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Humoral immune responses of antibacterial hemocyanin (Ab-Hcy) in mud crab, *Scylla serrata*

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ABSTRACT

Hemocyanins are large extracellular respiratory proteins distributed within the hemolymph of arthropods, molluscs and larval stages of certain insects. In the present study, we characterized the humoral immune functions of cation specific antibacterial hemocyanin (Ab-Hcy) from mud crab, *S. serrata*. The bacteriolytic activity of Ab-Hcy was against pathogenic and non-pathogenic to crustaceans which includes, *Bacillus* sp. N1, *B. flexus* N3, *E. coli*, *P. aeruginosa*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* and also expressed bacteriostatic activity against *E. coli*, *B. flexus* N3 and *V. harveyi*. The agglutination activity of Ab-Hcy ranged from 4-16 against *B. flexus* N3, *E. coli*, *V. harveyi* and *V. vulnificus* and Ab-Hcy also agglutinated with rat and human O erythrocytes (HA titer: 2). The Ab-Hcy lysed the panel of mammalian erythrocytes and strong hemolytic activity of 1.40, 1.28 and 1.22 units·min^{-1}·mg protein^{-1} against human A, B and O erythrocytes respectively. It was also found that the Ab-Hcy possess phenoloxidase (PO) activity by oxidizing L-DOPA. These results suggest that Ab-Hcy from the serum of mud crab, *S. serrata*, is capable of performing multiple humoral immune functions in addition to oxygen transportation.

Statement of relevance: This manuscript deals with the humoral immune functions, particularly antibacterial property of hemocyanin in crustacean. In this paper we report the different type of antibacterial activity and other humoral immune functions of a hemocyanin isolated from the serum of mud crab, *Scylla serrata*. This work will help to understand the role of respiratory molecule in immune system, also will be helpful improve disease free crustacean aquaculture industry.

And we also state this research work is relevant and suitable to publish in the Journal "Aquaculture".

1. Introduction

Crustaceans are the second largest group, next to insects, in the phylum Arthropoda, approximately >30,000 known species (Bachère et al., 2004). Crustacean production is a worldwide economic activity with prime importance in intertropical developing countries. It is important to note the intensification of crustacean aquaculture farming over the last few decades has been accompanied by the development of infectious diseases from viral, bacterial, and in some cases, fungal origin (Destoumieux-Garzón et al., 2001). In recent years, many investigations focused on crustacean immunity to overcome the above mentioned diseases and better productivity. Crustacean immunity has two distinct immune system i.e., humoral and cellular defense mechanism. Hemocytes play a central role in cellular immune system by phagocytosis, encapsulation and nodule formation, whilst humoral factors are agglutinins, killing factors, lysins, precipitins, cytokine-like molecules and clotting agents (Hauton, 2012; Smith and Chisholm, 1992; Söderhäll and Cerenius, 1992). Crustaceans lack of defined immunoglobulins and are incapable of adaptive immunity, but are known to contain repertoire humoral molecules that mediate various types of immune responses against non-self materials. These defense mechanisms depend completely on the innate immune system that is activated when pathogen-associated patterns recognize molecules, such as lectins, antimicrobial, clotting, and pattern recognition proteins, which turn and activate cellular or humoral immune mechanisms to destroy invading pathogens (Vazquez et al., 2009).

In crustaceans, the major protein components of hemolymph are copper containing hemocyanins. They typically represent up to 95% of the total amount of protein in the crustacean hemolymph (Horn and Kerr, 1969; Jayasree, 2001). Hemocyanins are extracellular giant copper containing protein found in the hemolymph of both molluscs and arthropods, performing multiple physiological functions, such as oxygen transport, osmoregulation, protein storage or enzyme activities (Decker et al., 2007; Markl, 2013; Paul and Pirow, 1997) and also contributing to development, homeostasis and immune defenses within marine invertebrates (Coates and Nairn, 2014).

