Peptides 30 (2009) 638–646

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide☆

Ke-Jian Wang *, Jing-Jing Cai, Ling Cai, Hai-Dong Qu, Ming Yang, Min Zhang

State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen, Fujian 361005, China

ARTICLE INFO

Article history:
Received 19 October 2008
Received in revised form 18 December 2008
Accepted 18 December 2008
Available online 27 December 2008

Keywords:
Pseudosciaena crocea
PC-hepc
Gene expression
Synthetic peptide
Antimicrobial activity

ABSTRACT

Hepcidin gene is widely expressed in various fish, suggesting that this antimicrobial peptide is a very important component in the innate immune system. Large yellow croaker (*Pseudosciaena crocea*) is one of the important economic species of marine-cultured fish but knowledge of its innate immune mechanism is lacking. In this study, we characterize a *P. crocea* hepcidin gene named as PC-hepc. It consists of an open reading frame of 258 bases encoding 85 amino acids and has a conserved sequence in common with other known hepcidins. The genomic DNA of PC-hepc contains three exons and two introns, the same organization as other reported hepcidins, indicating that PC-hepc is one member of the hepcidin family in fish. The tissue-specific expression of PC-hepc gene in normal fish and the expression pattern in LPS-challenged fish at the time course of stimulation were investigated. The expression of PC-hepc mRNA was significantly increased in the spleen, heart and stomach but not significantly induced in the liver after LPS challenge. An interesting finding is the demonstration of high amounts of PC-hepc transcripts in the kidney in normal fish and their maintenance through 48 h exposure to LPS challenge. The synthetic PC-hepc demonstrated a rather wide spectrum of antimicrobial activity in vitro against bacteria and fungi tested, and particularly showed strong activity against the principal fish pathogens, *Aeromonas hydrophila*, *Vibrio parahaemloyticus*, *Vibrio alginolyticus* and *Vibrio harvryi*. The study indicates that PC-hepc may play a role with a tissue-specific mode in the innate immunity of *P. crocea*.

1. Introduction

Since the first identification of hepcidin from human plasma ultrafiltrate [25] and urine [33], antimicrobial peptide hepcidin genes have been identified from various vertebrates. The biological functions of mammalian hepcidin are known to be involved in antimicrobial activity and in iron metabolism [31,33]. In comparison with their mammalian counterparts, fish hepcidin gene expression is mostly associated with bacterial infection or inflammation and only a few fish hepcidins were reported to be induced by iron overload [20,21,37]. Fish hepcidin cDNA and genomic DNA organization have been determined in many fish, including hybrid striped bass [38], winter flounder, Atlantic salmon [15], zebrafish [39], rainbow trout [45], olive flounder [23], Japanese flounder [20], channel catfish [6], red sea bream [9], Japan sea bass [36]), sea bass [37], black porgy [44], gilthead seabream [12]. Data mining of hepcidin sequences reported demonstrates that hepcidin genes are common in mammals and fish [12,15,25,33,36,38,44,45], suggesting that this antimicrobial peptide is a very important component in the innate immune system. Tissue-specific studies reveal that hepcidin transcripts are widely expressed in multiple tissues but the highest expression level is found predominantly in the liver of mammals and most fishes [12,15,20,22,23,25,33,37,38,44]. Hepcidin was originally named liver-expressed antimicrobial peptide (LEAP-1 and LEAP-2) [25,45]. However, exceptions exist in some fish species in which no significant hepcidin gene expression is found in the liver but in other tested tissues as reported in zebrafish [39] and in channel catfish [6]. This means that hepcidin gene in fish might not be always liver-expressed and the highest hepcidin transcripts in tissues may vary with fish species, thus, the role of fish hepcidins in innate immunity is probably limited and specific in tissues.

The antimicrobial activity of hepcidin has been investigated using the synthetic mature peptide of human hepcidin [33] and several fish hepcidins [12,20,22,28]. The in vitro antimicrobial activity of synthetic fish hepcidin has been studied in bass, *Morone chrysops* hepcidin [28], Japanese flounder, *Paralichthys olivaceus* hepcidin Hep-JF2 [20], tilapia, *Oreochromis mossambicus* TH1–5 and TH2–3 [22] and gilthead seabream Hep [12]. Most of them exhibited antibacterial or antifungal effects but the spectrum was variable. In view of the antimicrobial spectrum of hepcidin activity from different fish species there appears to be specificity among

☆ The nucleotide sequence data reported in this paper have been submitted to the GenBank. GenBank accession nos.: EF156401 (PC-hepc cDNA), EU443735 (PC-hepc DNA), and EU443734 (partial 18S mRNA cDNA).

* Corresponding author. Tel.: +86 592 2184658; fax: +86 592 2180655.
E-mail address: wkjian@xmu.edu.cn (K.-J. Wang).

0196-9781/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2008.12.014

the individuals, suggesting that hepcidin from different sources may have specific antimicrobial action. Accordingly, this attracts us to probe the antimicrobial characteristics and spectrum of activity for each newly revealed hepcidin peptide.

Large yellow croaker (*P. crocea*), a very important economic species of marine-cultured fish, frequently suffers from microbial infections. Little is known about the innate immune mechanism of the fish. This hinders the establishment of effective disease control measures in the breeding of large yellow croaker [46]. This study aimed, therefore, to analyze the *P. crocea* hepcidin (PC-hepc) cDNA and its genomic organization, elucidate the gene expression pattern in vivo, and to determine its antimicrobial activity using synthetic PC-hepc. The data may provide us valuable information on the antimicrobial role of PC-hepc as a molecular component in innate immunity of *P. crocea*.

2. Materials and methods

2.1. Determination of PC-hepc cDNA and genomic DNA sequences

2.1.1. Cloning of PC-hepc cDNA from large yellow croaker

RT-PCR and RACE (rapid amplification of the cDNA end) were performed to amplify the hepcidin cDNA sequence from large yellow croaker challenged by exposure to bacterial suspensions. Three normally cultured adults of large yellow croaker (~250 g) were obtained from a fish farm in Xiamen, Fujian province, China. The fish were immersed in a mixed suspension of *Staphylococcus aureus*, *E. coli*, *Bacillus subtilis* and *Micrococcus luteus* (approximately 10^8 CFU each) for 3–5 min on field. The inoculated fish were put in a large bag filled with marine water aerated with oxygen and brought to the laboratory for sampling. Livers were collected from fish after 3–5 min exposure and were frozen immediately in liquid nitrogen individually, and stored at −80 °C before use. The procedure for amplification of hepcidin gene from large yellow croaker followed that described in a previous study on black porgy hepcidins [44]. Primers S1 and A1 (Table 1) were designed based on the published cDNA sequence of white bass hepcidin [38]. Each liver (~50 mg) was ground quickly in liquid nitrogen and transferred to 1 mL TRIzol (Invitrogen). 18S rRNA cDNA was for the first time amplified from large yellow croaker based on a pair of degenerated primers (Genbank accession no. AB259837) and the determined sequence was released on Genbank (Genbank accession no. EU443734). 3′ RACE was performed with aliquots of RNA using the 3′–full RACE core set (TaKaRa) with S1 and 3′ adaptor primers. The procedure followed that described in a previous study [44]. The positive recombinants confirmed as 3′ RACE products incorporated in plasmids were sequenced.

2.1.2. Genomic DNA determination of PC-hepc

Genomic DNA was isolated using a MiniBEST Animal Tissue Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's instructions. Primers S1 (Table 1) and R5 (Table 1) were designed based on 5′ UTR (untranslated regions) and 3′ UTR of the cDNA sequences of large yellow croaker (GenBank accession no. EF156401). The PCR product was purified and its sequence analyzed as described by Yang et al. [44]. Intron positions were identified by comparison with the cDNA sequence.

