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Research Article

Hepcidin Gene Cloning and Expression Pattern in Turbot (*Scophthalmus maximus*) after *Vibrio. anguillarum* Infection

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Background: Antimicrobial peptides play crucial roles in organisms as the first line of defense against invading pathogens.

Objective: To isolate the hepcidin (*hepc1*) gene from the liver of turbot (*Scophthalmus maximus*) challenged with *Vibrio anguillarum* (GenBank accession number: AM113708), characterize it, and assess its expression level in various tissues.

Materials and Methods: The DNA sequence of hepcidin from *S. maximus* was determined from the total RNA extracted and reverse transcribed from this fish. The expression levels of tissue-specific hepcidin transcripts were determined using reverse-transcriptase polymerase chain reactions.

Results: Hepcidin levels increased in the livers, head kidneys and spleens of the fish. The transcriptional increase was especially noticeable in the liver after bacterial infection commencement. The presence of hepcidin and interleukin-beta (*IL-1β*) in blood leukocytes was compared at the transcription level and hepcidin transcripts were detected earlier than *IL-1β* transcripts after infection, indicating that hepcidin might serve as the first line of defense to kill bacteria and may also play a more direct and effective role than that of *IL-1β* during the initial stage of the innate immune response when turbot are exposed to bacteria invasion.

Conclusions: Hepcidin might serve as the first line of defense to kill bacteria and may also play a more direct and effective role than that of *IL-1β* during the initial stage of the innate immune response when turbot are exposed to bacteria invasion.

Keywords: Hepcidin, Infection, Interleukin-beta, *Scophthalmus maximus*, Turbot, *Vibrio anguillarum*

1. Background

Antimicrobial peptides are important components of the innate immune system (1-2). They are widespread among organisms, and a large number of these molecules have been identified in invertebrates and vertebrates as well as in plants (3,4). These peptides play an important role in protecting organisms against microbial invasion (5). They are particularly important in marine organisms because the ambient environment is awash with various pathogen types and microbial invasion is a real threat they face from time to time (6). The recombinant fish antimicrobial peptides might have great potential in Chinese aquaculture because the prevalence of bacterial diseases often causes heavy loses and antibiotic use can threaten the safety of aquaculture products. However, few antimicrobial peptides have been identified from teleost fish and their characteristics have not been extensively studied.

Hepcidin, a low molecular weight, cysteine-rich cationic peptide, which was originally isolated from human urine and ultrafiltrated plasma, exhibits antimicrobial activity *in vitro* (7,8). Hepcidin possesses eight positionally-conserved cysteine residues and, among the antimicrobial peptide family, it has a unique and distinctive cysteine bridge structure (9). With four disulfide bridges in its spatial structure, hepcidin resembles members of the defensin family and is functionally active against Gram-positive and Gram-negative bacteria as well as against yeasts (6,10). Hepcidin is a component of the innate immune system and thus constitutes the first line of defense against infections (7). It is also a key regulator of iron homeostasis in organisms (11-14). Studies aimed at characterizing hepcidin in mammals (15) and fish have resulted in the isolation and identification of this peptide in sea bass (16), winter flounder, Atlantic salmon (17)

and zebrafish (17).

Interleukin-beta (*IL-1β*), another pivotal innate immune factor that enables organisms to respond to pathogens, induces an inflammatory cascade, along with other defense responses against pathogen invasion (18-19). There is abundant evidence that fish immunocytes secrete *IL-1β*, a key modulator of the fish immune system, during bacterial infections, by enhancing leukocyte phagocytosis and bactericidal activity, as well as by stimulating tissue inflammation (20-21).

The antimicrobial activity of hepcidin may be useful in the fight against the bacterial and viral outbreaks that plague aquaculture species such as turbot, an important economic fish. However, little is understood about the mechanism of action of hepcidin in the immune system and its relationship with *IL-1β* in fish.

