Caspase-mediated apoptosis in crustaceans: Cloning and functional characterization of EsCaspase-3-like protein from *Eriocheir sinensis*

Min-Hao Wu, Xing-Kun Jin, Ai-Qing Yu, You-Ting Zhu, Dan Li, Wei-Wei Li, Qun Wang

Laboratory of Immunological Defense & Reproduction Biology, School of Life Science, East China Normal University, NO. 500 Dong-Chuan Road, Shanghai, China

**Article info**

**Abstract**

The caspase-3-like gene was cloned from *Eriocheir sinensis*, and its properties were characterized to identify the biological implications of this caspase in apoptosis in crab. Its deduced full-length protein sequence consists of 462 amino acid residues, including the prodomain and the large and small subunits. Moreover, several residues known to be critical in the caspase-3 catalytic center and binding pocket, as well as the active site pentapeptide motif Q220ACRG224, were identically present in the deduced EsCaspase-3-like protein. Subsequently, the recombinant EsCaspase-3-like (rEsCaspase-3-like) protein was expressed from *Escherichia coli* and obtained via affinity purification. Results of the in vitro enzymatic activity assays indicated that the rEsCaspase-3-like protein is capable of hydrolyzing the substrate Ac-DEVD-NA, suggesting a functional role in physiology. EsCaspase-3-like gene transcripts were found to be widely distributed in all tissues as detected by quantitative RT-PCR, being especially abundant in hematocytes and comparatively rare in muscles. Furthermore, EsCaspase-3-like, at both the mRNA and protein levels, was demonstrated to participate in the apoptotic process after stimulation by different pathogen-associated molecular patterns (PAMPs) in hemocytes. In conclusion, our findings suggest that the EsCaspase-3-like protein functions as an effector caspase and contributes to immune responses against pathogens.

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**1. Introduction**

Invertebrates, which have evolved into the most diverse group of animals in the world, lack a specific immune system and instead only harbor an innate immune system [1,2]. Apoptosis plays a crucial role in innate immunity by eliminating harmful, dangerous, damaged or unnecessary cells without causing an inflammatory response or tissue damage [7,8]. As a form of programmed cell death (PCD) [3,4], apoptosis also is essential in the normal development and homeostasis of all multicellular organisms [5,6]. Since 1972 when the concept of apoptosis was put forward [9], researchers have identified hundreds of genes involved in this process from different species [10]. An exciting breakthrough in the study of apoptosis came with the discovery of key molecules known as caspases or cysteine-dependent aspartate-directed proteases. In invertebrates, some caspases can also inactivate melanization [11]. Caspases are a conserved family of cysteine proteases which cleave after an aspartate residue in their substrates [4,12]. After the first identification of the caspase family member cell-death abnormality-3 (ced-3) in the nematode *Caenorhabditis elegans* [13], 14 distinct caspases have been identified from mammals [14]. While some of these caspases have non-apoptotic functions (caspase-1, -4, -5, -11 and -12), the apoptotic caspases are further categorized into initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, -7) [5]. Many invertebrate caspases were subsequently identified from *Geodia cydonium*, *Hydra vulgaris*, *Aiptasia pallida*, *C. elegans*, *Drosophila melanogaster*, *Strongylocentrotus purpuratus*, *Ciona intestinalis*, *Ciona savignyi*, *Macrobrachium rosenbergii*, *Lito- penaeus vannamei* and *Penaeus merguiensis* [15–17]. Although the duplications and diversities of the identified caspases are varied in each species, these specific caspases play similar roles to some extent and serve in conserved functions [49].

