Red Abalone Hemocyanin as an Alternative Hapten-Carrier for Vaccine Production

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RED ABALONE HEMOCYANIN AS AN ALTERNATIVE HAP TEN-CARRIER FOR
VACCINE PRODUCTION

by

Isaiah N. Mansour

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Marine Sciences)

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ABSTRACT

Vaccines protect millions of human lives per year from otherwise fatal illnesses. Vaccination promotes immunity by simulating infection with a non-pathogenic representative of the disease, an antigen, to prepare and train an immune response. Vaccine-safe antigens are often too small to elicit an immune response. These non-immunogenic molecules are referred to as haptens and are introduced to the immune system by hapten-carriers, large immune-stimulating proteins. Keyhole Limpet Hemocyanin (KLH), a respiratory protein of the giant keyhole limpet (*Megathura crenulata*), is the industry standard hapten-carrier. KLH is a potent yet safe immune-stimulant relied upon in research, available human medications, and emerging vaccines. KLH is currently valued at approximately $10,000 per gram, and economic analysis predicts the KLH market to exceed $35 million once new vaccines are approved. KLH production, however, is neither ecologically sustainable nor economically sensible. KLH is produced from live *M. crenulata* and cannot be synthesized. Over-fishing endangers wild *M. crenulata* populations, and only one company is authorized to aquaculture this species. A potential alternative hapten-carrier is the hemocyanin from the red abalone (*Haliotis rufescenes*). Aquaculture for this species is well established and globally profitable; their meat is considered a delicacy, and their shell is the source of the gem ‘Mother of Pearl’. Red abalone hemocyanin (RAH) is biochemically similar to KLH. This study adapts KLH purification techniques to RAH for comparison. KLH and RAH were determined to be approximately the same molecular weight, a critical characteristic for an alternative hapten-carrier. RAH isolation was optimized to rival commercial KLH purity. RAH, like KLH,
consists of two subunits between 350 kDa and 400 kDa. RAH elicits an antibody production response to conjugated haptens in mice. RAH is therefore a viable alternative to KLH as a hapten-carrier in vaccine production. There is precedent for abalone aquaculture in Maine.
DEDICATION

This project is dedicated to the oceans of planet Earth.

“Even if you never have the chance to see or touch the ocean, the ocean touches you with every breath you take, every drop of water you drink, every bite you consume. Everyone, everywhere is inextricably connected to and utterly dependent upon the existence of the sea.”

- Dr. Sylvia E. Earle
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INTRODUCTION

Background

Vaccines prevent millions of deaths each year from once-common and fatal diseases (1). Vaccination promotes immunity by preparing and training the immune system to recognize and eliminate pathogens, harmful germs and viruses (1). Surface molecules called antigens are specific to each pathogen species, allowing the immune system to identify and attack the pathogen (2). Antigen detection triggers the immune system to produce antibodies, molecules that are designed to neutralize specific pathogens (3). Antibodies bind to their counterpart antigens to inhibit their harmful effects or mark the pathogen for destruction (1). Antibodies are produced for each new pathogen encountered, and the host develops immunity to an illness once antibodies have been produced against antigens of the disease-causing pathogen (4).

It is important, however, that immunization does not result in infection. A successful and safe vaccine helps the immune system prepare antibodies against the pathogen without the patient becoming sick (1). Vaccines, therefore, typically introduce modified antigens that represent the pathogen to the immune system (1). Many antigens that are manipulated for vaccine safety are haptens, molecules too small to elicit an immune response (2). If the immune system cannot detect the hapten, antibody production will not occur, and immunity will never develop (1).

It is often necessary to deliver these haptens to the immune system in a recognizable form. Haptens are typically conjugated, or chemically attached, to large immune-stimulating proteins called hapten-carriers (5). Hapten-carriers are coated in the hapten and
introduced to the host intravenously (6). Immune system cells are activated to investigate the large foreign hapten-carrier and discover the haptens on its surface (7). These haptens are thereby effectively presented to the immune system and immunization can progress (5). Successful hapten-carriers are large, high-molecular-weight, and phylogenetically distant proteins that cause no harm to the host (3). Hapten-carriers are often used in conjunction with adjuvants, substances that modify the immune response complementary to an antigen to accelerate immunity development and extend protection duration (8). This may result in general upregulation of components of the immune response, such as antibody production, or modulation of the way immune system cell types interact (1).

The most efficient hapten-carrier and adjuvant in research and medicine is the oxygen carrying protein from a Californian sea snail, the giant keyhole limpet (*Megathura crenulata*) (2). Keyhole Limpet Hemocyanin (KLH), a respiratory protein serving comparable functions to hemoglobin in human blood, from this limpet is the industry standard for immunological research and vaccine production (2). KLH was discovered to be a potent, yet safe, immune stimulant in the 1950s (8). A single injection of KLH triggers intense immune system stimulation in nearly all vertebrates, including humans (8). KLH safely elicits both cellular and humoral immune responses in mammals and has thus been used in various immunological applications (5). By 1967 KLH was a standard for evaluating patient immunocompetence, or normal immune response efficacy (8). KLH is still used extensively in immunocompetence research and evaluation (2). Many antibody producers, businesses that prepare and supply antibodies to specific pathogens, prefer or require KLH as the hapten-carrier or complimentary adjuvant (2).
New immunotherapies use KLH as a hapten-carrier and adjuvant to bolster the patient immune system and treat existing illnesses (7). While preventative vaccines protect the patient from new sicknesses, immunotherapies and therapeutic vaccines treat present conditions (4). As an example, therapeutic vaccines use KLH to help the immune system recognize cancer cells as harmful (3). Immunotherapies are a rapidly growing pharmaceutical sector because of their ability to leverage natural immunological processes to fight current diseases that are not ordinarily recognized by the immune system (6).

KLH is also an immunotoxicology standard in monitoring the immune-suppressive side effects of candidate molecules for new drugs (7). Some diagnostic techniques for identifying illegal drugs and parasites in patients regularly utilize KLH (2). Beyond research products, KLH is used as a hapten-carrier in Immucothel, a leading bladder carcinoma treatment (9).

KLH has been used extensively as a multi-purpose immune-stimulant in clinical trials for decades but has recently garnered attention as a more efficient hapten-carrier in a variety of existing and emerging vaccines (9). There are approximately 20 different vaccines in late-stage clinical trials using KLH (10). These include preventative and therapeutic vaccines for illnesses such as Alzheimer’s, HIV/AIDS, Chron’s Disease, and a variety of cancers (10). Most of these KLH-incorporating vaccines are anticipated to enter the market in the next 3 years (11). KLH is currently valued at approximately $10,000 per gram, while economic analysis predicts the KLH market to exceed $35 million once the various vaccines are approved (10, 11). KLH production, however, cannot supply the growing demand (12).
KLH is produced from the hemolymph (blood) of live *M. crenulata* and cannot be synthesized (12). One company, Stellar Biotechnologies Inc., has monopolized *M. crenulata* aquaculture, creating a bottleneck in pharmaceutical availability (12). This company sets the price and pace of most KLH research, restricting access for scientists and patients (12). Other businesses have already overfished the giant keyhole limpet for KLH used in research and available therapies, endangering the species (12). The wild population cannot support the imminent pressure of increased demand for KLH in emerging vaccines (12). Stellar Biotechnologies Inc. openly warns of their limited capacity to supply KLH for the new vaccines in their 2017 Financial Report (12). Stellar Biotechnologies Inc. is sustained mainly by grants and shareholders (12). Failure to attract investment will cripple their manufacturing capability (12). KLH production is therefore neither ecologically sustainable nor economically sensible. An alternative hapten-carrier is needed for the future of vaccine development.

