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Macrobrachium rosenbergii mannose binding lectin: Synthesis of *Mr*MBL-N20 and *Mr*MBL-C16 peptides and their antimicrobial characterization, bioinformatics and relative gene expression analysis

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ABSTRACT

Mannose-binding lectin (MBL), an antimicrobial protein, is an important component of innate immune system which recognizes repetitive sugar groups on the surface of bacteria and viruses leading to activation of the complement system. In this study, we reported a complete molecular characterization of cDNA encoded for MBL from freshwater prawn *Macrobrachium rosenbergii* (*Mr*). Two short peptides (*Mr*MBL-N20: ^{20}AWNTYDYMKREHSLVKPYQG^{39} and *Mr*MBL-C16: ^{307}GGLFYVKHKEQQRKRF^{322}) were synthesized from the *Mr*MBL polypeptide. The purity of the *Mr*MBL-N20 (89%) and *Mr*MBL-C16 (93%) peptides were confirmed by MS analysis (MALDI-ToF). The purified peptides were used for further antimicrobial characterization including minimum inhibitory concentration (MIC) assay, kinetics of bactericidal efficiency and analysis of hemolytic capacity. The peptides exhibited antimicrobial activity towards all the Gram-negative bacteria taken for analysis, whereas they showed the activity towards only a few selected Gram-positive bacteria. *Mr*MBL-C16 peptides produced the highest inhibition towards both the Gram-negative and Gram-positive bacteria compared to the *Mr*MBL-N20. Both peptides do not produce any inhibition against *Bacillus* sps. The kinetics of bactericidal efficiency showed that the peptides drastically reduced the number of surviving bacterial colonies after 24 h incubation. The results of hemolytic activity showed that both peptides produced strong activity at higher concentration. However, *Mr*MBL-C16 peptide produced the highest activity compared to the *Mr*MBL-N20 peptide. Overall, the results indicated that the peptides can be used as bactericidal agents. The *Mr*MBL protein sequence was characterized using various bioinformatics tools including phylogenetic analysis and structure prediction. We also reported the *Mr*MBL gene expression pattern upon viral and bacterial infection in *M. rosenbergii* gills. It could be concluded that the prawn MBL may be one of the important molecule which is involved in antimicrobial mechanism. Moreover, *Mr*MBL derived *Mr*MBL-N20 and *Mr*MBL-C16 peptides are important antimicrobial peptides for the recognition and eradication of viral and bacterial pathogens.

1. Introduction

Crustaceans are the major single fishery commodity in world aquaculture trade. Among all crustaceans, freshwater giant prawn *Macrobrachium rosenbergii* has been considered as the sixth largest marketable aquaculture species in Asian countries [1]. *M. rosenbergii* is of worldwide interest because of its great capacity

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to be farmed commercially in freshwater [2]. They are also known as giant freshwater prawn which is widely distributed in Australia, China, Southeast Asia, Malaysia, Mexico, India and many other parts of world. In recent years, the hatcheries of freshwater crustaceans have been extremely aggravated by various bacterial and viral diseases such as white tail disease (WTD), white spot syndrome disease (WSSD), tail rot disease, etc., [3]. Hence, an efficient therapeutic agent is required to treat these diseases at molecular level.

Antimicrobial Peptides (AMPs) are naturally occurring defense molecules of plants and animals, which kill or defuse the invading pathogenic microbes. AMPs behave as amphipathic molecules, which vary in amino acid sequences, structures and their functional activities. They are smaller in size; approximately 15–100 amino acid residues long [4]. AMPs have various modes of action that includes permeabilizing the membrane of bacteria, fungi and viruses [5] and by interacting with macromolecules inside the cells of pathogens [6]. The broad activity spectrum and the relative selectivity towards microbial membranes make them interesting alternative for conventional antibiotics. In crustaceans such as *M. rosenbergii* [7–9], *Pacifastacus leniusculus* and *Scylla paramamosain* [10], *Penaeus monodon* [11], *Eriocheir sinensis*, *Fenneropenaeus chinensis* and *Litopenaeus setiferus* [12], many antimicrobial peptides were identified from various immune proteins such as crustin, anti-lipopolysaccharide factor, histone, etc.

Mannose-binding lectin (MBL), an antimicrobial protein, is an important component of innate immune system which recognizes repetitive sugar groups on the surface of bacteria and viruses leading to activation of the complement system [13,14]. MBL shows specificity for mannose, glucose, fucose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine which are present in the bacterial and fungal cell membranes [15]. MBL recognizes either the complete sugar molecules or part of the molecules or even glycosidic linkages [16]. MBL belong to collectins/collagenous lectin family [17,18]. MBL is an acute-phase protein produced by liver hepatocytes that increases in response to an infection or inflammatory conditions. MBL acts as an opsonin for phagocytosis by macrophages [19]. MBL also participates in several important biological phenomena such as pathogen recognition, protein synthesis and transport, signal transduction and cell–cell interaction [20].