Table 1
Primers (forward and reverse)/probes (5′-3′).

Primers (forward and reverse)/probes (5′-3′)	Use
18S	
18F: ACAAAGGGCAGGGACTTAATCA	Real time PCR
18R: TCCCATGAACGAGGAATTCC	Real time PCR
PC-hepc	
R5: GTGTTGTGGAGATCCCAGGTTG	Real time PCR, 5′RACE
Sy2: TTTGCTGCCGTTGCTGTCCTAGAATGAG	Real time PCR, 3′RACE
S1: CGAAGCAGTCAAACCCTCCTAAGATG	cDNA, DNA amplify
A1: CAACCTGCAGCAGACACCACATCC G	cDNA, DNA amplify

2.2. Computer analysis

The gene and predicted protein sequences were analyzed by ClustalX 1.83 (to perform the multiple alignments) [10]. Homology searches were performed using BLASTn and BLASTp by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The cleavage site for the signal peptide was predicted by the SignalP (http://www.cbs.dtu.dk/services/SignalP) [32]. The charge for the deduced mature peptide of PC-hepc was calculated by the ProtParam tool (http://cn.expasy.org/tools/protparam.html) [8].

2.3. In vivo expression of PC-hepc mRNA in multiple tissues

2.3.1. LPS challenge and tissue collection

Juveniles of large yellow croaker (~40 g) were obtained from a fish farm in Luoyuan, northeast Fujian Province, China. The challenge experiment was performed by intra-peritoneal injection in fifteen fish with approximately 20 μg LPS (Sigma, *E. coli* 0127:B8) in 100 μL of sterile physiological saline solution (PSS) per fish (50 μg/100 g) or in the equal quantities of fish with 100 μL LPS-free PSS as control. The fish were maintained in a cage in marine water after challenge. Tissue samples ($n = 3$) were obtained at 0 h (from the normal fish), 3, 6, 12, 24 and 48 h post-injection from the LPS-challenged and PSS mock-challenged fish. The tissues including the liver, spleen, kidney, intestine, brain, heart, gill, stomach and muscle were separately collected from each individual fish, and were frozen immediately in liquid nitrogen individually, and stored at −80 °C.

2.3.2. Quantitative evaluation of PC-hepc mRNA by real time PCR

The relative quantity of PC-hepc mRNA was evaluated using the comparative CT (Cycle threshold) method with a 7500 Real Time PCR System (AB Applied Biosystem) and Power SYBR Green PCR Master Mix (AB Applied Biosystem). The primers specific for PC-hepc gene and the endogenous control 18S rRNA are listed in Table 1. The cycling profile was as follows: at 60 °C for 2 min, at 95 °C for 10 min followed by 40 cycles of denaturing at 95 °C for 15 s, annealing and primer extension at 60 °C for 1 min. The validation experiment was performed using two diluted cDNA series from the control kidney to verify that efficiencies of the PC-hepc mRNA and the endogenous control 18S rRNA were approximately equal in real time PCR (data not shown). Expression of PC-hepc gene was normalized to an endogenous reference 18S rRNA and presented as ΔCT values. Comparison of gene expression between challenged samples and calibrator samples was derived from subtraction of calibrator ΔCT values from target ΔCT values to give a ΔΔCT value, and relative gene expression was calculated to determine fold difference ($2-\Delta\Delta CT$). Tissue-specific expression of PC-hepc gene was investigated in three normal fish, the tissues including the liver, spleen, kidney, intestine, brain, heart, gill, stomach and muscle. The expression patterns of PC-hepc gene at the time course of LPS challenge were investigated in the liver, spleen, kidney, heart, stomach, using 48 h challenged fish heart RNA pool as calibrator samples. All data were obtained in comparison with the same calibrator.

2.4. Synthesis of the PC-hepcidin peptides

The synthetic peptide (QGSPARCRFCCRCCPRMRGCGICCRF), coding for the predicted mature hepcidin, was produced by Shanghai Songon Biological Engineering Technology & Services Co,

Ltd. (Shanghai, China) with a purity >95%. The molecular masses and purity of the purified peptides were verified by mass spectroscopy and HPLC, respectively.

2.5. Antimicrobial assays

The antimicrobial activity of the synthetic PC-hepc was determined against a panel of microorganisms, including Gram-positive and Gram-negative bacteria, yeast and filamentous fungi. Antimicrobial assays were performed with twelve strains of bacteria including Gram-positive *M. luteus*, *S. aureus*, *B. subtilis*, *C. glutamicum*, *S. epidermidis*, *B. cereus*, and Gram-negative *A. hydrophila*, *E. coli*, *E. coli BL21(DE3) plysS*, *V. parahaemolyticus*, *V. alginolyticus*, *V. harvryi*, and the yeast *Pichia pastoris* GS115, *Candida albicans*, and the fungi *Aspergillus niger*, *Fusarium graminearum* and *Fusarium solani*.

The minimal inhibitory concentration (MIC) for liquid growth inhibition assay and minimal bactericidal concentration (MBC) were determined using a 96 well flat-bottom tissue-culture plate as previously described [7,28]. Briefly, the synthesized PC-hepc was dissolved and 2-fold serial dilutions (1.5–48 μM final concentrations) made in sterile MilliQ water. Logarithmic phase bacterial cultures were suspended in 10 mM NaPB to a final concentration of 1–2 × 10^6 CFU/mL. The assay mixture contained 50 μL diluted PC-hepc suspension, 30 μL diluted bacterial suspension and 20 μL Mueller-Hinton broth medium (Difco). Marine bacteria were suspended in saline peptone water (3% Tryptone, 3% NaCl, w/v) [14] to a final concentration of 0.6–1.2 × 10^6 CFU/mL. The assay mixture contained 50 μL diluted PC-hepc suspension and 50 μL diluted bacterial suspension. After 24 h of incubation at 28 °C, MIC was calculated as the lowest PC-hepc concentration yielding no detectable growth. Each assay was performed in triplicate. Samples without PC-hepc were used as blanks. For determination of MBC, bacteria were incubated in 96-well plates in the presence of varying concentrations of PC-hepc for 24 h at 28 °C, and aliquots of the cultures were then plated on Nutrient Broth agar, and bacterial growth was assessed after overnight incubation at either 37 or 28 °C for marine bacteria.

The yeasts *P. pastoris* GS115 and *C. albicans* were grown in YPG (Yeast extract 1%, peptone 1%, glucose 2%, agar 1.5%) for two days [14]. The fungi *A. niger*, *F. graminearum* and *F. solani* were cultured on potato dextrose agar for 10–14 days at 28 °C. 30 μL suspensions of yeast (∼10^3 CFU) or fungi (∼10^3 spores) were incubated with 50 μL different concentrations of synthetic PC-hepc and 20 μL of culture media (YPG without agar for yeast, PDB (Difco) at half-strength for fungi). The assay mixture was supplemented with 25 μg/mL chloramphenicol in every well to avoid bacterial contamination. MIC was determined after 48 h of incubation at 28 °C. The MIC value was expressed as an interval (a–b) [27], (a) being the highest concentration tested at which microbial growth can be observed, and (b) the lowest concentration tested that inhibited microbial growth or germination. Each assay was performed in triplicate. For determination of MBC, the contents of each well without visible germination were centrifuged for 3 min at 5000 rpm and resuspended in 50 μL of fresh PDB media, and plated on PDA plate. The plate was incubated at 28 °C for three day to monitor germination hyphae.

