



Recombinant medaka (*Oryzias melastigmus*) pro-hepcidin: Multifunctional characterization

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ARTICLE INFO

Article history:

Received 19 July 2011

Received in revised form 15 October 2011

Accepted 19 October 2011

Available online 25 October 2011

Keywords:

Marine medaka

Hepcidin

Prokaryotic expression

Antimicrobial activity

Antitumoral activity

ABSTRACT

Recently, two hepcidin variant genes (Om-hep1 and Om-hep2) were identified in a model fish marine medaka and both were highly induced in vivo with bacterial challenge, suggesting that the medaka hepcidin may have a similar function to other reported teleostean hepcidins. In the present study, the antibacterial, antiviral and antitumor activities of Om-hep1 were determined using its synthetic and recombinant pro-peptides. The recombinant pro-hepcidin1 was expressed in *Escherichia coli* and an effective method to produce recombinant Pro-Omhep1 was developed in order to obtain a right folded structure. The results showed that both the synthetic mature peptide and recombinant pro-peptide had similar antibacterial activity against Gram-positive and negative bacteria. In particular, both the synthetic mature Om-hep1 and recombinant Pro-Omhep1 inhibited the viral replication of white spot syndrome virus in the hematopoietic tissue cells of the crayfish *Cherax quadricarinatus*. Om-hep1 also presented antitumor activity on the cultured human hepatocellular carcinoma cells. In addition, the antimicrobial mechanism of Om-hep1 was measured and it was found that Om-hep1 was likely to be non-membranolytic. The recombinant Pro-Omhep1 performed better biological activity compared to the synthetic mature Om-hep1. This study suggested that Om-hep1 was likely to be an important multifunction protein involved in various resistance actions in the marine medaka immune system.

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1. Introduction

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune system and are found among all species of life. Naturally AMPs probably represent one of the first evolved and successful forms of chemical defense by eukaryotic cells against bacteria, protozoa, fungi, viruses and cancer cells (Hancock et al., 2006). To date, numerous AMPs have been characterized from fish, they represent functions of mediating innate immunity (Su, 2011). As a group member of the AMPs, hepcidin was first isolated from human blood (Krause et al., 2000) and named as liver-expressed AMP because its gene is dominantly expressed in the liver (Park et al., 2001). So far, hepcidin has been widely identified in many species widely from vertebrate to invertebrate. The function of hepcidin in the mammal is reported to be the master regulator of iron homeostasis. Unlike mammals (Pigeon et al., 2001; Zhang et al., 2004; Sang et al., 2006; Fu et al., 2007; Segat et al., 2008) and amphibians (Shi and Camus, 2006) whose hepcidin

genes are dominantly expressed in the liver, bony fish hepcidin genes are not only highly demonstrated in the liver (Douglas et al., 2003) but also in the kidney (Yang et al., 2007; Wang et al., 2009). Moreover, multiple hepcidin copies have been found in different fish species. These observations imply that fish hepcidin exists widely among marine fish and is more likely to exert immune-associated activity in bony fish.

Although many hepcidin genes have been amplified in bony fish and the gene expression patterns investigated in multiple tissues and various species of fish, the in vivo functions associated with immunity and the mechanism of hepcidin functioning in bony fish are not well described due to the fact that the tested samples were randomly collected from wild-growing fish or marine-cultured fish. However, two variants of marine medaka (*Oryzias melastigmus*) hepcidin Om-hep1 and Om-hep2 have been characterized (Bo et al., 2011). Both of them were induced with the bacterial challenge and their expression was significantly demonstrated in the liver, and in the early developmental stages of marine medaka embryos, suggesting that Om-hep1 and Om-hep2 might play an important role in innate defense through the life cycle of medaka. Thus, it was considered worthwhile to further characterize medaka hepcidin function so that future studies on the hepcidin mechanism could be carried out in a model fish. Medaka is a model fish with many advantages, such as their daily spawning activity, the transparency of their embryos, short generation time, obvious sexual dimorphism, and so on. The medaka genome is small, being about half the size of the zebrafish genome and the database of information on the reproductive

Abbreviations: Om-hep, *Oryzias melastigmus* hepcidin; Pro-hepcidin, predomain hepcidin; Pro-Omhep, predomain *Oryzias melastigmus* hepcidin; AMPs, antimicrobial peptides; CD, circular dichroism; WSSV, white spot syndrome virus; HepG2 cell, human hepatocellular carcinoma cell; IE gene, viral immediate-early gene; RLU, related light unit.

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biology, fertilization, gamete biology, developmental biology etc. is well developed (Kinoshita et al., 2009). Since this fish has been used for ecotoxicological studies for many years due to its many advantages as a research model (Kong et al., 2008), marine medaka has the potential to be a good model of immune studies of marine fish.

In previous studies, the antibacterial activity of fish hepcidin has been mostly tested using the synthetic mature hepcidin peptides which showed potent activity against both Gram-positive and Gram-negative bacteria (Hirono et al., 2005; Lauth et al., 2005; Huang et al., 2007; Yang et al., 2007; Cuesta et al., 2008; Wang et al., 2009). Compared to the synthetic peptide, the reported recombinant hepcidins have no obvious antimicrobial activity due to the putative reason of the incorrect folding peptide structure of four disulfides bonds (Zhang et al., 2005; Srinivasulu et al., 2008). However, elucidation of the fish hepcidin bioactive mechanism is essential to any understanding of its exact role in fish. A recent study demonstrates that AMPs inhibit bacteria in two ways: some AMPs perforate the plasma membrane leading to the rupture of cells, while others may inhibit bacterial growth by binding DNA in the bacteria cells (Broegden, 2005). A method in real-time measurement of cell permeabilization has been established by Virta et al. (1995), and the mechanism of antibacterial activity of AMPs has been successfully confirmed by the permeabilization examination of bacterial cells by Li et al. (2010). Membrane integrity assay is now used as a new effective method in the study of the AMP mechanism.

