

## Antimicrobial Peptide from the Crab, *Thalamita crenata* (Latreille, 1829)

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**Abstract:** Antimicrobial peptides are important in the first line of the host defense system of many animal species. In the present study antimicrobial peptide was isolated for the first time from the haemolymph of the crab *Thalamita crenata*. In antibacterial activity the highest zone of inhibition was observed in the haemolymph of *T.crenata* against *Proteus mirabilis* (17 mm) and lowest zone of inhibition was observed in against *Klebsiella oxytoca* and *Lactobacillus vulgaris* (14mm). But there was no activity against the tested fungal pathogens. On the basis of TLC observations on further confirmation with <sup>1</sup>H NMR peptide, fractions were subjected to tandem mass spectrometry (ESI /MS/MS), which resulted in the identification of peptides. The molecular mass of the purified haemolymph showed presence of several molecular mass range of m/z 88 to 507. To retrieve a sequence, the mass spectrometric data of the peptides were applied Mass difference between two adjacent peaks showed precisely fit the mass of an amino acid residue. Based on the arrangement of amino acid in the haemolymph of *T.crenata* species sample categorized peptide. The present study indicated that the haemolymph of *T.crenata* crabs may potential antibiotics.

**Key words:** Antimicrobial peptide % Crab % Haemolymph % Mass spectrometry % NMR

### INTRODUCTION

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural/ chemical features not found in terrestrial natural products. Antimicrobial peptides are important in the first line of the host defense system of many animal species. Their value in innate immunity lies in their ability to function without either high specificity or memory. Moreover their small size makes them easy to synthesize without dedicated cells or tissues and they rapidly diffuse to the point of infection. They have been defined molecules of less than 10 kDa which show stoichiometric, as opposed to enzymatic, antimicrobial properties [1]. Antimicrobial peptide defense in crustacean has long been suspected. In 1972, bactericidal activities were observed in Lobster *Homarus americanus* plasma [2] and hepatopancreas [3]. The isolated antifungal peptides from the plasma of the shrimp *Penaeus vannamei* and *P.stylirostris* [4]. On shrimp, *P.vannamei*, allowed full characterization of three members of a new family of antimicrobial peptides which were named as the penaeidins [5]. Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus* and its

response to infection with bacteria has been investigated [6]. The purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to batenecin-7, from the haemocytes of the shore crab, *Carcinus maenas* were studied by [7]. Partially characterized a cystine-rich 11.5kDa gram-positive specific antimicrobial peptide from *C.maenas* [8]. Callinectin is a cationic antimicrobial peptide of 3.7 kDa that represents the major antibiotic activity from the blue crab *Callinectes sapidus* [9].

Some of the brachyuran crabs have shown pronounced activities and may be useful in the biomedical area. The potential of marine crabs as a source of biologically active products is largely unexplored. Hence, a broad, based screening of marine crabs for bioactive compounds is necessary.

The recognition of pathogens and parasites by the invertebrate immune system may involve soluble proteins present in the haemolymph as well as proteins (receptors) localized at the surface of the hemocyte or other cells. The initial recognition may bring about communication to other population of cells through molecules that act as signals to stimulate a response. Thus it is obvious that no antimicrobial peptide study on the *Thalamita crenata* crabs has been attempted, hence the present study aimed

at isolation, purification and structural elucidation of bioactive peptides from the haemolymph of *T. crenata* crabs in the Vellar estuary.

## MATERIALS AND METHODS

Crabs were collected from the Vellar estuarine environment (Lat 11° 29'N; 79° 46'E) Southeast coast of India. Healthy male and female animals at different stages of development were used for experimental purposes and each animal was subjected to a single bleed collections at the time of use.

**Collection of Haemolymph:** Haemolymph were collected by cutting each walking legs of the animal with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the haemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1, V/V). Equal volume of physiological saline (0.85%, NaCl, w/v) was added to it. Haemolymph was centrifuged at 2000 rpm for 15 min at 4°C. Supernatant were collected by aspiration as hemocytes and stored at 4°C until use.