2. Objectives

Here, we report on the cloning of the hepcidin precursor (*hepc1*) from turbot, expression of the *hepc1* gene in the tissues of the turbot at the transcription level, and make a comparison of *hepc1* expression with that of *IL-1β* after *Vibrio anguillarum* infection.

3. Materials and Methods

3.1. Animals and Bacterial Infections

Healthy individuals of the marine teleost turbot (*Scophthalmus maximus*, 800 g mean weight) were obtained from an aquaculture farm in Qingdao (Shandong, China) and kept in a 260 L seawater tank at 20 °C for later studies. Fishes were infected with *V. anguillarum* by injecting a sublethal dose (10^8 cells.mL^{-1}) of this pathogen in 0.1 mL of NaCl buffer. Different tissue samples from the infected fish were collected at 0.5, 4, 24 h intervals post-infection and the tissues from uninfected fish were taken as the controls.

3.2. Leukocyte Collection

Turbot were anesthetized with benzocaine (Sigma, St. Louis, MO, USA) in 0.1 g.L^{-1} water. Peripheral blood was collected by puncturing the caudal veins of the anesthetized turbots with a heparinized syringe. Blood leukocyte-enriched fractions were obtained via a 50% Percoll density gradient (Pharmacia, Baie d'Urfe, Quebec, Canada), following the manufacturer's manual, and adjusted to 10^6 viable cells.mL^{-1} in Leibovitz-15 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% newborn calf serum, (Gibco), 100 U. mL^{-1} penicillin, 2.5 µg. mL^{-1} amphotericin B, and 0.1 mg. mL^{-1} streptomycin.

3.3. RNA Isolation and RT-PCR

Total RNA was isolated from leukocytes, head kidneys, spleens and livers using standard TRIzol Reagent (Invitrogen, USA). Bioscript M-MLV RNase H reverse transcriptase (200 U) was used to synthesize the first strand cDNA with an oligo (dT18) primer at 42 °C for 50 min. A pair of primers (WBHsp, WBHap) was designed based on the homology of known mammalian and fish hepcidin sequences (these sequences are *NM_205583*, *MH588516*, HQ711993, *NM_032541*, AF542965, *KT990221, KJ890396*) to obtain the complete coding sequence of the turbot hepcidin gene. Subsequently, hepcidin 1 precursor (Hepc1), *IL-1β* and *β-Actin* genes were PCR-amplified by reverse-transcriptase (RT)-PCR using the primers shown in **Table 1** and taking the prepared tissue cDNAs as the templates, with the PCR-amplified *β-Actin* gene serving as the internal control. Amplifications were performed in 50 µL volumes, the conditions of which were as follows: one cycle at 94 °C for 2 min, 35 cycles at 94 °C for 30 s, annealing temperatures of 55 °C (*β-Actin* gene), 52 °C (*hepc1*), or 50 °C (IL-1β gene) for 30 s, 72 °C for 1 min, followed by one cycle at 72 °C for 10 min. PCR products were separated on a 1.5% agarose gel containing 0.5 µg.mL^{-1} ethidium bromide (Sigma) and visualized under UV light. The results were photographed with the Alpha IS12200 Image Acquisition System. Band signals were scanned on a densitometric scanner and statistically analyzed using the Bandscan 5.0 program (22). The ratio of the target gene to the internal control gene represented the relative gene expression level.

Table 1. Primers and their sequences used in this study

Primers	Sequences (5′–3′)	Utilization
ActinF	CCCAGAGCAAGAGAGGTATC	Turbot β-Actin gene
ActinR	GCTGTGGTGGTGAAGGAGTAG	Turbot β-Actin gene
WBHsp	CAAACCCTCCTAAGATGAAG	Turbot *hepc1* gene
WBHap	AATCCTCAGAACCTACAGCA	Turbot *hepc1* gene
IL1sp	GCGACAGAATCCTCACCAAT	Turbot IL-1β gene
IL1ap	TTTGTAGAACAGAAATCGCACCA	Turbot IL-1β gene

