Molecular Immunology 101 (2018) 396–408

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Searching for crab-borne antimicrobial peptides: Crustin from *Portunus pelagicus* triggers biofilm inhibition and immune responses of *Artemia salina* against GFP tagged *Vibrio parahaemolyticus* Dahv2

Ravichandran Rekha[a], Baskaralingam Vaseeharan[a,*], Ramachandran Ishwarya[a], Mahalingam Anjugam[a], Naiyf S. Alharbi[b], Shine Kadaikunnan[b], Jamal M. Khaled[b], Mohammed N. Al-anbr[b], Marimuthu Govindarajan[c,d]

[a] Biomaterials and Biotechnology in Animal Health Lab, Department of Animal Health and Management, Alagappa University, Karaikudi, 630004, Tamil Nadu, India
[b] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, 11451, Saudi Arabia
[c] Unit of Vector Control, Phytochemistry and Nanotechnology, Department of Zoology, Annamalai University, Annamalainagar, 608 002, Tamil Nadu, India
[d] Department of Zoology, Government College for Women, Kumbakonam, 612 001, Tamil Nadu, India

ARTICLE INFO

Keywords:
In vivo
Antibacterial
Antibiofilm
Crustin
Haemolymph
Portunus pelagicus

ABSTRACT

Marine organisms represent a huge source of novel compounds for the development of effective antimicrobial drugs. The present study focus on the purification of the antimicrobial peptide crustin from the haemolymph of the blue swimmer crab, *Portunus pelagicus*, by blue Sepharose CL-6B matrix assisted affinity column chromatography. Crustin showed a single band with a molecular mass of 17 kDa in SDS-PAGE analysis. The XRD analysis exhibited peaks at 32° and 45° while a distinct peak with a retention time of 1.8 min resulted in high performance liquid chromatography (HPLC) pointing out the crystalline nature and purity of crustin, respectively. Crustin purified from *P. pelagicus* (*Pp*-Cru) showed immunological activities, triggering encapsulation, phagocytosis on Sepharose beads and yeast (*Saccharomyces cerevisiae*) respectively. Furthermore, encapsulation of GFP tagged *V. parahaemolyticus* in *Artemia salina* and challenging study were assessed under CLSM and the potential of *Pp*-Cru was examined *in vivo*. In addition, the growth reduction and biofilm inhibition potential of *Pp*-Cru on *Staphylococcus aureus, Enterococcus faecalis* (Gram- positive bacteria) and *Pseudomonas aeruginosa, Escherichia coli* (Gram-negative bacteria) was evidenced by inverted and confocal laser scanning microscopic analysis, revealing that 100 µg/ml of *Pp*-Cru can disrupt the biofilm matrix thereby the thickness of biofilm was significantly reduced. Overall, the present investigation might provide a sensitive platform to realize the significant function of *Pp*-Cru in crustacean immune mechanism as well as its potential to bacterial growth inhibitor. The functional properties of purified *Pp*-Cru antimicrobial peptide may lead to a superior understanding of innate immune response in *P. pelagicus* species, which suggest the promising application for drug development in aquaculture.

1. Introduction

Natural products, including those isolated from marine organisms, are outstanding sources of molecules multipurpose applications (Gobi et al., 2016; Benelli, 2016, 2018a,b,c; Anjugam et al., 2017; Ishwarya et al., 2018). An antimicrobial peptide (AMP) is a molecule acting as an integral component of the humoral part participated in the innate immune defense mechanism of vertebrates and invertebrates. AMPs represent an evolutionary and protection strategy of an organism against invading pathogens, including bacteria and viruses (Malanovic and Lohner, 2016; Abinaya et al., 2018). AMPs act as an effective defensive weapon and are present in higher concentration of haemocytes, playing an essential role in the flourishing evolution of intricate multicellular organisms. Since crustaceans lack in adaptive immune system responses, the AMPs have a major role as immune effectors against microbial infections (Antony et al., 2010). Crustacean-borne AMP has antibacterial activity which independently led to the lysis of bacterial cell wall. It can be able to function at very low concentrations as bactericidal agent against both Gram positive as well as Gram negative bacteria with haemocyte lysate supernatants (HLS) (Schnapp et al.,