Kill-curve studies: Kinetic studies were performed using *S. aureus* and *A. hydrophila* as described previously [40]. The PC-hepc (12 or 24 μM, twice the respective MBCs) was incubated with the bacteria as described above. At various time points, 5 μL was taken from the mixture, serially diluted in NaPB and plated on nutrition broth agar. Plates were incubated at 37 °C overnight and the colonies were counted. The percentage of CFU was defined relative to the CFU obtained in the control (100% CFU at 0 min) [27]. A fungicidal kinetic study was also carried out. The PC-hepc (24 or 48 μM, again twice the respective MBCs) was incubated with *F. solani* as mentioned above. At various times, 10 μL was taken from the mixture, serially diluted in NaPB and plated on PDA plate. Plates were incubated at 28 °C for 48 h and the colonies were counted.

3. Results

3.1. Determination of PC-hepc cDNA and genomic DNA sequences

The PC-hepc cDNA sequence was obtained and included a complete CDS in addition to a 3′ UTR, in which the polyadenylation signal appeared at 215 nt downstream of the stop codon (TGA) and 16 nt upstream of the poly (A) tail (Fig. 1). The deduced amino acid sequence of PC-hepc cDNA is 85 residues in length and consists of three domains: signal peptide (24 amino acids), prodomain (35 amino acids) and mature peptide (26 amino acids) (Fig. 1). The signal peptide cleavage site of the deduced PC-hepc was predicted between Ala 24 and Val 25 (Fig. 1). The mature peptide region of PC-hepc was predicted, consisting of 26 amino acid residues at the C terminus of ORF. The processed mature peptide of PC-hepc is predicted to be positively charged at neutral pH, having a theoretical pI of 8.76. It is thus a cationic protein.

The PC-hepc genomic DNA sequence was amplified and sequenced. The large yellow croaker hepcidin gene consists of two introns and three exons (Fig. 1). The first exon contains the 5′ UTR, the signal peptide and 5 amino acid residues of the prodomain peptide. The prodomain extends from exon 1 through exon 3. Two introns were inserted into the prodomain, thus separating the peptide into three exons. Exon 3 encodes the mature peptide and the 3′ UTR. PC-hepc genomic organization is indicated in Fig. 1, and was identical to most reported hepcidins.

A sequence alignment of PC-hepc precursor peptide of large yellow croaker and some other known and predicted hepcidin peptides was performed at the amino acid level (Fig. 2). The alignment showed that all listed hepcidins (71 hepcidin or hepcidin-like sequences searched on Genbank or published), including fish (48 hepcidin sequences) and mammalian (11 hepcidin sequences) were most characterized by eight cysteine residues conserved at identical positions in the mature peptide region (≈83%). However, there were 9 fish hepcidin sequences or ESTs with 4 cysteine residues in the mature peptide, 2 with 6 and 1with 7 as shown in the Fig. 2. The predicted signal peptide is highly conserved between PC-hepc and most other fish hepcidins (Fig. 2) except for zebrafish hepcidins (NP_991146, NP_001018873), channel catfish hepcidin (AAX39715), turbot hepcidin (CAI65386) and rainbow trout hepcidin (AAG30029) (Fig. 2). There was lower similarity in the signal peptide sequence of hepcidin between fish and mammals (Fig. 2). The signal peptide cleavage site of all deduced fish hepcidins was between Ala 24 and Val 25 or Gly 25 but the 25th amino acid in some hepcidins was substituted for other amino acids (Ala 24-Leu 25, or Ala 24-Ser 25, or Ala 25-Thr 26) as shown in Fig. 2. The deduced amino acid sequence of PC-hepc and bass hepcidin showed 71% homology when analyzed by Genbank blastp, the highest in comparison with each of other fish hepcidins listed (Fig. 2). Both hepcidin sequences are fully identical in the signal peptide.

3.2. Expression of PC-hepc gene in multiple tissues of normal and LPS-challenged P. crocea

The tissue-specific gene expression of PC-hepc in normal fish and gene expression pattern of PC-hepc in LPS-challenged fish were investigated using real-time PCR. PC-hepc mRNA transcripts were widely distributed in all tissues tested in normal fish including the liver, stomach, muscle, gill, heart, brain, intestine,

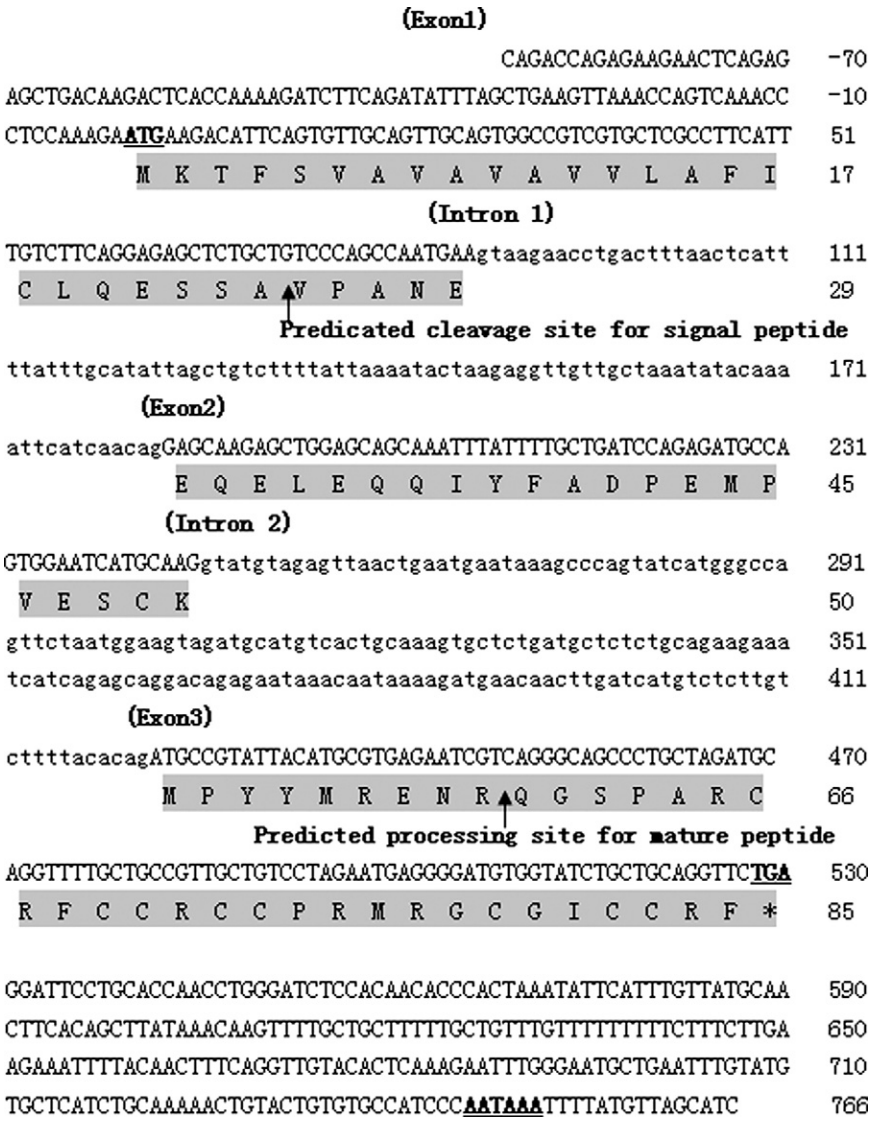

Fig. 1. Nucleotide sequence of cDNA, predicted amino-acid sequences and genomic DNA organization of the large yellow croaker hepcidin PC-hepc. The exon sequence is in boldface. The deduced amino acid sequences are translated and shadowed. Vertical arrows show the predicted positions of cleavage sites for signal peptide and mature peptide and the box indicates the putative motif RENR. The start codon (ATG), stop codon (TGA) and polyadenylation signal (AATAAA) are underlined.

spleen and kidney (Fig. 3a). The high amount of PC-hepc mRNA transcripts was demonstrated in the kidney and relative low levels of expression were observed in other tissues (Fig. 3a). Interestingly, the expression level in the liver was the least among all the tested tissues in normal fish (Fig. 3a).

