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Purification of antimicrobial peptides from *Paratelphusa jacquemontii* hemolymph in response to artificial bacterial infection

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Keywords

Paratelphusa jacquemontii, Hemolymph, bacterial infection.

Abstract

The present study is on purification of antimicrobial peptides from *Paratelphusa jacquemontii* hemolymph in response to artificial bacterial infection. This study showed three different antimicrobial proteins in the hemolymph of *P. jacquemontii* in response to artificial bacterial infection challenge by *V.parahaemolyticus* and *Staph. aureus*. The antimicrobial proteins expressed in response to the challenge were 66 kDa, 40kDa and 8kDa in SDS-PAGE gel. The expression antimicrobial proteins of 40kDa and 8kDa were observed only in response to *V.parahaemolyticus* infection and were not observed in *Staph. aureus* challenged hemolymph. In the present study all the purified antimicrobial proteins (AMP) showed antibacterial activity against the tested bacterial pathogens. In the present study, 66 kDa protein showed inhibition to both Gm-ve and Gm+ve bacteria tested. Among tested, *Pseudmonas aeruginosa* was most inhibited with 14mm zone of inhibition followed by *S.typhi* (12mm), *L.bulgaricus* (11mm). *S.paratyphi* (10mm) and *S.aureus* (10mm) with a zone of inhibition. Likewise, 40kDa purified protein most inhibited *Pseudmonas aeruginosa* (21mm), followed by *S.paratyphi* (14mm), *S.aureus* (13mm), *S.typhi* (12mm) *V.cholerae* (11mm), *K.pneumoniae* (10mm) and *K.oxytoca* (9mm). The 8kDa peptide showed highest inhibitory potential against *V.cholerae* (15mm) followed by *L.bulgaricus* (12mm), *Pseudmonas aeruginosa* (9 mm), *E.coli* (9mm),and *Staph. aureus* (8mm).

Introduction

Crustaceans compose a large, ancient and diverse animal group that includes many well-known, commercially exploited members, such as shrimp, crab, crayfish and lobster. Crustaceans have confronted a broad variety of challenges to their self-integrity during their long-standing existence, because their natural habitats are typically overloaded with infectious organisms, such as viruses, bacteria, fungi and other parasites. Indeed, they can harbor specific microbial communities in their surface and internal epithelia that have important roles in nutrition and defense (Gil-Turnes *et al.*, 1989).

In normal conditions, crustaceans maintain a healthy state and keep infections under control. Externally, they are covered by a hard, rigid exoskeleton that functions as an efficient physico-chemical barrier against mechanical injury and microbial invasion. Crustaceans lack adaptive immune system and they rely exclusively on their innate immune mechanisms that include both cellular and humoral responses (Iskratsch et al., 2009). The innate immune system of crustaceans is primarily related to their blood or hemolymph and is comprised of cellular and humoral responses. Humoral defenses include

pattern-recognition receptors/proteins that recognize pathogen-associated molecular patterns (PAMPs), the production of toxic oxygen and nitrogen metabolites, complex enzymatic cascades leading to melanization, clotting proteins and antimicrobial peptides.

Antimicrobial peptides are proteins (AMPs) are one of the major components of the innate immune defense and are ubiquitously found from all kingdoms from bacteria to mammals including fungi and plants. AMPs are primarily known as natural antibiotics because of their rapid and efficient antimicrobial effects against a broad range of microorganisms including gram positive and gram negative bacteria, yeasts, filamentous fungi and to lesser extent protozoans and enveloped viruses (Guani-Guerra *et al.*, 2010). Antimicrobial peptides have become recognized as important components of non-specific host defense or innate immune system in a variety of organisms ranging from vertebrates and invertebrates to plant species (Boman, 1995 and Hoffmann *et al.*, 1999). Antimicrobial peptides are important members of the host defense system and they have a broad ability to kill microbes. Large antimicrobial proteins (>100 a.a.), are often lytic, nutrient-binding proteins or specifically target microbial macromolecules. Small antimicrobial peptides act by disrupting the structure or function of microbial cell membranes. Hence the present study was on purification of antimicrobial peptides from *Paratelphusa jacquemontii* hemolymph in response to artificial bacterial infection.

Materials and Methods

Experimental animals and laboratory maintenance

Freshwater adult male crabs, with a body weight of 30±3 g (carapace width of 34±3 mm) were collected from the rice fields and irrigation canals around Oratha Nadu, Pattukottai Dist, Tamilnadu, India. Animals were housed 4-6 per plastic tank (length/width/ height=30:40:30 cm) with soil and fresh water and transferred to fresh medium daily. They were acclimatized to the laboratory conditions (Temperature - 27±1°C and relative humidity - 75%) for 7 days. Crabs were fed with goat meat throughout the period of maintenance in the laboratory. Only male crabs were used in the present study.

