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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 2×10^7 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.

2.7. Expression pattern of the abCaspase in abalones after bacterial challenge

The induction profile of the *abCaspase* expression after bacterial challenge was investigated in digestive gland, gill, gonad, hemocytes, hypobranchial gland, kidney, mantle and shell muscle tissues using real-time PCR. The *abCaspase* expression was determined using the $2^{-\Delta\Delta CT}$ method with healthy group data as the calibrator. Results were described as mean ± S.D. of three animals per group. Statistical analysis of differences was carried out using SPSS 13.0 and one-way analysis of variance. Student's *t*-test was used to determine the differences between the bacterial challenged and control groups. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Determination of the abCaspase cDNA sequence of H. diversicolor

The abalone caspase cDNA obtained was named *abCaspase* (GenBank accession number FJ628161). Its full-length was 2427 bp,

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1    GAGGATCGGACACGACAACGGAGGACGGTTGCAGCATCACCATGGCAGAAGGTTTAAAGATAGAGATTGAGGCAGGCGATGGAAAATCTAAT   92
1                                                M  A  E  G  L  K  I  E  I  E  A  G  D  G  K  S  N   17
93   AATTTAGTTATGGCCGCCGTTGTCACTGGAGGAGTCGACAATAAAGGTCAACCAAAGTCAGAAGGCATCAAATCAGATCTCTCAGCAACT   182
18    N  L  V  M  A  A  V  V  T  G  G  V  D  N  K  G  Q  P  K  S  E  G  I  K  S  D  L  S  A  T    47
183  AAGATAAAAATGAAGGGCCCCGGCAAAGATTCTCAACATAGAGATGTGAAGATGTTGGGTATTGTGGAAGGAGGGATGGCGTGTCAAGAC   272
48    K  I  K  M  K  G  P  G  K  D  S  Q  H  R  D  V  K  M  L  G  I  V  E  G  G  M  A  C  Q  D    77
273  GTCTCGGAAGATACTGACGGTAGAGGTCAAGGTCAAGAGGCTCGCTATAAAATGGACTCTGAACCGAGAGGGTTGTGTTACATATTTAGC   362
78    V  S  E  D  T  D  G  R  G  Q  G  Q  E  A  R  Y  K  M  D  S  E  P  R  G  L  C  Y  I  F  S   107
                                                                    P20
363  AACAAGACCTTCGAAGATAAACCTAAGCAAACGAGAATAGGAACCGAGAAGGACAGAGATGCGTTGACAGAAGTATGGAAAAAACTCCAT   452
108   N  K  T  F  E  D  K  P  K  Q  T  R  I  G  T  E  K  D  R  D  A  L  T  E  V  W  K  K  L  H   137
453  TTTGAGGTGGAATCATTTGACGACTTCAAAGCTGAAGGCATGGAAACAAGGATTAAGGAACTTGCCAAGATGGACCACACTGTCTATGAC   542
138   F  E  V  E  S  F  D  D  F  K  A  E  G  M  E  T  R  I  K  E  L  A  K  M  D  H  T  V  Y  D   167
543  TGTGTAGTCGTGATCTTCCTGACCCACGGGCGAGATGGAGCGGTGGAGGGTTCCGACAACGAGTGGCTGGACGTTAGTACCATCACATCT   632
168   C  V  V  V  I  F  L  T  H  G  R  D  G  A  V  E  G  S  D  N  E  W  L  D  V  S  T  I  T  S   197
633  TACTTCATGCCGTCGGTGTGTCCGTCACTGGCCAGCAAGCCGAAGCTGTTTTTCCTTCAGTCATGTCGAGGAAAGGAAATCCAAAAAGCC   722
198   Y  F  M  P  S  V  C  P  S  L  A  S  K  P  K  L  F  F  L  Q  S  C  R  G  K  E  I  Q  K  A   227
723  GTTCCGGTTCAAGGTGATGATGGCGGTAGTGATGCTGATGCTACAGAAACTATCGTTCAGGCTAACAAGATGGTTGCTCCAGAGGCAGAT   812
228   V  P  V  Q  G  D  D  G  G  S  D  A  D  A  T  E  T  I  V  Q  A  N  K  M  V  A  P  E  A  D   257
                                                                 P10
813  TTCTTCTTTGGGTTTGCTACAGTAGCGGGAATGCAGGCTTTAAGAAACCCGGAGACTGGCAGCTGGTTCATTCAGTCGCTTGTCCAAGCT   902
258   F  F  F  G  F  A  T  V  A  G  M  Q  A  L  R  N  P  E  T  G  S  W  F  I  Q  S  L  V  Q  A   287