The previous studies have reported several humoral immune functions of hemocyanin apart from the oxygen transportation. The clotting

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enzyme of the horseshoe crab, *Tachypleus tridentatus*, functionally converts hemocyanin to phenoloxidase (Nagai and Kawabata, 2000). Tachyplesin a major antimicrobial peptide from *T. tridentatus*, converted the hemocyanin to phenoloxidase (Nagai et al., 2001). Cold-adapted hemocyanin-derived phenoloxidase activity is important to the survival of crab *Erimacrus isenbeckii* (Kim et al., 2011). It is also notable that the hemocyanins of kuruma prawn *Penaeus japonicus* and crayfish *Gastrolith* functions as phenoloxidases in the presence of sodium dodecyl sulfate (SDS) (Adachi et al., 2008; Glazer et al., 2013).

Earlier studies also reported the crustacean hemocyanin appear to possess antimicrobial property in two distinguished forms: (i) antimicrobial proteins act as activators of hemocyanin and (ii) hemocyanin itself is the source of antimicrobial proteins (Decker and Jaenicke, 2004; Kawabata et al., 1995; Lee et al., 2003). Recently we have reported, the cation metals specific hemocyanin (305 kDa) with three or four possible subunits (70 to 98 kDa) from serum of mud crab, *Scylla serrata*, possesses a significant antibacterial activity against host/resident specific bacterial species including Gram-positive *B. flexus* N3 and Gram-negative *E. coli*, *V. harveyi* and *V. vulnificus* (Meiyalagan and Arumugam, 2015; Meiyalagan et al., 2016). The antibacterial activity caused by this hemocyanin is not clear, so it is necessary to know whether the activity is due to bacterial agglutination, bacteriolytic, bactericidal or bacteriostatic activity. Thus, in this study we attempted to investigate the specific type of antibacterial activity in addition to other possible humoral immune functions of the antibacterial hemocyanin from the mud crab, *Scylla serrata*.

2. Material and methods

2.1. Isolation of Ab-Hcy

Ab-Hcy was isolated by anion exchange chromatography. Briefly, serum (25 mg total protein) was passes through pre-equilibrated diethylaminoethyl cellulose (DEAE cellulose) matrix with PBS (10 mM; pH 7.0). The bound materials were eluted with gradient increase of 0.0–0.5 M NaCl and then protein content of every fractions (1 ml) were monitored at 280 nm. The antibacterial activity was tested and the fractions which invariably showed antibacterial activity were pooled together and used for further characterization (Meiyalagan et al., 2016).

2.2. Bacteriolytic activity

The bacteriolytic activity was assayed following Shugar (1952). 15 μg/ml of the isolated antibacterial hemocyanin (Ab-Hcy) was and mixed with bacterial suspension (0.5 O.D. at 570 nm), incubated for 90 min at 23 °C and centrifuged (3500 rpm for 10 min). The optical density of supernatant was read at 450 nm and the bacteriolytic activity was expressed as units·min^{-1}·mg $protein^{-1}$.

2.3. Bacteriostatic activity

The bacteriostatic activity was determined with modifications in method of Forrest (1972). Ab-Hcy (15 μg/ml) was mixed with bacterial suspension and this mixture was incubated at 37 °C for 10 h with frequent shaking. At 1 h intervals, to 0.1 ml of this mixture, 10 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml) was added and re-incubated for 30 min at 30 °C. The formazan development was read at 570 nm using ELISA plate reader (Power wave XS biotek).

2.4. Bacterial agglutination activity

The bacterial agglutinating activity was determined in U-bottom microtiter plate (Griener, Nürtingen, Germany) by serial two-fold dilution (Ueda et al., 1991). Briefly, 50 μl of Ab-Hcy (15 μg/ml) was mixed with an equal volume of TBS (50 mM tris-HCl; 115 mM NaCl; 10 mM CaCl2; pH 7.5). After dilution, 50 μl of bacterial suspension (0.5 O.D. at 570 nm) were added to each well, incubated for 1 h at room temperature and kept overnight at 10 °C, then the bacterial agglutinating activities observed under microscope.

2.5. Hemagglutination activity

The hemagglutination assays were performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany) by serial two-fold dilution of 25 μl of Ab-Hcy (15 μg/ml) with an equal volume of TBS. After dilution, 25 μl of RBC (1.5%) suspension we added to each well and incubated for 45 min at 26 °C.

The agglutination titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of bacteria/erythrocytes (Garvey et al., 1979). Controls for all assays consisted of substitution of the sample with TBS.

