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Short communication

Antimicrobial activity in the cuticle of the American lobster, *Homarus americanus*

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ABSTRACT

American lobster, *Homarus americanus*, continues to be an ecologically and socioeconomically important species despite a severe decline in catches from Southern New England and Long Island Sound (USA) and a high prevalence of epizootic shell disease in these populations. A better understanding of lobster immune defenses remains necessary. Cuticle material collected from Long Island Sound lobsters was found to be active against a broad spectrum of bacteria, including Gram-negative and -positive species. The antimicrobial activity was characterized by boiling, muffling, and size fractioning. Boiling did not significantly reduce activity, while muffling did have a significant effect, suggesting that the active component is organic and heat stable. Size fractioning with 3 and 10 kDa filters did not significantly affect activity. Fast protein liquid chromatography fractions were also tested for antimicrobial activity, and fractions exhibiting protein peaks remained active. MALDI mass spectrometry revealed peptide peaks at 1.6, 2.8, 4.6, and 5.6 kDa. The data presented suggest that one or several antimicrobial peptides contribute to antimicrobial activity present in the American lobster cuticle.

Lobsters, crabs, and other large crustaceans are ecologically important organisms, as they act as predators, scavengers, prey, and habitat modifiers [4]. Large crustaceans, especially the American lobster (*Homarus americanus*), are also of socioeconomic importance. American lobster is harvested on the continental shelf and continental margin of northeastern North America and has historically been one of the most valuable fisheries in coastal northeastern United States and the Canadian Maritime Provinces. In 2012, The U.S. lobster landings were 68 thousand tons, which was worth $424 million [18].

Since 1998, lobster landings in Southern New England (Massachusetts, Rhode Island, and Connecticut, USA) and Long Island Sound (New York, USA) have sharply declined and have not shown signs of recovery [16,23]. The decline of the Southern New England lobster fishery began dramatically with the 1999 lobster mortality event in western Long Island Sound. The lobster population and fishery has continued to be challenged by disease, particularly epizootic shell disease (ESD), a disease causing lesions in lobster cuticle [23]. The lesions make lobsters unmarketable and cause economic losses in the commercial lobster fishery [24]. When lesions become severe and affect the underlying membrane and soft tissue, they allow opportunistic infection, prevent proper molting, and potentially cause death [6].

In order to understand diseases affecting lobsters and other ecologically and economically important crustaceans, it is necessary to have a comprehensive understanding of their immune defenses. Like other invertebrates, crustaceans do not have adaptive immune capabilities and therefore rely on their innate defense mechanisms [25]. Traditionally, the invertebrate exoskeleton has been viewed as a nonliving, purely mechanical barrier against invading pathogens [27]. There is reason to believe that the exoskeleton plays a larger role in protecting the organism against infection, however, as phenoloxidase activity has been observed in arthropod exoskeletons [2,3,7,9]. Additionally, antimicrobial activity was found in the cuticle of northern shrimp and several crab species [11] and [13] reported antimicrobial activity in the shells of American lobster. In this study, we further document and characterize antimicrobial activity in the cuticle of the American lobster.

Eight lobsters were collected from western Long Island Sound and their carapaces were removed, scraped clean, rinsed with deionized water, dried and stored at −80 °C. A pooled water-soluble shell extract was prepared based on the procedure of [5]. Briefly, equal parts of shell from the 8 lobsters were frozen with liquid nitrogen and ground to a fine powder with a coffee grinder.

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Two grams of shell powder were added to 8 ml of potassium phosphate buffer (0.05 M, pH 7.2) and kept on ice while mixed for 1 h. After centrifugation (2500 g, 1hr, 4 °C), the supernatant was sterilized by filtration (0.22 μm) and stored at −80 °C until use, typically within 1 week.

