Antibacterial Activity and Preliminary Protein Profile of Rock Oyster (*Saccostrea cucullata*) Hemolymph

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ABSTRACT

Oysters have gained popularity among consumers worldwide. They are commonly consumed either fresh (with or without lime and herbs) or cooked. Many consumers believe that oyster hemolymph has the ability to stop growth of pathogens and inhibit infection. The aims of this study were to examine the bacterial inhibition of rock oyster *Saccostrea cucullata* hemolymph, and study protein alteration in four treatments: fresh hemolymph (FH), hemolymph boiled at 100 °C for 15 s (H1), hemolymph boiled at 100 °C for 5 min (H2), and fresh hemolymph combined with lime juice (FL). Protein patterns were studied by SDS-PAGE technique. The results showed that the FL treatment produced measurable inhibition of three bacterial species: *Vibrio parahaemolyticus* (AHPND), *Escherichia coli* (ATCC25922), and *Staphylococcus aureus* (ATCC25923). No antimicrobial activity was found in treatments FH, H1 or H2. The SDS polyacrylamide gels showed that acidity and heat altered protein band patterns of the oyster hemolymph. FH and H1 groups revealed 17 protein bands, while H2 and FL groups presented 10 and 11 protein bands, respectively. The results suggest that consuming oyster with lime juice could have an inhibitory effect on the growth of pathogenic bacteria compared with fresh and cooked ones.

Keywords: Bacteria, Hemolymph protein, Rock oyster, *Saccostrea cucullata*

INTRODUCTION

Oyster, a popular edible bivalve, is an excellent source of protein, enzymes, polysaccharides, minerals and vitamins (Cai *et al*., 2013; Carlson-Bremer *et al*., 2014; Qin *et al*., 2018). Oyster hemolymph is colorless and contains hemocyanin, which turns light blue when it is combined with oxygen (Schmitt *et al*., 2011). There is evidence of antimicrobial activity of oyster hemolymph, such as protection against bacterial, protist and viral infections (Pila *et al*., 2016; Dupont *et al*., 2020) caused by *Escherichia coli*, *Micrococcus luteus* and *Pseudomonas aeruginosa* (Watanachote *et al*., 2013). Prangtong and Tunkijjanukij (2004) reported that the hemolymph of white-jawed oyster *Crassostrea belcheri* retarded growth of *Stapphylococcus* sp., *Vibrio harveyi* and *V. alginolyticus*.

Consumption of raw oysters is popular worldwide, and has been found to be associated with enteric illness (e.g., Morse *et al*., 1986; Kohn *et al*., 1995). In Thailand, it is common to eat fresh oysters with seafood sauce to add flavor and/or with lime juice for freshness, flavor and medicinal properties. Cooked oysters are less popular despite the fact that cooking reduces the risk of enteric illness. Previous research suggested that heat (Froelich and Noble, 2016), acetic acid and citric acid (Drake *et al*., 2006; Singh *et al*., 2014) all reduced risk of enteric illness.

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Published studies on antibacterial activity of oyster have mostly used *Crassostrea* spp. hemolymph, with very few reports on *Saccostrea* spp. For this reason, the present study will provide important preliminary information on antimicrobial properties of the genus *Saccostrea*. This study was conducted to determine the antibacterial activity of rock oyster and reveal the protein band patterns in the hemolymph under treatments with high temperature at different durations and with lime juice to yield background information regarding the safety of seafood consumption.

MATERIALS AND METHODS

Preparation of oyster hemolymph

Thirty individuals of adult rock oyster *Saccostrea cuccullata* with 4–5 cm shell length were collected in Kung Krabaen Bay, Royal Development Study Center, Chanthaburi Province, Thailand. They were washed first with seawater and then tap water. By aseptic procedure, hemolymph was collected from the adductor muscle sinus in the pericardial cavity (Figure 1) using disposable sterile needles containing 3.8% trisodium citrate (ratio 1:9) as an anticoagulant. The collected hemolymph was transferred to a vial before storage at -20 °C for further study.

