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The role of ficolin-like protein (*Pc*FLP1) in the antibacterial immunity of red swamp crayfish (*Procambarus clarkii*)

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ABSTRACT

In invertebrates, ficolin-like proteins (FLPs) play important roles in innate immunity against pathogens. Previous studies primarily investigated the functions of FLPs in immune recognition, activation, and regulation. However, limited research has examined the functions of FLPs as immune effectors. In this work, a ficolin-like protein was identified in red swam crayfish (*Procambarus clarkii*) and designated as *Pc*FLP1. Quantitative RT-PCR and western blot were employed to analyze the distribution and expression profiles of *Pc*FLP1 in the tissues of the crayfish. The results indicated that *Pc*FLP1 was present in all tested tissues, including hemocytes, heart, hepatopancreas, gill, stomach, and mid-intestine. The expression level of *Pc*FLP1 was up-regulated in hemocytes, hepatopancreas and mid-intestines of the crayfish challenged with *Vibrio parahaemolyticus*. Further study demonstrated that *Pc*FLP1 could protect the hepatopancreatic cells of crayfish from *V. parahaemolyticus* infection. The recombinant *Pc*FLP1 enhanced bacterial elimination in crayfish, whereas the antibacterial action was inhibited after *Pc*FLP1 was knocked down. Furthermore, *Pc*FLP1 could bound to bacteria and inhibited bacterial replication. These results demonstrated that *Pc*FLP1 plays an important role in the anti-*Vibrio* immunity of red swamp crayfish.

1. Introduction

Red swamp crayfish (*Procambarus clarkii*) belongs to crustacean that mainly relies on innate immunity to prevent pathogen invasion (Bachère et al., 2004). The evolutionarily conserved pattern recognition receptors (PRRs) in many organisms can recognize pathogens (Medzhitov and Janeway, 2000). Increasing evidence indicates that fibrinogen-related proteins (FREPs), which act as PRRs, are associated with pathogen recognition and complement activation.The most studied FREPs are ficolins. In humans, ficolins primarily consist of M-fiarily (ficolin-1), L-ficolin (ficolin-2), and H-ficolin (ficolin-3) (Matsushita, 2013). A common function of ficolins in human, mouse, pig, xenopus and ascidian is their ability to bind specifically to N-acetyl-D-glucosamine (GlcNac) (Fujita et al., 2004). Usually, mammalian FREPs activate the complement system as PRRs (Matsushita and Fujita, 2002). In invertebrate, FREPs were first described in snail (*Biomphalaria glabrata*) (Adema et al., 1997). While, in crustaceans, FREP-like proteins were first described in *Pacifastacus leniusculus* (Söderhäll et al., 2009). Functions of FREPs in invertebrates have been shown to be involved in defense processes such as pathogen recognition and bacterial defense. *Mj*FREPs in *Marsupenaeus japonicus* were observed to cause agglutination of bacteria and Ca^{2+} while promoting phagocytosis of bacteria in hemocytes (Chai et al., 2012; Sun et al., 2014). In *P. leniusculus*, FREP regulating phenoloxidase (PO)-induced melanization has been identified (Söderhäll et al., 2009). Previous studies primarily focused on the functions of FREP in immune recognition, activation and regulation. Limited studies focused on the functions of FREP as an immune effector.

In the present study, a ficolin-like protein from red swamp crayfish was identified and named as *Pc*FLP1. Assays on tissues distribution showed that *Pc*FLP1 was expressed in all tested tissues. The expression level of *Pc*FLP1 was induced in the crayfish challenged with *Vibrio parahaemolyticus*. Next, bacterial clearance

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efficiency was analyzed after either injection of *Pc*FLP1 protein or silencing of *Pc*FLP1 in crayfish. Overall, this study investigated the functions of crayfish *Pc*FLP1 in innate immunity against *Vibrio* infection.

2. Materials and methods

2.1. Sequence analysis of Pc*FLP1*

The expressed sequence tag of *Pc*FLP1 was obtained through transcriptome sequencing of the hepatopancreas of crayfish. The result of BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the similarity of DNA sequences of *Pc*FLP1 was 44% with those of ficolin-like protein 2 (*P. leniusculus*). DNAMAN software was used for the protein prediction. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to identify the signal peptide of the protein. Based on the deduced amino acid sequences of *Pc*FLP1 and similar sequences of other species from NCBI, a phylogenetic tree was constructed with the MEGA 6 software using a neighbor-joining (NJ) algorithm (Kumar et al., 2008). Bootstrap trials were replicated 1000 times for the phylogenetic analysis. MEGA 6 and GENDOC were used to create the multiple sequence alignments.

