Acta Oceanol. Sin., 2021, Vol. 40, No. 6, P. 61–66
https://doi.org/10.1007/s13131-021-1776-4
http://www.aosocean.com
E-mail: ocean2@hyxb.org.cn

Antimicrobial peptide hepcidin contributes to host defense of *Centropristis striata* against *Vibrio harveyi* challenge

Xiaowan Ma[1], Ying Qiao[1]*, Yanxiang Shao[2], Chao Chen[2]

[1] Key Laboratory of Tropical Marine Ecosystem and Bioresource, Fourth Institute of Oceanography, Ministry of Natural Resources, Beihai 536000, China

[2] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

Received 29 April 2020; accepted 3 June 2020

Abstract

*Hepcidin*s are small cysteine-rich antimicrobial peptides that play a vital role in immunity against pathogen invasion. Here, a *hepcidin* (*Cshep*) from *Centropristis striata* was described, which is considered as a valuable aquaculture marine species in China. The open reading frame consisted of 273 bp. Eight conserved cysteine residues were identified. Phylogenetic analysis showed that *Cshep* had a relatively close relationship with the *hepcidin* from *Epinephelus moara*. Quantitative real-time PCR analysis demonstrated that *Cshep* was highly expressed in liver and significantly up-regulated when challenged with *Vibrio harveyi*. In addition, the synthetic Cshep peptide had a high antimicrobial activity against *V. harveyi*, but low against other pathogenic bacteria tested in this study. The killing kinetics analysis revealed that Cshep had a fast bactericidal effect on *V. harveyi*. These results suggested that Cshep may be involved in the immune response of *C. striata* against *V. harveyi* infection.

Key words: *Centropristis striata*, *hepcidin*, antimicrobial peptides, antimicrobial activity, *Vibrio harveyi*

Citation: Ma Xiaowan, Qiao Ying, Shao Yanxiang, Chen Chao. 2021. Antimicrobial peptide hepcidin contributes to host defense of *Centropristis striata* against *Vibrio harveyi* challenge. Acta Oceanologica Sinica, 40(6): 61–66, doi: 10.1007/s13131-021-1776-4

1 Introduction

Black sea bass (*Centropristis striata*) which belongs to the family of Serranidae, is considered as a highly valued marine teleost in commercial and recreational fisheries worldwide (Howell et al., 2003). The demand has increased greatly nowadays; however, the aquaculture of the fish is hampered by frequent disease outbreaks. It is necessary to find a safe approach to control the fish disease.

To date, antibiotics are still the major measure to treat infections caused by variety of bacterial pathogens in fish. However, the frequent, improper or large dose use of the antibiotics have resulted in the increasing of drug resistance bacteria which impedes the development of aquaculture in recent years and leads to drug accumulation in aquatic products which might threaten the health of customers (Gao et al., 2012). Developing an effective way to control bacterial resistance to antibiotics has become one of the most important challenges in the field of medicine. One solution is using antimicrobial peptide (AMP) for replacement.

AMPs are short, cationic peptides with an amphipathic secondary structure (Lemaitre et al., 1997). Their broad-spectrum antimicrobial activities have attracted increasing interest to use them as pharmaceuticals in medicine and aquaculture (Hancock and Sahl, 2006; Rajanbabu and Chen, 2011). To date, more than 3 000 AMPs have been discovered from six kingdoms (http://aps.unmc.edu/AP/main.php). In fish, a large number of AMPs have also been identified in recent years, including *hepcidin*, *defensin*, *piscidin* and *Nk-lysin*, etc. (Shabir et al., 2018).

Hepcidin was firstly isolated from human blood ultrafiltrate (Krause et al., 2000). It is a cysteine-rich peptide and is conserved in different species. Six to eight cysteine residues are found at conserved positions within *hepcidins* and considered to be essential for its antimicrobial activities (Hocquellet et al., 2012). In fish, *hepcidin* was initially identified in hybrid striped bass and later isolated (Shike et al., 2002) and characterized in many fish species (Huang et al., 2019; Xu et al., 2018). To date, plenty of studies show that *hepcidin* in fish plays a dual role in innate immunity and iron regulation (Jiang et al., 2017). Fish *hepcidin* exhibits a broad spectrum of bactericidal activities against both Gram-positive and Gram-negative bacteria (Liu et al., 2018). However, the characterization of *hepcidin* from *C. striata* has not been reported yet.