The expression pattern of PC-hepc gene was investigated in the spleen, heart, kidney, stomach and liver of *P. crocea* at the time course of 0 h (normal group), 3, 6, 12, 24 and 48 h post injection with LPS, using real time PCR. All the results are presented with 18S mRNA as an endogenous control. A significant up-regulation of PC-hepc expression was seen in spleen, heart and stomach after LPS challenge ($P < 0.05$) (Fig. 3b) but the gene expression pattern was different in these three tested tissues. The level of PC-hepc transcripts was significantly increased in the heart, spleen and stomach at 12, 24 and 48 h, respectively. The spleen displayed the higher expression level over 5 fold compared with control, the heart over 3 fold and stomach over 13 fold. Surprisingly, although no significant expression of PC-hepc gene was observed in the kidney of LPS-challenged fish at the time course of stimulation, the level of PC-hepc transcripts was maintained as high as detected in the kidney in normal fish (Fig. 3b). Moreover, no significant gene expression was detected in the liver after LPS challenge (Fig. 3b).

3.3. Antimicrobial activity of the synthetic PC-hepc

3.3.1. Antimicrobial assays

The antimicrobial activity of the synthetic PC-hepc was determined against a panel of microorganisms using MIC and MBC assays. The MIC and MBC values obtained are reported in Table 2. The synthetic PC-hepc was active against all of the Gram-positive and Gram-negative bacteria tested, including *M. luteus*, *S. aureus*, *B. subtilis*, *C. glutamicum*, *S. epidermidis*, *B. cereus*, and Gram-negative *A. hydrophila*, *E. coli*, *E. coli BL21*(*DE3*) *plysS*, *V. parahaemolyticus*, *V. alginolyticus*, *V. harvryi*. Particularly, Pc-hepc was strongly active against the important fish pathogens, *A. hydrophila* (3–6 μM), *V. parahaemolyticus* (3–6 μM), *V. alginolyticus* (12–24 μM), *V. harvryi* (6–12 μM). The synthetic peptide also showed activity at a low concentration against all Gram-positive bacteria tested, especially *M. luteus* (1.5–3 μM), *S. aureus* (3–6 μM), *B. subtilis* (3–6 μM), *C. glutamicum* (1.5–3 μM) and *S. epidermidis* (6–12 μM). In addition, PC-hepc displayed antimicro-

