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RESEARCH REPORT

Detection and preliminary characterization of antibacterial protein(s) in the serum of mud crab, *Scylla serrata*

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Abstract

Serum of mud crab, *Scylla serrata* has been found to possess significant antibacterial activity against some of the resident specific bacteria including *Bacillus* sp. N1, *Bacillus flexus* N3, *Escherichia coli* as well as crustacean pathogenic bacteria viz., *Vibrio harveyi, V. alginolyticus* and *V. vulnificus.* The physico-chemical characterization reveals the molecule responsible for antibacterial activity in the serum over 14 kDa, stable in the pH range of 6 to 8 and between the temperatures 20 to 40 ºC. Precipitation of respective molecule(s) with 75 % ammonium sulphate or the supernatant obtained after precipitating the protein with 10 % TCA indicated that the molecule(s) responsible for serum antibacterial activity appear to be proteinaceous in nature. Further studies demonstrated that antibacterial molecule(s) against *E. coli* and *V. harveyi* appeared to be trypsin and pronase resistant and the molecule(s) or domain responsible for antibacterial activity against *Bacillus* sp. N1 and *B. flexus* N3 appeared to be protease sensitive, thereby implicating possible involvement of multiple antibacterial factors in the serum of mud crab, *S. serrata*.

Key Words: mud crab; *Scylla serrata*; serum; antibacterial activity; protein

Introduction

Invertebrates, which contributes about 95 % of the extant species in the animal kingdom (Ratcliffe, 1985; Smith, 1991). Among Invertebrates, Crustaceans are the second largest group, next to insects, in the phylum arthropoda comprising approximately more than 30,000 known species and they have a predominant role in aquatic food chain, especially as primary and secondary consumers as well as food for human (Bachère *et al.,* 2000). Though, the invertebrate including crustaceans lack lymphocytes and functional immunoglobulins (IgGs), this should not rule out the potential for the existence of not only innate immune mechanisms known in mammalian system but also unique form of innate immune mechanisms that might have been discovered with evolution of the first vertebrates (Rowley and Powell, 2007). Invertebrates have developed unique defense mechanisms/modalities to detect microbial surface 'antigens like' lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, peptidoglycans (PGN), 1,3-β-D-glucans, toll like receptors mediated antibacterial peptides (Lemaitre *et al.,* 1996; Imler and Hoffmann, 2000; Underhill and Ozinsky, 2002) and respond through hemolymph coagulation (Iwanaga *et al.,* 1978), melanin formation (Sugumaran, 2002), agglutinin/lectin mediated complement activation (Fujita, 2002), generation of reactive oxygen intermediates (ROIs), nitric oxide (NO) (Raman *et al.,* 2008) and phagocytic system, encapsulation and nodule formation which co-operate with humoral immune reactors to kill invading pathogens (Söderhäll and Cerenius, 1992; Bogdan *et al.,* 2000). These mechanisms, which together compose the innate immune system, defend invertebrates from invading pathogens like bacteria, fungi and viruses (Iwanaga and Lee, 2005; Rowley and Powell, 2007).

Antimicrobial peptides/proteins are a major component of the innate immune defense system in marine invertebrates. These molecules are the first line of host defense in various species and the knowledge acquired over the last two decades on the identification and characterization of antimicrobial peptides/proteins in crustaceans has revealed their essential role in the immune response and also in the capacity of these animals to survive

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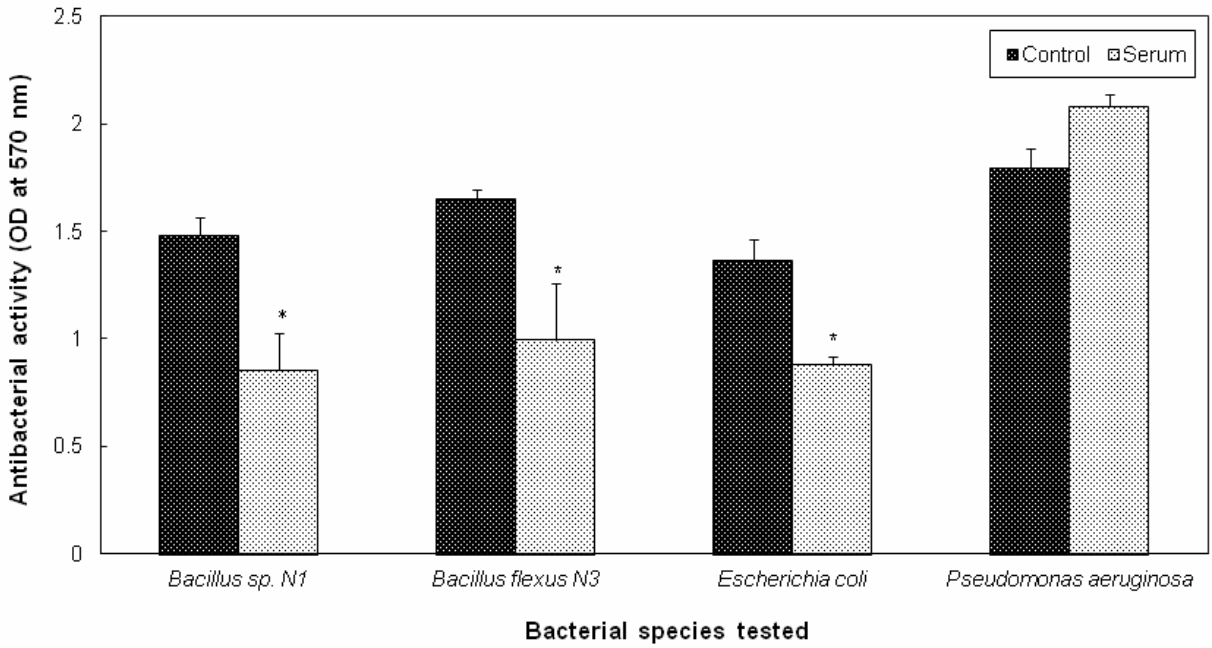