Abalones and limpets are taxonomically related species (13). Though abalones are in the Haliotidae family and *M. crenulata* is in the Fissurellidae family, both are vetigastropods, and the two types of sea snails share many characteristics (14). The hemocyanin of abalones has thus been investigated by immunologists as an alternative hapten-carrier to KLH in vaccine production and other immunological applications (14). The hemocyanins of various abalone species have been shown to be biochemically comparable to KLH (13). Importantly, the hemocyanin of most abalone species is approximately the same size, weight, and shape as KLH (15). These are critical characteristics for a successful alternative hapten-carrier (15). The carbohydrate composition, an important factor in hapten conjugation, of KLH and abalone hemocyanin
are also similar (16). Finally, abalone hemocyanin subunits may be more stable than KLH subunits after purification (14).

Hemocyanin from the red abalone (*Haliotis rufescenes*) has not been previously studied for use as a hapten-carrier. If *H. rufescenes* hemocyanin, which I refer to as RAH, is biochemically comparable to KLH, it may be a viable alternative hapten-carrier in vaccine production. The essential criteria are similarities of protein subunit size, shape, and weight (4). A competitive alternative hapten-carrier to KLH should be able to be extracted, isolated, and purified with comparable efficiency. Hapten carrying efficacy can be directly compared by conjugating the same hapten to both hemocyanins and testing their respective antibody production when injected into mice. Comparison of the respective antibody production against the hapten with the two hemocyanins will show if RAH is as effective as KLH. This study addresses the biochemical characteristics necessary to develop RAH as a KLH substitute, such as protein size. Maine is positioned to capitalize on abalone aquaculture for a variety of products, including RAH (17). Each abalone can be processed in a way that maximizes market diversity and minimizes waste— a sustainable advantage.

**Objectives**

1. To determine the requirements for sustained aquaculture of *H. rufescenes*.
2. To adapt KLH extraction, isolation, and purification techniques to RAH.
3. To compare the subunit structure and molecular weight of KLH and RAH.
4. To assess overt effects of hemolymph extraction on abalone health.
5. To determine the normal concentration of hemocyanin in *H. rufescenes* hemolymph.
6. To conjugate a hapten to both KLH and RAH.
7. To raise antibodies against the given hapten in mice and assess respective antibody production using KLH and RAH as hapten-carriers.

**Appendix Guide**

KLH and abalone hemocyanin structure and characteristics are discussed in Appendix A: KLH and Abalone Hemocyanin Biochemistry. Information pertaining to the differences in limpet and abalone harvest are discussed in Appendix B: Comparative Fishery Considerations. The economic landscape of KLH and various abalone products are discussed in Appendix C: Market Evaluation of Limpet and Abalone Products.
METHODS

Abalone Maintenance

Seven red abalones were supplied from a previous behavioral study at the University of Maine Aquaculture Research Center (ARC) in 2014. These abalones were originally from The Abalone Farm (Cayucos, CA) and were 4 years when incorporated into the present study. The previous research did not affect the health of these abalones. Thirty-five more red abalones were supplied from The Abalone Farm (Cayucos, CA) in 2015. These abalone were 2 years old at the time. All permits were filed and processed through the Aquaculture Research Institute (ARI) and the Maine Department of Marine Resources (DMR). The farm-raised abalones were originally grown in water directly from their natural California habitat and were fed a mixed diet of natural and processed feed prior to delivery at ARC for experimentation.

All abalones, once brought to ARC, were housed in approximately 150 gallons of artificial saltwater in ARC. The water was prepared using Crystal Sea Marine Mix. The primary housing enclosure was 2 meters long, 0.5 meters wide, and 0.65 meters deep. The airflow rate was typically 2 liters per minute. Salinity was maintained at 34-38 ppt, while the water temperature range was 8°C to 14°C (typically 11°C), depending on air temperature and water-chiller-system settings. These are normal temperatures and salinities for wild H. rufescenes. Water quality was monitored for concentrations of ammonia, nitrate, nitrite, phosphorus, calcium. Dissolved oxygen and pH levels were also monitored. Tank water exceeding 0.5 ppm phosphate, 0.5 ppm ammonium, 0.5 ppm nitrate, and 10 ppm nitrate was treated with a 50% water-change. Ambient light over the enclosure was programmed for 12 hour light cycle per day.
The abalones were originally fed “Abalone Broodstock Diet” from Adam and Amos Abalone Foods PTY LTD in Adelaide, Australia. Originally the abalones were fed a ‘chip’ formulation of this feed; this was switched to a ‘pellet’ formulation starting in 2016. Abalone were also occasionally fed nori, a dried seaweed. All abalones were fed 3 times a week, typically on Mondays, Wednesdays, and Fridays. Feed was not limited, and about 50 grams of food material were added to the tank each session. Each feeding began with a 25% tank water change to remove excess food. The variable nature of ARC systems resulted in occasional deviations from these parameters.

**Hemolymph Extraction**

Commercial KLH production often extracts the total hemolymph volume from the limpets resulting in death. Scientists at Stellar Biotechnologies Inc., however, published descriptions of non-lethal extraction techniques for limpet and abalone hemolymph (12). This information was integrated with a protocol designed by Erin E. Switzer, a 2013 University of Maine graduate student who studied abalone in the Bricknell Lab, and my previous experience with extracting hemolymph from horseshoe crabs (*Limulus polyphemus*) (18).

Large and active abalones were selected for hemocyanin extraction in advance. A selected individual was removed from the tank by sliding a flat object between its muscular foot and the aquarium wall and prying it upwards. The individual was then placed into an ice bath for 3 minutes. This slows the abalones, so they do not squirm during hemolymph extraction. The specimen is then held upside down in one hand, and a sanitary wipe was used to clean its foot.
A sterile 22-gauge needle attached to a 1 mL syringe was then carefully inserted into the pedal sinus, a major hemolymph cavity (18). The pedal sinus is located just below the anterior edge of the foot, where there are two visible lobes (18). The needle is inserted less than a centimeter into this area of the foot, at a 45-degree angle with the front of the animal, to reach the pedal sinus.