Besides antibacterial activity, fish hepcidin is reported to have antiviral activity and some fish hepcidins show inhibition of virus infection, but the antiviral mechanism of hepcidin is not very clear (Rajanbabu and Chen, 2010; Zhou et al., 2011). In addition, fish hepcidin was also found with antitumoral activity as described in a study of Tilapia hepcidins, which could alter the cancer cell membrane structure (Chang et al., 2011). In the present study, an efficient procedure to obtain a biologically active recombinant Om-hep1 in an *Escherichia coli* expression system was developed and concurrently, the mature peptide of Om-hep1 was synthesized for a comparative study of its function and mechanism with those of the recombinant peptide. Furthermore, the bioactivities against bacteria, viruses and tumor cells were evaluated using both the recombinant product and the synthetic peptide of Om-hep1.

2. Materials and methods

2.1. Synthesis of mature Om-hep1 peptide and acetyl hexapeptide-3

The predicted mature peptide of marine medaka (*Oryzias melastigma*) hepcidin 1 (Om-hep1) was synthesized with the solid-phase peptide synthesis method (Tash, China). Om-hep1 peptide consists of 26 amino acids (2.9 kDa) as follows: QSHLSMCSVCCNCKNYKGGCFCCRF. Acetyl hexapeptide-3, (trade name as Argireline, and sequenced as Ac-EEMQRR-NH₂), a negative of the solid-phase peptides synthesis method was also synthesized (Tash, China). The purity of the peptides was determined as 95%. The molecular masses and purity of the purified peptides were verified using mass spectroscopy and HPLC respectively. The synthetic peptides were dissolved in Milli-Q water for activity testing.

2.2. Prokaryotic expression of Pro-Omhep1

The cDNA coding for marine medaka pro-hepcidin 1 (Pro-Omhep1) was obtained from our previous work (GenBank accession no. HM990657). The coding region of Pro-Omhep1 was cloned into the pET-28a + vector (Novagen, Germany) using the primers listed in Table 1. The bold underlined bases in Table 1 showed *NdeI* and *EcoRI* endonuclease cleavage sites designed in the primers for this plasmid construction. Briefly, PCR was carried out using ExTaq polymerase (Takara, Japan) and the running program was: 4 min at 94 °C; 30 cycles of 40 s at 94 °C, 30 s at 65 °C (annealing temperature), 30 s at 72 °C; and then 10 min at 72 °C for the final extension. The PCR product was purified

Table 1
Nucleotide sequences of primers used in this study.

Primers	Sequence(5'–3')	using
OMF	TAT CATATG ATCCAGTCAATGGGGTCACAG	Pro-Omhep1 to pET-28a + vector plasmid construction
OMR	GTG GAAATTC TCAGAACCTGCAGCAGAAACCGC	
IEF	GGTATTGAGGTGATGAAGAGGGCG	WSSV <i>IE1</i> amplification
IER	TGACATGGGAACCACT GTTGAG	
18SF	GCTCTCGATTGTCGGTTATGC	Hpt cell 18S rRNA amplification
18SR	TAATTTGCGTGCCTGCTGC	

using the QIAquick Gel Extraction Kit (Qiagen, Germany). One microliter of PCR product was mixed with T4 DNA polymerase and *NdeI*–*EcoRI* digested pET-28a + vector at 16 °C overnight for ligation. The recombinant pET-28a+/Pro-Omhep1 plasmid was transformed into *E. coli* BL21 (DE3) *pLysS* cells and selected on LB supplied with 100 µg/mL of kanamycin. An overnight culture was expanded in 1 L LB media with 100 µg/mL kanamycin and incubated at 37 °C with shaking at 180 rpm to an optical density at 600 nm of about 0.3. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and harvested at 6 h after induction. The Pro-Omhep1 expression product (Pro-Omhep1) was constructed with an N-terminal hexa-histidine tag and marine medaka pro-hepcidin 1, i.e., an 87 amino acids (9.8 kDa) sequence, which is listed as follows: MGSSHHHHHHSSGLVPRGSHMIPVNGVTELEEASNDTPVAARHEMSMQSWMPNHIREKQRQSHLSMCSVCCNCK-NYKGGCFCCRF. The transformation and colonies mentioned above were confirmed using sequencing (Shenggong, China).

2.3. Air reducing-oxidation refolding of inclusion bodies and purification of recombinant Pro-Omhep1

The cells were resuspended in PBS (phosphate-buffered saline, pH 7.4) and lysed using sonication. The lysate was centrifugated at 16,000 g for 30 min at 4 °C. The supernatant and the inclusion bodies were analyzed using 15% SDS-polyacrylamid gel electrophoresis (SDS-PAGE). After the inclusion bodies were washed with 1% Triton X-100 (v/v), the cysteine/cystine assisted air reducing-oxidative refolding method was executed. Crude Pro-Omhep1 inclusion bodies were dissolved into reducing buffer (50 mM Tris-HCl, 100 mM NaCl, 8 M urea, pH 9.0, filtered with 0.45 µm filter) and β-mercaptoethanol as a reducing agent was added at a final concentration of 100 mM for 12 h at 4 °C. An equal volume of cysteine/cystine assisted oxidization buffer (50 mM Tris-HCl, 100 mM NaCl, 2 M urea, 20 mM cysteine, 0.1 mM cystine and 5% (v/v) glycerol, pH 9.0, filtered with 0.45 µm filter) was added and stirred in a open atmosphere at room temperature for 48 h (Gagliardo et al., 2008). Before the protein was purified, the protein solution was adjusted to 2 M urea concentration by adding diluent buffer (50 mM Tris-HCl, 100 mM NaCl, pH 9.0, filtered with 0.45 µm filter).

