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Research Article

BIOACTIVE PEPTIDES FROM THE GRAPSID CRAB *GRAPSUS STRIGOSUS* (SAKAI, 1976)

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ABSTRACT

Objective: Antimicrobial peptides (AMPs) are small peptides existing widely in mammals, amphibians and marine invertebrates they appear to be one of the factors in innate immunity. AMPs are widely expressed in organisms and have been linked to innate and acquired immunities in marine vertebrates.

Methods: An antimicrobial screening of *Grapsus strigosus*, a grapsid crabs hemolymph extracts were subjected to antimicrobial assay. Antimicrobial screening was done in ten consecutive human pathogenic bacterial and fungal pathogens using the standard disc diffusion method. The antibacterial test of the sample confirmed positive results against most of the pathogens used.

Results: In *G. strigosus,* maximum effect was recorded against *Vibrio cholera* (8 mm) and the lowest zone of inhibition against *Staphylococus aureus* (2 mm). In the positive control, maximum effect was recorded against *Vibrio parahemolyticus* (8 mm) and the lowest zone of inhibition against *Klebsiella pneumoniae* (4 mm). In antioxidant assay, the percentage of 2, 2-diphenyl-1-picrylhydrazyl scavenging activity was recorded as 1.21%. The protein content was estimated as 17.2 mg/ml. In thin layer chromatography, pink spots confirmed the presence of proteins. In sodium dodecyl sulfate polyacrylamide gel electrophoresis, five bands were detected in the gel that represents the presence of proteins in the range nearly 25.6-75.8 KDa.

Conclusion: The present study clearly shows that the various fractions was found to be rich in ninhydrin positive spots indicating the possibility of containing peptides, further confirmed by the presence of doublets in the region of its nuclear magnetic resonance spectrum. Hence, the present study indicated that the hemolymph of *G. strigosus* crabs may potential antibiotics.

Keywords: Crab, Hemolymph, Anti-microbial peptide, Thin-layer chromatography, Nuclear magnetic resonance.

INTRODUCTION

Marine invertebrates depend upon antimicrobial peptides (AMPs) as a major component of innate immunity, as they are rapidly synthesized and diffuse upon pathogen invasion [1]. AMPs are a major component of the innate immune defense system in marine invertebrates. Scygonadin was reported as a 10.8 KDa antibacterial protein isolated from the seminal plasma of *Scylla serrata* [2]. The invertebrates comprise over 95% of animal species and some live in environments rich in potentially harmful microorganisms. As a result, these animals have developed various competent strategies to defend their lives against invading pathogens [3]. The AMP molecules are the first line of host defense in various species, have a mass of ≤10 KDa and are readily synthesized and efficiently diffuse at the point of pathogen entry or infection, hence form an invaluable component of the innate immune system [4,5]. Cloning of a crustin-like, single whey- acidic-domain, an antibacterial peptide from the hemocytes of the European lobster, *Homarus gammarus* and its response to infection with bacteria has been investigated [6]. A survey revealed that the literature on AMP from the marine crab *Grapsus strigosus* has no studies so far. Hence, the present investigation was aimed at the isolation of AMP from this particular crabs. Peptides are known to exhibit good biological activity.

METHODS

Collection of hemolymph

Hemolymph was collected by cutting each walking legs of an animal with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the hemolymph 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. To remove hemocytes from the hemolymph sample was centrifuged at 2000 rpm for 15 minutes at 4°C. Supernatant was collected and stored at 4°C until use.

Microbial strains used

Antibacterial activity of crab hemolymph was determined against 10 different bacterial strains *viz.*, *Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi, Klebseilla oxytoca, Klebseilla pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Vibrio cholera, Vibrio parahemolyticus* and 10 fungal strains *viz.*, *Aspergillus niger, Aspergillus flavus, Alternaria alternaria, Candida albicans, Epidermophyton floccossum, Trichophyton mentagrophytes, Trichophyton rubrum, Pencillium* sp. *Rhizopus* sp. 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 was studied using the techniques described by Bauer *et al.* [7]. Antibacterial and antifungal activity was expressed in terms of zone of inhibition in mm using a scale and recorded.