2.6. Hemolytic activity

Hemolytic activity was determined as previously described by Nagatomo (1995). Briefly, Ab-Hcy (15 μg/ml) mixed with 0.5% (v/v) erythrocyte suspension and incubated for 1 h at 37 °C. The unbroken cells and cell debris were removed by centrifugation at 3500 rpm for 10 min. The optical density of the supernatant was read at 540 nm against suitable blank. The 0.5% (v/v) erythrocyte suspension was treated with double distilled water and PBS (10 mM, pH 6.0) as 100% and 0% hemolysis control, respectively.

2.7. Phenoloxidase (PO) activity

The phenoloxidase activity were assayed by mixing 200 μl of Ab-Hcy (15 μg/ml) with 200 μl of 5 mM L-DOPA prepared in tris-HCl buffer (50 mM, pH 7.5) and incubated for 20 min at 25 °C. The dopachrome formed was measured in Shimadzu (UV-160A) spectrophotometer against suitable reagent blank at 490 nm (Smith and Söderhäll, 1991).

2.8. Effect of PO inhibitors on the oxidation of L-DOPA

In this assay, Ab-Hcy with equal volume of 3 mM PTU (phenylthiourea) or 16 mM tropolone containing 16 mM H_2O_2 were incubated for 15 min at 25 °C. In controls, these chemicals were substituted with tris-HCl buffer (50 mM, pH 7.5). The reaction mixtures were then incubated with 5 mM L-DOPA for 20 min at 25 °C. The optical density of both control and experiments were measured spectrophotometrically at 490 nm and the results were expressed as Δ_A490 min^{-1}·mg $protein^{-1}$.

2.9. Statistical analysis

The difference between control and experimental values were analysed for statistical significance using paired sample Student's *t*-test (Bailey, 1995).

3. Results

3.1. Bacteriolytic activity

The antibacterial hemocyanin (Ab-Hcy) from the mud crab, *S. serrata* showed distinct bacteriolytic activity against all the bacterial species tested viz., *Bacillus* sp. N1, *B. flexus* N3, *E. coli*, *P. aeruginosa*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus*. The highest level of bacteriolytic activity were observed against *V. harveyi* and *E. coli* with corresponding values of 0.248 and 0.202 units·min^{-1}·mg protein (Fig. 1).

3.2. Bacteriostatic activity

Incubation of Ab-Hcy with bacterial species isolated from the injured/wounded cuticle of the crab up to 10 h, suppressed the growth of *B. flexes* after 4 h (Fig. 2A) and almost completely suppressed the growth of *E. coli* throughout period of incubation (Fig. 2B). Similarly Ab-Hcy significantly inhibited the growth of crustacean pathogenic bacteria. This bacteriostatic effect was found to be more pronounced against *V. harveyi* when compared to *V. vulnificus* (Fig. 2C & D).

3.3. Bacterial agglutination activity

Ab-Hcy agglutinated with all the four bacterial species viz., *B. flexus* N3, *E. coli*, *V. harveyi* and *V. vulnificus* with titers of 8, 16, 4 and 16 respectively (Table. 1). The results clearly show that the agglutination activities were recorded with *E. coli* and *V. vulnificus* (titer: 16).

3.4. Hemagglutination activity

This isolated antibacterial hemocyanin (Ab-Hcy) of *S. serrata*, agglutinated only with two types of erythrocytes (rat and human erythrocytes) and this agglutination was found to be weak (titer : 2) while Ab-Hcy completely failed to agglutinate with other mammalian erythrocytes mentioned in Table 2.

3.5. Hemolytic activity

The hemolytic activity (expressed as units·min^{-1}·mg $protein^{-1}$) of Ab-Hcy was observed to be effective against all the six erythrocyte types tested such as human A, B, O, buffalo, ox and sheep, ranging from 0.19 to 1.4 units·min^{-1}·mg $protein^{-1}$. Ab-Hcy very effectively lyses all the human erythrocytes tested including human A (1.4 ± 0.002 units·min^{-1}·mg $protein^{-1}$), human B (1.278 ± 0.005 units·min^{-1}·mg $protein^{-1}$) and human O (1.22 ± 0.003 units·min^{-1}·mg $protein^{-1}$) when compared with other mammalian erythrocytes including buffalo, ox and sheep RBCs (Table. 3).