An immobilized metal affinity chromatography system was used with a 5 mL HisTrap™ FF column (GE Healthcare Life Sciences, Sweden). The HisTrap™ FF column was linked to an AKTA Purifier100 workstation (GE Healthcare). After 15 mL equilibration buffer was loaded onto the column, the sample was applied onto the column at a flow-rate of 1.5 mL/min followed by washing with 15 mL of equilibration buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM imidazole, 2 M urea, pH 9.0) until the UV baseline was reached. Then, the bound protein on-column was refolded using a linear gradient from 2 M to 0 M urea, starting with the equilibration buffer and finishing with the binding buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM imidazole, pH 9.0). The refolding protein was eluted with elution buffer (50 mM Tris-HCl, 100 mM NaCl, 400 mM imidazole, pH 9.0). The collected elution (25 mM Tris-HCl, 50 mM NaCl, pH 9.0) fractions were dialyzed in dialysate buffer at 4 °C overnight, and then the peptide was desalted into Milli-Q water at 4 °C. Finally, Purified Pro-Omhep1 protein was lyophilized and stored at –80 °C. The protein

concentration was estimated using Bradford quantitative protein determination assay (Bradford, 1976).

2.4. Generation of anti-mouse Pro-Omhép1 antibody

Six Balb/c mice raised in the Animal Culture Centre of Xiamen University were used to prepare polyclonal antibody against Pro-Omhép1. For the first immunization injection, mice were subcutaneously injected with 200 µg Pro-Omhép1 mixed with Freund's Complete Adjuvant (Sigma, USA). The second injection was administered after an interval of 2 weeks, and a similarly 200 µg Pro-Omhép1 mixed with incomplete form Freund adjuvant (Sigma) was used. The blood of mice was collected 10 days after the second immunization and clotted for 60 min at 4 °C. Finally, the antiserum was collected from the blood at 6000 g for 10 min at 4 °C. The specificity of the polyclonal antibody was confirmed using Western-blot. Transformed bacteria protein was transferred to Hybond-N⁺ nylon membrane (GE Healthcare) using semi-dry blotting, and then blocking buffer (PBS, 1% Tween, 3% BSA) was added and it was incubated overnight at 4 °C. A 1:500 Anti-Pro-Omhép1 polyclonal antibody dilution in blocking buffer was added at 37 °C for 40 min incubation as the first antibody and a 1:3000 goat anti-mouse IgG to horseradish peroxidase dilution in blocking buffer was used as the second antibody at 37 °C for 45 min. The immunoreactive bands were revealed using the light emitting detection method of ECL.

2.5. Circular dichroism (CD) spectroscopy

CD spectra were recorded using a J-810 Circular Dichroism spectrometer (JASCO Corporation, Japan). Peptides were dissolved in Milli-Q water at a concentration of 120 µM, and the peptide solutions were scanned in 1 mm light path demountable cells over a 260 nm to 185 nm wavelength range at a sample interval of 1 nm. All data were collected using an average of four scans per replicate.

2.6. Bacterial cultures and bactericidal activity assay

The Gram-positive bacteria, *Corynebacterium glutamicum* and *Staphylococcus aureus*, and the Gram-negative bacteria, *E. coli* MC1061, *Aeromonas hydrophila*, and *Pseudomonas stutzeri*, were cultured using Mueller–Hinton broth media, and another two Gram-negative bacteria, *Vibrio alginolyticus* and *Vibrio parahaemolyticus*, were cultured on Difco marine medium. Determination of minimum inhibitory concentration (MIC) values was performed using liquid growth inhibition assays (Peng et al., 2010). Briefly, the synthetic peptide Omhép1 and the recombinant peptide Pro-Omhép1 were serially diluted in two 10 mM sodium phosphate buffers (pH 7.4). Bacteria were diluted in 10 mM NaPB (pH7.4) to 3~6 × 10⁵ CFU/mL. The assay mixture consisted of 50 µL diluted purified peptide, 30 µL diluted bacterial suspension, and 20 µL culture media. MIC was defined as the lowest concentration of peptide that could fully inhibit bacteria growth (Belén et al., 2007). MIC data were obtained from three independent experiments performed in triplicate.

2.7. Membrane integrity assay

The effect of synthetic and recombinant peptide Pro-Omhép1 on bacterial membrane permeability was determined using a whole-cell real-time assay employing *E. coli* MC1061, constitutively expressing a recombinant luciferase, as modified from a previous study (Virta et al., 1995). The experiment was performed as described previously (Li et al., 2010). The perforation of the plasma membrane caused influx of externally added D-luciferin into luciferase expressing cells and resulted in light emission. Briefly, 50 µL of MH medium containing 1 × 10⁷ *E. coli* cells (MC1061) and 2 mM D-luciferin potassium salt (Sigma, USA) were mixed with a 50 µL dilution of proteins. Measurements were made

according to the MIC of proteins against *E. coli* MC1061 (Pro-Omhép1: 12 µM, mature Omhép1: 25 µM). cecropin P1 (1 µM) (Sigma) was used as the positive control peptide and Argireline (25 µM) (Tash, China), was used as the negative control peptide. In order to make sure that the peptides did not inhibit luciferase activity, cecropin P1 (1 µM) was added after 5 min of incubation to the reactions conducted with Pro-Omhép1, mature Omhép1 and Argireline. Luminescence was monitored using a microplate reader (Tecan Infinite, China). All experiments were performed in triplicate to obtain valid statistical evaluation of the results.

