Fish & Shellfish Immunology 28 (2010) 134–142

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

A single WAP domain (SWD)-containing protein with antipathogenic relevance in red swamp crayfish, *Procambarus clarkii*

Zhi-Qiang Du, Xin-Cang Li, Zong-Heng Wang, Xiao-Fan Zhao, Jin-Xing Wang*

School of Life Sciences, Shandong University, Jinan, Shandong 250100, China

ARTICLE INFO

Article history:
Received 12 June 2009
Received in revised form
6 October 2009
Accepted 6 October 2009
Available online 17 October 2009

Keywords:
Whey acidic protein
Procambarus clarkii
Proteinase inhibitory activity

ABSTRACT

Single WAP domain-containing proteins (SWDs) are small proteins possessing a whey acidic protein (WAP) domain at the C-terminal region. In this study, a complementary deoxyribonucleic acid (cDNA) of SWD-containing protein was isolated from the hemocytes of red swamp crayfish, *Procambarus clarkii* called *Pc-SWD*. The full-length cDNA sequence is 998 bp. The deduced amino acid sequence consists of 74 residues with a signal peptide of 20 residues. The mature peptide has a single WAP domain which contains eight conserved cysteine residues forming a "four-disulphide core" (4-DSC). The predicted molecular mass of the mature protein is 5.97 kDa, with an estimated *p*I of 7.71. Tissue distribution analysis by reverse-transcribed polymerase chain reaction (RT-PCR) revealed that *Pc-SWD* transcripts were primarily found in the hemocytes, heart, hepatopancreas, gills, and intestine. The results of time course analysis demonstrated that expression of *Pc-SWD* was decreased at 6 h followed by a significant upregulation from 48 h to 72 h in hemocytes after white spot syndrome virus (WSSV) injection. A similar expression pattern was found in the hepatopancreas after WSSV injection. In addition, *Pc-SWD* expression was visibly upregulated in the gills from 6 h to 72 h after WSSV injection. The results of Western bolt revealed that Pc-SWD was constitutively expressed in the heart, hepatopancreas, gills, and intestine of unchallenged crayfish. A weak band was detected in the hemocytes and hemolymph of unchallenged *P. clarkii*. The Pc-SWD expression was upregulated in the hemocytes and gills after challenging with WSSV; however, no obvious change occurred in the heart and intestine. rPc-SWD could bind to both Gram-negative and -positive bacteria strongly. Moreover, rPc-SWD exhibited specific proteinase inhibitory activity against the secretory proteinase(s) from *B. subtilis* and *P. aeruginosa*. All these findings suggest that Pc-SWD possibly functions as an immunity effector in defense against the invasion of crayfish pathogens.

1. Introduction

Unlike other vertebrates, shrimps lack an adaptive immune system. Instead, they possess a well-developed innate immune system which protects them from microorganisms [1]. This includes humoral responses such as the modulation of melanization by the prophenoloxidase-activating system, clotting process, and production of antimicrobial peptides (AMPs); it likewise includes cellular immune responses such as phagocytosis, encapsulation of foreign materials, and cell agglutination [2].

As the endogenous antibiotic materials in the innate immune system, AMPs have been observed to provide an effective way of studying the innate immune system of crustaceans [3,4]. To date, a variety of AMP families has been described in penaeid shrimp, including penaeidins [5], antilipopolysaccharide factors (ALFs) [6], lysozymes [7–10], and crustins [11]. The crustin family (WAP domain-containing proteins), initially described in *Carcinus maenas* with activity against Gram-positive bacteria [12], has been proven to be an important antimicrobial peptide and has been described as an important element of crustaceans' innate immune system [11,13]. The single WAP domain-containing peptides (SWDs) detected in penaeid shrimp perform antimicrobial and antiproteinase activities [6,14,15]. The WAP domain is composed of approximately 50 amino acids, including 8 cysteine residues which form a 4-disulphide core (4-DSC) [16]. SWDs perform functions such as proteinase inhibitory [17] and antimicrobial activities [18].