In this study, a hepcidin was identified that had an antimicrobial function to *Vibrio harveyi*. The *hepcidin* from *C. striata* was cloned and analyzed for the structures and homologies with other vertebrates. Gene expression patterns in different tissues and the potential roles against *V. harveyi* infection were investigated. The antimicrobial activity and the killing kinetic of *V. harveyi*

Foundation item: The Youth Foundation of Guangxi Zhuang Autonomous Region, China under contract No. 2018GXNSFBA050032; the Innovation Driven Development Foundation of Guangxi under contract Nos AD19245135 and AD19245161; the Doctoral Research Startup Fund of the Fourth Institute of Oceanography, Ministry of Natural Resources under contract Nos 201803 and 201806; the Fund of Key Laboratory of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture and Rural Affairs, China under contract No. FREU2016-04.
*Corresponding author, E-mail: qiaoying0618@hotmail.com

were also examined in this study. It may contribute to further exploring functions of *hepcidin* genes and shed light on new methods for combatting bacterial diseases.

2 Materials and methods

2.1 *Fish rearing*

The black sea bass weighting (250±10) g and (22±2) cm in length were obtained from a nursery farm in Rizhao, Shandong, China. The fish were cultured in the aerated seawater (salinity 30, temperature (27±2)°C) in a 5 m×5 m tank for at least 15 d prior to experiment. All the fish used in the experiments was anesthetized using MS-222 for tissues collection and performed according to the Experimental Animal Management Law of China.

2.2 *Bacterial challenge experiment*

A total of 30 healthy *C. striata* were randomly assigned to the bacteria challenge experiment. Suspension (100 μL) containing 2.67×10^5 CFU/mL (lethal concentration 50, LC_{50}) of *V. harveyi* was intraperitoneally injected into the fish. CFU, acolony-forming unit, is a unit used in microbiology to estimate the number of viable bacteria or fungal cells in a sample. The *V. harveyi* strain used in the bacterial challenge experiment was isolated and purified from diseased *C. striata*. This strain was previously found to be highly pathogenic to *C. striata* (unpublished results). At 6 h, 12 h, 24 h, 48 h and 72 h postinjection, livers of five fish were collected on ice, stored in RNAstore (Qiagen) immediately and transferred to –80°C. Tissues collected at 0 h were injected with phosphate buffer (PBS) solution and served as control.

In addition, five individuals of healthy fish were randomly collected for brain, gill, heart, intestine, liver, spleen, head kidney, stomach and muscle tissues to explore the gene expression in different tissues.

2.3 *Total RNA, DNA isolation and cDNA synthesis*

Frozen tissues were transferred to a mortar and grounded to a fine powder. Total RNA was isolated from each tissue using TRIzol reagent (TaKaRa, Japan). The cDNA synthesis was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) using 1 μg of total RNA. Genomic DNA was extracted from muscle by using a Genomic DNA Isolation Kit (Qiagen) following the manufacturer's instructions.

2.4 *Full length cDNA and DNA cloning of a hepcidin gene from C. striata*

A transcriptome database of the liver of *C. striata* was constructed in previous work, and a hepcidin gene (*Cshep)* was identified based on the gene annotation. Based on the initial sequence of *Cshep* gene, specific primers were designed by Primer-Premier 5.0 and all the primers were listed in Table 1. Total RNA of livers from healthy individuals was used to extend 3′ and 5′ untranslated region (UTR) using rapid amplification of cDNA ends (RACE) method. The open reading frame (ORF) sequence and the whole DNA sequence were cloned based on the above result. PCR was performed in 50 μL reactions, containing 25 μL 2×Ex taq Buffer (Takara), 1 μL dNTP Mix (10 mmol/L), 1 μL Ex taq (Takara), 5 μL first strand of cDNA or genomic DNA, 3 μL primers. The PCR amplification was under the following condition: 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, 72°C for 10 min. All PCR products were gel-purified using a DNA extraction Kit (OMEGA, USA), cloned into pMD18-T Simple Vector (TaKaRa, Japan) and sequenced in Sangon Biotech (China).

2.5 *Sequence conservation and phylogenetic analysis*

Signal peptide characteristics were predicted using SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/). Molecular weight and isoelectric point prediction was performed using ProtParam tool (https://web.expasy.org/protparam/). Multiple sequence alignment was conducted using ClustalW (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) and analyzed in ESPript 3 (http://espript.ibcp.fr/ESPript/ESPript/). Phylogenetic tree of *Cshep* and other species were constructed with PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/). Gene intron-extron structure information and conserved domains were identified by BLAST. All the sequences used were retrieved from Genbank database.

