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# First molecular cloning of a molluscan caspase from variously colored abalone (*Haliotis diversicolor*) and gene expression analysis with bacterial challenge

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ABSTRACT

Mammal caspases have been demonstrated to possess important functions in apoptosis and immune signaling, but there is less knowledge available on abalone caspases. In the present study, a molluscan caspase gene, *abCaspase*, was cloned for the first time from the variously colored abalone (*Haliotis diversicolor*) and its full-length cDNA sequence was 2427 bp, with a 1008 bp of open reading frame encoding a protein of 336 aa. The molecular mass of the deduced protein was approximately 36.97 kDa with an estimated p*I* of 5.28. The predicted amino acid sequence of *abCaspase* contained two domains of p20 and p10 which were conserved in the caspase family, including the cysteine active site pentapeptide "QSCRG" and the histidine active site signature "HTVYDCVVVIFLTHG". Homology analysis showed that *abCaspase* shared high similarity with apoptotic caspases and it was grouped together with vertebrate caspase-8s and caspase-10s using phylogenetic analysis, suggesting that *abCaspase* belonged to a typical apoptotic caspase and might possess the characteristic of human caspase-8 and -10. The mRNA transcripts of *abCaspase* were widely distributed in various tissues of *H. diversicolor*. Expression of the *abCaspase* gene was significantly induced in the tissues tested, especially in the hemocytes, gill and mantle with bacterial challenge. This study suggested that *abCaspase* may be an initiator caspase associated with the induction of apoptosis which is potentially involved in the immune defense of *H. diversicolor*.



## 1. Introduction

Caspases are cysteinyl aspartate proteases that can cleave their substrates following an Asp residue, and play key roles at various stages of the apoptotic process [1,2]. In mammals, caspases have been demonstrated to possess important functions in apoptosis and immune signaling, and are categorized into three groups according to their functions: the initiator apoptotic caspases, the effector apoptotic caspases and the inflammatory caspases; and this seems to correlate with their phylogenetic relationship [3]. The initiator caspases are located up-stream in the caspase cascade and receive the apoptotic signals, and then transmit them to activate downstream effector caspases [4]. The effector caspases cleave a wide variety of cellular substrates, leading to the death of cells, and so function as the executioner of apoptosis [4,5].

Apoptosis is a highly regulated and conserved form of active cell death, which is essential for successful embryonic development and the maintenance of normal cellular and tissue homeostasis [6]. It also plays an important role in homeostasis and immune system function [7,8], acting as a protective response [9]. By activation of conserved apoptotic signaling pathways, damaged cells or unnecessary cells are eliminated during embryonic development, tissue remodeling, immune regulation and tumor regression. During the immune response, apoptosis is critical to ensure protective immunity and avoid lymphoid neoplasia and autoimmunity [7]. In some infections, the apoptosis of the infected cell can trigger rapid uptake by neighboring cells and promote the recruitment of phagocytes, finally resulting in rapid digestion of the apoptotic body packed with the pathogen [9,10]. On the other hand, many bacteria are able to trigger apoptosis in host cells [10], possibly serving to eliminate key immune cells or evade host defenses that can act to limit the infection [11].

Interaction between immune cells and parasites or pathogens usually triggers apoptosis in molluscs, however, some pathogens

*Abbreviations: abCaspase*, *H. diversicolor* Caspase homologue; h p.i., hours post-injection; ICE, interleukin-1β-converting enzyme; MW, molecular mass; NJ, neighbor-joining; p*I*, isoelectric point; RACE, rapid amplification of cDNA ends; SSH, suppression subtractive hybridization; TNF-α, tumor necrosis factor α.

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can inhibit this response and prevent host cell death [12]. Despite the description of the involvement of caspases in the apoptotic process of molluscan immune cells [12,13], no molluscan caspase homologue has been identified so far. Because they are regarded as important factors involved in modulating apoptosis, characterization of the caspases in mollusc would be important in understanding their roles in the mollusc immune system. In our previous study, a partial cDNA sequence of a caspase gene was isolated from a forward suppression subtractive hybridization (SSH) cDNA library from abalone, *Haliotis diversicolor*, with bacterial challenge [14]. In this study a complete cDNA sequence of the caspase gene (named *abCaspase*) was cloned, its mRNA distribution in tissues of normal abalone and its expression patterns after bacterial challenge were also determined.

## 2. Materials and methods

### 2.1. Animals

Live healthy female *H. diversicolor* (55 ± 5 mm in shell length) purchased from the Zhangpu abalone farm in Fujian Province, China, were acclimated in the laboratory at a salinity of 30‰ for seven days at 24 ± 1 °C seawater temperature before the experiments. Animals were reared in 80 L PVC tanks containing 60 L natural seawater treated with sand filtration, and fed daily with the marine alga *Gracilaria tenuistipitata*.