To establish antimicrobial activity in the shell, the shell extract was tested in a photometric antimicrobial assay based on the methods of [19] and adapted by Ref. [13]. Bacterial suspensions were prepared by inoculating sterile marine broth (Difco 2216, BD Biosciences, Franklin Lakes, New Jersey, USA) with a single colony of a particular bacterial species (grown on marine agar, Difco 2216) and incubated overnight at 37 °C on an orbital shaker (200 rpm). Bacterial cells were rinsed three times by spinning the suspensions at 300 g, 28 °C, for 5 min, discarding the supernatant, and resuspending the pellet with sterile phosphate buffered saline (PBS, 0.1 M, pH 7.4). After rinsing, the bacterial suspensions were diluted with sterile PBS to an OD_{570} of 0.1. Aliquots of 10 μl sterile shell extract were combined with 10 μl of bacterial suspension, and 30 μl of sterile PBS in sterile 1.5 ml microcentrifuge tubes. Three negative controls included: (1) 50 μl of PBS; (2) 40 μl of PBS and 10 μl of potassium phosphate buffer; and (3) 40 μl of PBS and 10 μl of shell homogenate. The positive growth control contained 10 μl of bacterial suspension, 10 μl of potassium phosphate buffer, and 30 μl of PBS. Assays were incubated at 28 °C for 30 min prior to the addition of 450 μl of ice cold marine broth. One hundred μl were transferred from each treatment to a well in a clear, flat-bottomed, 96-well microtitre plate (Falcon, Franklin Lakes, New Jersey, USA) and an initial absorbance reading was taken using a Wallac micro-plate reader (Wallac 1420 Multilabel Counter: Perkin Elmer, Wellesley, Massachusetts, USA) at 570 nm. Treatments were incubated at room temperature for 48 h before 100 μl of each treatment were transferred to a well in a new 96-well microtitre plate and the absorbencies were read. Antimicrobial activity was evaluated by calculating the percent decrease in bacterial growth from the positive control for each treatment.

The sterile shell extract possessed antimicrobial activity against several bacteria: *Vibrio parahaemolyticus* and *Vibrio anguillarum* (both being halophilic Gram-negative; from the culture collection at Stony Brook University's Marine Animal Disease Laboratory), *Escherichia coli* D31 (Gram-negative; from the culture collection at Yale University [17]), and *Staphylococcus aureus* (Gram-positive, ATCC 25923). The percent decrease in growth was highest in *S. aureus* (58 ± 5%, OD_{570} 0.13 ± 0.02 to 0.05 ± 0.01) and lowest in *V. anguillarum* (33 ± 5%, OD_{570} 0.18 ± 0.03 to 0.12 ± 0.01). The percent decrease in growth of *E. coli* was 48 ± 5% (OD_{570} 0.13 ± 0.02 to 0.05 ± 0.01). The overall growth of *V. parahaemolyticus* in controls was highest and the shell extract caused a 55 ± 3% decrease (OD_{570} 0.55 ± 0.01 to 0.25 ± 0.01) in the growth of this species (Fig. 1). *V. parahaemolyticus* was therefore used in the proceeding assays designed for the characterization of the antimicrobial activity. The activity against *V. parahaemolyticus* was seen to be dose dependent with a serial dilution of the extract decreasing in activity in a linear fashion ($R^2 = 0.90$, data not shown).

To determine heat sensitivity of the activity in the extract, the antimicrobial assay was performed with untreated shell extract prepared as described above (protein concentration 1,360 μg/ml), extract that had been boiled for 5 min at 100 °C (protein concentration 1,517 μg/ml), and shell extract prepared with lobster shell material that had been muffled in a furnace for 4 h at 450 °C (protein concentration 2 μg/ml). The untreated shell extract and the boiled shell extract significantly reduced bacterial growth when compared to the positive control. Boiling did not significantly decrease the antimicrobial activity of the extract when compared with the untreated extract (Tukey Honestly Significant Difference (HSD) pair wise test, $P > 0.05$, $n = 3$, Fig. 1), indicating that the component of the shell extract responsible for the detected antimicrobial activity is heat stable. The furnace treated extract caused a 19 ± 7% decrease in bacterial growth, which was a significantly smaller decrease in growth than was seen with the untreated shell extract (44 ± 2%). The reduced activity in the furnace treated extract indicates that most of the antimicrobial activity observed in the shell extract is likely due to an organic component, but residual activity in the furnace treated extract suggests that an inorganic component may also be contributing to the overall activity. This inorganic component may be present when the extract is untreated, or it may be released or transformed during the muffling process. Copper containing molecules, such as the pigment crustacyanin and the immune enzyme phenoloxidase, are found in the lobster cuticle. Copper ions released from these molecules during muffling may account for the residual activity observed, as Cu(II) has been shown to be bactericidal [15]. and [14] showed that lobster cuticle also contains manganese, lead and nickel of environmental origin, which may also contribute to antimicrobial activity. Additionally, [20] reported a similar antibacterial activity in furnace-treated (muffled) clam and oyster shells. These authors showed that baking causes the transformation of $CaCO_3$ in shell to CaO (lime) that reacts with water to produce $Ca(OH)_2$ and alkalize the solution, causing a bactericidal effect.