2.8. Inhibition of white spot syndrome virus (WSSV) replication by mature Omhép1 and Pro-Omhép1 in the crayfish hematopoietic tissue (Hpt) cells

Freshwater crayfish, *Cherax quadricarinatus*, were purchased from the freshwater fish farm, Zhangzhou, Fujian Province, China. The Hpt cells of crayfish were prepared in 96-well plates and cultured according to previous research (Söderhäll et al., 2003). The WSSV was isolated originally from infected *Marsupenaeus japonicus* in Xiamen, China (Xie et al., 2005). The WSSV (1 × 10⁶ copy /mL) was cultured at 25 °C for 30 min with 25 µM, 50 µM Omhép1 and 25 µM Pro-Omhép1. The Hpt cell cultures were inoculated with WSSV (1 × 10⁶ copy /well) suspensions for 40 min. The total RNA of viral infected Hpt cells was extracted using an RNAPrep pure micro kit (Tiangen, China). Reverse transcription PCR was carried out using PrimeScript™ RT reagent kit (Takara, China). The immediate-early gene *IE1* of WSSV was detected in the cultured cells using real-time PCR to determine the viral replication. The crayfish 18S rRNA was detected in parallel as an internal control. The primers for amplification are listed in Table 2 and the PCR cycling profile was set as follows: 94 °C for 3 min, 30 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; with a final extension at 72 °C for 10 min.

2.9. Tumor cell preparation and tumor cell viability assay

The HepG2 (human hepatocellular carcinoma) cell was obtained from the American Type Culture Collection (ATCC). HepG2 Cells were cultured in DMEM media (HyClone, Beijing China) containing 10% inactivated FBS at 37 °C in a 5% CO₂ humidified incubator. A Cell Counting Kit-8 (CCK-8) (Beyotime, China) was used to test cell viability (Yang and Zhou, 2008). HepG2 cells were trypsinized and a cell suspension containing 5.0 × 10⁴ cells/mL culture media was prepared. A volume of 0.1 mL cell suspension was added to each well of a 96-well plate coated with poly-HEMA coated plate. Cells were

Table 2
Summary of minimum inhibitory concentration (MIC) of mature Omhép1 and Pro-Omhép1.

Microorganisms	CGMCC NO. ^b	Minimal inhibitory concentration (µM) ^a	
		Pro-Omhép1	Mature Omhép1
<i>Gram-positive bacteria</i>			
<i>Corynebacterium glutamicum</i>	1.1886	3–6	12
<i>Staphylococcus aureus</i>	1.0363	1.5–3	12
<i>Gram-negative bacteria</i>			
<i>Escherichia coli</i> MC1061	ATCC: 25922 ^c	6–12	24
<i>Aeromonas hydrophila</i>	1.0927	1.5–3	12
<i>Pseudomonas stutzeri</i>	1.1803	3–6	12
<i>Vibrio alginolyticus</i>	1.1833	>48	>48
<i>Vibrio parahaemolyticus</i>	1.1615	>48	>48

^a MIC was determined as the lowest concentration of peptide causing 100% of the growth inhibition of the test organism compared to the growth control without any peptide present. Data were obtained from three independent experiments that were performed in triplicate.

^b CGMCC NO: China General Microbiological Culture Collection Number.

^c ATCC: American Type Culture Collection.

cultured for 24 h. One hundred microliters of protein (mature Om-hep1 or Pro-Omhep1) were added into each well. Respectively, the treatment concentrations were 0, 6.25, 12.5, 18.75, 25 μM of mature OM-hep1 and 0, 2.5, 5, 7.5, 10 μM of Pro-Omhep1 in culture media, which were similar to the range of MIC concentrations. Samples in wells containing only hepcidin peptides and media were maintained as controls. After 10 μL of the CCK-8 reagent was added to each well, the cells were incubated under 5% CO_2 for 4 h at 37 $^\circ\text{C}$. The absorbance of each well was measured at 450 nm in a microplate reader (Tecan, China). The experiments were performed in triplicate.

2.10. Statistical analysis

The cell viability percentage (%) was calculated for each concentration compared to the control wells. All results were represented as the mean \pm standard deviation of triplicates and analyzed for statistical significance using a simple *t*-test analysis. Values of $P < 0.05$ were considered to be significant differences for all treatments.

3. Results

3.1. Prokaryotic expression, purification and antibody preparation of Pro-Omhep1

The polypeptide Pro-Omhep1 was expressed with an N-terminal histidine tag as inclusion bodies in the cytoplasm of *E. coli*. SDS-PAGE analysis of the recombinant protein showed that almost all the Pro-Omhep1 was presented as inclusion bodies of the cell lysate and the band of peptide matched the predicted molecular mass of 9.8 kDa (Fig. 1A). Proteins without a histidine tail were removed by washing the resin with binding buffer and the proteins which remained bound to the resin were collected using elution buffer. The sharp UV protein absorbance peak at about 440 mL volume showed the collecting of elution buffer (Fig. 1B). SDS-PAGE analysis of purified product showed the clear band of recombinant Pro-Omhep1 at about 10 kDa, and western-blot resulted in a strong immunoreactive band at the same position. Another lighter band revealed a mass of approximately 20 kDa was probably a dipolymer of Pro-Omhep1 (Fig. 1C).

3.2. CD spectroscopy of Om-hep1

The CD spectra of synthetic mature Om-hep1 (Fig. 2A) in Milli-Q water were consistent with a structure that had some disorder, which indicated a different secondary structure of native human hepc20. The CD spectra of recombinant Pro-Omhep1 (Fig. 2B) had a series of β -turns, loops, and distorted β -sheets. The similarity of CD spectra

between recombinant Pro-Omhep1 and native hepcidin indicated the similarity of their structure and disulfide pairing.

