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The expression and purification of WSSV134 from white spot syndrome virus and its inhibitory effect on caspase activity from *Penaeus monodon*

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ABSTRACT

WSSV134 or VP36A protein of white spot syndrome virus was previously reported to be able to reduce apoptosis in Sf-9 cells transfected with caspase of *Penaeus monodon* (PmCasp). The protein was therefore believed to have a role in supporting the survival of WSSV inside the host cells during infection. However, the anti-apoptosis activity of WSSV134 involved in the inhibition of PmCasp is still unclear. In this study, we produced a recombinant WSSV134 (rWSSV134) and tested for its ability to inhibit PmCasp *in vitro*. The results from a caspase inhibition assay revealed that rWSSV134 could inhibit PmCasp in a dose-dependent manner. Since WSSV134 was predicted to contain three potential caspase binding sites, corresponding to the D54, D104 and D259, we then employed site-directed mutagenesis to investigate the involvement of these sites in PmCasp inhibition. D54A and D259A mutants could still inhibit PmCasp while D104A mutant lacks this activity. Our results confirmed that the WSSV134 is an inhibitor for PmCasp and that residue D104 is important for PmCasp inhibition.

1. Introduction

Apoptosis in shrimp is part of an innate immune system that helps defend against viral infection. Several studies have shown that shrimp activate apoptosis upon infection to prevent the virus from spreading inside the body [1–4]. Khanobdee et al. (2002) presented the evidence of apoptosis observed in the hemocytes, lymphoid organs and gills of YHV-infected shrimp *Penaeus monodon* through Light Microscopy that revealed chromatin condensation and DNA fragmentation inside the nuclease of shrimp cells [1]. Chayaburakul et al. (2005) examined *P. monodon* tissue using transmission electron microscopy and found the condensation of the chromatin in the lymphoid organ in the IHHNV-infected shrimp [2]. In addition, Sahtout et al. (2001) also found that *P. monodon* shrimp infected with WSSV also showed signs of apoptosis in several tissues, the abdominal epithelium, stomach epithelium, hepatopancreatic interstitial cells, gills and muscles [3]. The WSSV-induced apoptosis in *P. monodon* was later confirmed by Wongprasert et al. (2003) that WSSV infection induced several characteristic signs of apoptosis, i.e., nuclear disassembly, fragmentation of DNA into a ladder, and increased caspase-3 activity. Interestingly, it was shown by the same group that the apoptotic cells in all affected tissues were observed only in the cells without WSSV virions, whereas cells with virions are non-apoptotic, suggesting that WSSV has the ability to inhibit apoptosis in infected shrimp tissues [4].

Caspases are known to be a major component responsible for apoptosis [5]. Based on their roles in apoptosis, caspase proteins are divided into two groups, initiator caspases and effector caspases [6]. The initiator caspases have a long prodomain (>90 amino acids) containing specific protein-protein interaction motifs that are necessary for their activation, whereas the effector caspases usually have a short prodomain of only 20–30 residues [7]. Initiator caspases are normally activated by autoproteolysis in response to apoptotic signals and subsequently cleaves and activates the effector caspases [8–10]. Two different effector caspases, PmCasp

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and Pm caspase have been identified in *P. monodon* and shown to induce apoptosis in Sf-9 cells [11–13]. When *P. monodon* was infected with WSSV, the mRNA expression levels of PmCasp in the gills were increased at 48 h post infection [11], and indicated that PmCasp is up-regulated in response to WSSV infection. Caspase genes were also identified from other penaeid shrimp such as *P. japonicus* (PjCaspase) [14], *Litopenaeus vannamei* (*L. vannamei* cap-3 and Lvcaspase2-5) [15,16], *Fenneropenaeus merguiensis* (cap-3) [17], and from the freshwater prawn *Macrobrachium rosenbergii* [18].

To fight the host defense, many viruses have developed a strategy to inhibit caspase to avoid elimination by apoptosis [19–22]. WSSV449, also known as Anti-apoptotic protein-1 (AAP-1), of *P. monodon* was identified as an anti-apoptotic protein [23]. It was showed that AAP-1 blocked apoptosis in Sf-9 cell by inhibiting the activity of Pm caspase. This was further confirmed *in vitro* that the recombinant AAP-1 can directly inhibit Pm caspase [24]. In addition, AAP-1 contains three putative caspase-cleavage sites: VETD233G, DEVD272G, and LEHD303G. Site-directed mutagenesis showed that DEVD272G were the regions of AAP-1 important for Pm caspase inhibition [12,24]. Recently, WSSV134 or VP36A and WSSV322 were also reported to be an anti-apoptosis protein when it was found that the Sf-9 cells transfected with the vector expressing WSSV134 and WSSV322 become less apoptotic [13]. Also in the same study, WSSV134 was shown through Co-immunoprecipitation that it could interact with PmCasp, another effector caspase identified in *P. monodon* [13]. However, it is still unclear if the reduction of apoptosis by WSSV134 was due to the ability to inhibit PmCasp. To provide further insight, in this study we produced a recombinant thioredoxin-fused WSSV134 (rWSSV134) and performed a caspase inhibition assay to investigate the inhibitory effect of rWSSV134 on PmCasp. We found that rWSSV134 can inhibit PmCasp *in vitro* and that residue D104 might be important for PmCasp inhibition as evidenced by site-directed mutagenesis.