Preparation of experimental hemolymph

Oyster hemolymph was subjected to four different preparations: fresh hemolymph (FH), hemolymph boiled at 100 °C for 15 s (H1), hemolymph boiled at 100 °C for 5 min (H2), and fresh hemolymph mixed with lime juice (*Citrus aurantifolia*) (containing 7–9% citric acid) (FL) at the ratio 1:1. Before cutting, fresh limes were washed with distilled water to remove soil and other extraneous matter, then sterilized with 70% ethanol; a juice extractor, juice container and knife were also sterilized this way.

Preparation of bacterial cultures

Two bacterial species, *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC25923) were cultured in Tryptic Soy Broth (TSB), while TSB supplemented with 1.5% NaCl was used for *Vibrio parahaemolyticus* (AHPND) culture. The broth cultures were incubated at 37 ºC with 200 rpm shaking speed for 12 h. All culture samples were then diluted and absorbance was measured at 625 nm. Cultures with absorbance value in the range of 0.08–0.1 (108 CFU·mL^{-1}) were selected for the next step.

Antibacterial activity assay

Antibacterial activity of the untreated and treated oyster hemolymph was determined by agar well diffusion method with the three bacterial species: *V. parahaemolyticus* (AHPND), *E. coli* (ATCC25922), and *S. aureus* (ATCC25923). A sterile cotton swab was immerged into each broth culture. Subsequently, 5 mm diameter wells were punched into the agar medium using a cork borer at 5 wells per plate. Then, 50 µL of each oyster hemolymph preparation was transferred to a well. For antibiotics (control), polymyxin was used for *V. parahaemolyticus* (AHPND) and *E. coli* (ATCC25922); penicillin V potassium was used for *S. aureus* (ATCC25923) (concentrations of penicillin V potassium and polymyxin were 0.01 g·mL^{-1}). Next, the plates were incubated in the upright position at 37 °C for 24 h. After incubation, the diameters of the growth inhibition zones were measured from the edge of one zone to the other zone passing the center of the clear distance and recorded in mm. Three replicates were carried out for each sample against each of the test organisms.

$$\text{Inhibition zone (\%)} = \frac{(\text{Radius of clear zone-Radius of well})\times 100}{\text{Radius of clear}}$$

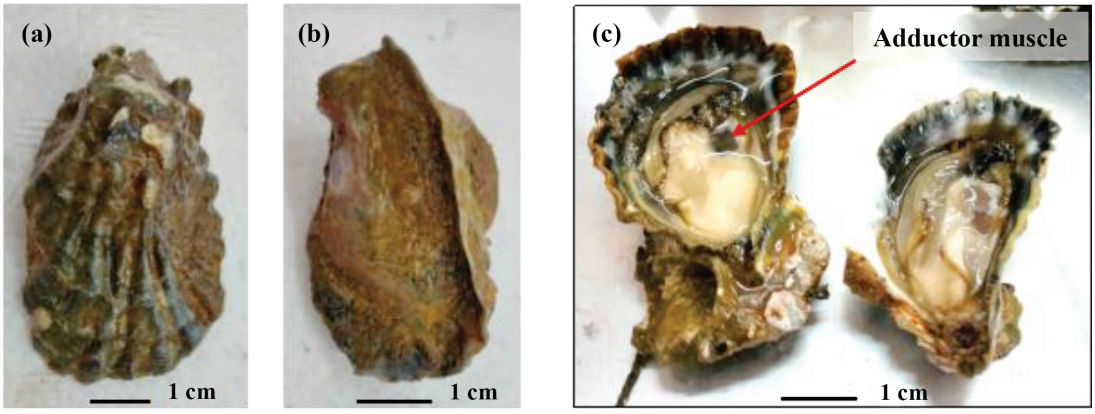

Figure 1. Posterior (a), anterior (b) and adductor muscle of *Saccostrea cucullata* (red arrow in c).