**Microbial Strains Used:** Antibacterial activity of crab haemolymph was determined against 9 different bacterial strains viz, *Staphylococcus aureus*, *Salmonella typhi*, *S.paratyphi*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Lactobacillus vulgaris* and *Klebsiella pneumoniae* and 6 fungal strains viz, *Aspergillus niger*, *Candida albicans*, *Rhizopus*, *Cryptococcus neoformans*, *A. flavus* and *Mucor* sp. These clinical strains were obtained from the department of Medical Microbiology (Raja Muthiah Medical College hospital) Annamalai University, Annamalai Nagar.

**Anti Microbial Assay:** The spectrum of antibacterial and antifungal activity were studied by using the techniques described by Bauer *et al.* [10]. Antibacterial and antifungal activity was expressed in terms of diameter of zone of inhibition was measured in mm using Vernier caliper or a scale and recorded.

**Thin-Layer Chromatography (TLC):** As the soluble fractions showed ninhydrin positive spots they were subjected to purification by gel permeation chromatography on sephadex LH 20 using methanol as eluent and monitored by TLC. Diagnostic thin layer chromatography was performed on methanol and chloroform extracts. They were also spotted and plates

developed in varying proportions. Detection was done with the specific color reagent ninhydrin, for detecting the compounds.

**NMR:** NMR samples were prepared by dissolving them after purification in a denatured solvent. Several deuterium lock solvents are available like CDCl<sub>3</sub>, CD<sub>3</sub>OD and D<sub>2</sub>O. NMR spectroscopy can provide a wealth of additional information about peptide in solution.

**ESI-QTOFXL MS/MS Spectrometry:** The mass spectrometer used was a QTOFXL MS/MS applied Biosystem instrument (Canada) equipped with analyst software application. The instrument was operated in positive ionization mode. The molecular mass of the purified haemolymph samples were determined by electrospray ionization mass spectrometer with an electrostatic ion spray source. The sample dissolved in MeOH: H<sub>2</sub>O containing trace of 0.1% TFA. It was directly infused at the constant flow rate of 10 µL/min in to the ion spray source using integrated syringe pump. The MS/MS products were produced by collision dissociation (CID) of selected precursor ions at collision energy between 25-40V and mass analyzed using TOF analyzer of the instrument.

## RESULTS

**Antimicrobial Assay:** Antibacterial activity of the haemolymph of the crab *T. crenata* was evaluated comparing to the positive control Erythromycin (C). Investigation against a range of nine different bacterial strains were used of which two gram-positive bacteria (*S. aureus* and *L. vulgaris*) and seven gram negative bacteria (*S. typhi*, *S.paratyphi*, *K. oxytoca*, *P. aeruginosa*, *E. coli*, *P. mirabilis* and *K. pneumoniae*).

The zone of inhibition in different bacterial strains against *T.crenata* haemolymph is shown in (Fig. 1). Among the various strains maximum diameter of (17mm) zone of inhibition was recorded in *P. mirabilis* strain and lowest zone of inhibition of (14 mm) was observed in *K oxytoca* and *L. vulgaris* strain. Among the tested nine pathogenic strains *S. aureus*, *S. typhi*, *S. paratyphi*, *E. coli* and *K. pneumoniae* shows negative activity and rest of them shown positive activity.

The antibacterial agent of Erythromycin showed activity against all the tested bacterial strains. The maximum activity against *P. aeruginosa* was (25 mm) and the minimum activity was observed against *L. vulgaris* (16 mm).

