Comparative Biochemistry and Physiology, Part B 158 (2011) 155–163

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular characterization of hepcidin AS-hepc2 and AS-hepc6 in black porgy (*Acanthopagrus schlegelii*): Expression pattern responded to bacterial challenge and in vitro antimicrobial activity

Ming Yang [a,b,1], Bei Chen [a,1], Jing-Jing Cai [a], Hui Peng [a], Ling-Cai [a], Jian-Jun Yuan [a], Ke-Jian Wang [a,*]

[a] State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen, Fujian 361005, China
[b] School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, China

ARTICLE INFO

Article history:
Received 23 August 2010
Received in revised form 3 November 2010
Accepted 4 November 2010
Available online 10 November 2010

Keywords:
Acanthopagrus schlegelii
Hepcidin
Bacterial infection
Gene expression
Antimicrobial peptides

ABSTRACT

There are more diversified isoforms of the hepcidin gene that exist in fishes than in mammals, and elucidating the differences between these isoforms should provide insight into the functioning of hepcidin in fishes. In our study, AS-hepc2 and AS-hepc6 hepcidin isoforms from black porgy were characterized for their in vivo expression patterns following bacterial challenge, and their in vitro antimicrobial activities against Gram-positive and Gram-negative bacteria as well as fungi. As a result, two isoforms were observed to be widely distributed in all the tissues tested. AS-hepc2 was a liver-expressed hepcidin peptide which was always highly more expressed in the liver than in the other tissues tested no matter whether this was before or after bacterial challenge. AS-hepc6 was detected mainly in the head kidney and trunk kidney of normal fish, but, in the challenged fish, its expression involved more tissues than just the kidneys. The mature peptides of AS-hepc2 and AS-hepc6 were modeled for 3D structure and then synthesized for antimicrobial assay. AS-hepc6 had a wider antimicrobial spectrum than AS-hepc2 and, in particular, had more potent antifungal activity. Our study indicated that the two hepcidin isoforms had different characteristics in terms of their expression patterns and antimicrobial activity, and they were assumed to play an overlapping role in the innate immune system of black porgy against invading pathogens.

1. Introduction

Hepcidin is a cysteine-rich antimicrobial peptide and putative iron hormone previously found in mammals and bony fish. To date, large numbers of hepcidin-like genes or expressed sequence tags have been identified in mammals and fishes, and also a few in birds and even amphibians (Shi and Camus, 2006; Ren et al., 2006; Zheng et al., 2006; Chiou et al., 2007; Fu et al., 2007; Segat et al., 2008). In mammals, only one hepcidin gene is identified in humans (Krause et al., 2000; Park et al., 2001) and two hepcidin genes are found clustered on the genome in mice (Nicolas et al., 2001; Pigeon et al., 2001). However, in fishes, two or more hepcidin gene copies have been identified in many fish species, including winter flounder *Pleuronectes americanus*, atlantic salmon (*Salmo salar*) (Douglas et al., 2003), zebrafish (*Danio rerio*) (Shike et al., 2004), japanese flounder or olive flounder *Paralichthys olivaceus* (Hirono et al., 2005; Kim et al., 2005), mosambique tilapia (*Oreochromis mossambicus*) (Huang et al., 2007), black porgy (*Acanthopagrus schlegelii*) (Yang et al., 2007), rockbream (*Oplegnathus fasciatus*) (Cho et al., 2009), and redbanded seabream (*Pagrus auriga*) (Martin-Antonio et al., 2009). These observations imply that the presence of multiple hepcidin isoforms is likely to be a more universal phenomenon in fishes than in other vertebrates.

The multiple hepcidin isoforms that existed in fish show isoform- or tissue-specific. In Japanese flounder (Hirono et al., 2005) and also in olive flounder (Kim et al., 2005), two hepcidin isoforms are expressed in a tissue-specific manner and respond differently to an iron overload or lipopolysaccharide (LPS) injection. Similarly, three types of hepcidin isoforms identified from winter flounder and Atlantic salmon are differentially expressed during bacterial challenge and larval development (Douglas et al., 2003). Also, three tilapia hepcidin genes are expressed in a tissue-specific manner in response to infection and iron overload (Huang et al., 2007). Moreover, four hepcidin isoforms from rockbream (Cho et al., 2009) are regulated in an isoform- and tissue-specific fashion during bacterial infection and iron overload or viral infection, and four hepcidin genes from the redbanded seabream are expressed in an isoform-specific fashion in embryogenesis and respond differently to LPS injection (Martin-Antonio et al., 2009).

To date, the reason why there are multiple hepcidin copies in some fishes has not been well explained. Padhi and Verghese (2007) suppose the reason for such a diversity of hepcidin isoforms in fishes to be positive Darwinian selection. Hilton and Lambert (2008) divide hepcidins into two groups according to their phylogenetic analyses

* Corresponding author. Tel.: +86 592 2184658; fax: +86 592 2180655.
E-mail address: wkjian@xmu.edu.cn (K.-J. Wang).
[1] These authors made equal contributions.

1096-4959/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2010.11.003

with a total of 68 reported hepcidin isoforms, and find that whereas only one hepcidin antimicrobial peptide, *HAMP1*, is usually present in each fish species, more diversified *HAMP2* homologues are detected in acanthopterygian fishes. Although the *HAMP2* sequences from different acanthopterygian fishes all share a much conserved amino acid constitution pattern, each isoform has its unique structural characteristics such as different isoelectric points (p*I*), thus suggesting that each isoform of *HAMP2* might play a unique or specific role in the innate immune system against pathogenic invaders.

Seven hepcidin isoforms from black porgy all belong to the *HAMP2* group (Hilton and Lambert, 2008; Martin-Antonio et al., 2009), among which the hepcidin 2 (AS-hepc2) gene and hepcidin 6 (AS-hepc6) gene are isolated from different tissues (Yang et al., 2007). The AS-hepc2 gene codes for a hepcidin peptide of 88 amino acids and the AS-hepc6 gene codes for a protein of 84 amino acids sharing 75% identified amino acids with AS-hepc2. In this study, AS-hepc2 and AS-hepc6 (the GenBank accession numbers are AY669377 and AY669381) were chosen to investigate the in vivo expression patterns during the course of bacterial challenge, and the in vitro antimicrobial activities using synthesized mature peptides. Our results should be helpful towards elucidating the different functions of hepcidin gene isoforms and so provide an insight into the necessity for, and the reasonability of, the existence of multiple hepcidins in individual fish.

2. Materials and methods

2.1. Bacteria preparation, fish challenge and tissue collection

The bacterial cocktail was prepared with *Staphylococcus aureus* CGMCC 1.363, *Escherichia coli* CGMCC 1.2389, *Vibrio parahaemeolyticus* CGMCC 1.1615 and *Micrococcus lysodeikticus* CGMCC 1.634, purchased from the China General Microbiological Culture Collection Center (CGMCC), Beijing, China. Each bacterial strain was cultured in lysogeny broth at 28–30 °C with shaking at 200 rpm overnight, then the four bacterial cultures were separately centrifuged, suspended, and finally mixed in sterile physiological saline solution (PSS) at a concentration of 10^{10} CFU mL^{-1} (each strain) for challenging.