Hemolymph protein profile analysis

Protein concentration analysis

Hemolymph protein analysis was done according to Lowry *et al.* (1951). Five µL of oyster hemolymph was mixed with 95 µL distilled water before adding 500 µL Lowry reagent (reagent A: 0.1 M sodium hydroxide and 0.09 M sodium carbonate; reagent B: 0.04 M potassium sodium tartrate and 0.03 M copper II sulfate), mixing well and then incubating for 15 min at room temperature. Subsequently, 50 µL Phenol Folin reagent was added and incubated for 30 min at room temperature. Absorbance was measured at 750 nm. Standard curve was generated using bovine serum albumin (BSA) concentrations ranging from 1–1,000 $\mu g \cdot \mu L^{-1}$.

Protein molecular weight analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein profile analysis according to the method of Laemmli (1970). In a 1:1 ratio, each sample of oyster hemolymph was mixed with sample buffer (0.5 M tris-HCl pH 7.4, 25% Glycerol, 10% SDS, bromophenol blue, and 5% Mercaptoethanol). The samples were separated in 10% stacking gels ($10\ mAh \cdot gel^{-1}$) followed by 12% resolving gels ($15\ mAh \cdot gel^{-1}$). The gels were stained for 12–18 h using Coomassie brilliant blue G-250, and destained (methanol and acetic acid) before silver nitrate staining. The molecular weight of the protein was determined by comparing the distances of the protein bands with standard proteins of molecular weight 16–250 kDa using the GelAnalyzer version 2010a program.

Statistical analysis

Data of bacterial inhibition by oyster hemolymph was subjected to one-way ANOVA. Differences in mean inactivation of bacteria by oyster hemolymph were evaluated by independent-sample t-test. All tests were considered significant at $p<0.05$. The analyses were done using the IBM SPSS Statistics Standard Program.

RESULTS AND DISCUSSION

Antibacterial activity of oyster hemolymph

The efficacy test against three bacterial species, namely *Vibrio parahaemolyticus* (AHPND), *Escherichia coli* (ATCC25922), and *Staphylococcus aureus* (ATCC25923), with four preparations of oyster hemolymph revealed that there was no observed antibacterial effect for fresh hemolymph (FH), hemolymph heated at 100 °C for 15 s (H1), or hemolymph heated at 100 °C for 5 min (H2) (Table 1). In contrast, lime juice mixed with

oyster hemolymph (FL) was significantly different ($p<0.05$) from the other treatments in inhibiting *V. parahaemolyticus* (AHPND), *E. coli* (ATCC25922) and *S. aureus* (ATCC25923), with mean bacterial inhibitory percentages of 73.08±2.38, 65.64±5.91, and 65.45±6.61, respectively. Clear zones indicating bacterial inhibition are shown in Figure 2.

The results showed that hemolymph mixed with lime juice had antibacterial activity, but hemolymph alone (heated or unheated) did not. This indicates that hemolymph and lime juice mixture produces a bacterial growth inhibitory effect (Tomotake *et al*., 2006; Hardoko and Yuliana, 2014). Enejoh *et al*. (2015) reported that the health benefits and antimicrobial activities of *Citrus aurantifolia* (lime) are associated with its high level of photochemical and bioactive components, such as coumarin, trichloroanisole, geranoxypsoralen, a-bergamotene, a-pinene, b-bisabolene, aterpineol, b-caryophyllene, dlimonene, berfapten, b-pinene, camphene, p-cymene, terpinene, apigenin, bergamottin, fenchol, ciral, germacreneB, citronellol, isoimperatorin, imperatorin, isovitexin, isopimpinellin,limonene, oxypeucedanin hydrate, kaempferol,rutin, nobiletin, o-cymene, quercetin, terpinolene, sabinene, and phellopterin. Oiken *et al*. (2016) also revealed the presence of steroids, flavonoids, alkaloids, saponins, terpenoids, reducing sugars and cardiac glycosides in *C. aurantifolia*. The juice concentrates had beneficial antimicrobial roles in controlling growth of Gram–positive (*S. aureus* and *Enterococcus faecalis*), Gram–negative (*Pseudomonas aeruginosa*, *E. coli*, and *Salmonella* spp.) and fungal species (*Candida albicans*, *Aspergillus niger*, and *Penicillum* spp.).