903  CTGCAAGCATCTACAAAGAGTGATCTGGACAGCATAATGACAACCGTACGTGGTAAAGTGAACAATCGTGAATGTGAAACAGTGGATGGA   992
288   L  Q  A  S  T  K  S  D  L  D  S  I  M  T  T  V  R  G  K  V  N  N  R  E  C  E  T  V  D  G   317
993  AAGTTTCTCAGACAAACAGCTGAGGTGCGGACGACGCTACTGAAGAAACTTTACTTCTGAAGAGGCTATGGCCTCACAGAATGGACAGTG  1082
318   K  F  L  R  Q  T  A  E  V  R  T  T  L  L  K  K  L  Y  F  *                                  336
1083 ATACCATTACCCTCCCGAAAAAAACTCAGCGCTCTGCACAATGTGCTTACATAGTCATTAAACATGAAGCGATGGATATAGAAAGAGATT  1172
1173 AGAGGTTAAGGTATGCCATCTGAAAAATGTGACAAAAAGAGAACTCCTGTGGCAGACACTGTATTTCATTGTGGTTTATAGTTACGTCAT  1262
1263 TGAGCAAGACAAAGTGTATCGGATTGGTTGTTTCAAATCACAGAAACGCGTCCATTTTATGTTTAGCATTAACACTTTCGTGTGTCTCGA  1352
1353 ACCGCACGATGGAGAATTTGCTCAGTGTGTCGGTTCAGTTTTAAGGAAGCAGTACAGATACACCTACACCTTTAAATACTTACTAGGCTT  1442
1443 AGATGGTAACGCTGGGCCTTGTCTACTGAGGCAGGGCTGCCTTCCTTGGGAGCATAGAAACCATGTCCAGGGGTGCGAATGGACTTGGTA  1532
1533 AGACGAACCCAGTAAATGTCCCCCTTGTCGGACTGTACTCCTACACAACGCTAACACGCCGTGATAAGTCTGGTATACCCTTCAAACAGC  1622
1623 GTTAAACCCAAACTCTCTCACCAACCCTCCCACCAGCTAACCCGCTCACCCACCGACTCACAAACTCAATAACCACCAACCCTTTTTCTC  1712
1713 AATTACTGGAATACCGTTCAAAACGGCGTTCAATCAAACTCACTCACTCATCAATCTACCCACCCACTAACCCAGTCGCTGACTCATTCA  1802
1803 CCCACCAACCCATATACCCATTAACTCAACCATTCACTCACACACCCACTCACTCAGTCCCAGGGGATAAAACTGGTAGACCATTCAAAT  1892
1893 TGGCGTTAAACCCAACTCATTCACTCACTCACTCACTGACCCACCCTTTCACCCAATACCTCTTCCATTCACGCACACCCCCACTCAGTC  1982
1983 ACTTACTCCCAGAAAATACTACAGGAATACCGTCGTTCAAAACGGCGTTAAACCCGACTCACTCACCAGTCTACCCGCCCACTAACCCAC  2072
2073 CCACCCACTGACCCATTAACCCAGTAACTCACCCATTCACTCACACGCACACACACTCACTCACTCACTCACTGGAGATACAACTGGATC  2162
2163 GCCGTTCAAAACGGCGCTAAACGCAGCTATATGTACGGCAATGTACATGTACTTACAAGTTGAATGTTCTGTGTAATATGTACAAAGTGG  2252
2253 TGATGAAGGACGTAGGTAGAGATGTCTTTGTAGTTAATCGTATAATTTTATATGTAAATGGAGTCCAGGTTAATATATGTGTCTCAAGGT  2342
2343 TCTCTTTTTTCAGAGAATGCCAATGCTGAATACATTCTACTAAAATTGAATACATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  2427
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Fig. 1. Complementary DNA and predicted amino acid sequences (GenBank accession number FJ628161) of *abCaspase* from *H. diversicolor*. The polyadenylation signal is underlined and the stop codon is indicated by "*". The large P20 subunit and small P10 subunit are marked in gray. The caspase family histidine active site and the pentapeptide in the cysteine active site are boxed.

which contained an open reading frame of 1008 bp with an encoding protein of 336 amino acid residues, a 5′-UTR of 41 bp, and a 3′-UTR of 1378 bp including a poly(A) tail with a putative polyadenylation signal presented at 1329 bp downstream of the stop codon (Fig. 1). The MW of the deduced protein was approximately 36.97 kDa with an estimated p*I* of 5.28. The ATG start codon was located within a favorable context for translation according to the Kozak rule [15]. ScanProsite analysis of the deduced amino acid sequence of the *abCaspase* showed that the caspase family p20 domain was located at residues 99–223, the caspase family p10 domain spanned residues 248–336, and the caspase family histidine signature HTVYDCVVVIFLTHG was presented between 163 and 177 amino acid residues (Fig. 1). A variant pentapeptide motif QSCRG as the caspase family cysteine active site was also found at residues 217–221(Fig. 1). These structures demonstrated characteristics typical of the caspase family.

