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Molecular Characterization of Cathepsin H gene from sevenband grouper, *Hyporthodus septemfasciatus* against nervous necrosis virus infection

Dilinaer Ainiwaer[*+], Jae-Young Pe[+], Kyung-Min Kang[*], Han-Gyeol Kim[*], Myung-Joo Oh[***††] and Jong-Oh Kim[*,**†]**

[*]*School of Marine and Fisheries Life Science, Pukyong National University, Busan, Korea*
[**]*Department of Microbiology, Pukyong National University, Busan, Korea*
[***]*Department of Aqualife Medicine, Chonnam National University, Yeosu, Korea*

Nervous necrosis virus (NNV) has caused significant economic losses in Korea's aquafarms due to mass mortality in sevenband groupers during the summer season. However, the mechanisms of NNV infection are not well understood. To investigate these mechanisms and search for antiviral proteins, we have established a differentially expressed genes (DEGs) from naive sevenband grouper and NNV-infected ones. Among the highly expressed proteins was Cathepsin H, a lysosomal cysteine proteinase that plays a role in lysosomal protein breakdown. The open reading frame (ORF) of Cathepsin H (SG.CH) was 987 nucleotides long that codes for a protein of 328 amino acids (approximately 20 kDa molecular weight). The gene showed a high degree of identity (98%) with the Cathpesin H from the giant grouper (*Epinephelus lanceolatus*). When the gene expression profile was analyzed in various tissues, the highest expression was found in the kidney. While following the NNV infection, the expression profile of the Cathepsin H gene displayed a significant upregulation in brain at 48 hours after infection. These experimental results can help understand the mechanism of NNV infection.

Key words: Cathepsin H, Cysteine protease, nervous necrosis virus, gene expression

Introduction

Aquaculture industry is a significant economic contributor in the world. However, the marine environment presents significant challenges to the aquaculture industry worldwide. Various factors such as viruses, bacteria, fungi and parasites are posing a severe threat to the global aquaculture industry. Viral nervous necrosis (VNN) is a disease that has a detrimental impact on marine fish. This disease is considered to be one of the most severe diseases affecting over 120 different species of cultured marine fishes across the globe (Costa *et al.,* 2016). The morbidity and mortality caused by VNN vary among different fish species. In korea, mass mortalities caused by VNN were reported from various cultured marine fish such as sevenband grouper (*Hyporthodus septemfasciatus*), rock bream (*Oplegnathus fasciatus*), red drum (*Sciaenops ocellatus*) and olive flounder (*Paralichthys olivaceus*) since 1990 (Sohn *et al.,* 1998; Oh *et al.,* 2005; Cha

[+]These authors contributed equally to this work.
[†]Corresponding author: Jong-Oh Kim
Tel: +82-51-629-5614; Fax: +82-51-629-5619
E-mail: jokim@pknu.ac.kr
[††]Corresponding author: Myung-Joo Oh
Tel. and Fax: +82-61-659-7173
E-mail: ohmj@jnu.ac.kr

et al., 2007).VNN outbreaks show clear seasonal characteristics and the peak mortality period being concentrated in the high-temperature summer months, whereas BFNNV has been reported to cause VNN at low temperature such as 4 to 15℃ (Chi *et al*., 2001).

The causative agent of VNN is Nervous Necrosis Virus (NNV) (Awang *et al.,* 1987; Yoshikoshi and Inoue *et al.,* 1990; Breuil *et al.,* 1991; Glazebrook *et al.,* 1990) a non-enveloped icosahedral virus belonging to the betanodavirus of *Nodaviridae* family. The virus has a diameter of 25-30 nm and a genome comprising two positive-sense single-stranded RNA segments called RNA1 and RNA2 (Mori *et al*., 1992). RNA1 encodes the RNA-dependent RNA polymerase (RdRp) protein, which is about 100 kDa in molecular weight (MW). RNA2 encodes the viral capsid or coat protein(CP), which is about 37 kDa in molecular weight (Nishizawa *et al.,* 1995). When RNA1 replicates, the RdRp directs the production of the subgenomic RNA3. RNA3 encodes two non-structural proteins: B1 and B2 (Johnson *et al.,* 2003). B1 encodes an 111 amino acid protein, 11 kDa molecular weight and B2 encodes a 75 amino acid protein, 8.5 kDa molecular weight (Johnson *et al.,* 2001; Ball and Johnson *et al.,* 1999; Schneemann *et al.,* 1998). B1 protein mediates a novel anti-necrotic death during early replication in fish cell systems (Chen *et al.*, 2009). B2 protein may play an important role in viral replication and proliferation and particle assembly (Janina and Tompson, 2016; Zhang *et al.*, 2022).