2.6 *The qPCR analysis of Cshep gene expression*

The qPCR was performed on a CFX Connect™ system (Bio-Rad, USA) using the *Power* SYBR™ Green PCR Master Mix (Thermo Scientific, USA). The qPCR primers design for *Cshep* and housekeeping gene are listed in Table 1. Melting curve analysis was performed at the end of each qPCR program to confirm the uniqueness of the product. The amplification program was as follows: 95°C for 3 min; 40 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 20 s; 75°C for 5 s. Melting curve analysis was performed at the end of each program. The expression level of *Cshep* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with *β-actin* as the internal control. All samples were analyzed in three replicates, and data are shown in terms of relative mRNA level to that of *β-actin* as means±SD.

2.7 *Antimicrobial assay*

The mature peptide of Cshep was chemically synthesized with over 95% purity (ChinaPeptides, China). Antimicrobial activity was determined by minimum inhibitory concentration (MIC) values and the minimum bactericidal concentration (MBC) values (Table 2). Briefly, the peptide was diluted to final

Table 1. Forward and reverse primers (5′-3′)

Name	Primers (forward and reverse)(5′-3′)	Primer application
B310-1 (GSP1)	GCTCCTGCACCCCGGT	5′RACE
B310-2 (GSP2)	GTGTCATTGCTCCCTG	5′RACE
B310-3 (GSP3)	GGCGGAGCTCTCCAGAAT	5′RACE
C232-1	GAAATGTCAATGGAATCGAGGATG	3′RACE
C232-2	GCCAGATCACATCAGGCAGAAGCG	3′RACE
QB310F1	CCGACACCCATGAGAAAG	DNA amplify/ORF
QB310R1	AGGTAGTTTTCTTGGGGTTA	DNA amplify/ORF
*β-actin*_qPCR_F	GTGCTGTCTTTCCCTCCATC	qPCR
*β-actin-actin*_qPCR_R	CTCTTGCTCTGGGCTTCATC	qPCR
*Cshep*_F	TCGAGGATGATGCCAGATCA	qPCR
*Cshep*_R	TGCGGGAATCTTCAGAACCT	qPCR

Table 2. Antimicrobial activity of the synthetic Cshep

Microorganisms	MIC/(μmol·L^{-1})	MBC/(μmol·L^{-1})
V. harveyi	3.0–6.0	6.0–12.0
P. damselae	6.0–12.0	12.0–24.0
V. parahaemolyticus	24.0–48.0	24.0–48.0
A. hydrophila	12.0–24.0	24.0–48.0

Note: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

concentrations from 1.5 μmol/L to 96 μmol/L with sterile Milli-Q water. The microorganisms (*V. harveyi*, *V. parahaemolyticus*, *Aeromonas hydrophila* and *Photobacteria damselae*) were diluted with 10 mmol/L PBS (pH=7.4) to 0.001 8 Optical Density and incubated with serial dilutions of peptides in a 1:1 ratio. Samples without peptides were considered as blanks. After 24 h of incubation at 28°C in a 96-well plate, the MIC values on which the lowest concentration of peptide inhibiting growth of the microorganisms were determined. Then 5 μL of the mixtures were dropped onto Mueller-Hinton agar (MHA) plate and recorded MBC value after overnight incubation at 28°C. All the values were averaged on three independent measurements.

2.8 *Kinetics of bacterial killing*

According to the antimicrobial assay results above, the evaluating of killing kinetics was performed using *A. hydrophila*, *V. harveyi*, *V. parahaemolyticus* and *P. damselae* incubated with 2×MBC Cshep. The procedures were performed according to the antimicrobial assay. Aliquots of 5 μL of the peptide and bacteria mixtures were removed at various intervals (0, 3 min, 6 min, 9 min, 12 min, 15 min, 20 min, 25 min, 30 min, 60 min, 120 min, 180 min, 240 min, 300 min and 360 min) and diluted with 10 mmol/L PBS onto MHA plate. After overnight incubation at 28°C or 37°C, the recovered colonies were counted by colony forming amount (experimental group) divided by colony forming amount (control group), 0 min colony forming proportion is 100%. Each assay was repeated three times.