Pathirana *et al*. (2018) reported that by the agar well diffusion method, the crude extract of lime (*C. aurantifolia*) inhibited the growth of both Gram–negative and Gram–positive bacteria. The inhibition zones were 28, 9, 8, and 8 mm for the inhibitory effect against *Vibrio cholerae*, *Enterobacter* sp., *Citrobacter* sp. and *E. coli*, respectively. However, the lime extract was ineffective against *Shigella* sp., *Salmonella* sp. and *Klebsiella* sp. In addition, lime was recommended as an effective agent in preventing *Vibrio* sp. infection, which is consistent with the results for the hemolymph and lime mixture in this study. Jayana *et al*. (2010) revealed the effect of different concentrations of lime extract (75, 50, 25, and 5%) against *V. cholera*, which resulted in inhibition zones of 31, 24, 17, and 9 mm, respectively. Tomotake *et al*. (2006) suggested that citric acid in lime juice was responsible for inhibiting the growth of *V. parahaemolyticus*. Okeke *et al*. (2015) applied both agar well diffusion and macro-broth dilution methods and found anti-bactericidal activity against *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 12600, *E. coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 10145, *S. aureus* ATCC 25923,

Table 1. Antibacterial activities of rock oyster *Saccostrea cucullata* hemolymph.

Species of bacteria	**Average percentage of bacterial inhibition in oyster hemolymph**				
	Antibiotics (Control)	**Fresh hemolymph (FH)**	**Hemolymph heated at 100 °C for 15 s (H1)**	**Hemolymph heated at 100 °C for 5 min (H2)**	**Fresh hemolymph mixed with lime juice (FL)**
Vibrio parahaemolyticus (AHPND)	86.56 ± 0.13^{Aa}	ND	ND	ND	73.08 ± 2.38^{Ba}
Escherichia coli (ATCC25922)	73.64 ± 0.06^{Bb}	ND	ND	ND	65.64 ± 5.91^{Bb}
Staphylococcus aureus (ATCC25923)	71.29 ± 0.29^{Bb}	ND	ND	ND	65.45 ± 6.61^{Bb}

Note: ND = not detected; means in the same row superscripted with different capital letters are significantly ($p<0.05$) different; different lowercase superscripts show significant ($p<0.05$) difference between means in the same column.