A 1 mL sample of hemolymph was extracted at a time and added to a sterile Eppendorf tube on ice, for a total of 2 mL. Each animal experienced one to five attempts per bleeding until a total 2 mL of hemolymph was obtained. Specimens were returned to the tank after providing 2 mL of hemolymph or by the fifth attempt. The animals that were bled were monitored for changes in behavior for the duration of the study. Ten abalone were bled during this study, four of which were bled on two separate occasions. Nitrile gloves were worn throughout these processes to prevent sample contamination. Each 2 mL sample was separated into three 600 uL sub-samples for all subsequent experiments.
Hemocyanin Isolation

Initially the extracted raw hemolymph was added to Eppendorf tubes on ice that contained a marine anticoagulant (2.63 g sodium chloride, 1.8 g glucose, 0.088 g tris-sodium citrate, 0.055 g citric acid, and 0.029 g EGTA per 100 milliliters of nanopore water). As the experiment progressed this marine anticoagulant was found to provide no advantage during the process and was removed from the protocol. Extracted raw hemolymph was then added to empty Eppendorf tubes on ice.

All hemolymph samples were subject to 30 minutes of 800 g centrifugation at 4°C immediately after collection. This was to prepare cell-free hemolymph; large particulates such as whole cells and contaminants were centrifuged down into the pellet at the bottom.
of the tube. The supernatant, or remaining solution above the coalesced pellet, was then removed and placed into a new Eppendorf tube on ice. The supernatant samples were then subjected to 3 hours of 15,000 g centrifugation at 4°C. This resulted in the formation of an opalescent blue hemocyanin pellet in the bottom of the centrifuge tube. The supernatant was then removed, and the hemocyanin pellet resuspended in 150 µL hemocyanin stabilization buffer (HSB), a solution used as reported in other studies (19). The formula for this solution is 50 mM Tris HCl, 5 mM CaCl₂, 5 mM MgCl₂, pH 7.4. The hemocyanin concentration was determined via Bicinchonic Acid (BCA) analysis using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Catalog No. 23225).

Published KLH experiments use a 1.0% solution of ammonium molybdate to separate the one hemocyanin subunit by selectively dissociating the other (14). The intact subunit can then be purified through size exclusion methods, such as polyacrylamide gel electrophoresis (PAGE) or gel filtration chromatography (GFC). I prepared a PEG 0.2% Mr 1,000 solution with the pH adjusted to 5.9 at room temperature using 1 N NaOH. This solution is referred to as AMPEG. The dissociated hemocyanin subunit can be gathered and chemically re-associated. Samples were exposed to AMPEG by direct buffer exchange (hemocyanin pellet formation via centrifugation, supernatant removal, and administration of AMPEG), overnight cassette dialysis against AMPEG, and buffer exchange spin columns (ThermoFisher Scientific, Catalog No. 89849) (2). The marine anticoagulant, however, was not effective with RAH and was omitted in later experiments.

Hemocyanin Purification

I purified hemocyanin in three different ways. First, following the KLH literature closely, dissolved RAH samples in AMPEG were subject to gel filtration chromatography.
Gel filtration chromatography was performed precisely as according to Harris et al. 1995; “in the presence of AMPEG solution at 4°C through Bio-Gel A-15m, 200-400 mesh (Biorad Laboratories). The column, 1 m in height and 15 mm diameter (bed volume 180 cm³), was thoroughly equilibrated with the AMPEG solution, prior to the application of the pelleted and AMPEG-dialyzed KLH sample” (19). Commercial protein standards (Biorad Laboratories) were used to calibrate the column and determine the molecular weight ranges of the fractions eluted. The protein concentrations and identities were determined via spectrophotometry.

All RAH samples were subjected to native and SDS denaturing PAGE (NuPAGE ®) following the protocol included for Tris-Acetate Mini Gels (MAN0003679). RAH was purified from native PAGE gels through the excision and elution protocol of Tech Tip #51 (ThermoFisher Scientific, TR0051.1). This consisted of cutting the separated hemocyanin protein band out of the gel, grinding it into an elution solution, and incubating it at 37°C overnight. The protein was then recovered from the supernatant of the centrifuged sample, and subject to follow-up native PAGE to determine purification efficacy. Alternatively, RAH samples were ‘cleaned’ by serial centrifugation in protein desalting spin columns with a molecular weight cut-off of 7 kDa (ThermoFisher Scientific, MAN0011469). These centrifuge tubes function as filters that remove particulates lighter than the given molecular weight cut-off. Many RAH contaminants were small, so repeated (3x) filtration with these units was successful in enhancing RAH purity.

The most efficient purification protocol utilized RAH samples that were not exposed to marine anticoagulant nor AMPEG. These hemocyanin pellets, dissolved in HSB, were subject to filtration with the desalting spin columns, repeated three times, using
more HSB as the exchange buffer. This procedure was inspired by and modeled after the similar and effective serial filtration regime of Zanjani et al. 2016 (20). The subunit sizes of these efficiently purified RAH samples were directly compared to those of KLH from a commercial KLH-hapten conjugation kit (ThermoFisher Scientific, MAN0011175) through the previously described native PAGE conditions.

**Hapten-carrier Efficacy**

A commercially available Imject™ KLH hapten conjugation kit (ThermoFisher Scientific, Catalog No. PI77600) was utilized for hapten conjugation with KLH and RAH. This kit includes industry grade KLH for hapten conjugation in research. Other necessary materials were Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) (ThermoFisher Scientific, Catalog No. 22322), Imject Maleimide Conjugation Buffer (ThermoFisher Scientific, Catalog No. 77164), and 20 mg of sulfhydryl-containing hapten. The hapten chosen was thiosalicylic acid (Acros Organics, Mfr. No.138891000). Thiosalicylic acid was chosen because of its low molecular weight and available sulfhydryl groups.

The RAH samples used for conjugation were never exposed to marine anticoagulant nor AMPEG; the hemocyanin pellet was simply dissolved in HSB and filtered of contaminants through serial centrifugation with desalting spin columns (ThermoFisher Scientific, Catalog No. 89849). The hemocyanin concentration of these samples was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Catalog No. 23225). The previous methods provided insight to proper hemocyanin processing and led to the identification of a reliable experimental procedure.
The hapten conjugation process was then repeated with control samples of Phosphate Buffered Saline (PBS) and no protein. Without a hapten-carrier, the thiosalicylic acid was removed from the sample during an excess filtration step. A substitute 20 mg of thiosalicylic acid was then added to the finished solution, which had been processed like the hemocyanin samples.

These samples were then sent to Pocono Rabbit Farm and Laboratory (PRFL), an antibody production facility in Canadensis, PA. PRFL prepared 6 mice for this study. Two positive control mice were injected with the KLH-thiosalicylic acid conjugate, two experimental mice were injected with the RAH-thiosalicylic acid conjugate, and two negative control mice were injected with the PBS-thiosalicylic solution. PRFL uses a proprietary antibody production regime called the Mighty Quick Protocol to raise mouse antibodies in 28 days. This is an accelerated process using a proprietary immune-stimulant (Mighty Quick Stimulant). PRFL is a KLH consumer; they use KLH predominately for their experiments. Therefore, Mighty Quick Stimulant is not anticipated to interfere with hemocyanin immune modulation.

