Fish & Shellfish Immunology 54 (2016) 489–498

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Involvement of a LysM and putative peptidoglycan-binding domain-containing protein in the antibacterial immune response of kuruma shrimp *Marsupenaeus japonicus*

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ARTICLE INFO

Article history:
Received 13 February 2016
Received in revised form
22 April 2016
Accepted 29 April 2016
Available online 30 April 2016

Keywords:
Lysin motif
Marsupenaeus japonicus
Antimicrobial peptide gene
Bacterial clearance

ABSTRACT

Lysin motif (LysM) is a peptidoglycan and chitin-binding motif with multiple functions in bacteria, plants, and animals. In this study, a novel LysM and putative peptidoglycan-binding domain-containing protein was cloned from kuruma shrimp (*Marsupenaeus japonicus*) and named as *Mj*LPBP. The cDNA of *Mj*LPBP contained 1010 nucleotides with an open reading frame of 834 nucleotides encoding a protein of 277 amino acid residues. The deduced protein contained a Lysin motif and a transmembrane region, with a calculated molecular mass of 31.54 kDa and isoelectric point of 8.61. *MjLPBP* was ubiquitously distributed in different tissues of shrimp at the mRNA level. Time course expression assay showed that *MjLPBP* was upregulated in hemocytes of shrimp challenged with *Vibrio anguillarum* or *Staphylococcus aureus*. *MjLPBP* was also upregulated in hepatopancreas after white spot syndrome virus and bacteria challenge. The recombinant protein of *Mj*LPBP could bind to some Gram-positive and Gram-negative bacteria and yeast. Further study found that r*Mj*LPBP bound to bacterial cell wall components, including peptidoglycans, lipoteichoic acid, lipopolysaccharide, and chitin. The induction of several antimicrobial peptide genes and phagocytosis-related gene, such as anti-lipopolysaccharide factors and myosin, was depressed after knockdown of *MjLPBP*. *Mj*LPBP could facilitate *V. anguillarum* clearance in vivo. All the results indicated that *Mj*LPBP might play an important role in the innate immunity of shrimp.
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1. Introduction

Kuruma shrimp (*Marsupenaeus japonicus*) is one of the most important aquaculture shrimp species worldwide. However, the frequent disease outbreaks of shrimp caused by bacteria and viruses have resulted in high mortality of shrimp and devastating economic losses, which seriously restricted the development of shrimp culture industry. Given the important economic value of shrimp, *M. japonicus* has become an important research model for innate immunity in crustacean. Researchers have paid increasing attention to the shrimp innate immune system for developing novel methods of disease control [1]. Shrimp, similar to other invertebrates, lacks an adaptive immune system and relies on the innate immune system for protection against bacterial and viral diseases [2,3]. The innate immune defense system serves as the first line of defense and consists of humoral response and cellular response [4]. The humoral response mainly includes synthesis of antimicrobial peptides, whereas the cellular response involves phagocytosis, encapsulation, and nodule formation [4–6]. The host immune response is initiated when microbial components called pathogen-associated molecular patterns (PAMPs) are recognized by the host pattern recognition receptors (PRRs) [7,8]. In invertebrates, several PRRs have been identified, such as peptidoglycan recognition proteins, Toll receptors, Gram-negative binding proteins, thioester-containing proteins, Down syndrome cell adhesion molecules, and C-type lectins [9,10].

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http://dx.doi.org/10.1016/j.fsi.2016.04.134
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Lysin motif, an ancient and widely distributed protein domain, usually contains approximately 40 amino acid residues and functions in peptidoglycan and chitin binding [11]. Lysin motif-containing proteins have been extensively studied in plants, and they function as the second major pattern recognition proteins and mediate plant symbiosis and immunity [11]. In rice, Lysin motif-containing proteins LYP4 and LYP6 functioned as dual PRRs in the perception of bacterial peptidoglycan and fungal chitin, as well as signal transduction of the innate immune reaction [12,13].

Although numerous nucleotide sequences of Lysin motif-containing proteins from animals are deposited in GenBank database, only few have been thoroughly studied and their exact functions remain unclear. Only chitinase containing several Lysin motifs in *Caenorhabditis elegans* [14] and a Lysin motif-containing protein of red swamp crayfish *Procambarus clarkii* [15] were identified and studied. The Lysin motif-containing protein from red swamp crayfish was a member of the intracellular Lysin motif and putative peptidoglycan-binding domain-containing protein 2 family; this protein functioned in the antibacterial innate immune reaction by recognizing different microorganisms [15]. Therefore, further studies are needed to explore the function of Lysin motif-containing proteins in animals.

In this study, a new Lysin motif and putative peptidoglycan-binding domain-containing protein was identified from kuruma shrimp, *M. japonicus*, and designated as *Mj*LPBP. This protein of interest is a new member of the Lysin motif and putative peptidoglycan-binding domain-containing protein 3 family, and it contains a Lysin motif and a transmembrane region. *MjLPBP* was distributed in hemocytes, heart, hepatopancreas, gills, stomach, and intestine, and it was upregulated in hemocytes and hepatopancreas in shrimp after *V. anguillarum*, *S. aureus*, and white spot syndrome virus (WSSV) challenge. The recombinant extracellular region of *Mj*LPBP displayed binding ability to Gram-negative bacteria, Gram-positive bacteria, and yeast by binding to microbial cell wall components, including peptidoglycan (PGN), lipoteichoic acid (LTA), lipopolysaccharide (LPS), and chitin. Knockdown of *MjLPBP* impaired the expression of some antimicrobial peptide genes and phagocytosis-related gene in response to *V. anguillarum* or *S. aureus* infection. *Mj*LPBP facilitated *V. anguillarum* clearance in vivo. All these results suggested that *Mj*LPBP might play a key role in the innate immunity against bacteria of kuruma shrimp.