To determine the size of the molecule(s) involved in the antimicrobial activity of untreated shell homogenates, size exclusion cartridges (Millipore, Billerica, Massachusetts, USA) were used to produce three subsamples of the extract: extract that could not pass through a 10 kDa filter (>10 kDa), extract that did pass through a 10 kDa filter (<10 kDa), and extract that passed through a 3 kDa filter (<3 kDa). Antimicrobial activity in the <10 kDa and <3 kDa extract fractions was not significantly different from the activity observed in the full extract (Fig. 1). The antimicrobial activity of the extract unable to pass through a 10kD filter was lower than that of the full extract (Fig. 1). Additionally, the protein concentration of the >10 kDa fraction (4800 μg/ml due to protein concentration on the cartridges) was much higher than that of the full extract (1360 μg/ml), <10 kDa fraction (76.8 μg/ml), or the <3 kDa fraction (9.5 μg/ml). These data suggest the component of the extract responsible for antimicrobial activity is less than 10 kDa and likely less than 3 kDa. The respiratory pigment hemocyanin, which is present in crustacean cuticle [1] and can generate antimicrobial peptides [8], may be linked to the antimicrobial activity detected in our study.

Fast protein liquid chromatography system (BioLogic LP system, Bio-Rad Laboratories, Inc. Philadelphia, Pennsylvania, USA) using a cation exchange column (HiTrap Q-sepharose FF column, 1.6 × 2.5 cm, 5 ml, GE Healthcare, Uppsala, Sweden) showed 5 protein peaks in the cationic fraction of the sterile untreated shell extract. Samples collected at time points 3 (protein concentration 144.9 μg/ml), 4 (426.5 μg/ml), 5 (218.5 μg/ml), 6 (310.2 μg/ml), and 10 (238.0 μg/ml) (samples collected every minute beginning after void volume collection) correspond to protein peaks in the chromatograph (data not shown). The void volume peak (collected from 0 to 5 min, protein concentration 344.0 μg/ml) corresponds to anionic organic compounds and inorganic compounds in the shell extract. Antimicrobial activity observed in FPLC fractions 3 and 4 was not significantly different from that observed in the full extract (Fig. 1). Fractions 5, 6, and 10 exhibited lower antimicrobial activity that was highly variable and not significant. The void volume actually increased bacterial growth by 25%, perhaps by providing nutrients in the cuticle without the antimicrobial component. These results suggest that the component(s) with antimicrobial activity in the shell extract is cationic in nature.

To assess whether activity observed in FPLC fractions 3 and 4 was from a small molecule, size exclusion cartridges were used to