2. Materials and methods

2.1. The construction of expression plasmid

The expression plasmid of rWSSV134 was constructed by using pET15b-Thio which was modified by the insertion of thioredoxin and a TEV cleavage site before the multiple cloning site into pET15b (+) (Novagen). The specific primers used for constructing the rWSSV134 expressing plasmid were F-WSSV134-WT (**CGC** GGA TCC GCA TTA CAG GAA AAG GAT ATA) and R-WSSV134-WT (**CCG** CTC GAG TCA AAC TAC TAC TAT ACA TA) for forward and reverse primer respectively. All of the primers contained flanking non-complementary sequences (bold type), including appropriate restriction sites (underlined) so that the desired restriction sites would be included in amplicons. The WSSV134-encoding sequence was amplified with *KOD Hot start* DNA polymerase (Novagen). The PCR was carried out in a 50 μl reaction containing 100 ng DNA template, 0.2 μM of each primer, 0.2 mM of dNTP mix, 1.5 mM $MgSO_4$ and 0.02 U/μl of KOD Hot start DNA polymerase in 1× PCR reaction buffer. The following PCR protocol was used: pre-denaturing at 95 °C for 2 min; denaturation at 95 °C for 20 s, annealing at 57 °C for 10 s, extension at 70 °C for 15 s for 25 cycles. The PCR product was verified by 1% Agarose Gel Electrophoresis and then purified by using Gel/PCR purification kit (Geneaid). The gene product was digested with *Bam*HI and *Xho*I and ligated into *Bam*HI and *Xho*I sites of pET15b-Thio vector. The ligation product was transformed into *E. coli* strain XL1-Blue (Stratagene) and plating on a LB agar with 100 μg/mL of ampicillin. Plasmid from positive clones was extracted and purified using Presto™ Mini Plasmid Kit (Geneaid). The resulting plasmids were verified by PCR checking, using the condition and primers as above and DNA sequencing (Macrogen).

2.2. Protein expression and purification

After transformation of the recombinant plasmids into *E. coli* BL21 (DE3), BL21-RIL, Rosetta (DE3) and Origami, rWSSV134 were expressed by induction with IPTG. Bacteria were cultured in LB media supplemented with 100 μg/mL of ampicillin and then incubated at 37 °C until the OD_{600} reached about 0.6–0.8. Protein expression was induced by adding IPTG at a final concentration of 1.0 mM and incubated at 20 °C for 4 h. The bacterial cells were then harvested by centrifugation and resuspended with lysis buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 15 mM Imidazole, and 0.5% Triton X-100) and disrupted by sonicator (Total time 2 min, Pulse 5.0, 70% Amplitude). The cell lysates were centrifuged at 20,000 × g, 4 °C for 20 min to collect supernatants. The fusion proteins were purified from supernatants by Ni-NTA affinity chromatography using 2.5 × 20 cm Econo-column (BIO-RAD) packed with 2 mL of Ni-NTA sepharose (Qiagen) and washed with 1 L of washing buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, and 15 mM imidazole). The fusion proteins were recovered in 5 × 5 ml of elution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 300 mM imidazole). The fusion proteins were transferred to Tris buffer (20 mM Tris-HCl pH 8.0, and 50 mM NaCl) by using PD-10 Desalting Columns (GE Healthcare) and concentrated by Amicon Ultra centrifuge filter (Merck Millipore) to reduce the volume to approximately 500 μl. Size exclusion chromatography was used to determine the uniformity of fusion proteins. In brief, 500 μl of fusion proteins were injected into HiLoad™ 16/600 Superdex™ 200 column (GE-Healthcare Life Science) and eluted at 2 mL/min with Tris buffer (20 mM Tris-HCl pH 8.0, and 50 mM NaCl). The purified proteins were analysed by SDS-PAGE (12% w/v) followed by Western blot analysis. The expression of PmCasp was carried out by transforming pET15b carrying PmCasp encoding sequence into BL21 (DE3) as previously described [11]. The soluble fraction of the cell lysate was passed through Ni-NTA column. The elution fraction from the Ni-NTA column was analysed by SDS-PAGE.

2.3. Western blot analysis of rWSSV134

The fusion proteins were analysed by SDS-PAGE (12% w/v). The gel was then subjected to Western blot analysis. In brief, the proteins were transferred onto nitrocellulose membrane in electroblotting buffer (25 mM Tris-HCl pH 8.0, 190 mM glycine, and 20% methanol) at a constant voltage of 80 V for 1 h. The nitrocellulose membrane was immersed in blocking buffer (PBS pH 7.4 containing 5% (w/v) skimmed milk) at room temperature for 1 h, followed by incubation with anti (His) HRP-conjugated antibody (Qiagen) diluted 1:3000 in blocking buffer. After washing three times with PBST, a signal was detected with TMB substrate solution (Sigma) according to the supplier's protocol.

2.4. Site-directed mutagenesis of rWSSV134

Single point mutations were introduced by PCR-base mutagenesis, using the QuikChange Site-Directed Mutagenesis procedure (Stratagene) by which a target amino acid residue was replaced with Alanine. The forward mutagenic primers were D54A (CAA AAT AAG ACA AAT TGT GGC TAA AAT ACG ATC CCA AAC AAC), D104A (GAG TGG GGA CTT TAT TGC TGG CCG TAA AAA GCT C), D259A (CAA CTG TTT TAT AGA GGC AGC TAT GAG TAG CCT CTA CAT GG) and reverse primers were reverse complement with the forward strand. The rWSSV134 construct was used as the template for mutant