The deduced protein sequence of *abCaspase* showed a maximum identity of 43.1% with the cartilaginous fish (*Leucoraja erinacea*, ACH96579) caspase-8 based on the BLASTp results. Based on the homology analysis of the amino acid sequences between the *abCaspase* and mammalian caspases, it was found that the *abCaspase* shared 35.9% and 38.0% identity with human caspase-8 and -10, relatively higher than that with any other mammalian caspase. In addition, the *abCaspase* shared higher identity with those caspases involved in the apoptotic response than those in the inflammatory response. An NJ phylogenetic tree of amino acid sequences of *abCaspase* was reconstructed with different members of the caspase superfamily selected from vertebrates and

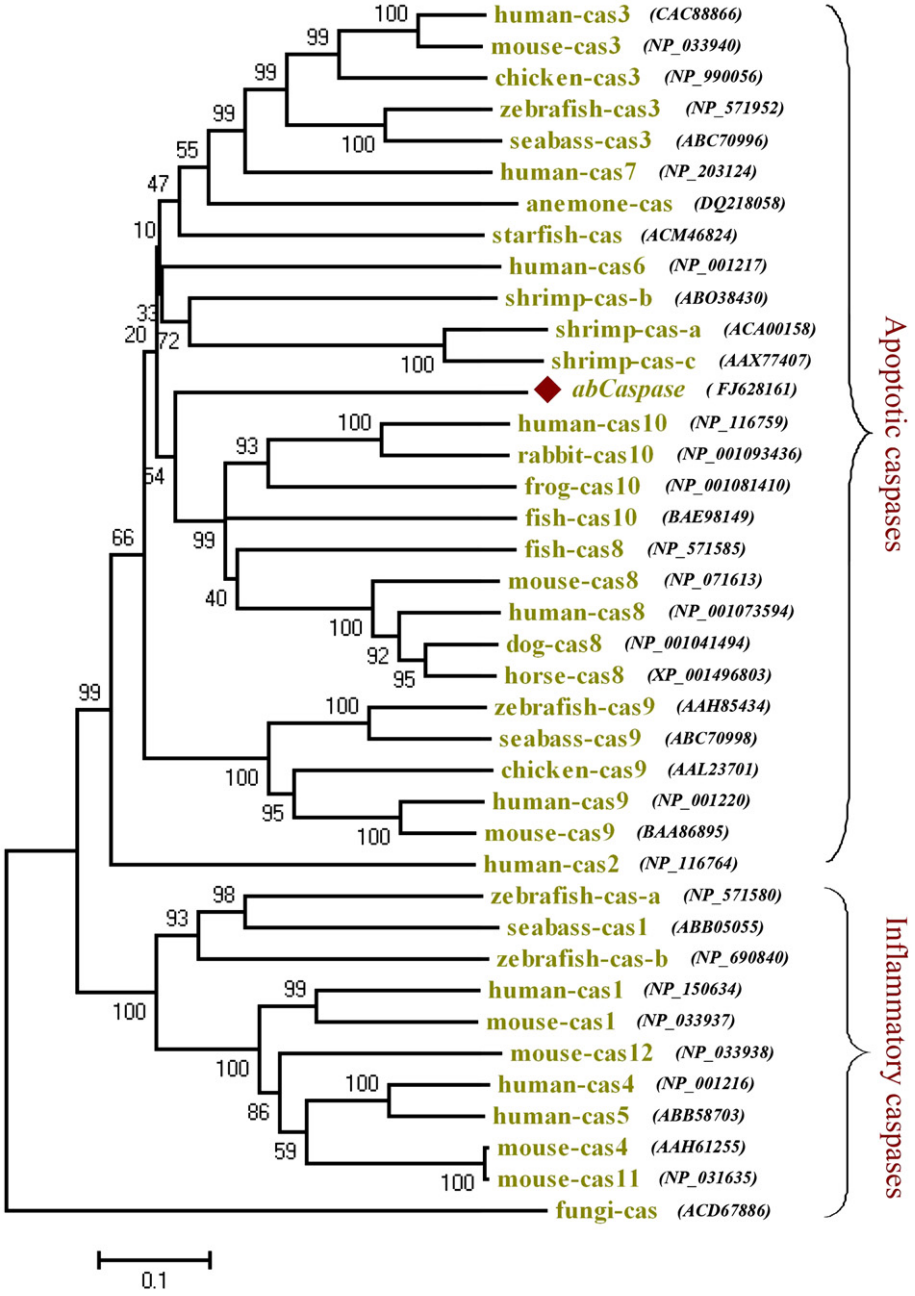

Fig. 2. Phylogenetic analysis of deduced amino acid sequences from *abCaspase* and other caspases obtained from GenBank using MEGA 4.0. Italics in brackets represent the GenBank accession number of the sequences. *AbCaspase* is marked by "◆". Numbers next to the branches indicate bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates.

invertebrates using MEGA V4.0.2 software. A caspase of the fungus *Pichia angusta* (ACD67886) was rooted to build the phylogenetic tree. As shown in Fig. 2, all selected caspases were divided into two distinct clades in the phylogenetic tree and *abCaspase* was in a branch of apoptotic caspases and apart from the inflammatory caspases. Within the apoptotic caspases branch, all the sequences of caspase-8, caspase-9, caspase-10 as well as caspase-3 from different species were clustered in their groups respectively. The *abCaspase* was branched in a unique subfamily constructed by caspase-8s together with caspase-10s, as shown in Fig. 2.

To reveal possible evolutionary relationship of the *abCaspase* with the vertebrate caspase-8s and -10s, their protein domain

