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A fibrinogen-related protein identified from hepatopancreas of crayfish is a potential pattern recognition receptor

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ABSTRACT

Fibrinogen-related protein (FREP) family is a large group of proteins containing fibrinogen-like (FBG) domain and plays multiple physiological roles in animals. However, their immune functions in crayfish are not fully explored. In the present study, a novel fibrinogen-like protein (designated as PcFBN1) was identified and characterized from hepatopancreas of red swamp crayfish *Procambarus clarkii*. The cDNA sequence of *PcFBN1* contains an open reading frame (ORF) of 1353 bp encoding a protein of 450 amino acids. Sequence and structural analysis indicated that PcFBN1 contains an FBG domain in C-terminal and a putative signal peptide of 19 amino acids in *N*-terminal. Semi-quantitative PCR revealed that the main expression of *PcFBN1* was observed in hepatopancreas and hemocyte. Temporal expression analysis exhibited that *PcFBN1* expression could be significantly induced by heat-killed *Aeromonas hydrophila*. Tissue distribution and temporal change of *PcFBN1* suggested that *PcFBN1* may be involved in immune responses of red swamp crayfish. Recombinant PcFBN1 protein binds and agglutinates both gram-negative bacteria *Escherichia coli* and gram-positive bacteria *Micrococcus lysodeikticus*. Moreover, binding and agglutination is Ca^{2+} dependent. Further analysis indicated that PcFBN1 recognizes some acetyl group-containing substance LPS and PGN. RNAi experiment revealed that PcFBN1 is required for bacterial clearance and survival from *A. hydrophila* infection. Reduction of PcFBN1 expression significantly decreased the survival and enhanced the number of *A. hydrophila* in the hemolymph. These results indicated that PcFBN1 plays an important role in the innate immunity of red swamp crayfish as a potential pattern recognition receptor.

1. Introduction

Under natural conditions, pathogens invade crustacean via gill or digest system [1], and therefore gill or digest system are the first lines of defense against pathogen infection. In addition to their defense function as physical barriers, it needs to be investigated more detailedly whether gill or digest system also play a role in humoral immunity or cell immunity. At present, most researches on crustacean immunity focus on hemocyte and hemolymph. Much less was known of other organ or tissues about their immune function. Recent studies indicated that the transcripts of some immune-related genes were accumulated in hepatopancreas in response to pathogen infection [2–4]. Hepatopancreas is the main digestive gland of crayfish, and was reported to play a role in immune function of crustacean. However, more evidences are needed to fully understand the immune function of hepatopancreas. In the present study, we identified a new fibrinogen-related protein from hepatopancreas of red swamp crayfish which was responsive to *Aeromonas hydrophila* challenge. Investigation of its role in defending against bacteria will contribute to understanding the immune function of hepatopancreas.

Fibrinogen-related protein family (FREP) is an evolutionarily conserved protein family containing fibrinogen-like (FBG) domains in the C terminal [5]. They are found universally in mammals and invertebrates, and function as immune molecules or coagulation factor [6]. In mammals, one important member of FREP family is ficolin which is composed of an FBG domain in the C terminal and a collagenous region [7,8]. Ficolins recognize invading pathogens and trigger complement system via the lectin-dependent pathway. As a result, the invading pathogens are finally cleared from the animal via complement-induced cell lysis or other cellular immune responses [9]. A number of reports suggested that both plasma L- and

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H-ficolins also play a role in the clearance of dying host cells. The FBG domain of ficolin consists of 200–250 residues including 24 invariant, mostly hydrophobic amino acids and 40 highly conserved residues. In addition, four cysteines involving forming intra-chain disulfides are also highly conserved [9]. There are different ficolin types with different ligand binding specificity in the same species. Two types of ficolin are present in mice (ficolin-A and ficolin-B) and pig (ficolin-α and ficolin-β), but there are three types in human: L-ficolin, *M*-ficolin and H-ficolin [10]. All these ficolins were reported to recognize carbohydrate, interact with microorganisms or dead cell bodies of host and clear them.

In invertebrates, many FREPs were identified from different species. They commonly contain an FBG domain in the C terminal, but they differ from each other in N terminal. Some FREPs contain a collagen-like region in N terminus [11], but some others lack the collagen-like domain [12]. There are also some FREPs combining immunoglobulin domains in N terminus and FBG domains in C terminal [6,13–15]. Great diversity was also observed in the FBG domain between FREP proteins from the same or different species [5]. The structural diversity of FREP proteins may provide different binding specificity or diverse function. Two FREPs have been identified from *Mollusca* scallop and have exhibited obvious properties of pattern recognition receptors (PRR) [16,17]. A FREP protein of *Drosophila melanogaster* encodes a lateral inhibitor of R8 cell differentiation [18]. BbFREP identified from *Branchiostoma belcheri* was reported to bind to both gram-negative and gram-positive bacteria [19]. Similar FREP was also identified from *Armigeres subalbatus* as lectin which binds to bacteria *Micrococcus luteus* and *Escherichia coli* [20]. FBNs from *Anopheles* were found to mediate host defense against bacteria and malaria parasites invasion [5]. FREPs from snail *Biomphalaria glabrata*, recognize various pathogens ranging from prokaryotes to eukaryotes, and that different FREPs show specific function to the pathogen encountered [21]. TLs-5 from horseshoe crab agglutinates gram-positive, gram-negative bacteria and all types of human erythrocytes by specifically recognizing acetyl group. They can also enhance the antimicrobial activity of a horseshoe crab-derived big defensin [12]. In crayfish *Pacifastacus leniusculus*, two ficolin-like proteins (FLPs) were identified, and were found to be involved in clearing a gram-negative bacterium from circulation [11].