3.4. Sequencing and Bioinformatic Analysis

PCR products were characterized by agarose gel electrophoresis and purified using a gel extraction kit (TIANGENE, China). The recovered products were sequenced by the Shanghai Shenggong Co. The amino acid sequences were deduced from the sequencing results using DNAStar 5.0 software (23), which was also used to perform the multiple sequence alignments calculated by Clustal W (24). The secondary structure of the deduced protein was predicted by Omiga 2.0 software (25). Protein hydrophilicity was predicted by DNAStar (https://www.dnastar.com/) (23). The signal peptide cleavage site was predicted using the signalP 3.0 server (26) (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight (MW) and isoelectric point (PI) of the protein was determined by the Compute pI/Mw tool in the ExPASy sever (27) (http://au.expasy.org/tools/pi_tool.html).

4. Results

4.1. The Turbot hepc1 Sequence

The turbot hepcidin1 precursor (*hepc1*) cDNA was amplified from the liver of a turbot challenged with *V. anguillarum* after 24 h of infection (AM113708). The cloned hepcidin1 cDNA was 293 bp long with an ORF of 273 bases and a coding capacity of 90 amino acids (**Fig. 1**).

4.2. Bioinformatic Analysis of the Sequence

The deduced amino acid sequence of the turbot *hepc1* cDNA was compared with hepcidin sequences from other fish species and mammals. The results indicate that the hepcidin sequences from the different sources had highly similar C-terminal end segments, all sharing the same feature of eight cysteine residues **(Fig. 2, processing site 2**). The highest amino acid sequence

