VOL.65 NO.02 JULY-DECEMBER 2023 • PRINT ISSN 0025-3146 • ONLINE ISSN 2321-7898

JMBAI

JOURNAL OF THE MARINE BIOLOGICAL ASSOCIATION OF INDIA

MBAI
Marine Biological Association of India

Available online at: www.mbai.org.in doi:10.6024/jmbai.2023.65.2.2380-05

Antimicrobial activity of the crude peptide extracts of flower tail shrimp, *Metapenaeus dobsoni*

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Received: 07 July 2022 Revised: 26 Dec 2022
Accepted: 29 Dec 2022 Published: 15 Nov 2023

Original Article

Abstract

Antimicrobial peptides (AMPs) are widely distributed in various organisms, including plants and mammals. The present study was undertaken to isolate and purify antimicrobial peptides from *Metapenaeus dobsoni* (Md), Flower tail shrimp. The crude peptides were tested for antibacterial activity against 12 pathogens by disc-diffusion assay. The remarkable antibacterial activity could be observed towards the 11 pathogens (7-20 mm), and three were selected for further assays. For purification, crude peptide extract was subjected to solid phase extraction and cation exchange chromatography. The fractions Md5-3, Md40-1, Md80-3 showed the highest activity towards *Staphylococcus aureus*, Md5-3, Md40-4, Md40-6, Md80-1 against *Vibrio alginolyticus* and Md5-3, Md40-6, Md80-1 against *Bacillus cereus*. *M. dobsoni* could be a promising source of bioactive peptides. This basal study suggests that *M. dobsoni* is a source of antimicrobial peptides (AMP), which could be applied to aquaculture fields as therapeutic agents.

***Keywords**: Antimicrobial peptides, Metapenaeus dobsoni, innate immunity, bacterial pathogens, shrimps*

Introduction

Life in deep pelagic waters usually faces elevated pressure, low temperature, absolute darkness and high salinity (Sperstad *et al.,* 2011). Marine organisms are a reserve of biologically active metabolites with pharmaceutical and nutraceutical properties. Among these, the marine bioactive peptides in all organisms have gained attention owing to their broad-spectrum health-promoting benefits (Rauf *et al.,* 2021). The emergence of drug-resistant pathogens has triggered the search for alternatives to conventional antibiotics like antimicrobial peptides (Zasloff, 2002). The peptides from marine organisms exhibit antimicrobial, calcium binding, anticoagulant, cardiovascular protective, antidiabetic, immunomodulatory and appetite suppression functions (Harnedy and FitzGerald, 2012; Cheung *et al.,* 2015; Matos and Rosa, 2022).

After insects, crustaceans are the most diverse and widespread animals in the biosphere (Verdon *et al.,* 2016). Antimicrobial peptides from crustaceans started in the mid 90's in the last century with the discovery of two gene-coded AMPs in the haemolymph of brachyuran crab (Schnapp*et al.,* 1996) and penaeid shrimps (Destoumieux *et al.,* 1997). Many AMPs such as crustins, Anti-lipolysaccharide factors, penaeidins, arasin, and hyastatin have been identified from different crustacean species (Kang *et al.,* 2007; Stensvag *et al.,* 2008; Sperstad *et al.,* 2009; Imjongjirak *et al.,* 2011; Sruthy *et al.,* 2017; Anju *et al.,* 2020). Apart from these gene-encoded AMPs, there are haemocyanin-derived fragments (Destoumieux-Garzon *et al.,* 2001) and histone-derived fragments (Patat *et al.,* 2004). Astacidin 1, a cationic 1.9KDa peptide derived from the C-terminal part of crustacean haemocyanin, has been shown to possess antibacterial activities (Lee *et al.,* 2003).