* Corresponding author.
E-mail address: vaseeharanb@gmail.com (B. Vaseeharan).

https://doi.org/10.1016/j.molimm.2018.07.024
Received 26 February 2018; Received in revised form 9 July 2018; Accepted 14 July 2018

1996). A number of studies focused on the antimicrobial potential of AMPs from the haemolymph of crustaceans, such as the green crab *Carcinus maenas* (Schnapp et al., 1996), mangrove crab *Episesarma tetragonum* (Sivakamavalli et al., 2013), giant tiger prawn *Penaeus monodon* (Tonganunt et al., 2008; Supungul et al., 2008). Moreover, blue swimmer crab *Portunus pelagicus*, mud crab *Scylla tranquebarica* (Afsal et al., 2013), American lobster *Homarus americanus* (Battison et al., 2008), and hermit crab *Clibanarius clibanarius* (Rethna Priya et al., 2015) have been also investigated to identify novel antimicrobials. In addition, the AMPs have also been reported in some molluscs like *Mytilus edulis, Ruditapes decussatus* and oyster *Mytilus galloprovincialis* (myticins, mytilin) (Mitta et al., 2000).

The universally accepted definition of crustin refers to a cationic amphipathic secondary structure, rich in cysteine, with low molecular weight and four disulphide cores containing one whey acidic protein (WAP) domain. Crustin can be found in the haemolymph of crustaceans are considered as a source of antibacterial proteins. It includes glycine and glutamine, alanine or threonine at the cleavage sites during the signal transduction sequences (Smith et al., 2008). In crustaceans, AMP crustin was first purified with the haemolymph of *Carcinus maenas* with the molecular mass of 11.5 kDa (Relf et al., 1999). Several studies showed that crustaceans have the ability of producing bioactive antimicrobial peptides, especially in crab sector and this predominantly account for prevention of microbial infections in aquaculture. The vital demands to expand new strategies of novel antibacterial drugs are still highly significant to control biofilm-forming pathogens. It is conceivable that *Pp*-Cru with 17 kDa cysteine rich protein has antibacterial properties by performing as agent modifying membrane permeability and causing bacterial cell wall damage. Hence, the present work focused on antimicrobial peptide crustin isolated from the crab *P. pelagicus*.

It may have a great prospective for the improvement of highly effective drugs (Uhlig et al., 2014). In the last few years, new antibacterial agents have developed from important immune recognition components of crustaceans like, crustin (Antony et al., 2010), α_2-macroglobulin (Gollas-Galvan et al., 2003), lectin (Sivakamavalli and Vaseeharan, 2014), as well as other antimicrobial peptides (Sivakamavalli et al., 2013; Veeruraj et al., 2008; Sivakamavalli et al., 2015), penaeidin (Shanthi and Vaseeharan, 2012), pattern recognition proteins (Anjugam et al., 2016) anti-lipopolysaccharide factors (ALF) (Antony et al., 2010), prophenoloxidase (Ishwarya et al., 2016). Crustin plays a leading role in the immune system and diverse applications have been demonstrated (Smith et al., 2008). While the antimicrobial activities of AMPs have been the main crucial point, some of the crustacean molecules are effective against bacterial plus viral pathogens (Yasin et al., 2000). In this regard, our study focuses on the purification of the antimicrobial peptide crustin with the haemolymph of the blue swimmer crab, *P. pelagicus*, by Sepharose CL-6B matrix assisted affinity column chromatography. Crustin showed a single band with a molecular mass of 17 kDa through SDS-PAGE, then it was characterized by XRD, FTIR, CD, and HPLC analysis.The previous studies reports the immunity in arthropods has phagocytosis and encapsulation process evaluated with hemocyte granules (Ratner and Vinson, 1983; Bayne, 1990). Hence the impact of *Pp*-Cru on immunological activities, *i.e.*, encapsulation, phagocytosis on Sepharose beads and yeast *Saccharomyces cerevisiae,* was studied. In addition, the micro-crustacean *Artemia salina* was selected as a model invertebrate and evaluated for *Pp*-Cru encapsulation experiments of GFP tagged *V. parahaemolyticus*. In addition, the growth inhibition and biofilm inhibition potential of *Pp*-Cru was evaluated on *S. aureus, E. faecalis*, *P. aeruginosa,* and *E. coli* bacteria, relying to inverted and confocal laser scanning microscopic analyses, as well as live and dead cell assays.