Fig. 1 Serum antibacterial activity against bacterial colonies (*Bacillus* sp. N1, *Bacillus flexus* N3, *Escherichia coli* and *Pseudomonas aeruginosa*) isolated from the scraps of injured/wounded cuticle of the mud crab, *Scylla serrata*. Each bar represents mean ± SD from three determinations. The difference in antibacterial activity between control and whole serum treated bacteria were statistically significant at $*p < 0.05$ level.

infection. Antibacterial peptides or proteins have been most extensively studied (Yeaton, 1981; Vasta and Marchalonis, 1985; Olafsen, 1995; Smith and Chisholm, 2001) due to the following reasons: (1) they occur in the hemolymph/serum of almost every crustacean species examined and (2) interact directly with foreign materials, particularly the potential microbial pathogens and thereby appear to serve as humoral recognition function in second line of defense.

Scylla serrata, is an economically important marine invertebrate distributed throughout the west Pacific and Indian Oceans. It is the most important edible crab for commercial culture in the Indo-West Pacific region and commands a high price in both the domestic and export markets (Samonte and Agbayani, 1992). These crabs are in intimate contact with an environment rich in pathogenic bacteria, and are prone to infection by microbes at various stages of growth, losses due to disease can be enormous (Hudson and Lester, 1994). Hence, it is necessary to understand the existing defense mechanisms in such animals and find ways of enhancing their natural immunity against infectious pathogens. In addition, from the identified antimicrobial peptides/protein families, only a few investigators have tested these molecules against crustacean pathogens such as marine *Vibrios* that may cause severe infections to these animals. This study thus describe the serum antibacterial activity against resident specific as well as crustacean pathogenic bacteria and physico-chemical characterization of the naturally occurring antibacterial molecule in the serum of mud crab, *S. serrata*.

Materials and Methods

Isolation and identification of bacteria

The mud crab, *Scylla serrata* collected from the backwaters of Pulicat Lake, were brought to laboratory, maintained in sea water (35 ± 2 ‰) and acclimatized for at least 3 to 4 days. The samples were collected from injured cuticular region of the crab, from these samples four distinct bacterial colonies were isolated and identified using a series of morphological, biochemical as well as molecular (16s rDNA) studies as *Bacillus* sp. N1, *Bacillus flexus* N3, *Escherichia coli* and *Pseudomonas aeruginosa* (data not shown) using Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

Serum antibacterial activity

From the cut end of dactylus region of the walking leg of the crab, the hemolymph was collected in the eppendorf tubes held on ice and allowed to clot at room temperature (RT). The clot was vigorously disturbed by using a glass rod, centrifuged (450*g*, 10 min, 4 °C) and the resulting clear supernatant (=serum) was used immediately. The serum antibacterial activity was tested by colorimetric method using MTT [3-(4, 5- dimethylthiazol-2-yl) - 2, 5- diphenyltetrazolium bromide] against bacterial species isolated from *S. serrata* viz., *Bacillus* sp. N1, *Bacillus flexus* N3, *Escherichia coli and Pseudomonas aeruginosa* as well as other known crustacean pathogenic bacteria procured from CIBA, Chennai, including *Vibrio harveyi, V. vulnificus, V. alginolyticus, V. parahemolyticus*and *V. anguillarum* (Freimoser *et al.,* 1999; Sheena *et al.,* 2003).