Bacterial challenge/Injection of bacteria

Each group (contains 3 Nos.) of crabs were injected with 100 µl of a suspension containing about 10^6 CFU/ml bacteria (*S.aureus* and *V.parahaemolyticus*) in phosphate buffered saline (PBS). Control crabs (3 Nos.) received 100 µl of PBS only. Injections were made inserting the needle of 1-ml sterile syringe into the third walking leg. After injection, control and injected animals were immediately transferred to three different plastic tanks with fresh water and soil. Note: *Staphylococcus aureus* and *V. parahaemolyticus* were isolated from infected crabs.

Antimicrobial activity of haemolymph

Antagonistic assay was done by an agar-well diffusion method in aerobic condition. Collected haemolymphs were tested for the antibacterial activity. Human bacterial pathogens such as *Staphylococcus aureus, Vibrio cholerae, Salmonella paratyphi, Klebsiella pneumoniae, Salmonella typhi, Proteus mirabilis, Klebsiella oxytoca, E.coli* and *Pseudomonas aeruginosa* spreaded on Muller Hinton agar plates. The pathogenic strains were obtained from CMC, Vellore. Fish pathogens such as *V.harveyi, V.parahaemolyticus, V.vulnificus, V.anguillarum,V.mimicus, V.alginolyticus, A.hydrophila* and *Yersinia ruckeri* obtained from CIFE (Mumbai) and Fisheries collage (Tuticorin) were also used. The spectrum of antibacterial activity was studied by using the techniques described by Bauer *et al.*, 1996. Antibacterial activity was expressed in terms of diameter of zone of inhibition was measured in mm using scale and recorded.

Purification of antimicrobial peptides

Ammonium sulphate precipitation and dialysis

The crude hemolymph samples were collected from both control and infected, *Paratelphusa jacquemontii* male crabs and centrifuged at 15,000 rpm for 10min. To the serum, varying concentration of ammonium sulphate was added slowly with stirring. Dialysis was followed in a tubular cellulose membrane against phosphate buffer for 24 hrs at 4°C. The resultant concentrated protein was checked for inhibitory activity and based on the result 60% ammonium sulphate was added to the hemolymph. The partially purified protein was lyophilized in a Vertis lyophilizer and was stored at -20°C for further analysis.
The lyophilized powder was resuspended in 10 mmol/L phosphate buffer (pH 7.2) and checked in 15% SDS-PAGE. The crude sample showed protein ranges from ~6.5-158kDa were separated on a 10kDa molecular weight cut-off (MWCO) membrane. Sample was separated by the centrifugation filters, centripepes (Millipore, India) having cut-off value of 10 kDa, by centrifugation for 45min. (3000×g at 4°C). After separation the filtrate (< 10 kDa) and rentate (>10 kDa)

were kept in a separate vial for further purification. The antibacterial activity was observed for both filtrate and rentate. As the both showed antibacterial activity they were further purified on gel filtration chromatography.

Gel filtration chromatography

The filtrate and rentate were purified on a column of Sephadex G-10 and G-100 (SIGMA, USA), respectively and eluted with phosphate buffer containing 0-1.5mol/L NaCl and also by using DEAE Sephadex A-50 column. Each fraction was assayed for antimicrobial activity. The active fractions were pooled together and concentrated by lyophilization. The protein contents were determined by measuring the absorbance at 280 nm or by the method of Bradford (1976). The resolution of purification was checked on 15% SDS-PAGE gel. Purified fractions were again tested for its activity against human bacterial pathogens.

Results and Discussion

Many antimicrobial peptides show a broad activity against microorganisms and have a remarkable specific activity to prokaryotic cells and with low toxicity to eukaryotic cells. These phenomena and the mode of action demonstrate that antimicrobial peptides may be developed as therapeutic agents for use as pharmaceuticals or in agricultural applications (Zasloff, 1992).