Antibody production began on April 9th, 2018, when PFRAL injected the six mice with the respective samples; two with KLH conjugated hapten, two with RAH conjugated hapten, and two with the hapten in a PBS solution. Serum samples were extracted, and ELISA was performed after the 28-day incubation period to quantify antibody production. ELISA measures antibody production by determining the maximum dilution of the sera that reacts to the antigen. Titers are the dilutions of the mouse sera that react to the given antigen. PRFL measures titers by performing an initial dilution of the mouse sera at 1:300, and then in three-fold (1:3) dilutions across 11 wells on a 96 well microtiter plate. The titer
steps, or dilution factors, are referred to as the reciprocal of the dilution (i.e. 300, 2,700, 8,100, etc.). The antibody response was measured only for the hapten, thiosalicylic acid, as that was the molecule I was attempting to vaccinate against. The antibody response to the hapten carrier itself should be minimal; effective vaccine stimulates a response to the desired antigen, in this case thiosalicylic acid, not the carriers. ELISA was therefore performed using the RAH-thiosalicylic acid conjugate solution with the KLH-thiosalicylic conjugate injected mice and vice versa; one hemocyanin-hapten solution was used as the antigen for ELISA of the other hemocyanin. This isolates the results to the immune response generated solely against the hapten because antibodies produced against KLH will not react to RAH and vice versa. If RAH is a competitive hapten-carrier alternative for vaccine production, RAH-thiosalicylic acid conjugated samples and as KLH-thiosalicylic acid conjugated samples should elicit a similar antibody production, measured with titers, against thiosalicylic acid in mice. Both samples should theoretically result in far greater antibody production than the PBS-thiosalicylic acid solution, which does not have a way to present the small dissolved haptens to the immune system. This control was used as the antigen for control mouse sera; the PBS-hapten solution measures the immune response against the hapten alone. The results of ELISA testing will indicate if RAH can be used instead of KLH in a variety of immunology applications.
RESULTS

Abalone Aquaculture

Seventeen of the total 42 abalone experienced mortality during this study. Five abalone succumbed to heat-stress during separate instances of temperature regulation malfunctions. All abalone were temporarily moved to several smaller enclosures for two weeks during system repairs in 2016. Seven abalone escaped these temporary enclosures and desiccated in the facility. The water quality, temperature, salinity, and light parameters of the primary and temporary housing were identical. The major difference was that the abalone were split into three groups housed in smaller circular tanks. All enclosures were open, as abalone had not been observed to escape before. It is unclear why the abalone left these aquaria, but none ever escaped from the circular-topped original tank throughout the entire study. The cause of death is unknown for five abalone that were found deceased in the enclosure during this study. None of the bled abalone experienced mortality.

The behavior of bled abalones was normal compared to that of wild and aquaculture animals. Abalone were nocturnal, active when hungry, and perceptive of motion in and around the tank. Their typical defensive posture is to withdraw into their shell and fasten themselves to the wall of the tank; the general vitality of an individual can be gauged by the vigor with which it can hold onto the tank wall. The health of seemingly lethargic abalone was routinely assessed via this method. The abalone exhibited a gradient of space utilization; many abalone would typically crowd one side of the tank while fewer were further away.

Abalones rapidly produce fecal matter, and excess food partially dissolves, necessitating consistent cleaning and vigilance. All abalones grew through the course of
the study. Nutrients from abalone feed are incorporated into the shell, and shell color is influenced by feed composition. The 2016 switch in feed resulted in a change of new shell growth color. Variations in water quality (i.e. a temperature increase to over 17°C or a nitrate concentration above 0.5 ppm) were rare and addressed immediately. Most abalone mortality was attributed to system malfunctions and escapes. This general aquaculture regime was therefore successful for 4 years of abalone maintenance. Abalone maintenance required 4 hours of direct care per week.

Hemolymph Extraction

The extraction of hemolymph was a skill honed and refined throughout this study. The hemolymph extraction protocol was reliable, but finding the pedal sinus is far more difficult in practice than in theory. The dimensions of each abalone are different, and the contour of muscles and viscera in this invertebrate continuously stretch, contract, or otherwise change the shape of the pedal sinus. If the needle is too shallow or too deep it will be buried in tissue and no hemolymph will be drawn. Once the needle is properly in the pedal sinus, however, hemolymph flows readily into the syringe. By the end of the study the pedal sinus could be found quickly, and the number of necessary punctures were greatly reduced. The four abalone that were bled during two experimental sessions had four months to recuperate between sessions.

Hemocyanin Isolation

The two-phase centrifuge regime of producing a cell-free supernatant, then a predominately hemocyanin pellet, was consistently successful. Abalone hemocyanin accounts for 90% of total hemolymph proteins (2). The hemocyanin subunits of other
abalone species are known to have a molecular weight of at least 350 kDa, which is significantly heavier than most other hemolymph components (2). Hemocyanin, therefore, pellets out of suspension before most other hemolymph components. Despite this, a minimum of 2.5 hours in the centrifuge at 15,000 g was required to start pelleting hemocyanin. The hemocyanin pellet is visually distinguishable by its opalescent blue color. The mass of the pellet was not determined. BCA analysis at this point in the purification process consistently reflected a hemocyanin concentration of approximately 3 mg/mL (Table 2).

AMPEG was designed to prepare KLH subunits for size exclusion purification methods such as polyacrylamide gel electrophoresis (PAGE) or gel filtration chromatography. This buffer selectively dissociates only one of the two hemocyanin subunits. The subunits are similar in size—one is approximately 350 kDa while the other is approximately 400 kDa. These subunits could not, therefore, be separated from each other by size exclusion purification methods without this step. AMPEG, however, has been known to occasionally denature all hemocyanin in solution and cause precipitate formation (11). This is usually triggered by temperature variation or excess agitation of the sample (11). All RAH samples prepared with AMPEG exhibited protein denaturation and precipitate formation; the solutions turned cloudy gray and fine white particulates formed. This reaction occurred when AMPEG was introduced via direct buffer exchange, overnight cassette dialysis, and buffer exchange spin columns. This may be an inherent reaction between AMPEG and RAH, or there may have been a handling issue with the introduction of this buffer.
The marine anticoagulant was a precautionary measure to prevent hemolymph from clotting against bacterial exposure in the hemolymph. Once the process of RAH purification was refined using the marine anticoagulant in the original sample Eppendorf tubes, the procedure was replicated omitting the marine anticoagulant. The same results were achieved, and the marine anticoagulant was deemed unnecessary.

Figure 3. Shows the standard curve generated for BCA analysis. Bicinchonic Acid (BCA) analysis was performed using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Catalog No. 23225).

Table 1. Shows the protein concentration determined via BCA of 6 representative hemocyanin samples (SPL1-SPL6). These were typical results when the hemocyanin concentration was measured after the RAH isolation protocol. The concentration was determined by the standard curve generated for BCA analysis; the spectrophotometric absorbance at 562 nm. Bicinchonic Acid (BCA) was incorporated into the equation \( y = 1.1551x - 0.0154 \) for the variable ‘x’. The hemocyanin concentration of *H. rufescenes* hemolymph is thus approximately 3 mg/mL.