Black porgy (100–200 g, 15–20 cm) were obtained from TongAn marine-culture fish farm in Xiamen, Fujian Province, China. Fish were acclimatized to laboratory conditions for one week and then healthy fish were chosen for the experiments. The fish were divided into a bacteria injected group and a control group. Prior to injection, normal fish tissues were collected from healthy fish ($n = 3$). Blood cells were first sampled from the caudal vein using a syringe and then liver, spleen, head kidney, trunk kidney, heart, brain, stomach, intestine, gill and skin samples were collected separately from each individual fish, frozen immediately in liquid nitrogen, and stored at −80 °C. At 0 h, the bacteria injected group was injected with 50 μL of live bacterial cocktail and the control group was injected with 50 μL of sterile PSS by intra-peritoneal injection below the pectoral fin of the fish. The bacterial challenge experiment was performed for 48 h, and blood cells, liver, spleen, head kidney, trunk kidney, stomach, intestine and gill were collected at 6 h, 24 h and 48 h post-injection, and stored at −80 °C.

2.2. Real time quantitative PCR analysis of the AS-hepc2 and AS-hepc6 genes

2.2.1. Total RNA extraction and reverse transcription

Tissue samples were separately ground quickly in liquid nitrogen and transferred to 1 mL TRIzol solution (Invitrogen). Blood cells from 200 μL of fresh whole blood were directly transferred to 1 mL TRIzol solution. Total RNA was then isolated according to the manufacturer's instructions and evaluated for quality and quantity using an Ultrospec 2100 Pro UV/visible spectrophotometer (Amersham Biosciences). The reverse transcription reactions were performed using a PrimeScript™ Reverse Transcription Reagent Kit for Perfect Real Time (TaKaRa), using 0.5 μg of total RNA and oligo dT and random 6 primers in a DNA Engine (Bio-Rad) as follows: 15 min at 37 °C, 5 s at 85 °C, and holding at 4 °C. The reverse transcription product was diluted 50 to 100 fold in pure sterile water before use for real time PCR assays.

2.2.2. Primer and probe design

Due to the similarity of the sequences of the AS-hepc2 and AS-hepc6 genes, two specific TaqMan probes and the corresponding primers were manually designed for the AS-hepc2 and AS-hepc6 genes using the Primer Express 3.0 software at distinctly different regions so as to differentiate their gene expression patterns. The sequences of primers and TaqMan probes are listed in Table 1. The forward primers for AS-hepc2 and AS-hepc6 were designed to span an intron, thus avoiding amplification of the target genes in the genomic DNA.

2.2.3. Relative quantity of AS-hepc2 and AS-hepc6 mRNA

For each cDNA sample, the AS-hepc2, AS-hepc6 and 18S rRNA genes were amplified in separate reaction tubes in duplicate using TaqMan Universal PCR Master Mix (Applied Biosystem) in a 7500 Real Time PCR System. The relative AS-hepc2 and AS-hepc6 gene expression was quantified using 18S rRNA gene as an endogenous control gene. The efficiencies of the AS-hepc2, AS-hepc6 and 18S rRNA genes were verified to be approximately equal (slope difference <0.1) by the validation experiment, which was performed using a serial dilution of liver cDNA (1/50, 1/100,1/200, 1/400, 1/800, 1/1600). The

Table 1
Primers and probes for real time PCR.

Gene name	Primer and probe	Amplicon length	Application
AS-hepc2	Forward primer: 5′ TGGAAGATGCCGTATAACAACAGA 3′ Reverse primer: 5′ GGAGCAGGAATCCTCAGAACCT 3′ TaqMan probe: 5′ FAM-CAGCCCCGCTGGTTGTCGCTT-TAMRA 3′	121 bp	Target gene
AS-hepc6	Forward primer: 5′ GCCAGAGGACTCCTGGAAGAT 3′ Reverse primer: 5′ AGCAGAGACCACATCCTCTCATG 3′ TaqMan probe: 5′ FAM-ATGGCAGCAGACGCTGGAGGTGT-TAMRA 3′	98 bp	Target gene
18S rRNA	Forward primer: 5′ TCCCATGAACGAGGAATTCC 3′ Reverse primer: 5′ ACAAAGGGCAGGGACTTAATCA 3′ TaqMan probe: 5′ FAM-CGCGAGCTTATGACCCGCGC-TAMRA 3′	69 bp	Endogenous control

PCR cycling profile was set as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s, and annealing and primer extension at 60 °C for 1 min.

For each cDNA sample, the gene expression of AS-hepc2 or AS-hepc6 was first normalized to the 18S rRNA gene, and presented as ΔC_T values. Liver at the 0 h time point (healthy liver) was used to calibrate, and the $\Delta\Delta C_T$ value was obtained by subtracting the mean ΔC_T values of the 0 h liver from the ΔC_T value of the target sample. The relative gene expression of the target sample was presented as the fold difference to 0 h liver and calculated using the formula ($2^{-\Delta\Delta CT}$).

2.2.4. Absolute quantity of AS-hepc2 and AS-hepc6 mRNA

Two serial dilutions of recombinant pMD_{18}-T plasmid constructed with either the AS-hepc2 PCR product or the hepc6 PCR product at known concentration were prepared as templates for absolute standard curves. The PCR reactions for the AS-hepc2 or AS-hepc6 genes in the cDNA samples tested were simultaneously performed with the reactions in the standard templates in duplicate using TaqMan Universal PCR Master Mix in a 7500 real time PCR System following the same procedure described in Section 2.2.3. From the C_T value and the absolute quantity standard curve, the quantity of AS-hepc2 and AS-hepc6 genes in the tested samples was calculated and presented as absolute cDNA copy numbers per μg RNA.

2.3. Antimicrobial activity of AS-hepc2 and As-hepc6 peptides

2.3.1. Synthesis of mature peptides

The putative mature regions of the AS-hepc2 and AS-hepc6 peptides were synthesized by Sangon Biotech (Shanghai, China). MALDI-TOF MS and HPLC analyses were performed to identify the synthetic peptides. The peptides were suspended in serial dilutions with milli-Q water for the antimicrobial assays.

2.3.2. Microbial culture and preparation

All strains for the antimicrobial assays were purchased from the CGMCC with the exception of the yeast strain, *Pichia Pastoris* GS115, which was bought from Invitrogen Biotech. Three marine bacteria, *Vibrio harveyi*, *V. alginolyticus* and *V. parahaemolyticus*, and all the other bacteria were cultured overnight at the appropriate temperature (28 °C or 37 °C) to the logarithmic phase for antimicrobial assays on marine agar 2216 (Difco) or Muller–Hinton agar. Yeast strains were grown on YPG agar (yeast extract 1%, peptone 1% and glucose 2%) at 28 °C for 2 d for the experiments. Filamentous fungi were grown on potato dextrose agar at 28 °C for 7 d to spore germination.

2.3.3. Antimicrobial assays

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the AS-hepc2 and AS-hepc6 peptides were determined in triplicate in a 96-well culture plate using the liquid growth inhibition assay referred to in previous studies (Bulet et al., 1993; Peng et al., 2010). Briefly, microorganisms were washed off a slope agar culture, diluted in 10 mM sodium phosphate buffer (NaPB, pH 7.4), and then about 10^4 CFU of bacteria/well and 10^3 CFU of yeast or fungal spores/well were incubated with serial dilutions of synthesized peptide (beginning at 60 μM) at 28 °C or 37 °C in the presence of the appropriate growth media. For yeast and fungal spores, chloramphenicol (30 μg mL^{-1}) was added to avoid bacterial contamination. MIC was determined after 24 h of incubation with bacteria or 48 h of incubation with yeast and fungal spores. Then, 50 μL aliquots of the reactive solutions were plated on the appropriate agar and incubated at the appropriate temperature (28 °C or 37 °C). MBC was determined after incubation overnight for bacteria, 2 d for yeast and 3–4 d for fungal spores.