### 2.2. Preparation of a mixture of the five bacterial strains

A bacterial suspension was prepared as described previously [14]. Five bacterial species including two Gram-negative bacteria (*Escherichia coli* CGMCC 1.2389 and *Vibrio parahaemeolyticus* CGMCC 1.1615), and three Gram-positive bacteria (*Staphylococcus aureus* CGMCC 1.89, *Micrococcus lysodeikticus* CGMCC 1.634 and *Staphylococcus epidermidis* CGMCC 1.2429) were prepared for the bacterial challenge. The bacterial strains were cultured separately overnight, in 5 mL Difco™ marine broth 2216 at 30 °C for *V. parahaemeolyticus* strains, and in 5 mL LB at 37 °C for the other strains. The bacteria were collected by centrifugation (3000 × g for 10 min at 4 °C) and suspended in sterile saline solution (0.85% NaCl) at a concentration of $2 \times 10^7$ cfu/mL. Equal volumes of the five bacterial suspensions were mixed to provide the stock bacterial suspension for injection.

### 2.3. Bacterial challenge and preparation of samples

For bacterial challenge, abalones were injected with 25 μL of the mixed bacterial suspension via the front of the foot. Abalones injected with an equal volume of sterile saline solution (0.85% NaCl) were prepared as the control treatment. For each time period, three abalones were used in each treatment including bacterial challenge, saline control and normal control groups. Sampling was performed at time intervals of 3, 6, 12, 24, 36, 48, 60 and 72 h after injection. Hemolymph was separately collected by cutting the foot, and hemocytes were then isolated by centrifugation at 800 × g, at 4 °C for 10 min and placed immediately in liquid nitrogen for later use.

Gill, mantle, gonad, foot, epipodium, hypobranchial gland, digestive gland, hemocytes, shell muscle, and kidney tissues were separately collected from each individual abalone, and frozen immediately in liquid nitrogen, followed by storage at −80 °C.

### 2.4. Determination of the abCaspase cDNA sequence

In our previous study, a partial cDNA sequence (HDr4CJ332) with homology to caspase was identified from the SSH cDNA library from *H. diversicolor* hemocytes challenged with bacteria [14]. To obtain the full sequence of HDr4CJ332, rapid amplification of cDNA ends (RACE) 5′ and 3′ was performed. Briefly, total RNAs were extracted from the hemocytes of the bacterial challenged abalones using TRIZOL reagent following the manufacturer's instructions (Invitrogen) and quantified with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Sweden). Based on the partial caspase sequence (HDr4CJ332), the RACE PCR were carried out with primers CasAS1 (5′-TCCATCCACTGTTTCACA TTCACGA-3′) and CasAS2 (5′-AATCTGCCTCTGGAGCAACCATCTT-3′) for 5′, or CasS1 (5′-ATCTGGACAGCATAATGACAACCGT-3′) and CasS2 (5′-CGTGGTAAAGTGAACAATCGTGAAT-3′) for 3′. The first-strand cDNA synthesis and the RACE reactions were performed using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. Expected PCR products were purified from gel using a Qiaquick Gel Extraction Kit (Qiagen), and ligated into the T/A cloning vector pMD18-T (TaKaRa) followed by transformation into *E. coli* X-Blue competent cells. The positive clones identified using PCR were sequenced at least twice using ABI 3730 automated sequencers (Applied Biosystems, USA) at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China).

### 2.5. Sequence analysis

The amino acid sequence was deduced using DNAStar 5.0. Homology searches were performed using BLASTn and BLASTp in NCBI. Molecular mass (MW) and isoelectric point (p*I*) were predicted using the ProtParam tool (http://www.expasy.ch/tools/protparam.html). The protein motifs were analyzed with PROSITE (http://www.expasy.ch/prosite/). Direct comparison between two sequences was performed using BLAST (bl2seq) from NCBI. Multiple sequence alignment was carried out with the ClustalX 1.83 software. The neighbor-joining (NJ) method was used to reconstruct a phylogenetic tree with 1000 bootstrap replicates using MAGA v4.0 software.

### 2.6. Tissue distribution analysis of the abCaspase mRNA in normal abalones

Three healthy individual abalones were used for tissue distribution analysis. The *abCaspase* mRNA transcripts were measured in gill, mantle, gonad, foot, epipodium, hypobranchial gland, digestive gland, hemocytes, shell muscle, and kidney tissues using quantitative real-time PCR. Total RNAs were separately extracted from individual tissues as described above. For cDNA synthesis 0.5 μg of total RNA was reverse-transcribed in a final volume of 10 μL using a PrimeScript™ RT reagent kit (Perfect Real Time) (TaKaRa) following the manufacturer's instructions. Real-time PCR was performed in a reaction mixture of 20 μL containing cDNA obtained from 10 ng of total RNAs, 10 pmol of each gene-specific primer Cas-F (5′-TTACCCTCCCGAAAAAAACTC-3′) and Cas-R (5′-GAAA CAACCAATCCGATACAC-3′) and 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, UK). Reaction mixtures were incubated for 2 min at 50 °C and then denatured for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The actin gene (GenBank: EF587284) was selected as a reference gene and it was amplified using Actin-F (5′-ACCACGGGTATTGTTCTTGAC-3′) and Actin-R (5′-CGGTGGTGGTGAAGGAGTAAC-3′). Since the lowest expression level was observed in hemocytes, the relative expression in other tissues was obtained based on the comparison with that of hemocytes. Results were reported as mean ± S.D. of three animals per group.