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Pellino-1 derived cationic antimicrobial prawn peptide: Bactericidal activity, toxicity and mode of action

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ABSTRACT

The antimicrobial peptides (AMPs) are multifunctional molecules which represent significant roles in the innate immune system. These molecules have been well known for decades because of their role as natural antibiotics in both invertebrates and vertebrates. The development of multiple drug resistance against conventional antibiotics brought a greater focus on AMPs in recent years. The cationic peptides, in particular, proven as host defense peptides and are considered as effectors of innate immunity. Among the various innate immune molecules, functions of pellino-1 (Peli-1) have been recently studied for its remarkable role in specific immune functions. In our study, we have identified Peli-1 from the cDNA library of freshwater prawn *Macrobrachium rosenbergii* (*Mr*) and analyzed its features using various *in-silico* methods. Real time PCR analysis showed an induced expression of *Mr*Peli-1 during white spot syndrome virus (WSSV), bacteria (*Vibrio harveyi*) and lipopolysaccharide (LPS) from *Escherichia coli* challenge. Also, a cationic AMP named *Mr*DN was derived from *Mr*Peli-1 protein sequence and its activity was confirmed against various pathogenic bacteria. The mode of action of *Mr*DN was determined to be its membrane permeabilization ability against *Bacillus cereus* ATCC 2106 as well as its DNA binding ability. Further, scanning electron microscopic (SEM) images showed the membrane disruption and leakage of cellular components of *B. cereus* cells induced by *Mr*DN. The toxicity of *Mr*DN against normal cells (HEK293 cells) was demonstrated by MTT and hemolysis assays. Overall, the results demonstrated the innate immune function of *Mr*Peli-1 with a potential cationic AMP in prawn.

1. Introduction

Macrobrachium rosenbergii is a commercially valuable food source and holds the sixth place among the marketable aquaculture species in Asian countries. The total production of *M. rosenbergii* was recently reported as 1.4 million tonnes globally. However, frequent occurrence of bacterial and viral infections such as white spot syndrome, white tail disease, luminescent larval syndrome, bacterial necrosis and white post larval disease in the hatcheries resulted a huge economic crisis in the aquaculture industry (Hameed and Bonami, 2012; Chaurasia et al., 2016a). Therefore, the recent research has been focusing on understanding the immune mechanisms underlying the host-pathogen interaction. Recently, few immune genes that are potentially involved against the prawn diseases have been reported in *M. rosenbergii* (Kumaresan et al., 2016). However, the participation of pellino proteins in immune functions has been poorly understood in *M. rosenbergii*.

Pellino proteins are evolutionary conserved proteins and demonstrated to play a crucial role in immune functions. Though

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some pellino protein functions have remained obscure, it was evident from recent studies that pellino has its function as scaffold proteins in Toll-like receptor/interleukin-1 receptor (TLR/IL-1R) signaling pathway (Schauvliege et al., 2006). Previously, pellino was identified to bind with IL-1R associated kinase 1 (IRAK-1) homolog; however, its participation in the activation of IL-1R was confirmed recently. Members of the pellino family include pellino 1, 2 and 3 with splicing variants pellino 3a and 3b (Moynagh, 2009) and each protein member has been determined with various functions in the living system. Pellino-1 functions as an indispensable player of TLR/IL-1R (Schauvliege et al., 2007). Similarly, interaction between pellino-2 and pelle-like kinase/interleukin-1 receptor-associated kinase-1 (mPLK/IRAK-1) was observed in mouse (Yu et al., 2002). The third member, pellino-3 is involved in the activation of MAPK pathway (Jensen and Whitehead, 2003).

The significant functions of pellino proteins have been well studied in various organisms since it has been discovered in *Drosophila melanogaster*. These proteins have been proved as novel players of the TLR signaling cascade which is critically involved in innate immune response (Honda et al., 2005). Moreover, the TLRs have been considered as one of the prominent classes of pattern recognition receptors to distinguish the pathogen associated molecules (Mogensen, 2009). As TLRs involved in microbial recognition, the invasion of pathogens results with activation of TLR signaling pathways (Takeda and Akira, 2004) which further induce the role of pellino. Bennett et al. (2012) demonstrated a reduction in cytokine production in the innate immune system due to silencing of pellino-1. Also, studies suggested the involvement of pellino-1 in positive regulation of NF-κB in pacific white shrimp *Litopenaeus vannamei*. The gene expression of pellino-1 in *L. vannamei* was observed to be modulated during bacterial and viral infections (Li et al., 2014). All these studies suggested the participation of pellino-1 in immunity and functions against pathogenic invasion. Thus we predicted that pellino-1 (Peli-1) from *M. rosenbergii* (*Mr*) may also involve in antimicrobial functions against pathogenic organisms.