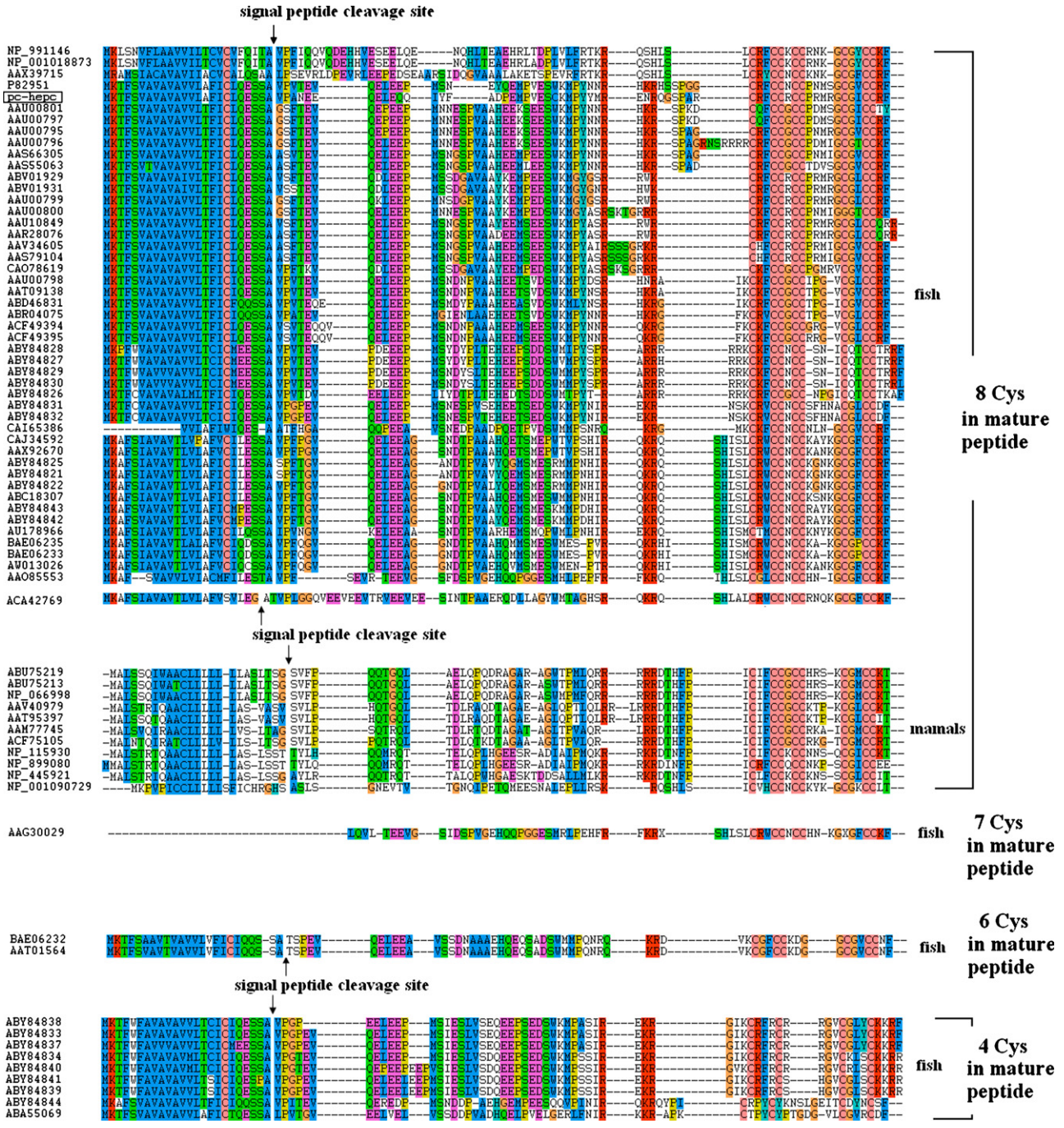

Fig. 2. ClustalX alignment and comparison of the amino acid sequence deduced from *Pseudosciaena crocea* hepcidin precursor cDNA with some similar protein sequences of hepcidin. The amino-acid sequence deduced from large yellow croaker (*Pseudosciaena crocea*, GenBank accession number EF156401) is marked as PC-hepc. Other similar proteins are predicted and obtained from GeneBank, as follows: Red seabream hepcidins (*Pagrus major*, AAS66305, AAV34605, AAS79104, AAU10849, AAR28076); Antarctic eelpout hepcidins (*Lycodichthys dearborni*, ABY84842, ABY84843, ABY84844); Black porgy hepcidins (*Acanthopagrus schlegelii*, AAU00795, AAU00797, AAU00801, AAU00798, AAU00796, AAU00800, AAU00799); Patagonian blennie (*Eleginops maclovinus*, ABY84822, ABY84826); Bastard halibut hepcidins (*Paralichthys olivaceus*, BAE06233, BAE06235, AAT01564, BAE06232); turbot hepcidins (*Scophthalmus maximus*, AAX92670, CAJ34592, CAI65386); large yellow croaker hepcidin (*Pseudosciaena crocea*, ABY84821); Pogonophryne scotti hepcidins (*Pogonophryne scotti*, ABY84821, ABY84840); Maori chief hepcidins (*Notothenia angustata*, ABY84825, ABY84831, ABY84832, ABY84839, ABY84841, ABY84827); Japanese sea perch (*Lateolabrax japonicus*, AAS55063, AAT09138); Gilthead seabream hepcidins (*Sparus aurata*, ABV01931, CAO78619, ABV01929, European perch hepcidin (*Perca fluviatilis*, ABR04075); Nile tilapia hepcidin (*Oreochromis niloticus*, ABD46831); Ploughfish hepcidin (*Gymnodraco acuticeps*, ABY84834); Antarctic toothfish hepcidins (*Dissostichus mawsoni*, ABY84833, ABY84838, ABY84837, ABY84830, ABY84829); zebrafish hepcidins (*Danio rerio*, NP_991146, NP_001018873); striped beakperch hepcidins (*Oplegnathus fasciatus*, ACF49394, ACF49395); Yellow grouper hepcidin (*Epinephelus awoara*, ABA55069); Atlantic cod hepcidin (*Gadus morhua*, ACA42769); Blackfin icefish hepcidin (*Chaenocephalus aceratus*, ABY84828); Xenopus tropicalis hepcidin (*Silurana tropicalis*, NP_001090729); Takifugu obscurus hepcidin (*Takifugu obscurus*, ABX57735); human hepcidin (*Homo sapiens*, NP_066998); brown rat hepcidin (*Rattus norvegicus*, NP_445921); house mice hepcidins (*Mus musculus*, NP_115930, NP_899080); dog hepcidins (*Canis familiaris*, AAV40979, AAT95397); Swine hepcidin (*Sus scrofa*, AAM77745); Rainbow trout hepcidin (*Oncorhynchus mykiss*, AAG30029); Atlantic salmon hepcidin (*Salmo salar*, AAO85553); Channel catfish hepcidin (*Ictalurus punctatus*, AAX39715); Yak hepcidin (*Bos grunniens*, ACF75105); Gorilla hepcidin (*Gorilla gorilla*, ABU75219); Guinea Baboon hepcidin (*Papio papio*, ABU75213); Medaka liver hepcidin (AU178966); Winter flounder liver hepcidin (AW013026) and HEPC_MORCS Hepcidin precursor (*Morone chrysops* × *Morone saxatilis*, P82951).

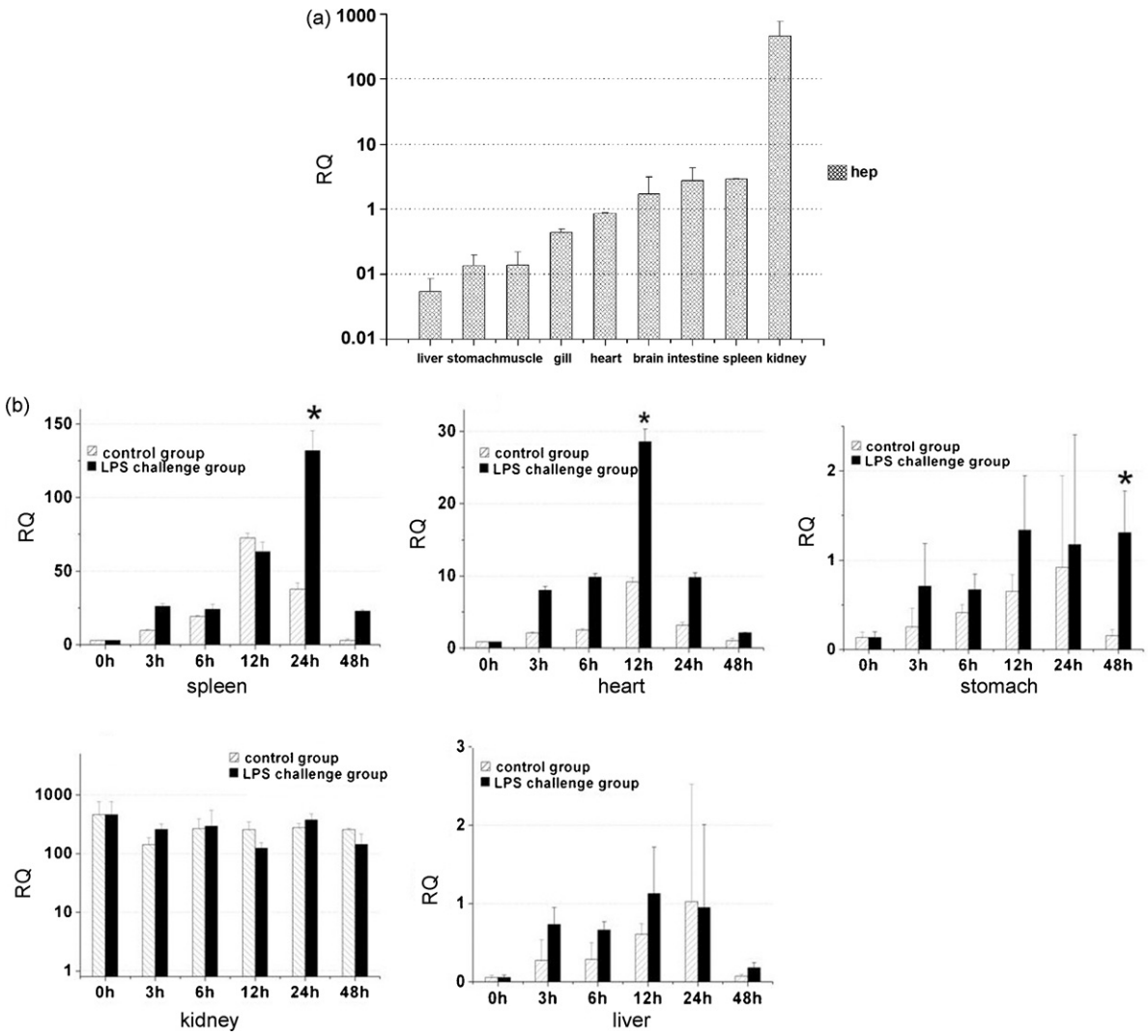

Fig. 3. (a) Distribution of PC-hepc gene transcripts in various tissues in normal large yellow croaker analyzed using real time PCR. 18s gene was used as endogenous control. Among the tissues assayed, the relative quantification (RQ) value of the liver located on the left side of the abscissa is the minimum and the RQ value of kidney located on the right side of the abscissa is the maximum and is about 460 much higher than those of other tissues assayed. (b) Quantitative analysis of the PC-hepc gene expression in tissues of LPS-challenged *P. crocea* using real-time PCR. All data were normalized relative to 18s rRNA and represented by the means ±S.E. Results shown are the mean of three individual RNA samples of fish ($n = 3$) with LPS challenge. The gene expression of PC-hepc in control fish inoculated with LPS-free PSS was shown in parallel. The '0h' group includes normal *P. crocea* without any treatment ($n = 3$). The data with "*" indicate statistical significance ($P < 0.05$, one-way ANOVA).

bial activity against the fungi *A. niger*, *F. graminearum* and *F. solan* with the value of MIC 12–24 μM, but was not active at a concentration over 48 μM against the yeasts *P. pastoris GS115* and *C. albicans*. Additional experiments were conducted to examine the bactericidal effects of PC-hepc. The bactericidal activity of the peptide was assessed by plating cultures, and the number of CFUs was counted after overnight incubation at 37 °C.