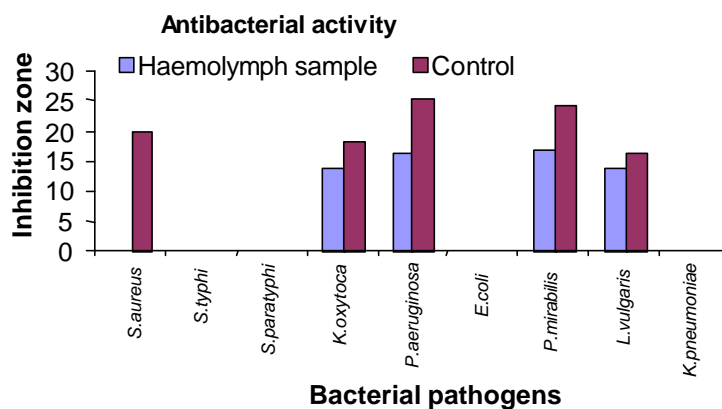


Fig. 1: Antibacterial activity of *Thalassidroma crenata* crab haemolymph

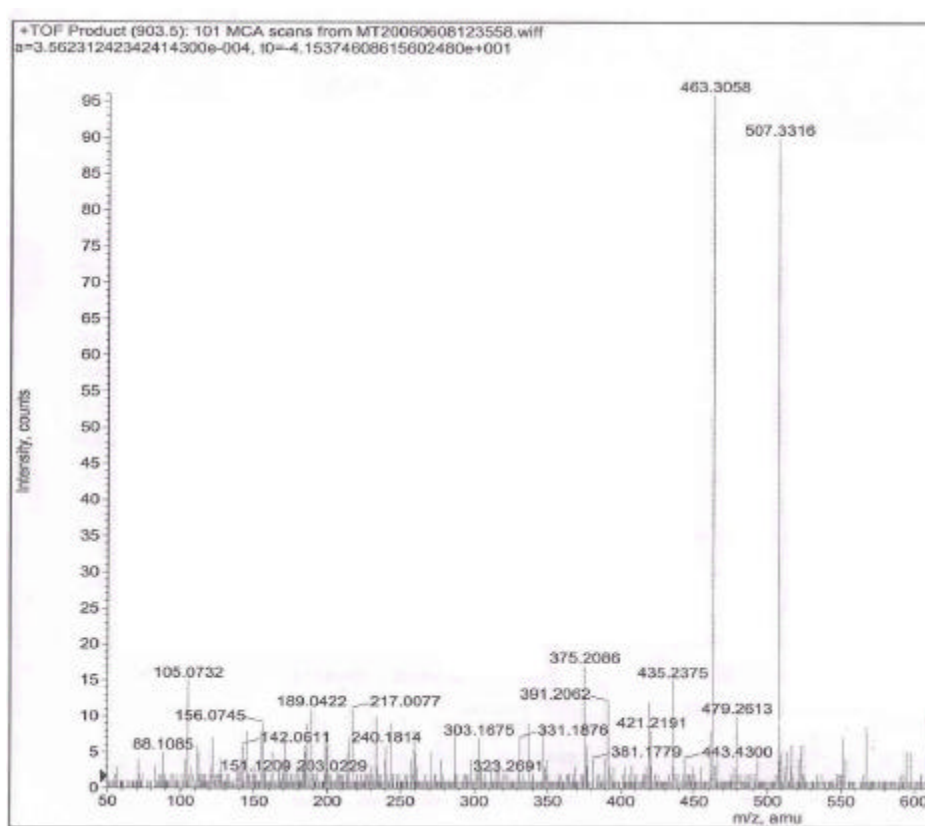


Fig. 2: MS/MS spectrum at m/z 507 of peptide

Antifungal activity of the haemolymph of *T. crenata* crab was used for the present study. The positive control agents Fluconazole (C) showed activity against all the fungal strains tested. The maximum activity against *A. niger* was (17 mm) and the minimum activity was observed against *C. albicans* (14 mm). Concerning the antifungal activity of the crab haemolymph there was no activity against the tested strains.

**Thin-Layer Chromatography:** Thin-Layer chromatography profiling was done for the haemolymph samples in two different solvent systems of A and B were used. The solvent system A consisted of methanol: chloroform (1:9) and B was a combination of butanol, acetic acid and water (B: A: W) in proportions of 5:1:4. The plates developed in both the solvent systems showed light pink spots when sprayed in ninhydrin.

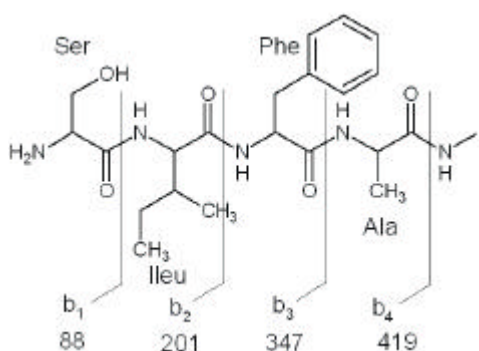


Fig. 3: Structure and Mass Fragmentation of Peptide

The plate with fractions developed in BAW as the solvent system and spayed with ninhydrin, showed pink spots indicating the presence of amino acids and peptides.