Crabs, shrimps, prawns, Antarctic krill and crayfishes are the primary crustacean resources. Over the past few decades, shrimp consumption has substantially increased, and various products have been commercially developed. Shrimp is the largest single commodity in terms of value (Destoumieux-Garzon *et al.,* 2016). Crustaceans are covered externally by a hard, rigid exoskeleton which performs as an efficient physiochemical barrier against microbial invasion and mechanical injury (Rosa and Barracco, 2010).

The present study deals with the isolation of antimicrobial peptides from the Flower tail shrimp, *M. dobsoni* and testing their antibacterial potential.

Material and methods

Sample collection

The organism used for the study was *Metapenaeus dobsoni*, commonly known as flower tail shrimp. Live specimens of *M. dobsoni* were collected from the *Kalamukku* Fishing Harbour, Cochin, Kerala. The samples were sacrificed humanely and were stored at -80 ^{0}C until use.

Peptide extraction

The peptide was extracted as per Rivillas and Garcia (2007) using the acetic acid-acetone precipitation method. Samples were cut into small pieces, weighed and subjected to homogenization with 10% acetic acid in a ratio of 1:5 (W: V). The homogenized sample was kept overnight at 4 ^{0}C and thereafter centrifuged at 13500 rpm at 4 ^{0}C for 30 minutes. The residue was discarded, and to the supernatant were added two volumes of acetone. This was followed by overnight incubation at 4 ^{0}C. The sample was centrifuged at 13500 rpm at 4 ^{0}C for 30 minutes. The supernatant was discarded, and the protein pellet was collected and stored at -80 ^{0}C until use.

Initial activity screening

The crude peptide sample was tested against various Gram-positive and Gram-negative bacteria (Table 1) for its antimicrobial activity by Disc diffusion assay (Bauer *et al.,* 1966). The crude peptide sample was reconstituted in sterile Milli Q and loaded on 6 mm discs (Whatman No. 1) using a micropipette with a sterile tip, with each disc containing 4 mg of the peptide. These impregnated discs were placed on nutrient agar plates seeded with microbial pathogens (1x 10^8 cells/ml), kept at 4 ^{0}C for 30 minutes and then incubated overnight at 30 ^{0}C. The plates were then observed for the zone of inhibition and the activity was recorded in terms of the diameter of the zone of inhibition.

Table 1. Antimicrobial activity of the sample against pathogenic strains

No.	Microbial strains	Inhibition Zone (mm)
1	*Staphylococcus aureus* (MTCC 3061)	20 ± 0.5
2	*Bacillus cereus* (MCCB 101)	15 ± 1
3	*Edwardsiella tarda* (MTCC 2400)	15 ± 1.2
4	*Vibrio alginolyticus* (VKF 44)	15 ± 1
5	*Pseudomonas aeruginosa* (MCCB 119)	11 ± 2
6	*Vibrio cholerae* (MCCB 129)	10 ± 2.5
7	*Vibrio proteolyticus* (M10W1)	10 ± 1.5
8	*Vibrio parahaemolyticus* (MCCB 133)	09 ± 2.9
9	*Vibrio vulnificus* (WV13)	09 ± 1
10	*Aeromonas hydrophila* (MCCB 113)	08 ± 2.2
11	*Escherichia coli* (MTCC 483)	07 ± 2
12	*Vibrio harveyi* (MCCB 284)	——-

Purification of peptide

The crude peptide extracts were reconstituted in sterile Milli Q and subjected to solid-phase extraction using Sep-Pak® C-18 cartridges (Waters, USA). The cartridges were conditioned with methanol and equilibrated with 0.1% Tri Fluoro Acetic acid (TFA). The sample was loaded onto the cartridge, followed by a wash with 0.1% TFA. The analyte trapped in the cartridges were eluted with 6ml each of 5%, 40%, and 80% acetonitrile in 0.1% TFA. Fractions were labelled as 5%, 40%, and 80% fractions, lyophilized and stored at - 20 ^{0}C until use.