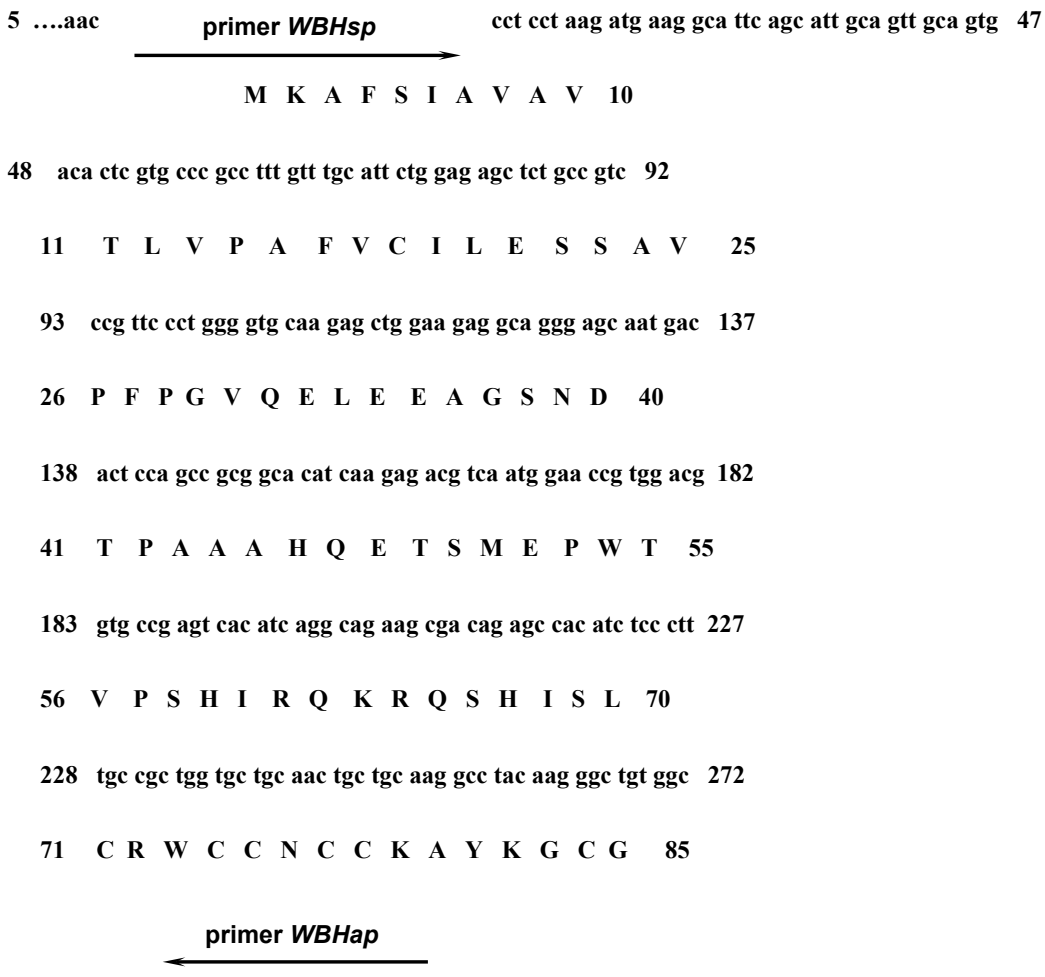

Figure 1. Hepc1 cDNA and the predicted amino acid sequence.

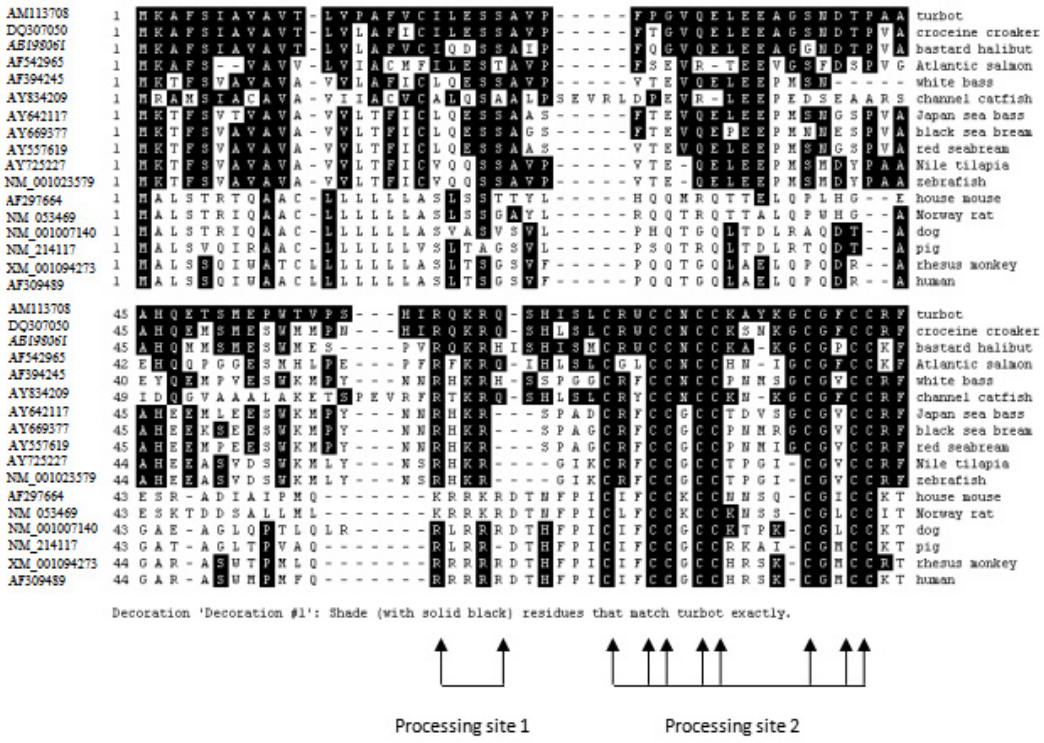

Figure 2. Alignment of the amino acid sequences deduced from turbot hepcidin (*hepc1*) gene (AM113708) with those of croceine croaker (DQ307050), bastard halibut (AB198061), Atlantic salmon (AF542965), white bass (AF394245), channel catfish (AY834209), Japan sea bass (AY642117), black sea bream (AY669377), red seabream (AY557619), Nile tilapia (AY725227), zebrafish (NM_001023579) , house mouse (AF297664), Norway rat (NM_053469), dog (NM_001007140), pig (NM_214117), rhesus monkey (XM_001094273) and human (AF309489). Propeptide convertase acting site is indicated as processing site 1 and cystine-rich domain is indicated as processing site 2.