2.3.4. Kinetics of bactericidal activity

The time course of bactericidal activity was determined using a modified method referred to in a previous study (Tencza et al., 1997). Briefly, synthetic AS-hepc2 and AS-hepc6 peptides at their respective MBC were incubated with the logarithmic phase cultures of *E. coli* or *S. aureus* in the presence of Muller–Hinton broth following the same procedure described in the antimicrobial assay. Then, aliquots of the mixtures were removed at each time point and immediately diluted 1:80 with 10 mM NaPB (pH = 7.4), then 100 μL of each bacterial dilution was plated on Muller–Hinton agar and incubated overnight. The colonies were counted and the percentages of the recovered bacterial CFUs to the initial CFUs (without peptide) were plotted as a function of peptide exposure time.

2.4. Statistical analysis

The relative quantity of AS-hepc2 and AS-hepc6 mRNA in each tested cDNA sample was represented as a fold difference to that in the healthy livers. SPSS (version 10.5) software was used for statistical analysis. Data were presented as the mean relative quantity ± standard error of the mean (S.E.M.), calculated from three fish (each sampled in duplicate). Prior to statistical analysis, raw data were log-transformed because of the low homogeneity. A comparison of gene expression between bacteria and PSS injection fish was made using Independent-sample t tests and those with a significant difference ($P<0.05$) were indicated by '*'.

Differences of relative gene expression between two treatment groups and among four time points were evaluated using two-way ANOVA followed by the Fisher's Least Significant Difference (LSD) test. The difference between groups was considered significant at the 0.05 level and the P value was shown by '$P^{\dagger}$', while the differences between time points at the $P<0.05$ or 0.01 level were considered significant and indicated by '*' or '**'.

The bacterial recovery index in the bactericidal kinetic curves was represented as the mean ± standard deviation (S.D.) of three independent assays and the data were analyzed using one-way ANOVA followed by the Tukey post hoc test. The same letters (a, b, c, d, and e) indicate no significant difference between time points and different letters indicate statistically significant differences ($P<0.05$) between time points.

3. Results and discussion

3.1. Different expression patterns of AS-hepc2 and AS-hepc6 genes

3.1.1. Tissue distribution in normal fish

AS-hepc2 and AS-hepc6 genes were detected in all tissues tested, suggesting that they were widely present in the tissues of black porgy. However, it was observed that the two gene isoforms had different tissue-specific expression patterns in normal fish (Fig. 1). For AS-hepc2, three classes of relative expression were presented according to the quantity: higher relative expression in the liver; lower expression in the skin, head kidney, blood, heart, brain and spleen (0.01-0.001 fold of that in the liver); and the negligible expression in the stomach, gill, intestine and trunk kidney (below 0.001 fold). However, four classes of gene expression were presented for AS-hepc6: higher relative expression in the head kidney and trunk kidney (about 100 fold that in the liver); moderate expression in the stomach, intestine, blood, spleen and liver (1-10 fold); lower expression in the gill and heart (0.1-1 fold); and the lowest expression in the brain and skin (0.01-0.1 fold). In general, AS-hepc2 was expressed predominantly in the liver in normal fish, but AS-hepc6 was highly expressed in the head kidney and trunk kidney.

3.1.2. AS-hepc2 response to bacterial challenge

On comparing the AS-hepc2 gene expression between the bacteria injected with the PSS mock injection fish using the Independent-sample t test method, it was found that AS-hepc2 was significantly induced in the liver, spleen, trunk kidney, head kidney at 6 h. The significant expression was further detected in the liver and stomach at 24 h and in the gill at 48 h, while in the trunk kidney the level of AS-

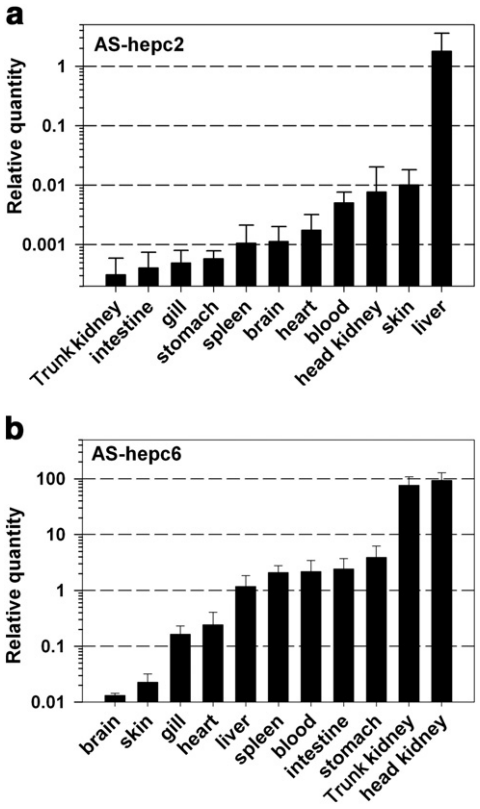

Fig. 1. Tissue distribution of AS-hepc2 and AS-hepc6 genes in normal black porgy (a, AS-hepc2; b, AS-hepc6). The quantity of AS-hepc2 or AS-hepc6 mRNA was normalized using 18S rRNA, and the relative index was determined using liver as the calibrator tissue. Data are presented as mean ± S.E.M. (3 fish, in duplicate) fold difference to the healthy liver.

hepc2 expression maintained significant at 24 h and 48 h (Fig. 2a–c). The relative quantity of AS-hepc2 mRNA in the liver was always more than 100 fold higher than that in the other tissues at any challenge time point, showing the predominantly higher expression of the AS-hepc2 gene in the liver than in the other tissues no matter whether taken before or after bacterial challenge.

Therefore, we proposed that the AS-hepc2 was a liver-expressed isoform in the black porgy. This was consistent with the previous observations on human and white bass hepcidins, which are expressed predominantly in the liver and the expression could be highly induced after bacterial infection and thus they could be considered as liver-expressed antimicrobial peptides (Krause et al., 2000; Park et al., 2001; Shike et al., 2002). Similarly, Hepc-JF1 in Japanese flounder (Hirono et al., 2005), hepcidinII in olive flounder (Kim et al., 2005), HAMP3 and HAMP4 in redbanded seabream (Martin-Antonio et al., 2009), and other single hepcidin genes in sea bass (Rodrigues et al., 2006) and turbot (Chen et al., 2007) are also reported to be specifically expressed in the liver.

Two-way ANOVA was performed to evaluate the response of AS-hepc2 to bacterial injection or PSS mock injection during the time course in each tissue tested. The results showed that in the liver, trunk kidney, spleen, gill and stomach, bacterial injection, distinguished from the PSS injection ($P<0.05$), had a significant effect on AS-hepc2 expression, indicating that the expression change was triggered just by bacterial infection but not by the injection treatment. A similar up-regulated expression trend was presented in the liver, trunk kidney, spleen and gill in response to bacterial infection: highly induced expression at 6 h, and diminished induction at 24 h, and then enhanced induction at 48 h as shown in Fig. 2d–g.