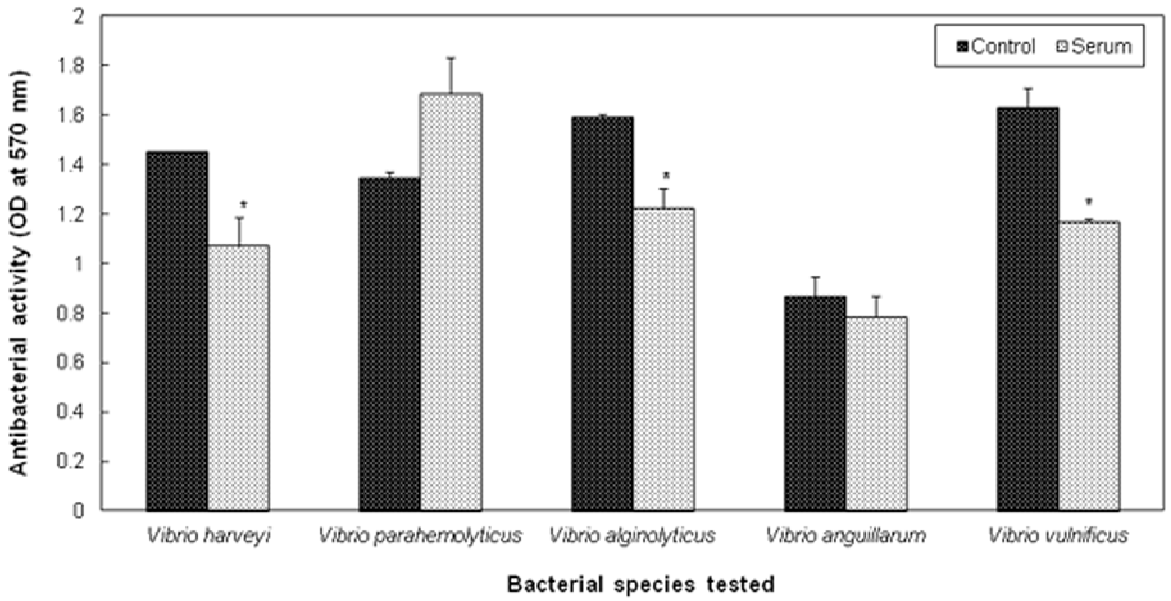

Fig. 2 Serum antibacterial activity against crustacean pathogenic bacteria (*Vibrio harveyi, V. alginolyticus* and *V. vulnificus).* Each bar represents mean ± SD from three determinations. The difference in antibacterial activity between control and whole serum treated bacteria were statistically significant at $*p < 0.05$ level.

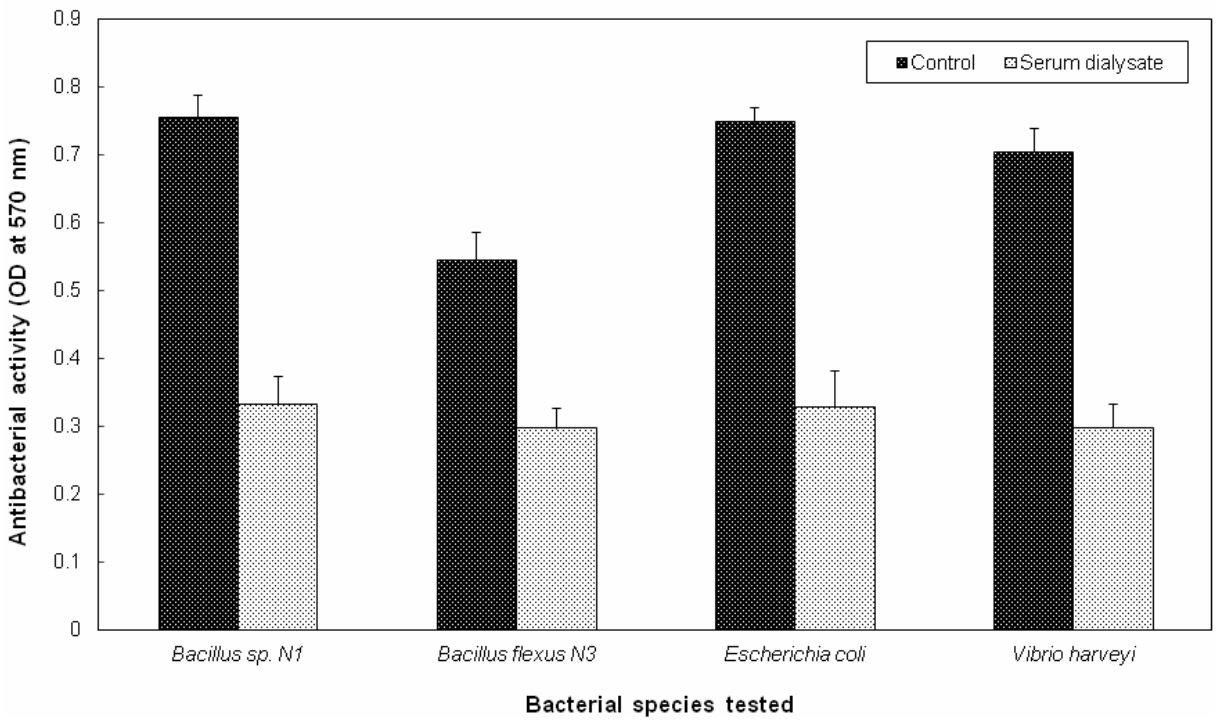

Fig. 3 Antibacterial activity in the serum of serum dialysate (MWCO 12 to 14 kDa) of the mud crab, *Scylla serrata*. Each points represents mean ± SD from four determinations.