identity score deduced for Hepc1 was 96.7% for the turbot (*Scophthalmus maximus*) hepcidin precursor (AY994074), and the cDNA sequence identity score between them was 98%. The RX(K/R)R structure, a motif typical of mammalian proprotein convertase active sites (22), was identified in most of the species listed in **Figure 2** (processing site 1). The SignalP server predicted that approximately 24 amino acids at the N-terminal end of Hepc1 comprised the signal peptide domain. This region was predicted to be hydrophobic by DNAStar. About 21 amino acids at the C-terminus end were predicted by software Omiga2.0 as being capable of forming a β-sheet structure, and were designated as the mature peptide after comparison with those from other species.

4.3. Expression of hepc1 in the Tested Tissues and the IL-1β Gene in Leukocytes Based on RT-PCR Analysis

The transcriptional expression of *hepc1* was analyzed by RT-PCR in leukocytes, head kidneys, livers and spleens of the fish after infection with *V. anguillarum*. **Figure 3** shows a representative result from the RT-PCR tests. Prior to infection, *hepc1* expression was almost at the same level in the livers, spleens and kidneys. After pathogen invasion, however, increased *hepc1* expression was detected in all the tested tissues at 4 h post-infection, a trend that continued until 24 h. *hepc1* was more abundant in the liver than in the spleens and head kidneys of the fish (**Fig. 3**). *hepc1* expression was detected at increasing levels in the leukocytes during the time course after pathogen challenge (**Fig. 4**). The relative amount of *hepc1* expression in the different tissues was liver > spleen > head kidney > leukocyte at 24 h post bacterial infection.

However, leukocyte-specific *IL-1β* transcript levels remained constant at 0 h (uninfected) and 0.5 h post-infection in the host (**Fig. 4**). Surprisingly, the increasing *hepc1* expression level was detected as early as 0.5 h post-infection, whereas the level of *IL-1β* remained unchangeable at this time (**Fig. 4**). Thereafter, both *hepc1* and *IL-1β* expression levels continued to increase over time and the increase in *IL-1β* transcripts was more rapid than for *hepc1* from 0.5 h to 4 h after bacteria invasion.