3.3. Antimicrobial activity and cell permeabilization of Om-hep1

The antimicrobial activity of both recombinant peptide Pro-Omhep1 and synthetic mature peptide Om-hep1 was examined using the method of minimal inhibitory concentration. Both peptides showed the similar antimicrobial activity: they all inhibited the growth of Gram-positive bacteria (*C. glutamicum*, *S. aureus*) and the growth of some of Gram-negative bacteria (*E. coli* MC1061, *A. hydrophila*, *Pseudomonas stutzeri*), but not the two strains of *Vibrio* bacteria. Interestingly, the recombinant Pro-Omhep1 had a stronger antimicrobial activity than the synthetic mature peptide (Table 2).

Light emission kinetics for *E. coli* MC1061 after treatment with cecropin P1, Pro-Omhep1, mature Om-hep1 and Argireline are shown in Fig. 3A. The strong light peak after cecropin P1 addition was the result of membrane permeabilization due to the activity of the membrane-active control peptide cecropin P1. The presence of Pro-Omhep1 and mature Om-hep1 slightly reduced the light intensity compared to the Argireline negative control. Results showed that Pro-Omhep1 and mature Om-hep1 did not enhance the permeability of the membrane, as they were similar to the negative control. Subsequently, cecropin P1 was added to the membrane-inactive samples and the light emission was followed for another 5 min to exclude the direct effects of the OM-hep1 peptides on assay function (Fig. 3B). The light peak of Argireline samples indicated that the membranes were still intact and that the membrane assay was not inhibited. The resulting light emission of Pro-Omhep1 and mature Om-hep1 were comparable to the kinetics of themselves alone, the light emission reaction system have been disturbed.

3.4. Anti-WSSV activity of the recombinant Pro-Omhep1 and synthetic Om-hep1

Pro-Omhep1 (25 μM) and Om-hep1 (25 μM , 50 μM) were incubated with the WSSV-infected cells prepared from crayfish Hpt. Meanwhile, the positive control with WSSV alone and negative control without WSSV in the wells set up in parallel. The cells cultured with WSSV were harvested after 3 h incubation, and then the total RNA of these cells was extracted, and reverse transcription of cell mRNA performed. WSSV immediate-early gene *IE1* was detected as a signal of WSSV infection. Hpt cell 18S rRNA was examined as a reference gene to confirm the cell activity. No *IE1* DNA band was detected using the mRNA sample from the WSSV-infected cells, which were treated with both Pro-Omhep1 and Om-hep1 proteins (Fig. 4).

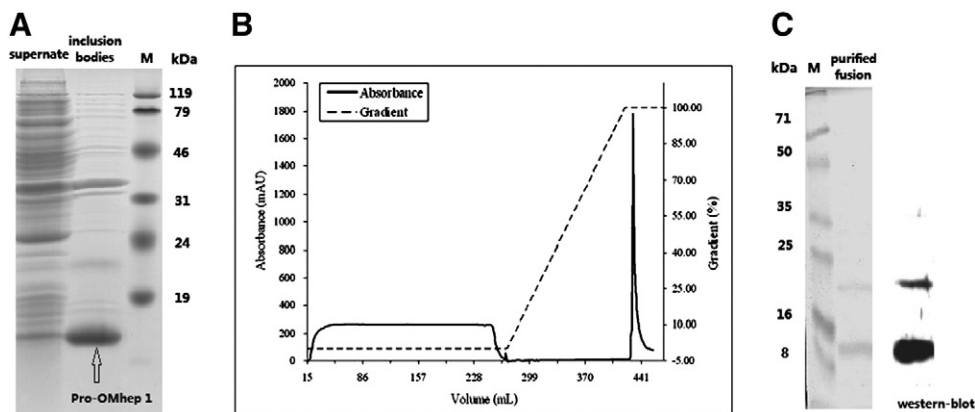


Fig. 1. Expression, purification and western-blot analysis of recombinant Pro-Omhep1. (A) Expression of recombinant Pro-Omhep1. Left panel shows the position of the molecular-mass markers (kDa). (B) Purification of Pro-Omhep1 (C) SDS-PAGE analysis of purified product and western-blot analysis of the purified recombinant Pro-Omhep1.

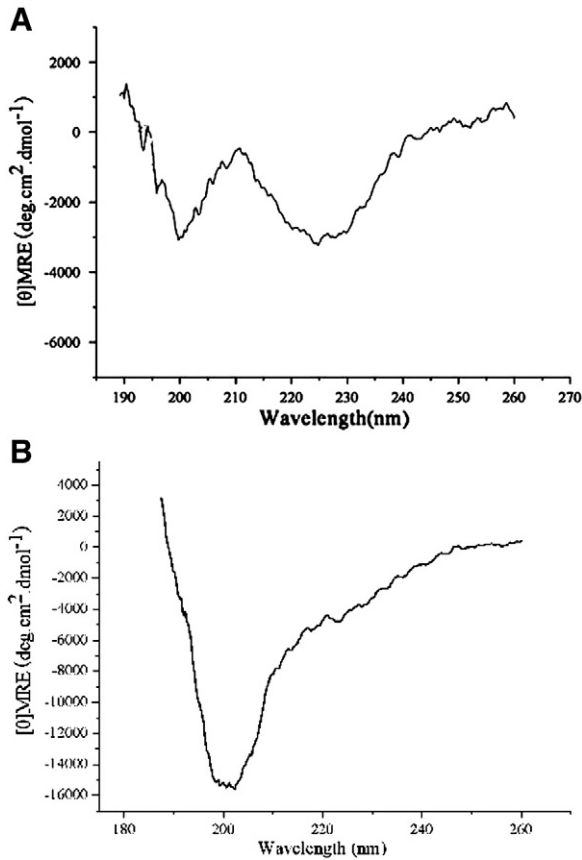


Fig. 2. Circular dichroism (CD) spectrum of OM-hep1. (A) The CD spectra of synthetic mature Om-hep1 were recorded in Milli-Q water at a concentration of 120 μM . (B) The CD spectra of recombinant Pro-Omhep1 were recorded in Milli-Q water at a concentration of 120 μM . Data were collected using an average of four scans per replicate.

