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*Lv*serpin3 is involved in shrimp innate immunity via the inhibition of bacterial proteases and proteases involved in prophenoloxidase system

Yongjie Liu, Tao Liu, Fujun Hou, Xianzong Wang, Xiaolin Liu*

College of Animal Science and Technology, Northwest A&F University, Shaanxi, Yangling, 712100, China

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ABSTRACT

Serine protease inhibitor, represented by serpin, plays an important inhibitory role on proteases involved in the immune responses. To clarify the immune characterizations of serpin, a novel serpin (*Lv*serpin3) encoding for 410 amino acids with a 23-amino acid signal peptide and a serpin domain was identified from the Pacific white shrimp *Litopenaeus vannamei*. *Lvserpin3* expressed strongest in hepatopancreas, and was significantly up-regulated in the early stage upon *Vibrio anguillarum*, *Micrococcus lysodeikticus* or White Spot Syndrome Virus (WSSV) infection. Suppression of *Lvserpin3* by dsRNA led to a significant increase in the transcripts of *LvPPAF*, *LvproPO* and phenoloxidase (PO) activity, and also led to the high cumulative mortality. The recombinant *Lv*serpin3 protein (r*Lv*serpin3) inhibited the proteases secreted by *M. lysodeikticus* and *Bacillus subtilis*, and further exhibited inhibitory role on the growth of *B. subtilis* and *M. lysodeikticu*. Moreover, r*Lv*serpin3 was found to be able to block the activation of prophenoloxidase system. Taken together, the results imply that *Lv*serpin3 may be involved in shrimp innate immunity via the inhibition of bacterial proteases and proteases involved in prophenoloxidase system.

1. Introduction

Serine proteases play a significant role in mammals' physiological processes, especially in digestion, coagulation and the complement system [1–3]. Most of the processes are specific and irreversible, and mediated by proteolytic cascades [4]. To avoid the damage of the host caused by the redundant proteases, organisms synthesize varieties of protease inhibitors to prevent needless proteolysis [5]. As one of the members of serine protease inhibitors (SPIs), serpins contribute a great deal to the balance of proteases [6,7].

Serpins are a super family of proteins with molecular weight of 40–50 kDa. The reactive center loop (RCL), which contains a scissile bond between amino acid residues P1 and P1′, is located near the C-terminus [8,9]. When the bond is cleaved by a target protease, the serpin undergoes a conformational change and traps the protease in an inactive state, and finally results in irreversible inhibition of the protease [10]. The P1 residue plays a key role in determining the target specificity [11].

Recently, a large amount of studies on serpins have been reported, and the vast majority of them focus on the inhibitory role of the prophenoloxidase (proPO) system. In insects, *Drosophila melanogaster* serpin-28D effectively inhibits the activation of proPO [12]. *Ms*serpin-1, -3, -4, -5, -6, -7 from *Manduca sexta* block proPO activation by inhibiting proPO activating protease (PAP) or hemolymph protease (HP) [13–18]. Studies of serpins in crustacean increased recently. For instance, *Es*serpin in Chinese mitten crab *Eriocheir sinensis* was reported to be able to suppress the growth of *Escherichia coli* and inhibit the activation of proPO [19]. *Pt*Serpin from the swimming crab *Portunus trituberculatus* was also found to be involved in the inhibition of bacterial growth and proPO system activation [20]. *Pm*SERPIN8 and *Pm*SERPIN3 from the tiger shrimp *Penaeus monodon* showed inhibition role on the shrimp proPO system [21,22]. Of particular note was that *Mj*Serp1 from the kuruma shrimp *Marsupenaeus japonicas* functioned as a direct effector in the bacterial clearance [23]. However, detailed study about serpins for the inhibitory roles in the protease cascades in *Litopenaeus vannamei* is insufficient.

In this study, we characterized a novel serpin (namely *Lv*serpin3) from *L. vannamei*. Tissue distribution and temporal expression patterns against pathogens were investigated. Then, double stranded RNA mediated RNA interference was performed to study

* Corresponding author.
E-mail address: liuxiaolin@nwsuaf.edu.cn (X. Liu).

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the regulating role of *Lv*serpin3 on protease inhibition. Furthermore, the recombinant protein was expressed in vitro and used for investigation on bacterial growth inhibition and blocking of the proPO system.

2. Materials and methods

2.1. Shrimps and pathogens infection

Healthy *L. vannamei* (10–15 g) were collected from a local shrimp farm in Zhanjiang (Guangdong, China). The shrimps were cultured in tanks with aerated seawater for a week before processing.

Samples of hemocytes, gill, hepatopancreas, eyestalk, stomach, muscle, intestine, nerve, heart and testis were collected for tissue distribution analysis. For immune challenge experiments, 20 μl of live *Vibrio anguillarum* (4×10^8 CFU/ml), *Micrococcus lysodeikticus* (4×10^{10} CFU/ml) or WSSV suspension (80 copies/μl) were injected into the abdomen of shrimp. Shrimps received an injection of 20 μl PBS, were employed as the control. At 0, 2, 6, 12, 24 and 48 h post injection (hpi), three shrimps were randomly chosen for hemolymph collection using 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). Then hemocytes were separated by centrifugation at 800 g for 10 min at 4 °C [24] and subjected to total RNA preparation.