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Hemocyanin Purification

The gel slurry used to prepare the gel filtration chromatography column took nearly six hours to ‘pack’ or settle, yet exhibited inconsistent compaction. Slight temperature
variations resulted in changes to the density of the slurry, interrupting the experiment. Bubbles often formed before the gel was caste, which interfere with the filtration. Stabilizing the flow rate through the column was consistently challenging, even when using a peristaltic pump. This original protocol included casting and calibrating the column in AMPEG, which was found to later interfere with the spectrophotometry. This interference was reported by other KLH researchers. Gel filtration chromatography was therefore eliminated as a viable means of RAH purification.

Native and SDS denaturing PAGE were successful for determining the subunit composition and molecular weight of RAH. RAH was the most prevalent protein in the samples, though consistent contaminants persisted prior to purification. The two RAH subunits were identified as being nearly identical in molecular weight, both at approximately 375 kDa. Often the protein bands of these subunits would appear as a single smudged band, but in rare cases the subunits were separated (Figure 5 and Figure 7). These samples were prepared the same way, so it is unclear what caused the subunit separation. Gel images showing the RAH subunits and contaminants are shown in Figures 4-7. The approximately 800 kDa protein band was suspected to be aggregates of RAH subunits, as a similar configuration is found in KLH research. Gel excision and protein elution of the 375 kDa and 800 kDa protein bands resulted in the concentration and isolation of 375 kDa protein with relatively little contaminates via PAGE. This shows that the 800 kDa protein band was indeed aggregated hemocyanin subunits. RAH samples that were ‘cleaned’ via serial centrifugal filtration with desalting spin columns were consistently purified from most contaminants. PAGE results of these samples clearly show RAH to be composed of two approximately 375 kDa subunits. The remaining contaminants were consistent through
all samples, though less concentrated. In one instance the eluted protein sample from excised 375 kDa and 800 kDa bands was subjected to the ‘cleaning’ process, resulting in the most purified sample of the study.

Commercial KLH from the Imject™ kit was compared to the most efficiently ‘cleaned’ RAH samples on one native PAGE gel (Figure 6). The resulting gel showed the two KLH subunits were separated in size by about 50 kDa, while the two RAH subunits were closer in molecular weight. Both RAH subunits were intermediate to the size range of the two KLH subunits. Both KLH subunits function as effective hapten-carriers, meaning the RAH subunits are in the proper size range for an alternative hapten-carrier. Both experimental RAH and commercial KLH contained the same contaminants, in similar concentrations. This gel is shown in Figure 6. This RAH purification rivals industry grade KLH purification for hapten conjugation.
Figure 4. Native PAGE of RAH samples. Lanes 1: Crude RAH. 2: ‘cleaned’ RAH. 3: molecular weight markers (RAH is between 480 kDa and 242 kDa).

Figure 5. SDS denaturing PAGE of RAH and KLH samples. Lanes 1, 2, 3, 4, 6, 7, 9, 10: ‘cleaned’ RAH after one cycle of filtration via desalting spin columns. Samples become more purified after three cycles. 5: molecular weight markers (RAH is just below 400 kDa). 8: crude (unprocessed) hemocyanin (raw hemolymph). Clear separation of two RAH subunits are shown in wells 9 and 10.

Figure 6. Native PAGE of RAH and KLH samples. Lanes 1, c, d, e: KLH (Commercial hapten carrier). 2-9, a, b: ‘cleaned’ RAH. Both RAH subunits are between KLH subunits in size. Commercial KLH and experimental RAH have similar impurities.

Figure 7. Native PAGE of RAH samples. Lanes 1, 3, 4: ‘cleaned’ RAH. 2, 6, 7: excised and eluted RAH. 5: molecular weight markers (RAH is between 480 kDa and 242 kDa). 8-10: crude (unprocessed) hemocyanin (raw hemolymph). Smudged separation of two RAH subunits are shown in wells 8 and 10.
Hapten-carrier Efficacy

Thiosalicylic acid was conjugated to KLH and RAH respectively using the Imject™ conjugation kit. Control samples of thiosalicylic acid in PBS were prepared in the same way as the protein samples and ensured to contain the hapten. These solutions were successfully shipped to PFRAL. PFRAL performed a BCA assay on the samples prior to beginning their analysis, to confirm their protein concentration. The KLH sample was expected to have a concentration of 3.9 mg/mL and was found to have a concentration of 3.6 mg/mL. The RAH sample was expected to have a concentration of 1.3 mg/mL and was found to have a concentration of 1.25 mg/mL. The conjugation process had high efficiency.

Sera was collected from all six mice at days 0 (prior to injection to establish a baseline of inherent antibody production against the hapten), 21, and 28 of the incubation period. PFRAL identified the day-28 samples as being the most accurate gauge of antibody production. Therefore, only day-28 ELISA results are reported. Sera was tested using the other hemocyanin-hapten conjugated solution as the antigen, i.e. ELISA was performed on KLH-thiosalicylic acid conjugate injected mouse sera using RAH-thiosalicylic acid solution and vice versa. The titer steps were determined by diluting the sera 1:300 in PBS. Serial threefold dilutions of this solution are used to create establish the titers (300, 2,700, 8,100… 1,986,000). The maximum titer (dilution factor) of the sera that reacts to the antigen is a measure of antibody production. These titers are compared as ranked ‘steps’ (i.e. the first titer of 1:300 is the ‘1st titer step’, while a titer of 24,300 is the ‘5th titer step’). Each ‘titer step’ represents a geometric threefold difference; three ‘titer steps’ is a 27-fold difference and so on.
Serum samples were collected from all six mice on May 11\textsuperscript{th}. Sera was shipped on ice to the University of Maine and has been stored for potential future analysis. The hapten-carrier solutions were prepared at different concentrations but were standardized for the hemocyanin mass in each by PFRAL. A 200 ug injection of hapten-carrier solution was administered on day 1 of incubation (this first ‘day’ beginning at the moment of injection), and 100 ug injections were administered intermittently through the study through the consistent PFRAL procedure. All injections were prepared to have a concentration of approximately 1 mg/mL. The KLH-thiosalicylic conjugated solution originally had a concentration of 3.5 mg/mL. 0.057 mL and 0.029 mL of this sample were mixed with 0.15 mL of adjuvant and brought to a volume of 0.25 mL per animal with PBS, respectively for the 200 ug and 100 ug injections. The RAH-thiosalicylic conjugated solution originally had a concentration of 1 mg/mL. 0.2 mL and 0.1 mL of this sample were mixed with 0.15 mL of adjuvant and brought to a volume of 0.25 mL per animal with PBS, respectively for the 200 ug and 100 ug injections. The control solution of thiosalicylic acid in PBS had an original hapten concentration of 2mg/mL. 0.1 mL and 0.05 mL of this sample were mixed with 0.15 mL of adjuvant and brought to a volume of 0.25 mL per animal with PBS, respectively for the 200 ug and 100 ug injections. Antibody production was assessed via an ELISA procedure performed in duplicate for each dilution and included a no-antibody control to ensure the absence of an independently reactive agent in the antigen solution.