The NNV can be categorized into five genotypes based on its properties, which are Striped jack NNV (SJNNV), Tiger puffer NNV (TPNNV), Barfin flounder NNV (BFNNV), Red-spotted grouper NNV (RGNNV), and Turbot NNV (TNNV) (King *et al*., 2011). When a fish is infected with NNV, it will typically exhibit neurological symptoms such as abnormal swimming behavior (Bandín *et al.,* 2020). These symptoms arise due to vacuolation and necrosis of the central nervous system. Larvae are particularly vulnerable to NNV, with mortality rates being extremely high. The RGNNV TN1 strain induces host apoptosis which precedes the onset of necrosis in a grouper liver cell line (GL-av) (Chen *et al.*, 2017).

Cathepsins are enzymes that are involved in the degradation of proteins, and the term "cathepsin" comes from the Greek word meaning "to digest" (Willstätter and Bamann, 1929). Cathepsins were first discovered in the late 1920s, and since then, more than 20 types of cathepsins have been identified in all living organisms (Scarcella *et al.*, 2022). Interestingly, Cathepsins are also involved in the process of virus release and spreading by enhancing and modifying the activity of the host enzyme Heparanase (HPSE) (Hadigal *et al*., 2020; Scarcella *et al.*, 2022). Cathepsin H is a type of lysosomal cysteine proteinase that is an essential part of the innate immune system's response to pathogens. Cathepsin H is a member of the C1 family of papain-like enzymes, which includes various proteases found in different organisms (Barrett *et al.*, 2004; Kuester *et al*., 2008; Nakanishi *et al*., 2003).

In this study, we cloned Cathepsin H gene from sevenband grouper and analyzed the transcript expression level in each tissue of the sevenband grouper which is infected with NNV (RGNNV genotype).

Materials and Methods

The juvenile sevenband groupers with a mean body weight of 7.8±0.5 g used in the experiment were purchased from aquaculture farms which had no prior infection with NNV. To ensure that the fish were not infected with NNV, real time PCR was conducted prior to the start of the experiments as per a previous study (Kim *et al.*, 2016). The striped snakehead (SSN-1) cell line was used to culture the NNV isolate obtained from a sevenband grouper fish farm in Yeosu, Korea in 2008. To propagate NNV, SSN-1 cells were grown at 25°C in 75-cm^2 cell culture flasks containing Leibovitz's (1X) L-15 medium (Gibco,

USA) supplemented with 10% fetal bovine serum (FBS) (Corning, USA) and 1% penicillin/streptomycin (Corning, USA). The supernatant of harvested virus samples were filtered with 0.45 μm filter and aliquoted in small volumes and stored at -80°C until use. Gene-specific primers were designed based on the previously identified full-length cDNA sequence of the cathepsin H gene (Kim *et al.*, 2017) for cloning and quantitative real-time PCR (Table 1). RNA was extracted from the brain tissue of healthy sevenband grouper using RNeasy® Plus Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. SuPrimeScript RT premix (GeNetBio, Korea) was used to perform reverse transcription to obtain cDNA. The obtained cDNA was amplified using the primer set provided in Table 1 and stored at -20°C until further use. The PCR reaction was conducted in a final volume of 20 μl, consisting of AccuPower™ PCR premix (Bioneer, Korea), 1 μl of each gene specific primer pair, 2 μl cDNA and ddH_2O. PCR conditions were as follows: pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 5 min. A final extension step was performed at 72°C for 5 min. The PCR product was purified and was ligated with pGEM®-T Easy vector (Promega, USA) for overnight at 4°C. The ligated DNA was then transformed into DH5α competent cells (BioFACT, Korea). The transformed colonies were cultured in LB broth containing ampicillin (50 mg/ml) for 16 hours and the plasmid DNA was extracted with QIAprep Spin Miniprep kit (Qiagen, Germany).