Effect of dialysis on the serum antibacterial activity

Serum samples (500 µl) were extensively dialysed using dialysis tubing with MW exclusion limit of 12,000 to 14,000 Da against TBS (50 mM tris-HCl; 115 mM NaCl; 10 mM $CaCl_2$; *p*H 7.5; 300 mOsm). The resulting dialysates were centrifuged (400*g*, 5 min, 4 °C) and the antibacterial activity of the supernatant was determined against four bacterial species including *Bacillus* sp. N1, *B. flexus* N3, *E. coli* and *V. harveyi*.

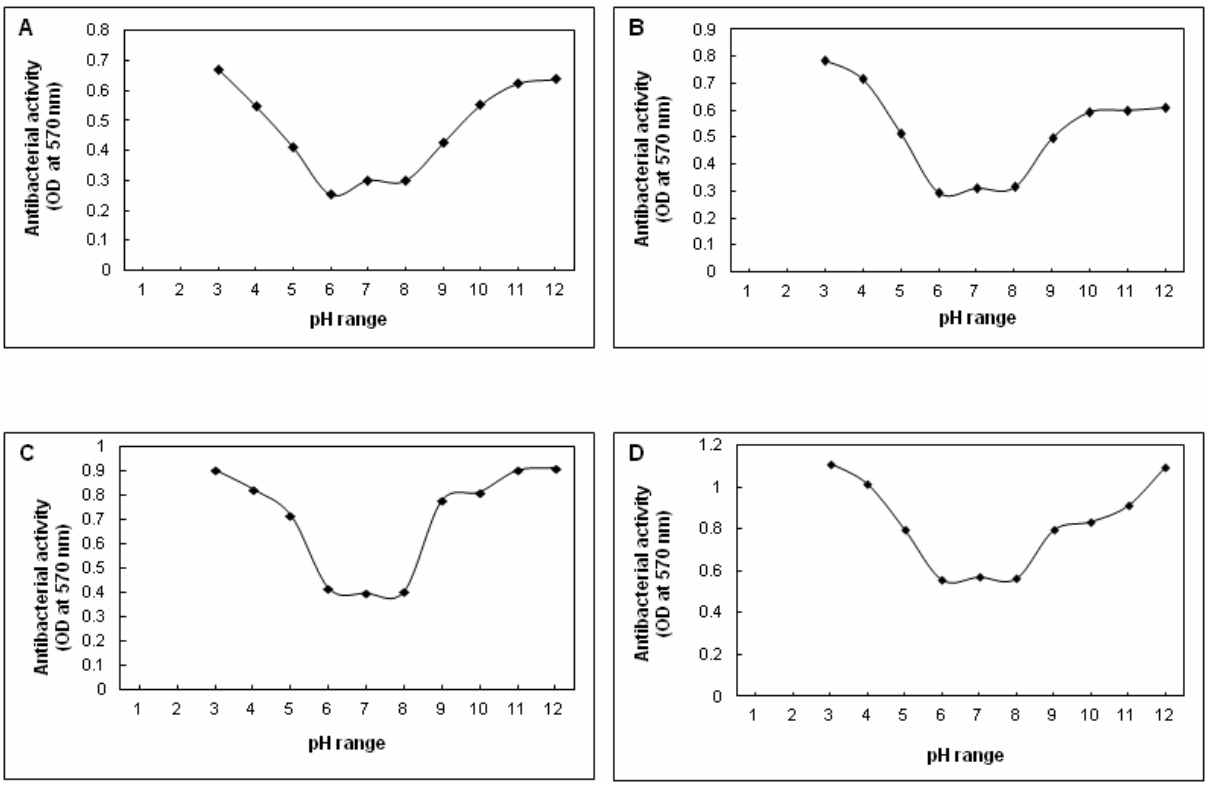

Fig. 4 pH stability of serum antibacterial activity of mud crab, *Scylla serrata.* Results represents consistent performance from three determinations. A. *Bacillus* sp. N1; B. *B. flexus* N3; C. *E. coli*; D. *V. harveyi*