3.6. Phenoloxidase activity

The oxidation rate of the Ab-Hcy phenolic substrate (L-DOPA) was be 0.0198 ± 0.004 units·min^{-1}·mg $protein^{-1}$ and the oxidation of L-DOPA by Ab-Hcy was significantly ($p < 0.005$) inhibited by PTU a known phenoloxidase inhibitor. On the other hand, Ab-Hcy treated with tropolone did not significantly affect the oxidation of L-DOPA (0.0125 ± 0.003 Δ_{490} min^{-1}·mg $protein^{-1}$) when compared to buffer treated control (Table 4).

4. Discussion

Hemocyanins the large extracellular negatively charged proteins (Jaenicke and Decker, 2003) and distributed within the hemolymph of arthropods, molluscs and larval stages of certain insects (Decker and Jaenicke, 2004; Pick et al., 2008, 2009). Previously, many investigators have demonstrated multiple humoral defense functions of hemocyanin decapod crustaceans including agglutination (=lectin like), phenoloxidase activity and hemolytic activity (Decker et al., 2001; García-Carreño et al., 2008; Lee et al., 2004; Yan et al., 2011a, 2011b; Zhang et al., 2009). Recently, antibacterial protein isolated from the serum of mud crab, *S. serrata* was demonstrated for its antibacterial activity against *B. flexus* N3, *E. coli*, *V. harveyi* and *V. vulnificus*. This antibacterial protein was characterized as a native hemocyanin with native molecular weight of 305 kDa, appeared to possess at least three to four subunits, capable of binding to various other metals including Ca^{2+}, Zn^{2+} and Fe^{2+} (Meiyalagan et al., 2016).

The bacteriolytic activity which is, one of the attributes of antibacterial activity, in crustaceans have been reported by many investigators, however, most of these studies in decapod crustaceans have been restricted to the ability of isolated antibacterial factors as it cause lysis of Gram-positive bacteria particularly against *Micrococcus luteus* (de-la-Re-Vega et al., 2006; Destoumieux et al., 1997; Fenouil and Roch, 1991; Hikima et al., 2003; Tyagi et al., 2007). In the present study, analysis of bacteriolytic activity of Ab-Hcy against both Gram-positive and Gram-negative bacteria revealed Ab-Hcy from *S. serrata* had significant bacteriolytic activity against both resident specific, crustacean pathogenic or non-pathogenic bacteria including *Bacillus* sp. N1, *B. flexus* N3, *E. coli*, *P. aeruginosa*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* (Fig. 1).

The measurement of the growth of the bacterial species viz., *B. flexus* N3, *E. coli V. harveyi* and *V. vulnificus* up to 10 h simultaneously incubated with Ab-Hcy presented highest bacteriostatic activity against *E. coli*, *B. flexus* N3 and *V. harveyi* (Fig. 2). These findings also derives support from the earlier works in the shrimp *Penaeus monodon* (Destoumieux