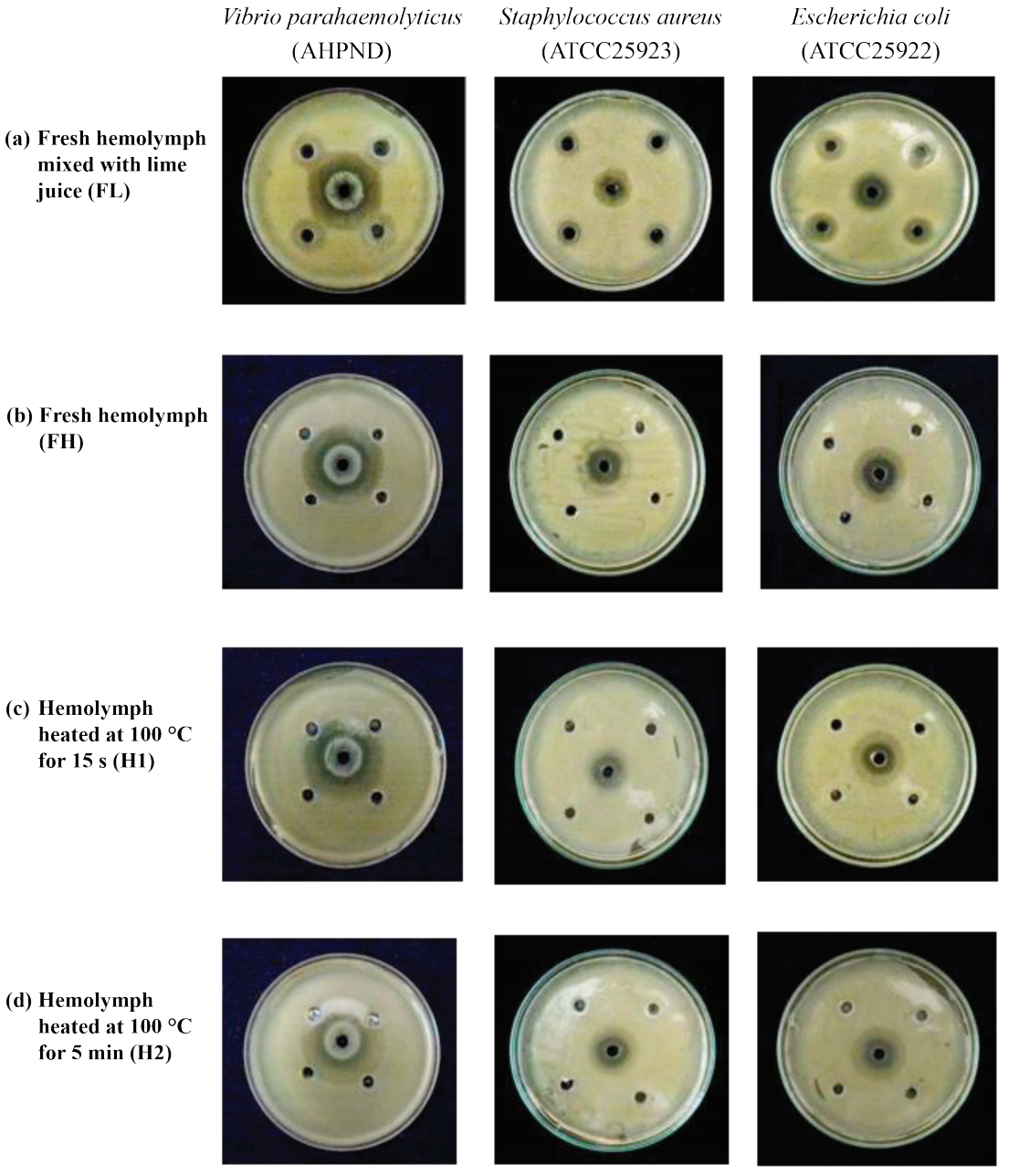

Figure 2. Antimicrobial activity of rock oyster hemolymph against *Vibrio parahaemolyticus* (AHPND), *Staphylococcus aureus* (ATCC25923), and *Escherichia coli* (ATCC25922): (a) fresh hemolymph mixed lime juice (FL); (b) fresh hemolymph (FH); (c) hemolymph heated at 100 °C for 15 s (H1); and (d) hemolymph heated at 100 °C for 5 min (H2). The central well contained an antibiotic (positive control) and outer wells contained the hemolymph treatment.

and *E. coli* ATCC 25922 (Shakya *et al*., 2019) by lime juice (*Citrus limonum*). The present results are also in line with Chothonglang (2019), who confirmed that cockle (*A. granosa*) hemolymph mixed with lime juice was found to inhibit *V. parahaemolyticus*, *E. coli*, and *S. aureus*.

Protein patterns of oyster hemolymph

The number and molecular weight of protein bands of four preparations of oyster hemolymph are shown in Table 2 and Figure 3. FH and H1 showed 17 protein bands at the same molecular weights, while H2 and FL presented different numbers of bands (10 and 11 protein bands, respectively) but with similar molecular weights. It was clearly seen that the protein profiles of the oyster hemolymph were changed after treating with heat or lime juice. However, among the four haemolymph treatments, seven protein bands were shared by all treatments: 18, 20, 25, 26, 40, 56, and 61 kDa.

