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Molecular cloning, expression of a galectin gene in Pacific white shrimp *Litopenaeus vannamei* and the antibacterial activity of its recombinant protein

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ABSTRACT

Galectins play crucial roles in innate immune responses in invertebrate by recognizing and eliminating microinvaders. In this study, a cDNA encoding a galectin in the Pacific white shrimp (*L. vannamei*) was identified and characterized. A recombinant variant of this lectin, r*Lv*galectin, was expressed in the model organism *P. pastoris* and its expression was confirmed by Western blot. Biochemical assays indicated that the recombinant protein antibacterial r*Lv*galectin activity and was expressed in all of the organism's tested tissues Injection of the bacterium *V. alginolyticus* into *L. vannamei* induced hemocytes upregulation of *Lvgalectin*. The recombinant *Lvgalectin* protein (r*Lvgalectin*) could bind various microorganism including Gram-positive bacteria, Gram-negative bacteria and yeast. And it revealed antimicrobial activity against the test Gram-positive bacteria, Gram-negative bacteria, but did not inhibit the growth of fungus *Pichia pastoris*. Moreover, r*Lvgalectin* could significantly enhance the clearance activity of *V. alginolyticus* in *vivo*. In *vivo* challenge experiments showed that the recombinant r*Lvgalectin* protein can significantly reduce the mortalities of *V. alginolyticus* injection. Furthermore, Compared to their wild-type counterparts, *Lv*galectin-silenced shrimp exhibited increased mortality and hemocyte apoptosis, decreased bacterial clearance ability and total hemocyte counts, and stronger expression of *Lvp53*, *LvproPO*, *LvPEN3*, and *LvCrustin* following *V. alginolyticus* challenge. Taken together, these results suggest that galectin is important in the innate immune response of shrimp to pathogens infection.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most important commercial crustacean aquaculture species worldwide and especially in China (Wang et al., 2014). However, with the rapid development of intensive culture, frequent outbreaks of aquaculture diseases have extremely effected the sustainable development of pacific white shrimps farming (Peng et al., 2015). Previous studies suggested that bacterial infections were the main causes of shrimp diseases, and that *Vibrio alginolyticus* was one of the most important bacterial pathogens in diseased shrimp in China (Thepnarong et al., 2015; Xu et al., 2013).

Like most invertebrates, shrimps lack a true adaptive immune system and depends heavily on the innate immune system (Cao et al., 2014; Cha et al., 2015b; Zhao et al., 2013). Antimicrobial consists of cellular and humoral immune responses. The cellular immune response includes apoptosis, tumor formation, and phagocytosis, whereas the humoral immune response includes the p53 pathway, prophenoloxidase (proPO) system, and production of multiple antimicrobial peptides (Liu et al., 2013). Galectins are a family of β-galactoside-binding lectins sharing conserved carbohydrate recognition domains (CRD) and they have been found in a

Abbreviations: *P. pastoris*, *Pichia pastoris*; *L. vannamei* (*Lv*), *Litopenaeus vannamei*; *V. alginolyticus*, *Vibrio alginolyticus*; *B. subtilis*, *Bacillus subtilis*; *S. aureus*, *Staphylococcus aureus*; *A. veronii*, *Aeromonas veronii*; BSA, bovine serum albumin; YPD, yeast extract peptone dextrose medium; BMGY, buffered minimal glycerol-complex medium; BMMY, buffered minimal methanol-complex medium; YPDS, yeast extract peptone dextrose sorbitol medium; TBST, Tris-buffered saline with 0.05% Tween 20; PVDF, polyvinylidene difluoride; CFU, colony-forming units; PBS, phosphate buffered saline; EDTA, ethylene diamine tetraacetic; PMSF, phenylmethanesulfonyl fluorid; TCBS, thiosulfate–citrate–bile salts–sucrose; MIC, minimum inhibitory concentration.

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wide variety of eukaryotic organisms ranging from fungi to mammals (Houzelstein et al., 2004; Liu et al., 2013; Rajan et al., 2013; Song et al., 2011). In mammalians, fifteen kinds of galectins have been identified so far, which based on the primary structure and polypeptide architecture of the subunits, galectins are classified as "proto", "chimera", and "tandem-repeat" types (Cedeno-Laurent and Dimitroff, 2012; Song et al., 2011). Up to 15 distinct galectins have been found in vertebrates and demonstrated to play multiple functions in development, embryogenesis and regulation of innate and adaptive immunity (Brandt et al., 2010; Hemmrich et al., 2007; Hou et al., 2015; Hsieh et al., 2008). For example, the stimulation of the mitochondrial apoptotic pathway by galectin-1 initiated ceramide release and the requirement of prophenoloxidase system for ceramide-induced apoptosis prompted us to investigate the role of the p53 pathway for cell death (Bremer et al., 2006; Hahn et al., 2004). Previously research demonstrated that galectin-1 can induce cell death of T cells and thymocytes, while other groups have shown that galectin-1 can kill B cells, breast cancer cells and prostate cancer cells (Kurushima et al., 2005; Suzuki et al., 2012). Galectins introduces chemotaxis, activation, oxidative activity and degranulation of eosinophils (Bremer et al., 2006; Paz et al., 2001). Besides, recent findings show that galectins could bind glycans on the surface of pathogenic microbes, indicating their role as pattern recognition receptors (PRRs) (Shi et al., 2014). Galectin-1 expression on the surface of endothelial cells is increased by endothelial cell activation in vitro with lipopolysaccharide (LPS) or lipoteichoic acid (LTA) (He and Baum, 2006). While in invertebrates, especially mollusks, there are also several galectins with unique quadruple-CRD (Song et al., 2011). However, few studies about galectins were reported in invertebrates compared with relatively detailed studies in vertebrates. A galectin (*Aigalctin*) of unique CRD organization from the bay scallop *Argopecten irradians*, can recognize a great number of environmental bacterial species and strains (Song et al., 2011). Another two galectins with quadruple CRDs were characterized from pearl oyster *Pinctada fucata* (*Pf*galectin) and eastern oyster *Crassostrea virginica*, and their mRNA were remarkably up-regulated by pathogens challenge (Liu et al., 2013; Rhee et al., 2012). A similar galectin (*Mj*galectin) that is up-regulated by microbial challenge and can recognized microbial pathogens described in the kuruma shrimp *Marsupenaeus japonicas* (Shi et al., 2014). Besides, it could be binding to the shrimp hemocyte surface, *Mj*galectin functions as an opsonin for microbial pathogens, promoting their phagocytosis (Shi et al., 2014).