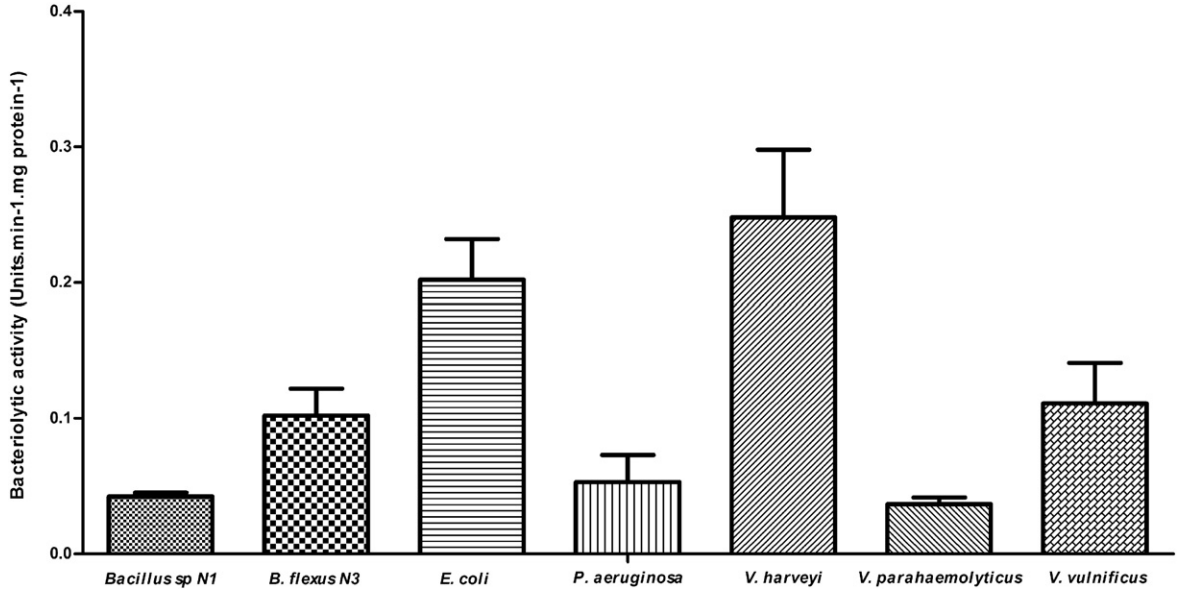

Fig. 1. Bacteriolytic activity of isolated antibacterial protein (Ab-Hcy) of mud crab, *Scylla serrata*. 15 μg/ml of Ab-Hcy with bacterial suspension (0.5 O.D. at 570 nm), incubated for 90 min at 23 °C and O.D. was measured at 450 nm. The bacteriolytic activity was expressed as units·min^{-1}·mg $protein^{-1}$. Each bar represents the mean ± SD from three determinations.