**Molecular Mass Characterization:** Based on the investigation of Thin Layer Chromatography and NMR studies ninhydrin positive spots indicating the presence of peptides. On the basis of solubility, chloroform soluble fraction was selected for further studies by ESI/MS analysis.

The sequences of the amino acid in peptides were proposed on the basis of the collision dissociation experiments and homology of known peptides supported by the fragmentation pattern. The ESI-MS of the ninhydrin was positive chloroform fraction. The result showed presence of several molecular mass range of m/z 88 to 507 (Fig. 2). To retrieve a sequence from the mass spectrometric data of the peptides were applied the criteria mass difference between two adjacent peaks showed precisely fit the mass of an amino acid residue. Based on the arrangement of amino acid in the haemolymph of *T. crenata* species sample was categorized peptide in (Fig. 3).

## DISCUSSION

In recent years, great attention has been paid to study the bioactivity of natural products due to their potential pharmacological utilization. The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction. Many of these organisms have been antimicrobial properties, although most of the anti bacterial agents that have been isolated from marine

sources have not been active enough to complete with classical anti microbial obtained from micro organisms [11]. However, majority of marine organisms are yet to be screened for discovering useful antibiotics.

The recognition of the pathogens and parasites by the invertebrate immune system may involve soluble proteins present in the haemolymph as well as proteins (receptors) localized at the surface of the hemocyte or other cells. The recognition stimulus and the secondary signals trigger signaling factors or induced transcription of genes for production of antimicrobial proteins [12]. In the present study, the crab haemolymph showed antimicrobial activity against a range of different pathogenic strains of both gram positive and gram negative bacteria. The results suggest that brachyuran crabs were not involved in the economy of finfish resources. It can also produce anti bacterial substances instantly to combat bacterial infection. Similar result was observed in the haemolymph of some mangrove crabs against clinical pathogens [13]. Induction of antibacterial compounds was also observed in case of sarcotoxin 1 [14] and sapecin [15], in *Sarcophagi peregrine*, moricin [16], lebobcin [17] and ceropin-B in *Bombyx mori* [18].

Antibacterial activity has been reported earlier in the haemolymph of the blue crab *C. sapidus*. It was highly inhibitory to gram-negative bacteria [19]. Although there were several reports on antibacterial activity in seminal plasma [20-24] few antibacterial peptides have been reported in *Scylla serrata*.

The present study indicated that antibacterial activity of the highest zone of inhibition was observed in the haemolymph of *T. crenata* against *P. mirabilis* and lowest zone of inhibition was observed in the haemolymph of *T. crenata* against *K. oxytoca* and *L. vulgaris*. On the base of antifungal activity, there was no activity against the tested pathogenic strains. It is an interesting finding that crab, being marine animals has the ability to dispose the bacteria upon infection. As the bacterium is a human pathogen, it is important that sea water should be free from this type of bacteria.

With meager amounts of material no single analytical technique is capable of complete characterization of peptide structure. As a consequence, structure elucidation is usually performed by using various techniques, of which mass and NMR spectrometry are two of the most powerful methods. Mass spectrometry has been a primary technique in peptide structure analysis for more than three decades, with the information available depending strongly on instrumentation and ionization methods.

The appearance of peptides CID spectra, especially those of (M+H)<sup>+</sup> ions are highly dependent on the collision energy [25].

Recently marine peptides have opened a new perspective for pharmaceutical developments. The present study clearly shows mass m/z 507, antimicrobial peptide in the haemolymph of *T. crenata* crabs. Structure was assigned to the peptide with mass m/z 507. It was fractionated and its various fractions was found to be rich in ninhydrin positive spots indicating the possibility of containing peptides. It was further confirmed by the presence of doublets in the region of its NMR spectrum. In conclusion, the present study indicates that the haemolymph of crab would be a good source of antimicrobial agents and would replace the existing in adequate and cost effective antibiotics.

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