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Interaction study of a novel *Macrobrachium rosenbergii* effector caspase with B2 and capsid proteins of *M. rosenbergii* nodavirus reveals their roles in apoptosis

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ABSTRACT

Apoptosis is an essential immune response to protect invertebrates from virus infected cells. In shrimp, virus infection has been reported to induce apoptosis. *Macrobrachium rosenbergii* (*Mr*) was considered to be a disease-resistant host when compared to penaeid shrimps. Caspase-3 was classified as an executioner caspase which played a key role in virus-induced apoptosis. In this study, an *effector caspase* gene of *M. rosenbergii* (*Mrcasp*) was cloned and characterized. The open reading frame (ORF) of *Mrcasp* was 957 nucleotide encoding 318 amino acid with a deduced molecular mass of 35.87 kDa. RT-PCR analysis showed the presence of *Mrcasp* in all examined tissues. The phylogenetic tree indicated that *Mr*casp was closely related with caspase 3 of shrimp. The functions of the *Mr*casp, B2 and capsid proteins of *M. rosenbergii* nodavirus (*Mr*NV) were assayed in *Sf*-9 cells. The results showed that *Mr*casp induce apoptotic morphology cells; however, capsid protein of *Mr*NV could inhibit apoptotic cells whereas B2 could neither induce nor inhibit apoptotic cells by DAPI staining. The protein interaction between *Mr*casp and viral *Mr*NV structure revealed that *Mr*casp did not bind to B2 or capsid protein whereas B2 and capsid proteins could bind directly to each other. This study reported a novel sequence of a full-length *Mrcasp* and its functional studies indicated that *Mr*casp could induce apoptotic cells. Our data is the first report demonstrating the direct protein–protein interaction between capsid protein and B2 protein of *Mr*NV.

1. Introduction

White tail disease (WTD) caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) is a major cause of 100% mortality in post-larvae (PL) but not in adult freshwater prawn *M. rosenbergii* [1]. *Mr*NV was first reported in the French West Indies [2] and thereafter in China [3], India [4,5], Taiwan and Thailand [6]. *Mr*NV is a small, icosahedral, non-enveloped virus. The genome is formed by two single stranded RNA (ssRNA) called RNA1 and RNA2 [2]. *Mr*NV RNA1 is the larger genome (3.2 kb) that encodes RNA-dependent RNA polymerase (RdRp) and B2 protein. B2 protein is encoded in the sub-genomic RNA3 and its function is a suppressor in post-transcriptional gene silencing whereas the RNA2 (1.2 kb) encodes a capsid protein in size of 43 kDa [7,8]. There are several methods for detection of *Mr*NV such as RT-PCR, multiplex RT-PCR [6], *Taq*Man realtime RT-PCR, *in situ* hybridization, dot blot hybridization [9], double antibody sandwich enzyme-linked immunosorbant assay [10], monoclonal antibodies based assay [11], loop-mediated isothermal amplification [12,13], and high resolution melt duplex RT-PCR [14].

The crustaceans have an innate immune system to protect themselves from bacterial, fungal and viral pathogens [15]. The innate immune system comprises cellular and humoral immune

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responses. The cellular immune responses include apoptosis, encapsulation, phagocytosis and nodule formulation whereas humoral immune responses include the prophenoloxidase system, the clotting cascade, the antimicrobial peptides and antiviral peptides [16]. Among the cellular immune responses, apoptosis is also an important mechanism for removal of unwanted or dangerous cells such as virus-infected cells [17]. In shrimp, virus infection may induce apoptosis based on morphological and biochemical assays such as white spot syndrome virus (WSSV) or yellow head virus (YHV) infection can lead to an increased number of apoptotic cells [18–20]. The morphological change of apoptosis such as DNA fragmentation in hemocytes of WSSV-infected *Penaeus monodon* and *Penaeus indicus* was observed by using flow cytometry and agarose gel electrophoresis [21]. Thereafter, the majority of cells are believed to die via apoptosis pathway. In shrimp, apoptosis was suggested to be the cause of mortality as a terminal result of viral infection [22].

The major players of apoptosis which induced morphological changes of cell are a group of caspases, a family of structurally related cysteine proteases [23]. Caspases are divided into two groups: initiator caspases and effector caspases [23,24]. Usually, caspases are inactive proenzymes or zymogen and become activated under a process of activation during apoptosis [25,26]. All caspases can be activated by autoprocessing by other caspase family members to produce an enzymatic active form [23,27,28]. Caspase-3 is an important terminal effector caspase in apoptosis and involved in virus-induced apoptosis [29,30]. There are several reports of caspase 3 in shrimp such as banana prawn *Penaeus* (*Fenneropenaeus*) *merguiensis* [28], *P. monodon* [30] and whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei* [31]. Wongprasert et al. [20] discovered that caspase-3 activity increased in WSSV-infected *P. monodon*.