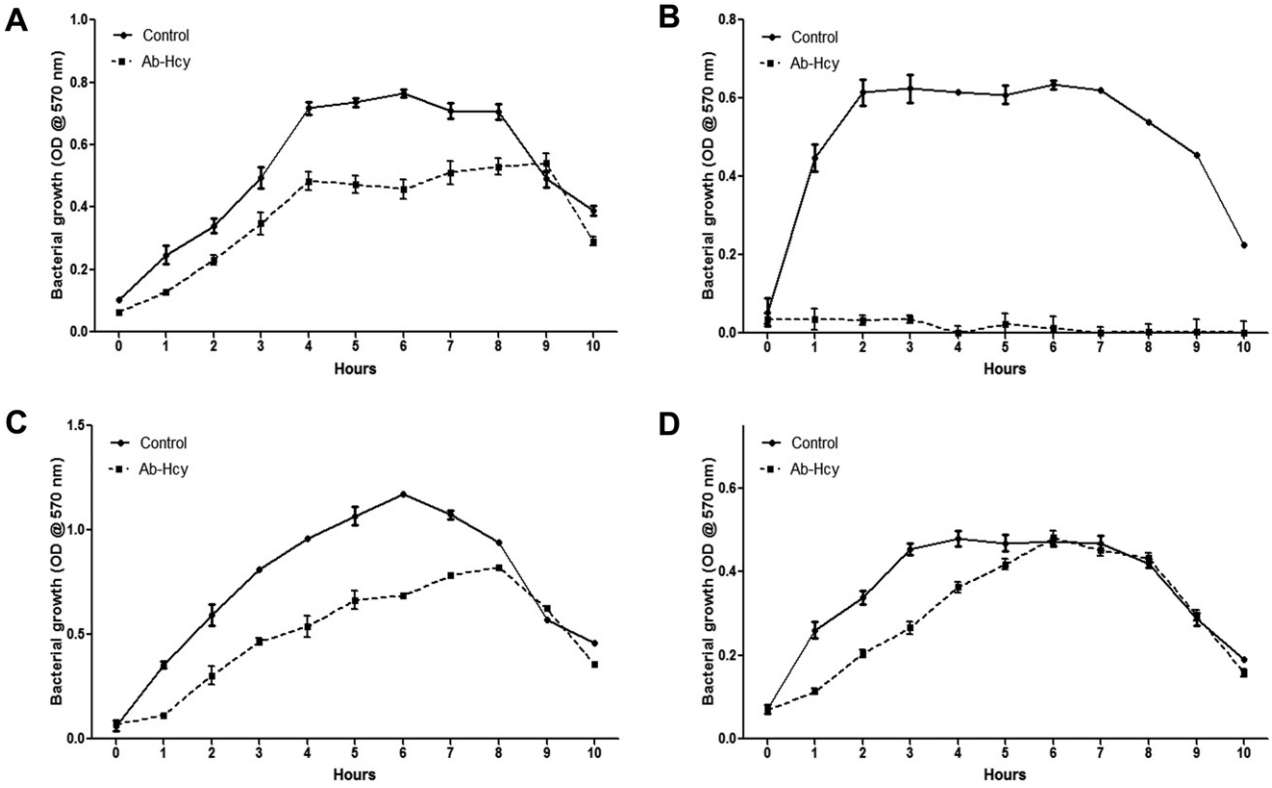

Fig. 2. Bacteriostatic effect of isolated antibacterial protein (Ab-Hcy) of mud crab, *Scylla serrata*. Ab-Hcy (15 μg/ml) was mixed with bacterial suspension, incubated at 37 °C for 10 h with frequent shaking. At 1 h intervals, in 0.1 ml of this mixture, 10 μl of MTT (5 mg/ml) added and re-incubated for 30 min at 30 °C. The formazan development was read at 570 nm using ELISA plate reader (Power wave XS biotek). Each point represents mean ± SD from three determinations. A. *B. flexus* N3; B. *E. coli*; C. *V. harveyi*; D. *V. vulnificus*.

et al., 1997), where these authors have shown that an antibacterial peptide isolated from the shrimp did not kill bacteria (*M. luteus*) but rather inhibited its growth by bacteriostatic effect. Similarly, a synthetic tiger shrimp penaeidin-like antimicrobial peptide showed both bacteriostatic/bactericidal activity against both Gram-positive and Gram-negative bacteria (Chiou et al., 2005).

The observations from the previous studies and results from the present investigation revealed the bacteriostatic effect of the isolated antibacterial protein (Ab-Hcy) considerably differed either compared to bacteriolytic activity or bactericidal activity. This also indicating that Ab-Hcy showed all the three types of activities viz., bactericidal, bacteriolytic and bacteriostatic activity. However, the type of antibacterial activity appeared to depend on the type of bacterial species and assay method used.

The literature pertaining to the bacterial agglutination, the hemolymph agglutinins from the shrimp *Penaeus indicus* (181 kDa), banana shrimp *Fenneropenaeus merguiensis* (316 kDa) showed strong agglutination activity against various *Vibrio* species, two *Pseudomonas* species and mammalian erythrocytes including human A, B, O, rat, rabbit and buffalo (Jayasree, 2001; Jayasree et al., 2000; Rittidach et al., 2007), where the bacterial agglutination activities reported were restricted with Gram-negative bacteria.

Table 1
Bacterial agglutinating activity of Ab-Hcy from mud crab, *Scylla serrata*.

Bacterial species tested[a]	Bacterial agglutinating activity[b,c]
Bacillus flexus N3	8
Escherichia coli	16
Vibrio harveyi	4
Vibrio vulnificus	16

[a] Optical density of each bacterial suspension was adjusted to 0.5 O.D. at 600 nm.
[b] Data represent median values from five determination of each bacterial species.
[c] Controls for all assays consisted of substitution of the sample with TBS.

In the present study, Ab-Hcy (305 kDa) caused agglutination with both Gram-positive (*B. flexus* N3) and Gram-negative bacteria (viz., *E. coli*, *V. harveyi* and *V. vulnificus*) and weak agglutination with rat and human O erythrocytes (HA titer: 2). Notably, bacterial agglutination activity found in serum against *P. aeruginosa*, *V. parahaemolyticus* and *V. anguillarum* could not be detected in Ab-Hcy. On the other hand, Ab-Hcy showed highest bacterial agglutination activity against *V. vulnificus* (titer: 16) while, serum did not agglutinated with this species of bacteria. The serum of *S. serrata* agglutinated various RBC types tested and the agglutination titers observed were ranging from 2 to 64, with highest titer of 64 for rat, mouse and buffalo, and did not agglutinate with goat erythrocytes (data not shown).