2. Materials and methods

2.1. Collection of haemolymph from P. pelagicus

Physically well, blue crabs, *P. pelagicus,* were obtained from the east and coastal area of Mandapam, Ramanathapuram district, (9.2770 °N, 79.1252 °E) Tamilnadu, India, with support of fishermen at the morning hours between 7.00 a.m. to 9.30 a.m.. The *P. pelagicus* crabs (250 g of 2 crabs) were rinsed by distilled water to eliminate dust particles and planktonic materials. Haemolymph (50 ml) was collected from the chelate legs using 10 ml of syringe with pre-cooled anticoagulant buffer contains dextrose 10.25 g, trisodium citrate 4 g, citric acid 0.28 g, NaCl 2.10 g, Distilled water 500 ml, pH 7.5 (Ishwarya et al., 2016). The *P. pelagicus* plasma or haemocyte lysate supernatants (HLS) was equilibrated with Tris buffer and centrifuged at 4000 rpm for 10 min at 4 °C and the final haemolymph supernatant was reserved in freeze condition at −80 °C for further analysis.

2.2. Purification of crustin from P. pelagicus

AMP crustin was purified from the *P. pelagicus* haemolymph as described by (Sivakamavalli et al., 2015) with minor changes. Briefly, *P. pelagicus* plasma was equilibrated with 20 ml of Tris buffer then applied to Blue-Sepharose CL-6B column (11 × 1 cm). The eluted 1 ml fractions were collected in tubes and then column was rinsed with TBS at a flow speed of 1 ml min^{-1}. Purified sample was collected as the maximum concentrated fractions from the 1.2 ml of Eppendorf tube. After the purification, *Pp*-Cru was pooled and dialyzed against double distilled water for overnight.

2.3. Characterization of purified crustin from P. pelagicus

2.3.1. SDS -PAGE

Estimated molecular weight of the purified *Pp*-Cru and the purity was evaluated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS –PAGE- 12% separating gel and 5% spacer gel) followed by (Laemmli, 1970) using vertical gel electrophoresis system. The sample was prepared with *Pp*-Cru and sample buffer (1:1 ratio), and then vortexed for 5 min. Primarily, the sample was boiled for 10 min and cooled at 37 °C. Protein sample (20 µl) with loading dye was loaded into polyacrylamide gel electrophoresis at constant electricity (150 V) for 3 h. The molecular mass of the purified *Pp*-Cru was determined by standard protein molecular weight markers (Biorad). After the process of electrophoresis, the gel was treated with Coomassie brilliant blue (GE Healthcare Bio-Sciences, India) and fixed at gentle shaking. The quantity of protein was assessed by the method of (Lowry et al., 1951) using Bovine Serum Albumin (BSA) as a standard. Absorbance was measured at 540 nm to calculate the concentration of *Pp*-Cru and the sample was lyophilized for further studies.

2.3.2. High performance liquid chromatography (HPLC) and circular dichroism (CD)analysis

High performance liquid chromatography applied a high pressure to drive the *Pp*-Cru solutes through the column accurately and fastly. The concentrated fractions of *Pp*-Cru were applied and further purified through HPLC parting, then were carried with C_{18} column (7.8 mm × 30 cm) formerly added with Tris buffer at a run speed of 0.8 min HPLC instrument (AKTA HPLC purifier system, GE Healthcare, Pharmacia), was used for the analysis. In HPLC, C_{18} column with linear gradient between 0.05 % trifluroacitic acid in water 80 % of acetonitrile. The confirmation of *Pp*-Cru secondary structure was predicted through circular dichroism (CD) Jasco-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan). The measurements analyzed at wavelength were recorded between 190–260 nm, by quartz cuvette with path length 1 cm^{-1}. The purified *Pp*-Cru prepared in 10 mM sodium phosphate buffer, was taken for atomic spectra determinations. The spectral range