Antioxidant assay

To measure the antioxidant activity of the hemolymph of crab *G. strigosus*, 0.1 ml of the sample was taken in a vial and then 0.5 ml of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was added. Then these sample mixture was incubated for 30 minutes in a dark place. After an incubation period, the DPPH scavenging activity was measured using the spectrophotometer in 620 nm.

Estimation of hemolymph protein

The amount of protein was measured according to the Lowry *et al*., (1951) [8] method with different concentrations of bovine serum albumin as a standard. Biuret reagent as a color reactant and concentration was calculated in response to the absorbance at 540 nm in a spectrophotometer.

Thin-layer chromatography (TLC)

TLC profiling was done for the hemolymph samples of *G. strigosus* in a solvent system was a combination of butanol, acetic acid, and water (BAW) in proportions of 5:1:4. The plates, when developed in the solvent systems, showed light pink spots when sprayed with ninhydrin. The plate with fractions developed in BAW as the solvent and sprayed with ninhydrin, showing pink spots indicating the presence of amino acids and peptides.

Purification

Lyophilized crab hemolymph purified through GFC was reconstituted in 10 ml of 0.1M phosphate buffer solution (PBS), containing 5 mM ethylenediaminetetraacetic acid, PH 6.0. The sample was applied to a Sephadex G 25 (Superfine, Amersham Biosciences, 2.6 cm × 90 cm) gel filtration column was previously equilibrated with 0.1 M PBS. The absorbance of elute was monitored at 280 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to find out the molecular weight active fractions of the sample. SDS-PAGE was performed in 12% separating gels, according to the method described by Laemmli (1970) [9].

Nuclear magnetic resonance (NMR)

NMR is usually observed when nuclei of certain atoms are placed in a static magnetic field and exposed to a second oscillating magnetic field. Nuclei of the atoms, which are considered to spin, experience this phenomenon, depending upon whether they possess spin of ½ is they, act like tiny bar magnets. One such nucleus is proton. NMR samples were prepared by dissolving them after purification in a deuterated solvent. Several deuterated solvents are available like $CDCl_3$, CD_3OD, and D_2O. NMR spectroscopy can provide a wealth of additional information about peptide in solution.

RESULTS

Anti-microbial assay

The antibacterial activity of the hemolymph of the crab *G. strigosus* was used for the present study. The positive control tetracycline (c) was also used. Investigation against a range of ten different bacterial strains was used of both Gram-positive and Gram-negative bacteria. The zone of inhibition in different bacterial strains is shown in Fig. 1. In *G. strigosus*, maximum diameter of the zone of inhibition was recorded in *V. cholera* (8 mm) and the lowest zone of inhibition against *S. aureus* (2 mm). In the positive control maximum effect was recorded against *V. parahemolyticus* (8 mm) and the lowest zone of inhibition against *K. pneumoniae* (4 mm). Antifungal activity of the hemolymph of the crab *G. strigosus* against the fungal strains did not show any activity against the tested strains.

Anti-oxidant assay

The free radical scavenging activity of protein from crab *G. strigosus* hemolymph was assessed by the DPPH assay. The result shows that the percentage of DPPH scavenging activity was recorded as 1.21%.

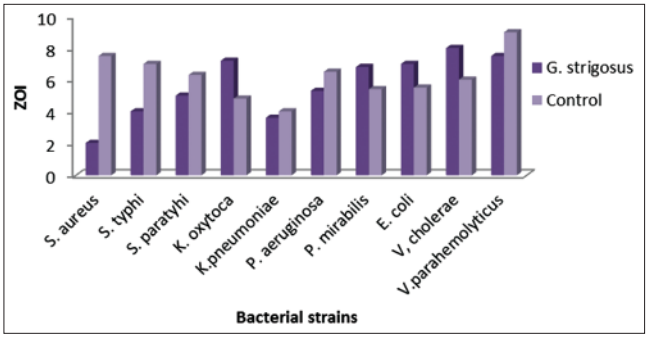

Fig. 1: Antibacterial activity of *Grapsus strigosus* crab hemolymph

Hemolymph protein estimation

The protein content of the hemolymph of *G. strigosus* was estimated. Protein concentration of the hemolymph is measured using a spectrophotometer. The amount of protein present in the hemolymph was estimated as 17.2 mg/ml.