3 Results

3.1 *Sequences analysis and phylogenetic analysis of Cshep gene*

Cshep gene was identified from *C. striata* transcriptome database and the nucleotide sequence has been submitted to NCBI Genbank database under the accession number of MK353156. The ORF consisted of 273 bp which encoded a 90 amino acids. The corresponding molecular weight of *Cshep* was 9.96 kDa and the isoelectric point was 7.48. The genomic DNA sequence of ORF region of the *Cshep* was 535 bp, consisting of 3 exons and 2 introns (Fig. 1). Eight conserved cysteine residues were identified from Cshep through multiple alignments with other vertebrate *hepcidins* (Fig. 2). Phylogenetic tree showed that the *Cshep* was in a branch position with *Epinephelus moara*, *E. coloides*, *Alphestes immaculatus*, *Lycodichthys dearborni* and *Trachidermus fasciatus* (Fig. 3).

3.2 *Cshep gene was highly expressed in the liver of C. striata and up-regulated in the liver under V. harveyi attack*

The distribution of *Cshep* gene in the nine different tissues of *C. striata* was assessed using qPCR. The length of the qPCR amplified fragment is 128 bp. The transcript of *Cshep* was predominantly expressed in the liver and had significantly low expression level in other tested tissues (Fig. 4).

To understand the possible biological role of *Cshep* under *V. harveyi* challenge, the relative expression levels of *Cshep* were detected in the liver using qPCR. The expression level of *Cshep* was significantly up-regulated in liver for 6 h and 12 h postinjection (Fig. 5). The 18 h postinjection, the expression level was recovered to control levels.

3.3 *Cshep shows high antimicrobial activity against V. harveyi*

The antimicrobial activity of Cshep is listed in Table 2. The results indicated that the growth of tested bacteria were inhibited by the Cshep peptide. In the assay, four pathogenic bacteria were performed, including *A. hydrophila*, *V. harveyi*, *V. parahae-*

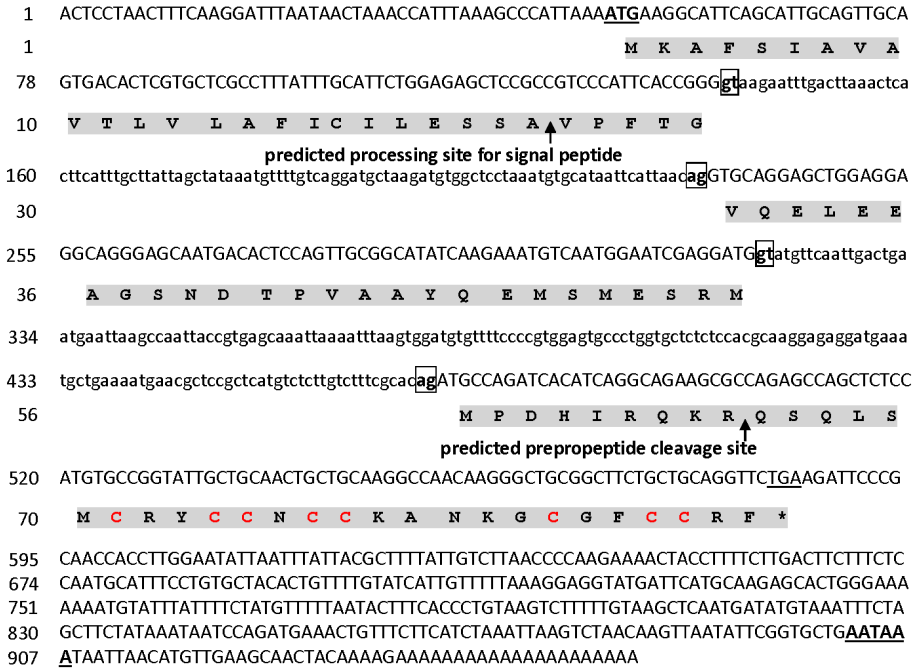

Fig. 1. Nucleotide and amino acid sequence of *Cshep* full length cDNA.