Several antimicrobial peptide have been characterized from insects and chelicerates, but a few peptides demonstrated in crustaceans from the shore crab *Carcinuns meanas* (Relf *et al.,* 1999) and the shrimp *Penaeus vanamei* (Destoumieux *et al*., 1997; 1999). Recentaly, two antibacterial peptides of low molecular masses named astacidin 1and 2 were purified and charaterized from crayfish *Pacifastacus leniusculus* hemolymph. Astacidin 1 with 16 amino acid residuces had no homology to other hitherto discribed antimicrobial peptides whereas, astacidin 2 with 14 amino acid residues has high similarity to the proline rich-peptide, metalnikowin 1 purified from the hemipteran insect, *Palomena prasina* (Charlet *et al.,* 1996). Few researchers have worked on antimicrobial activity of low molecular weight peptides isolated from crustaceans. Okino *et al.,* 1995 purified and characterized a 27KDa lectin from hemocytes of horse shoe crabs. Schnapp *et al*., 1996 isolated a 6.5KDa protein from *C.maenas* haemocytes which was found to be hydrophobic, cationic and prolinc rich.

The present study showed expression of three different antimicrobial proteins in in the hemolymph of *P. jacquemontii* in response to artificial bacterial infection i.e. (challenged by *V.parahaemolyticus* and *S.. aureus*. The antimicrobial proteins were purified using various purification techniques and the purified proteins were separated on SDS-PAGE which revealed that the molecular weight of the purified proteins were 66 kDa, 40kDa and 8kDa (Figs. 1-4). The expression of antimicrobial proteins of 40kDa and 8kDa were observed only in response to *V.parahaemolyticus* infection and were not observed in *S.. aureus* challenged hemolymph.

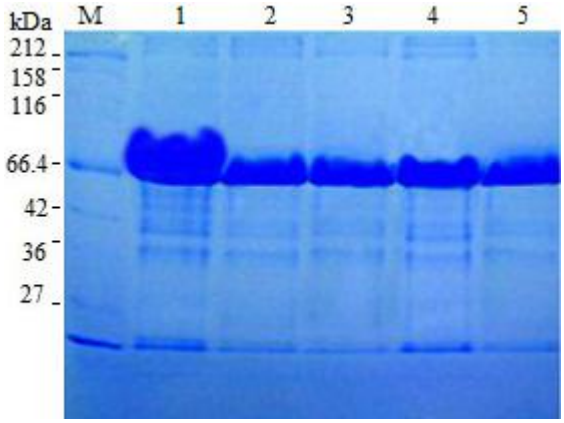

Fig. 1: Haemolymph protein pattern of *P. jacquemontii* challenged by *S.aureus*

Lane 1: Challenged - after 1hr., Lane 2: Challenged - after 1 day, Lane 3: Challenged - after 10 days, Lane 4: Control (Injected with PBS alone), Lane 5: Unchallenged control animal serum and Lane M: Standard protein molecular weight marker

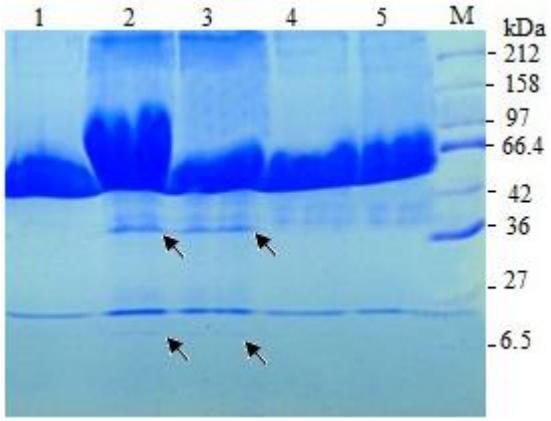

Fig. 2: Haemolymph protein pattern of *P. jacquemontii* challenged by *V.parahaemolyticus*

Lane 1: Unchallenged control animal serum, Lane 2: Challenged - after 1hr.
Lane 3: Challenged - after 1 day, Lane 4: Challenged - after 10 days, Lane 5: Control (Injected with PBS alone) and Lane M: Standard protein molecular weight marker

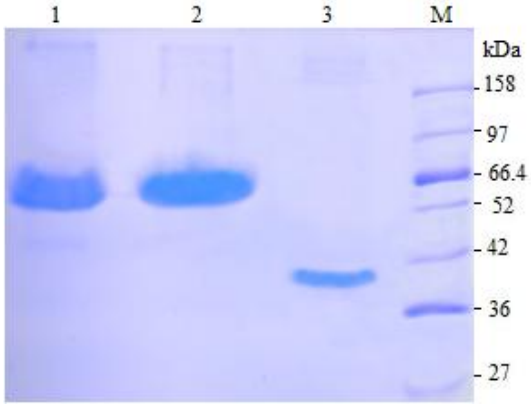

Fig. 3: Purified antimicrobial proteins of *P. jacquemontii* challenged by *S. aureus* and *V.parahaemolyticus* on SDS-PAGE (66 kDa & 40 kDa)