3.3.2. Kinetics of killing of S. aureus, A. hydrophila and F. solan by PC-hepc

In the kinetic study, a highly sensitive strain of *S. aureus* was used to evaluate bactericidal activity of the synthetic PC-hepc (Fig. 4a). The bactericidal activity of this peptide was assessed by plating cultures and the number of CFUs was countered overnight incubation at 37 °C. When *S. aureus* was incubated with the synthetic PC-hepc at a concentration 1–2 times the MBC value (12–24 μM), approximately 90% bacteria were killed at 9 min and nearly all the bacteria were killed around 30 min (Fig. 4a). Similarly, when *A. hydrophila* was incubated with the synthetic PC-hepc at a concentration 1–2 times the MBC value (12–24 μM), over 90% bacteria were killed at 3 min and nearly all the bacteria were killed at 15 min (Fig. 4b). In addition, the fungicidal activity was also investigated in *F. solan*. When *F. solan* was incubated with PC-hepc at a concentration 1–2 times the MBC values (24–48 μM), approximately 90% fungi were killed at 15 min and nearly all the fungi were killed around 180 min (Fig. 4c). Thus the killing of the target microbes is time dependent.

4. Discussion

In the study, the cDNA and genomic DNA sequences of a new homologous hepcidin gene PC-hepc cloned from *P. crocea* were revealed. PC-hepc consists of an ORF of 258 bases with a coding capacity of 85 amino acids and possesses eight cysteines in the mature region which are conserved in human, mice and the majority of fish hepcidins. The putative amino acid sequence of PC-hepc shows similarity with other known hepcidins. Almost all the fish hepcidins were much more conserved in the signal peptide region except for the hepcidins of zebrafish, channel catfish, turbot and rainbow trout (Fig. 2). The PC-hepc gene has a similar genomic organization (three exons and two introns) to the reported mammalian and fish hepcidins [12,15,33,34,36,38,44]. These

Table 2
Antimicrobial activity of the synthetic PC-hepc.

Microorganisms	CGMCC no.[a]	MIC[b] (μM)	MBC[c] (μM)
Gram-negative bacteria			
Aeromonas hydrophila	1.1801	3–6	6–12
Escherichia coli	1.2389	12–24	12–24
Escherichia coli BL21(DE3)plysS	Gene power[d]	3–6	12–24
Vibrio prahaemloyticus	1.1615	3–6	12–24
Vibrio alginolyticus	1.1833	12–24	>48
Vibrio harvryi	1.1593	6–12	6–12
Gram-positive bacteria			
Micrococcus luteus	1.634	1.5–3	1.5–3
Staphylococcus aureus	1.363	3–6	6–12
Bacillus subtilis	1.108	3–6	6–12
Corynebacterium glutamicum	1.1886	1.5–3	1.5–3
Staphylococcus epidermidis	1.2429	6–12	12–24
Bacillus cereus	1.447	12–24	12–24
Yeast			
Pichia pastoris GS115	Invitrogen[e]	>48	NT[f]
Candida albicans	2.2411	>48	NT[f]
Fungi			
Aspergillus niger	3.316	12–24	12–24
Fusarium graminearum	3.349	12–24	12–24
Fusarium solani	3.584	12–24	12–24

MIC values are expressed as the interval of concentration [a] − [b], where [a] is the highest concentration tested at which microbial growth can be observed, and [b] is the lowest concentration tested yielding no detectable microbial growth ($n = 3$).
[a] CGMCC no. indicates China General Microbiological Culture Collection number.
[b] MIC: minimal inhibitory concentration.
[c] MBC: minimal bactericidal concentration, the values of MIC and MBC were expressed as an interval (a − b), (a) being the highest concentration tested at which cells were able to grow and (b) the lowest concentration tested that inhibited microorganism growth or that killed more than 99.9% microorganisms.
[d] *Escherichia coli* BL21 (DE3) plysS was purchased form Gene Power Lab Ltd.
[e] *Pichia pastoris GS115* was purchased from Invitrogen.
[f] N, not tested.

results indicate that PC-hepc is a member of the hepcidin family. However, the putative motif RENR recognized in the sequence was particular and different from typical propeptide convertases RX(K/R)R found in many fish [15,38,44]. Sequence analysis indicates that most of hepcidin amino acids from both mammals and fish contain eight cysteine residues in the mature peptide, forming four disulfide bonds but 12 fish hepcidin sequences lack one to four cysteine residues in the mature peptide. This suggests that not all of the hepcidin molecules identified from fish have a characteristic structure of eight cysteine residues in the mature peptide. To date, the reason why there are multiple hepcidin copies in some fish is not clear. It is tempting to think different fish hepcidins may have different functions [19] and the additional copies serve to support innate immunity in fish [15,20]. Mammalian hepcidin has bifunctions involved in antimicrobial activity and in iron metabolism [31,34], but in fish the two biological functions are separated which yields insights into the evolution of hepcidin [13]. It seems reasonable that many hepcidin variants existing in fish are associated with the adaptive differentiation. In a recent study, Xu et al. [43] suggest adaptive evolution of the reduced cysteine variant of hepcidin in response to the polar environment.

An interesting finding is the demonstration of the highest amount of PC-hepc transcripts in kidney, conversely PC-hepc mRNA in liver was at the lowest level among tested tissues. The tissue-specific expression is different from that of most reported fish hepcidins. Earlier studies show that hepcidin transcripts are usually demonstrated at a higher level in the liver in normal fish such as in uninfected Atlantic salmon [15], in normal black porgy [44] and in unchallenged Japanese flounder [20], and sometimes in the kidney [20,44]. During LPS challenge we found that the expression level of PC-hepc gene was significantly enhanced in the spleen, heart and stomach but no significant expression was detected in the liver and kidney. This expression pattern is different from that of human, mouse and many reported fish hepcidins. The Japanese flounder Hep-JF1and Hep-JF2 are both induced to a higher level in the liver with LPS challenge and Hep-JF2 was also expressed in the kidney [20]. In addition, the olive flounder *Paralichthys olivaceus* HAMP2 was highly induced in liver, gill, kidney (head), and spleen upon LPS stimulation [23], while transcripts of another variant, HAMP1, did not show any significant change in tissues tested although it is widely expressed in multiple tissues of normal fish. Similarly, the mouse HAMP1 on LPS challenge [18] and white bass HAMP with bacterial challenge [38] are also reported to increase gene expression predominantly in the liver, while the Atlantic salmon Sal1 and Sal2 transcripts were both up-regulated in multiple tissues with bacterial challenge [15]. As Hilton summarized recently, hepcidin transcript levels in fish challenged with bacteria were increased primarily in the liver and a limited extent in the kidney or other tissues [19]. The discrepant expression pattern between PC-hepc and other fish hepcidins corresponds with the description of Hirono et al. [20]; that is, the regulation of hepcidin gene expressed in fish is highly divergent among species. It is also noteworthy that PC-hepc mRNA expression in the kidney was not significantly changed during the period of 48 h after LPS challenge but maintained a high level of transcripts similar to that in unchallenged normal fish, suggesting that PC-hepc was constitutively expressed in the kidney. Similar to Sal1 which is an Atlantic salmon hepcidin that was constitutively expressed in blood cells [15], the high level of PC-hepc transcript in the kidney before and after LPS challenge may be a precautionary measure against impending infection. In addition, the high levels of transcripts of PC-hepc constitutively expressed in kidney, but not in liver [12,15,20,22,23,37,38,44], suggest that PC-hepc may not be liver-expressed. A few studies indicated that fish kidney is also the site where hepcidin gene is highly expressed besides the liver [20,22,44], and the level of hepcidin transcripts in the kidney remained high before and after challenge [44]. The evidence from large yellow croaker may further support the previous observations that the kidney might be involved in hepcidin metabolism [26,44]. The present study also showed that PC-hepc gene was up-regulated in specific organs after LPS stimulation and the expression pattern between them was different as shown in Fig. 3b in which PC-hepc transcripts in heart were highly increased at 12 h after challenge while in spleen at 24 h and stomach at 48 h. The difference in tissue-specific expression pattern probably exhibits the antimicrobial properties and other physiological functions of AMPs described previously [11,24].