Many pathogens are known to produce extracellular proteinases which are reported to cause various diseases [19]. Several reports suggest that a major function of proteinase inhibitors is to combat the proteinases of pathogens [6]. Certain proteinase inhibitors have been observed in crustaceans such as Kazal-type inhibitors [20,21], serpin [22], pacifastin [23], alpha 2-macroglobulin families [24,25], and whey acidic protein (WAP) domain-containing proteins [16,26].

At present, several SWDs have been observed in penaeid shrimp, namely, *Penaeus monodon* [27,28], *Litopenaeus vannamei* [29],

* Corresponding author. Tel./fax: +86 531 88364620.
E-mail address: jxwang@sdu.edu.cn (J.-X. Wang).

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doi:10.1016/j.fsi.2009.10.009

Marsupenaeus japonicus [30], and *Fenneropenaeus chinensis* [15]. No SWDs have been reported in crayfish. In this paper, a new full-length cDNA of the SWD protein has been obtained from red swamp crayfish, *Procambarus clarkii* (*Pc-SWD*). The expression profiles have been examined in various tissues in response to the infection of the white spot syndrome virus (WSSV), and the binding and proteinase inhibitory activities have been investigated. The results revealed that *Pc-SWD* is an immunity-relevant gene which may play an important role in the innate immune system by fighting against pathogenic organisms.

2. Material and methods

2.1. Immunity challenge and collection of tissues

P. clarkii (approximately 10–15 g each) were obtained from a market in the Chinese city of Jinan in Shandong Province. These were cultured temporarily in laboratory tanks filled with fresh water. In WSSV-challenge experiments, WSSV (3.2×10^7 per crayfish) was injected into the abdominal segment of the crayfish [31]. The hemolymph was extracted from the ventral sinus at different time points (6, 12, 24, 48, and 72 h) after WSSV injection using a 1 ml sterile syringe preloaded with 100 μl anticoagulant (10% sodium citrate, pH 7).

The hemolymph was centrifuged immediately at 800 × g for 5 min (4 °C) to isolate the hemocytes. The hemolymph and hemocytes from the unchallenged crayfish were collected using the same method and were used as the control. Other tissues such as the heart, hepatopancreas, gills, and intestine were collected at different time points (6, 12, 24, 48, and 72 h) after WSSV infection for ribonucleic acid (RNA) extraction and tissue fixation. The abovementioned tissues extracted from the unchallenged crayfish were collected and used as the control.

2.2. RNA extraction and cDNA synthesis

The total RNA of a variety of tissues (hemocytes, heart, hepatopancreas, gills, stomach, and intestine) from the control group or the WSSV-challenged crayfish was extracted using Biozol reagents (Hangzhou, China) following the manufacturer's instructions. This was then dissolved in the diethylpyrocarbonate (DEPC)-treated water. Electrophoresis on 1% RNase-free agarose gel was performed to test the RNA quality. The first-strand cDNA synthesis was performed in 25 μl reaction volume containing 5 μg RNA, 1 μl M-MLV reverse transcriptase (Promega USA), 1 mM dNTP mixture using SMART F (5′-tacggctgcgagaagacgacagaaggg-3′), and oligo anchor R (5′-gaccacgcgtatcgatgtcgact$_{16}$v-3′) at 42 °C for 2 h.

2.3. Gene cloning

The specific forward and reverse primers (F1:5′-atgaagctcttgct gctctcag-3′; R1:5′-gacacgttccaagcattcgtg-3′) were designed based on the nucleotide sequences of an expressed sequence tag (EST) obtained from random cDNA library sequencing. The F1 and 3′ anchor R primer (5′-gaccacgcgtatcgatgtcgac-3′) were used to amplify 3′ end of the cDNA. The 5′ PCR primer (5′-tacggctgcgagaag acgacagaa-3′) and R1 were used to amplify 5′ end of the cDNA. Polymerase chain reaction (PCR) amplification was conducted as follows: a cycle of 94 °C for 3 min and 35 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s; this was followed by an additional extension at 72 °C for 10 min.