For signal sequence prediction, the SignalP program was used (http://www.cbs.dtu.dk/services/SignalP-3.0). Conserved sites and domains were predicted by NCBI Conseved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The amino acid sequence of reported Cathepsin H was aligned using ClustalW to create a multiple-sequence alignment. The aligned sequences were then used to construct a phylogenetic tree using MEGA-X software with a neighbor-joining method and 1,000 bootstrap replicates (Kumar *et al.,* 2018).

Juvenile sevenband grouper was injected with 100 μl NNV (SGYeosu08 isolate, RGNNV genotype) at a dose of 10^4 $TCID_{50}$/fish (Krishnan *et al.*, 2018) whereas negative control fish were injected with L-15 medium at 100 μl/fish. Following injection, tissues (spleen, heart, gill, brain, eye, liver, kidney, and blood) were collected at 24, 48, 72, 96, 120 hours post infection. Total RNA from each tissue was extracted and cDNA was synthesized as described above. A quantitative RT-PCR (qRT-PCR) assay was performed to determine expression levels of cathepsin H in each tissue on the Exicycler™ 96 Real time Quantitative Thermal Block (Bioneer, Korea). The PCR reaction was performed in a 20 μl volume containing 10 μl Accupower™ 2X Greenstar RT PCR Mastermix, 1 μl of each specific primer pair, 1 μl cDNA, 7 μl ddH_2O. The PCR conditions were 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 sec, annealing/extension at 60°C for 40 sec. The gene specific primers for the Cathepsin H were de-

Table 1. Primers used in this study

Primer	Sequence (5'-3')	Used for	Reference
SG.CH-F	CGAGAAGTTCGAGAAGGAAGC	cloning	In this study
SG.CH-R	GATGAGCTGCTTCACACCAAG	cloning	
qSG.CH-F	CAACGGACCGTATCCAGACT	qPCR	
qSG.CH-R	CGAGAAGTTCGAGAAGGAAGC	qPCR	
qEF-1a-F	CGAGAAGTTCGAGAAGGAAGC	qPCR	Krishnan *et al.*, 2018
qEF-1a-R	GATGAGCTGCTTCACACCAAG	qPCR	

signed using the Primer3Plus tool (https://www.primer3plus. com/) and their sequences are provided in Table 1. To determine the expression level of Cathepsin H, an internal control gene EF1a was amplified using primers EF1a_F and EF1a_R (Krishnan *et al.*, 2019). The expression level was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) (Livak and Schmittegen, 2001) with EF1α as an internal reference.

Fig. 1. The cDNA and deduced amino acid sequence of *H.septemfasciatus* Cathepsin H. Cathepsin H gene consists of a 988 bp ORF encoding 327 amino acids. Start (ATG) and Stop (TGA) codon is indicated by red font. The signal peptide domain is indicated by an arrow, and the Inhibitor I29 domain is indicated by blue highlighted. The Peptidase_C1 superfamily domain is indicated by yellow highlighted, the predicted active site is indicated by red circle and the predicted S2 subsite is indicated by blue circle.

Results and Discussions

Cathepsins are proteases that are mainly present in acidic endo/lysosomal compartments, and are responsible for a range of intracellular processes including protein degradation, energy consumption (Yadati *et al*., 2020; Wang *et al.*, 2018). In addition, Cathepsins have been shown to play a significant role in the immune response. It is involved in various aspects of the immune system, including antigen processing and presentation, regulation of immune cell activation and migration, and modulation of immune signaling pathways against virus infection (Jevnikar *et al.*, 2013, Scarcella *et al.*, 2022).

