Buffers which destabilise hemocyanin may do so for a variety of reasons. Acetate buffer at pH 4.5 was destabilising, as expected, due to having a pH close to the isoelectric point of the protein. Glycine/NaOH buffer at pH 8.5 however was also destabilising, despite being far away from the isoelectric point. PBS buffer, which was a purchased standard at pH 7.4, also destabilised the hemocyanin, potentially due to the other ions formulated into this standard buffer.

Each of the buffers which destabilised hemocyanin was also observed to promote aggregation of the sample, with visible aggregates presenting when stored for 24 hours. This further indicates their lack of suitability.

Figure 5-2, shows buffers which were stabilising. These buffers were all formulated by hand to a pH of 7.4 and had no additional ions beyond the buffering agent and its titrant.

![Figure 5-2 DSC traces of Haliotis Rubra hemocyanin in buffers which promote stability](image_url)
It can be seen for stabilising buffers that the denaturation temperature of the Haliotis Rubra hemocyanin changed with the buffer identity (Table 5-2), indicating that the buffering species is actively involved in the denaturation reaction.

Table 5-2 Comparative denaturation temperatures for Haliotis Rubra hemocyanin in various buffers prepared to 50mM

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>73.8</td>
</tr>
<tr>
<td>Phosphate</td>
<td>76.8</td>
</tr>
<tr>
<td>Tris</td>
<td>78.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>79.0</td>
</tr>
</tbody>
</table>

HEPES was found to be the most stabilising buffer for this hemocyanin formulation; however TRIS also provided a suitable buffer environment. Phosphate, whilst stabilising, was not as effective. Thus in the combined final formulation, HEPES will be used to provide the greatest stabilising effect, however for the investigation of other factors such as ionic strength, a TRIS based buffer was utilised, as it was the first buffer identified to provide a stable formulation.
5.1.2. Effect of ionic strength

Once a suitable buffer had been chosen, with TRIS being the first suitable buffer identified, we set to investigate the optimal ionic strength for a stable sample. In order to have effective buffering a minimum buffer concentration of 50 mM of the buffer was used. Sodium Chloride was then added to increase the ionic strength up to 500 mM. As *Haliotis Rubra* is a sea based animal an ionic strength of 500 mM, similar to sea water, was anticipated to increase stability.

Salts are generally used to increase the ionic concentration and conductivity of protein formulations. It was discovered in initial denaturation studies that hemocyanin in pure water rapidly aggregated and denatured, such behaviour potentially arising from the lack of ions in solution which could stabilise the protein. Hence, all further formulations were created with a minimum of 100mM NaCl as well as the buffering agent.

![Figure 5-3 Aggregation of hemocyanin in high salt concentration TRIS buffer](image)

As it can be seen in Figure 5-3, the *Haliotis Rubra* hemocyanin denatured and aggregated at low temperatures, indicating a heavily reduced stability. The observed rapid drop in apparent heat capacity is the result of protein aggregation, and is characteristically observed when the hemocyanin sample aggregates. Visual observation of the aggregated sample after testing confirms that aggregation occurred. Whilst this concentration has been found to promote stability for some
proteins[50], the effects can vary, in this case being highly destructive. Lower salt concentrations were then used to find an optimal point.

As hemocyanin formulations lacking any salt have previously been seen to aggregate in a similar manner, the range of salt concentrations for this study was amended to 50-200 mM allowing us to maintain a sample without aggregation through the denaturation transition.

![Figure 5-4 Changes in hemocyanin denaturation rates with change in ionic concentration](image)

In Figure 5-4 it can be seen that whilst the peak position for the hemocyanin denaturation transition is unchanged by the salt concentration, the volume of the peaks, and their activation energy change dramatically. A maximum stability was recorded at 100 mM and this concentration was therefore adopted for further formulations.
5.1.3. Effect of salt valence

Whilst initial tests were conducted with simple monovalent salts, it was anticipated that adding salts of different valences may lead to different effects on the stability of hemocyanin due to the cross bonding effect of diivalent ions. Taking the previously determined optimal salt concentration, we then substituted divalent anions and cations to determine their effect on hemocyanin stability.