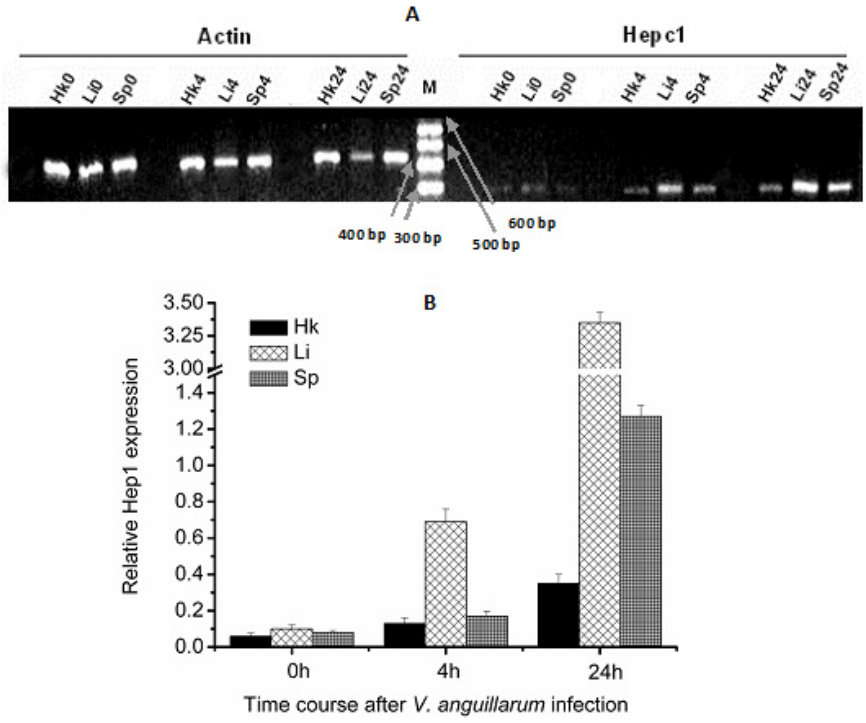

Figure 3. Hepcidin mRNA levels determined by semi-quantitative RT-PCR (A). Tissues assayed were head kidney (Hk), Liver (Li) and spleen (Sp) (B). Samples were taken 4 and 24 h after infection, with the uninfected fish as a control (denoted by 0). Quantitative densitometry of the specific Hepc1 bands are expressed as a percentage of the corresponding β-actin. Data shown are mean ± SEM of the three experiments.