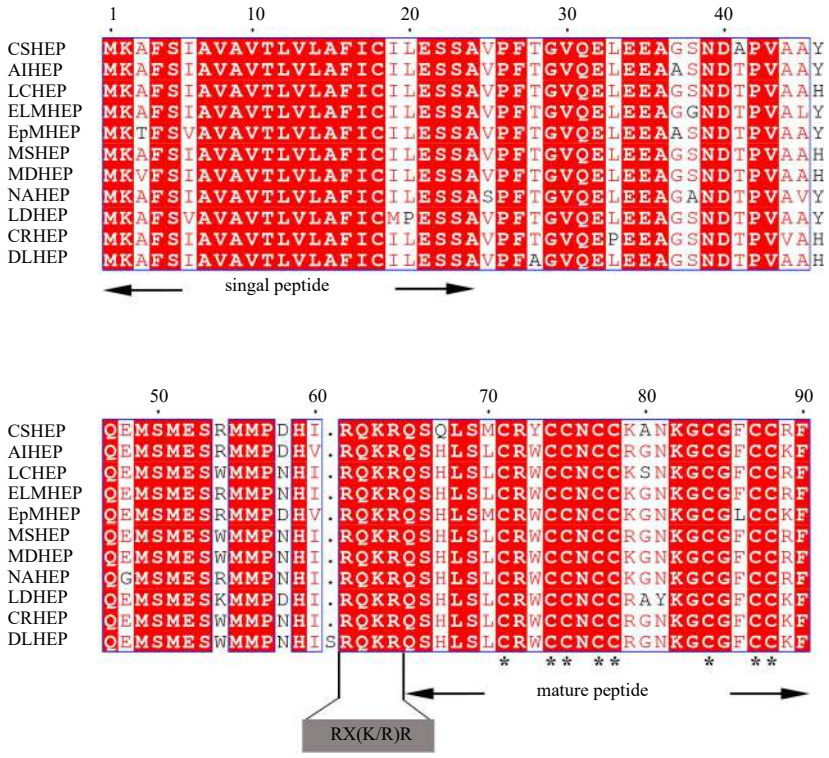

Fig. 2. *Hepcidins* sequences alignment.

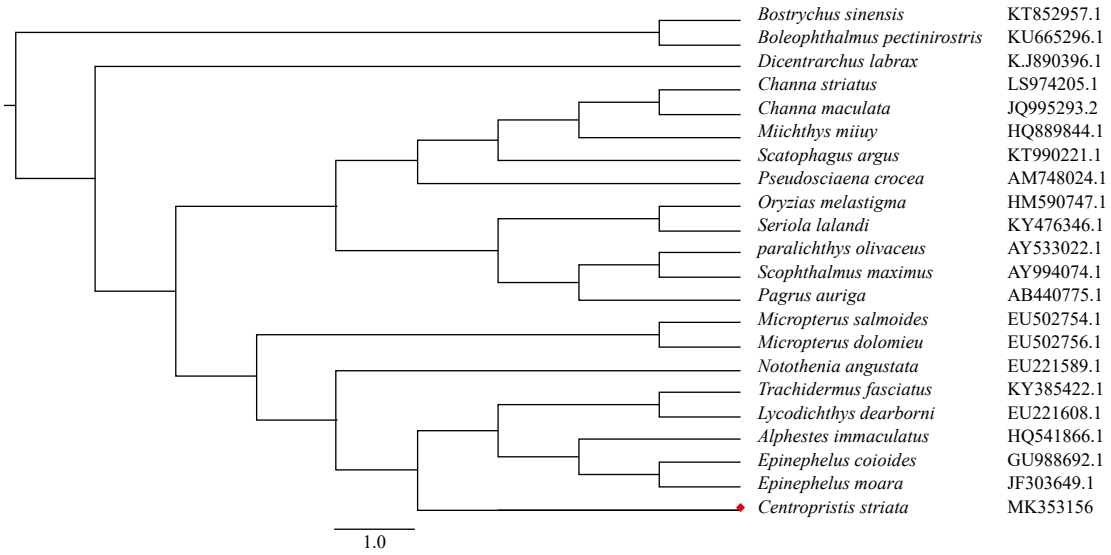

Fig. 3. Phylogenetic tree of *hepcidins* amino acid sequences constructed with Neighbor-Joining method using MEGA v6.06.

molyticus and *P. damselae*. Generally, Cshep has low antibacterial activity against all detected microorganisms except *V. harve*yi. The results showed that 3–6 μmol/L of Cshep peptide would inhibit the growth of *V. harveyi*.