3.5. Effect of Pro-Omhep1 and mature Om-hep1 peptides on tumor cell viability

The viability percentage of HepG2 cells decreased in the wells with added Om-hep1 peptide in a dose dependent manner. The growth of tumor cells was significantly inhibited when cells were incubated at concentrations of 18.75 μM and 25 μM mature Om-hep1 (simple *t*-test, $P < 0.05$). Approximately nearly 40% inhibition percentage was

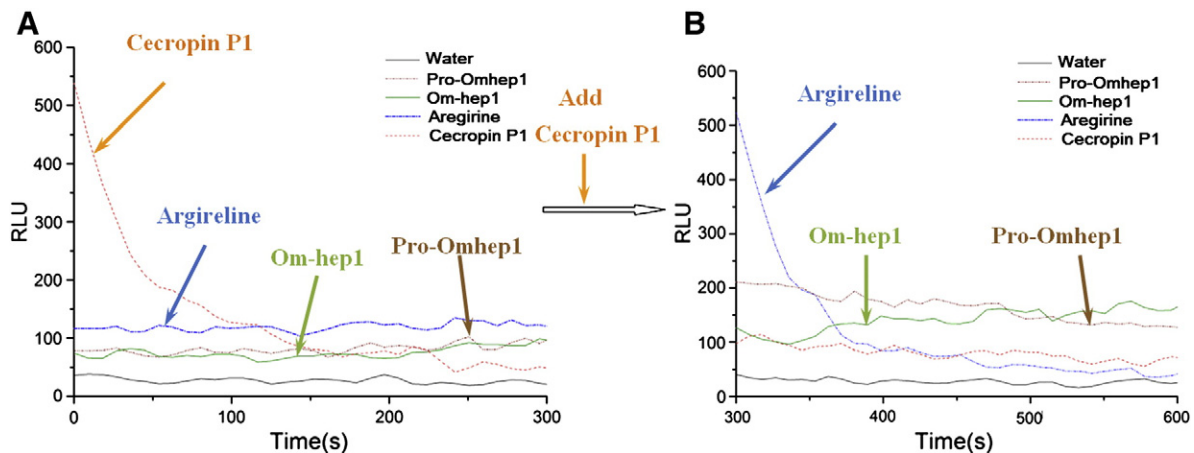


Fig. 3. Light emission kinetics of *E. coli* MC1061 cells when treated with AMPs. The treatment of membranes by membranolytic peptide led to D-luciferin permeation and resulted in emission of light. (A) The light emission from adding 12 μM Pro-Omhep1, 25 μM mature Om-hep1, 25 μM Argireline, and 1 μM cecropin P1. Related light unit (RLU) was detected as time developed. (B) Subsequently, cecropin P1 was added to the membrane-inactive samples, and the light emission was followed for another 5 min. RLU was detected as time developed. All detections were performed in triplicates.

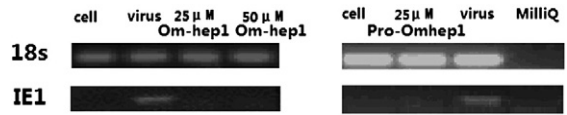


Fig. 4. Antiviral activity by Pro-Omhep1 and mature Om-hep1. PCR amplifications were carried out to examine the effect of WSSV infection in Hpt cells.

observed when cells were incubated with 25 μM mature Om-hep1. The inhibition percentage of HepG2 cells was directly proportional to the concentration of mature Om-hep1. Moreover, the decrease percentage of cell viability decreased by approximately 40% when they were incubated with 5 μM , 7.5 μM and 10 μM Pro-Omhep1 peptides. The results were all significantly different from that of the control cells (Fig. 5). Less Pro-Omhep1 were used to inhibit the growth of HepG2 cells than mature Om-hep1, recombinant Pro-Omhep1 showed a better antitumor activity compared to synthetic mature Om-hep1.

4. Discussion

Hepcidin is an AMP with eight cysteine residues folding four-disulfide bridges, and its particular structure makes it difficult for it to be synthesized with a right refolded configuration. In previous studies of the functions of hepcidin, one common way to obtain the peptide was to synthesize its short mature peptide using chemical methods (Park et al., 2001; Pigeon et al., 2001), another way to obtain a large quantity of peptide for research was through expression of its mature peptide by prokaryotic expression system (Zhang et al., 2005). In fish, it is interesting to note that the antimicrobial activity of synthetic hepcidins from different species was somewhat different. For example, the synthetic hepcidins of white bass (*Morone chrysops*) (Douglas et al., 2003) are only active against Gram-negative bacteria, but some hepcidins from black porgy (Yang et al., 2007), gilthead seabream (Cuesta et al., 2008), Japanese flounder (Hirono et al., 2005), bass (Lauth et al., 2005), tilapia (Huang et al., 2007) and large yellow croaker (Wang et al., 2009) have potent activities against both Gram-positive and Gram-negative bacteria. In addition, there were differences in activity between isoforms or variants of hepcidin even from the same fish, such as AS-hepc-2 and AS-hepc-6 (Yang et al., 2011). On the other hand, studies using recombinant hepcidin were far fewer than those using synthetic peptides. Mouse hepcidin1 is expressed in *E. coli* and its recombinant peptide shows biological activity in promoting ferroportin degradation in macrophages (Gagliardo et al., 2008), conversely the recombinant Japanese flounder