3.1.3. AS-hepc6 response to bacterial challenge

The AS-hepc6 gene was significantly induced in the liver, spleen and gill at 6 and 24 h post challenge (Fig. 3a–c), compared to fish from the control group (independent-sample t test). Comparing the relative quantity in all tissues, a high AS-hepc6 mRNA value was

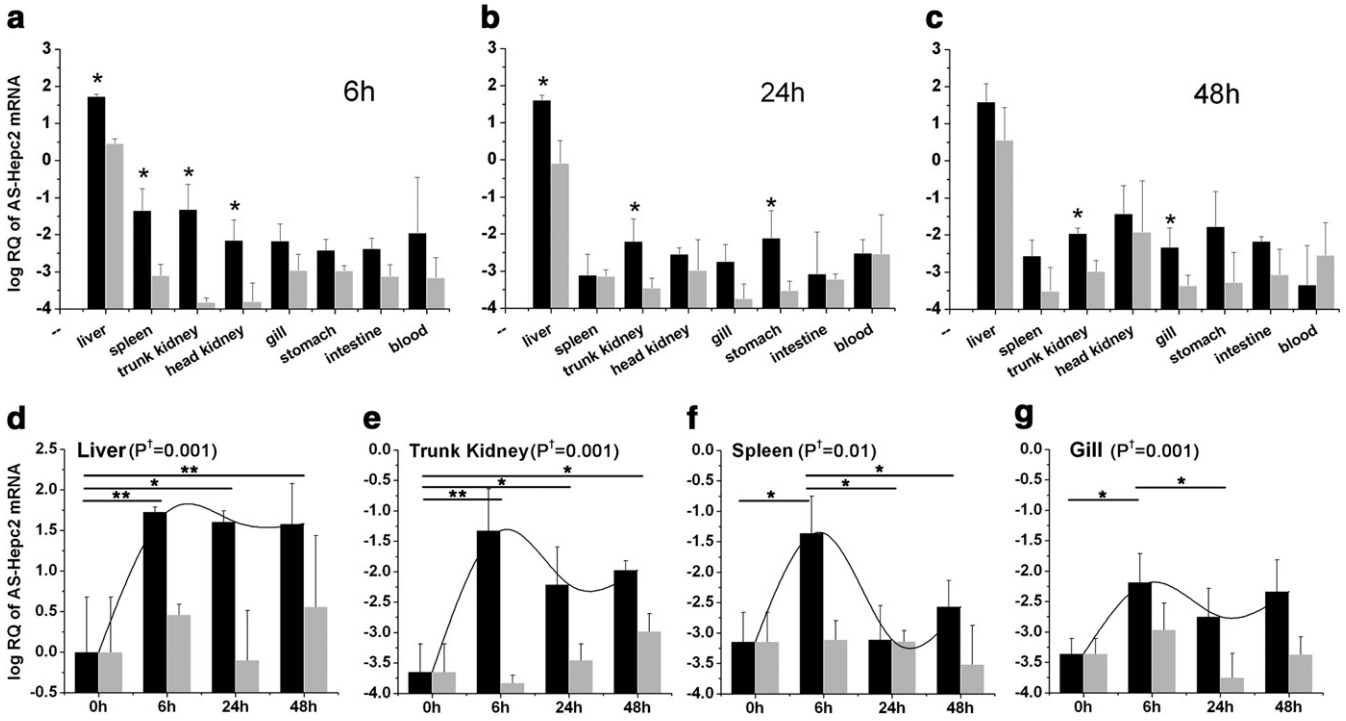

Fig. 2. The expression profiles of the AS-hepc2 gene in multiple tissues of black porgy following bacterial challenge. Black columns represent the bacterial injection group, and gray columns the PSS injection group. Data are expressed as the mean ± S.E.M. (3 fish, in duplicate) fold difference to the healthy liver. In a, b and c, '*' indicates tissues with statistical significance between two groups ($P<0.05$, independent-sample t test). In d, e, f and g, the difference between groups was considered significant at the 0.05 level and the P value is shown by 'P†'. '*' and '**' indicate significant difference between two time points ($P<0.05$ and $P<0.01$, two-way ANOVA followed by the LSD test).

detected in the trunk kidney, head kidney and spleen at all time points and, in particular, a further higher quantity was observed in the blood cells from both groups at 24 h and 48 h.

As a result, and according to the quantity of transcripts in every tissue, AS-hepc6 was thought not to be a liver-expressed hepcidin isoform. Our observation on AS-hepc6 was similar to that of the research on catfish hepcidin (Bao et al., 2005), which is expressed in a wide range of tissues in normal fish and is highly distributed in the spleen and head kidney but not in the liver. Particularly, it was very interesting to note that although the AS-hepc6 gene was not significantly expressed in the head kidney and trunk kidney during bacterial challenge, the gene expression level in those two tissues was still maintained at a level as high as that in the normal fish, showing the constitutive expression of AS-hepc6 in the kidney, consistent with the observation on a hepcidin isoform in large yellow croaker (Wang et al., 2009).

Among the eight tissues tested, in the spleen and gill (Fig. 3d and e) AS-hepc6 expression was significantly induced at all time points by bacterial injection but not from PSS mock injection (two-way ANOVA). In contrast, in the other tissues, AS-hepc6 gene change in expression could be differentiated between time points but could not be distinguished between the bacterial and PSS injection. For example, in blood cells (Fig. 3f), AS-hepc6 mRNA was dramatically up-regulated at 6 h, 24 h and 48 h compared to that at 0 h, but the induction was not just in response to bacterial injection but also PSS injection, implying that AS-hepc6 expression might be triggered by the injection treatment.

Since the initial discovery of hepcidin in human and hybrid bass (Krause et al., 2000; Park et al., 2001; Shike et al., 2002), many tissues are revealed to be involved in hepcidin synthesis, including the liver, kidney, spleen and pancreas (Ilyin et al., 2003; Krijt et al., 2004). It has been reported that fish hepcidin can be detected in the blood cells of Atlantic salmon (Douglas et al., 2003) and turbot (Chen et al., 2007), and also in peripheral blood leukocytes of the Japanese flounder (Hirono et al., 2005) and gilthead seabream (Cuesta et al., 2008). In the black porgy, blood cells were more active in the up-regulated expression of AS-hepc6 than any other tissue tested, responding to the injection treatment, and the expression level of AS-hepc6 increased about 1000 fold at 24 h compared to 0 h, suggesting that blood cells, as well as trunk kidney, head kidney or spleen, might be an important site for AS-hepc6 synthesis in the black porgy.

3.1.4. The absolute copy number

The absolute copy number of the AS-hepc2 and hepc6 mRNA transcripts was estimated in the liver and blood cells in order to elucidate the comparative constitution of AS-hepc2 and hepc6 gene isoforms in black porgy. According to the quantity, we noted that AS-hepc6 mRNA transcripts greatly outnumbered AS-hepc2 in the normal fish as observed in Fig. 4. In the liver, AS-hepc2 was much less than AS-hepc6 in the normal fish (163.5 times), however, after bacterial challenge AS-hepc2 was highly induced from 6 to 48 h and the transcript number was up to a similar level as AS-hepc6 at 48 h (AS-hepc2 to 6 ratio was 1:1.5).

In blood cells, the AS-hepc6 background expression level was approximately 12,000 times higher than AS-hepc2. After bacterial challenge, AS-hepc6 expression was further induced and the level was 51,951, 4,967,081 and 724,422 times higher than AS-hepc2 at 6, 24 and 48 h, suggesting the predominant abundance of AS-hepc6 gene transcripts in blood cells.