3.4 *Cshep shows a rapid killing process against microorganisms*

To further investigate the rate of bactericidal activity against the bacteria, the kill curve of Cshep was subsequently measured (Fig. 6). Cshep exhibited a marked bactericidal effect. Approximately 75% reduction of *V. parahaemolyticus* was achieved in 15 min and almost 100% strains in 2 h. A total of 88% of *V. harveyi* and *A. hydrophila* were reduced only in 15 min. In addition, it took only 25 min for Cshep to kill almost 100% of *V. harveyi*, however, in the case of *A. hydrophila* it took about 3 h. The time for Cshep to reach 80% and 100% reduction of *P. damselae* was 15 min and 1 h, respectively.

4 Discussion

The innate immune system of fish is essential for the survival in the environment rich in microbes (Magnadóttir, 2006). Antimicrobial peptides are one of the vital components of fish's innate immunity against pathogens (Silphaduang and Noga, 2001;

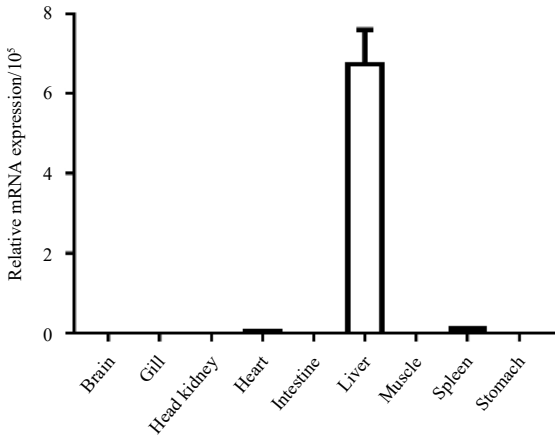

Fig. 4. The relative expression level of *Cshep* in nine tissues of healthy *C. striata*.

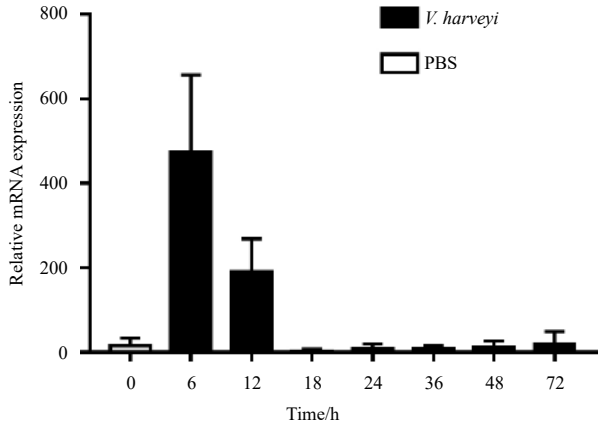

Fig. 5. The relative expression level of *Cshep* in the liver after stimulation with *V. harveyi* at different time points.

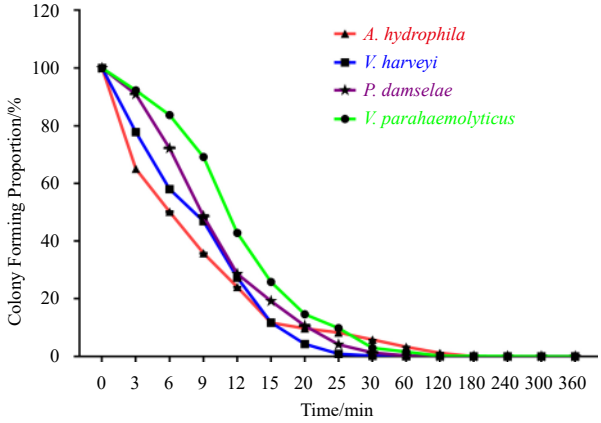

Fig. 6. Kinetics of the bacterial activity of Cshep peptide against *A. hydrophila*, *V. harveyi*, *P.damselae* and *V. parahaemolyticus*.

Zhu et al., 2013). *Hepcidin* is a key player in regulating innate immunity in fishes, protecting them from pathogenic attacks. The broad-spectrum antimicrobial activity of *hepcidin* provides new insights into the breeding and development of new antimicrobial agents by overcoming drug resistance problems.

Analysis of *Cshep* cDNA and DNA showed that *Cshep* is highly conserved with *hepcidin*s from other species. Similar to other fish, *Cshep* has three exons and two introns, and the relative position and length were conserved among them (Jordan et al., 2009). Usually, prepropeptide of *hepcidin* from fish and mammals contain 83–96 amino acids (Xu et al., 2018; Huang et al., 2019), and Cshep contains 90 amino acids. The number of amino acids of signal peptide is 24 in different fish species (Xu et al., 2018; Huang et al., 2019), which is the same for Cshep.

