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Short communication

Identification of 10 transcripts of FREP in penaeid shrimp *Litopenaeus vannamei*

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ABSTRACT

Fibrinogen-related proteins (FREPs) are widely distributed in vertebrates and invertebrates and known having Fibrinogen-related domains (FReDs) which function in multiple aspects, especially in innate immune response as pattern recognition receptors. However, there is no any report about FREP in penaeid shrimp *Litopenaeus vannamei*. Here, totally 10 transcripts of FREP were isolated and named *Lv*FREP1.1, 1.2 until 1.10. All of the 10 transcripts have high identity in their 3′ ends and encode conserved FReDs. Since the 10 transcripts are highly similar we could not design any primers that can amplify a single transcript. We chose a pair of primers corresponding to part of *Lv*FREP1.1 and *Lv*FREP1.5 to examine their expression. Tissue distribution indicated *Lv*FREP1.1 and *Lv*FREP1.5 mRNA locates in hemocytes, gills, intestine, hearts and slightly in nerve. The expression of *Lv*FREP1.1 and *Lv*FREP1.5 behaves differently post bacteria and virus infection. Besides, recombinant *Lv*FReD could agglutinate bacteria *Vibrio harveyi* in the presence of Ca^{2+}. These initial data presents the diversity of FREPs in penaeid shrimp and also push us to further explore their roles in shrimp immune response.

1. Introduction

Shrimp are considered lacking adaptive immunity and relying on innate immunity. Although they do not have immunoglobulin against specific pathogens, they have been evolved diversified strategies against infection including humoral immunity and cellular immunity. Once they are infected, the pattern recognition receptors (PRRs) of shrimp can recognize pathogen-associated molecular patterns (PAMPs) on the surface of pathogens, which thus activate a series of immune reactions such as phagocytosis, encapsulation, nodule, prophenoloxidase system or the release of antimicrobial peptides. So far, PRRs which have been identified in shrimp include lectins, Down syndrome cell adhesion molecule, scavenger receptors, galectins, fibrinogen-related proteins and so on (see review [1,2]).

Fibrinogen-related proteins (FREPs) are named for containing a fibrinogen-related domain (FReD), which are widely found in vertebrates and invertebrates. FReD is around 200 amino acid residues in which 24 conserved residues exist. FReD locates in the C-terminal of FREPs while the N-terminal of FREPs is much divergent. For example, human ficolin has an N-terminal collagen domain as well as the C-terminal FReD [3] while molluscan FREPs have one or two immunoglobulin (Ig) domains in their N-terminus besides the C-terminal FReDs [4,5]. However, many FREPs in invertebrates do not have clearly conserved domain in the N-terminus such as shrimp *Marsupenaeus japonicas* FREP1 [6]. So far, many FREPs family members have been found in different species which might result from genome-wide duplication events [7]. For example, 428 FREPs have been found in *Branchiostoma floridae*, 22 FREPs in *Drosophila melanogaster* and 59 FREPs in *Anopheles gambiae* [7].

Functions of FREPs include immune recognition and defense, participating in development and allorecognition [7,8], while in vertebrates, some FREPs acquire the role in mediating coagulation, starting appear in urochordates and jawless fish [9,10], which is believed to be the result of genome duplication. Horseshoe crab Tachylectin, a kind of FReD containing protein has shown the ability to agglutinate bacteria and human erythrocytes [11]. There are two FREPs found in penaeid shrimp *Marsupenaeus japonicas* (*Mj*FREP) both of whose expression are up-regulated post bacteria challenge. The recombinant *Mj*FREP1 and *Mj*FREP2 could bind bacteria in a

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calcium dependent way. Besides, *Mj*FREP2 could shift to membrane or secret out of cell post bacteria challenge and it can also promote bacteria phagocytosis by hemocytes [6,12]. Two ficolin-like proteins which were identified in freshwater crayfish *Pacifastacus leniusculus* function as pattern recognition receptors in immune response [13]. A melanization inhibiting protein (MIP) which contains FReD was demonstrated functioning in regulating phenoloxidase (PO)-induced melanization in crayfish *Pacifastacus leniusculus* [14] and shrimp *penaeus monodon* [15].

The diversity of FREPs has been found in different species and extensively introduced in Gastropod *Biomphalaria glabrata*. So far, *Biomphalaria glabrata* genome contains 14 FREP subfamilies, among which FREPs 3, 12, and 13 are further alternatively spliced in different forms [16–18]. Besides, gene conversions and point mutations increase the diversity of certain FREP members like FREP3 in *Biomphalaria glabrata* [19]. However, only two FREPs were reported in penaeid shrimp *Marsupenaeus japonicas* and one FREP in *penaeus monodon*. Here, by using reverse transcription-PCR method, we identified 10 transcripts of FREPs in penaeid shrimp *Litopenaeus vannamei*, among which sequence losses occur in the middle and 5′ ends which might be caused by alternative splicing. Besides, we examined their immune responses post pathogens infection and analyzed its bacteria-agglutination activity.