Antimicrobial peptides represent a new and broad class of promising drug due to its potency and safest ability to neutralize or kill the pathogenic microbes compared to the traditional antibiotics. These short peptides have been identified in all forms of life and inbuilt in the living system to thwart against pathogenic invasion (Gordon et al., 2005). Besides, the threat of raising multiple drug resistance strains has been aggravated in the recent years, especially in aquaculture. The significant investigations and discovery of AMPs increased the curiosity for the search of more potent and novel AMPs which can serve as a potent alternate for the conventional antibiotics. As the invertebrates majorly depend on the innate immune components, AMPs play a vital role to function against the invading pathogens which made them an abundant source of AMPs (Pasupuleti et al., 2012). The production of these AMPs is considered as one of the important mechanisms in injured or infected organism. Exclusively, vast range of AMPs has been discovered from hemolymph or haemocytes of the crustaceans in normal as well as in infected state (Ganz, 2003; Rosa and Barracco, 2010). Followed by, the isolation of proline-rich antimicrobial peptides from *Carcinus maenas* (Schnapp et al., 1996), Penaeidin from *Penaeus vannamei* (Destoumieux et al., 1997), Crustins from *Hyas araneus* (Sperstad et al., 2009), Defensin from *Panulirus japonicas were reported in crustaceans*. Most of the identified peptides from crustaceans are cationic and reported with broad spectrum of antimicrobial activity (Rosa and Barracco, 2010).

Among the identified peptides, the cationic peptides continue to dominate due to its potential therapeutic and broad spectrum of activity; eventually, several of them are in clinical and preclinical studies. Notably, majority of the effective AMPs that are identified from natural sources were cationic (Hancock and Diamond, 2000; Hancock and Sahl, 2006). Additionally, studies on *M. rosenbergii* emerged a variety of AMPs with demonstrated bactericidal activities (Arockiaraj et al., 2014, 2015; Chaurasia et al., 2015a). Though, pellino has been reported to involve in immune function against pathogens, the region responsible for the antibacterial activity remains unclear.

Hence, in this study, we have demonstrated the induced expression of *Mr*Peli-1 by real time PCR analysis against viral and bacterial challenge. Also, the ability of peptide *Mr*DN against various pathogenic organisms was evaluated further. Its toxicity was assessed on HEK293 cells and in peripheral blood erythrocytes. Followed by, the mode of action of *Mr*DN on bacteria was elucidated from different analysis, such as membrane disruption, scanning electron microscopic (SEM) analysis and DNA binding ability.

2. Materials and methods

2.1. *Identification of* M. rosenbergii *pellino-1 from cDNA library*

A cDNA sequence encoding *Mr*Peli-1 was identified from the previously established *M. rosenbergii* cDNA library by GS-FLX™ technology (Arockiaraj et al., 2013a) using total RNA extracted from the haemocyte, hepatopancreas, muscle, gills and brain of *M. rosenbergii*. The identified cDNA sequence was translated to the corresponding amino acid sequence and the cDNA sequence was submitted to EMBL database.

2.2. *In-silico characterization*

The 5′ and 3′ untranslated region (UTR), open reading frame (ORF) and the respective protein sequence of *Mr*Peli-1 cDNA were obtained from Expasy translate tool (http://web.expasy.org/translate/). The physico-chemical properties of *Mr*Peli-1 protein were determined by ProtParam tool of ExPASy (http://www.expasy.org/tool/protparam/). The homologous protein sequences of *Mr*Peli-1 were identified from BLAST (http://blast.ncbi.nlm.nih.gov/Blast) and the identity and similarity of *Mr*Peli-1 with those identified sequences were studied. Further, multiple sequence alignment of the *Mr*Peli-1 protein sequence was performed with five other homologous sequences from invertebrates and vertebrates using BioEdit (Version 7.0.0). Phylogenetic tree was built using the Neighbor-Joining method in MEGA 5.05 to understand the evolutionary relationship and genetic distance between the species. Finally, to understand the structural features of *Mr*Peli-1, the protein sequence was submitted to I-Tasser server (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and the obtained 3D model was further analyzed in PyMol program.

2.3. *Pathogens*

The bacteria used for the antimicrobial assays were purchased from microbial type culture collection and gene bank (MTCC), American type culture collection (ATCC) and some are clinical isolates (CI). Following are the bacterial strains used for the study: *Aeromonas hydrophila* MTCC 1739, *Bacillus cereus* ATCC 2106, *B. mycoides* MTCC 8920, *B. subtilis* ATCC 6051, *Escherichia coli* ATCC 25922, *E. coli* ATCC 9637, *E. coli* CI 2931, *E. coli* MTCC 10312, *Klebsiella pneumoniae* ATCC 27736, *K. pneumoniae* CI 1876, *K. pneumoniae* CI 2493, *K. pneumoniae* CI 7367, *Micrococcus luteus* MTCC 6164, *Pseudomonas aeruginosa* ATCC 25668, *P. aeruginosa* ATCC BAA 427, *P aeruginosa* CI 556, *P. aeruginosa* CI 1068, *P. aeruginosa* CI 2606, *P. aeruginosa* 15159, *P. fluorescens* MTCC 2269, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 335922, *S. aureus* ATCC 700699, *S. aureus* ATCC 9144, *S. aureus* MTCC 9542, *Serratia marcescens* MTCC 3124, *Salmonella enterica* MTCC 1166.