M. rosenbergii was found to be the host that tolerant to WSSV infection and able to operate the clearance of WSSV [32]. Moreover, adult *M. rosenbergii* was resistant to *Mr*NV and XSV but the exact mechanism was still unknown [1]. Recently, *Mr*casp3c was identified from the *M. rosenbergii* transcriptome database using Illumina Solexa Genome Analyzer technique and was found to be up-regulated after challenge with the infectious hypodermal and hematopoietic necrosis virus (IHHNV). However, the phylogenetic tree of *Mr*casp3c revealed the highest similarity to caspases 3C of *Hydra vulgaris* and *H. magnipapillata* instead of closely related to shrimp caspases [33]. Recently, a caspase-3-like protein was isolated and characterized from a Chinese mitten crab *Eriocheir sinensis* and the results revealed that EsCaspase-3-like protein shared the highest similarity to that of *Mr*casp3c [34]. This data indicated that caspase-3 isoforms may exist in crustacean similar to other invertebrate such as *H. vulgaris* [35], coral *Stylophora pistillata* [36], and mussel *Mytilus galloprovincialis* [37].

The present study aims to isolate an effector caspase of *M. rosenbergii* (*Mrcasp*) and characterize the protein interaction between *Mr*casp, B2 and capsid proteins of *Mr*NV by using *Sf*-9 cells.

2. Materials and methods

2.1. Shrimp RNA preparation

Juvenile *M. rosenbergii* of approximately 1.5 g each were purchased from a local farm in Suphan Buri province, Thailand. Total RNAs were extracted from various *M. rosenbergii* tissues (hemocytes, hepatopancreas, muscle, gills, heart, pleopods, stomach, and intestine) using the Trizol reagent (Invitrogen) following the manufacturer's manual. RNA concentration was measured by Quant-iT™ RNA as described by the manufacturer's instruction (Molecular Probes) with Fluoroskan Ascent (Labsystems).

2.2. Cloning of the full-length cDNA of Mrcasp

A partial sequence of *Mrcasp* was initially obtained by PCR using *M. rosenbergii* cDNA generated by SuperScript III First-Strand Synthesis kit (Invitrogen) and a pair of degenerate primers called CAP-F2N and CAP-R2N. Briefly, the degenerate primers were designed from conserved domains obtained from an alignment of known caspase-3 sequences of *P. vannamei* (ABW69658.1), *Penaeus* (*Fenneropenaeus*) *chinensis* (ADG84879.1), *P. merguiensis* (AAX77407.1) and *P. monodon* (ABI34434.1) using ClustalW2 program. Sequences of degenerate primers for *M. rosenbergii caspase* are shown in Table 1. A PCR reaction volume of 50 μl contained; 3 μl of cDNA template, 4 μl of 10 mM dNTPs, 1 μl of 50 μM of each primer, 0.5 μl of *Taq* DNA polymerase (5 units/μl, Invitrogen), 5 μl of 10 × PCR buffer, and 35.5 μl of distilled water. The PCR was performed for 1 cycle at 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were cloned into pGEM®T Easy Vector System (Promega) and then sequenced.

To obtain the full-length cDNA of *Mrcasp*, 3′ and 5′ rapid amplification of cDNA ends (RACE) were performed using a Smarter RACE cDNA amplification kit (Clontech) using the gill RNA as a template. Specific primers for 3′- and 5′-RACE and adapter primers from the kit were used (Table 1) in touchdown PCR conditions: 5 cycles at 94 °C for 30 s, 72 °C for 1 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 1 min; 30 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 1 min and cooling at 4 °C. PCR products were purified by MinElute®Gel Extraction kit (QIAGEN) and cloned into pGEM®-T Easy Vector System for DNA sequencing. After sequence analysis, the full-length *Mrcasp* cDNA was amplified by PCR using *Pfx* DNA polymerase enzyme (Invitrogen) and specific primers Exp_CAS_F-BamHI and Exp_CAS_R-XhoI (Table 1).

2.3. Sequence analysis of the full-length Mrcasp gene

The full-length cDNA and deduced amino acid sequences of *Mrcasp* were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the Expasy Translate Tool (http://web.expasy.org/translate/). The protein sequence motifs were analyzed with ScanProsite (http://prosite.expasy.org/scanprosite). Multiple sequence alignment was carried out using ClustalW (www.ebi.ac.uk) and phylogenetic trees were constructed by the Neighbor-joining (NJ) method using MEGA 6 software.

2.4. Detection of Mrcasp expression in different tissues of M. rosenbergii

Total RNA from various tissues of *M. rosenbergii* were used to study expression of *Mrcasp* by RT-PCR using SuperScript™ III One-Step RT-PCR System (Invitrogen) and specific primers called Cas_ExpF and Ncas_ExpR (Table 1), producing an expected product of 444 bp. *M. rosenbergii β-actin* gene primers generating a partial gene product of 337 bp designed based on GenBank accession number AY626840.1 was used as an internal control. The RT-PCR protocol consisted 50 °C for 30 min, 95 °C for 5 min and 4 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and final extension at 72 °C for 10 min. The amplified RT-PCR products of *Mrcasp* and *β-actin* were analyzed on a 1% agarose gel, stained with ethidium bromide and observed under the ultraviolet light.

2.5. Construction of recombinant Mrcasp for polyclonal antibody production

The open reading frame of *Mrcasp* obtained from PCR using specific primers Exp_CAS_F-BamHI and Exp_CAS_R-XhoI