In the experiment, the purpose of using high temperature was to help reduce microorganisms in raw oysters. Under the heat treatments, the proteins could be denatured and lose their natural conformation. The five-minute heat treatment and lime mixture were able to induce protein hydrolysis in the hemolymph similar to the oyster protein hydrolysates by pepsin and trypsin, which cleave most proteins into smaller peptides. Different heat levels influence the degree of degradation and breakdown of muscle protein (Palka and Daun, 1999; Bax *et al*., 2012; Singh *et al*., 2014). Temperatures between 53–63 ºC cause the deterioration and towering of collagen fibers and proteins found in sarcoplasm with molecular weights ranging from 17 kDa (myoglobin) to 92.5 kDa (phophorylase b). The heat and acidic treatments in this study resulted in the disappearance of the protein bands with molecular weight above 69 kDA, and changes of the band profiles below 61 kDa (Figure 3). Certain protein bands of this study could be compared to SDS-PAGE results from other oyster species. The 25-kDa band from the study by Nuchchanart *et al*. (2007) was related to sarcoplasmic calcium binding protein (SCP) from the tropical oyster *Crassostrea belcheri*; however, this protein band was not different among the treatments in this study. The hemolymph protein bands in this study that were similar to those from previous research included the bands at 18.6 kDa (Xue *et al*., 2004), 39 kDa (Xue *et al*., 2012), and 68 kDa (Ziegler *et al*., 2002) from *C. viginica* oyster, and the 19.4-kDa band from *C. gigas* oyster (Scotti *et al*., 2007). The present results also concurred with the report by Chothonglang (2019), who examined the protein band pattern change of cockle *A. granosa* hemolymph. The SDS-PAGE results also showed different effects of the heat and acidic treatments on the hemolymph protein band profiles. High temperatures initially destroy the hydrogen bonds between the polypeptide chains, and heating for a longer period (5 min) can damage the peptide bonds and result in shorter peptides. Treatment of

Table 2. Number and molecular weight of proteins in rock oyster *Saccostrea cucullata* hemolymph, analyzed by GelAnalyzer version 2010a.

Sample	Molecular weight (kDa) of oyster hemolymph protein	Number of protein bands
Fresh hemolymph (FH)	14, 18, 20, 23, 24, 25, 26, 40, 49, 52, 56, 61, 69, 78, 116, 210, 297	17
Hemolymph heated at 100 °C for 15 s (H1)	14, 18, 20, 23, 25, 26, 27, 40, 43, 49, 52, 56, 61, 71, 116, 130, 232	17
Hemolymph heated at 100 °C for 5 min (H2)	18, 20, 25, 26, 27, 40, 43 ,56, 61, 232	10
Fresh hemolymph mixed with lime juice (FL)	15, 18, 20, 25, 26, 27, 39, 40, 52, 56, 61	11