Cation exchange chromatography

The 5%, 40% and 80% Sep-pak® fractions were subjected to purification by cation exchange chromatography using FPLC (Fast Protein Liquid Chromatography) (Duo flow, Bio-Rad). The column used was UNOTMQI (QIBio-Rad) with Solution A (25 mM Tris-HCl) and Solution B (1 M NaCl in 25 mM Tris-HCl) as mobile phase. The column and baseline were equilibrated with solution A, followed by sample loading. Linear gradient elution was achieved using 0-50% of solution B in 40 ml of mobile phase. The flow rate was maintained at 2 ml/min. Absorbance was monitored at 215, 225, 260 and 280 nm wavelength using a Quad Tec detector (Bio-Rad). Each peak was collected as separate fractions, which were lyophilized and stored at -20 ^{0}C until use.

Broth microdilution Assay

Broth microdilution assay was employed to test the antimicrobial activity of the active fractions obtained from cation exchange chromatography. The bacterial suspensions were prepared for the assay, and it was carried out in a 96-well microtiter plate. The strains were tested for purity by repeated streaking on sterile nutrient agar plates, followed by incubation for 48 hours. The isolated colonies were then picked and streaked onto nutrient agar slants. The slants were incubated at room temperature for 24 hours. To this, a sufficient quantity of 4-(2hydroxyethyl)-1-piperazineethane –sulfonic acid (HEPES) buffer was added. The bacterial cells were scrapped off using a sterile inoculation loop and mixed well. The optical density (OD) of the sample was adjusted so that 10 μl of the bacterial suspension contained approximately 100 Colony Forming Units (CFU).

The antimicrobial activity of the antimicrobial peptide was determined by the broth microdilution assay by Huang *et al.* (2006) with slight modifications. The assay was carried out in a 96-well microtiter plate. Mid-logarithmic phase bacteria were diluted to 10^4 CFU/ml in 50 mM HEPES buffer (pH 6.8), 10 μl FPLC fractions and 10 μl of the diluted bacterial suspension were loaded into the microtiter well and incubated at room temperature for two hours. HEPES buffer served as the Blank. A set of negative and positive controls were maintained for the assay. The negative control comprised 10 μl of bacterial suspension and10 μl of 50 mM Tris-HCl. 10 μl of bacterial suspension along with 10 μl of different concentrations of two-fold serially diluted antibiotic Ampicillin (*Vibrio alginolyticus*) and Chloramphenicol (*Staphylococcus aureus* and *Bacillus cereus*) served as a positive control. Those bacteria that showed the highest zone of inhibition in the disc-diffusion assay were selected for liquid broth inhibition assay (Table 1). After incubation for 2 hours at room temperature, 80 μl of MH (Mueller-Hinton) broth was added to each well. Absorbance was measured using a Microplate Reader (Thermo Scientific) at a wavelength of 600 nm after incubation at 37 ^{0}C for another 16 hours with shaking at 100 rpm. Inhibition % = 100 – Growth Percentage, where Growth % = (OD of Test/ OD of negative Control) x 100.

Results

Antimicrobial activity

Antimicrobial activity was found against both Gram-negative and Gram-positive microorganisms (Table 1). *S. aureus* was found to be inhibited maximum (20 mm dia. clear zone) by the crude peptide extract, followed by *B. cereus* (15 mm), *V. alginolyticus* (15 mm), *E. tarda* (15 mm), *P. aeruginosa* (11 mm) and no activity against *V. harveyi*.

Purification of peptide

The solid phase (Sep-pak) fractionation of the crude peptide from *M. dobsoni* yielded three fractions, *ie*., 5, 40 and 80%. These Sep-pak fractions were further purified by cation exchange chromatography using the FPLC system. 5% Sep-pak fraction yielded three fractions which were denoted as Md5-1, Md5-2 and Md5-3 (Fig. 1). FPLC of 40% fraction yielded six fractions as Md40-1, Md40-2, Md40-3, Md40-4, Md40-5, and Md40-6 (Fig. 2). FPLC of 80% Sep-pak fraction yielded 4fractions- Md80-1, Md80-2, Md80-3 and Md80-4 (Fig. 3).

