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Identification, characterization and functional analysis of a serine protease inhibitor (*Lvserpin*) from the Pacific white shrimp, *Litopenaeus vannamei*

Yongjie Liu [a], Fujun Hou [a], Shulin He [a], Zhaoying Qian [a], Xianzong Wang [a], Aitao Mao [b], Chengbo Sun [b], Xiaolin Liu [a,*]

[a] College of Animal Science and Technology, Northwest A&F University, Shaanxi Yangling 712100, China
[b] Fisheries College, Guangdong Ocean University, Guangdong Zhanjiang 524025, China

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ABSTRACT

As important arthropod immune responses, prophenoloxidase (proPO) activation and Toll pathway initiation are mediated by serine proteinase cascades and regulated by serpins. Herein, a serine protease inhibitor (*Lvserpin*), encoding for 415 amino acids with calculated molecular weight of 46,639 Da and isoelectric point of 7.03 was characterized from the Pacific white shrimp *Litopenaeus vannamei*. Multiple sequence alignment revealed that *Lv*serpin shared the highest similarity with *Penaeus monodon* serpin6 (87%). Quantitative real-time PCR (qRT-PCR) results showed that the transcripts of *Lvserpin* were detected in all the examined tissues and most highly expressed in gill. The expression profiles of *Lvserpin* were greatly fluctuated upon infection of *Vibrio anguillarum*, *Micrococcus lysoleikticus* or White Spot Syndrome Virus (WSSV). Double stranded RNA-mediated suppression of *Lvserpin* resulted in a significant increase in the transcripts of two clip-domain serine proteinases (*PPAE* and *PPAF*), prophenoloxidase (*proPO*), antilipopolysaccharide factor (*ALF*), *Crustin* and penaeidin3 (*Pens3*) and also increased the high cumulative mortality post *V. anguillarum* injection. Besides, the recombinant *Lv*serpin protein (r*Lv*serpin) was purified and exhibited inhibitory activity against trypsin. Also the r*Lv*serpin showed inhibition on prophenoloxidase activation and bacterial growth. Hence, we proposed that the *Lvserpin* played important role in the shrimp innate immunity.

1. Introduction

In recent years, with the enlargement of the cultivation scale, shrimp diseases have become increasingly prominent, and it has become a major bottleneck restricting of the development of aquaculture (Akira et al., 2006; Lightner, 2005). Lacking of adaptive immunity compel shrimp to defense against the invasion of pathogens depending on the innate immunity, which function through the cellular and humoral responses (Lee and Söderhäll, 2002; Li and Xiang, 2013a; Tassanakajon et al., 2013). Actually, hemolymph coagulation, complement activation, melanization, phagocytosis, encapsulation and synthesis of antimicrobial peptides involved in the two immune responses are mediated by serine proteinase cascades (Cerenius and Söderhäll, 2004; Jiravanichpaisal et al., 2006; Söderhäll et al., 2003). And yet, these activated proteinase cascades needed regulation by serine proteinase inhibitors (SPIs) to prevent the damage to the host (Kanost, 1999). At present, there are several families of SPIs in biological system, for instance, Kazal, Kunitz, Bowman-Birk, serpin, α-macroglobulin, etc. (Kanost, 1999; Rimphanitchayakit and Tassanakajon, 2010).

Serpins acting as suicide-like substrates participate in the regulation process and irreversibly inhibit their specific target proteinases (Silverman et al., 2001). So far, they have been found in all groups of organisms only with the exception of fungi. Serpin encodes a protein of 400 amino acid residues in length with high molecular weight of 40–60 kDa. All of these proteins have a common structure folded by eight to nine alpha helices and three sets of beta sheets. The reactive center loop (RCL), core feature of serpins, is an exposed protein motif composed of about 20 amino acids located near C-terminus. This motif contains a scissile bond between two residues, called P1 and P1′, which is cleaved by the target proteinase (Wilczynska et al., 1995). The cleavage in the reactive site induces a large conformational change. Then a stable serpin-proteinase complex was formed and resulted in the inactivation of the protease activity. The P1 residue determines the primary selectivity of a serpin, as well as determines the target specificity (Huntington, 2011; Mangan et al., 2008).

In insects, the serpins have been characterized biochemically and shown regulation on proPO activation or synthesis of

* Corresponding author. Tel.: +86 029 87054333; fax: +86 029 87092164.
E-mail addresses: liuxiaolin@nwsuaf.edu.cn, xiaolinliu2000@sina.com (X. Liu).

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antimicrobial peptides (Kanost, 1999). Serpins in *Aedes aegypti* (Zou et al., 2010), *Tenebrio molitor* (Jiang et al., 2009; Park et al., 2011), *Manduca sexta* (Christen et al., 2012; Suwanchaichinda et al., 2013) and *Drosophila melanogaster* (Tang et al., 2008) have been found to be associated with innate immune system through the activation of prophenoloxidase (proPO). Regulation of the Toll pathway by serpins has also been observed in *A. aegypti* (Shin et al., 2006), *T. molitor* (Jiang et al., 2009; Park et al., 2011), *M. sexta* (An et al., 2010, 2011) and *D. melanogaster* (Ahmad et al., 2009). In *D. melanogaster*, serpin-28D controls the activation of prophenoloxidase (Scherfer et al., 2008). Serpin-27A functions as a negative inhibitor of proPO activation by inhibiting the proPO activating enzyme (PPAE) (Nappi et al., 2005). In *M. sexta*, serpin-3 blocks proPO activation by inhibiting PAP-1 and PAP-3 in the hemolymph (Christen et al., 2012). Serpin-5 appears to negatively regulate expression of antimicrobial peptide genes by inhibiting the proteinase HP6 (Tong and Kanost, 2005). Furthermore, three novel serpins (SPN40, SPN55 and SPN48) from the hemolymph of *T. molitor* block the Toll signaling cascade by making specific serpin-serine protease pairs (Jiang et al., 2009).