Determination of molecular weight by using SDS-PAGE

The hemolymph of *G. strigosus* showed antibacterial activity was subjected to SDS-PAGE to estimate the molecular weight of active proteins present in it. Different standards were used to determine the molecular weight of hemolymph proteins. The stained gel revealed that the hemolymph contained a simple population of proteins. There were different molecular weight markers used. Five bands were detected in the gel that represents the presence of proteins in the range nearly 25.6-75.8 KDa (Fig. 2).

NMR and TLC

The hemolymph sample showed antibacterial activity was subjected to TLC and NMR studies. After spotting the sample on TLC plate, then spraying with ninhydrin, it was observed that pink spots. As it is well-known that peptides are known to pink spots with ninhydrin and since it has yield it was taken for further investigation leading to the conformation of the peptides. Ninhydrin positive indicating the possibility of counting peptides, further by the presence of doubles in the region of δ 6-8 in its ^1H NMR spectrum (Fig. 3). Based on the investigation of TLC and NMR studies showed ninhydrin positive spots indicating the presence of peptides.

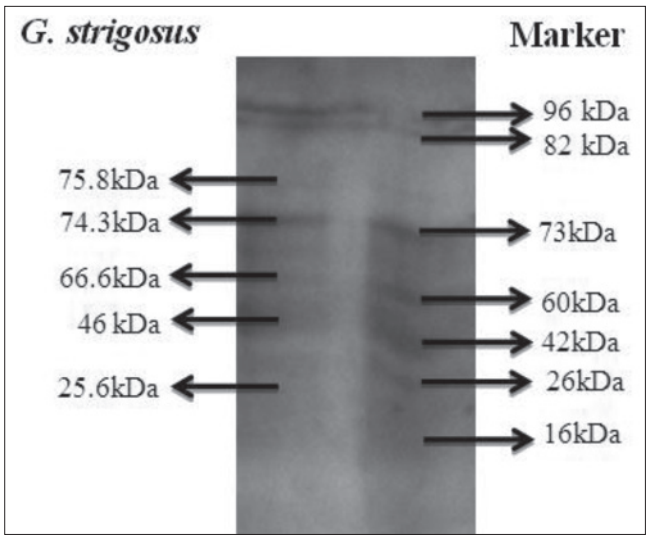

Fig. 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of *Grapsus strigosus*

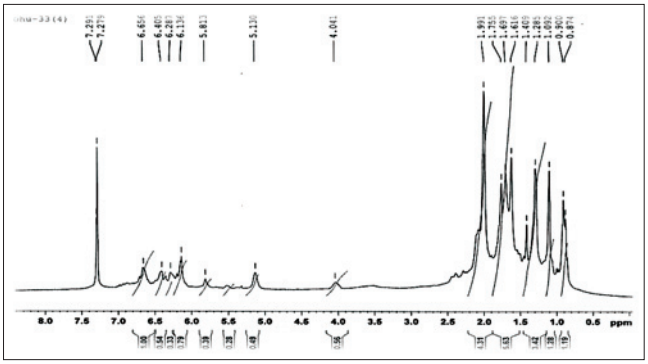

Fig. 3: ^1H nuclear magnetic resonance spectrum of peptides in *Grapsus strigosus*

DISCUSSION

2010 *Crustaceans* compose a large, ancient and diverse animal group that includes many well-known, commercially exploited members, such as shrimp, crab, crayfish, and lobster. AMPs or proteins are one of the major components of the innate immune defense and are ubiquitously found in crustaceans. 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, yeast, filamentous fungi and, to a lesser extent, protozoans and enveloped viruses [10-12]. In the present study, in the case of antibacterial activity of the crab *G. strigosus*, maximum zone of inhibition was recorded in *V. cholera* and the lowest zone of inhibition against *S. aureus.* In the positive control, maximum effect was recorded against *V. parahemolyticus* and the lowest zone of inhibition against *K. pneumoniae.* Antifungal activity of the hemolymph of the crab *G. strigosus* against the fungal strains did not show any activity against the tested strains. Similar result was observed with the hemolymph of some brachyuran crabs against clinical pathogens [13-20].