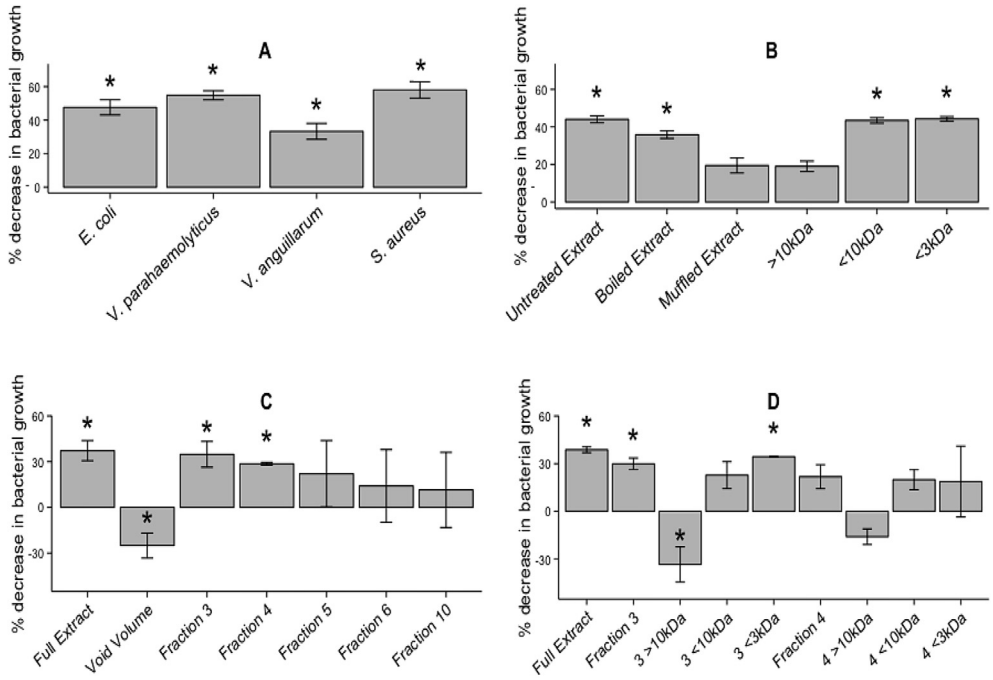

Fig. 1. (A) Antimicrobial activity of lobster shell extract against gram-positive and -negative bacteria. (B) Antimicrobial assay of lobster shell extract against *V. parahaemolyticus*. Extract that did not pass through a 10 kDa filter is labeled >10 kDa, extract that passed through the 10 kDa filter is labeled <10 kDa, and extract that passed through a 3 kDa filter is labeled <3 kDa. (C) Antimicrobial assay of FPLC fractions of lobster shell extract against *V. parahaemolyticus*. (D) Antimicrobial assay of size-fractionated FPLC fractions of lobster shell extract against *V. parahaemolyticus*. The asterisks indicate significant difference from positive control (Tukey Honestly Significant Difference (HSD) pair wise test, $P < 0.05$, $n = 5$ in A and $n = 3$ in B, C and D).

create three subsamples of each fraction as described above (>10 kDa, <10 kDa, and <3 kDa). The antimicrobial activity of the FPLC fraction 3 that passed through the 3 kDa (protein concentration 52.3 μg/ml) and 10 kDa (67.5 μg/ml) filters was not significantly different from that of the full FPLC fraction 3 or untreated sterile shell extract (Fig. 1). The FPLC fraction 3 that was unable to pass through a 10 kDa filter (protein concentration 186.8 μg/ml) increased bacterial growth. FPLC fraction 4 and its subsamples followed the same pattern as FPLC fraction 3, but it was less pronounced, more variable, and not significant.

To determine the molecular sizes of proteins eluted from the FPLC exchange column (fractions 3, 4, 5, 6, &10), a 15% Tris/Tricine SDS PAGE polyacrylamide gel electrophoresis was conducted under reducing conditions (adapted from Ref. [22]). The molecular masses of the proteins were determined using a protein ladder (Bio-Rad, Hercules, California, USA) and protein bands were visualized using silver staining. Gel electrophoresis of the FPLC fractions revealed two faint bands with a molecular weight smaller than 10 kDa in fractions FPLC 3 and 4 that were not seen in the other fractions (data not shown). This indicates the presence of small peptides (<10 kDa) in FPLC fractions 3 and 4, but not in FPLC fractions 5, 6, and 10.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was utilized to determine the molecular weight of small peptides in the sterile shell extract [12]. Sterile shell extract that passed through a 10kD size exclusion cartridge was cleaned up on a Zip Tip (Millipore, Billerica, Massachusetts, USA) and the peptides eluted onto a MALDI target with alpha cyano-4-hydroxycinnamic acid (10 mg/ml in 50% Methanol, 0.1% trifluoroacetic acid). MALDI spectra were acquired with an Applied Biosystems Voyager DE-STR mass spectrometer (Carlsbad, California, USA) from m/z 1000–10,000. The mass spectrometry showed 4 protein peaks at 1.6 kDa, 2.8 kDa, 4.6 kDa, and 5.6 kDa, with the most intense of these peaks at 5.6 kDa (Fig. 2).