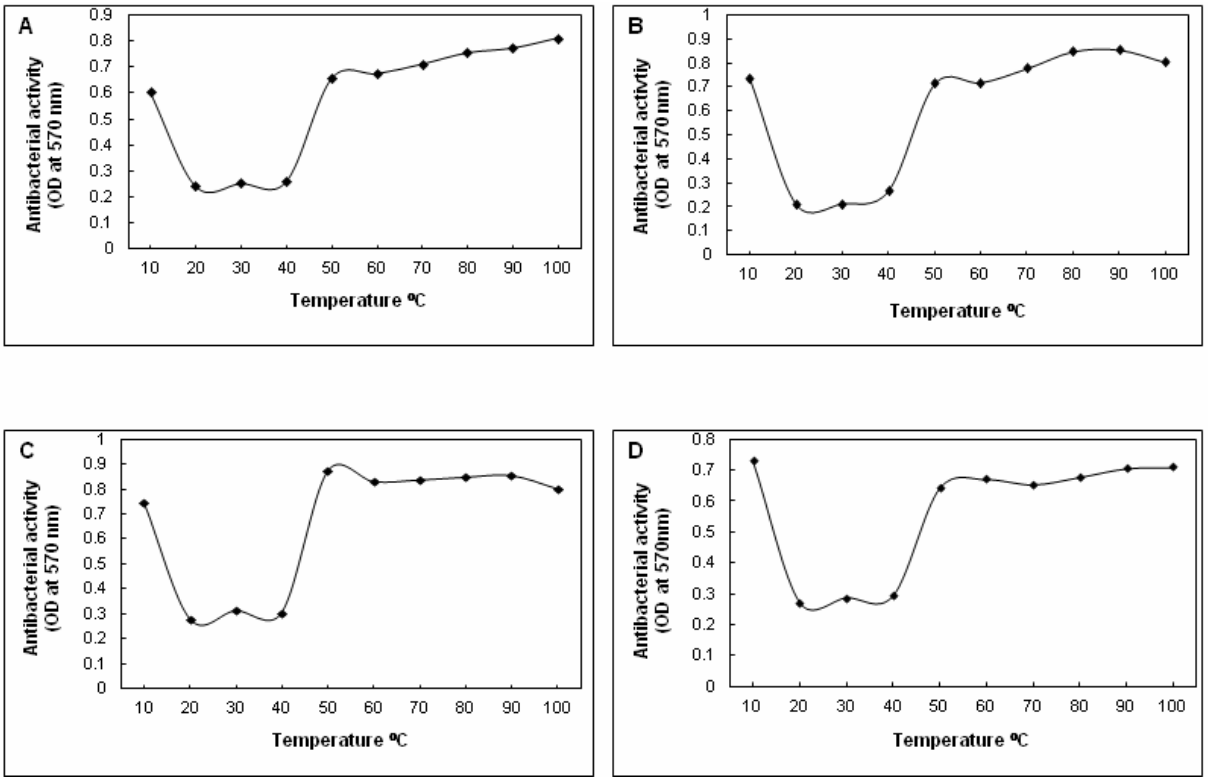

Fig. 5 Thermal stability of serum antibacterial activity of mud crab, *Scylla serrata.* Results represent consistent performance from three determinations. A. *Bacillus* sp. N1; B. *B. flexus* N3; C. *E. coli*; D. *V. harveyi*

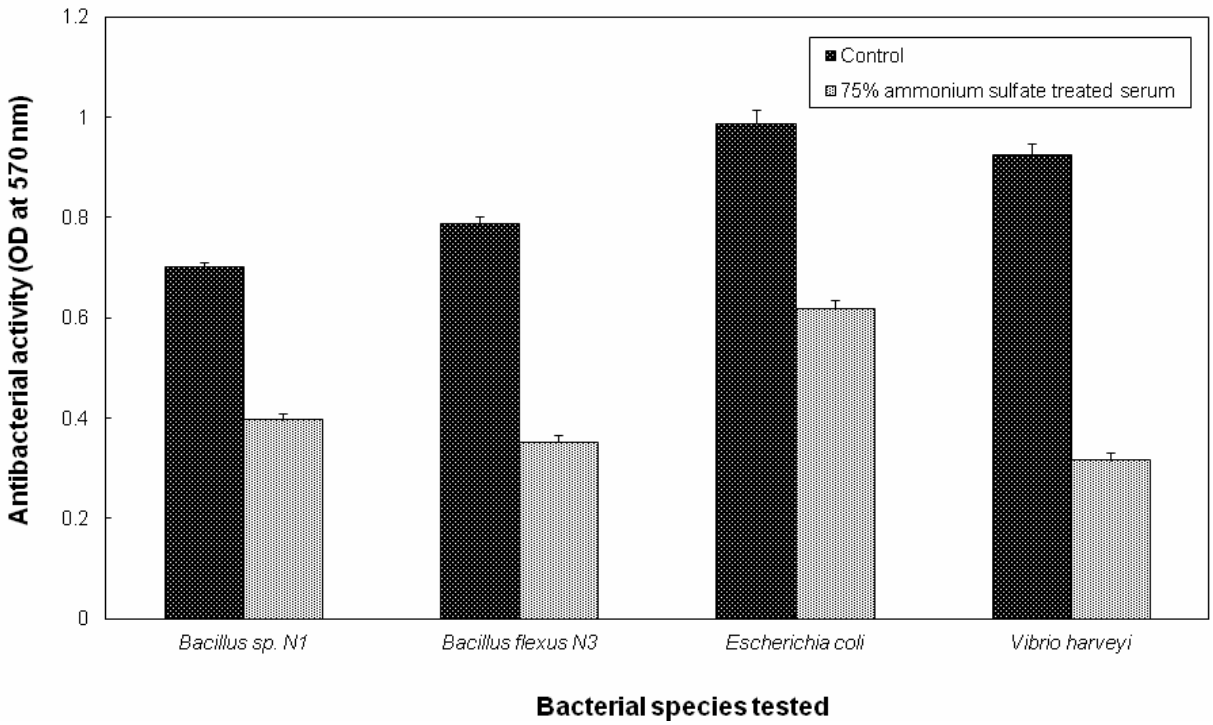

Fig. 6 Effect of protein precipitating agent (75 % ammonium sulphate) on serum antibacterial activity of the mud crab, *Scylla serrata*. Each points represents mean ± SD from four determinations.