The first KLH-thiosalicylic acid conjugate treated mouse reacted until the 9\textsuperscript{th} titer, a dilution of 1:1,968,300. The second KLH-thiosalicylic acid conjugate treated mouse reacted until the 8\textsuperscript{th} titer, a dilution of 1:656,100. The first RAH-thiosalicylic acid conjugate treated mouse reacted until the 6\textsuperscript{th} titer, a dilution of 1:72,000. The second RAH-
thiosalicylic acid conjugate treated mouse reacted until the 5th titer, a dilution of 1:24,300.
No reaction occurred for any dilution of the PBS-thiosalicylic acid treated mice sera. The
shows that KLH is potently immunogenic, RAH is moderately immunogenic, and
thiosalicylic acid without a carrier is not immunogenic. This analysis was for ‘proof-of-
concept’ and the study was not designed large enough to be statistically sound. Methods of
statistical analysis were therefore not performed; only preliminary trends were observed.
The ELISA results are shown in Figure 8.

Figure 8. Shows the antibody response, in titer dilutions, to thiosalicylic for the KLH, RAH, and
PBS carrier-treated mice. The antibody titers produced in two KLH-hapten treated mice were
1,968,000 and 656,000, 9 and 8 dilutions respectively. The antibody titers produced in two RAH-
hapten treated mice were 72,000 and 24,000, 6 and 5 dilutions respectively. No antibodies were
produced against thiosalicylic acid in both PBS-hapten treated mice.
DISCUSSION

Abalone maintenance at ARC provided insight to strategies and issues for their aquaculture. The general protocol was successful; water quality, temperature, salinity, flow rate, and enclosure lighting were conducive to abalone health and growth. These parameters were consistent through most of the study, with few deviations due to system malfunctions or reconfigurations. The mortality of abalone due to heat shock shows that the upper thermal tolerance of this species is 17°C. Exposure to this warmer water resulted in mortality after 2 days in each instance. Therefore, a commercial abalone production facility must maintain water temperature below 17°C and have contingency plans to cool the water quickly in emergency situations.

No abalone escaped from the open-topped original tank, nor were any observed above the water-line. The seven abalone that escaped the temporary housing exhibited no erratic behavior prior to their escape. All seven escapees left their tanks in a period of 4 days. No abnormal water quality, temperature, salinity, or other parameters were observed for that period. Abalone hatchery protocols suggest that abalone prefer tanks with corners and straight sides, as supported by experimental observations (21). Netting was used over these temporary tanks after the 4 days of escape to eliminate the possibility of further loss. Netting was also used when the abalone were returned to the original tank, but was removed when no abalone was observed above the water-line for two weeks.

The five abalone that died of unknown causes were always the smallest (in shell length) of the group. There is a possibility that they were out-competed for food, but some of the bodies displayed symptoms of Withering Foot Syndrome (WFS). This disease is
caused by the bacterium *Candidatus xenohaliotis californiensis*, which infects the abalone digestive tract and inhibits digestion enzyme production (22). The body mass of the abalone rapidly diminishes as it starves (22). Observed symptoms included this shrinking of abalone foot and mantle size. WFS is a global problem for abalone harvest in the wild and aquaculture, and especially common in California (22). The abalone most likely were infected with WFS before arrival at ARC.

Most abalone losses were clearly attributed to maintenance issues, and the remaining losses were likely natural or caused by a common industry pathogen. General parameters such as 35-38 ppt salinity, 12°C water, and thresholds for molecules such as ammonia, nitrite, and nitrate were established for long-term abalone maintenance. This is an important result, showing that abalone maintenance is feasible in recirculating aquaculture settings. Abalone farms are typically coastal and use seawater directly from the local environment. Sea water is pumped directly into these facilities, then returned to the environment. This is a high-risk process; toxic algae, pollution, diseases, and parasites are often introduced to farmed abalone via the natural sea water. The global abalone farming industry struggles with sometimes catastrophic crop losses due to wild diseases, like WFS and parasites such as shell-boring annelids, introduced through the natural sea water (21). Recirculating aquaculture allows the producer to have more control of what is or is not in the water. Abalone raised in recirculating conditions are thus safer, and the process is more stable. This study shows that abalone can be housed in long-term recirculating conditions, with artificial sea water. Assessing the efficacy of recirculating aquaculture maintenance is critical to the development of an abalone farm in Maine, because abalone cannot be exposed to natural waters in this state. As a foreign species from
the Pacific, it is illegal to allow abalone any means of escape, whether larval or adult, into Maine waterways. These abalone, and any diseases or parasites they carry, may become invasive species if released in Maine, damaging the local ecosystem. An abalone farm in the state would therefore be responsible for preparing and disposing its own artificial seawater in a way that does not introduce abalone material to the natural environment.

Hemolymph extraction was initially challenging, though refined into a skill through this study. Locating the pedal sinus on an abalone diagram and a live specimen are different endeavors. The ice-bath method of cooling the abalone prior to hemolymph extraction was essential to successful hemolymph extraction. It is nearly impossible to extract hemolymph from a moving specimen. None of the abalone bled through the study died or displayed adverse health effects. 2 mL of hemolymph is therefore a ‘safe’ volume for extraction; this process is not anticipated to kill or harm a healthy abalone.

The hemocyanin-isolation process was modeled directly from KLH literature. The initial 30-minute 800 g 4°C centrifugation was consistent in the literature and shown to effectively remove whole cells and large debris from the sample. The second centrifugation step, however, was experimentally refined. The first samples of this study were centrifuged at 15,000 g and 4°C for 30-minute intervals. At the end of each interval the samples were checked for the formation of a visible hemocyanin pellet. The pellets consistently formed after 2.5 hours at this speed, but were visibly larger after an additional interval. 3 hours was therefore determined to be the standard centrifugation time for hemocyanin pellet formation in subsequent experimentation.

The hemocyanin concentration after centrifugal isolation and resuspension in HSB was approximately 3 mg/mL (Table 2). The normal hemocyanin concentration in red
abalone hemolymph is thus approximately 3 mg/mL. The KLH concentration of *M. crenulata* hemolymph is also approximately 3 mg/mL. This is the first experimental similarity between RAH and KLH: the concentration of each protein is the same in the hemolymph of the respective species. Theoretically, therefore, the same amount of RAH and KLH can be produced from equal hemolymph volumes from each species.

Removing the marine anticoagulant from the refined protocol had no negative effect on the final RAH purity. Use of the marine anticoagulant was therefore discontinued. Abalone exhibit a slow clotting process, and therefore an anticoagulant is not necessary.

I could not adequately configure gel filtration chromatography to purify the RAH. Methods were designed from Harris *et al.* 1995 and followed carefully (19). The gel slurry would settle inconsistently and change density during column calibration, despite using the recommended materials. Issues with column preparation, such as bubble formation, remain mysterious. Various methods of troubleshooting, including changing the experimental temperature of the column and mobile phase and the use of a pump to control the flow rate, were unsuccessful in standardizing successful column setup. Control proteins of known molecular weight could not be consistently recorded.