3.2. Different antimicrobial activity of AS-hepc2 and AS-hepc6 peptides

3.2.1. Peptide characterization

The AS-hepc2 and AS-hepc6 genes coded for hepcidin peptides of 88 amino acids and 84 amino acids, respectively. The signal peptide cleavage site (Emanuelsson et al., 2007) and RX(K/R)R motif typical of propeptide convertases (Douglas et al., 2003) were recognized in the two deduced peptides, and as a result, the two peptides were presumed to consist of three domains: signal peptide, predomain and mature peptide (Yang et al., 2007). Two mature peptides (24 and 20

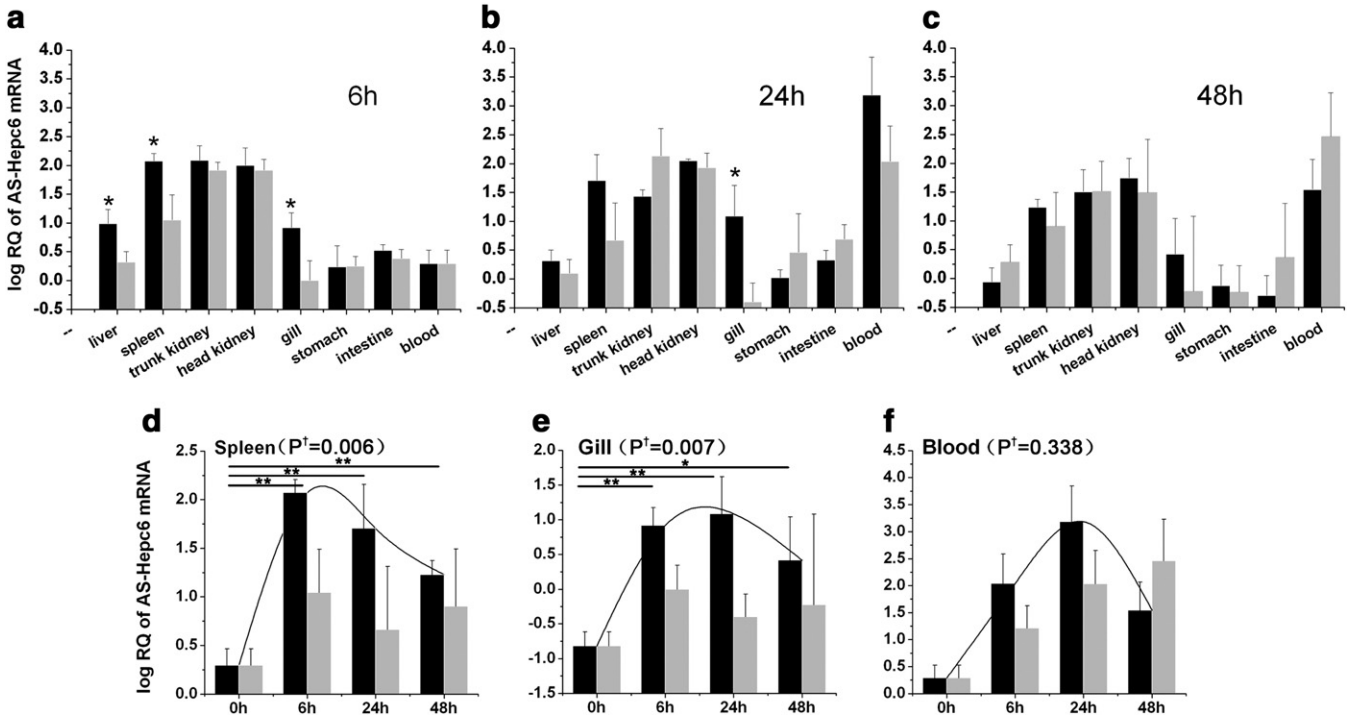

Fig. 3. The expression profiles of the AS-hepc6 gene in multiple tissues of black porgy following bacterial challenge. Black columns represent the bacterial injection group, and gray columns the PSS injection group. Data are expressed as the mean ± S.E.M. (3 fish, in duplicate) fold difference to the healthy liver. In a, b and c, '*' indicates the tissues with statistical significance between two groups ($P<0.05$, independent-sample t test). In d, e and f, the difference between groups was considered significant at the 0.05 level and the P value is shown by 'P†'. '*' and '**' indicate significant difference between two time points ($P<0.05$ and $P<0.01$, two-way ANOVA followed by the LSD test).

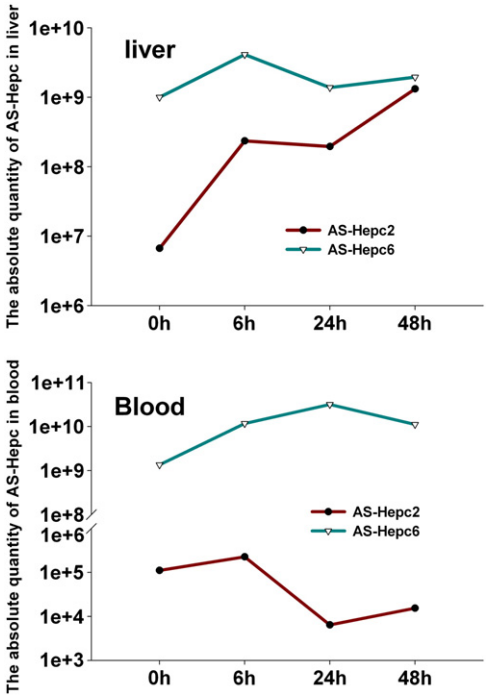

Fig. 4. The absolute quantity of AS-hepc2 and AS-hepc6 mRNA in liver and blood cells of black porgy during the time course of bacterial challenge. Data were calculated from the absolute standard curves using the real time PCR method, and are represented as the mean of cDNA copy number·μg^{-1} RNA (3 fish, in duplicate).

amino acids) derived from the C-terminal of AS-hepc2 and hepc6 prepropetides were analyzed using ExPASy Protein Tools (Gasteiger, et al., 2005) and the Antimicrobial Peptide Database (Wang and Wang, 2004), and the characteristics are listed in Table 2. It was found that AS-hepc6 peptide took more positive net charges and had a higher p*I* value and hydrophobic residue ratio than AS-hepc2.

The 3D structure of the two peptides was built using the modeling tools at the SWISS-MODEL website (Arnold et al., 2006), and bass hepcidin21 (PDB 1S4W) was used as a template. AS shown in Fig. 5, two β-sheets and four disulfide bonds were identified in the two peptides and AS-hepc6 had a stronger positive electric field than the AS-hepc2. In AS-hepc2, the β1-sheet comprised amino acids from Arg^6 to Cys^8, and the β2-sheet comprised amino acids from Gly^{19} to Cys^{21}. In AS-hepc6, the β1-sheet comprised amino acids from Arg^2 to Cys^4, and the β2-sheet comprised amino acids from Gly^{19} to Cys^{21}. Each peptide had eight cysteine residues forming four disulfide bonds which were connected following the pattern Cys^1–Cys^8, Cys^2–Cys^7, Cys^3–Cys^6, and Cys^4–Cys^5, the same pattern as that reported in human hepcidin (25 or 20 amino acids, Hunter et al., 2002) and bass hepcidin (21 amino acids, Lauth et al., 2004). It is reported that the connectivity pattern of four disulfide bonds between the two anti-parallel strands, especially an unusual vicinal disulfide bond of Cys^4–Cys^5 located at the turn of the hairpin points might be a possible crucial domain in the activity of the molecule (Kemna et al., 2008).