Generally, two pathways lead to apoptosis. The first is an extrinsic one mediated by caspase-8 (-10) that is activated by cell surface death receptor proteins and induces the activity of effector...
caspases. Meanwhile, the intrinsic apoptosis pathway is mediated by the activation of caspase-9 (-2), which itself is activated when cytochrome c is released from the mitochondria, thereby signaling the initiation of the caspase cascade. Both pathways result in apoptosis via caspase-3, the executor in the cell. During activation prior to and apoptotic process, caspase-3 is cleaved into two active heterodimers, a large and a small subunit [18]. Caspase-3 has many downstream substrates, such as DNA-PK, P53, PARP, DAS-GDI, procaspase-3 and procaspase-9 [14,19,20]. Because all caspase-3 substrates contain DEVD sequences in common, synthetically modified tetra peptides (e.g., Ac-DEVE-NA) can be used as the specific substrate and inhibitor of caspase-3 [21–23]. Caspase-3 has a conserved motif “QACRG”, the P10 and P20 domains (large and small subunits, respectively) and a pro domain without the Death Effector Domain (DED, which caspase-8 and -10 contain) or the Caspase-Recruitment Domain (CARD, which caspase-1, -2, -4, -5 and -9 contain).

In the present study, an expressed sequence tag (EST) sequence from our cDNA library was identified as an apoptosis-related molecule based on partial matching to the coding sequence of a caspase. After cloning, characterization and testing the hydrolytic activity of this caspase, we named it Eriocheir sinensis caspase-3-like (EsCaspase-3-like) based on the observed characteristics and function. The hydrolytic activity of the recombinant EsCaspase-3-like (rEsCaspase-3-like) protein against synthetic peptide substrates and EsCaspase-3-like mRNA tissue distribution were determined. We also investigated the expression profiles in both EsCaspase-3-like mRNA expression levels and enzyme activities.

2. Materials and methods

2.1. Animal immune challenge and sample collection

Healthy Chinese mitten crabs (n = 200; 60 ± 10 g wet weight) were collected from a local farm in Shanghai, China. After acclimation at 20–25 °C for a week in filtered, aerated freshwater, crabs were placed on ice until they were anesthetized before being dissected. Hemolymph was drawn from the hemocoel in the arthrodial membrane of the last pair of walking legs using a syringe (approximately 2.0 mL per crab) with an equal volume of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl, 10 mM EDTA) [24]. The hemocytes were collected from the hemolymph by centrifugation at 600 × g for 10 min. Hepatopancreas, gills, muscle, stomach, intestine, heart, testis, ovary, thoracic ganglia and brain were collected, snap frozen in liquid nitrogen and stored at −80 °C prior to nucleic acid analysis. Tissues of each type were pooled from 15 crabs and ground with a mortar and pestle prior to cloning and expression analysis.

For stimulation of pathogen associated molecular patterns (PAMPs) more than 120 crabs were divided equally into four groups (sex ratio, 1:1). Crabs of the three experimental groups were injected into the arthrodial membrane of the last pair of walking legs with approximately 100 μl of lipopolysaccharides (LPS from Escherichia coli, Sigma–Aldrich, St. Louis, MO, USA), 100 μl of peptidoglycan (PG from Streptococcus aureus, Sigma–Aldrich) or 100 μl of zymosan (Glu from peptidoglycan (PG from Streptococcus aureus, Sigma–Aldrich) or 100 μl of lipopolysaccharides (LPS from Escherichia coli, Sigma–Aldrich) resuspended (500 mg/ml) in ESS (E. sinensis saline, 0.2 M NaCl, 5.4 mM KCl, 10.0 mM CaCl2, 2.6 mM MgCl2, 2.0 mM NaHCO3; pH 7.4) [24–26]. Meanwhile, the fourth (control) group received 100 μl ESS (pH 7.4). More than five crabs were randomly selected at each time interval of 0 (as blank control), 2, 6, 12 and 24 h after injection with each type of PAMP. Hemocytes were harvested according to the methods above and stored at −80 °C after the addition of 1 μl of Trizol reagent (Invitrogen, Carlsbad, CA, USA) for subsequent RNA extraction.