PCR products were in-gel purified using the gel purification kit (Shenergy Biocolor, Shanghai, China) following the manufacturer's instructions. This was followed by ligation into the pMD-18T vector (TaKaRa) and transformation into competent DH5α cells. The positive recombinants were identified through blue-white colour selection in ampicillin-containing LB plates and PCR screening using two specific primers, F1 and R1, respectively. Positive clones were sequenced by Sangon companies (Shanghai, China).

2.4. Sequence BLAST and phylogenic analysis

The similarity between Pc-SWD and other single WAP domain-containing proteins was analyzed using the Web-based Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Translation of the amino acid sequences and prediction of the deduced protein were performed on ExPASy (http://www.expasy.org). Signal peptides were then predicted using the Simple Modular Architecture Research Tool (SMART) (http://www.smart.embl-heidelberg.de/). Meanwhile, alignment with the homogenous sequences from other species was conducted using MEGA software 4.0 [32]. Phylogenic analysis was likewise conducted using Neighbor Joining (NJ) methods of MEGA based on the amino acid sequences. To assess reliability, 1000 bootstraps were selected for the NJ tree.

2.5. RT-PCR

Total RNAs were isolated from the different tissues at different time points (6, 12, 24, 48, 72 h) after WSSV infection using Unizol reagent. 5 μg of the total RNA was reverse-transcribed into first-strand cDNA using for the PCR template. Semiquantitative RT-PCR was employed to investigate *Pc-SWD* expression patterns using specific primers (Pc-SWD-RT-F:5′-tgttgtcttgttgctggctgtg-3′; Pc-SWD-RT-R:5′-gcgcctctactggct gaaataat-3′). The PCR program was the initial predenaturation at 94 °C for 3 min followed by the 24 cycles (94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s) and the final additional extension step at 72 °C for 10 min. The constitutively expressed *18 S RNA* was likewise amplified as inner standard. The experiment was repeated three times using three independent samples. The RT-PCR products were run on 2% agarose gels, stained by ethidium bromide (EB), and photographed over ultraviolet (UV) light using Quantity One software (Bio-Rad, Hercules, CA). By using this software, the PCR product bands' intensities of absorbance were read from the photographs. Band density was converted to digital data using the Quantity One software. The ratio of *Pc-SWD* to *18S RNA* was calculated using as the relative expression level of *Pc-SWD*. Subsequently, the average of the ratios from the three independent experiments was calculated and utilized to construct the histograms. The data obtained were subjected to statistical analysis followed by an unpaired sample *t*-test. The significant difference was accepted at $P < 0.05$, while the extremely significant difference was accepted at $P < 0.01$.

2.6. Recombinant expression, purification, and antiserum preparation of recombinant Pc-SWD

The mature Pc-SWD was amplified by the primers (Pc-SWD-exp-F:5′-tac tca gaa ttc cag cgc aca cac tat gct-3′; Pc-SWD-exp-R:5′-tac tca ctc gag cct gac ctt agt tca tgc ac-3′, *Eco* RIand *Xho*Isites were underlined). pGEX-4T-1(+)-*Pc-SWD* was generated by subcloning mature Pc-SWD cDNA (171 bp) into the *EcoR* I and *Xho* I sites of pGEX-4T-1. This constructed plasmid was transformed into competent cells of *Escherichia coli* BL21 for recombinant expression.

Overnight culture transformants (2 ml) were transferred into the 200 ml ampicillin-containing Luria-Bertani broth for the large-scale culture. When the A_{600} value was up to 0.6, the final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce *Pc-SWD* expression. Cultured at 37 °C for 4 h, the bacteria were collected by centrifugation at 6000 × g for 5 min and re-suspended in 20 ml of PBS containing 0.2% Triton X-100. The collected bacteria were lysed by ultrasonic [33]. The soluble fraction was saved and used for