Within crustaceans a diverse range of serpins have been identified, including *Pacifastacus leniusculus* (Liang and Söderhäll, 1995), *Penaeus monodon* (Homvises et al., 2010), *Fenneropenaeus chinesis* (Liu et al., 2009), *Marsupenaeus japonicas*, *Eriocheir sinensis* (Wang et al., 2013), *Scylla paramamosain* (Chen et al., 2010) and *Portunus trituberculatus* (Wang et al., 2012b). In *S. paramamosain*, *Spserpin* was found to be up-regulated in response to lipopolysaccharide challenge (Chen et al., 2010). *EsSerpin* identified from hemocyte of *E. sinensis* were reported have the ability on inhibition of bacterial growth and regulation of prophenoloxidase-activating system (Wang et al., 2013). The first Penaeidea shrimp *serpin* was reported in *P. monodon*, called *PmserpinB3*, and its transcript was found to be up-regulated upon bacterial infection (Somboonwiwat et al., 2006). To date, 8 different *serpin* genes had been identified from *P. monodon*. Among them, *Pmserpin3* and *Pmserpin8* showed inhibition on prophenoloxidase system (Somnuk et al., 2012; Wetsaphan et al., 2013). Moreover, *Fcserpin*, identified from *F. chinensis*, exhibited the function to defense against invading pathogens (Liu et al., 2009).

In view of the important functions of serpins, a novel *serpin*, namely *Lvserpin*, was identified from *Litopenaeus vannamei*. The tissue expression pattern and temporal response after injection with different pathogens were investigated. The function of *Lvserpin* in regulating the transcription of proPO-AS related genes and Toll pathway dependent AMPs were investigated by dsRNA-mediated RNA interference. Besides, *Lvserpin* was expressed in *Escherichia coli*, and the purified r*Lv*serpin was used for the investigation on inhibition of proteinase activity, bacterial growth and prophenoloxidase activation.

2. Materials and methods

2.1. Shrimp and immune challenge

The Pacific white shrimp, *L. vannamei* (average body mass of 10–15 g) were obtained from Hengxing Company in Guangdong province, China, and acclimatized in tanks for a week before processing. The salinity (30‰) and temperature (24–28 °C) were maintained at normal levels, and the shrimps were fed with commercial diet at 5% of body weight every day.

For tissue distribution analysis, samples of hemocytes, gill, hepatopancreas, eyestalk, stomach, muscle, intestine, nerve, heart and testis were collected. For the challenge experiment, three pathogens were selected: *Vibrio anguillarum*, *Micrococcus lysoleikticus* and White Spot Syndrome Virus (WSSV). The bacterial challenge group was injected with 20 μl of live *V. anguillarum* (4×10^8 CFU/ml) or *M. lysoleikticu* (4×10^{10} CFU/ml) suspended in Phosphate Buffered Saline (PBS), respectively. The WSSV challenge group was performed by injection into the last abdominal segment of 20 μl tissue suspension prepared from WSSV-infected shrimp. Both the control groups were injected with equal volume sterilized PBS. Then, hemolymph from three shrimps of bacterial challenge group and control group was collected at 0, 2, 6, 12, 24, and 48 h post injection (hpi). For WSSV challenge group, the hemolymph from three shrimps was collected at 0, 2, 6, 12, 24, 48 and 72 hpi, The shrimp hemolymph was collected into a sterilized syringe with an equal volume of anticoagulant modified Alsever solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0), and centrifuged (800*g* at 4 °C for 10 min) immediately to collect hemocytes (Rodriguez et al., 1995). All the samples were preserved in liquid nitrogen immediately for RNA extraction.

2.2. Production of dsRNA

Preparation of dsRNA was done in vitro using T7 RiboMAX Express (Promega, USA) following the manufacturer's instructions. Briefly, T7 promoter was incorporated to gene specific primers at the 5′ terminus (Table 1) to produce sense and anti-sense strand separately. PCR products were purified, quantified and transcribed to yield single stranded RNAs. Equal amount of single stranded RNAs were mixed together and annealed to produce double stranded RNA which are further purified and quantified for RNA interference experiment. Then, ten microgram (1 μg/g shrimp) of dsRNA for *Lvserpin* or *LvEGFP* gene were injected to shrimp and hemocytes was collected from 0, 24, 36 and 48 hpi. Moreover, PBS was also injected to serve as control.

2.3. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from selected tissues with Trizol Reagent (Invitrogen, USA) as described in the manufacturer's protocol. The quality of RNA was verified on 1% agarose gels. Also, RNA concentration was determined by measuring the absorbance at 260 nm with a UV-spectrometer (Bio-Rad, USA). Then, 500 ng of the obtained RNA was used to synthesize cDNA using the PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) in accordance with the manufacturer's instructions.

2.4. Identification of Lvserpin cDNA sequence

According to the full-length cDNA sequence of *Pmserpin6* (GeneBank accession number: GQ260129.1), sequence specific primers (Ser-F; Ser-R) were designed to amplify the Open Reading Frame (ORF) (Table 1). The amplification reaction was run as follows: 95 °C for 5 min; 38 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. The purified PCR products were cloned into the pUC-18T vector (CWBIO, China), then, transformed into the competent cells of *E. coli* DH5α. The potentially positive recombinant clones were identified by colony PCR. Then, the positive recombinants were picked for sequencing.

2.5. Bioinformatics analysis

The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) was used to analyze the nucleotide sequences. Multiple sequence alignments were created using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The protein domains were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.emblheidelberg.de/). Signal peptide and nuclear localization