2.2. Vibrio challenge and tissue collection

V. parahaemolyticus (VP100, wild type) was isolated in our lab. The crayfish (approximately 9 g) was obtained from a farm in Hanchuan City, Hubei Province, China. The crayfish were raised in an aerated water tank for 2 weeks before carrying out experiments. The total RNAs from six tissues (hemocyte, heart, hepatopancreas, gill, stomach and mid-intestine) were extracted and the protein samples including cell free hemolymph were prepared from healthy crayfish. The specified tissues were all pooled from three healthy crayfish. The hemolymph was extracted from abdomen and the anticoagulant buffer [0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM ethylene diamine tetra acetic acid (EDTA), pH 4.6] (Söderhäll and Smith., 1983) was used. Then, the abdominal segment of each crayfish was injected with 25 μL of *V. parahaemolyticus* (10^7 CFU/mL). Hemocytes, hepatopancreas and mid-intestines were collected at 0, 6, 12, 24 and 48 h post-injection (hpi) for RNA and protein extractions as described before. RNAiso Plus (Takara, Dalian, China) was used in accordance with manufacturer's protocol. First-strand cDNAs were reverse-transcribed using the kit with gDNA Eraser (Aidlab, Beijing, China) before they were stored at −20 °C. RIPA buffer (Beyotime, Beijing, China) was used to prepare total proteins following manufacturer's protocol. Protein samples were then boiled with SDS loading buffer (KeyGEN, Nanjing, China) for 5 min and stored at −20 °C until use.

2.3. Expression and purification of recombinant Pc*FLP1*

In the experiment on prokaryotic recombinant expression, the primers (*Pc*FLP1-EX-F/R, Table 1) were used to amplify the fragment of *Pc*FLP1 (1071 bp), and its predicted molecular mass was 40.3 kDa. The fragment was linked to the vector pET32a. The recombinant *Pc*FLP1 was expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) cells. His-*Pc*FLP1 was found mainly in the supernatant and was purified according to the described method (Wang et al., 2009). The polyclonal antibody against *Pc*FLP1 was generated in rabbit using purified his-*Pc*FLP1 as antigen.

Table 1
Oligonucleotide primers used in the study..

Primer name	Sequence (5'-3')
RT-PCR assay	
*Pc*FLP1-RT-F	CGACAAGACCGTCAAGGC
*Pc*FLP1-RT-R	ATCTGTGTTCGTTTTTTATTTT
18s-RT-F	TCTTCTTAGAGGGATTAGCGG
18s-RT-R	AAGGGGATTGAACGGGTTA
Recombinant expression	
*Pc*FLP1-EX-F	TACTCAGGATCCATGCAAGAGAAGCAGCGC
*Pc*FLP1-EX-R	TACTCACTCGAGTCAGACGTTGTGGTCGTG
RNAi assay	
GFP–RNAi-F	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFP–RNAi- R	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC
*Pc*FLP1-RNAi-F	GCGTAATACGACTCACTATAGGGCCGAGGAGAGTAAAGG
*Pc*FLP1-*RNAi-R*	GCGTAATACGACTCACTATAGGTCCGATCTCTGATGTTGAA

2.4. Tissue distribution and expression profile analysis of Pc*FLP1*

Quantitative real-time PCR (qRT-PCR) and western blot were used to examine the tissue distribution and the expression profiles of *Pc*FLP1. For qRT-PCR, a pair of primers (*PcFLP1*-RT-F/R, Table 1) was used to detect the transcriptional levels of *Pc*FLP1 with 18S rRNA (with primers 18S-RT-F/R, Table 1) as an internal control. The qRT-PCR procedure was initially set at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The specificity of the amplified products was confirmed through DNA melting analysis. SPSS software was used to analyze the obtained data. The experiment was repeated three times, and the results were analyzed using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). SDS-PAGE was used to separate protein samples and the proteins were then transferred onto nitrocellulose (NC) membranes followed by blocking in nonfat milk (5% in TBS: 8.8 g NaCl, 20 mL 1 M Tri-HCl, add ddH_2O to 1 L, PH 7.4)for 2 h. The NC membrane was incubated with the first antibody (rabbit anti-*Pc*FLP1 or β-actin antiserum) for 2 h, washed three times with TBST (0.02% Tween 20 in TBS), and incubated with the secondary antibodies, 800CW donkey anti-rabbit IgG (H+L) (Gene Company). After washing with TBST three times, the machine IRDye (LI-COR, Inc, USA) was used to obtain the image.

2.5. Pathological changes of hepatopancreas of crayfish challenged with V. parahaemolyticus

The *V. parahaemolyticus* was diluted to 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 CFU/mL with PBS (Google, Wuhan). Different concentrations of *V. parahaemolyticus* (25 μL) were injected into each crayfish (3 crayfish in one group). Hepatopancreas were collected from each crayfish at 24 hpi, and fixed with 4% paraformaldehyde solution. All samples were then sent to the company (Google, Wuhan) for pathological valuation, including hepatopancreas sectioning, hematoxylin and eosin (H&E) staining, and examination under microscope. Six healthy crayfish were injected with 50 μg of his-*Pc*FLP1 before they were randomly divided into 2 groups. One hour after his-*Pc*FLP1 injection, each crayfish from one group was injected with 25 μL *V. parahaemolyticus* (10^5 CFU/mL), while in the other group, each crayfish was injected with 25 μL *V. parahaemolyticus* (10^6 CFU/mL). His-tag and his-Trim were used as controls.

2.6. Immunofluorescence assay of Pc*FLP1*

The hemolymph was collected from three healthy crayfish. The hemocytes were isolated by centrifugation (700*g*, 10 min, at 4 °C), washed with PBS, incubated in 0.2% Triton X-100 at 37 °C (5 min), and washed with PBS 3 times. After blocking with 2% nonfat milk (1 h, 37 °C), hemocytes were incubated 2 h with anti-*Pc*FLP1