![Figure 5-5: Effect of changing both anion and cation valence on the stability of Haliotis Rubra hemocyanin](image)

It was observed that whilst changing the valence of anions had a very minor effect on the stability of hemocyanin (Figure 5-5), the presence of divalent cations in the hemocyanin formulation dramatically increased the prominence of the initial denaturation peak at the expense of the second denaturation peak.

A number of possible explanations for this behaviour are hypothesised. As was previously discussed in Chapter Four, it is possible that the first and second denaturation peaks represent a number of different transitions and without isolating hemocyanin into individual chain subunits it is impossible to prove what each transition represents. However, if the two denaturation peaks represented the individual unfolding of separate subunits, then the data in Figure 5-5 would suggest that divalent cations highly stabilised one subunit at the expense of the other.
If, on the other hand, the first transition represents the breaking of the hemocyanin quaternary structure and the second represents a subsequent unfolding step, then this data would suggest that divalent anions help maintain the quaternary structure of the hemocyanin during the entire denaturation, thereby leaving less free subunits to unfold subsequently. As the prominence of the initial unfolding transition is increased so dramatically, the second hypothesis appears more likely. However, until individual subunits of the *Haliotis Rubra* hemocyanin can be isolated it may be impossible to prove which of these mechanisms is in effect.

### 5.1.4. Effect of sugar concentration

Sugars are frequently added to protein formulations to increase stability. As was discussed in the literature review (Chapter 2), this is attributed to an increase in ordering of water around the protein as the sugar is excluded from its surface. We therefore anticipate that increasing sugar concentrations will only favour stability for the protein. Sucrose was chosen as the model sugar for this experiment, due to its frequent use in protein formulation.

![Figure 5-6](image-url)  
*Figure 5-6 Effect of increasing sugar content on hemocyanin stability*
It can be seen in Figure 5-6 that the denaturation temperature of the protein is not dependant on the sugar concentration. This may be anticipated, as the excluded sugar is not directly changing the structure of the hemocyanin. The shape, and therefore the activation energy of the denaturation, however, is seen to change.

Table 5-3 Activation Energy parameters for *Haliotis Rubra* hemocyanin in solutions at various sucrose concentrations

<table>
<thead>
<tr>
<th>Sucrose Concentration (mg mL⁻¹)</th>
<th>$T_m$ (°C)</th>
<th>Activation Energy (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>75.03</td>
<td>472</td>
</tr>
<tr>
<td>1</td>
<td>75.11</td>
<td>530</td>
</tr>
<tr>
<td>10</td>
<td>74.96</td>
<td>545</td>
</tr>
<tr>
<td>100</td>
<td>75.09</td>
<td>557</td>
</tr>
<tr>
<td>Sol Lim</td>
<td>75.15</td>
<td>371</td>
</tr>
</tbody>
</table>

Up until 100 mg mL⁻¹ the denaturation activation energy and hence protein stability increases. It was found, however, that increasing that sugar concentration further, up to the solubility limit (, resulted in a stability decrease (Table 5-3). This may be a result of sugar precipitation directly affecting the hemocyanin. A sugar concentration of 100 mM will therefore be applied to further samples.

**5.1.5. Effect of sugar identity**

It was anticipated that all sugars stabilised hemocyanin by the same mechanism, and hence should yield similar gains to formulation stability, however as the regulatory approvals and costs vary between sugars we have chosen to investigate a number of options. Hemocyanin in Tris, was formulated with 100 mM of glucose, fructose, xylose and arabinose.
In Figure 5-7 we see that all the monosaccharides tested provided a similar and effective increase to hemocyanin stability.

The size/Mw of the sugars was also examined. Here, we have compared a monosaccharide (glucose), a disaccharide (sucrose) and a trisaccharide (raffinose) to compare the effect of the sugar size on its stabilising effect. The molar concentration, not mass of sugar was maintained as the osmotic forces excluding water from the protein surface are dependent on the molar concentration of sugar osmolyte in the solution.
It can be seen that the effect of changing sugar molecular weight was more dramatic than the change between various monosaccharides, which may be expected as the chemical difference between the monosaccharides is extremely minor, whereas the mass and hence intermolecular forces between other polysaccharides may be different.