Here, a new FBG-containing protein was identified from hepatopancreas of *Procambarus clarkii* and designated as PcFBN1. Different from FLPs of *Pacifastacus leniusculus*, PcFBN1 lacks collagenous region, but possess intergrin binding motif RGD. The objective of this research is to address the role of PcFBN1 playing in immune defenses for the further understanding immune function of hepatopancreas in crayfish defense responses.

2. Materials and methods

2.1. Experimental animal and bacteria

Red swamp crayfish, *Procambarus clarkii*, purchased from Chengyang aquatic market of Qingdao, were reared in 50 L plastic boxes with aerated water at room temperature. Each box contained 30 crayfish and the boxes were cleaned in time to avoid polluting the water. Crayfish were fed with minced clam meat and temporarily reared for at least two weeks before being used to make all the crayfish in a similar physiological status.

2.2. cDNA cloning and sequence analysis

The encoding cDNA fragment of *PcFBN1* was amplified from cDNA of hepatopancreas using gene specific primers 5′-ATGTGCTGGTGTAGAGCGTG-3′ and primer 5′-CTAGTCCCCGGAACAGTCCC-3′ which were designed according to mRNA sequence obtained from TSA (Transcriptome shotgun Assembly) database by BLAST algorithm. Hepatopancreas RNA was isolated using RNA Isolation reagent (TaKaRa, Japan), and used as a template to synthesize first strand cDNA according to the procedure in a previous report [22]. The resultant PCR fragment was cloned into pMD19-T simple vector (TaKaRa, Japan) and sequenced.

Protein sequence similarity analysis was performed by BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment was created with ClustalX1.83 program and Multiple Align Show (http://www.bioinformatics.org/sms/multi_align.html). The structural feature of PcFBN1 protein was analyzed by the Expert Protein Analysis System (http://www.expasy.org/) and Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

2.3. Quantification of PcFBN1 gene expression

The expression pattern of *PcFBN1* gene in different tissues was determined by semi-quantitative RT-PCR. Total RNA was isolated from eyestalk, nerve, gill, hemocyte, hepatopancreas, heart, skeletal muscle, antennal gland and intestine. First strand cDNA synthesis was performed as described above, and 5 × diluted cDNA mix was used as template for PCR. Gene specific primers 5′-ATCTCCTTGTCCAGCTCTTA-3′ and 5′-CTCAATCACCTTGTTACTCTTC-3′ were used to amplify a 371 bp *PcFBN1* gene fragment for semi-quantitative RT-PCR.

The temporal expression of *PcFBN1* in hepatopancreas after challenged with gram-negative *A. hydrophila* (heat-killed) and gram-positive *Micrococcus lysodeikticus* (heat-killed) was evaluated by quantitative real-time PCR. Fifty microliter of heat-killed *A. hydrophila* (4.5×10^5 cfu mL^{-1}) and *M. lysodeikticus* (4.5×10^5 cfu mL^{-1}) in crayfish saline buffer (CFS, 0.2 M NaCl, 5.4 mM.

KCl, 10 mM CaCl2, 2.6 mM MgCl2, 2 mM NaHCO3, pH = 6.8) [23] was injected via the base of the fourth walking leg. The crayfish received an injection of the equal volume of CFS were employed as a control. Hepatopancreas were collected from 5 randomly sampled individuals at each time point 0, 3, 6, 9, 12, 15, 18, 21, 24 h after injection. The RNA extraction and cDNA synthesis were conducted as described above. *PcFBN1* gene-specific primers 5′-GCCAAGAAGAGTAACAAGGT-3′ and 5′-CGACACAGTAACATTCTCAC-3′ were used to amplify a 126 bp *PcFBN1* fragment for quantitative real-time PCR. Quantitative real-time PCR and data analysis were performed as previously described [24].

2.4. Recombinant expression and purification of PcFBN1 protein

cDNA fragment encoding mature peptide of *PcFBN1* was amplified with primer 5′-*CCATGG*GGACCCATGTCCGGCAGCAA-3′ and 5′-*CTCGAG*CTAGTCCCCGGAACAGTCCC-3′ from hepatopancreas cDNA and subcloned into pMD19-T simple vector. After amplification of the recombinant pMD19-T in *E. coli* Top10, the target fragment was ligated into pET-30a (+) and transformed into BL21 (DE3) for recombinant protein expression. The positive clone screened by PCR and sequencing was inoculated in 200 mL lysogeny broth (LB) containing 50 μg mL^{-1} kanamycin for shaking culture at 37 °C. When OD_{600} of the culture reached 0.8, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 0.1 mmol L^{-1} and incubated for 10 h at 30 °C to induce the expression of target protein. The recombinant PcFBN1 protein was purified by a Ni^{2+}-chelating Sepharose column. The concentration of purified PcFBN1 protein was quantified by BCA (Bicinchoninic Acid) method.