3.2.2. Antimicrobial spectrum

Antimicrobial activity against bacteria and other microbes is reported in humans and in several fish species using synthetic mature peptides (Park et al., 2001; Hirono et al., 2005; Lauth et al., 2005; Huang et al., 2007; Cuesta et al., 2008; Wang et al., 2009). In our study (Table 3), synthesized AS-hepc2 and hepc6 peptides were both active against Gram-positive *S. aureus*, *M. lysodeikticus*, *S. epidermidis*, *C. glutamicum* and *B. subtilis*, and Gram-negative *E. coli*, *Aeromonas hydrophila* and *V. harveyi*. However, *B. cereus* and *S. epidermidis* were

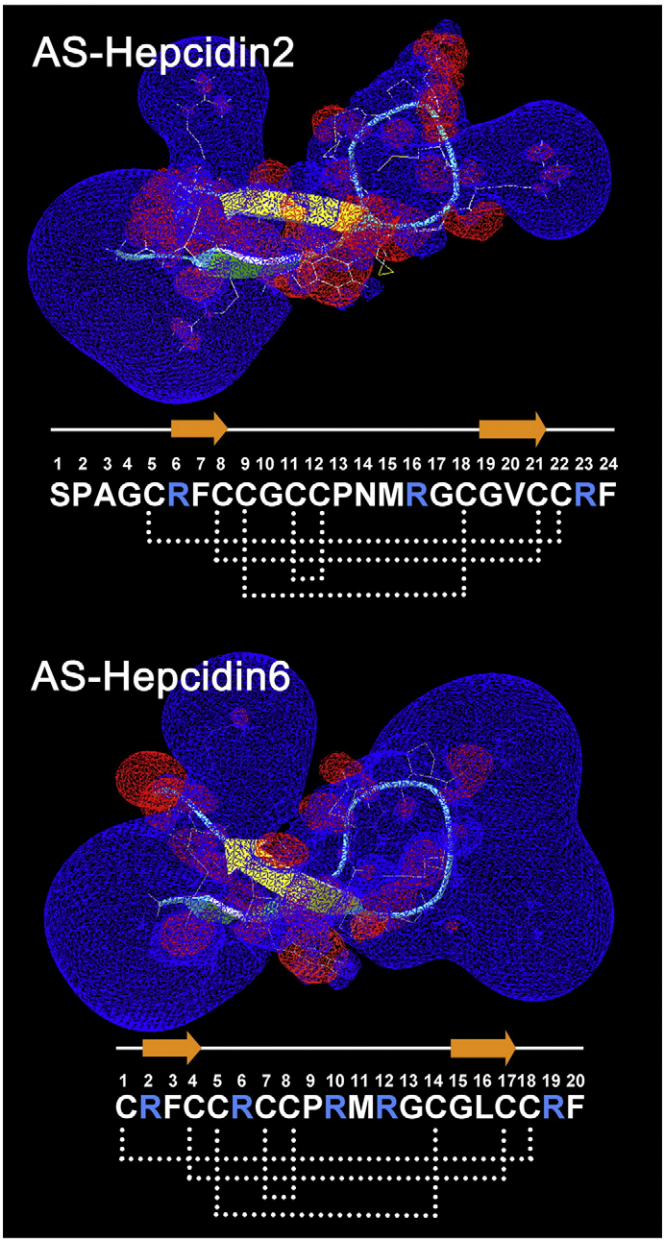

Fig. 5. Three-dimensional structural analysis of AS-hepc2 and AS-hepc6 mature peptides. The 3D structures were built with reference to bass hepc21 (PDB 1W6S) at the SWISS-MODEL website. Two β-sheets are represented by arrows. Disulfide bonds are positioned by dashed lines. Blue letters indicate positive residues. The electrostatic potential was calculated using the Poisson–Boltzmann equation. The positive potentials are shown in blue and negative potentials are in red.

Table 2
Characteristics of AS-hepc2 and AS-hepc6 mature peptides.

Peptide	Amino acid sequence	Length	Molecular weight	Net charge	p*I*	Total hydrophobic ratio	Protein-binding potential (Boman index) kcal/mol
AS-hepc2	SPAGCRFCCGCCPNMRGCGVCCRF	24	2531.0	+3	8.53	54%	1.1
AS-hepc6	CRFCCRCCPRMRGCGLCCRF	20	2373.9	+5	9.01	60%	2.46

Table 3
Antimicrobial activities of synthetic AS-hepc2 and AS-hepc6 mature peptides.

Microorganism	CGMCC NO.[a]	MIC (μM)		MBC (μM)	
		AS-hepc2	AS-hepc6	AS-hepc2	AS-hepc6
Gram-positive bacteria					
Staphylococcus aureus	1.363	1.25–2.5	2.5-5	10	20
Micrococcus lysodeikticus	1.634	2.5–5	2.5–5	10	10
Corynebacterium glutamicum	1.1886	5–10	2.5–5	20	20
Bacillus subtilis	1.3343	20–40	5–10	40	20
B. cereus	1.1846	>60	40–60	NT[b]	60
S. epidermidis	1.2429	>60	20–40	NT	>60
Gram-negative bacteria					
Escherichia coli	1.2389	5–10	10–20	40	60
Aeromonas hydrophila	1.2017	10–20	10–20	40	40
Vibrio harveyi	1.1593	20–40	40–60	60	60
V. alginolyticus	1.1833	>60	>60	NT	NT
V. parahaemolyticus	1.1615	>60	>60	NT	NT
Fungi					
Aspergillus niger	3.316	40–60	20–40	60	40
Fusarium graminearum	3.3490	>60	20–40	NT	40
F. solani	3.5840	>60	20–40	NT	40
Candida albicans	2.2411	>60	>60	NT	NT
Pichia pastoris GS115	Invitrogen[c]	>60	>60	NT	NT

The highest concentration tested with micrograms was 60 μM. MIC is expressed as the interval '*a*-*b*', where '*a*' is the highest concentration tested at which microorganisms are growing and '*b*' is the lowest concentration that causes 100% visual growth inhibition after 24 h of incubation with bacteria or 48 h of incubation with yeast and fungal spores. MBC represents the lowest concentration at which no microorganism grew on the appropriate plate after incubation with the MIC assay mixture overnight for bacteria, 2 d for yeast and 3–4 d for fungal spores.

[a] CGMCC NO. indicates China General Microbiological Culture Collection center number.

[b] NT, not tested.

[c] Strain from Invitrogen Biotech.

only sensitive to AS-hepc6 peptide. Our results showed that the two peptides were active against two typical fish-pathogenic Gram-negative bacteria *A. hydrophila* and *V. harveyi* and one other Gram-negative bacterium *E. coli*. It is reported that native human Hepc22 and Hepc25 (Park et al., 2001) and native and synthetic bass hepcidins (Shike et al., 2002; Lauth et al., 2005) are all active against *E. coli*. However, our results showed that both synthetic peptides were also active against Gram-positive bacteria, which were different from the observations on human and bass hepcidins but consistent with the observations on the synthetic hepcidins from the Japanese flounder (Hirono et al., 2005) and large yellow croaker (Wang et al., 2009).

AS-hepc6 peptide displayed antifungal activity in spore germination assay against *A. niger*, *F. graminearum* and *F. solani*, while AS-hepc2 only displayed it against *A. niger*, and neither peptide was active against the yeasts *P. pastoris GS115* and *C. albicans*. Our observation, that both peptides were active against *A. niger* but neither were active against yeast strains, was also consistent with the observation of a synthetic bass peptide (Lauth et al., 2005).

Overall, the MICs of the synthetic peptides against bacteria and fungi ranged from 1.25 to 60 μM, and their MBCs ranged from 10 to 60 μM. Although the synthetic AS-hepc2 and AS-hepc6 peptides possessed inhibitory or killing activity against microorganisms in a similar range of concentrations, there was a difference in the antimicrobial activity between them. Twelve (75%) of the total 16 microbial species tested were sensitive to AS-hepc6 peptide while eight (50%) species were sensitive to AS-hepc2 peptide. AS-hepc6 had a wider antimicrobial spectrum than AS-hepc2, and in particular, it had more potent antifungal activity than AS-hepc2.