2.2. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from E. sinensis tissues, samples as described in Section 2.1, using Trizol® reagent (RNA Extraction Kit, Invitrogen), followed by the manufacturer’s protocol. The extracted RNA was treated with DNase I (Qiagen, Hilden, Germany) to remove potential genomic DNA contamination and purified using the RNeasy Mini Kit (Qiagen). Integrities of representative RNA samples were visualized via agarose gel electrophoresis assays. Thereafter, the quality and concentration of total RNA were estimated using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Only the RNA samples with an A260/A280 ratio between 1.8 and 2.0 were used for the subsequent analysis. Total RNA (5 μg) isolated from hemocytes were reverse transcribed by using the SMARTer® RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) for cDNA cloning. Total RNA (4 μg) from different types of tissues and hemocytes after PAMP stimulations were reverse transcribed by using the PrimeScript™ real-time PCR kit (Takara, Shiga, Japan) for real-time PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) expression analysis.

2.3. Cloning of full-length EsCaspase-3-like cDNA

In order to obtain the full-length cDNA of E. sinensis caspase-3-like, we employed 5’ and 3’ RACE methods (SMARTer RACE cDNA Amplification kit, Clontech) to extend its partial cDNA sequence using gene-specific primers (Table 1) designed based on the original comparative transcriptomic sequencing from hemocytes (unpublished data). The 3’ RACE PCR reaction was carried out in a total volume of 50 μl containing 2.5 μl (800 ng/μl) of the first strand cDNA reaction as a template, 5 μl of 10 × Advantage 2 PCR buffer, 1 μl of 10 mM dNTPs, 5 μl (10 mM) gene-specific primer (Table 1), 1 μl of Universal Primer A Mix (UPM; Clontech, USA), 34.5 μl of sterile deionized water and 1 μl of 50 × Advantage 2 polymerase mix (Clontech, USA). For the 5’ RACE, UPM was used as the forward primer in PCR reactions in conjunction with the reverse gene-specific primer (Table 1). PCR amplification conditions for both the 3’ and 5’ RACE were as follows: 5 cycles at 94 °C for 30 s, 72 °C for 30 s, followed by 26 cycles at 94 °C for 10 s, 50 °C for 10 s, and 72 °C for 1 min. The amplified fragments were purified and sequenced.

Table 1

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>Primers by application</th>
<th>Sequence (5’–3’)</th>
</tr>
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<tr>
<td>Full-length cloning</td>
<td>5’RACE-1</td>
<td>TAGTTACCCTTACGTAGATGCC</td>
</tr>
<tr>
<td></td>
<td>5’RACE-2</td>
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<td></td>
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<td>ShortUP</td>
<td></td>
<td>CTAAACGACTTCATATAGGCA</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td></td>
<td></td>
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<td>EsCaspase-3-like</td>
<td>F</td>
<td>CATGGTGATGAGAATGAC</td>
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<tr>
<td></td>
<td>R</td>
<td>TTGATGAATGAGAAGC</td>
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<td>F</td>
<td>CTCTGCTGGTCTGATCCATAC</td>
</tr>
<tr>
<td></td>
<td>R</td>
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<tr>
<td>β-actin-F</td>
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<td></td>
</tr>
<tr>
<td>β-actin-R</td>
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</tbody>
</table>

23-Mer CGACTCACTATAGGGAGAGCGGC
24-Mer CCGCCGACTTAAATGACATCAACAGGCGCT
T7 promoter CTTATACGACTCAGTATAGG
T7 terminator CACCGCTGCAATAAACTAGC
3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min [27]. PCR amplicons were size separated and visualized on an etidium bromide stained 1.2% agarose gel. Amplicons of expected sizes were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), inserted into a pZero/Back/Blunt Vector (Tiangen, Beijing, China) and finally transformed into TOP10 E. coli. Positive clones containing inserts of an expected size were two-way sequenced using 23-mer and 24-mer primers (Table 1).

2.4. Sequence analysis

The homologous conserved domains and signal peptides were identified by using both the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de) and Center for Biological Sequence Analysis (CBS, http://www.cbs.dtu.dk/). The entire deduced amino acid sequence from the P20 to P10 domain of EsCaspase-3-like was compared against other vertebrate and invertebrate orthologs using the BLAST program (http://blast.ncbi.nlm.nih.gov). These multiple sequence alignments were analyzed by ClustalX and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). An unrooted neighbor-joining (NJ) phylogenetic tree was constructed with MEGA 5.0 software.