Some RAH samples were exposed to AMPEG prior to GFC so that one subunit could be separated from the other. AMPEG, however, interferes with spectrophotometer function, preventing protein identification and analysis. This problem was reported by other KLH researchers, who had to use more complex means of hemocyanin identification after GFC elution (11). These issues suggest that neither AMPEG nor GFC are useful in RAH purification.
Native and SDS PAGE of crude (unprocessed) *H. rufescenes* hemolymph confirms that RAH is the most abundant hemolymph component. Serial centrifugation of isolated RAH successfully removed most contaminants, and drastically reduced the concentration of others. This ‘purified’ RAH was shown to be composed of two subunits, approximately 375 kDa each. Sometimes the subunits appeared as a single smudge, and other times there was clear separation. This occurred in samples prepared and handled the same way, so it is unclear what caused the occasional separation, which was observed in native and denaturing settings.

KLH is also known to be composed of two subunits. One subunit is approximately 350 kDa, while the other is approximately 400 kDa. Industry grade KLH for hapten-carrying uses a mixture of both hemocyanin subunits. The emerging vaccines typically utilize only one subunit, but both are effective. There is no biochemical need to isolate one subunit for vaccine production, especially since KLH aggregates are typically sorted by subunit (2). RAH is therefore within the molecular weight range of KLH formulations for hapten-carrying and vaccine production.

‘Purified’ RAH was directly compared to a commercial formulation of KLH hapten-carrier via native PAGE. The results show that both RAH subunits are between the two KLH subunits in molecular weight. This suggests that both RAH subunits are viable alternative hapten-carriers, alone or together. The commercial KLH and experimental RAH were shown to have similar contaminants. The non-hemocyanin protein bands match from both types of samples. The RAH purification protocol therefore succeeded in producing RAH that rivals commercial KLH in purity.
The PFRAL assessment of antibody production via ELISA shows that RAH is a viable hapten carrier. A solution of thiosalicylic acid in PBS was administered to mice as a negative control. No antibodies whatsoever were raised against thiosalicylic acid in these mice. Thiosalicylic acid is therefore a proper hapten, it is too small to elicit an immune response. Mice treated with the RAH-thiosalicylic acid conjugate, however, exhibited antibody production of 5 to 6 titers. This was described as a moderate antibody response by scientists at PFRAL. The KLH-thiosalicylic acid conjugate treated mice exhibited the greatest antibody production in this study. The titers for KLH-treated animals were 2 to 3 titers higher than the RAH-treated animals, a 9 to 27-fold difference, respectively. KLH is therefore more potent than RAH as a hapten carrier. For immunological applications, however, RAH may still be useful. The supervisor for this project at PFRAL explained that while RAH did not perform at the same level as the KLH, it did provoke a reasonable immune response. This scientist elaborated that if the present results are corroborated with a more statistically sound study using an industry standard hapten, RAH would be a suitable alternative to KLH for antibody production.

It is possible that the similarity of sizes between the RAH subunits contributed to the lesser hapten carrier efficacy compared to KLH. KLH subunits are ~350 kDa and ~400 kDa, there is a ~50 kDa difference between their sizes. RAH subunits were found to both be ~375 kDa. The KLH subunits aggregate either in groups of just KLH1, or predominately KLH2 with some KLH1 present (14). The less distinct RAH subunits may all aggregate together, leaving less surface area available for hapten conjugation than that of the two aggregate assemblies of KLH. This can be tested through the identification and implementation of a selective dissociation buffer for one of the RAH subunits, as has been
developed for KLH. It is also possible that in some commercial applications, a moderately immune-stimulating molecule may be preferable to one of the strongest known immune-stimulating molecules. RAH is therefore immunogenic enough to be a viable alternative hapten carrier, but also can be used for other purposes that KLH is too potent for.
CONCLUSION

Sustained *H. rufescenes* maintenance is feasible in recirculating aquaculture. Netting or a cover is recommended for abalone housing. Heat stress begins at approximately 17°C in *H. rufescenes* and can be fatal within 48 hours. Response protocols to temperature variation are therefore critical to responsible abalone maintenance. Hemolymph extraction of 2 mL does not negatively impact overt abalone health. Hemocyanin concentration in *H. rufescenes* hemolymph is approximately 3 mg/mL, the same as in *M. crenulata* hemolymph. Successful protocols for RAH isolation and purification were created through this study. Serial centrifugation of isolated RAH with desalting spin columns removes most contaminants after three cycles. This processed RAH rivals commercial KLH in purity. RAH was found to be composed of two subunits, each approximately 375 kDa. KLH is also composed of two subunits, 350 kDa and 400 kDa respectively. Both KLH subunits are trusted hapten-carriers, and a mixture is often used in immunology applications. RAH therefore has the proper structure and molecular weight to be a competitive substitute to KLH. Hapten conjugation to both hemocyanin types shows that RAH is an effective hapten carrier. RAH successfully initiated a moderate immune response to a hapten that was otherwise non-immunogenic. Industry evaluation considers the RAH-mediated immune response to be suitable for use as an alternative hapten carrier to KLH. The KLH-mediated immune response was 2 to 3 titers greater than that of RAH. The less potent immune reaction to RAH may be useful in commercial circumstances. The viability of abalone maintenance in recirculating aquaculture, development of successful RAH processing techniques, and comparable biochemical characteristics suggest that RAH
may be a successful alternative hapten-carrier to KLH in vaccine production. The global aquaculture of *H. rufescenes* is an additional advantage to RAH production.
Maine is the ‘seafood state’ and excels in aquaculture. The Maine seafood market has a history of successfully introducing new food products to the East Coast, then throughout the United States. A major expense for abalone farms is feed; abalone eat seaweed and algae. Maine is a leading East Coast state in seaweed aquaculture and wild harvest. Partnership with local seaweed producers provides a unique opportunity for reducing the cost of abalone feed in Maine. There is precedent and interest for abalone aquaculture in Maine; the Maine Technology Institute awarded $17,000 to a start-up abalone farm in 2012 (22). Dominant New Zealand abalone farms were concerned that abalone aquaculture would be successful in the state and recruited the leading entrepreneur from the MTI grant to New Zealand, preventing development in Maine. The CEO of Campbell Science, an abalone beta-gulcuronidase distributor, laments that there are no abalone farms capitalizing on biomedical products, adding that the East Coast would be an ideal market location. Other abalone products, such as meat and pearls, would enable consistent profitability while RAH undergoes approval for immunology and vaccine applications. Business incubation space was secured at the Mount Desert Island Biological Laboratory in 2018 for the development of an abalone farm in Maine. Business and scientific developments are advancing rapidly. The mission of this company is to sustainably develop biomedical products from marine invertebrates while utilizing the entire organism to maximize market diversity and minimize waste.
REFERENCES