Crabs are the very good resource of antimicrobial proteins with a wide range of antimicrobial properties which is highly supported in the hemolymph study of *Charybdis lucifera* [21]. In the present investigation, the percentage of protein concentration of hemolymph the crab *G. strigosus* was recorded as 17.2 mg/ml. Ravichandran *et al.,* (2009) reported similar type of findings in various brachyuran crabs. But the concentration of protein in the hemolymph shows wide interspecific variation among decapod crustaceans, ranging from as low as 28 mg/ml in *Carcinus maenas* [22] to as high as 22.2 mg/ml in *Ucaminax* [23] of this hemolymph protein, consists of the respiratory pigment hemocyanin [24,25].

The present investigation showed that the protein from *G. strigosus* crab hemolymph exhibited DPPH scavenging activity. The reducing power ability of the protein greatly depends on the presence of reductones which have exhibit antioxidant potential by breaking the free radical chain by donating a hydrogen atom [26]. The result of the present study reveals that the strongest H_2O_2 scavenging activity was observed for protein at various concentrations when compared to scavenger of hydrogen peroxide. In the present study protein of the *G. strigosus* hemolymph shows the maximum activity as 1.21%. The maximum activity has observed in *G. strigosus* crab hemolymph protein can be a good source for antioxidant activity. The present result suggests that the hemolymph of crab *G. strigosus* protein might be a potent agent for scavenging of nitricoxide. This result indicating the proteins are the best source of antioxidant compounds.

In the male-female crab, hemolymph of *C. lucifera* showed different protein bands between the molecular weight of 45 KDa and 25 KDa (Rameshkumar *et al.*, 2009a). In the present investigation, purified hemolymph that showed antibacterial activity was subjected to SDS-PAGE to estimate the number and molecular weight of proteins present. After electrophoresis, clear bands were detected in the gel which represented proteins of molecular weight between 25.6 and 75.8 KDa which is similar to the antibacterial peptides in the hemolymph of the range of 1-100 KDa from *Callinectes sapidus* [27]. Schagger and Vonjagow (1987) explained tricine SDS-PAGE for the separation of proteins in the range from 1 to 100 KDa, which fits well with a range of antibacterial peptides [28]. The findings of AMPs isolated from the crab *Thalamita crenata* was found to be 56.8 KDa, which is analogous to the present investigation [21].

The solution structure of AMPs tachystain A from the Japanese horseshoe crab (*Tachypleus tridentatus*) was determined by two-dimensional NMR measurements and distance restrained simulated annealing calculations [29]. In the present study, various fractions were 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.

CONCLUSION

The research on marine organisms is still in its infancy considering the vast resources that still remains untapped with the majority of marine organisms yet to be screened for the discovery of useful antibiotics. In conclusion, the isolation of AMP from *G. strigosus* as well as its antimicrobial activity is reported here for the first time. The biological significance of the presence of the AMPs in the crab hemolymph is still unclear. Considerable effort is being put into investigating the therapeutic potential of these peptides. Furthermore, studies have to be performed to check whether the potency can be improved by synergy with conventional antibiotics or other antibacterial proteins. So far, numerous AMPs have been characterized from marine invertebrates. In this work, an AMP was purified and characterized from hemolymph of the crab *G. strigous* that lives in harsh marine environment may contain potential antibiotics. 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. A thorough understanding of chemical structure and biological activity will lead to the formulation of novel drugs with specific actions.