2.5. Construction of recombinant expression vector for EsCaspase-3-like

The open reading frame (ORF) of EsCaspase-3-like was amplified from the hemocyte cDNA template with a pair of specific primers into which BamH I and XhoI endonuclease sites were introduced (Table 1). The target PCR products were double used publicly described using BamH I and XhoI (New England Biolabs, Ipswich, MA, USA), purified using the Universal DNA Purification Kit (Tiangen, Beijing, China) and then finally fused with the corresponding cohesive ends of the pET32a plasmid (Novagen, Darmstadt, Germany) with T4 ligase (New England Biolabs). The positive recombinant plasmids were confirmed by two-way DNA sequencing with a T7 primer pair (Table 1).

2.6. Expression and purification of rEsCaspase-3-like proteins

The positive transformants of E. coli BL21-DE3 (Tiangen, China) were selected to express recombinant proteins. The pET32a plasmid without any inserted fragment was selected as the blank control. The positive transformants were extensively cultured in ampicillin-containing LB broth until the logarithmic phase (OD600 value ~ 0.6). Thereafter, isopropyl β-D-thiogalactopyranoside (IPTG) was added into the culture medium (final concentration of 1 mM) to induce the expression of EsCaspase-3-like proteins. After culturing for 4 h at 37 °C, the rEsCaspase-3-like proteins were collected and purified following the instructions of the Ni-NTA Purification System (Invitrogen). The purified proteins were eluted with elution buffer and dialyzed in refolding buffer for renaturing. The protein samples were examined by 12% SDS-PAGE and visualized with Coomassie Blue R250.

2.7. Hydrolyzing activity assays of rEsCaspase-3-like protein in vitro

To determine the precise biological function of the EsCaspase-3-like protein, its potential hydrolytic activity was detected using the Caspase-3, 8 and 9 Activity Assay Kit (Beyotime, Shanghai, China) under the manufacturer’s manual. In brief, the concentration of purified rEsCaspase-3-like protein was adjusted to 0.1 mg/mL [28]. The reaction contained the following: 70 mL reaction buffer, 20 mL of rEsCaspase-3-like protein and 10 mL 2 mM substrate (Ac-DEVD-pNA specific for caspase-3; Ac-IETD-pNA specific for caspase-8; Ac-LEHD-pNA specific for caspase-9). Subsequently, the reaction solution was gently mixed and incubated at 37 °C for 4 h. SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA) was used to monitor the absorbance at 405 nm. The different absorbance values represent the cleavage and release of pNA. The enzyme activity and substrate specificity of rEsCaspase-3-like were determined by comparing the hydrolytic activity against Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA.

2.8. Transcriptional analysis by qRT-PCR

The qRT-PCR assay was performed using SYBR Premix Ex Taq (TaKaRa, Japan) with EsCaspase-3-like gene-specific primer pairs designed in the P20 domain (Table 1), which produced a 120-bp amplicon. The PCR reaction was conducted using the CFX96™ Real Time System (Bio-Rad, Hercules, CA, USA) as follows: 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. Internal control PCR reactions for β-actin were performed in separate tubes, as described above with the exception of an alternative gene-specific primer pair (Table 1), which was designed based upon a cloned E. sinensis β-actin cDNA fragment to produce a 276-bp amplicon. All qRT-PCR experiments were performed in triplicate using independently extracted RNA. Both for the tissue transcription assay and the immune challenge transcription assay, qRT-PCR amplifications were carried out in a total reaction volume of 50 μL, including 25 μL 2 × SYBR Premix Ex Taq (TaKaRa), 2 μL diluted cDNA template (100 ng/μL), 21 μL PCR grade water and 1 μL of each primer. Results were calculated using the 2−ΔΔCt method comparative Ct method [29].