Table 5-4 Effect of Sugar identity on the stability of Haliotis Rubra hemocyanin

<table>
<thead>
<tr>
<th>Sugar identity</th>
<th>Sugar Size g mol⁻¹</th>
<th>Tₘ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fructose</td>
<td>180.16</td>
<td>80.9</td>
</tr>
<tr>
<td>arabinose</td>
<td>150.13</td>
<td>80.9</td>
</tr>
<tr>
<td>glucose</td>
<td>180.16</td>
<td>81.0</td>
</tr>
<tr>
<td>xylose</td>
<td>150.13</td>
<td>81.0</td>
</tr>
<tr>
<td>sucrose</td>
<td>342.30</td>
<td>81.2</td>
</tr>
<tr>
<td>raffinose</td>
<td>504.42</td>
<td>81.5</td>
</tr>
</tbody>
</table>

It was still found, however, that the identity of sugar was far less important than the concentration of sugar within the sample when affecting the hemocyanin denaturation activation energy. Sucrose has been chosen, instead of Raffinose, as the stabilising agent for further formulations. Whilst Raffinose was slightly superior in stabilising the hemocyanin, we found this difference to be minor and sucrose
has the advantage of being a much cheaper and more frequently used adjuvant than raffinose. This will allow for easier drug approval for the combined formulation, an important factor.

5.1.6. Combined hemocyanin formulation
Based on the above data for the most stabilising buffer, salt, and sugar conditions, a final combined formulation was created. In it 50mM HEPES at pH 7.4 with 100 mM NaCl and 100 mM sucrose was formulated with 1 mg mL\(^{-1}\) *Haliotis Rubra* hemocyanin. The stability of this formulation, compared with a base sample of hemocyanin in pure water is shown in Figure 5-9.

![DSC curve for Haliotis Rubra hemocyanin in the most stable formulation identified, containing 50mM HEPES at pH 7.4 with 100mM NaCl and 100mM sucrose with 1mg mL\(^{-1}\) Haliotis Rubra hemocyanin.](image)

The final combined formulation was found to be highly stable with a denaturation temperature of 78.9\(^\circ\)C and a denaturation activation energy of 726 kJ mol\(^{-1}\), a stabilisation of 38%. 

Gavin Marshall PhD Thesis
Table 5-5 Comparative stability of Haliotis Rubra hemocyanin in water and in an optimised formulation

<table>
<thead>
<tr>
<th></th>
<th>$T_m$ (°C)</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemocyanin in water</td>
<td>73.7</td>
<td>311</td>
</tr>
<tr>
<td>Final Hemocyanin formulation</td>
<td>78.9</td>
<td>726</td>
</tr>
</tbody>
</table>

This combined formulation of Haliotis Rubra hemocyanin is the most stable we have yet recorded. It remains yet to be seen, however, if this will translate to reduced aggregation rates or a long term stability of the sample, which will be crucial for the therapeutic testing of the protein.

The aggregation rate of the final sample was then measured following the same method used in Chapter 4, as shown in Figure 5-10.

![Figure 5-10](image.png)

**Figure 5-10** Aggregation behaviour of Haliotis Rubra hemocyanin in water compared with an optimised formulation

It can be seen that the formulated sample aggregates much less readily than pure hemocyanin in water. Whilst the pure sample begins aggregating around 72°C, the formulated one has not undergone any aggregation up to the maximum temperature of the zetasizer machine (78°C). This improvement in reducing aggregation rates will be crucial in the long term stability of the protein, as any aggregation of the sample will lead to irreversible loss of protein from the formulation.
5.2. Stability of hemocyanin after storage

Whilst we have now confirmed that an improved formulation could enhance the stability of hemocyanin in the short term, we are yet to determine if this translates to the long term stability required of a pharmaceutical product. In order to assess this, the completed Haliotis Rubra hemocyanin samples were then stored for a period of nine months and then retested.

