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A single WAP domain (SWD)-containing protein with antiviral activity from Pacific white shrimp *Litopenaeus vannamei*

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ABSTRACT

The single whey acidic protein (WAP) domain (SWD)-containing proteins, also called type III crustins, are a group of antimicrobial peptides (AMPs) in crustaceans. At present, a number of SWDs have been identified in shrimp, which showed essential antibacterial activities. However, the roles of SWDs in antiviral immune responses have not been reported up to now. In this study, a novel SWD (LvSWD3) was identified from Pacific white shrimp, *Litopenaeus vannamei*, which contained a typical single WAP domain homologous to those of other crustacean SWDs. Although lacking the pro and arg-rich region between the signal peptide and the WAP domain, LvSWD3 was closely clustered with other shrimp SWDs in the phylogenetic tree. Similar to many shrimp SWDs, the highest expression of LvSWD3 was detected in hemocytes. The LvSWD3 expression exhibited only limited changes after challenges with *Vibrio parahaemolyticus*, Poly (I:C) and lipopolysaccharide, but was significantly up-regulated after white spot syndrome virus (WSSV) infection. Silencing of LvSWDs significantly accelerated the death of the WSSV-infected but not the *V. parahaemolyticus*-infected shrimp. The recombinant LvSWD3 protein did not show proteinase inhibitory and antibacterial activities but could significantly postpone the death of WSSV-infected shrimp and reduce the viral load in tissues. These suggested that LvSWD3 was a novel SWD with antiviral activity.

1. Introduction

Innate immunity is central to defense against invading pathogens in invertebrates, which generally lack acquired immunity [1]. Antimicrobial peptides (AMPs), a unique and diverse group of peptides with inhibitory activities against a spectrum of microorganisms, are key components of the innate immune system in invertebrates [2,3]. At present, a large number of AMPs, such as antilipopolysaccharide factors (ALFs), penaeidins, and crustins, have been identified in various invertebrate animals [4–7]. The crustin family from crustaceans is characterized by a C-terminal whey acidic protein (WAP) domain of approximately 50 amino acids, which contains eight Cys residues in a conserved arrangement that form a four-disulfide core (4-DSC) [8]. Based on the domain structure between the N-terminal signal sequence and the WAP domain, crustins can be divided into three subgroups I, II and III [8]. The type I and II crustins have Cys-rich, and Gly/Cys-rich regions in the C-terminus, respectively, while the type III crustins possess only one WAP domain and thus are also called a single WAP domain-containing peptides (SWDs) [9,10].

Since first identified in mouse milk protein in 1981 [11], the WAP domain has been found in various proteins containing or not containing other active domains in vertebrates and invertebrates [12,13]. These proteins exhibit a wide variety of functions, including blockage of calcium transport, inhibition of sodium/potassium ATPase, metastasis, anti-proteinase and antimicrobial effects [14–17]. It has been known that many double WAP domain-containing proteins (DWDs) could be involved in antiviral responses. Secretory leukocyte proteinase inhibitor (SLPI), a well characterized DWD, has been reported to have anti-human immunodeficiency virus type 1 (HIV-1) activity [18]. A Study in *Marsupenaeus japonicus* also demonstrated that expression of a shrimp DWD gene could be activated during white spot syndrome virus (WSSV) infection [19]. As a group of single WAP domain-containing proteins, a growing number of SWDs have been identified and characterized in various crustaceans, including *Penaeus monodon* [20], *Litopenaeus*

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Table 1
Summary of primers used in this study.

Primers	Sequences(5' to 3')
For cDNA cloning	
LvSWD3-5'RACE1	TTCAGCAATTGGCATGCACTCC
LvSWD3-5'RACE2	CGATCAGAACCTCCTTAAGGCT
LvSWD3-3'RACE1	GAGAACAAGCAAGGCGAACA
LvSWD3-3'RACE2	CAGGAAGGTGCCGTGATGT
RT-PCR analysis	
LvSWD3-F	CAGGAAGGTGCCGTGATGT
LvSWD3-R	GGACAGCACTTGTAGCCGTATT
Lv*EF-1α*-F	CCTATGTGCGTGGAGACCTTC
Lv*EF-1α*-R	GCCAGATTGATCCTTCTTGTTGAC
WSSV-VP28-F	TGAGGTTGGATCAGGCTACTTC
WSSV-VP28-R	CCGCATCTTCTTCCTTCATCTG
dsRNA production	
T7-dsLvSWD3-F	GGATCCTAATACGACTCACTATAGGGAGAACAAGCAAGGCGAACA
T7-dsLvSWD3-R	GGATCCTAATACGACTCACTATAGGGGACAGCACTTGTAGCCGTATT
T7-GFP-F	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAG
T7-GFP-R	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCATGCC
T7-dsLvDorsal-F	GGATCCTAATACGACTCACTATAGGCTGTTGACCCACCTTACCGAC
T7-dsLvDorsal-R	GGATCCTAATACGACTCACTATAGGATCTTTGACCTCATAGAAACGGAC
T7-dsLvSTAT-F	GGATCCTAATACGACTCACTATAGGTCAGTATGCCCAGTCCTT
T7-dsLvSTAT-R	GGATCCTAATACGACTCACTATAGGCCTAACTCTTTCCGTCTCC
T7-dsLvIRF-F	GGATCCTAATACGACTCACTATAGGCTTTCACCAATGTCCCGATG
T7-dsLvIRF-R	GGATCCTAATACGACTCACTATAGGCGGCGATGTCGTAGGAATG
protein expression	
PET32a + -LvSWD3-F	CGC**GGATCC**GGCCCAATGGCATTCGAC
PET32a + -LvSWD3-R	CCC**AAGCTT**ATTATTCAGCAATTGGCATGCACTC