2.9. Hydrolyzing activity analysis of EsCaspase-3-like protein in hemocytes after challenge

An in vivo experiment was conducted to determine whether the EsCaspase-3-like protein exerts hydrolytic activity in immune cells and to infer its exact role in apoptosis. Hemolymph was sampled at 0, 6, 12, 24 and 48 after stimulation with PAMPs or the saline control (ESS). The collected hemolymph was resuspended in 100 mL lysis buffer and incubated on ice for 15 min. After centrifuging the lysate centrifuged at 16,000 × g for 15 min at 4 °C, the supernatant was transferred to a new tube. The concentration of the supernatant was measured using the Bradford Protein Assay Kit (Beyotime) and adjusted to 2 mg/mL with lysis buffer. The hydrolytic activity of the EsCaspase-3-like protein was detected with the Caspase-3 Activity Assay Kit (Beyotime) as described by the manufacturer’s manual.

2.10. Statistical analysis

Statistical analysis was performed using SPSS software (Ver20.0). The data are represented as the mean ± standard error (S.E.). Statistical significance was determined by one-way ANOVA and post hoc Duncan multiple range tests. Significance was set at P < 0.05 or P < 0.01.

3. Results

3.1. Cloning and characterization of EsCaspase-3-like gene

The Caspase-3-like cDNA obtained from E. sinensis was designated as EsCaspase-3-like (GenBank accession numbers KC140109). The 1868-bp full-length sequence of EsCaspase-3-like contains a 1449-bp ORF that encodes a 462-amino acid (aa) protein, a 303-bp...
5′ UTR and a 116-bp 3′ UTR, as well as a typical polyadenylation signal sequence (AATAAA) (Fig. 1). The deduced protein sequence of EsCaspase-3-like was predicted to have both a large subunit (P20, residues 196-226 aa) and a small subunit (P10, residues 337-427 aa) by using the SMART program. Compared with caspase-3 sequences from other animal species, the putative cleavage sites which produce mature P20 and P10 subunits are present at Pro196-His197-Gly198-Leu199-Cys200 and Val423-Gln424-Leu425-Val426-Arg427, respectively. Moreover, a putative conserved site “GSWYI” is present in P10, and the active-site pentapeptide motif (QACRG) is also in the P20 large subunit of the EsCaspase-3-like protein.

3.2. Multiple sequence alignments and phylogenetic analysis

Multiple sequence alignment was carried out on the EsCaspase-3-like amino acid sequence with orthologs from other animal species. EsCaspase-3-like was shown to share the highest similarity with caspase 3C from M. rosenbergii. NJ phylogenetic trees were produced based on analysis of P10 and P20 from representative invertebrate and vertebrate caspase-3 sequences from Protein Blast results (Figs. 2 and 3).

3.3. Expression, purification and hydrolyzing activity assay of rEsCaspase-3-like protein

The predicted molecular weight of EsCaspase-3-like is approximately 53 kDa. Since the pET-32a plasmid is fused with the extra 109-aa Trx™ thioredoxin protein [30], the molecular mass of the expressed target rEsCaspase-3-like protein increased to ~70 kDa. The coding frames were examined by nucleic acid sequencing of the recombinant plasmids, and the purified recombinant proteins were confirmed by 12% SDS-PAGE and visualized with Coomassie Blue (Fig. 4(A)). The results of the hydrolyzing activity assay of the purified rEsCaspase-3-like protein indicated that its activity for the caspase-3 substrate, Ac-DEVD-pNA, was 1.14 units per mg protein, while it had a comparatively lower activity level against the caspase-8 substrate, Ac-IETD-pNA (0.12 units per mg protein), as well as the caspase-9 substrate, Ac-LEHD-pNA (0.11 units per mg protein) (Fig. 4(B)).

3.4. Tissue distribution of EsCaspase-3-like gene

The specific tissue expression of EsCaspase-3-like in various tissues was widely observed in all the detected tissues of E. sinensis.
EsCaspase-3-like gene expression was relatively higher in hemocytes, intestine and testis, but lower in muscle, while its expression level in gills, hepatopancreas, heart, muscle, stomach, thorax, ovaries and brain was moderate (Fig. 5).