Antimicrobial activity

The antibacterial activity of FPLC fractions of *M. dobsoni* crude peptide extract was tested by broth microdilution assay. Three bacterial strains which were sensitive to the crude peptide extract were selected for testing the growth inhibition, *ie*., *S. aureus*, *V. alginolyticus* and *B. cereus* (Table 2). Fractions Md40-4 and Md40-6 showed about 100% growth inhibition against *V. alginolyticus*. All the 5%, 40% and 80% fractions

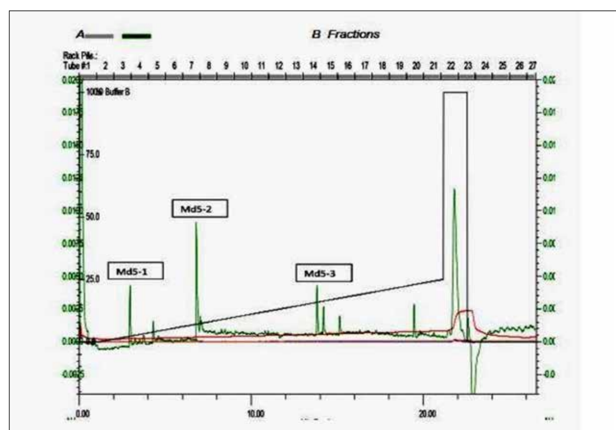

Fig. 1. Peaks obtained by 5% of Sep-pak fraction using cation exchange chromatography from *M. dobsoni*

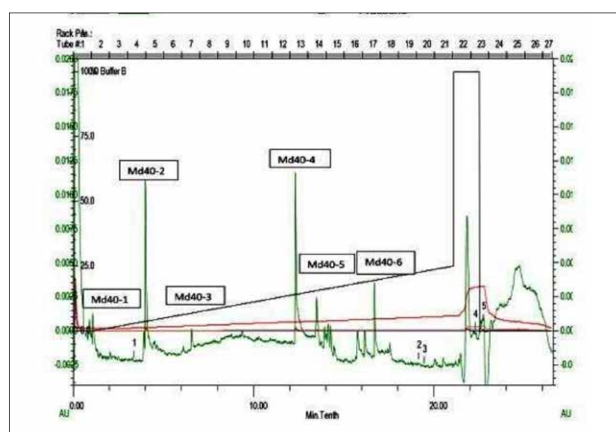

Fig. 2. Peaks obtained by 40% of Sep-pak fraction using cation exchange chromatography from *M. dobsoni*

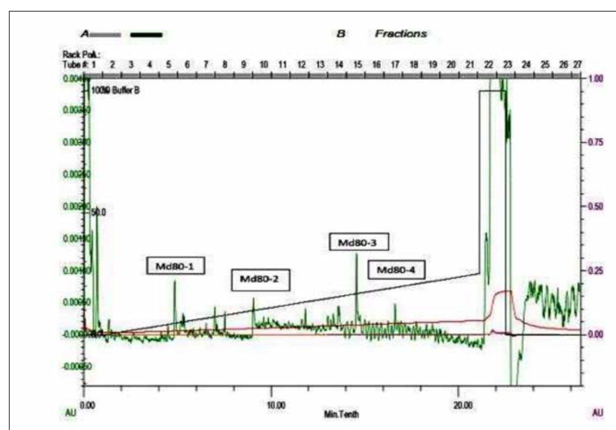

Fig. 3. Peaks obtained by 80% of Sep-pak fraction using cation exchange chromatography from *M. dobsoni*

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Table 2. Antimicrobial activity of FPLC fractions from *M. dobsoni*