![DSC traces of Haliotis Rubra hemocyanin formulations after nine months of storage as either a liquid or a freeze dried solid](image)

Figure 5-11 DSC traces of Haliotis Rubra hemocyanin formulations after nine months of storage as either a liquid or a freeze dried solid

Figure 5-11 shows that whilst there was some degradation of the hemocyanin over the storage period, a large portion of the sample retained its native state, producing a reasonable DSC trace. Freeze drying, whilst further reducing the stability of the sample, also retained most of the stable hemocyanin.
This was verified via particle sizing in Figure 5-12 where the particle size of fresh hemocyanin is compared to those measured for the samples stored for nine months.

![Particle sizes of Haliotis Rubra hemocyanin formulation and its stored forms](image)

**Figure 5-12 Particle sizes of Haliotis Rubra hemocyanin formulation and its stored forms**

It can be seen that a large degree of aggregation has occurred over the nine months of storage, with the hemocyanin in liquid form performing worse than the freeze dried sample. This is, however, expected due to the inherent instability of the hemocyanin material. However, as the sample did not form extremely large aggregates, as is observed in all other denatured samples, we suggest that much of this sample is still in its native form. Further, as DLS can be over-influenced by larger particles, it is possible that far more of the hemocyanin is in the didecameric form of 43nm that appears from this data. Thus there is a large portion of the protein which is not aggregated. Considering that this sample also showed strong DSC peaks and is hence still folded, we anticipate that the sample is still active.
5.3. Conclusions

Haliotis Rubra hemocyanin is a promising antiviral candidate, however in its pure form a lack of stability means that its efficacy trials would be compromised by a lack of active protein during testing. We have, therefore, formulated the protein in order to stabilise its native state and create a sample which does not denature or aggregate under normal storage conditions.

The effect of buffers, salts and sugars have been investigated as candidate stabilising adjuvants. Whilst it was found that all sugars promoted stable formulations, likely due to the excluded volume effect, with a maximum suitable concentration only limited by the solubility of the sugar. Buffers were found to have specific interactions with the protein, resulting in either a strongly destabilising effect or a promotion of stability and increase of denaturation temperature. Both TRIS and HEPES were strongly stabilising. Salts were found to stabilise the protein at some concentrations, as long as divalent cations were not included, with an optimum concentration of 100mM observed.

In order to combine the stabilising adjuvants into a single formulation we identified the optimum mixture to be a 50mM HEPES at pH 7.4 with 100 mM NaCl and 100 mM sucrose was formulated with 1 mg mL$^{-1}$ Haliotis Rubra hemocyanin. This resulted in a stabilised formulation with a denaturation temperature of 78.9°C and a denaturation activation energy of 726 kJ mol$^{-1}$, a stabilisation of 38%.

This sample was then stored for nine months, with the sample retaining its thermal stability and oligomeric state. The sample was found to have aggregated to some degree, however degraded to a much lesser extent than was observed for unformulated samples. As we have formulated the protein into a solvent with FDA approved adjuvants and proven long term stability, the hemocyanin sample is now suitable for therapeutic testing. Creating a stable hemocyanin formulation will allow for viable drug testing and will show the true efficacy of native Haliotis Rubra hemocyanin.
Chapter 6: Conclusions and Further Directions
CHAPTER 6. Conclusions and Further Directions

6.1. Conclusions

The aim of this work was to characterise the hemocyanin from Haliotis Rubra and develop a suitable formulation for pharmaceutical applications. Our research group has been studying this protein as it has been demonstrated to have activity against Herpes Simplex Viruses, which our research group has been studying. [1, 2, 92] This activity is very promising as the current treatment options for Herpes viruses are limited [13]. This activity is also not entirely surprising as recent studies have identified antiviral behaviours in hemocyanins from different species. The activities of these various hemocyanins appear to be species dependant however[9, 80] and as such we have found that this warrants individual studies on the hemocyanins from Haliotis Rubra individually.

Both the characterisation and formulation of the hemocyanin from Haliotis Rubra are tasks of considerable difficulty due to the extremely large size of the protein (over 7 MDa). The vast majority of existing techniques for both the characterisation and formulation of proteins are suited to much smaller molecules, meaning a key aspect of this work was developing suitable measurement and formulation methods for large proteins.