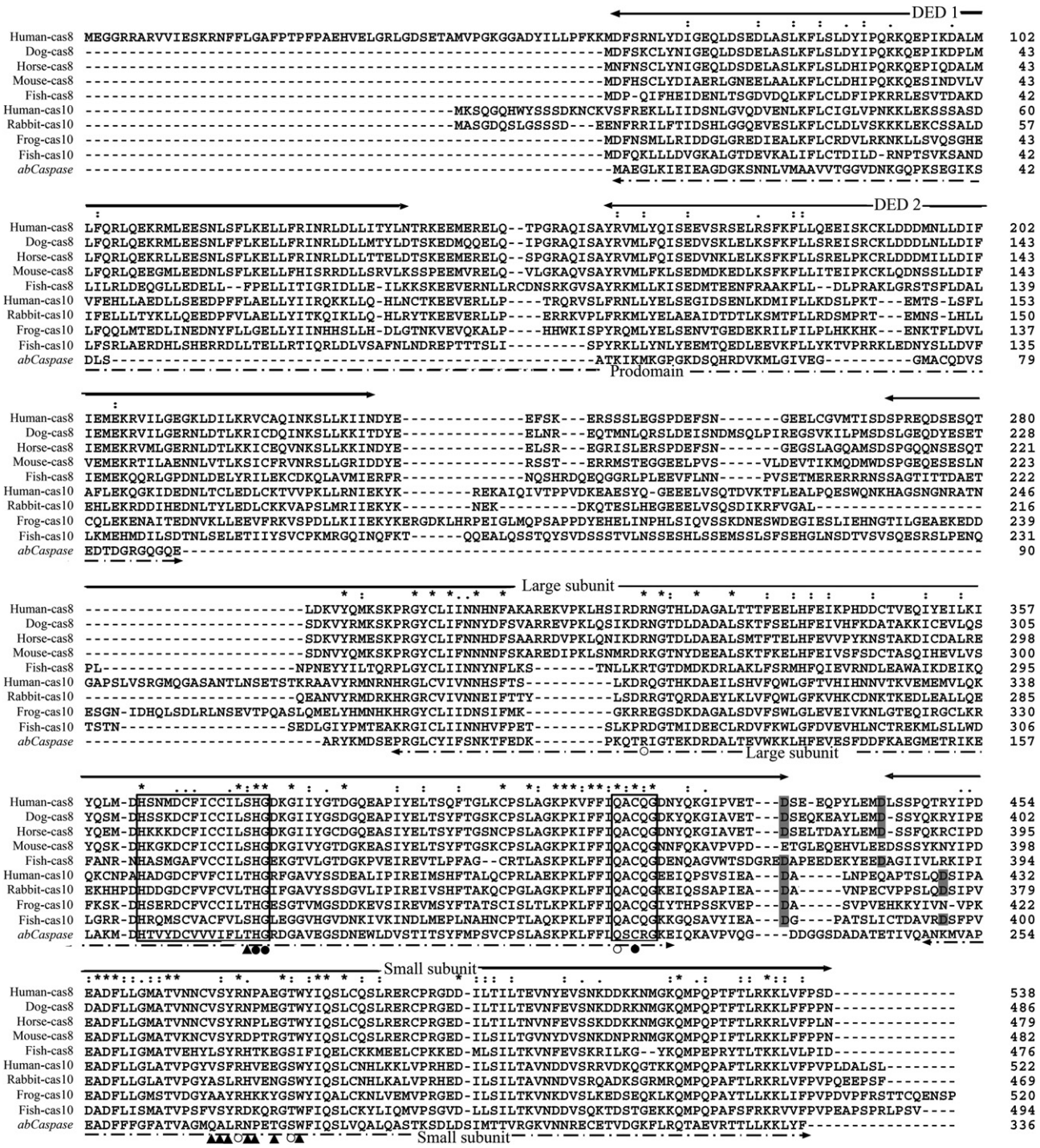

Fig. 3. Multiple alignment of amino acid sequences of *abCaspase* and vertebrate caspase-8s and -10s. The caspase family histidine active site and the pentapeptide in the cysteine active site are boxed in a continuous line. The putative cleavage sites at aspartic acid residues are shaded in gray. The continuous arrowheads indicate the domain structure of human-cas8, and the dashed arrowheads indicate the domain structure of *abCaspase* predicted by PROSITE. The residues putatively involved in catalysis are indicated with filled circles. The residues constituting the binding pocket for the carboxylate of the P1 Asp are marked by unfilled circles. Filled triangles represent the residues adjacent to the P2–P4 amino acids. Amino acid residues are numbered to the right of each sequence. Caspase amino acid sequences are obtained from GenBank as follows: human-cas8: NP_001073594, dog-cas8: NP_001041494, horse-cas8: XP_001496803, mouse-cas8: NP_071613, fish-cas8: NP_571585, human-cas10: NP_116759, rabbit-cas10: NP_001093436, frog-cas10: NP_001081410, fish-cas10: BAE98149.

structures were compared by scanning with PROSITE. All the vertebrate caspase-8s and caspase-10s contained two DED domains in their N-terminal region as well as one P20 large subunit and one P10 small subunit in the C-terminal region. However, the *abCaspase* lacked the N-terminal DED domains but contained the P20 and P10 subunits in the C-terminal region (Supplementary Fig. 1). Multiple alignment of the deduced protein sequences between the *abCaspase* and the vertebrate caspase-8s and -10s was performed. The result showed that a putative prodomain was predicted in two separated regions which were