In our previous study, it was confirmed that Cathepsin H, a type of cathepsins, was highly expressed in the NNV-infected brain (Kim *et al.*, 2017). To characterize the Cathpsin H, we cloned the Cathepsin H gene from the sevenband grouper and investigate its genomic structure and expression, and explore its potential role in NNV infection. The open reading frame (ORF) of sevenband grouper Cathepsin H is 988 bp long, with 327 amino acids in the predicted protein. It had a signal peptide of 18 amino acid at the beginning and two active sites motifs at amino acid residues 130 and 136, as well as two predicted S2 subunit sites at amino acid residues 180 and 181. The conserved domains of the protein are located at amino

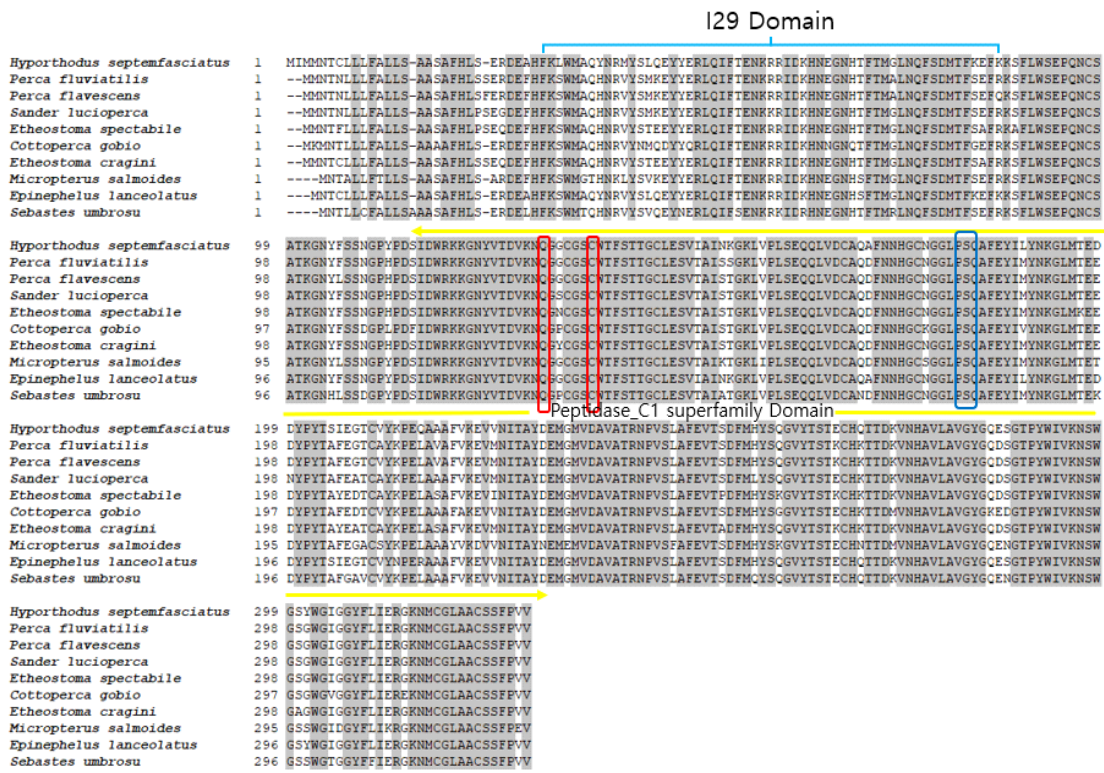

Fig. 2. The Multiple alignment of the cathepsin H amino acid sequence with other known Cathepsin H sequence of fish species. The grey box depict that 100% identical regions. The GenBank accession numbers of the aligned Cathepsin H sequences are as follows: *Perca fluviatilis*, XM_039809038.1; *Perca flavescens*, XM_028585153.1; *Sander lucioperca*, XM_031282928.2; *Etheostoma spectabile*, XP_032379604.1; *Etheostoma cragini*, XM_03487 8269.1; *Cottoperca gobio*, XP_029289776.1; *Micropterus salmoides*, XM_038706811.1; *Epinephelus lanceolatus*, XM_033635558.1; *Sebastes umbrosu*, XM_037768626.1

acid residues 30-85, which belong to the inhibitor I29 superfamily, and at residues 112-327, which belong to the peptidase_C1 superfamily (Fig. 1).