Lane 1: Purified 66kDa haemolymph protein from *P. jacquemontii* challenged by *S. aureus*, Lane 2: Purified 66kDa haemolymph protein from *P. jacquemontii* challenged by *V.parahaemolyticus*, Lane 3: Purified 40kDa haemolymph protein from *P. jacquemontii* challenged by *V.parahaemolyticus* and Lane M: Standard protein molecular weight marker

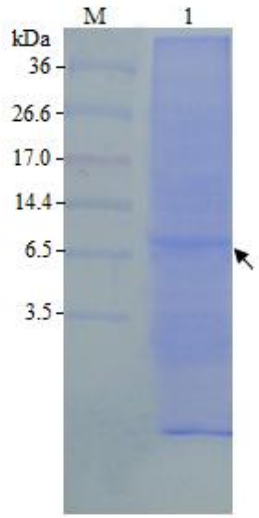

Fig. 4: Purified antimicrobial peptide (8kDa) of *P. jacquemontii* challenged by *V.parahaemolyticus* on SDS-PAGE

Lane 1: Purified antimicrobial peptide (8kDa) and Lane M: Standard protein molecular weight marker

In the present study all upregulated and newly formed proteins showed varying antibacterial activity. This result indicated the presence of "specific immune response" to invading pathogen. In the present study, 66 kDa protein showed inhibition to both Gm-ve and Gm+ve bacteria tested. Among tested, *Pseudmonas aeruginosa* was most inhibited with 14mm zone of inhibition, followed by *S.typhi* (12mm), *L.bulgaricus* (11mm). *S.paratyphi* (10mm) and *S.aureus* (10mm) with a zone of inhibition. Likewise, 40kDa purified protein most inhibited *Pseudmonas aeruginosa* (21mm), followed by *S.paratyphi* (14mm), *S.aureus* (13mm), *S.typhi* (12mm) *V.cholerae* (11mm), *K.pneumoniae* (10mm) and *K.oxytoca* (9mm). The 8kDa peptide showed highest inhibitory potential against *V.cholerae* (15mm) followed by *L.bulgaricus* (12mm), *Pseudmonas aeruginosa* (9 mm), *E.coli* (9mm),and *Staph. aureus* (8mm) (Tables 1-3). Schnapp *et. al*., 1996 isolated a 6.5 KDa antimicrobial peptide which was active against *Psychrobacter immobilis* and *Micrococcus luteus*, Gm +ve bacteria as well as to *E.coli*, a Gm-ve bacterium.

Table – 1: Antibacterial activity of 66 kDa protein (10µg/ml) isolated from haemolymph of *S.aureus* challenged *P. jacquemontii*

Pathogens	Zone of inhibition (mm)
S.typhi	12
P.aeruginosa	14
S.paratyphi	9
L.bulgaricus	11
S.paratyphi	10
S. aureus	10

Table - 2: Antibacterial activity of 40 kDa protein (10µg/ml) isolated from haemolymph of *V.parahaemolyticus* challenged *P. jacquemontii*

Pathogens	Zone of inhibition (mm)
P.aeruginosa	21
V.cholerae	11
S. aureus	13
K.oxytoca	9
K.pnemoniae	10
S.typhi	12
S.paratyphi	14

Table - 3: Antibacterial activity of 8 kDa peptide (10µg/ml) isolated from haemolymph of *V.parahaemolyticus* challenged *P. jacquemontii*

Pathogens	Zone of inhibition (mm)
P.aeruginosa	9
E.coli	9
V.cholerae	15
S. aureus	8
L.bulgaricus	12

Rameshkumar *et al*., 2009 studied the antimicrobial effect of proteins isolated from hemolymph of crab *Charybdis lucifera* collected from Vellar estuary. Inhibitory activity of protein isolated from male and female crabs showed slight variation against tested bacterial pathogens. Antifungal activity was not found except to *F. moniliforme*. Jayasankar and Subramoniam (1999), extracted seminal plasma of the mud crab *S.serrata* and checked its antibacterial activity. Hoq *et al.*, 2003 isolated and characterized antibacterial peptides from *Scylla serrata*. Ramesh kumar, 2007 isolated an antimicrobial peptide from the crab *Thalamita crenata*. Schnapp *et al.*, 1996 reported the presence of several constitutive antibacterial proteins, active against both Gram-positive and Gram-Negative bacteria, in the haemocytes of the shore crab, *Carcinus maenas*. Though these studies o marine crabs, the present study was AMPS on a fresh water edible crab.

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