located in positions corresponding to the two DED motifs of human caspase-8 (Fig. 3). The two subunits in the C-terminal were highly homologous and conserved (Fig. 3). The histidine active site and the cysteine active site in the caspase family were found to be located in the corresponding position of the large subunit. It was noted that the *abCaspase* contained a unique pentapeptide QSCRG instead of the QACQG which were shared by all the vertebrate caspase-8s and -10s (Fig. 3). The conserved amino acid residues His^{176}, Gly^{177} and Cys^{219} of the *abCaspase* corresponding to the human caspase-8 His^{376}, Gly^{377}, and Cys^{419} are predicted to be involved in catalysis [16,17] (Fig. 3). Furthermore, the residues involved in forming a binding pocket for the carboxylate side chain of the P1 aspartic acid were also conserved. One exception was a conservative and alternative change of Thr to Ser in the Thr^{478} position of human caspase-8 (Fig. 3). However, residues forming the P2-P4 binding pockets were less conserved, suggesting a specificity in substrate selection (Fig. 3).

3.2. Tissue-specific expression of the abCaspase

Tissue-specific expression of *abCaspase* was analyzed using real-time PCR in the various tissues of the normal *H. diversicolor*. As shown in Fig. 4, the *abCaspase* mRNA transcripts were demonstrated in all tissues tested, but the amount of transcripts in each tissue varied. The highest expression level was present in the gill and epipodium, and a relatively high expression was also observed in digestive gland, hypobranchial gland and mantle tissues. The *abCaspase* gene transcripts showed a relatively low expression in the other tissues tested, particularly in the hemocytes.

