Optimizing an Approach for the Identification of Antimicrobial Peptides in the American Lobster, *Homarus americanus*, via Transcriptomics and LC-MS/MS-Based Proteomics

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The decapod crustacean, *Homarus americanus* (commonly known as the American lobster), is an immensely valuable species as an economic resource in the coastal Northeast. In the state of Maine, lobster harvesting alone was a near $500 million industry in 2015. However, anthropogenic challenges, such as global warming and pollution, have been implicated as physiological stressors capable of impairing the lobsters' immune response[1]. The infamous Long Island Sound Lobster Mortality Event of 1999 was one of the first studied major lobster die-offs that scientists correlated to above-average water temperatures, which contributed to an inhospitable environmental condition capable of suppressing the lobster immune response against the protozoan parasite, *Paramoeba*[2,3]. The lasting effects of the mortality event are still present today in Long Island Sound as the lobster harvest continues to decrease to just one-sixth of the pre-1998 levels[4]. Given the economic importance of lobster fishing in the Northeast and the potential future anthropogenic challenges, it will be important to first identify at a molecular level the biological components driving lobster immune response.

Antimicrobial peptides (AMPs) are a major biological component contributing to the lobster's first line of immune response via nonspecific defense against a variety of pathogens. Structurally, AMPs are small, cationic, and amphipathic peptides endogenously synthesized with molecular weights of approximately less than or equal to 10 kDa and are composed of 15-100 amino acids. Biochemically, AMPs function against pathogens, such as bacteria, via an initial electrostatic attraction between its cationic portion and the negatively charged phospholipid bilayer of the bacterial membrane. At a closer proximity, the amphipathic structure of the AMP can then subsequently permeabilize the membrane to reduce bacterial integrity and overall survival.

AMPs function within a biological pathway called the prophenoloxidase (proPO) system, which is a major hemocyte driven immune response pathway activated committedly upon pathogen recognition. Crustaceans have three types of hemocytes located in its hemolymph known as hyaline, semi-granular, and granular cells; the latter two distinctively contain vacuoles[6]. AMPs are located in these vacuoles and, downstream of the proPO system activation, these semi-granular and granular cells can be promoted to exocytose its vacuoles and release AMPs to mount an immune response[7,8].

The Stemmler Lab has identified two types of AMPs in the lobster (a *defensin* and a family of *crustins*) via a joint approach of transcriptomics and liquid-chromatography tandem mass spectrometry-based (LC-MS/MS) proteomics[9]. However, the former approach to sample preparation was crude because the hemolymph extracted from the lobster was immediately heated at 100 °C to avoid coagulation and proteolytic degradation. While AMPs were detected, this crude approach resulted in protein/peptide loss and it was unclear if plasma or hemocytes were the source of the detected AMPs.

This summer, I have optimized an approach that avoids heating the hemolymph, prevents coagulation, extracts greater amounts of proteins/peptides of interest, and allows for further downstream application and instrumental analysis. My methods include the use of an anticoagulate[10] added to the hemolymph upon extraction, which allows the hemocytes to be pelletized and subjugated to lysis for protein/peptide extraction via homogenization in conditions of urea and ammonia bicarbonate. These solvent conditions also allow for later preparatory steps such as reduction, alkylation, and trypsin digestion prior to LC-MS/MS analysis[11]. Microscopy and cellular staining via methylene blue confirmed that my modified approach successfully isolated the intact hemocytes (Fig. 1). Biochemical techniques such as a Lowry Assay and SDS-PAGE confirmed quantitatively and qualitatively respectively that my approach extracted significantly more proteins/peptides than the prior approach (Fig. 2). Furthermore, SDS-PAGE also demonstrated that my lysis conditions of urea and ammonia bicarbonate were more suitable for the extraction of lower molecular weight proteins/peptides such as AMPs relative to commercial lysis buffers. Future directions will be to combine SDS-PAGE and LC-MS/MS via in-solution or in-gel digestion protocols, density centrifugation to separate the hemocytes by size and shape, and chromatography to separate proteins/peptides for sample cleanup prior to instrumental analysis.

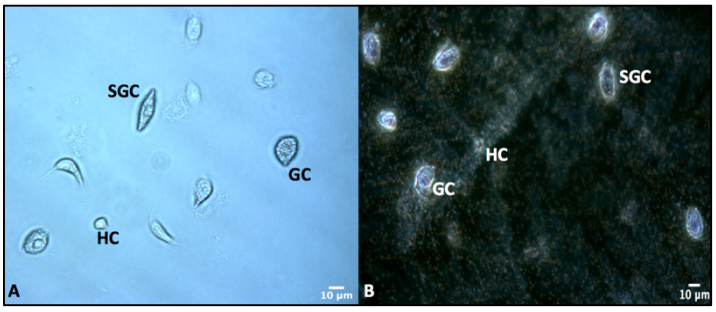

Figure 1. Hemocytes from the hemolymph of *Homarus americanus*. **(A)** Hyaline (HC), semi-granular (SGC), and granular (GC) hemocytes. **(B)** Hemocytes stained with methylene blue. (Total Magnification = 400x, Scale Bar = 10 µm)

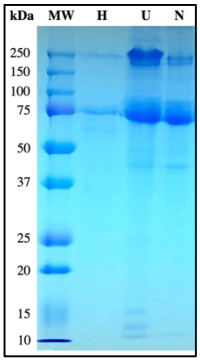

Figure 2. SDS-PAGE (4-12% acrylamide) gel fixed with Coomassie blue protein staining of the heated hemolymph (H) and proteins extracted with urea (U) or a commercial lysis reagent (N). U demonstrated the greatest protein extraction capability as evident by the overall band coverage and signal intensity and of particular importance for this study, the isolation of proteins/peptides <15 kDa such as antimicrobial peptides.

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