Nucleotides in bold represent the restriction sites introduced for cloning.

vannamei [21,22], *M. japonicus* [23], *Fenneropenaeus chinensis* [24] and *Procambarus clarkia* [25]. Previous studies have suggested that the SWD proteins from *P. monodon*, *P. clarkii* and *F. chinensis* have inhibitory capacity towards the hydrolysis activity of proteinases derived from bacteria and exhibit inhibitory activity against Gram-positive or -negative bacterial infection [20] [24], [25]. However, although it has been known that the expression of many crustacean SWDs could be up-regulated upon virus infection [25], their roles in antiviral immune responses have not been determined up to now.

The Pacific white shrimp, *L. vannamei* is now the major aquaculture shrimp in the world, which accounts for over two-third of all aquaculture shrimp production every year [26]. The shrimp aquaculture is threatened by diverse bacterial, fungal and viral pathogens, which have caused serious economic losses. More and more studies have focused on the immune system of *L. vannamei* [27]. Because of its important evolutionary status, *L. vannamei* has become a useful model for studying the immune system of invertebrates [28]. As key effectors of the humoral immunity in vertebrates, AMPs have attracted growing attentions in shrimp. A series of AMPs with remarkable antibacterial activities have been characterized in *L. vannamei* [29–31]. In this study, a novel SWD peptide termed LvSWD3 was identified from *L. vannamei*. We demonstrated that LvSWD3 did not show proteinase inhibitory and antibacterial activities but could significantly inhibit the infection of WSSV, the major viral pathogen threatening shrimp aquaculture. To our knowledge, this is the first report of the antiviral activity of a SWD, which could enrich the knowledge of the crustacean immunity.

2. Materials and methods

2.1. Shrimp and pathogens

L. vannamei (~5 g) were collected from a shrimp farm in Zhanjiang, Guangdong Province. Five percent of shrimp were randomly sampled to ensure to be free of WSSV and *Vibrio parahaemolyticus* by PCR as previously described [32,33]. Before experiments, shrimp were acclimated at ~28 °C for at least 7 days in a recirculating water tank system filled with air-pumped seawater (2.0% salinity). Preparation of the stocks of *V. parahaemolyticus* and WSSV was performed as previously described [34].

2.2. Cloning of LvSWD3 gene

An expressed sequence tag (EST) containing a putative WAP domain-encoding sequence was retrieved from a *L. vannamei* transcriptome data [35]. The 3′ and 5′ ends of LvSWD3 mRNA were determined by rapid amplification of cDNA ends (RACE) using a SMARTer RACE cDNA Amplification kit (Clontech, Japan). The PCR amplified products were cloned into pMD-20 T Vector (Takara, Japan) and sequenced.

2.3. Bioinformatics analysis

The amino acid sequences of many SWD homologues were retrieved from the National Center for Biotechnology Information (NCBI) databases. Sequence alignments were generated by Clustal W 1.8 with parameters as previously described [36]. Phylogenetic tree was constructed using MEGA 5.0 software with the neighbor-joining (NJ) method using a Poisson model and other parameters as follows: substitution type (amino acid), rates among sites (uniform rates), gap missing deletion treatment (complete deletion), and number of bootstrap replication (1000 replicates).

2.4. Real-time PCR

To examine the tissue distribution of LvSWD3, the hepatopancreas, hemocytes, gill, heart, pyloric caecum, stomach, nerve, eyestalk, epithelium, muscle and intestine tissues were taken from healthy *L. vannamei*. Each tissue sample was pooled from 15 individual shrimp. For the challenge experiments, healthy *L. vannamei* were intramuscularly injected at the second abdominal segment with 50 μl PBS buffer containing lipopolysaccharide (LPS, 2 μg), poly I:C (2 μg), *V. parahaemolyticus* (10^6 CFU) or WSSV (10^6 copies). Shrimp injected with PBS were used as control. The hemocytes were sampled at 0, 4, 12, 24, 48, 72 and 96 h post injection and subjected to total RNA extraction, cDNA