2. Materials and methods

2.1. Immune challenge of shrimp

Kuruma shrimp (approximately 10 g each) were purchased from a seafood market in Jinan, Shandong Province, China. The shrimp were cultured in lab tanks which were filled with circulating air-pumped seawater. For bacterial immune challenge, *V. anguillarum* or *S. aureus* (2×10^6 cells per shrimp) were injected at the abdominal segment of each shrimp. For viral infection, 50 μL of WSSV (2×10^5 copy, extracted from infected crayfish) was injected. The same volume of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) was injected to shrimp of another group, which was used as the control.

2.2. Sequence analysis

The sequence of *MjLPBP* was obtained via hemocyte transcriptome sequencing of shrimp. Total RNA was extracted from the hemocytes of WSSV-challenged shrimp for transcriptome sequencing by BGI (Beijing Genomics Institute, Shenzhen, China). Sequence translation and deduced protein characterization were performed with ExPASy program (http://www.expasy.ch/). Protein homology analysis was analyzed by BLASTp program in the National Center for Biotechnology Information server (http://blast.ncbi.nlm.nih.gov/). The protein domain was predicted with SMART program (http://smart.embl-heidelberg.de/). The phylogenetic tree was constructed using MEGA 5.05 software [16].

2.3. Gene expression analysis by semi-quantitative and real-time quantitative RT-PCR (qRT-PCR)

Hemolymph was extracted from the ventral sinus by a sterile syringe containing 1/3 volume of anticoagulant buffer (450 mM NaCl, 10 mM KCl, 10 mM EDTA, and 10 mM Hepes pH 7.45). It was then centrifuged at 800 *g* for 10 min at 4 °C to collect hemocytes. Tissues including heart, hepatopancreas, gills, stomach, and intestine were collected from at least three shrimp for RNA extraction. Total RNAs were extracted from different samples, such as hemocytes, heart, hepatopancreas, gills, stomach, and intestine of normal shrimp. After digestion by RNase-free DNase I, total RNAs were reverse transcribed using first-strand cDNAs, which were diluted tenfold in nuclease-free water and used as templates for semi-quantitative RT-PCR. *Mj*LPBPRT-F (5′-GTC TGA TGG GCA AGG GAA TA-3′) and *Mj*LPBPRT-R (5′-CCA GGA CGG AGT TGG GTT T-3′) were used as primers. PCR was conducted as follows: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 53 °C for 45 s, and 72 °C for 30 s; and 72 °C for 10 min. The sequence amplified with primer *Mj*LPBPRT-F and *Mj*LPBPRT-R was ligated into the pMD-18T vector (TaKaRa, Dalian, China) and sequenced to confirm its correctness. β-*actin* was used as the internal control.

Total RNAs were extracted from hemocytes and hepatopancreas of shrimp at 3, 6, 12, 24, and 48 h after PBS, *V. anguillarum*, *S. aureus*, or WSSV challenge. After treatment by RNase-free DNase I, total RNAs (about 5 μg each) were used to reverse transcribe the first-strand cDNAs used as templates for qRT-PCR after 20-fold dilution. β-*actin* was used as the internal control. The PBS-challenged group was used as the mock challenge group. qRT-PCR was accomplished in C1000 thermal cycler (Bio-Rad, USA) with a total volume of 20 μL containing 10 μL of 2 × Ultra SYBR mixture (with ROX, CWBio, Beijing, China), 2 μL of 1:20 diluted cDNA, 4 μL of 1 μM forward primer, and 4 μL of 1 μM reverse primer. The amplification conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and melting from 65 °C to 95 °C. Amplification was repeated in triplicate. The qRT-PCR data were analyzed by $2^{-\Delta\Delta CT}$method to calculate the relative expression level of *MjLPBP* in response to bacterial or viral infection. GraphPad Prism software (GraphPad, San Diego, CA, USA) was used to construct figures.

2.4. Recombinant protein expression and purification

The nucleotide fragment encoding the extracellular region of *Mj*LPBP was amplified by *Mj*LPBPExF (5′-ATC TAC GGATCC ATG AGT TCT TCC CTC TGG-3′) and *Mj*LPBPExR (5′-ATC TAC AAGCTT GCT GAT GCC CCA GTC TGC CCC-3′). After digestion by *Bam*H I and *Hin*d III, the fragment was ligated into pET32a (+) vector which was digested by *Bam*H I and *Hin*d III (Novagen, WI, USA). The recombinant *Mj*LPBP-pET32a plasmid was sequenced to verify the correctness of the full length *Mj*LPBP in the plasmid. Recombinant *Mj*LPBP-pET32a was transformed into *Escherichia coli* Rosetta cells, and recombinant protein expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranosid at 37 °C for 5 h. Recombinant *Mj*LPBP (r*Mj*LPBP) was expressed into inclusion bodies and then refolded following previously described methods [15]. The empty pET32a (+) vector without any insert

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