3.3. Analysis of the abCaspase gene expression pattern in abalones with bacterial challenge

The *abCaspase* gene expression pattern in the different abalone tissues was evaluated after bacterial challenge using real-time PCR. As shown in Fig. 5, the *abCaspase* gene was significantly expressed in both hemocytes and other tissues tested in comparison with that of the saline control. A significant expression (8.5-fold, $P < 0.05$) of *abCaspase* mRNA was observed in hemocytes 3 h post-injection (h p.i.), and it maintained a high level until 36 h in the bacterial challenged group. The *abCaspase* expression showed 6.6-fold, 5.3-fold, and 11.2-fold control ($P < 0.05$) at 6, 24, and 36 h p.i. It was interesting to note that the expression level of *abCaspase* in hemocytes decreased to the control level at 48 h, but later the gene expression increased again and showed 2.5-fold control ($P < 0.05$) at 72 h (Fig. 5). Moreover, the *abCaspase* mRNA was relatively more highly expressed in the hemocytes, gill and mantle than in other tissues. The *abCaspase* gene was induced in the gill and mantle at 3 h p.i. and was maintained at a high level until 36 h in the bacterial challenge group. In gonad tissue, it was found that the *abCaspase* expression was induced at 3 and 6 h p.i., fell to the control level from 12 to 60 h, and then increased again at 72 h p.i. (Fig. 5). The *abCaspase* gene was also significantly expressed in the kidney at 12, 36 and 72 h p.i., and in the shell muscle at 3 and 24 h p.i. (Fig. 5). In contrast, the induction of the *abCaspase* gene was observed only at 3 h p.i., showing 4.1-fold and 7.3-fold control in the digestive gland and hypobranchial gland (Fig. 5). Together, these results suggested that tissue- and time-dependent expression occurred in abalones after bacterial challenge.

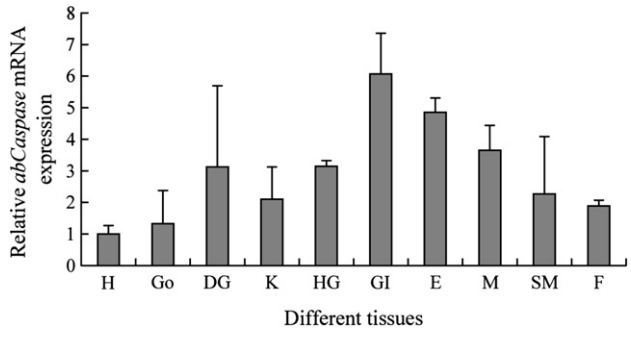

Fig. 4. Distribution of *abCaspase* mRNA transcripts in 10 tissues of normal variously colored abalone analyzed using quantitative real-time PCR. The relative mRNA expression of each tissue was compared to hemocyte expression in order to determine the tissue-specific expression. Each bar represents the mean ± SD of three replicates. H – hemocytes; Go – gonad; DG – digestive gland; K – kidney; HG – hypobranchial gland; GI – gill; E – epipidium; M – mantle; F – foot; SM – shell muscle.

4. Discussion

Programmed cell death during larval metamorphosis, and apoptotic processes in immune cells have been revealed in molluscs [13,18,19], but no molluscan caspase homologue has been reported so far. In this study, a new caspase homologous gene from *H. diversicolor* was cloned and its gene expression was investigated. To the best of our knowledge, this was the first caspase gene to be identified from a mollusc. The abalone caspase sequence obtained (*abCaspase*) had the caspase family signature and the conserved cysteine active site pentapeptide as well as the typical p20 and p10 domains of the caspase family. In addition, homology analysis showed that *abCaspase* shared higher identity with those caspases involved in the apoptosis response than those in the inflammatory response and phylogenetic analysis indicated that *abCaspase* was grouped in a distinct branch on the phylogenetic tree with caspase-8s and caspase-10s, suggesting that the *abCaspase* might be the homologue of either mammalian caspase-8 or caspase-10. In humans, caspase-8 and caspase-10 share a similar protein structure and both can activate downstream caspases to initiate apoptosis [17,20]. Due to both genes being located closely in the same chromosome, they are thought to arise from one gene by tandem duplication [5,21]. It is reported that vertebrates have more genes than other animal taxa because of gene duplication [22,23], and the numbers of caspases seem to have increased with increasing evolutionary complexity [3]. In view of the above, we hypothesized that the evolutionary abruption of caspase-8 and caspase-10 might not occur in abalone, so that *abCaspase* as the current isoform in abalone would be the ancestral orthologue of vertebrate caspase-8 and caspase-10.