In the phylogenetic analysis of the present study, *Cshep* was brunched together with the HAMP1 class of *A. immaculatus* (Masso-Silva et al., 2011) and orange-spotted grouper (Zhou et al., 2011). HAMP1 class peptide occurs as a single copy that shares a considerable degree of homology with mammalian counterparts, and plays a dual role in innate immunity and iron regulation (Hilton and Lambert, 2008). The placing together of *Cshep* with other HAMP1 Isoforms might suggest that it is belonging to the HAMP1 class. The phylogenetical classification into the same cluster indicated that they might exhibit the similar function or expression patterns throughout the evolution.

In this study, the expression level of *Cshep* was the highest in the liver compared to other tissues. It was also detected in spleen and heart, however, far less than in liver. Previous studies on other fish species also found that the highest expression of the *hepcidin* mRNA occurs in the liver (Huang et al., 2019; Wang et al., 2009; Xu et al., 2018). In *Monopterus albus*, HAMP2 transcripts were abundant in kidney, spleen and intestine, while HAMP3 and HAMP4 were mainly expressed in liver (Li et al., 2011). In *Sebastes schlegelii*, the highest expression of *hepcidin* was observed in the liver, followed by head kidney, stomach, and skin (Kim et al., 2008).

In some cases the expressions of *hepcidin* in spleen and heart were also observed. Like in *Brachymystax lenok*, the expression of *Blhepc* in the liver was higher than that in other tissues, but showed a decreasing trend in the order of skin, eye, brain, pectoral fin, and muscle (Xu et al., 2018; Huang et al., 2019; Wang et al., 2009). An amount of *PC-hepcidin* mRNA transcripts was also demonstrated in the spleen and heart in *Pseudosciaena crocea* (Wang et al., 2009). In this study, *Cshep* was found at low levels in spleen and heart, so further studies about the role that *hepcidin* may play are warranted. However, these results indicated that *hepcidin* was constitutive in other tissues not only liver.

Vibrio harveyi is gram-negative bacteria that is highly pathogenic to a large number of marine fish (Farmer et al., 2005; Ransangan and Mustafa, 2009). In the breeding process, *V. harveyi* was the major pathogen for the *C. striata* and caused exophthalmia in this study. In order to examine the expression profile of *Cshep* under *V. harveyi* infection, this study intraperitoneally injected the bacteria and assessed using qPCR. In this study, *Cshep* mRNA expression was up-regulated during the early immune response and immediate recovery to control levels, which is similar to those reported in other fishes (Chen et al., 2018). It could be concluded that immune response of *Cshep* to *V. harveyi* was rapid in the liver of black sea bass.

In vitro studies have attributed antimicrobial activity to fish hepcidins. Usually, synthesized hepcidin showed broad antimicrobial activity against different Gram-negative bacteria. In *Platichthys stellatus*, the MIC value of PsHepicidin against *V. harveyi* was 62.9 μmol/L (Liu et al., 2018), 6–12 μmol/L in *P. crocea* (Wang et al., 2009), >96 μmol/L in *E. coioides* (Qu et al., 2013) and 12.5 μmol/L in *Boleophthalmus pectinirostris* (Chen et al., 2018). Different from previous work, Cshep showed a relatively high antimicrobial activity against *V. harveyi* in this study.

To further study the rate of bactericidal activity, the kinetic ef-

fect of Cshep against different microorganisms was also observed. The kinetics studies indicated that bacterial-killing was time dependent and the antimicrobial effects were exerted within 15 min of exposure to the tested microorganisms. What's more, it took less than half an hour for Cshep to kill all *V. harveyi*. The highly potent antimicrobial activity of Cshep against *V. harveyi* would make it possible as a substitute of antibiotics in the medical industry in future.

In conclusion, this study described and characterized a *hepcidin* from *C. striata* in this paper. The results demonstrated that *Cshep* was highly expressed in liver and significantly up-regulated after the *V. harveyi* challenging. In addition, the synthetic Cshep peptide had a high antimicrobial activity against *V. harveyi*, but low in other pathogenic bacteria tested in this study. The killing kinetics analysis revealed that Cshep had a fast bactericidal effect on *V. harveyi*. Above results suggested that Cshep might be involved in the immune response of *C. striata* against *V. harveyi* infection.

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