3.5. Temporal expression and hydrolyzing activity analysis of EsCaspase-3-like protein in hemocytes after PAMP immune challenge

Expression patterns of the EsCaspase-3-like transcript upon induction with various PAMPs were measured by qRT-PCR. The expression of EsCaspase-3-like in the hemocytes was significantly higher than the blank control after 2, 6 and 12 h after LPS stimulation ($P < 0.05$) (Fig. 6(A)), but recovered to the normal level after 24 h. After PG stimulation, the expression level of EsCaspase-3-like was slightly raised after 6, 12 and 24 h ($P < 0.05$) (Fig. 6(B)). Once challenged with Glu, EsCaspase-3-like expression was significantly increased after 2, 6, 12 and 24 h ($P < 0.05$) (Fig. 6(C)).

The enzymatic activities of EsCaspase-3-like protein in the hemocytes samples were then detected by the caspase3 substrate hydrolyzing assay. Both LPS and Glu stimulation increased the hydrolyzing activity of EsCaspase-3-like in the hemocytes during the 24 h after stimulation, which then decreased at 48 h. However, the enzyme activity increased during the 12 h after stimulation and then decreased at 24 h by PG stimulation. (Fig. 6(D)).

4. Discussion

In the present study, we cloned and characterized an E. sinensis caspase. The deduced amino acid sequence of the EsCaspase-3-like protein was found to be highly homologous with that of the previously reported M. rosenbergii Caspase 3C [17] (Fig. 2). Interestingly, neither the DED (found in caspase-8 and -10) nor CARD (found in caspase-1, -2, -4, -5 and -9) structure was found in the EsCaspase-3-like protein. In addition, the BLAST and motif analysis showed that the EsCaspase-3-like protein shares a similar domain arrangement with other known caspase-3 proteins, including a putative prodomain followed by a large subunit (P20 subunit) and a small subunit (P10 subunit)[31]. In most cases, caspase exists in an inactive form and is activated by two successive proteolytic cleavages. The first occurs between the large and small subunits, and the second between the large subunit and the prodomain[16,32,33]. Importantly, the active-site pentapeptide motif Q220ACRG224 and G366SWYI370 are also conserved in the EsCaspase-3-like protein.
Caspase-3-like protein with a thioredoxin protein tag was ~70 kDa as verified by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, protein standard; lane 2, total proteins of *E. coli* transformed with pET32α-EsCaspase-3-like, without induction; lane 3, proteins of *E. coli* transformed with pET32α-EsCaspase-3-like, induced with IPTG; lane 4, rEsCaspase-3-like purified with the Ni-NTA Purification System. A) Expression, purification and hydrolyzing activity assay of rEsCaspase-3-like protein. B) Hydrolyzing activity assay. The hydrolyzing activities of rEsCaspase-3-like were determined using Caspase-3, -8 or -9 Activity Assay Kits. The activities of rEsCaspase-3-like were determined measuring hydrolyzing activity against Ac-DEVD-pNA (caspase-3 substrate), Ac-IETD-pNA (caspase-8 substrate) or Ac-LEHD-pNA (caspase-9 substrate). The activities are presented as the fold change in activity compared to that of rEsCaspase-3-like against Ac-LEHD-pNA. Values are means ± standard error; *P < 0.05.

**Fig. 4.** Expression, purification and hydrolyzing activity assay of rEsCaspase-3-like protein. A) Protein samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, protein standard; lane 2, total proteins of *E. coli* transformed with pET32α-EsCaspase-3-like, without induction; lane 3, proteins of *E. coli* transformed with pET32α-EsCaspase-3-like, induced with IPTG; lane 4, rEsCaspase-3-like purified with the Ni-NTA Purification System. B) Hydrolyzing activity assay. The hydrolyzing activities of rEsCaspase-3-like were detected with Caspase-3, -8 or -9 Activity Assay Kits. The activities of rEsCaspase-3-like were determined measuring hydrolyzing activity against Ac-DEVD-pNA (caspase-3 substrate), Ac-IETD-pNA (caspase-8 substrate) or Ac-LEHD-pNA (caspase-9 substrate). The activities are presented as the fold change in activity compared to that of rEsCaspase-3-like against Ac-LEHD-pNA. Values are means ± standard error; *P < 0.05.