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REFERENCES

1. Reddy KV, Aranha C, Gupta SM, Yedery RD. Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: *in vitro* and *in vivo* studies. Reproduction 2004;128(1):117-26.
2. Huang WS, Wang KJ, Yang M, Cai JJ, Li SJ, Wang GZ. Purification and part characterization of a novel antibacterial protein scygonadin, isolated from the seminal plasma of mud crab, *Scylla serrata*. J Exp Mar Biol Ecol 2006;339:37-42.
3. Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. Immunobiology 2006;211(14):213-36.
4. Reddy KV, Yedery RD, Aranha C. Antimicrobial peptides: Premises and promises. Int J Antimicrob Agents 2004;24(6):536-47.
5. Cole AM. Antimicrobial peptide microbicides targeting HIV. Protein Pept Lett 2005;12(1):41-7.
6. Hauton C, Brockton V, Smith VJ. 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. Mol Immunol 2006;43(9):1490-6.
7. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol 1996;45:493-6.
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
9. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680-5.
10. Bulet P, Stöcklin R, Menin L. Anti-microbial peptides: From invertebrates to vertebrates. Immunol Rev 2004;198:169-84.
11. Yount NY, Bayer AS, Xiong YQ, Yeaman MR. Advances in antimicrobial peptide immunobiology. Biopolymers 2006;84(5):435-58.
12. Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. Antimicrobial peptides: General overview and clinical implications in human health and disease. Clin Immunol 2010;135(1):1-11.
13. Rameshkumar G, Ravichandran S, Aravindhan T. Antimicrobial proteins from the crab *Charybdis lucifera* (Fabricius, 1798). Middle East J Sci Res 2009;4:40-3.
14. Ravichandran S, Kannupandi T, Kathiresan K. Mangrove leaf processing by sesarmid crabs. Ceylon J Sci 2006;35:107-13.
15. Ravichandran S, Anthonisamy S, Kannupandi T, Balasubramanian T. Habitat preference of crabs in Pichavaram mangrove environment, Southeast coast of India. J Fish Aquat Sci 2007;2:47-55.
16. Ravichandran S, Anthonisamy S, Kannupandi T, Balasubramanian T. Leaf choice of herbivorous mangrove crabs. Res J Environ Sci 2007;1:26-30.
17. Ravichandran S, Rameshkumar G, Anthonysamy A, Kumaravel K. Antibacterial activity from the hemolymph of some brachyuran crabs. Am Eurasian J Sci Res 2009;4:26-33.

18. Ravichandran S, Wahidulla S, D'Souza L, Rameshkumar G. Antimicrobial lipids from the hemolymph of brachyuran crabs. Appl Biochem Biotechnol 2010;162(4):1039-51.
19. Veeruraj A, Ravichandran S, Rameshkumar G. Antibacterial activity of crab hemolymph on clinical pathogens. Trends Appl Sci Res 2008;3:174-81.
20. Anbuchezhian RM, Ravichandran S, Ramesh Kumar G, Ajithkumar T. Influence of crab hemolymph on clinical pathogens. Adv Bio Res 2009;3:104-9.
21. Rameshkumar G, Ravichandran S, Kaliyavarathan G. Antimicrobial peptide from the crab, *Thalamita crenata* (Latreille, 1829). World J Fish Mar Sci 2009;1:74-9.
22. Gilles JP, Kanost M, Trenczek T. Biological mediators of insect immunity. Rev Entomol 1997;42:611-43.
23. Pequex A, Vallota AC, Gilles R. Blood proteins as related to osmoregulation in crustacean. Comp Biochem Physiol 1979;64:433-5.
24. Uglow RF. Hemolymph protein concentrations in portunid crabs-1. Studies on adult *Carcinus maenas*. Comp Biochem Physiol 1969;30:1083-90.
25. Zatta P. Zinc transport in the hemolymph of the shore crab *Carcinus maenas*. J Mar Biol Assess 1984;64:801-7.
26. Roopa, Borar S, Thakral J, Kalia AN. Antioxidant potential fraction from methanol extract of aerial parts of *Ruellia prostra poir* (*Acanthaceae*). Int J Pharm Sci Res 2011;2:1015-22.
27. Khoo L, Robinette DW, Noga EJ. Callinectin, an antibacterial peptide from blue crab, *Callinectes sapidus*, hemocytes. Mar Biotechnol (NY) 1999;1:44-51.
28. Schagger H, Vonjagow G. Tricine-sodium dodecyle sulphate - Polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368-79.
29. Fujitani N, Kawabata S, Osaki T, Kumaki Y, Demura M, Nitta K, *et al.* Structure of the antimicrobial peptide tachystatin A. J Biol Chem 2002;277(6):23651-7.