2. Materials and methods

2.1. Shrimp culture and immune challenge

Pacific white shrimp *Litopenaeus vannamei* (5–8 g) were cultured in tanks filled with filtered seawater and fed three times per day with commercial diets. Immune challenge experiments were according to previous studies [20,21]. Briefly, Shrimps were divided into four groups and every shrimp in different groups were separately injected with 20 μl Gram-negative bacteria *Vibrio anguillarum* (*V. anguillarum*, 1.6×10^8 cfu/shrimp), Gram-positive bacteria *Micrococcus lysodeikticus* (*M. lysodeikticus*, 1.0×10^{10} cfu/shrimp) or white spot syndrome virus (WSSV, 80 copies/μl). PBS-injected group was treated as control. Shrimp without any injection were sampled as "0" point. Post injection, shrimp were sampled at 6, 12, 24 and 48 h and different tissues were isolated and preserved for RNA extraction.

2.2. Complementary DNA cloning of FREP in shrimp Litopenaeus vannamei

Through blasting EST database of Pacific white shrimp (taxid: 6689) in NCBI using *Marsupenaeus japonicas* FREP1 cDNA (GenBank accession no. JN100568), we got partial sequence which is highly similar with the 3′ end of *Mj*FREP1. A further 5′ end Rapid Amplification of cDNA ends (RACE) method with the universal primer (5′CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT3′) and FREP1-R primer (5′ TCACCTCACAGCATGCAGAA3′) was used to get other partial sequence according to RACE protocol of Clontech.

2.3. Sequence analysis

Open reading frame was predicted by ORF finder of NCBI and signal peptide was predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Conserved domains were analyzed by SMART Server (http://smart.emblheidelberg.de/). DNA and protein sequences of the different forms were aligned by Megalign software. Phylogenetic analysis of FREPs from different species were analyzed by MEGA 5 software.

2.4. Tissue localization of LvFREP1 and its expression post pathogen infection

Shrimp tissues were extracted including hemocytes, eyestalk, nerve, gill, muscle, hepatopancreas, heart, stomach and intestine. Method for hemocytes isolation was described in previous study [22]. For immune challenge experiment, shrimp hemocytes were sampled from the 4 groups at indicated time and prepared for RNA extraction. Total RNA was extracted from the above tissues and cDNA was prepared following the related manuals. According to the 3′ end conserved region of *Lv*FREP1, we designed primers which, after PCR, always produce several bands, so we re-designed a pair of primers (Forward 5′CACAAGCCCAACGAGAAGC3′, Reverse 5′GCGGGAAAAAGTGGAATCC3′) according to the 5′ end conserved region of *Lv*FREP1.1 and *Lv*FREP1.5, which makes only one single band. For tissue distribution of *Lv*FREP1 and *Lv*FREP1.5, PCR products were loaded into 1% agarose gel and after separated by electrophoresis, they were examined under UV and photoed for analysis. For examining *Lv*FREP1.1 and *Lv*FREP1.5 expression post immune challenge, quantitative real-time PCR was applied by the Bio-Rad CFX 96 Real-Time PCR Detection System. 2 × UltraSYBR Green Mix (CWBIO, China) was used in 20 μl PCR reaction systems. *Litopenaeus vannamei* β-actin (GenBank No. AF300705) was used as internal control. The comparative Ct method was used and comparison analysis between pathogen infection groups and PBS group was examined by Excel *t*-test.

2.5. Protein expression, purification and bacteria agglutination assay

Partial cDNA sequence of *Lv*FREP1 which encodes partial FReD (*Lv*FReD) was constructed in PET-32a plasmid by double-restriction enzyme cleavage and ligation steps. The recombinant plasmid was transformed into *E.coli* BL21 (DE3) to express the encoding FReD fusioned with a Thioredoxin (Trx) tag. Protein expression and purification were same with our previous study [21]. To test whether the expressed FReD could agglutinate bacteria, *in vitro* agglutination assay was conducted using bacteria *Vibrio harveyi* (*V.harveyi*) with or without 10 mM $CaCl_2$. Detailed steps were same with previous study [21].

3. Results and discussion

3.1. Isolation of 10 transcripts of FREP cDNA in shrimp Litopenaeus vannamei

By RT-PCR method combining with 5′ end RACE, we obtained multiple bands between nearly 500 bp and 2000 bp (Fig. 1A) and after sequencing, totally 10 transcripts were isolated, all encoding fibrinogen-related domains (FReDs). Since they are highly similar with FREP1 in shrimp *Marsupenaeus japonicas* (*Mj*FREP1), we named them *Litopenaeus vannamei* FREP1 (*Lv*FREP1) with *Lv*FREP1.1, 1.2, 1.3 until 1.10 representing the 10 truncated forms, respectively (Genbank accession numbers are KR338960, KR338961, KR338962, KX132076, KX132077, KX132078, KX132079, KX132080, KX132081, KX132082). Among them, *Lv*FREP1.1 is the longest transcript with 1719 nucleotides. Multiple alignment of the 10 transcripts showed all of their 3′ ends (around 330 nucleotides) are nearly the same except 7 nucleotides substitutions in *Lv*FREP1.2, 1.4, 1.7. These nucleotides substitution might be a kind of somatic diversification which has been found in *B. glabrata* FREP family [7].While, sequence losses which might be caused by alternative splicing exist in their 5′ ends except *Lv*FREP1.4 and 1.5 compared with *Lv*FREP1.1. Both *Lv*FREP1.4 and 1.5 are consistent with *Lv*FREP1.1 in their 5′ ends but they have sequence losses in the