		% Inhibition		
No.	Fraction	*S. aureus*	*V. alginolyticus*	*B. cereus*
1	Md5- 1	31.67 ± 3.2	56 ± 5	65.45 ± 3.9
2	Md5- 2	64.43 ± 3	0	60.78 ± 2.5
3	Md5- 3	68.48 ±	78.26 ±	84.85
4	Md40- 1	65.53 ± 1	0	0
5	Md40- 2	52.94 ± 3.5	20.6 ± 1.6	90.25 ± 3.4
6	Md40- 3	12.15 ± 2.6	96.02 ± 2.9	71.84 ± 4.1
7	Md40-4	11.29 ± 1.9	100 ± 0.5	83.31 ± 2.6
8	Md40-5	27.91± 3.6	87.90 ± 2.5	88.53 ± 3.12
9	Md40-6	28.25 ± 2.5	100 ± 2.1	95.4 ± 1
10	Md80- 1	59.55 ± 1	98.56 ± 1.5	98.73 ± 1.4
11	Md80- 2	69.43± 5.1	53.91 ± 2	86.58 ± 2.2
12	Md80- 3	72.78 ± 4	86.04 ± 3.7	84.9 ± 1
13	Md80- 4	60.44 ± 2.5	79.19 ± 4.6	85.76 ± 1

except Md40-1 showed above 60% inhibition against *B. cereus*. Md80-3 exhibited the highest inhibition (72.78%) towards *S. aureus* than the other fractions.

Discussion

Aquatic organisms are treasure houses of molecules with specific and potent bioactivity. Crustaceans live in intimate contact with their aqueous environment, which is highly populated with various microorganisms. To survive in such a confrontational environment, they have a healthy immune mechanism (Afsal *et al.,* 2013). Though crustaceans mainly rely on the innate immune system for defence, antimicrobial peptides play a significant role. AMPs possess different functions, including antimicrobial and immunomodulatory activities (Yount *et al.,* 2006). Recently, AMPs are getting noticed for their activity without developing drug resistance (Yang *et al.,* 2015). The present study reports the isolation and antimicrobial potential of bioactive peptides from *M. dobsoni*, the flower tail shrimp.

There are earlier reports of antimicrobial peptides from crustaceans with remarkable antimicrobial activity (Schnapp *et al.,* 1996; Donpudsa *et al.,* 2010; Sun *et al.,* 2010; Archana *et al.,* 2021). Different organs and tissues like haemolymph, haemocytes, gills, eggs, exoskeleton, internal organs, seminal plasma (Jayasankar and Subramoniam, 1999) and hepatopancreas (Mori and Stewart, 1978) have been found to possess antibacterial factors (Haug *et al.,* 2002). Major antibacterial activity was reported in the haemolymph/haemocytes (Noga *et al.,* 1996; Haug *et al.,* 2002). The crude peptide fractions from *M. dobsoni* showed significant antibacterial activity against *S. aureus*, *V alginolyticus* and *B. cereus*. The whole shrimp was used for the extraction of the peptide. So, AMPs from different tissues were involved in the bacterial inhibiting activity.

The crude protein extract from *M. dobsoni* showed activity against the pathogens tested. Among 5% Md fractions, the potent fraction was Md5-3. Among all the 40% fractions, except Md40-1, all the other fractions showed above 70% inhibition towards *B. cereus*. Md80 (1-4) fractions possessed inhibition above 50% for all three strains, which indicates that Md80 fractions exhibit the highest potential for antimicrobial activity among other Md fractions (5 and 40%).

