



## Short communication

Cloning and characterization of a novel *caspase-8*-like gene in *Crassostrea gigas*

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## ABSTRACT

Cysteine-dependent aspartate-directed proteases, or caspases, play key roles in apoptosis and immune defense. In this study, we cloned the first *caspase-8*-like gene (*CgCaspase8-2*) identified in the pacific oyster, *Crassostrea gigas*. The 2572-bp cDNA encodes a putative protein of 714 amino acids that contains two tandem death effector domains (DEDs) at the N-terminal, and P20 and P10 domains at the C-terminal. The conserved pentapeptide motif QACQG was also identified in the deduced *CgCaspase8-2* protein. Phylogenetic analysis indicated that *CgCaspase8-2* was clustered with initiator caspases in the invertebrate subgroup, but the similarity between *CgCaspase8-2* and other invertebrate *caspase-8*s was low. *CgCaspase8-2* protein was localized in the cytoplasm, and over-expression of *CgCaspase8-2* in HEK293T cells induced cell death, suggesting a role in apoptosis. Quantitative real-time PCR results demonstrated that *CgCaspase8-2* was widely expressed in various tissues and developmental stages, with the highest *CgCaspase8-2* expression levels detected in hemolymph and the blastula stage. Furthermore, *CgCaspase8-2* transcripts showed no change in response to a bacterial challenge but exhibited notable up-regulation post-poly (I:C) challenge, suggesting that *CgCaspase8-2* is specifically involved in immune responses against viruses. In summary, *CgCaspase8-2* is involved in both apoptotic and immune function.

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## 1. Introduction

Apoptosis was first described in 1972 by Kerr [1]. It plays a key role in immune system homeostasis and function, pathogen defense, and self/non-self recognition [2]. Apoptosis is divided into intrinsic and extrinsic pathways based on the nature of initiating signals. Intrinsic apoptosis involves mitochondria and the endoplasmic reticulum, and occurs in response to cytotoxins and environmental stress [3]. The extrinsic pathway is activated through interactions between extracellular ligands and death receptors (DRs, e.g., Fas/CD95, TNFR, and DR4/DR5 [4]), which then initiate a

cascade through recruiting downstream adaptors that trigger caspases [5–7]. This pathway has been characterized in the amphioxus [8] and is therefore highly conserved in chordates.

*Caspase-8*, an initiator caspase, plays a critical role in the regulation of programmed cell death [9]. It is involved in the extrinsic apoptotic pathway through its association with Fas-associated protein with death domain (FADD) to form the death-inducing signaling complex (DISC). Apoptosis is executed when DISC triggers *caspase-3* [10]. Moreover, *caspase-8* has been implicated in other roles beyond apoptosis [11–13], especially in immune function [2,14–17]. Specifically, *caspase-8* appears to mediate *caspase-1* processing and innate immune defense in response to a bacterial blockade of NF-kappa B and MAPK signaling [14]. Studies investigating the immune function of this enzyme have focused on members of Mollusca in particular. Notably, the role of *caspase-8* in anti-bacterial response has been demonstrated in *Haliotis rufescens*, *Haliotis discus*, *Haliotis diversicolor*, *Mytilus galloprovincialis*, and *Crassostrea hongkongensis* [18–22].

The Pacific oyster, *Crassostrea gigas*, is the first species of Mollusca to undergo whole-genome sequencing [23]. This oyster is of

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vital economic and ecological importance, with the highest production of all cultivated aquatic species [24]. However, *C. gigas* cultivation has suffered drastically from high mortality in recent years, due to a complex interaction of biotic and abiotic factors, with pathogen infection playing a key part [25]. The apoptosis system has been reported to be highly involved in molluscan immunity [4], but except for the characterization of some apoptosis-related genes [2,4,18–22,26], we know little about the apoptotic pathway in this model organism, and indeed in marine mollusks as a whole.

To date, one *CgFADD* and *CgCaspase-3* gene, respectively located up- and downstream of the extrinsic apoptotic pathway, have been cloned and characterized in *C. gigas* [4,26]. Recently, a *caspase-8*-like gene (*ChCaspase-8*) has also been characterized in the closely related *C. hongkongensis* [21], prompting a homologous comparison to *C. gigas* that yielded two *caspase-8*-like genes. One gene was extremely similar to *ChCaspase-8* and was named *CgCaspase-8-1* (unpublished data), while the other was named *CgCaspase-8-2*. Owing to the importance of *caspase-8* in the extrinsic apoptotic pathway and immune response, these findings are of particular relevance to research on programmed cell death and molluscan immunity.

In this report, we cloned *CgCaspase8-2* to better understand its role in *C. gigas*, as preliminary analyses indicated differences from *ChCaspase-8* (unpublished data). We first compared its deduced protein sequence with other invertebrate and vertebrate model species, including with *C. hongkongensis*, to understand its function in a phylogenetic context. Next, we examined the subcellular localization of *CgCaspase8-2* protein and its ability to induce cell death. Furthermore, we detected *CgCaspase8-2* mRNA expression levels in multiple tissues, at different developmental larval stages, as well as post-bacterial and poly (I:C) challenges.

## 2. Materials and methods

### 2.1. Study subjects, tissue collection, and cell culture

Live Pacific oysters were bought from Qingdao, Shandong province, China, and cultured in seawater tanks at  $22 \pm 1^\circ\text{C}$  for 7 days before treatment. To detect the tissue distribution of *CgCaspase8-2* transcripts, samples of hemolymph, adductor muscles, mantles, gills, gonads, and labial palp were collected from six healthy oysters. To investigate the role of *CgCaspase8-2* at different developmental stages, we collected larval samples of the following stages: fertilized egg (0 h post-fertilization), blastula (5 h post-fertilization), gastrula (8 h post-fertilization), trochophore (12 h post-fertilization), D-shaped veliger (24 h post-fertilization), umbo veliger (8 days post-fertilization), and pediveliger (35 days post-fertilization) larva. Seawater temperature was maintained at  $23 \pm 1^\circ\text{C}$ .