In the study, we observed that the synthetic PC-hepc exhibited different activity against both the Gram-positive and Gram-negative bacteria tested, it was particularly active against the Gram-negative bacteria *A. hydrophila*, *V. parahaemolyticus*, *V. alginolyticus* and *V. harvryi*. These are the main pathogens of fish and invertebrates [1,3,4,5], for example, *V. harveyi* is a serious pathogen of marine fish and invertebrates [4], and *V. parahaemolyticus* is an enteric pathogen usually responsible for acute human gastroenteritis associated with the consumption of contaminated seafood [35]. PC-hepc also had potent activity against *S. aureus* and *S. epidermidis*. This is an interesting result because over half of *S. aureus* strains in clinics are now estimated to be resistant to antibiotics [41], and in particular *S. epidermidis* has emerged as a major cause of nosocomial infections and of nosocomial bacteraemia [17]. In view of the hepcidin antibacterial activity, there appear to be differences between the synthetic peptides from different fish species [20,27]. Strain specificity in hepcidin activity was also observed in other types of AMPs such as dermaseptins [30] and penaeidin [14]. The results of our kinetic studies with PC-hepc showed that bacterial-killing was time

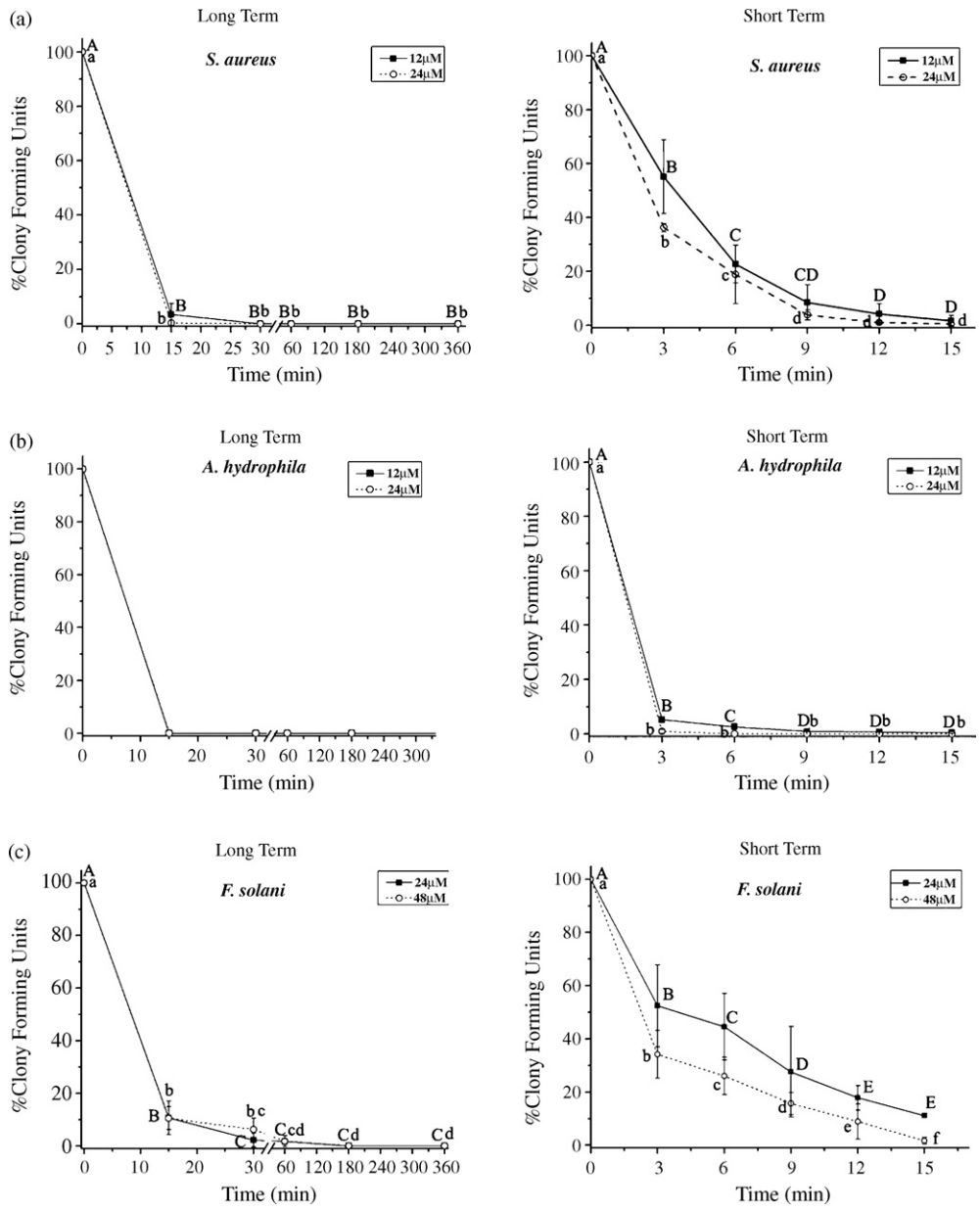

Fig. 4. Kinetics of killing of *S. aureus*, *A. hydrophila* and *F. solan* by PC-hepc. PC-hepc at a final concentration 1–2 times of the MBC value (12–24 μM) or water (control) was added to an exponential growth phase culture of *S. aureus* (a), and 1–2 times of the MBC (12–24 μM) of *A. hydrophila* (b) in NaPB and plated on nutrition broth agar. Fungicidal kinetic studies were also undertaken using *F. solani* with a concentration 1–2 times of the MBC value (24–48 μM) (c). The percentage of CFU was defined relative to the CFU obtained in the control (100% CFU at 0 min). Data at each time point represents the mean ± S.D. of 3 determinations ($n = 3$) and they were analyzed by 2 way ANOVA followed by Tukey post hoc test. The same letters (a, b, c, d, e, f) or (A, B, C, D, E) indicate no significant difference between different time points and different letters indicate statistically significant differences ($P \leq 0.05$) between time points.

dependent and it exerted its antimicrobial effects within 15–30 min of exposure to *S. aureus* and *A. hydrophila*. This relatively rapid bacteria-killing process might have resulted from the action of the cationic PC-hepc on the cytoplasmic membrane of the bacteria [16,42]. It is also interesting to note that PC-hepc was capable of killing more than 95% *F. solan* at 30 min and nearly 100% were killed at 180 min (Fig. 4c). Fungal infections in human and animals have increased in recent twenty years but few effective drugs are now available for fungal diseases [2,29]. The antifungal activity of PC-hepc reminds us that AMPs with the capability of killing fungi may be candidates for treatment of fungal infections. Nevertheless, PC-hepc did not show activity against the yeasts *Pichia pastoris* GS115 and *Candida albicans*. This is consistent with previously described results [28].

In summary, *P. crocea* hepcidin gene (PC-hepc) was identified and the cDNA and genomic DNA sequences were revealed in this study. PC-hepc transcripts are widely distributed in various tissues of *P. crocea* and the gene expression pattern was somewhat specific (such as in kidney) in comparison with other reported fish hepcidin genes. The synthetic PC-hepc exhibited antimicrobial activity against bacteria and fungi tested, particularly showed strong activity against both the principal fish pathogens and the antibiotic-resistant strains, thus suggesting that this peptide has potential for use in veterinary medicine and aquaculture. Taken together, our study indicates that PC-hepc is involved in the innate immunity of large yellow croaker and probably as an important effective component in specific tissues in response to invading microorganisms.

Acknowledgments

This work was supported by a grant (2007AA091406) from the National High Technology Research and Development Program of China (863 Program) and a grant (2008N0043) from Fujian Science and Technology Department. We thank Professor John Ross Egerton for assisting in preparing of this manuscript.