Based on multiple alignments with other fish species, it was confirmed that the amino acid sequence of Cathepsin H gene is highly conserved in fish including freshwater and marine fish (Fig. 2). Phylogenetic tree analysis revealed the evolutionary relationship among fish species, with the Cathepsin H of *Epinephelus lanceolatus* being the closest (Fig. 3). It suggests that despite the high conservation of the cathepsin H gene, it exhibits a closer evolutionary similarity to marine fish species compared to freshwater fish species. The expression levels of Cathepsin H gene were analyzed in various healthy sevenband grouper tissues. The results showed that the kidney had the highest expression level of Cathepsin H among all the tissues analyzed from healthy fish as depicted in (Fig. 4). This finding demonstrates a similar pattern of Cathepsin H expression to that observed in Rock bream (Kim *et al.*, 2013), Half-smooth tongue sole (Wang *et al.*, 2023). Not only Cathepsin H gene, but also other genes which is related to innate immunity such as interferon regulatory factor 3 (IRF3), IRF7, and peroxiredoxin 1 (PRDX1) were up-regulated in kidney, spleen, liver tissues in Asian seabass and Hongkong grouper (Ganeshalingam *et al.,* 2023; Krishnan *et al.,* 2019). Recently, research has indicated that histone proteins, traditionally recognized for their role in regulating gene expression, are also involved in the innate immune response to pathogens (Over and Michaeals, 2014). It has also been showed that the Histone H2A gene of sevenband grouper expression level was highly expressed in spleen after NNV infection (Lee *et al.,* 2022). The relatively high expression of these genes in the kidney and spleen can be attributed to the fact that these organs are recognized as key immune organs involved in various

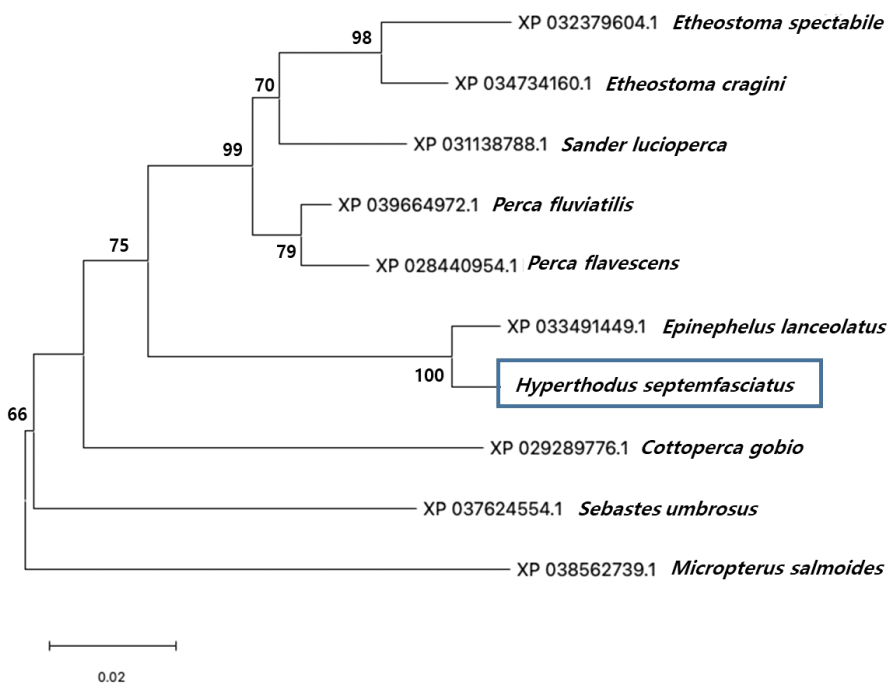

Fig. 3. Phylogenetic analysis of sevenband grouper Cathepsin H complete amino acid sequences. Phylogenetic tree was constructed by Neighbour Joining (NJ) method using MEGA-X. The number show the boot-strap percentile from 1000 replicates.