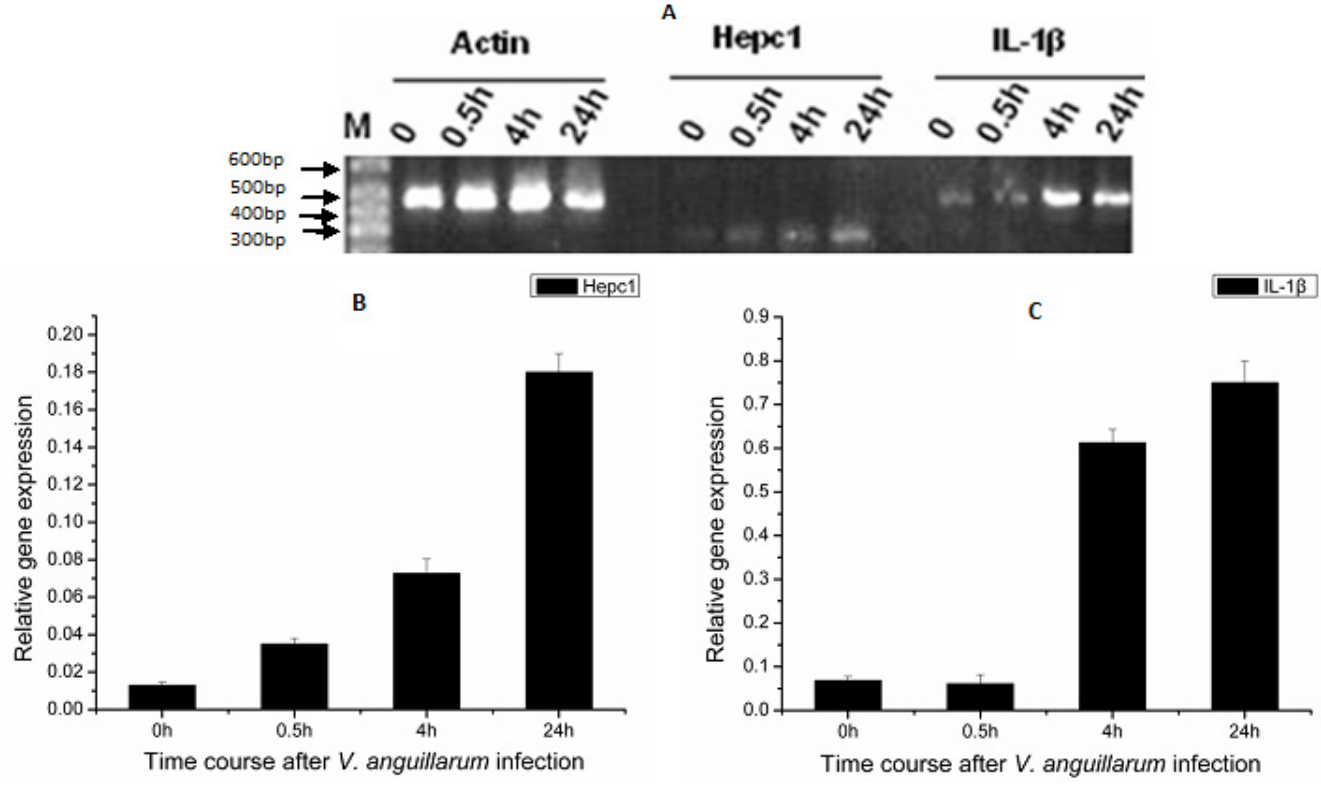

Figure 4. The course of Hepc1 (B), IL-1β mRNA (C) expression in turbot leukocytes infected after 0.5, 4 and 24 h, with the uninfected fish as a control (denoted by 0) (A). Quantitative densitometry of the specific Hepc1 bands isexpressed as a percentage of the corresponding β-actin. Data shown are mean ± SEM of the three experiments.

5. Discussion

Our results have shown that turbot Hepc1 shares strikingly similar amino acid sequences with hepcidin from several different mammals and fish, and that hepcidin from these different sources shares a high degree of homology in the eight-cysteine domain (**Fig. 2**). Preprohepcidin usually comprises three parts: a signal peptide, a proregion, and a mature peptide (28). A signal cleavage site in Hepc1 between Ala24 and Val25 was predicted by Signal P. The 1 to 25 amino acid region at the N-terminal end was predicted to be hydrophobic by DNAStar. The resulting signal peptide has a basic residue (Lys3) at the N-termini end, followed by a hydrophobic region containing Ala and Val, which is typical of a signal peptide (15). Similarly, a signal peptide cleavage site for white bass and zebrafish preprohepcidin was also predicted to occur between Ala24 and Val25 (15,29), suggesting that a conserved mechanism exists for proteolytic cleavage of the hepcidin signal peptide among fish.

The NH2-termini of mature peptides have been assigned based on the amino acid sequence of human hepcidin (29), and the proximity to the RX(K/R)R motif is characteristic of processing sites in propeptide convertases (30). A similar cleavage site for Hepc1 was predicted in the present study (**Fig. 2, processing site 1**). With its high homology to bass and zebrafish hepcidin, the mature *S. maximus* Hepc1 peptide should comprise 21 amino acids starting at Leu70. But it is different from the predicted hepcidin mature peptide from turbot, which is a 26-amino acid peptide according to Chen SL et al (26). Other evidence supports our conclusion. For example, a 21 amino-acid mature hepcidin peptide was also predicted in hybrid striped bass and Japan sea bass (15,22). If this is correct, the predicted mature hepcidin peptide should have a MW of 9881.52 Da and pI of 7.64 based on the calculation by ExPASy. The mature peptide should confer a totally positive charge. This property enables antimicrobial peptides to permeate tissues resulting in the leakage of plasma membranes from the invading microorganisms (8). The 21 amino acid region in the C-terminal end was predicted by Omiga2.0 to form a β-sheet structure, thereby predicting the mature peptide exactly. The mature human hepcidin peptide forms a simple hairpin structure, where the two arms are linked by four disulfide bridges (27). The mature Hepc1 peptide appears to have the same eight-cysteine-like structure (**Fig. 2, processing site 2**). Chen, *et al*. also isolated a form of hepcidin from turbot (GenBank accession No. AY994074) (31). Their deduced hepcidin peptide differs from ours in having three different amino acids. The 14th, 81st, 89th amino acids reported by Chen, *et al*. are Leu, Asn, and Lys, while in the present study the same sites in Hepc1 are Pro, Tyr, and Arg. The data from Chen *et al*. suggest that the mature hepcidin peptide they cloned consists of 26 amino acids, with the 81st and the 89th amino acids differing from our data for the mature peptide. These different results imply that the two hepcidin genes from turbot may be different members of the hepcidin family. Shi, *et al*, compared some hepcidin EST (expressed sequence tag) sequences from various fish and found that there was a CCR/KF structure at the C-terminal end of hepcidin (32). Although the amino acid composition of the hepcidin molecule cloned herein and the GenBank database hepcidin (AY994074) differ, each of the two sequences also conform to the above-mentioned structure. Three types of mature hepcidin peptides have been isolated from human urine. Their amino acid lengths are 25, 22 and 20. Their relative molecular weights are 2789Da, 2436Da and 2192Da (5). The mature peptides of our own (Hepc1) and the database hepcidin (AY994074) are 21 and 26 amino acids, respectively. According to this calculation, their molecular weights are 2468Da and 2943Da. The two different hepcidin sequences indicate the possible existence of hepcidin isomers in turbot.