pH and thermal stability

Serum samples (250 µl) were dialyzed against the following buffers (200 mM) at pH ranging from 3 to 12: acetate buffer (pH 3 to 6), tris-HCl buffer (pH 7 to 9) and glycine-NaOH buffer (pH 10 to 12) and re-equilibrated by dialysis against TBS. The dialysates were centrifuged (400*g*, 5 min, 4 °C) and the resulting supernatant were tested for antibacterial activity. Thermal stability of serum antibacterial activity was examined by holding 150 µl serum samples for 30 min at temperatures ranging from 10 to 100 °C. All samples were centrifuged (400*g*, 5 min, and 4 °C) and the clear supernatant was used to determine the antibacterial activity against *Bacillus* sp. N1, *B. flexus* N3, *E. coli* and *V. harveyi*.

Effect of deproteinising agents and proteases

The ice cold serum samples were mixed with 75 % saturated $(NH_4)_2SO_4$ and incubated for 45 min at 10 °C, centrifuged (400*g*, 5 min, 4 °C), resulting precipitates were finally dissolved in TBS, dialyzed and used for antibacterial assays. Similarly, 400 µl serum samples was mixed with an equal volume of ice-cold TCA solution (10 %) and incubated for 45 min at 10 °C, supernatant was collected, dialyzed and used to antibacterial activity. The serum samples (250 µl) were mixed with an equal volume of trypsin or pronase (final concentration 6 $mg.ml^{-1}$), incubated for 3 h at 37 °C, centrifuged (400*g*, 5 min, 4 °C) and the supernatants were tested for antibacterial activity. Controls consisted of serum mixed with heat inactivated (10 min, 100 °C) enzymes.

Statistical analysis

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

Results

Serum antibacterial activity

The serum (1.224 mg protein) of healthy mud crab, *S. serrata* significantly inhibit the growth of the resident specific bacteria (isolated from injured cuticle of crab) viz., *Bacillus sp*. N1, *B. flexus* N3 and *E. coli* ($p < 0.05$) but did not affect the growth of *Pseudomonas aeruginosa*. Similarly, the serum also inhibit the crustacean pathogenic bacteria including *Vibrio harveyi, V. alginolyticus* and *V. vulnificus* ($p < 0.05$) but serum did not influencing the growth of *V. parahemolytics* and *V. anguillarum* (Figs 1, 2). These observations reveals the presence of antibacterial molecule in the serum of this crab not only against resident specific Gram-positive (*Bacillus* sp.) and Gram-negative (*E. coli*) bacteria but also against certain other crustacean pathogenic bacteria.

Based on these results obtained from the serum antibacterial activity tested, three resident specific bacteria including *Bacillus* sp. N1, *B. flexus* N3, *E. coli* and one of the crustacean pathogenic