Bioactive peptides can be prepared by various methods depending on their species, amino acid compositions and differences in nucleotide sequence. Moreover, some methods may cause the loss of activity of the peptides (Agyei *et al.,* 2016). The purification procedure of bioactive compounds includes FPLC gel filtration, Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) using the C18 column. The solid phase extraction method separates compounds according to their hydrophobicity. Different separation methods need to be used to suit the active peptides with different characteristics. Separation methods that are commonly used include- High-Performance Liquid Chromatography (HPLC), Ion exchange chromatography, Gel chromatography, Reversed Phase (RP)–HPLC, High-Speed Counter Current Chromatography (cc), Membrane separation (Microfiltration, Ultrafiltration, Nanofiltration, Affinity-membrane filtration (Jin *et al.,* 2021). In recent years, many researchers have used membrane filtration as the first purification step (Cho *et al.,* 2003). HPLC was reported to purify marine organisms like cyanobacterium (Lopez *et al.,* 2016), sponge (Youssef *et al.,* 2014), abalone (Nguyen *et al.,* 2013), and so forth. In recent years, HPLC is usually being combined with qualitative equipment such as mass spectrometry (MS) and liquid chromatography, followed by tandem mass spectrometric detection (LC–MS/MS) as the standard method for the characterization of peptide sequences (Alomirah *et al.,* 2000; Singh *et al.,* 2014; Vijaykrishnaraj and Prabhasankar, 2015). Nowadays, MALDI also was used frequently (Chen *et al.,* 2009). In the case of unknown peptides, the purification work requires finding the appropriate resin, pH and polarity conditions. AMPs characteristics are known to be mainly cationic and less often anionic, as well as hydrophobic or amphipathic. Crude peptide fractions from *M. dobsoni* were extracted and characterized by a three-step protocol–acetic acid- acetone precipitation, solid phase extraction by Sep-pak column (C-18cartridges) and cation exchange chromatography using FPLC system, and the peptide fractions exhibited considerable antimicrobial activity. Recently, the implementation of ion-exchange chromatographic methods (IEX) was in higher demand for the separation, detection and structural determination of proteins (Levison, 2003). The IEX

method has charged functional groups that bind molecules with an opposite charge. While an increased concentration of similarly charged molecules, the already bound molecule from the resin was displaced (Wang *et al.,* 2017). In our study, we used the cation exchange chromatographic method for the purification of Sep-pak fractions from *M. dobsoni* crude extract. A salt gradient was used for the elution of peptides. By adjusting the pH and ionic concentration of the mobile phase, proteins could be separated.

A study on humans revealed that the bioactive peptides could bring down hypertension linked to their angiotensin I- converting enzyme (Zhao *et al.,* 2009). Angiotensin I- converting enzyme was inhibited by bioactive peptides from mussels, tuna frame and shrimp (Je *et al.,* 2005; Lee *et al.,* 2010; Kleekayai *et al.,* 2015). Marine bioactive peptides possess various biological activities like neuroprotective, antidiabetic, anticancer, antiviral, antioxidative, and immuno-modulating perspectives. Marine bioactive peptides provide an opportunity for marine pharmaceutics to expand owing to their novel and unique structural and functional properties (Rauf *et al.,* 2021). Thus, the existing manufacturing techniques need further improvement to find out more marine bioactive peptides. Furthermore, the most critical problem is applying the bioactive peptides to human health and nutrition because most of the research stay in the stages of *in vitro* experiment or animal experiment due to the time consumption and cost problems (Wang *et al.,* 2017).

Conclusion

In conclusion, the current study demonstrated that peptides from *M. dobsoni*, showed antibacterial activity. Identification and characterization of the peptide would be highly promising, and the antimicrobial peptides from *M. dobsoni* can be potential agents in aquaculture and human therapeutics.

Acknowledgements

The authors are grateful to the Director, Centre for Marine Living Resources and Ecology (CMLRE), and Ministry of Earth Sciences (MoES), Govt. of India for the research grant (MoES/10-MLR/01/2012) and scientific support for the work. The authors also thank the Director of the National Centre for Aquatic and Animal Health (NCAAH) for scientific/ technical support of the work. The first author gratefully acknowledges DST (Department of Science and Technology), Govt. of India, for the award of a fellowship.

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