3.2.3. Bactericidal kinetics

Kinetic curves of bactericidal activity were plotted using Gram-positive *S. aureus* and Gram-negative *E. coli* at MBC and are shown in Fig. 6.

AS-hepc6 peptide (Fig. 6b) killed about 90% of Gram-positive *S. aureus* in around 15 seconds, and nearly 99% were killed within 15 min of incubation with the bacterium. Approximately 100% killing of *S. aureus* was achieved after 80 min. AS-hepc2 (Fig. 6a) immediately killed 10% of *S. aureus* within 15 s after being added to the culture and maintained the kinetics within 10 min of incubation. Later, the bactericidal activity was potent and 98% killing was achieved at 60 min, approximately 99% at 90 min and nearly 100% at 120 min.

AS-hep2 and AS-hepc6 showed more gentle and slow bactericidal activity against *E. coli* than against *S. aureus* over the time course. Within one hour of incubation with AS-hepc6 (Fig. 6d), around 90% of *E. coli* were killed, and with extension of the incubation time for 1.5 h and 2 h, approximately 99% and 100% were killed. However, in the case of AS-hepc2 (Fig. 6c), 90% were killed after 90 min of incubation and no live bacterium (100% killing) was observed at 3 h.

The present study indicated that AS-hepc6 had a wider antimicrobial spectrum than AS-hepc2 and correspondingly both AS-hep2 and AS-hepc6 were found to be cationic antimicrobial peptides (Yang et al., 2007).The cationic antimicrobial peptides to kill bacteria are likely to be attracted to the anionic phospholipids and phosphate groups on lipopolysaccharide (LPS) of Gram-negative bacteria and the teichoic acids on the surface of Gram-positive bacteria (Brogden, 2005). Some researchers investigated the importance of cationic charge in the derivatives synthesized from antimicrobial peptides (Nan et al., 2009; Kluver et al., 2005) and the study on the synthetic derivatives of human β-defensin-3 reported that the distribution of positively charged amino acid residues played a crucial role in the antimicrobial potency of hβD-3 peptides (Kluver et al., 2005). In the study, based on the secondary structure of both peptides, it was predicted that AS-hepc6 had more positively charged amino acid residues than AS-hepc2 and the positive electricity field of AS-hepc6 was broader than AS-hepc2 (Fig. 5). Taking into account of the MIC results, the discrepancy in predicted structures might be one of the reasons why AS-hepc6 showed wide antimicrobial potentiality than AS-hepc2.

4. Conclusions

From our results, it was found that AS-hepc2 and AS-hec6 isoforms had individual and distinct expression characteristics in several respects. In the normal fish, AS-hepc2 mRNA transcripts were highly demonstrated in the liver while AS-hepc6 in the head and trunk kidneys. After bacterial challenge, the two genes showed different tissue-specific and time-specific modes in response to bacterial challenge or just the injection treatment. Compared with the relative

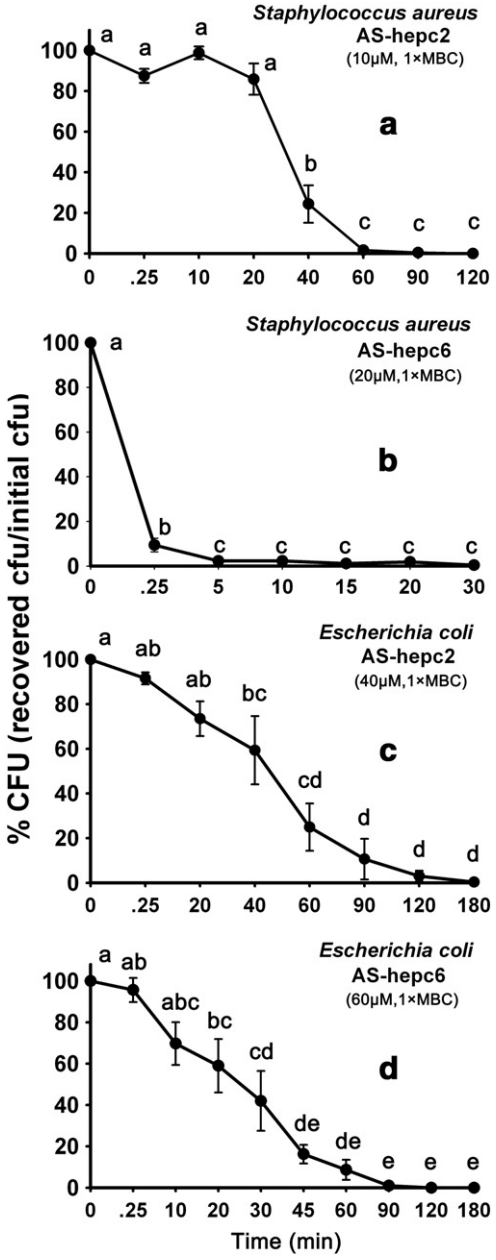

Fig. 6. Bactericidal kinetic curves of synthesized AS-hepc2 and AS-hepc6 mature peptides. The minimal bactericidal concentration against Gram-positive *S. aureus* and Gram-negative *E. coli* was used. Data are represented as the mean ± S.D. (n = 3) bacterial recovery index. The same letters indicate no significant difference between different time points, and different letters indicate statistically significant differences between time points. ($P<0.05$, one-way ANOVA followed by the Tukey post hoc test.)

quantity, AS-hepc2 was more highly expressed in the liver than in the other tissues tested no matter before or after bacterial challenge, while AS-hepc6 expression involved more tissues than just the head kidney and trunk kidney after bacterial challenge. Our results indicated that AS-hepc2 was likely to be a liver-expressed hepcidin peptide, while AS-hepc6 was expressed in multiple tissues including blood cells. The mature peptides of AS-hepc2 and AS-hepc6 had different peptide characteristics and both synthetic peptides were active against bacteria and fungi, but took a selective action against different microorganisms. Our study implied that diverse hepcidin isoforms may play overlapping roles in the innate immune defense of fish and the existence of multiple hepcidin isoforms might be a strategy for fish survival in the complex aquatic environment.

Acknowledgements

This work was supported by grants (2007I0022 and 2008N0043) from Fujian Science and Technology Department, a grant (2007AA091406) from the National High Technology Research and Development Program of China (863 Program) and a grant for Young Scientist Visiting Fellowship from MEL of China. Professor John Hodgkiss is thanked for his assistance with English and Dr. Kunming Xu for comments on statistical analysis.

References

Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.

Bao, B.L., Peatman, E., Li, P., He, C.B., Liu, Z.J., 2005. Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection. Dev. Comp. Immunol. 29, 939–950.

Brogden, K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3, 238–250.

Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., Hoffmann, J.A., 1993. A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution. J. Biol. Chem. 268, 14893–14897.

Chen, S.L., Li, W., Meng, L., Sha, Z.X., Wang, Z.J., Ren, G.C., 2007. Molecular cloning and expression analysis of a hepcidin antimicrobial peptide gene from turbot (*Scophthalmus maximus*). Fish Shellfish Immunol. 22, 172–181.

Chiou, P.P., Lin, C.M., Bols, N.C., Chen, T.T., 2007. Characterization of virus/double-stranded RNA-dependent induction of antimicrobial peptide hepcidin in trout macrophages. Dev. Comp. Immunol. 31, 1297–1309.