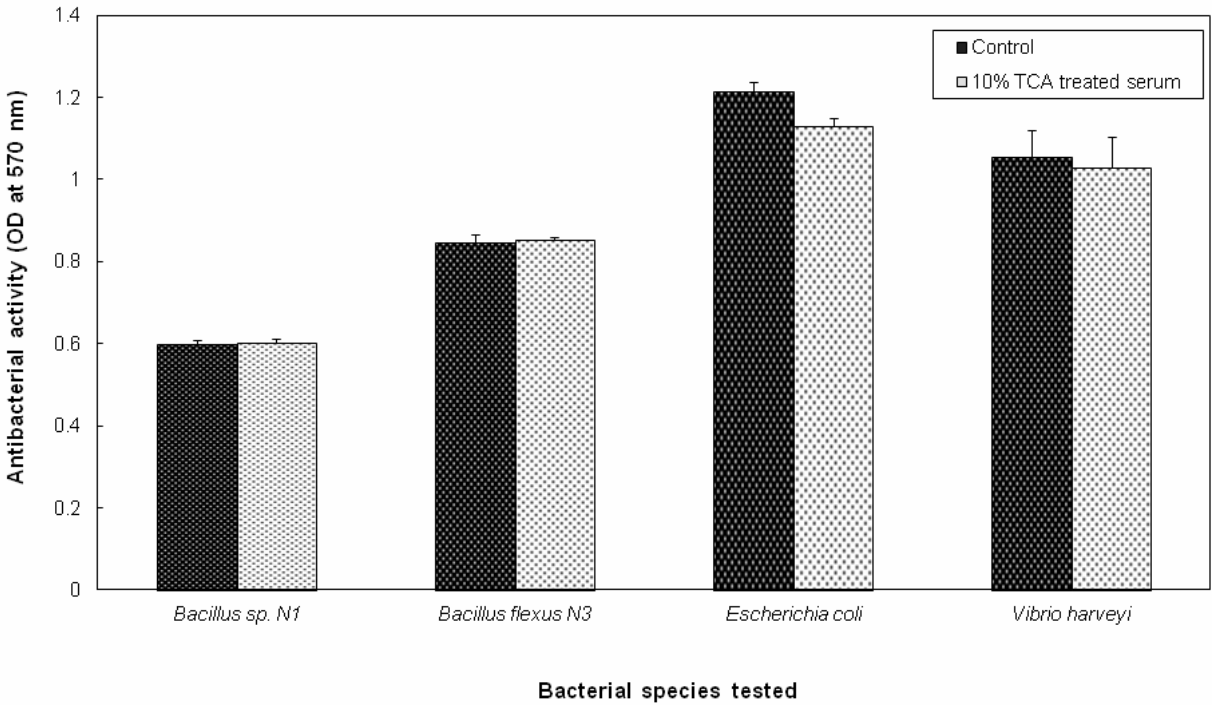

Fig. 7 Effect of protein denaturing agent (10 % TCA) on serum antibacterial activity of the mud crab, *Scylla serrata*. Each points represents mean ± SD from four determinations.

bacteria *(V. harveyi)* were chosen for further preliminary characterization of serum antibacterial activity (factor) of mud crab, *S. serrata*.

Physico-chemical properties

The dialyzate obtained, after an extensive dialysis of serum of *S. serrata,* using dialysis tubing with a molecular weight cut-off (MWCO) of 12 to 14 kDa, showed effective antibacterial activity all the four bacterial species tested including, *Bacillus* sp. N1, *B. flexus* N3*, E. coli* and *V. harveyi* (Fig. 3).

pH and thermal stability

The serum samples dialyzed against the buffers at different (pH 3 to 12), the antibacterial activity was stable only between the pH range 6 to 8 and no activity was found on either side of this pH range against all the four bacteria tested (Fig. 4). When serum samples were held at various temperatures (10 °C to 100 °C) for 30 min, the serum antibacterial activity against all the four bacterial species tested were found to be stable only between 20 to 40 ºC and the activity was completely lost at both the extremes of temperature (Fig. 5).

Effect of deproteinising agents and proteases on serum antibacterial activity

The buffer re-dissolved precipitate, obtained after precipitation of serum proteins using 75 % ammonium sulphate, inhibited the growth of all the four bacterial species tested (Fig. 6). On the other hand, the dialyzed supernatant, obtained after precipitating serum proteins with 10 % TCA, did not inhibit the growth of any of the four bacterial species tested (Fig. 7). In addition, when serum samples were treated with proteases like trypsin and pronase E (6 mg. ml^{-1}; 37 °C; 3 h), serum incubated with pronase E completely inhibited the growth of *E. coli* and *V. harveyi* while no antibacterial activity were found against *Bacillus* sp. N1 and *B. flexus* N3. While the serum samples incubated with tripsin failed to inhibit the growth of all the four bacterial species tested (Fig. 8).

Discussion

The earlier investigations reported the presence of antibacterial proteins/peptides in a variety of tissues including seminal plasma, hemolymph or serum of crustaceans (Schnapp *et al*., 1996; Destoumieux *et al.,* 1997; Majumder *et al*., 1997; Jayasankar and Subramoniam, 1999; Hoq *et al*., 2003; Amparyup *et al*., 2008) and they were found to be effective against both Gram-positive and Gram-negative bacteria. In the present study, the undiluted serum of mud crab, *S. serrata* appear to contain antibacterial factor(s) especially against *Bacillus* sp. N1, *B. flexus* N3 and *E. coli* but not *P. aeruginosa*. Similarly, when the undiluted serum was tested against other *Vibrio* colonies also showed antibacterial activity against *Vibrio harveyi, V. alginolyticus* and *V. vulnificus,* while it failed to show antibacterial activity against *V. parahemolyticus* and *V. anguillarum*. These results clearly indicate that the serum of the mud crab appeared to possess effective antibacterial activity not only against resident specific Gram-positive