2.2. Total RNA extraction and cDNA synthesis

Total RNA of collected hemocytes were extracted with Trizol Reagent (Invitrogen, USA) as described in the manufacturer's protocol. After a determination of the concentration by a UV-spectrometer (Bio-Rad, USA), 500 ng of the obtained RNA was used to synthesize the first-strand cDNA using the Prime-Script RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) in accordance with the manufacturer's instructions.

2.3. cDNA cloning and sequence analysis of Lvserpin3

Based on the full-length cDNA sequence of *PmSERPIN3* (GenBank accession number: KC577446), gene specific primers (SPN3-F/R; Table 1) were designed to amplify the Open Reading Frame (ORF). The PCR products were purified with Universal DNA Purification Kit (TIANGEN, China) and further cloned into the pGEM-T vector (Promega, USA). Three positive clones were selected for sequencing. Bioinformatics analysis of *Lv*serpin3 was done following our previous study [25]. Briefly, multiple sequence alignment was created using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Signal peptide was predicted with SignalP3.0 program (http://www.cbs.dtu.dk/services/SignalP/). The protein motif features were predicted by Simple Modular Architecture Research Tool (http://smart.emblheidelberg.de/). The phylogenetic tree was constructed based on the amino sequences alignment by the neighbor-joining (NJ) algorithm embedded in MEGA5.0 program with bootstrap trial 1000 replicates.

2.4. Expression analysis by quantitative real-time PCR (qRT-PCR)

The tissue distribution and expression profiles upon immune challenge were performed by qRT-PCR in a CFX Manager system (Bio-Rad, USA). Gene-specific primers S3t-F/R and Actin-F/R (Table 1), which amplified a product of 244 bp and 260 bp in length respectively, were designed to determine the relative expression of *Lvserpin3*. The total reaction volume consisted of 10 μl SYBR Green Super mix (CWBIO, China), 2 μl of diluted cDNA, 0.4 μl of each primer (10 pmol/μl) and 7.2 μl nuclease-free water. The PCR program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Melt curve analysis (65 °C–95 °C: increment 0.5 °C for 5 s) was performed at the end of each PCR thermal profile to assess the specificity of amplification. The relative expression to controls was determined by the $2^{-\Delta\Delta CT}$ method [26]. All data represented means ± standard deviation and were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SPSS 16.0 program. Differences were considered statistically significant at $P < 0.05$.

2.5. mRNA expression analysis of dsRNA injected shrimp post V. anguillarum

Double stranded RNA was done in vitro using T7 RiboMAX Express (Promega, USA) following the manufacturer's instructions. The detailed steps were performed as previous study [27]. The purified RNA was dissolved in DEPC water, and adjusted to the concentration of 500 ng/μl. Then 20 μl of dsRNA for *Lvserpin3* or

Table 1
Sequences of primers used in this research.

Primer	Objective	Tm (°C)	Sequence (5′–3′)
SPN3-F	ORF amplification	55	ATACAGAGTGAAAGGGCGAGAAC
SPN3-R			ATTTATTGCTGACCATCCATTCA
T7S3-F	Serpin3-dsRNA	62	*TAATACGACTCACTATAGGGAGA*AGAAAGAAACCAATGCCAAGATC (T7 promoter sequence, italic)
S3-R			GTTGCCAGAAATGCCTGAAAG
S3-F			AGAAAGAAACCAATGCCAAGATC
T7S3-R			*TAATACGACTCACTATAGGGAGA*GTTGCCAGAAATGCCTGAAAG (T7 promoter sequence, italic)
T7EGFP-F	EGFP-dsRNA	58	*TAATACGACTCACTATAGGGAGA*GTGCCCATCCTGGTCGAGCT (T7 promoter sequence, italic)
EGFP-R			TGCACGCTGCCGTCCTCGAT
EGFP-F			GTGCCCATCCTGGTCGAGCT
T7EGFP-R			*TAATACGACTCACTATAGGGAGA*TGCACGCTGCCGTCCTCGAT (T7 promoter sequence, italic)
S3t-F	Real-time RT-PCR	60	TGGCTAAGGCAGATTTGTATGAG
S3t-R			GCCTCACTGCCCTTTTCATTA
PPAF-F	Real-time RT-PCR	60	GCAAGAGGAACTCGCAAGGCTTC
PPAF-R			CGGCTGTGAGAACGATGGATGGA
proPO-F	Real-time RT-PCR	60	CATAGAACACGGCCCTGAG
proPO-R			AATGTCGTACCTGGCGATAAT
Actin-F	Real-time RT-PCR	60	CACGAGACCACCTACAACTCCATC
Actin-R			TCCTGCTTGCTGATCCACATCTG
rSer3-F	Protein expression	65	CCG*GAATTC*CAGGCCCCGACCGCTTTCCCA (*Eco*R I, italic)
rSer3-R			CCC*AAGCTT*AGGCTTGACAAGTCGCCCAACAAA (*Hin*dIII, italic)