Cho, Y.S., Lee, S.Y., Kim, K.H., Kim, S.K., Kim, D.S., Nam, Y.K., 2009. Gene structure and differential modulation of multiple rockbream (*Oplegnathus fasciatus*) hepcidin isoforms resulting from different biological stimulations. Dev. Comp. Immunol. 33, 46–58.

Cuesta, A., Meseguer, J., Esteban, M.A., 2008. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. Mol. Immunol. 45, 2333–2342.

Douglas, S.E., Gallant, J.W., Liebscher, R.S., Dacanay, A., Tsoi, S.C.M., 2003. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev. Comp. Immunol. 27, 589–601.

Emanuelsson, O., Brunak, S., von Heijne, G., Nielsen, H., 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protoc. 2, 953–971.

Fu, Y.M., Li, S.P., Wu, Y.F., Chang, Y.Z., 2007. Identification and expression analysis of hepcidin-like cDNAs from pigeon (*Columba livia*). Mol. Cell. Biochem. 305, 191–197.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., 2005. Protein identification and analysis tools on the ExPASy Server. In: Walker, J.M. (Ed.), The Proteomics Protocols Handbook. Humana Press, Totowa, NJ, pp. 571–608.

Hilton, K.B., Lambert, L.A., 2008. Molecular evolution and characterization of hepcidin gene products in vertebrates. Gene 415, 40–48.

Hirono, I., Hwang, J.Y., Ono, Y., Kurobe, T., Ohira, T., Nozaki, R., Aoki, T., 2005. Two different types of hepcidins from the Japanese flounder *Paralichthys olivaceus*. FEBS J. 272, 5257–5264.

Huang, P.H., Chen, J.Y., Kuo, C.M., 2007. Three different hepcidins from tilapia, *Oreochromis mossambicus*: analysis of their expressions and biological functions. Mol. Immunol. 44, 1922–1934.

Hunter, H.N., Fulton, D.B., Ganz, T., Vogel, H.J., 2002. The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. J. Biol. Chem. 277, 37597–37603.

Ilyin, G., Courselaud, B., Troadec, M.B., Pigeon, C., Alizadeh, M., Leroyer, P., Brissot, P., Loreal, O., 2003. Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload. FEBS Lett. 542, 22–26.

Kemna, E., Tjalsma, H., Willems, H., Swinkels, D., 2008. Hepcidin: from discovery to differential diagnosis. Haematologica 93, 90–97.

Kim, Y.O., Hong, S., Nam, B.H., Lee, J.H., Kim, K.K., Lee, S.J., 2005. Molecular cloning and expression analysis of two hepcidin genes from olive flounder *Paralichthys olivaceus*. Biosci. Biotechnol. Biochem. 69, 1411–1414.

Kluver, E., Schulz-Maronde, S., Scheid, S., Meyer, B., Forssmann, W.G., Adermann, K., 2005. Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. Biochemistry 44, 9804–9816.

Krause, A., Neitz, S., Magert, H.J., Schulz, A., Forssmann, W.G., Schulz-Knappe, P., Adermann, K., 2000. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 480, 147–150.

Krijt, J., Cmejla, R., Sykora, V., Vokurka, M., Vyoral, D., Necas, E., 2004. Different expression pattern of hepcidin genes in the liver and pancreas of C57BL/6N and DBA/2N mice. J. Hepatol. 40, 891–896.

Lauth, X., Babon, J.J., Stannard, J.A., Singh, S., Nizet, V., Carlberg, J.M., Ostland, V.E., Pennington, M.W., Norton, R.S., Westerman, M.E., 2005. Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and in vivo hepatic response to bacterial infections. J. Biol. Chem. 280, 9272–9282.

Martin-Antonio, B., Jimenez-Cantizano, R.M., Salas-Leiton, E., Infante, C., Manchado, M., 2009. Genomic characterization and gene expression analysis of four hepcidin

genes in the redbanded seabream (*Pagrus auriga*). Fish Shellfish Immunol. 26, 483–491.
Nan, Y.H., Park, K.H., Park, Y., Jeon, Y.J., Kim, Y., Park, I.S., Hahm, K.S., Shin, S.Y., 2009. Investigating the effects of positive charge and hydrophobicity on the cell selectivity, mechanism of action and anti-inflammatory activity of a Trp-rich antimicrobial peptide indolicidin. FEMS Microbiol. Lett. 292, 134–140.
Nicolas, G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, A., Vaulont, S., 2001. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc. Natl Acad. Sci. USA 98, 8780–8785.
Padhi, A., Verghese, B., 2007. Evidence for positive Darwinian selection on the hepcidin gene of Perciform and Pleuronectiform fishes. Mol. Divers. 11, 119–130.
Park, C.H., Valore, E.V., Waring, A.J., Ganz, T., 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J. Biol. Chem. 276, 7806–7810.
Peng, H., Yang, M., Huang, W.S., Ding, J., Qu, H.Q., Cai, J.J., Wang, N., Zhang, K.J., 2010. Soluble expression and purification of a crab antimicrobial peptide scygonadin in different expression plasmids and analysis of its antimicrobial activity. Protein Expres. Purif. 70, 109–115.
Pigeon, C., Ilyin, G., Courselaud, B., Leroyer, P., Turlin, B., Brissot, P., Loreal, O., 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J. Biol. Chem. 276, 7811–7819.
Ren, H.L., Wang, K.J., Zhou, H.L., Yang, M., 2006. Cloning and organisation analysis of a hepcidin-like gene and cDNA from Japan sea bass, *Lateolabrax japonicus*. Fish Shellfish Immunol. 21, 221–227.
Rodrigues, P.N., Vazquez-Dorado, S., Neves, J.V., Wilson, J.M., 2006. Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*). Dev. Comp. Immunol. 30, 1156–1167.
Segat, L., Pontillo, A., Milanese, M., Tossi, A., Crovella, S., 2008. Evolution of the hepcidin gene in primates. BMC Genomics 9, 120.
Shi, J.S., Camus, A.C., 2006. Hepcidins in amphibians and fishes: antimicrobial peptides or iron-regulatory hormones? Dev. Comp. Immunol. 30, 746–755.
Shike, H., Lauth, X., Westerman, M., Ostland, V., Carlberg, J., Van Olst, J., Shimizu, C., Bulet, P., Burns, J., 2002. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur. J. Biochem. 269, 2232–2237.
Shike, H., Shimizu, C., Lauth, X., Burns, J.C., 2004. Organization and expression analysis of the zebrafish hepcidin gene, an antimicrobial peptide gene conserved among vertebrates. Dev. Comp. Immunol. 28, 747–754.
Tencza, S.B., Douglass, J.P., Creighton, D.J., Montelaro, R.C., Mietzner, T.A., 1997. Novel antimicrobial peptides derived from human immunodeficiency virus type 1 and other lentivirus transmembrane proteins. Antimicrob. Agents Chemother. 41, 2394–2398.
Wang, K.J., Cai, J.J., Cai, L., Qu, H.D., Yang, M., Zhang, M., 2009. Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide. Peptides 30, 638–646.
Wang, Z., Wang, G., 2004. APD: the antimicrobial peptide database. Nucl. Acids Res. 32, D590–D592.
Yang, M., Wang, K.J., Chen, J.H., Qu, H.D., Li, S.J., 2007. Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B.). Fish Shellfish Immunol. 23, 1060–1071.
Zheng, W., Liu, G., Ao, J., Chen, X., 2006. Expression analysis of immune-relevant genes in the spleen of large yellow croaker (*Pseudosciaena crocea*) stimulated with poly I. C. Fish Shellfish Immunol. 21, 414–430.