The first section of this work involved the both the purification and characterisation of the hemocyanin, as this was felt to be a necessary first step in the development of a stable formulation. A range of techniques for the purification of the protein were examined in Chapter 3; again the large size of the protein presented several unique challenges. It was found that by applying an innovative filtration process it was possible to produce a relatively high-purity hemocyanin solution.

As previously noted the characterisation of such large proteins is a topic which is not widely addressed in the open literature and hence it was necessary to develop suitable experimental techniques to address this important challenge. A range of techniques including Differential Scanning Calorimetry, Photon Correlation Spectroscopy, Mass Spectrometry and Gel Electrophoresis were used to determine the size, charge and sequence of the Haliotis Rubra hemocyanin. Through mass spectrometry we discovered that Haliotis Rubra hemocyanin was closely related to that of Haliotis
Tuberculata, and surprisingly that it did not share much homology to that of the widely studied Megathura Crenulata explaining why we did not observe much similarity between the stability properties of the hemocyanins from these organisms. Using dynamic light scattering we also discovered that the hemocyanin has a solvated diameter of 43 nm, similar to the measured size of Megathura Crenulata didecamers, suggesting that a similar structure was also present in the Haliotis Rubra hemocyanin. Further, the isoelectric point of the hemocyanin was found to be 5.97, determining that we must avoid this pH in Haliotis Rubra hemocyanin formulations.

The second stage of the thesis examined the stability of the hemocyanin. Once more, the size of the protein presented a challenge in terms of identifying suitable measurement techniques; however, it was found that Digital Scanning Calorimetry (DSC) was able to provide suitable information about the protein stability. Such knowledge is undoubtedly crucial; as it allows the effect of any additives to be quantified, which is a key focus of the work.

As was investigated in Chapter Two, much has already been said on the stability of proteins going back as far as the 1950s [25, 34]. Despite the similarity in structure of all proteins, as folded polypeptides, we see a range of stability parameters which varies far more than those for synthetic polymers. The expected irreversibility of hemocyanin denaturation reactions also creates a challenge in analysing the thermodynamic properties inherent in stability. We, therefore identified the method laid out by Sanchez-Ruiz [40], which analyses the activation energy barrier to unfolding as a proxy for thermodynamic stability as the most viable framework for analysing the stability of this irreversible transition.

Previous studies into the stability of hemocyanins have also been used as a basis for our investigation. We found that whilst all hemocyanins observed have been seen to unfold irreversibly, they show a remarkably large range of both denaturation temperatures (63.1-89.9°C [43, 91]) and activation energy profiles (383.3 – 597 kJ mol⁻¹ K⁻¹ [43, 74]), giving a wide range of parameters within which we might expect to find Haliotis Rubra hemocyanin stability. This highlights the difference between hemocyanins which may explain their disparate therapeutic effects as well as their varying stability.
Before any changes to formulation could be considered, the stability of pure hemocyanin in water was investigated. This allowed us to develop a baseline reading for stability, and to test and validate our methods. The denaturation of hemocyanin was confirmed as an entirely irreversible reaction, and was therefore characterised by its kinetic parameters. The method of Sanchez-Ruiz was applied in order to quantify the activation energy with an activation energy of 464-591 kJ mol$^{-1}$ which was consistent with that measured for other hemocyanins. This data was the core of our publication “Thermal denaturation and protein stability analysis of Haliotis rubra hemocyanin” [2].

It was also expected that aggregation accompanied the denaturation of hemocyanin and as such we set to monitor this using dynamic light scattering. DLS allowed us to observe the aggregation of hemocyanin from monodisperse 32nm particles into large aggregates, and it was confirmed that this occurred simultaneously to the denaturation temperature.

