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Short communication

Molecular cloning, immunohistochemical localization, characterization and expression analysis of caspase-8 from the blunt snout bream (*Megalobrama amblycephala*) exposed to ammonia

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ABSTRACT

Caspase-8 is an initiator caspase that plays a crucial role in some cases of apoptosis by extrinsic and intrinsic pathways. Caspase-8 structure and function have been extensively studied in mammals, but in fish the characterization of that initiator caspase is still scarce. In this study, we isolated the caspase-8 gene from *Megalobrama amblycephala*, one of the most important industrial aquatic animals in China using rapid amplification of cDNA ends (RACE). The 2034 bp full-length *M. amblycephala* caspase-8 cDNA sequence contained an ORF of 1467 bp encoding a polypeptide of 489 amino acid residues, a 5′-UTR of 102 bp and a 3′-UTR of 462 bp. The caspase-8 amino acid sequences contained two highly conservative death effector domains (DEDs) at N-terminal, the caspase family domains P20 and P10, caspase-8 active-site pentapeptide and potential aspartic acid cleavage sites. Phylogenetic analysis revealed that *M. amblycephala* caspase-8 were clustered with the caspase-8 from other vertebrate. Real-time quantitative PCR analysis revealed that caspase-8 transcripts were detected in liver after exposure to ammonia. Meanwhile using Western blot analysis, caspase-8 cleaved fragment was detected and significant alteration of procaspase-8 level was found with the same ammonia treatment condition. Furthermore, the result of immunohistochemical detection showed that remarkable changes of immunopositive staining were observed after ammonia treatment. Accordingly, the results signify that caspase-8 of fish may play an essential role in ammonia induced apoptosis.

1. Introduction

Apoptosis is critically important for the survival of multicellular organisms as it destroys damaged or infected cells that may interfere with normal function. The central molecules of signaling pathway leading to apoptosis are the caspases. Members of the caspase family can be subdivided into initiator and effector caspases depending on their placement within the cascade of apoptosis signal transduction. Initiator caspases comprise caspase-2, -8, -9 and -10, which are capable of activating downstream caspases (executioners) after cleavage either directly through proteolysis or indirectly via a secondary messenger mechanism [1–3]. Among the initiator caspases, caspase-8 is an initiator caspase involved in the early steps of apoptosis by the receptor pathway triggered by Fas, tumor necrosis factor receptor type 1 (TNFR1), and related death receptors of TNF superfamily (reviewed in Ref. [4]). Once activated, caspase-8 is able to directly activate, by proteolytic cleavage, downstream effector caspases such as procaspases-3 and -7, leading to the cell dismantling and death. Caspase-8 can also amplify the apoptotic signal through an indirect pathway involving the cleavage and activation of BID [5]. Truncated Bid protein translocates to the mitochondria, triggering the release of cytochrome c into the cytosol where it will lead to the activation of Apaf-1/caspase-9 apoptosome [6]. Besides the role of caspase-8 in apoptotic death signaling, in recent years, several non-apoptotic functions of caspase-8 have been reported, such as embryonic development, monocyte differentiation into macrophages and regulation of T cell development [7]. The emerging roles of caspase-8 show the importance of this molecule and its participation in the balance between survival and apoptotic cell death.

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Some previous studies demonstrated that fish caspase-8 could be triggered by pathogenic infection, heavy metal exposure and ammonia exposure [8–10], indicating that caspase-8 might play important roles in many important biological events. Ammonia is one of the serious problems in fish culture especially in recirculation systems, aquaria and fish ponds. In intensive fish farming where fishes are held in high densities, a gradual accumulation of ammonia in water may occur especially when water exchange is restricted [11,12]. It is well accepted that high ammonia level is physiologically harmful to aquatic animals [13,14]. Ammonia can cause oxidative stress in organisms, through increasing the concentration of reactive oxygen species (ROS) [15]. Overproduction of ROS can damage important biomolecules, such as DNA, proteins and lipids, and initiate a cascade of events, bringing about impaired cellular function [16]. However, the precise mechanism of ammonia-induced apoptosis is not yet fully understood.

Up to now, molecular cloning and functional expression of caspase-8 are the currently hot topic in fishes. both caspase-8 were cloned in *Danio rerio* [17], *Dicentrarchus labrax* [18] and *Cyprinus carpio* [9] and so forth. Nevertheless, there was no caspase-8 report on blunt snout bream (*Megalobrama amblycephala*) which accounts for an important proportion in Chinese aquaculture. Aquaculture of this fish has expanded rapidly during the last decade because of fast growth rate, high feed efficiency ratio, tender flesh and high disease resistance. Since prevalence of disease in cultured *M. amblycephala* is increasing, especially during summer months, high temperatures and elevated ammonia concentrations seem to trigger disease outbreaks that result in high mortality [19], we predicted that *M. amblycephala* is relatively sensitive to ammonia exposure due to ammonia-induced apoptosis pathway. In this work, we sequenced and characterized the *M. amblycephala* caspase-8 gene and studied the expression of caspase-8 in tissues of resting and ammonia stimulated fish.