Our attempt to analyse the hemolytic activity, an attribute to the serum antibacterial protein in decapod crustaceans, the isolated Ab-Hcy exhibited the hemolytic activity against the panel of mammalian erythrocytes (viz., buffalo, ox, sheep, human A, B and O), of which, strong hemolytic activity was observed against human A, B and O

Table 2
Hemagglutinating activity of Ab-Hcy from mud crab, *Scylla serrata*.

RBC types tested	Hemagglutinating activity[a,b]
Rat	2
Mouse	0
Rabbit	0
Buffalo	0
Sheep	0
Ox	0
Goat	0
Human A	0
Human B	0
Human O	2

[a] Data represent median values from five determination of each RBC type tested.
[b] Controls for all assays consisted of substitution of the sample with TBS.

Table 3
Hemolytic activity of Ab-Hcy from the mud crab, *Scylla serrata*.

RBC types	Hemolytic activity (units·min^{-1} mg protein^{-1})[a]
Buffalo	0.19 ± 0.003
Ox	0.272 ± 0.008
Sheep	0.33 ± 0.005
Human A	1.4 ± 0.002
Human B	1.278 ± 0.005
Human O	1.22 ± 0.003

[a] Results represent consistent performance from four determinations using samples from different preparations against each RBC type tested.

erythrocytes. Previously, extracts of hemolymph fractions or other tissues such as eggs, muscles and exoskeleton of various crustaceans including *Pandalus borealis*, *Pagurus bernhardus*, *Hyas araneus* and *Paralithodes camtschatica*, are known to cause lysis of human erythrocytes (Haug et al., 2002). Recently, hemocyanin from the shrimp, *Litopenaeus vannamei* or mud crab, *S. serrata* showed hemolytic activity against chicken erythrocytes or human A, B, AB, O, mouse, rabbit and chicken erythrocytes respectively (Yan et al., 2011b; Zhang et al., 2009).

Phenoloxidase, a copper containing enzyme, is known to play an important role in the host defense system of many invertebrates and its functions by initiating the biosynthesis of melanin for wound healing and pathogen encapsulation (Hoffmann, 1999; Söderhäll and Cerenius, 1998). Nagai and Kawabata (2000) have reported a clotting enzyme of the horseshoe crab, *Tachypleus tridentatus*, which functionally converted hemocyanin to phenoloxidase activity. Similarly, a chitin binding antimicrobial peptide of this horseshoe crab induced conversion of hemocyanin into an intense phenoloxidase activity (Nagai et al., 2001). Thus, these observations clearly indicate that the hemocyanin, which possesses antimicrobial activity, can show or produce phenoloxidase activity through a proteolytic cleavage by a clotting enzyme. In the present study, we have found that the antibacterial protein isolated from the serum of mud crab, *S. serrata* also possess phenoloxidase activity by oxidizing L-DOPA, this activation is inhibited by known PO PTU and tropolone confirming the activation of PO is one of defense mechanisms in humoral immune function.

Thus, taking together all the findings, it may be safely concluded that the antibacterial hemocyanin (Ab-Hcy) from the serum of mud crab, *S. serrata*, is capable of performing multiple antimicrobial activities including bacteriostatic, bacteriolytic, bacterial agglutinating, hemolytic as well as phenoloxidase activity, a feature often reported for either native or subunits of hemocyanin molecule(s) in the hemolymph of decapod crustaceans.

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Table 4
Effect of phenylthiourea (PTU) and tropolone on the oxidation of L-DOPA by Ab-Hcy of mud crab, *Scylla serrata*.

Samples tested	Δ_{490} min^{-1}·mg protein^{-1}[a]
Ab-Hcy + L-DOPA	0.0198 ± 0.004
Ab-Hcy + PTU + L-DOPA	0.0021 ± 0.003*
Ab-Hcy + tropolone + L-DOPA	0.0125 ± 0.003

[a] Oxidation of L-DOPA was determined spectrophotometrically at 490 nm and the values are given as mean ± SD of three determinations using samples from different preparations.

* The difference between the oxidation of L-DOPA by Ab-Hcy with PTU are statistically significant ($^*p < 0.005$) compared to Ab-Hcy + L-DOPA.

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