Phosphate buffer solution (PBS, pH 7.2) containing *Vibrio alginolyticus* (strain no. MVM425) and polyinosinic-polycytidylic acid dsRNA (poly (I:C), InvivoGen) were used for the bacterial and simulated-viral challenges, respectively. For *V. alginolyticus*, many anti-bacterial genes has been reported to be upregulated after challenge [21,27–29]. The *V. alginolyticus* bacterial culture concentration was determined by measuring the optical density at wavelength 550 nm (1 unit at OD 550 is equivalent to  $5 \times 10^8$  bacteria/mL) [25], while the working concentration of poly (I:C) was 1.0 mg/mL. We randomly divided 150 oysters into control (50 oysters) and experimental groups (100 oysters). Control oysters received 100- $\mu\text{L}$  PBS injections into the adductor muscle. Experimental oysters were equally subdivided into bacterial or simulated-viral challenge groups and injected with 100- $\mu\text{L}$  *V. alginolyticus* or poly (I:C), respectively. Since a number of immune-related genes in

oyster were detected in hemolymph after bacterial and viral challenges [4,27,29,30], the hemolymph of six oysters was randomly sampled from both experimental groups at 0, 6, 12, 24, 48, and 72 h post-challenge.

HeLa cells (ATCC, Manassas, USA) and HEK293T cells (ATCC, Manassas, USA) were grown in RPMI-1640 medium (HyClone, Logan, UT, USA) and DMEM/High Glucose medium (HyClone), respectively [26]. The former cell line is used for cell viability assays and the latter for visualizing subcellular localization. Both media were supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL). Cells were cultured under a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ .

### 2.2. RNA extraction and cDNA cloning

RNA of *C. gigas* samples were extracted using TRIzol reagent (Invitrogen, USA) according to manufacturer protocol. cDNA was reverse-transcribed from 1  $\mu\text{g}$  of total RNA in a 20- $\mu\text{L}$  reaction mixture, using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan), following manufacturer protocol. Based on the predicted *caspase-8* sequence in the *C. gigas* genome (GenBank accession number: CGI\_10023960), we designed primers cas8F1 and cas8R1 to obtain the middle sequence of *CgCaspase8-2*. We then used the amplified fragment and applied RACE (rapid amplification of cDNA end) technology to obtain the 3' and 5' ends of *CgCaspase8-2*. For the 3' end, first-round PCR primers were cas8-3F1 and oligo (dT)-adaptor, while second-round primers were cas8-3F2 and anchor primer (AP). For the 5' end, first-round primers were cas8-5F1 and oligo (dG)-adaptor, while second-round primers were cas8-5F2 and AP. The 5' end cDNA was purified using the DNA purification kit (Shenggong, China) and a poly (C) tail was appended using terminal deoxynucleotidyl transferase (TaKaRa, Japan). All sequences for the primers used are listed in Table 1.

### 2.3. Sequence analysis of *CgCaspase8-2*

For *CgCaspase8-2*, the open reading frame (ORF) and amino acid sequences were predicted with the ORF finder and show translate tool in the sequence manipulation suite (<http://www.bioinformatics.org/sms/index.html>), respectively. The calculated molecular mass and the theoretical isoelectric point (pI/Mw) were

**Table 1**  
Primers used in this study.

| Name               | Sequence                                       | Function |
|--------------------|------------------------------------------------|----------|
| cas8F1             | ATGAATGATATAGAGACAGACAATAG                     | clone    |
| cas8R1             | TCACCTGAAGAAGAGTTTTTCC                         | clone    |
| cas8-5F1           | CACGGACGTCAGAGTAAAC                            | 5'RACE   |
| cas8-5F2           | CTCCGCCCTCTGTATCTT                             | 5'RACE   |
| Oligo (dG)-adaptor | GGCCACGCGTCGACTAGTACG10                        | 5'RACE   |
| AP                 | GGCCACGCGTCGACTAGTAC                           | RACE     |
| cas8-3F1           | ACAGGACTGGCGGAAACC                             | 3'RACE   |
| cas8-3F2           | CAGGGAAGCTGGTTCGTCAA                           | 3'RACE   |
| Oligo (dT)-adaptor | GGCCACGCGTCGACTAGTACT16                        | 3'RACE   |
| cas8F2             | GGACTCAGATCTCGAGATGAATGATA<br>TAGAGACAGACAATAG | clone    |
| cas8R2             | GAAGCTTGAGCTCGAGCTGAAGAAG<br>AGTTTTTCC         | clone    |
| cas8RTF            | CAGGGAAGCTGGTTCGTCAA                           | qRT-PCR  |
| cas8RTR            | CCCTCCAGTCGGGCGTTA                             | qRT-PCR  |
| GAPDHF             | TTCTCTTGGCCCTCTTGC                             | qRT-PCR  |
| GAPDHR             | CGCCCAATCCTTGTTCCT                             | qRT-PCR  |
| RS18F              | GCCATCAAGGGTATCGGTAGAC                         | qRT-PCR  |
| RS18R              | CTGCCTGTTAAGGAACCACTCAG                        | qRT-PCR  |
| EFF                | AGTACCAAGGCTGCACAGAAAG                         | qRT-PCR  |
| EFR                | TCCGACGTATTCTTTCGCGATGT                        | qRT-PCR  |

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