This study has demonstrated antimicrobial activity in the shell of American lobster for the first time, as the sterile shell extract consistently exhibited antimicrobial activity against 4 species of bacteria, including Gram-positive and -negative species. The data presented indicate that American lobster cuticle possesses an intrinsic antimicrobial activity and therefore plays a larger role in the defense against invading pathogens than a simple physical barrier. A previous study by [11] also reported broad-spectrum antimicrobial activity in the exoskeletons of several crustacean species, including Northern Shrimp (*Pandalus borealis*), Hermit Crab (*Pagurus bernhardus*), Spider Crab (*Hyas araneus*), and King Crab (*Paralithodes camtshatica*). This activity was not fully characterized, but it was found to be heat resistant and sensitive to enzyme proteinase K [11]. The antimicrobial activity discovered here, in the shell of American lobster, is also heat resistant and appears to be partly due to an organic, cationic component less than 10 kDa and likely less than 3 kDa.

The characteristics of the activity described here are similar to those of antimicrobial peptides (AMP). AMPs are usually small (<12 kDa), cationic peptides that exhibit broad-spectrum antimicrobial activity at very low concentrations [26]. Structural characteristics of AMPs and their small size make them stable and heat resistant. Small AMPs sometimes display an extended linear form without secondary or tertiary folding unless in contact with a bacterial membrane ([10,21]). If the activity in the lobster shell is due to a small AMP, this would explain the persistence of the activity after boiling. Although AMPs have not previously been reported to exist in crustacean exoskeletons, they are wide spread internal defense factors in crustaceans [26]. Because these defense molecules are ubiquitous internally, it is possible that AMPs may be present in the crustacean cuticle as well. The chitin-binding capabilities of the shrimp AMP, penaeidin (2.7–8.3 kDa), supports this idea [7]. Further work should be done to fully characterize the small peptides and antimicrobial activity detected in lobster cuticle and

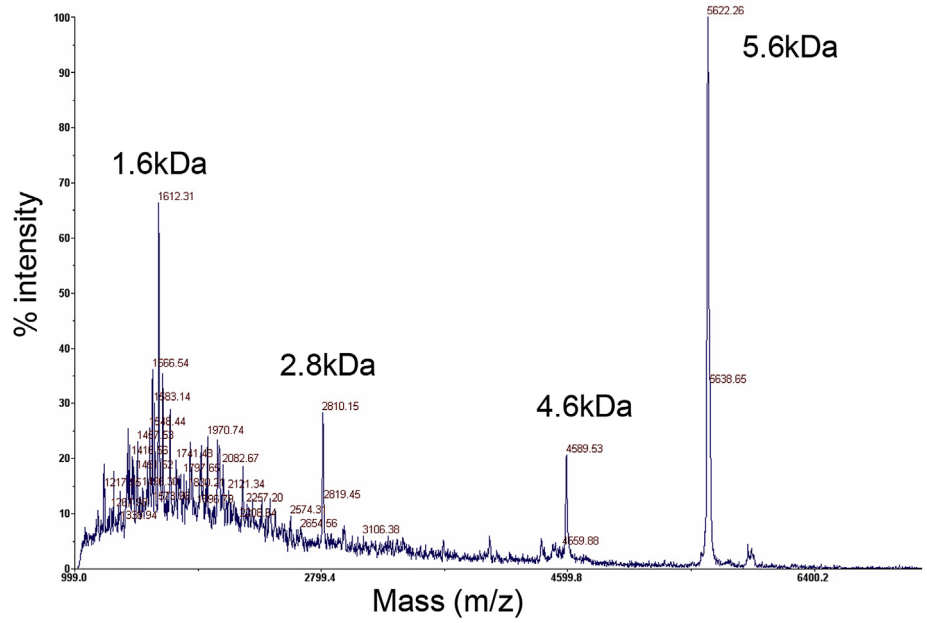

Fig. 2. MALDI mass spectra of shell extract filtered with a 10 kDa size exclusion cartridge. Four discrete peaks were detected.

assess the activity against both model pathogens, as well as microbes associated with ESD.

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