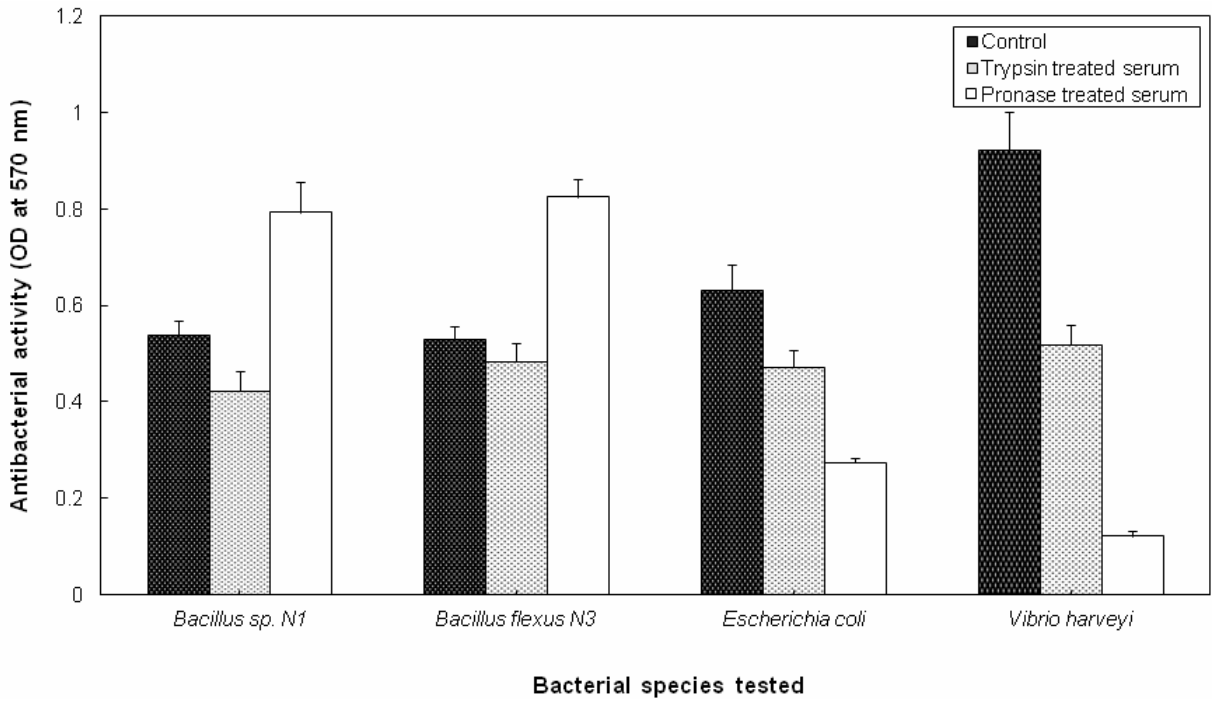

Fig. 8 Effect of proteases on serum antibacterial activity of the mud crab, *Scylla serrata*. The serum treated with proteases (6mg.ml^{-1}) showed antibacterial activity against *Escherichia coli and Vibrio harveyi.* Each points represents mean ± SD from four determinations.

(*Bacillus* sp.) and Gram-negative (*E. coli*) bacteria, but also against certain specific species of *Vibrio* isolates from other crustaceans.

In order to study the physical/chemical characteristics of the molecule responsible for serum antibacterial activity, a dialysis experiment was carried out using dialysis tubing with MWCO 12 to 14 kDa. These observations clearly showed that the molecule responsible for antibacterial activity against these bacterial species over 14 kDa, indicating the presence of larger antibacterial molecules in serum of mud crab *S. serrata*. While most of the antibacterial activity reported in the serum of decapod crustaceans are in the range of 3.7 to 12 kDa (Schnapp *et al.,* 1996; Khoo *et al.,* 1999; Relf and Chisholm, 1999; Herbinière *et al.,* 2005; Battison *et al.,* 2008), there are few reports including the serum of *S. serrata* with larger native antibacterial molecule(s) with a size of 247 kDa (Makesh, 2006) or inducible antibacterial protein with a size of 36 to 64 kDa (Hoq *et al.,* 2003).

Further analyses on the physical properties of antibacterial factor(s) present in the serum revealed that this antibacterial factor(s) against all the four bacterial species tested was stable in the pH range of 6 to 8 and between the temperatures 20 to 40 ºC. The protein present in the granular and semi-granular hemocytes able to kill both Gram-positive and Gram-negative bacteria including some *Vibrio* strains known to pathogenic to crustaceans with activity optimal at pH 6 - 9 stable to 50 ºC (Hikima *et al.,* 2003; Tyagi *et al.,* 2007). In contrast, Relf and Chisholm (1999) reported that the antibacterial protein from the granular hemocytes of the shore crab, *Carcinus maenas* is stable after heating to 100 ºC. Presence of antibacterial activity against all the four bacterial species in the precipitation obtained with 75 % ammonium sulphate or the supernatant obtained after precipitating the protein with 10 % TCA indicated that the molecule(s) responsible for serum antibacterial activity appear to be proteinaceous in nature.

The pre-treatment of serum samples with exogenous proteases namely trypsin or pronase did not affect the serum antibacterial activity against *E. coli* and *V. harveyi*. On the other hand, the proteases treated serum failed to show antibacterial activity against *Bacillus* sp. N1 and *B. flexus* N3. These results clearly indicated that while the molecule(s) responsible for antibacterial activity against *E. coli* and *V. harveyi* appeared to be trypsin and pronase resistant, the antibacterial molecule(s) or domain responsible for antibacterial activity against *Bacillus* sp. N1 and *B. flexus* N3 appeared to be protease sensitive, thereby implicating possible involvement of multiple antibacterial factors. Further, these inferences also indicated that the serum molecule(s) responsible for antibacterial activity against *E. coli* and *V. harveyi* or *Bacillus* sp. N1 and *B. flexus* N3 possibly appear to be larger sized heterogenous protein comprising non-specific protease sensitive and resistant molecule either with

different domains of native molecule(s) or subunits of a native antibacterial molecule(s). Thus, this study provides the evidence that serum of mud crab, *S. Serrata* possess multiple antibacterial protein molecules against host specific/resident specific bacteria as well as crustacean pathogenic bacteria. The isolation and characterization of these respective antibacterial molecules are required to understand the humoral immune mechanism of the crustaceans.

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