Caspases are produced in cells as inactive zymogens, which need to be proteolytically cleaved at internal Asp sites to generate the active form. Vertebrate caspase-8 and -10 have two DED motifs in the prodomain, and these motifs are responsible for the autoactivation of inactive proenzymes into active forms [24] but they were not found in the *abCaspase* amino acid sequence. Recently, a caspase (designated as *PjCaspase*) without DED was cloned from the marine shrimp *Marsupenaeus japonicus*, and the authors suggest that the prodomain preceding the p20 subunit of *PjCaspase* might reveal an ancient mechanism of caspase activation [25]. In the present study, the putative prodomain was analyzed in the *abCaspase*, which may strengthen the argument that the DED

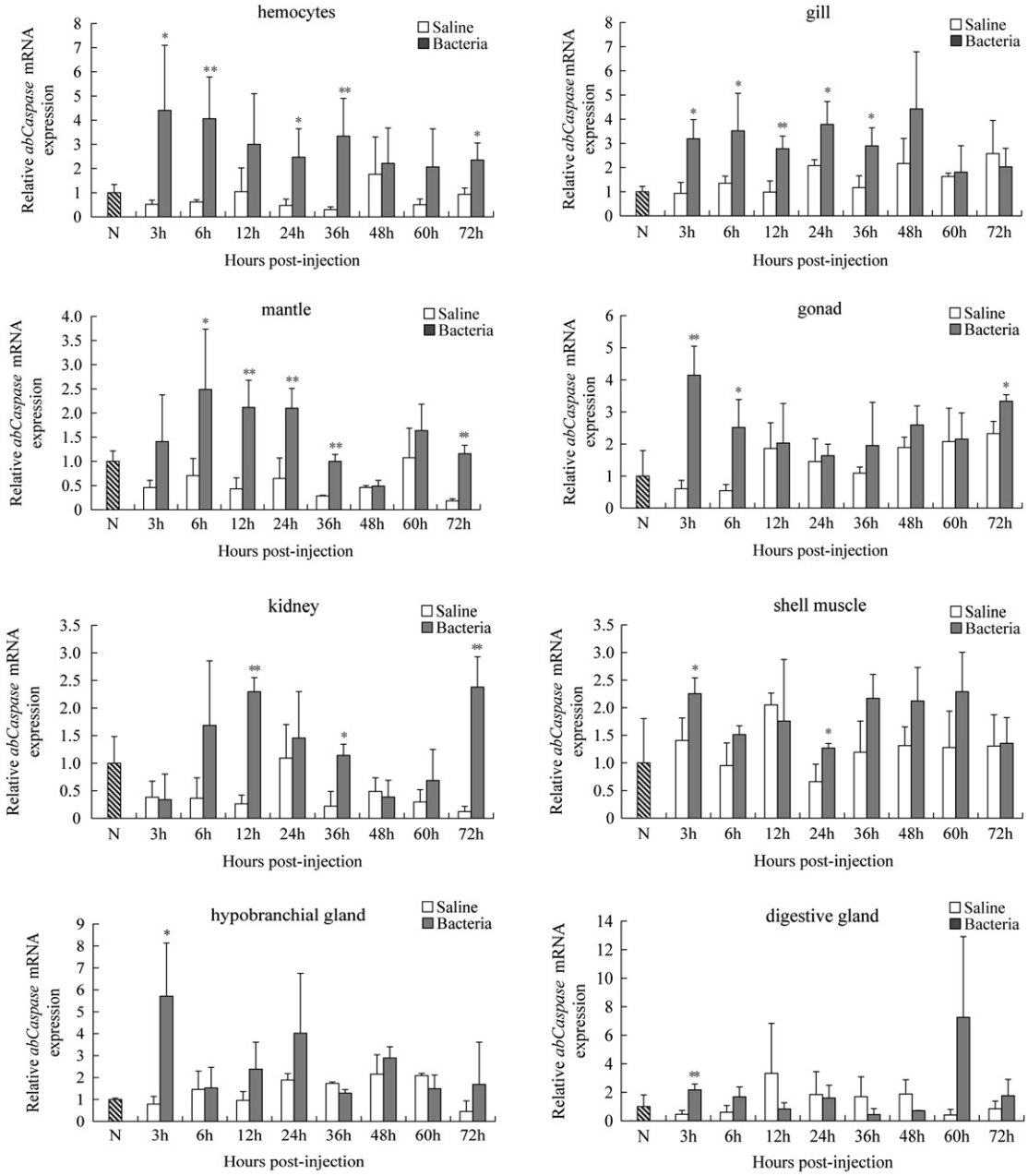

Fig. 5. Message RNA expression of *abCaspase* in abalone hemocytes, gill, mantle, gonad, kidney, shell muscle, hypobranchial gland and digestive gland after bacterial challenge. "N": the normal group. Each bar represents the mean ± SD of three replicates. Statistical analysis of differences between the saline control and the bacterial challenged groups was carried out using one-way analysis of variance with SPSS 13.0 software. Differences are considered statistically significant (*) at $P < 0.05$, and very significant (**) at $P < 0.001$.