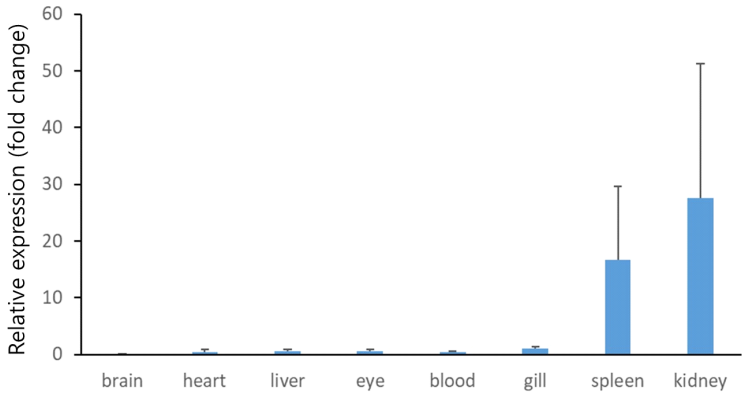

Fig. 4. The graphical result of the sevenband grouper Cathepsin H gene expression in various tissues from healthy sevenband grouper by real time PCR.

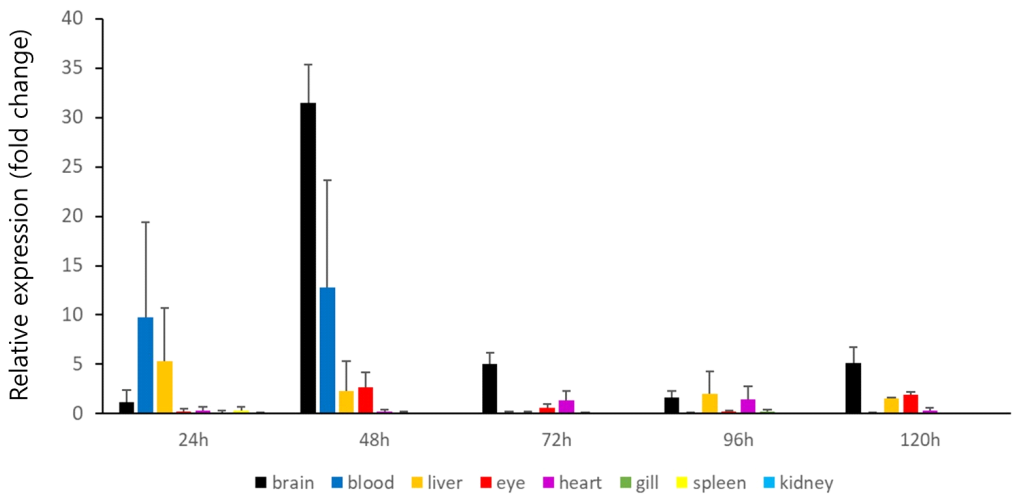

Fig. 5. The relative mRNA expression of sevenband grouper Cathepsin H after NNV infection. The sevenband grouper Cathepsin H expression from various tissues (brain, blood, liver, eye, heart, gill, spleen and kidney) at 24, 48, 72, 96 and 120 hours post NNV infection. Each value represents a mean value ± SD of replicates (n=3).

immune processes.

The expression profile of the Cathepsin H gene displayed a significant upregulation (over 30 fold change) in brain at 48 hours after infection (Fig. 5). It is considered to be deeply related to the fact that the main infectious tissue of NNV is the brain. Interestingly, the eye, which is also a major tissue affected by the infection, did not exhibit a similarly high expression level. On the other hand, kidney and spleen showed no change in expression level of Cathepsin H after NNV infection, which was also confirmed in RSIV-infected rock bream (Kim *et al.*, 2013).

To summarize, our results suggest that Cathepsin H could be involved in an important role in the immune response of the sevenband gropuer, particularly during NNV infection. However, further research is necessary to fully understand the function of Cathepsin H during NNV infection.

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