**Fig. 5.** Tissue distribution of EsCaspase-3-like transcript. The EsCaspase-3-like transcript was examined in various tissues by qRT-PCR. The β-actin gene from *E. sinensis* was used as an internal control. Values are means ± standard error; *P < 0.01.

Crustacean hemocytes play important roles in the host immune response, including recognition, phagocytosis, cytotoxicity cell communication and melanization [35]. Hence, we detected the EsCaspase-3-like gene expression in hemocytes induced by different PAMPs. We observed that the EsCaspase-3-like mRNA expression significantly increased after stimulation with PAMPs. The peaks of EsCaspase-3-like expression induced by LPS and Glu were observed in hemocytes after 12 h, consistent with a previous determination that the first distinct phase of the immune response occurs approximately in the first 12 h after challenge in *E. sinensis* [36,37]. But in our previous studies, initiator caspases gene *Es-Casp* reached the highest level at eight hours after challenge, may be owing to initiator caspases mediate effector caspases [37]. The expression level of EsCaspase-3-like has a dramatic increase from 6 h to 12 h, which may be caused by the regulation of some transcription factors, like STAT family protein. These transcription factors may be up-regulated by PAMPs, or down-regulated by the factors that induced by PAMPs, and participate in the positive or negative regulation of EsCaspase-3-like mRNA expression post PAMPs challenge, but the mechanism of this phenomenon still remains unclear and need to be studied in the future.

Notably, the observed regulation of EsCaspase-3-like by PC stimulation was different from that of other stimulations. This result supports the idea that effector caspases respond differently to immuno-stimuli, which may be related to the affinity with different apoptotic pathways [38,44]. Similar phenomena occur across species from mammals [39,40] to fish (e.g., *Paralichthys noheardi*). Therefore, we inferred that this *E. sinensis* caspase belongs to the caspase-3 subfamily.

In order to confirm the subfamily classification and biological function of EsCaspase-3-like, a recombinant expression vector for this protein was constructed. The rEsCaspase-3-like protein was expressed in *E. coli* and purified to homogeneity via Ni-NTA Purification System subsequently. The molecular mass of the EsCaspase-3-like protein with a thioredoxin protein tag was ~70 kDa as observed on a 12% SDS-PAGE gel, similar to the earlier reported caspase 3C from *M. rosenbergii* [17]. Furthermore, the activity of rEsCaspase-3-like was highly specific towards Ac-DEVD-pNA, which is a substrate of the caspase-3 subfamily. The same result was found for previously reported recombinant caspase-3 proteins from zebrafish [34], prawn [17] and croaker [28]. Thus, the combined evidence supports EsCaspase-3-like as a functional analogue of the vertebrate caspase-3.

Apoptosis occurs in almost all organs of all multicellular organisms to eliminate damaged or unnecessary cells. In healthy organisms to eliminate damaged or unnecessary cells. In healthy
that different caspase-specific responses to bacterial and other pathogens exist. Importantly, the enzyme activity of caspase-3 was also found to be up-regulated by PAMPs in hemocytes. However, differences were found between changes in EsCaspase-3-like mRNA levels and enzyme activity after stimulation. EsCaspase-3-like mRNA peaked at 12 h after stimulation with LPS and Glu, while its enzyme activity reached the highest levels at 24 h. This discrepancy indicated a regulatory mechanism for Ac-DEVD-pNA. EsCaspase-3-like activity of the sample at 0 h was defined as 1 and used as a relative index to compare with that of samples at other times. Values are means ± standard error; *P < 0.05.

In conclusion, the crustacean EsCaspase-3-like protein was identified as a caspase in this study with strong evidence to support that it is a typical effector caspase. We successfully obtained the functional rEsCaspase-3-like protein and demonstrated its high specificity for Ac-DEVD-pNA as a substrate. We examined the regulation of this caspase gene by PAMPs in crustaceans and also found that the enzyme activity of EsCaspase-3-like to be up-regulated by PAMPs in hemocytes. Finally, our work contributes to the knowledge of evolutionary conservation and the role of apoptosis in crustacean innate immunity.

References