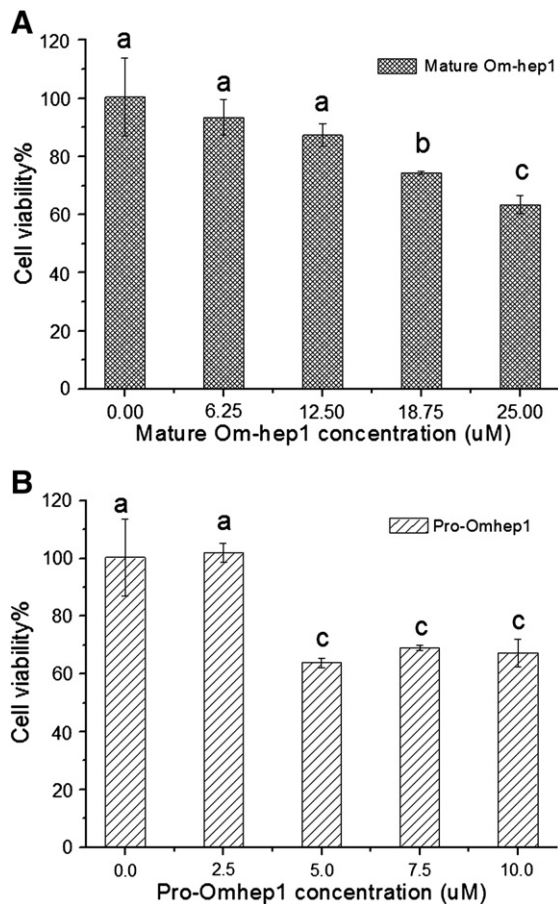


Fig. 5. Effect of Pro-Omhep1 and mature Om-hep1 peptides on the percentage of HepG2 cell viability. The bars indicate the mean \pm S.D. Three independent samples were used to obtain valid statistical evaluation of the results. Means with different letters in this figure were regarded as different significant levels at $P < 0.05$ (simple *t*-test).

hepcidin from an *E. coli* expression system is less active than the synthetic one as a result of the incorrect refolding structures (Srinivasulu et al., 2008).

Presumably, whether the recombinant peptide maintains biological activity is dependent on its refolding as a correct structure. In the present study, an effective *E. coli* expression system which could produce biologically active recombinant Pro-Omhep1 was constructed in the form of a precursor essential for hepcidin interaction (Nemeth et al., 2006), i.e., pro-hepcidin, which is processed to produce the biologically active mature peptide (Valore and Ganz, 2008). In addition, an efficient procedure for refolding and purification of the recombinant Pro-Omhep1 was developed. The present study demonstrated that both recombinant Pro-Omhep1 and synthetic Om-hep1 had antibacterial activity against Gram-positive bacteria tested (*C. glutamicum*, *S. aureus*) and Gram-negative bacteria tested (*E. coli* MC1061, *A. hydrophila*, *Pseudomonas stutzeri*) except some, such as the vibrios (*V. alginolyticus*, *V. parahaemolyticus*). Interestingly, the antibacterial activity of the recombinant Pro-Omhep1 was much better than that of the chemical synthetic mature peptide Om-hep1. The result was inconsistent with the observation using the recombinant Japanese flounder hepcidin which is less active than the synthetic one (Srinivasulu et al., 2008). To understand whether the recombinant product of Pro-Omhep1 has a right refolded configuration, the circular dichroism spectroscopy was used for analyzing Pro-Omhep1. The CD spectrum result was compatible with some structurally known β -sheet defensins (Nunes et al., 2011) and native humane hepcidin (Park et al., 2001), confirming that the recombinant Pro-Omhep1

obtained a better similar refolding of native hepcidin structure than did synthetic Om-hep1. This result indicated that our strategy of disulfide bond formation could be useful.

The membrane integrity assay was a method to estimate whether an antibacterial peptide exerts its role directly on the membrane of bacterium. The light emission peaks of membrane active AMP, cecropin P1, had confirmed the membrane integrity assay was useful (Li et al., 2010). As observed in the study, Pro-Omhep1 and mature Om-hep1 reduced the light intensity in all membrane integrity assays, suggesting that the bacterial growth had been inhibited by a different way from cecropin P1. The results indicated that the antibacterial activity of Om-hep1 was not directly with membrane pore formation. However, previous studies in tilapia hepcidin 1–5 on cell membranes of HeLa cells by microscopy showed that hepcidin had similar functions of lytic peptides (Chang et al., 2011). In the present study, the antibacterial mode of Om-hep1 was presented the same as that of *Sp*Strongylocins, which were identified from sea urchins (Li et al., 2010). However, the mechanism of hepcidin activities needed further more evidence.

It is interesting to note that AMPs can inhibit viral infection. Cathelicidins, the host defense peptides, such as human LL-37 and porcine Protegrin-1, inhibit the HIV replication cycle at the early stage of viral infection (Steinstraesser et al., 2005). In fish, it is also noted that AMPs were involved in antiviral activities. Some fish hepcidins are acknowledged to have antiviral activities: the β -defensin (BD)-1 peptide shows resistance against viral hemorrhagic septicemia virus infection in rainbow trout (Falco et al., 2008); tilapia hepcidin 1–5 enhances the survival of CHSE-214 cells infected with infectious pancreatic necrosis virus (Rajanbabu and Chen, 2011); orange-spotted grouper EC-hepcidins strongly inhibit the replication of Singapore grouper iridovirus (Zhou et al., 2011). These encouraging results shed light on the potential application of fish hepcidin in aquaculture and veterinary medicine in the future. Our present study further provided the evidence of fish hepcidin against virus. WSSV is the main pathogen of a viral infection-white spot syndrome of penaeid shrimps (Liu et al., 2011). The virus is highly virulent and has a wide host range, thus commonly leading to 100% mortality within days in the case of cultured penaeid shrimps. In our study, where WSSV was first incubated with Pro-Omhep1 and mature Om-hep1 and then the mixture was used to infect the cultured cells prepared from crayfish Hpt, it was observed that the viral replication was strongly inhibited because the transcripts of WSSV immediate-early gene *IE1* were not detected using RT-PCR amplification in the Hpt cells. The result indicated that Om-hep1 was likely to interfere with the infectious activity of WSSV. This study will extend the antimicrobial spectrum of fish hepcidin to viral protection.