As the stability of hemocyanin in water was far too low for drug trials we then set about improving that stability through formulation of the protein. Whilst buffers such as PBS have been successfully applied to formulations of other hemocyanins, we found that PBS led to rapid aggregation of *Haliotis Rubra* hemocyanin. Whilst these buffers may have been used in drug formulations, their use would have resulted in no hemocyanin activity, due to unstable proteins. Instead we identified that both TRIS and HEPES buffers could promote stability, both increasing the denaturation temperature of the protein and decreasing aggregation rates. In order to create a formulation plan, we investigated which adjuvants have previously been used in protein formulations and the mechanisms by which they work. Buffer and salt identities were considered in order to investigate the effect of specific ion interactions and the varying charge ratios on the solvation properties of *Haliotis Rubra* hemocyanin. Sugars were also anticipated to be a worthy addition to high stability formulation, as their addition is known to increase protein stability by changing the free energy of the hemocyanin water system. This allowed us to design a series of formulations to narrow down which interactions lead to a stabilised hemocyanin formulation.

In order to create an optimal formulation, however, other adjuvants such as salt and sugar additions were then considered. It was found that, as expected, sugar additions increased the hemocyanin stability as well as promoting delayed aggregation. This was published in “Formulation of abalone hemocyanin with high antiviral activity and stability” [1]. As the sugar identity did not greatly influence...
the stability gain, we attribute this stabilisation to the preferential hydration effect, whereby the sugar addition changes the free energy of water in the formulation, leading to a higher overall energy barrier for the unfolding reaction. Salt concentration was also found to play a key role in the stability of hemocyanin, with an optimal salt concentration of 100 mM identified. Divalent cations were also found to dramatically alter the denaturation reaction character, reducing the prominence of the second peak. As this likely relates to reducing the stability of one hemocyanin region, divalent cations were avoided in further formulations.

This lead us to a final formulation, combining the stabilising elements investigated which consisted of 1 mg/mL Haliotis Rubra hemocyanin in 50 mM HEPES, 100 mM NaCl and 100 mM sucrose. This formulation displayed greatly increased thermal stability over hemocyanin in water alone, with an increase in denaturation temperature of 5.2°C, to 78.9°C and an increase in activation energy of 415kJ mol⁻¹ K⁻¹ to 726kJ mol⁻¹ K⁻¹. This stability increase was reflected in aggregation behaviour, where the sample was found not to heavily aggregate when placed in temperatures up to 80°C and over a period of up to nine months in refrigeration. These stability increases highly improve our chances of retaining stable active hemocyanin throughout drug trials. With a stable Haliotis Rubra hemocyanin sample we will be able to determine activity of hemocyanin against Herpes Simplex Viruses, showing the true efficacy of the native protein, and avoiding the risk of reduced activity due to sample degradation.

6-1 Composition and properties of the final improved Haliotis Rubra hemocyanin formulation.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Stability Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>50mM HEPES</td>
<td></td>
</tr>
<tr>
<td>Ionic Salts</td>
<td>100mM NaCl</td>
<td></td>
</tr>
<tr>
<td>Non Ionic additions</td>
<td>100mM Sucrose</td>
<td></td>
</tr>
<tr>
<td>Denaturation Temperature</td>
<td>78.9°C</td>
<td>5.2°C</td>
</tr>
<tr>
<td>Activation Energy</td>
<td>726 kJ mol⁻¹ K⁻¹</td>
<td>415 kJ mol⁻¹ K⁻¹</td>
</tr>
<tr>
<td>Aggregation onset Temperature</td>
<td>&gt;80°C</td>
<td>70°C</td>
</tr>
</tbody>
</table>
6.2. Future directions

This study has developed a thermo-stable formulation of Haliotis Rubra hemocyanin, suitable for therapeutic testing. There are many other questions, however, which may be asked about Haliotis Rubra hemocyanin and its products. We suggest here four broad paths further research may follow, namely;

- Further investigation of hemocyanin stability properties, such as resistance to chemical denaturation and techniques complementary to DSC.
- Investigation of Haliotis Rubra hemocyanin subunits.
- Modification of hemocyanin proteins via conjugation, deglycosylation or partial digestion.
- Genome sequencing and recombinant expression of hemocyanin or its components.