In this study, Shrimp are susceptible to diverse pathogens that cause considerable damage to the aquaculture industry (Cao et al., 2014). It is therefore important to understand the roles of proteins such as the galectins in their innate immune responses in order to support the development of new strategies for protecting shrimp against pathogens (Cha et al., 2015b). We reported the cloning and characterization of a full-length cDNA of a galectin gene from the Pacific white shrimp, *Litopenaeus vannamei*, which we designated *Lvgalectin* (*L. vannamei* galectin). The main objective of the present study was to investigate its tissue distribution and expression profiles in response to *V. alginolyticus* challenges, and examine the binding activity, clearance activity and anti-pathogen activity of recombinant *Lvgalectin* protein against various bacteria and fungus. Furthermore, we also present data on the expression of four genes known to influence innate immune responses (*Lv*p53, *Lvcrustin*, *LvproPO*, and *LvPEN3*) in wild-type and galectin-silenced *L. vannamei* individuals. These results indicated that *Lvgalectin* is important in the innate immune response of shrimp to *V. alginolyticus* infection. Finally, we present results showing that *Lv*galectin expression can be induced by immune challenge and may play an important role in the innate immune response of shrimp to *Vibrio alginolyticus* infection.

2. Materials and methods

2.1. Bacterial infection of shrimp and total RNA extraction

Healthy pacific white shrimp, *L. vannamei*, approximately 3–5 g in body weight and 4–5 cm in length, were collected from a shrimp farm in Panyu in the Guangdong Province of China. The shrimp were cultured in a recirculating water tank system containing air-pumped seawater (4‰ salinity) at 23–26 °C and fed commercial feed at 5% of body weight twice a day, as previously described (Wang et al., 2012). Three individuals were randomly selected for tissue collection. Hemolymph was extracted from the ventral sinus of the shrimp using a 2 ml syringe containing 0.2 ml of anticoagulant (NaCl, 450 mM KCl, 10 mM EDTA, 10 mM HEPEs, 10 mM; pH 7.4) and centrifuged for 10 min at 4 °C (800 g) for hemocyte collection. In addition, seven other tissues (heart, hepatopancreas, gills, stomach, eyestalks, muscles, and gastropod) were collected for RNA extraction.

L. vannamei individuals used for *V. alginolyticus* challenge were randomly divided into two groups. The shrimp of the challenged group were injected with 3×10^8 CFU/ml of *V. alginolyticus* in 20 μl of PBS, and those of the control group were injected with 20 μl of PBS. Hemocytes and hepatopancreases were collected at 0, 1, 3, 6, 12, 24, and 48 h post-injection from at least three individuals of the challenged and control groups for RNA extraction.

The collected total RNA was reverse transcribed into first-strand cDNA using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Japan) for gene cloning. The cDNA samples were prepared for qPCR analysis using a PrimeScript RT Reagent Kit (TaKaRa, Japan). The cDNA template was prepared by RACE using a SMART RACE cDNA Amplification Kit (Clontech, USA) for the rapid amplification of cDNA ends.

2.2. Cloning of Lvgalectin from L. vannamei

A 143 bp cDNA fragment of *Lvgalectin* was obtained by PCR using the cDNA templates and degenerate primers *Lvgalectin*-F1 and *Lvgalectin*-R1 (Table 1). The full-length cDNA of *Lvgalectin* was obtained by RACE based on the cDNA fragment. The primers *Lvgalectin* 5′RACE1 and *Lvgalectin* 3′RACE1 (Table 1) were used for the first round of 5′ and 3′-end RACE-PCR with a thermal cycler under the following conditions: denaturation at 94 °C for 3 min; 7 cycles of 94 °C for 30 s, 60 °C for 30 s (decreased by 1 °C per cycle), and 72 °C for 1 min; 32 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The conditions for the second round of 5′- and 3′-end PCR using *Lvgalectin* 5′ RACE2 and *Lvgalectin* 3′ RACE2 (Table 1) were the same as those for the first round. The PCR products were cloned into the pMD19-T vector (TaKaRa, China) and then sequenced.

2.3. Sequence and bioinformatics analysis

Sequences were analyzed using the BLAST program on the servers of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to perform multiple sequence alignments. The deduced amino acid sequence of *Lvgalectin* was analyzed using the simple modular architecture research tool (http://smart.embl-heidelberg.de). A neighbor-joining phylogenic tree was constructed by MEGA 5.0 (http://www.megasoftware.net) based on the deduced amino acid sequences of galectin proteins from various species. Bootstrap sampling was reiterated 1000 times.

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