Many studies demonstrated that both native and synthetic AMPs exhibited a broad spectrum of antitumor activity against cancer cells (Hoskin and Ramamoorthy, 2008; Zoysa et al., 2009). AMPs play a major role in the selective disruption of cancer cell membranes. These antitumor AMPs include α -helix structure proteins such as BMAP-28 isolated from *Bos taurus* and β -helix structure proteins such as TachyplesinI identified from *Tachyplesus tridentatus* (Hoskin and Ramamoorthy, 2008). A fish hepcidin tilapia TH1-5 shows inhibitory activity on the proliferation of tumor cells and reduces the colony formation in a soft agar assay, whereas tilapia hepcidin TH2-3 shows potent activity against human fibrosarcoma cells (Rajanbabu and Chen, 2010). As a β -helix structural AMP, hepcidin has not only antibacterial activity but also the antitumor activity, which is a new interesting function reported in recent studies. In the present study, Pro-Omhep1 and mature Om-hep1 showed strong antitumor activity and in HepG2 cells the antitumoral activity of Pro-Omhep1 and mature Om-hep1 peptides was supported by cell viability assay and approximately 40% of tumor cells were significantly inhibited. The present study provided additional evidence that the hepcidin fish and different species have the capability of resisting the proliferation of cancer cells and have good potential use in the clinical treatment of cancers.

Previous studies report that mammalian hepcidin has dual functions, one involved in antimicrobial activity and the other in iron metabolism (Sang et al., 2006; Segat et al., 2008). However, the existence of multiple hepcidin variants in acanthopterygian fish suggests that the additional hepcidin may have different functions (Hirono et al., 2005; Huang et al., 2007). Based on their hepcidin mRNA distribution, their response to microbial invasion, and the sequence clustering in phylogenetic trees, fish hepcidins could fall into two classes: HAMP1 and HAMP2 (Hilton and Lambert, 2008). HAMP1 is an orthologue of mammalian hepcidin, and HAMP2 seems to only support innate immunity (Hilton and Lambert, 2008). Because the prepropeptide *PI* of Om-hep1 was 7.5 and consulted to the phylogenetic trees analysis (Bo et al., 2011), OM-hep1 was likely to be a HAMP1 paralogue. More evidence from previous studies show that fish HAMP1 has strong antimicrobial activity against microorganisms and shows a similar antimicrobial activity to the mammalian hepcidins (huhepc25) (Table 3). JFL4 (Srinivasulu et al., 2008) and Hep-JF2 (Hirono et al., 2005) were HAMP1 homologues isolated from olive flounder show antimicrobial activities. Two Nile tilapia hepcidin TH2-3 and TH1-5 (Rajanbabu and Chen, 2010), respectively corresponding to HAMP1 and HAMP2, show multiple biological activities. Similar to other fish HAMP1, Om-hep1 showed antibacterial, antiviral and antitumor activities, indicating that this peptide had a strong biologically active function.

In conclusion, a recombinant product of Pro-Omhep1 with disulfide bonds was appropriately obtained from *E. coli* expression system and the recombinant Pro-Omhep1 showed even better potent activity against bacteria, viruses and cancer cells, than the synthetic mature peptide Om-hep1. Om-hep1 had a non-lytic mechanism as evaluated by membrane integrity assay. Taken together, Om-hep1 was likely to be an important multifunctional protein involved in innate immunity defense in the marine medaka immune system.

Table 3
A comparison of Om-hep1 with other hepcidins.

Hepcidin	Source	Peptide region	Activity	Class	Accession code
<i>Synthetic hepcidin</i>					
Om-hep1	<i>Oryzias melastigmus</i>	Mature	Gram(G)+,G – bacteria, virus, tumor cells	HAMP1	HM990657
TH1-5	<i>Oreochromis mossambicus</i> (Rajanbabu and Chen, 2011)	Mature	G+,G – bacteria, fungi, virus, tumor cells	HAMP2	ABD46831
TH2-3	<i>Oreochromis mossambicus</i> (Rajanbabu and Chen, 2010)	Mature	G – bacteria, tumor cells	HAMP1	XP_003450578
Ec-hepcidin1			<i>Epinephelus coioides</i> (Zhou et al., 2011)	Mature	G+,G – bacteria, fungi, virus
–	ADC93804				
<i>Recombinant hepcidin (prokaryotic expression)</i>					
Pro-OMhep1	<i>Oryzias melastigmus</i>			Pro	Gram(G)+,G – bacteria, virus, tumor cells
HAMP1	HM990657				
JFL4	<i>Paralichthys olivaceus</i> (Srinivasulu et al., 2008)	Mature	<i>Bacillus subtilis</i> , <i>Escherichia coli</i>	HAMP1	BAE06235
huhepc25	<i>Homo sapiens</i> (Park et al., 2001)	Mature	Gram(G)+,G – bacteria, ferroportin	HAMP1	NP_066981

–: Ec-hepcidin1 has only four Cys residues.

Acknowledgements

This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT, IRT0941), the Minjiang Scholar Program to K.-J. Wang et al. (2009) and the grant (2007AA091406) from the National High Technology Research and Development Program of China (863 Program). Special thanks to Prof. Matti T. Karp of Tampere University of Technology of Finland and Dr. Chun Li for providing us with the *E. coli* (MC1061) transferred with vector pCSS962 with the luciferase gene. The WSSV stock was kindly provided by Professor Xun Xu of the Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration (SOA) in China. We thank Professor John Hodgkiss of The University of Hong Kong for his assistance with English corrections.

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