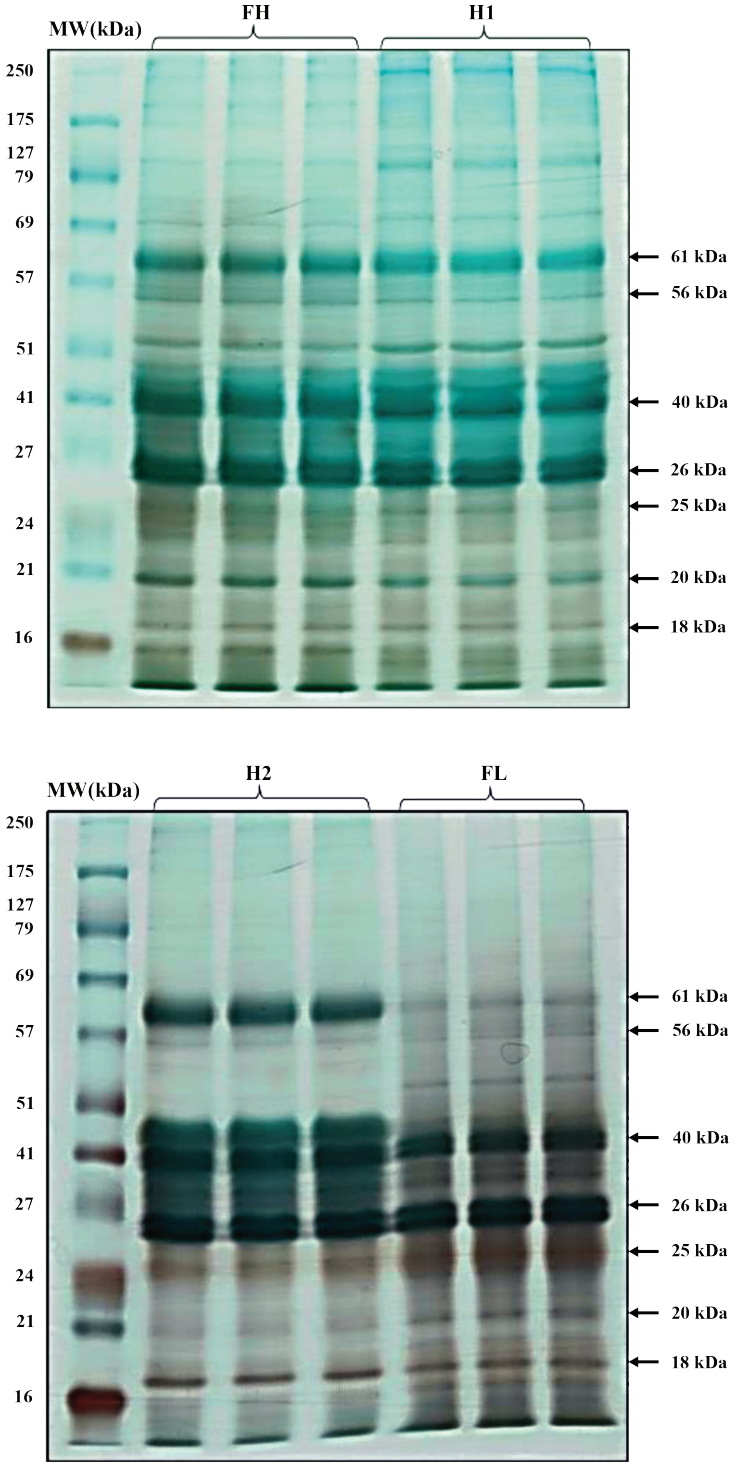

Figure 3. Patterns of protein bands in fresh oyster hemolymph (FH), heat-treated oyster hemolymph at 100 ºC for 15 s (H1), heat-treated oyster hemolymph at 100 ºC for 5 min (H2) and oyster hemolymph mixed with lime juice (FL), with arrows indicating molecular weight bands common to all treatments.

hemolymph with lime juice resulted in adjusting its pH and exceeding the isoelectric point, and thus may have changed the structure and functional properties of the protein. Thus, the antibacterial activity of hemolymph mixed with lime juice in this study could very well be due to the pH influence on bacterial growth. However, the antibacterial activity of the pH-altered hemolymph proteins should be further investigated.

CONCLUSION

This study has demonstrated bacterial inhibition by hemolymph of rock oyster *Saccostrea cucullata*, and revealed the protein pattern of the hemolymph following several treatments. Absence of growth inhibition against *Vibrio parahaemolyticus* (AHPND), *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC25923) was observed for fresh hemolymph and hemolymph heated at 100 °C for 15 s and for 5 min, while fresh hemolymph mixed with lime juice showed significant ($p<0.05$) inhibition of all three bacterial strains. Treatments of oyster hemolymph with either lime juice or heat resulted in alteration of its protein profile.

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