2. Materials and methods

2.1. Animal and ammonia exposure experiment

M. amblycephala juvenile with average (±SD) weight of 15.15 ± 0.85 g (n = 200) were obtained from Yixing fish farm of Fresh-water Fisheries Research Center, Chinese Academy of Fishery Sciences. and acclimated for two weeks in 250 L cycling-filtered plastic tanks containing continuously circulating aerated water at 24 ± 1 °C (pH 7.6; 6.5 mg/L dissolved oxygen). During the acclimation period, the commercial fish diet (35% protein, supplied by Tongwei Group Foods, Sichuan, China) was employed to feed twice a day until 24 h before the experimental treatments. In the ammonia challenge tests, two different ammonia concentrations treatment of 0 (normal freshwater as control) and 25 mg/L were used according to preliminary experiment in our laboratory [20]. During the exposure experiment, the pH values of water aquaria were every 6 h registered using a pH meter. The total ammonia nitrogen levels were measured by nesslerization [21] and adjusted by adding NH_4Cl solution every 6 h. For each treatment, liver from three individuals were sampled at 0, 3, 6, 12, 24 and 48 h after ammonia exposure respectively, then the samples were snap-frozen in liquid nitrogen. The liver tissues from different treatment groups and different tissues samples were preserved in liquid nitrogen and then stored at −80 °C for RNA extraction.

2.2. RNA extraction, reverse transcript and part of caspase-8 cDNA cloning

Total RNA was extracted using a TRizol reagent kit (Biostar, Shanghai, China) according to the manufacturer's protocol. Total RNA was incubated with RNase-free DNase I (Roche, USA) to remove the contaminating genomic DNA. The synthesis of the first strand cDNA was performed by using superscript™ III RNAse H^- reverse transcriptase (Invitrogen, USA) to transcribe poly $(A)^+$ RNA with oligo-d (T) 18 as the primers. Reaction conditions were complied with the manufacturer's instruction.

A large of genes in the liver of *M. amblycephala* have been discovered by the expressed sequence tag (EST) and annotation analysis [22]. One EST (isotig07265) in the library was homologous to the caspase-8 of *D. rerio* (ADM86391.1). To obtain *M. amblycephala* caspase-8 (Macaspase-8) cDNA, the primers designed from EST were used to amplify the partial cDNA caspase-8 of the *M. amblycephala*. The PCR reactions were performed as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and elongation at 72 °C for 3 min, followed by a 10 min extension at 72 °C and cooling to 4 °C. The PCR fragments were subjected to electrophoresis on a 1% agarose gel for length difference and cloned into the pMD-18T vector (Takara, Japan). After transformed into the competent cells of *Escherichia coli* DH5<alpha>, recombinant bacteria were identified by blue/white screening and confirmed by PCR. Three of the positive clones were sequenced in both directions and these resulting sequences were verified and subjected to cluster analysis in NCBI.

2.3. cDNA cloning of Macaspase-8

Total RNA was isolated from mixed tissues of prawns using RNAiso Plus Reagent (TaKaRa, Japan) according to the manufacturer's protocols. First strand cDNA was synthesized using Reverse Transcriptase M-MLV Kit (TaKaRa, Japan). The 3′-RACE and 5′-RACE were performed using 3′-full RACE Core Set Ver.2.0 Kit and 5′-full RACE Kit (TaKaRa, Japan) to get cDNA 3′ and 5′ ends of Macaspase-8. All the primers used in the clone were listed in Table 1. The PCR products were purified using Gel Extraction kit (Sangon, China), and sequenced by ABI3730 DNA Analyzer after insertion into PMD-18T vector.

2.4. Sequence analysis of Macaspase-8

The Macaspase-8 gene sequence was analyzed using the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Multiple alignment of Macaspase-8 was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A phylogenetic tree of caspase-8 was constructed by MEGA5.1 using the neighbor-joining method (http://www.megasoftware.net). The reliability of the estimated tree was evaluated by the bootstrap method with 1000 pseudo-replications.

Table 1
Description of primers used in this study.

Primer	Primer sequence (5′–3′)
Macaspase-8-F1 (3′RACE out primer)	GTATGACTGGATTCACGCTGGAC
Macaspase-8-F2 (3′RACE in primer)	TTGATCTCGCTGTCTCTGAGAG
Macaspase-8-R1 (3′RACE out primer)	CTCTCTAGTGTGGCGAAAGGAC
Macaspase-8-R2 (3′RACE in primer)	TGTCCATCTGCTGTCCATACAC
Macaspase-8-F CDS amplification (Nde I)	catATGGATCCTAAGACGTTTCACG
Macaspase-8-R CDS amplification (Xho I)	GCTGGTTCTTCCCATGGACctcgag
Mscaspase-8-F (real-time primer)	AGAGGCTTGGGGAAGACAACC
Mscaspase-8-R (real-time primer)	CAAGCGAGGCAACAGAAGAGC
β-Actin F (real-time primer)	TCGTCCACCGCAAATGCTTCTA
β-Actin R (real-time primer)	CCGTCACCTTCACCGTTCCAGT