References

[1] Alcaide E, Amaro C, Todoli R, Oltra R. Isolation and characterization of *Vibrio parahaemolyticus* causing infection in Iberian toothcarp *Aphanius iberus*. Dis Aquat Org 1999;35:77–80.
[2] Alcouloumre MS, Ghannoum MA, Ibrahim AS, Selsted ME, Edwards Jr JE. Fungicidal properties of defensin NP-1 and activity against *Cryptococcus neoformans* in vitro. Antimicrob Agents Chemother 1993;37:2628–32.
[3] Austin B, Austin DA. A review: bacterial pathogens of fish. J Appl Bacteriol 1985;58:483–506.
[4] Austin B, Zhang XH. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett Appl Microbiol 2006;43:119–24.
[5] Balebona MC, Andreu MJ, Bordas MA, Zorrilla I, Morinigo MA, Borrego JJ. Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.). Appl Environ Microbiol 1998;64:4269–75.
[6] Bao B, Peatman E, Li P, He C, Liu ZJ. Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection. Dev Comp Immunol 2005;29:939–50.
[7] Belén LG, Wimal U, Richard LG, Jose FM. Parallel evaluation of antimicrobial peptides derived from the synthetic PAF26 and the human LL37. Biochem Biophys Res Commun 2007;356:107–13.
[8] Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, et al. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 1993;14:1023–31.
[9] Chen SL, Xu MY, Ji XS, Yu GC, Liu Y, Cloning. Characterization, and expression analysis of hepcidin gene from Red Sea Bream (*Chrysophrys major*). Anti Agen Chem 2005;49(4):1608–12.
[10] Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003;31:3497–500.
[11] Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, et al. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. J Biol Chem 1996;271:2935–40.
[12] Cuesta A, Meseguer J, Esteban M. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. Mol Immunol 2008;45:2333–42.
[13] De Domenico I, Nemeth E, Nelson JM, Phillips JD, Ajioka RS, Kay MS, et al. The hepcidin-binding site on ferroportin is evolutionarily conserved. Cell Metab 2008;8:146–56.
[14] Destoumieux D, Bulet P, Strub JM, Van Dorsselaer A, Bachere E. Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. Eur J Biochem 1999;266:335–46.
[15] Douglas SE, Gallant JW, Liebscher RS, Dacanay A, Tsoi SC. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev Comp Immunol 2003;27:589–601.
[16] Duclohier H, Molle G, Spach G. Antimicrobial peptide magainin I from Xenopus skin forms anion-permeable channels in planar lipid bilayers. Biophys J 1989;56(5):1017–21.
[17] Eiff C, Peters G, Heilmann C. Pathogenesis of infections due to coagulase negative staphylococci. Lancet Infect Dis 2002;2:677–85.
[18] Ilyin G, Courselaud B, Troadec MB, Pigeon C, Alizadeh M, Leroyer P, et al. Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload. FEBS Lett 2003;542:22–6.
[19] Hilton KB, Lambert LA. Molecular evolution and characterization of hepcidin gene products in vertebrates. Gene 2008;415:40–8.
[20] Hirono I, Hwang JY, Ono Y, Kurobe T, Ohira T, Nozaki R, et al. Two different types of hepcidins from the Japanese flounder *Paralichthys olivaceus*. FEBS J 2005;272:5257–64.
[21] Hu X, Camusb AC, Aonoa S, Morrisona EE, Dennisa J, Nusbaumc KE, et al. Channel catfish hepcidin expression in infection and anemia. Comp Immunol Microbiol Infect Dis 2007;30:55–69.
[22] Huang PH, Chen JY, Kuo CM. Three different hepcidins from tilapia. Oreochromis mossambicus: analysis of their expressions and biological functions. Mol Immunol 2007;l44:1932–44.
[23] Kim YO, Hong S, Nam BH, Lee JH, Kim KK, Lee SJ. Molecular cloning and expression analysis of two hepcidin genes from olive flounder *Paralichthys olivaceus*. Biosci Biotechnol Biochem 2005;69:1411–4.
[24] Koczulla R, von Degenfeld G, Kupatt C, Krötz F, Zahler S, Gloe T, et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. J Clin Invest 2003;111(11):1665–72.
[25] Krause A, Neitz S, Magert HJ, Schulaz A, Forssmann WG, Schulz-Knappe P, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147–50.
[26] Kulaksiz H, Theilig F, Bachmann S, Gehrke SG, Rost D, Janetzko A, et al. The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. J Endocrinol 2005;184:361–70.
[27] Lauth X, Shike H, Burns JC, Westerman ME, Ostland VE, Carlberg JM, et al. Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass. J Biol Chem 2002;277:5030–9.
[28] Lauth X, Babon JJ, Stannard JA, Singh S, Nizet V, Carlberg JM, et al. Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and in vivo hepatic response to bacterial infections. J Biol Chem 2005;280:9272–82.
[29] Lupetti A, Danesi R, van't Wout JW, van Dissel J, Senesi TS, Nibbering PH. Antimicrobial peptides: therapeutic potential for the treatment of Candida infections. Expert Opin Investig Drugs 2002;11:309–18.
[30] Mor A, Hani K, Nicolas P. The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem 1994;269:31635–41.
[31] Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, et al. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci USA 2002;99:4596–601.
[32] Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Prot Eng 1997;10:1–6.
[33] Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001;276:7806–10.
[34] Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2000;276:7811–9.
[35] Pinto AD, Ciccarese G, Corato RD, Novello L, Terio V. Detection of pathogenic *Vibrio parahaemolyticus* in southern Italian shellfish. Food Control 2008;19:1037–41.
[36] Ren HL, Wang KJ, Zhou HL, Yang M. Cloning and organisation analysis of a hepcidin-like gene and cDNA from Japan sea bass *Lateolabrax japonicus*. Fish Shellfish Immunol 2006;21:221–7.
[37] Rodrigues PN, Vazquez-Dorado S, Neves JV, Wilson JM. Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*). Dev Comp Immunol 2006;30:1156–67.
[38] Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, et al. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur J Biochem 2002;269:2232–7.
[39] Shike H, Shimizu C, Lauth X, Burns JC. Organization and expression analysis of the zebrafish hepcidin gene, an antimicrobial peptide gene conserved among vertebrates. Dev Comp Immunol 2004;28:747–54.
[40] Supungul P, Tang S, Maneeruttanarungroj C, Rimphanitchayakit V, Hirono I, Aoki T, et al. Cloning, expression and antimicrobial activity of crustinPm1, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. Dev Comp Immunol 2008;32(1):61–70.
[41] Vizioli J, Salzet M. Antimicrobial peptides from animals: focus on invertebrates. Trends Pharmacol Sci 2002;123:494–6.
[42] Wu M, Maier E, Benz R, Hancock REW. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and the cytoplasmic membrane of *Escherichia coli*. Biochemistry 1999;38:7235–42.
[43] Xu Q, Cheng CC, Hu P, Ye H, Chen Z, Cao L, et al. Adaptive evolution of hepcidin genes in Antarctic Notothenioid fishes. Mol Biol Evol 2008;25:1099–112.
[44] Yang M, Wang KJ, Chen JH, Qu HD, Li SJ. Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii B.*). Fish Shellfish Immunol 2007;23:1060–71.
[45] Zhang YA, Zou J, Chang CI, Secombes CJ. Discovery and characterization of two types of liver-expressed antimicrobial peptide 2 (LEAP-2) genes in rainbow trout. Vet Immunol Immunopathol 2004;101:259–69.
[46] Zheng W, Liu G, Ao J, Chen X. Expression analysis of immune-relevant genes in the spleen of large yellow croaker (*Pseudosciaena crocea*) stimulated with poly I:C. Fish Shellfish Immunol 2006;21:414–30.