motif was evolved later, reflecting a demand for specific recruitment in receptor complexes [3]. In humans, mature caspase-8 and caspase-10 were generated from procaspase-8 by cleaving the sites of VETD^{433}S and LEMD^{443}L and from procaspase-10 by cleaving IEAD^{415}A and SLQD^{428}S between the large and small subunits, respectively [20,26]. These cleavage sites are quite conserved among vertebrate species (Fig. 3). However, these cleavage sites were not predicted in the sequence of the *abCaspase* in the present study. Caspases play the central protease functions in apoptosis that cleave target proteins at specific sites with typical aspartic acid residues [27]. Previous studies show that the several critical amino acid residues of human interleukin-1β-converting enzyme (ICE)/caspase-1 are involved in catalysis (His^{237}, Gly ^{238} and Cys^{285}) and in forming a binding pocket (Arg^{179},Gln^{283},Arg^{341} and Ser^{347}) for P1 aspartic acid in the substrate based on the X-ray crystal structure of ICE/caspase [28,29]. These residues are conserved in all ICE/CED-3 family members including human caspase-8 and -10 [16,17,20]. Consistently the residues forming a P1 binding pocket were found in the predicted amino acid sequence of *abCaspase*, reflecting the absolute requirement for an Asp at position P1 for substrate cleavage [30].

Caspases are reported to be widely distributed in fish and shrimp. Caspase-3 and caspase-9 are constitutively expressed in various tissues of sea bass (*Dicentrarchus labrax*) [31,32], and caspase-1 expression is found in various tissues of gilthead seabream (*Sparus aurata*) [4]. In addition, caspase-3 expression is detected in various tissues, especially in the hemocytes and lymphoid organ of white shrimp *Litopenaeus vannamei* [33]. In the present study, the

abCaspase mRNA was widely demonstrated in various tissues, indicating that the *abCaspase* was constitutively expressed in various tissues of normal abalone. The universal distribution of the *abCaspase* might imply its importance in eliminating injured or unwanted cells and in maintaining homeostasis via modulating apoptosis [11]. Recently, the first molluscan tumor necrosis factor α (TNF-α) homologue has been cloned from disk abalone and it is constitutively expressed in all the tissues tested, including the gill, mantle, muscle, digestive tract, hepatopancreas and hemocytes. It was interesting to note that the AbTNF-α is most expressed in the gill of disk abalone [34], and coincidentally the mRNA transcripts of *abCaspase* were highly demonstrated in the gill of *H. diversicolor* in our study. TNF in vertebrates is a major death factor involved in the extrinsic pathway of caspase activation and induce apoptosis [16,35] and is also thought to play an important role in cell signaling in molluscs [12]. Similar to its vertebrate homologues, TNF-α expression was induced under bacterial infections [34], and it is able to induce apoptosis in molluscan hemocytes [36]. It is known that bacteria can trigger apoptosis by modulating caspase expression. In *D. labrax* L., the expression level of caspase-3 and -9 in phagocytes increases after *Photobacterium damselae* spp. *piscicida* infection, leads to the induction of apoptosis and results in the key pathogenicity mechanism of *phdp* [31,32]. In our study, the *abCaspase* gene was significantly expressed in various tissues after bacterial challenge. In particular, the expression pattern of *abCaspase* in the gills of abalone challenged with bacteria corresponded well to that of AbTNF-α in disk abalone [34], suggesting a potential interaction between them. Whether the expression of *abCaspase* in hemocytes and other tissues associated with the apoptotic process in *H. diversicolor* is similar to the TNF-α homologue described in molluscan hemocytes needs further investigation.

In conclusion, a molluscan caspase gene, *abCaspase*, was identified for the first time from *H. diversicolor* and it belonged to a typical apoptotic caspase. The *abCaspase* mRNA transcripts were demonstrated in various tissues of normal abalone and it was significantly expressed in tissues with bacterial challenge, especially hemocytes. 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*.

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Appendix. Supplementary material

Fig. S1. Domain organization of *abCaspase* and vertebrate caspase-8s and -10s. The amino acid sequence of each caspase was obtained from GenBank; italics in brackets represent the GenBank accession number. Protein motifs analyzed are performed with PROSITE. The position of each domain is indicated with respect to a ruler.

Note: Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsi.2009.12.016.

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