APPENDICES
Appendix A: KLH and Abalone Hemocyanin Biochemistry

Hemocyanin, stemming from the Greek ‘haima’ meaning blood, and ‘kyanus’ meaning blue pigment, is a respiratory protein found commonly in invertebrates (8). This large glyco-metallaprotein, discovered by French naturalist Leon Fredriq in 1878, is responsible for the transportation of oxygen molecules through arthropods and mollusks (8). There are typically 7 or 8 oxygen binding sites on the protein, dubbed functional units, where binuclear copper is bound to a branching side chain of amino acids and readily binds with oxygen molecules brought in by the respiratory system (23). The oxygenated copper has a light absorbance near 340 nanometers, causing the rich opalescent blue color of mollusk blood, or hemolymph (24). Dull grey samples of hemolymph indicate denatured proteins (2). The Functional Units are referred to as ‘functional unit a-h’, depending on the hemocyanin type (23). In KLH the functional units are made up of dimeric morphological units, with individual molecular masses of 45-65 kDa, and themselves make up three-tiered decameric subunits of the protein (24). These subunits comprise hollow cylindrical oligomers with molecular masses of 350 to 450 kDa and external diameters of 3.7 nanometers (25). Two or more of these decamer structures have the propensity to aggregate and adjoin “face to face with the collar complexes at each end” to form didecamers and multididecamers (26). This is the native form of hemocyanin (26). The didecamers of gastropod hemocyanin can have total height of 40 nanometers and a mass of 7.5 to 8 MDa (24). Molluscan hemocyanins are the largest oxygen-carrying proteins in the animal kingdom (4). Hemocyanin production in gastropods is influenced by organism strength and stress, as well as water temperature, pH, cations, O₂, calcium and magnesium (27).
A complete KLH didecamer is comprised of two isomorphic decamer subunits, dubbed KLH1 and KLH2 (25). These subunits are 60% identical at the protein level (23). Both KLH1 and KLH2 have 8 distinct Functional Units, each replicated 20 times through the manifolds of the decamer (24). KLH1 has a molecular mass of 390 kDa, whereas KLH2 has a molecular mass of 360 kDa (25). Recent studies suggest that captive Keyhole Limpets have drastically reduced KLH1 concentrations, and some individuals lack KLH1 altogether (26). In most aquaculture circumstances, KLH1 and KLH2 exist in a 1:2 ratio within each individual Keyhole Limpet (26).

Mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine were shown to be present in KLH1 by gas liquid chromatography (16). Fucose was also detected in KLH2 (16). Both KLH1 and KLH2 are 4% carbohydrate by mass (16). This unique carbohydrate composition contributes to the immune-stimulating power of KLH (2).

Conveniently, KLH2 dissociates into its functional units in an environment of pH of 5.7 to pH 5.9 (14). KLH1, however, stays intact in these acidic solutions (26). This allows for the separation of KLH1 from KLH2 with size exclusion methods (26). The KLH1 can be extracted and the remaining KLH2 can be re-associated by increasing the solution pH (26). The source of KLH biosynthesis has not yet been identified. Little genomic work on M. crenulata has been conducted. Recent interest in molluscan hemocyanin has begun to produce research on abalone hemocyanin as well (4).

Hemocyanin from abalone species such as Haliotis tuberculata, Haliotis rubra, and Haliotis diversicolor have been studied extensively in recent years (4). These hemocyanins are known to be produced by pore cells and some have been genomically sequenced (28, 29). These hemocyanins are also composed of two subunits, both approximately 390 kDa.
(2). Hemocyanin from these abalone species have similar carbohydrate composition to KLH (4). *H. tuberculata* hemocyanin has been shown to have comparable structural integrity to KLH but does not dissociate as well in reduced pH environments (14). This abalone hemocyanin is therefore considered more stable than KLH in dissociating conditions (14).
Appendix B: Comparative Fishery Considerations

The giant keyhole limpet has a narrow population range, from the coastlines of northern Mexico to northern California (30). Wild populations have been overfished for KLH production (31). Giant keyhole limpet aquaculture is limited to one company (12). Most techniques and innovations for giant keyhole limpet aquaculture are proprietary (12). Giant keyhole limpets are omnivorous and require a more protein-rich diet than other marine gastropods (30). Aquaculture feed for the giant keyhole limpet is therefore more expensive to produce than plant-based feeds.

The red abalone has a large population distribution, from the coastlines of southern Mexico to northern Canada (31). Wild populations are stable, and commercial aquaculture has maintained continuously lucrative production (31). There are many companies that aquaculture red abalone, yet the techniques and innovations for this process are accessible to new businesses (32). Red abalone are herbivorous, and their feed is relatively cheap (32). Red abalone is therefore a more ecologically sustainable and economically sensible aquaculture species than the giant keyhole limpet.
Appendix C: Market Evaluation of Limpet and Abalone Products

Red abalone aquaculture is globally established and successful (33). Abalone meat is a delicacy in many parts of the world (34). Approximately 250 tons of red abalone are produced in the United states per year, predominately from California and Hawaii (30). This annual production is valued to exceed $2 million (33). Red abalone is particularly profitable in the seafood industry, with a typical market price of $70/kg (33). Abalone shells are also prized for the gemstone ‘Mother-of-Pearl’ (12). This unique form of nacre is valued in fashion, design, and artisan products (34). Aquacultured red abalone can be induced to form horn-shaped pearls, with the same distinct iridescent blue and green pattern (34). These pearls often sell for more than $400 each (34). These shells and other aspects of abalone biology are considered to have great biotechnological value.

Abalone shells are also coveted by structural scientists studying material strength (35). MIT and DARPA study abalone shells for inspiration and emulation in new ceramics (36). Abalone entrails are the primary source of beta-glucuronidase, an enzyme used in toxicology and drug testing. This commercially important abalone biotechnology is valued at $20/mL. Abalone hemocyanin has also been found to exhibit anti-viral qualities, prompting its use in emerging herpes medications (37). Preliminary research shows that abalone hemocyanin mitigates herpes proliferation, offering a new approach to patient treatment. Limpets, including M. crenulata, have no commercial value beyond KLH (4).
AUTHOR’S BIOGRAPHY

Isaiah N. Mansour was born in Fairfield, Connecticut on July 19th, 1995. He was raised in Fairfield, Connecticut and graduated from Fairfield Warde High School and the Bridgeport Regional Aquaculture Science and Technology Education Center in 2012. Majoring in Marine Sciences, Isaiah has a dual concentration in Marine Biology and Aquaculture, with a minor in Fisheries Sciences. Isaiah is a member of the Alpha Tau Omega leadership development Fraternity. Isaiah has contributed to campus life at UMaine through his involvement in Student Government (Vice President of Student Entertainment, 2015-2017), the Jazz Ensemble (bass player, 2013-2016), the Sophomore Owls (2014-2015), and the Honors College. In his free time Isaiah enjoys reading, playing bass, and meditation. Isaiah has received funding for this project through the Center for Undergraduate Research (CUGR, 2014), Maine EPSCoR SEANET (2015), the NASA Maine Space Grant Consortium (2016), INBRE Functional Genomics Junior Research Fellowship (2016), The Mount Desert Island Biological Laboratory (MDIBL, 2016, 2017), The Carolyn E. Reed Thesis Fellowship (2017), and the Charlie Slavin Thesis Fellowship (2018). Upon graduation Isaiah will be working as a research assistant in the Yin Laboratory at MDIBL. Isaiah has also secured business incubation space at MDIBL to pursue the establishment of an abalone farm in Maine.