There is indirect evidence that *hepc1* may be an essential element of the innate defense systems of turbot, because the expression of *hepc1* in the liver and immune tissues can be induced dramatically by bacteria challenge. That the expression of *hepc1* was detectable soon after the bacterial infection commenced in turbot suggests that the host may use hepcidin as the first line antimicrobial agent *in vivo* to directly kill invading microorganisms. However, direct evidence that fish hepcidin functions as an endogenous antimicrobial agent has yet to be established (33).

Like human and bass hepcidin, turbot hepcidin is expressed predominantly in the liver. Hepcidin is translated in the liver as a 84 amino-acid pre-pro-peptide and the 25 amino-acid peptide is the predominant form in the urine after processing and excretion through human kidneys (5,12). Pigeon *et al*. discovered that the 84 amino acid preprohepcidin synthesized in the liver was transported through the hepatocyte basolateral membrane into the blood stream (11). Our semi quantitative result suggests that expression of the *hepc1* gene in the liver increased by almost 30 fold 24 h after infection. In contrast with the liver, the expression of *hepc1* was relatively low in the immune tissues we tested. It is possible that as a precursor peptide, Hepc1

produced in turbot liver may be transported through the hepatocyte basolateral membrane into the blood stream. Hence, turbot hepcidin may exert antibacterial affects in other forms such as the predicted mature peptide in different immune tissues when pathogen invasion has occurred. The expression of *hepc1* was higher in the spleen than in the head kidney and leukocytes at 24 h post-infection. This result is similar to that reported previously for hepcidin gene expression in bass induced by bacterial challenge (34).

IL-1β can be produced by monocytes, macrophages, polymorphonuclear phagocytes, fibroblasts and other cells. However, mature *IL-1β* is mainly secreted by blood macrophages (35). It has already been shown that leukocytes are responsible for secreting *IL-1β* into the turbot blood circulation (34). The expression of *IL-1β* was constant in the uninfected host and at 0.5 h in the infected host. *Hepc1* expression was found to continually increase at 0.5 h post-infection. As an immune regulator, *IL-1β* can enhance leukocyte phagocytosis and anti-bactericidal activity, inducing leukocyte proliferation and differentiation in response to infectious insults (36). *IL-1β* cannot kill pathogens directly, whereas hepcidin can exert antimicrobial activity, as has been shown in humans. Thus, it is possible that hepcidin performs its function earlier than *IL-1β* in activating the innate immune system in turbot, by performing a direct action that can kill bacteria during their invasion. In our experiments, the expression of *IL-1β* increased more rapidly than that of Hepc1 from 0.5 h to 4 h after bacterial challenge, suggesting that the immune defense effect would have been boosted in the fish.

6. Conclusion

Our vision is to constructed heterologous expression system in yeast, which can include several kinds of antimicrobial peptides sequences such as hepcidin, moronecidin, *et al*. Due to the antimicrobial peptides coming from kinds of fishes, it cannot produce resistance like antibiotics. Actually, the yeast have been widely used as a good feed additives in aquiculture. It could enhance the aquiculture disease resistance and nutrition.

Acknowledgments

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