MBL consist of three peptide chains including a collagen helix, α-helical coiled-coli and a carbohydrate recognition domain (CRD) [21–23]. The CRD is approximately 120 amino acids long in MBL [24,25] along with a distinctive double-loop stabilized by two conserved disulfide bridges and a conserved polar and hydrophobic interaction. There are four Ca^{+2} binding sites recognized from CRDs of diverse species, in that the Ca^{+2} binding site 2 is connected with carbohydrate-binding activity [26]. CRD is involved in glycan interaction which activates complement via lectin pathway. Thus the antimicrobial activity of MBL depends on both the N terminal and C terminal region.

In this study, a cDNA sequence encoded for MBL from *M. rosenbergii* (designated as *Mr*MBL) was identified from the cDNA library of *M. rosenbergii* constructed using genome sequencing (GS) FLX technology and their physical and chemical properties were studied. The predicted *Mr*MBL protein sequence was characterized using various bioinformatics tools including phylogenetic analysis and structure prediction. We also reported the *Mr*MBL gene expression pattern upon viral [white spot syndrome baculovirus (WSBV) and *M. rosenbergii* nodovirus (*Mr*NV)] and bacterial (*Aeromonas hydrophila* and *Vibrio harveyi*) challenges. Moreover, to understand the antimicrobial property of *Mr*MBL protein, two peptides were synthesized from the protein at N (N-terminal peptide: *Mr*MBL-N20) and C (C-terminal peptide: *Mr*MBL-C16) terminals and various anti-microbial activities including anti-microbial assay, bactericidal assay and hemolytic assay were performed.

2. Materials and methods

2.1. Identification, cloning and sequencing of MrMBL

A giant freshwater prawn cDNA library which was normalized using CloneMiner™ cDNA library construction kit (Invitrogen) and Trimmer Direct Kit: cDNA Normalization Kit (BioCat GmbH) was established by utilizing RNA extracted from the giant prawn tissue pool including muscle, gills, hemocyte, hepatopancreas and brain [27–29]. A partial cDNA sequence encoding MBL was identified from the giant prawn cDNA library during screening. Further, its full length was obtained by an internal sequencing method utilizing an ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit (Life Technologies) using the following forward primer: *Mr*MBL F1 5′ TGG CTT TGC CTT CTG GTA TG 3′. Then, the untranslated region (UTR), translated region and its corresponding polypeptide region of *Mr*MBL were analyzed.

2.2. Bacterial culture

The following bacteria were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India for the antimicrobial assays: Gram negative *A. hydrophila*, *Escherichia coli*, *Vibrio parahaemolyticus*, *V. alginolyticus* and *V. harveyi* and Gram positive *Bacillus subtilis*, *B. licheniformis*, *B. coagulans*, *Streptococcus pyogenes*, *Micrococcus luteus* and *Listeria monocytogenes*.

2.3. Synthesis and purification of MrMBL-N20 and MrMBL-C16

The peptides (*Mr*MBL-N20: ^{20}AWNTYDYMKREHSLVKPYQG^{39} and *Mr*MBL-C16: ^{307}GGLFYVKHKEQQRKRF^{322}) used in this study were synthesized by Mimotopes Pvt. Ltd (Australia). The purity of the *Mr*MBL-N20 (89%) and *Mr*MBL-C16 (93%) peptides were confirmed by MS analysis (MALDI-ToF), provided by the manufacturer. Peptides were diluted in endotoxin free water and used as stock (3 mM) or stored at −20 °C until further use. The stock solution was used for the antimicrobial assays.

2.4. MIC assay of MrMBL-N20 and MrMBL-C16

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial required to inhibit the growth of a micro organism after certain period of incubation. The study was performed as reported by Lehrer et al. [30] with slight modifications. In brief, the Gram negative and positive bacteria were cultured overnight in 10 mL of 3% (w/v) trypticase soy broth, whereas *Vibrio* sps were cultured in marine broth. Then, they were washed in 10 mM Tris buffer solution (pH = 7.4). Followed by, 6.6 μL bacterial cultures at a concentration of 1-2 × 10^9 CFU/mL was added underneath of 1% (w/v) low-electro endosmosis type agarose gel which contains 0.03% (w/v) trypticase soy broth and 0.02% (v/v) Tween 20 (Sigma). Then, the gel was casted into petri plates. Four millimeter (diameter) wells were perforated in the gel after solidification, followed by 6 μL of peptide was added into each well. The petri plates were incubated for 3 h at 37 °C to enable dispersion of the peptides. Then, the gel was concealed with 15 mL of molten overlay which made of 6% trypticase soy broth and 1% low-electro endosmosis type agarose gel or with marine broth which made of 4% of marine broth and 1% low-electro endosmosis type agarose gel. Then, the plates were incubated at 37 °C for 18–24 h. After incubation, the minimal inhibitory concentration (MIC) of the peptides was observed as a clear zone around each well and determined (diameter, mm). One hundred μg ampicillin and the equal amount of DEPC treated nuclease free de-ionized water was