Many of these avenues were attempted during the course of this research, however due to the size and denaturing behaviour of the protein they were unsuccessful. The techniques which were attempted will be briefly described below, along with potential solutions if they are known. Due to the success in developing a suitable formulation for the protein it is now possible to perform trials to determine its efficacy both in vitro and in vivo. Workers from our research group are currently pursuing this issue.

In order to complement the thermal denaturation data we have collected in this study, chemical denaturation studies would be of interest. This would involve an isothermal titration calorimetry method, to determine the effect of a denaturant, such as urea, would have on the protein. Whilst chemical denaturation was avoided in this study, due to being less directly applicable to the expected conditions for our hemocyanin drug tests, a chemical denaturation curve may allow us to quantify the solvation effects on protein stability, giving a more complete picture of the hemocyanin stability. Other studies into hemocyanin stability have applied techniques other than DSC, including circular dichroism, FTIR spectroscopy, and fluorescence spectroscopy to study thermal denaturation. Whilst these techniques are expected to yield similar data to that from DSC (allowing for an activation energy analysis), they would provide a validation of the DSC technique we applied. Both CD and FTIR methods were considered in this study, and preliminary trials were conducted. These tests, however, did not yield reasonable data. The use of a temperature controlled FTIR flow cell may allow us to collect accurate data on the denaturation reaction, however there are currently no suitable cells available for...
use within Sydney University, or the surrounding universities. Similarly, the CD technology available did not have the precise temperature control required to measure accurate denaturation temperatures. This equipment is highly specialised and unfortunately, due to a lack of interest in this field locally, was not available. Application of these techniques could corroborate the DSC data used in this study.

As has been described earlier, *Haliotis Rubra* hemocyanin forms a didecameric quaternary structure which can be separated into monomeric components at high pH. Investigation of these pure subunits may answer many of the open questions left in this research, including assignment of the two separate denaturation peaks as well as providing a sample to test the relative antiviral activity of subunits compared with the complete structure. Whilst we were successful in breaking the hemocyanin into subunits, as evidenced in electrophoresis and DLS experiments, separating the subunits proved much more challenging. Systems including high molecular weight filtration, chromatography and self-cast Agarose gel electrophoresis were attempted, however as the hemocyanin subunits were much more prone to aggregation than the didecameric structure we could not isolate them long enough for a stability study. Further formulations, modifications or purification techniques may allow the isolation of subunits, as has been done for hemocyanins from other sources.

Protein modification may be one avenue to further study *Haliotis Rubra* hemocyanin. As the hemocyanin is heavily glycosylated, it is theorised that the surface sugars on the protein may have some impact on the therapeutic effects of the protein. We therefore attempted enzymatic deglycosylation to test this theory. Despite multiple attempts, the deglycosylation reaction would not proceed. This may be due to the tight barrel structure of the protein obscuring access for enzymes to the sugar moieties. Similarly, PEGylation was considered as a method for stabilising the hemocyanin, and more importantly the hemocyanin subunits. PEGylation of the subunits was unable to inhibit aggregation, and hence no viable samples could be obtained to test if the PEGylation proceeded. When repeated for the whole hemocyanin, no PEGylation was detected. This, however, may instead be due to the extreme size of the relative to the PEG reagents, which may hide the change in molecular mass. Successful deglycosylation or PEGylation may allow us to understand the effect the hemocyanin surface has on its therapeutic activity.
Finally, obtaining the primary sequence for *Haliotis Rubra* hemocyanin would allow for a more systematic approach to study of the protein. This would most likely involve sequencing the genome for the organism, as the peptide length is too long for peptide sequencing. Whilst the unique value of this study comes from the necessary sourcing of our protein from a blood sample, utilising a recombinant protein source would allow tagging and would bring our methods more in line with modern proteomics studies. As it may be difficult to reproduce the complex folding or post translational modifications of hemocyanin within a recombinant source, this may be best used to study the therapeutic effects of pure unmodified hemocyanin subunits.
References


Keller, H., et al., *Abalone (Haliotis tuberculata) hemocyanin type 1 (HtH1) Organization of the <400 kDa subunit, and amino acid sequence of its functional units f, g and h*. European Journal of Biochemistry, 1999. 264.


