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Molecular Cloning, Recombinant Expression, and Antimicrobial Activity of EC-hepcidin3, a New Four-Cysteine Hepcidin Isoform from *Epinephelus coioides*

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Hepcidin, a cysteine-rich antimicrobial peptide, is widespread in fish and shows multiple activities, including antimicrobial, antivirus, and antitumor. Here, a new four-cysteine hepcidin isoform gene, EC-hepcidin3, was cloned from the marine-cultured orange-spotted grouper (*Epinephelus coioides*). The complete cDNA sequence consisted of 603 bases with an open reading frame (ORF) of 270 bases. The genomic DNA sequence was composed of two introns and three exons, and its 312-bp upstream region had multiple putative transcription factor binding sites. Soluble recombinant protein EC-proHep3 containing a His-tag at the C-terminus was obtained from expression plasmid pET-28a/EC-proHep3 in *Escherichia coli* Rosetta. It was purified by immobilized metal affinity chromatography (IMAC), and it showed antibacterial activity *in vitro*. Kinetic studies indicated that recombinant EC-proHep3 has strong, rapid activity against *Staphylococcus aureus* and *Pseudomonas stutzeri*. The results indicate that EC-hepcidin3 might be an effective component in the innate immune system of groupers.

Key words: *Epinephelus coioides*; four-cysteine hepcidin; recombinant expression; *Escherichia coli* Rosetta; antimicrobial activity

Hepcidin, a cysteine-rich antimicrobial peptide (AMP), has been identified in mammals, birds, amphibians, and fish.[1–4] Since it was first reported in bass,[5] large numbers of hepcidin genes have been identified in various species of fish.[1,6–14] One of the discrepancies between mammal and fish hepcidins is that there are variants of hepcidin in one species, such as the seven variants identified in the black porgy,[8] and various fish hepcidin isoforms are found not only highly expressed in the liver but also in the kidney[6,8] in contrast with mammal hepcidins. The phenomenon that multiple hepcidin isoforms present in a fish is thought to be necessary in the fish immune defense system to confront the risk from the complex aquatic environment in which they live.[15]

Most mature peptides of fish hepcidin have the typical eight cysteine residues at conserved positions and form four disulfide bonds. This kind of hepcidin has obvious biological activities against gram-positive and gram-negative bacteria, viruses, and tumors.[16] The four-cysteine hepcidin was first reported in the Antarctic notothenioid.[17] Recently two more four-cysteine hepcidins were reported in a marine-cultured fish, the orange-spotted grouper, which has been found to possess antimicrobial and antiviral activities through the use of synthetic mature peptides.[18]

To date the feasibility of obtaining large quantities of hepcidin is through the *E. coli* prokaryotic and the *P. pastoris* eukaryotic recombinant expression system, all reported products are recombinant eight-cysteine hepcidins.[19–24] Neither the prokaryotic nor the eukaryotic expression system has been reported to express four-cysteine hepcidins. To determine the future application of four-cysteine hepcidins in fishery aquaculture, it is indispensable to know whether hepcidin with four cysteine residues can be expressed effectively in an engineering system.

In this study, a new four-cysteine hepcidin gene, EC-hepcidin3, was cloned from the orange-spotted grouper. Its cDNA sequence and genomic organization were determined. The recombinant EC-proHep3 was successfully expressed in *E. coli*, and it showed potent activity against certain bacteria. This study indicates that four-cysteine hepcidin is active against bacteria, and thus is probably an effective component in the innate immunity of fish.

Materials and Methods

Sampling. Normal live *Epinephelus coioides* were obtained from the Zhangpu Fish Farm (Fujian Province, China). The liver of the fish was collected and frozen immediately in liquid nitrogen, followed by storage at −80 °C.

Determination of EC-hepcidin3 cDNA and genomic DNA (gDNA) sequences.

Cloning of the EC-hepcidin3 cDNA sequence. Total RNA was extracted from the liver using TRIZOL reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), and quantified with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden).

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Abbreviations: AA, amino acid; AMP, antimicrobial peptide; *E. coli*, *Escherichia coli*; gDNA, genomic DNA; IMAC, immobilized metal affinity chromatography; pI, isoelectric point; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; MW, molecular mass; ORF, open reading frame; RACE, rapid amplification of the cDNA ends; UTR, untranslated region

Table 1. Primers Used in This Study

Primers	Sequences (5′–3′)
EA-S	CTGCTTACCAACTAAACCACTTCAACCCTC
EA-A	TGGTTGTGGAAGTCTAATCCTGGGG
EC-A	AGTCGGGTAGCAGTAAGGA
ECH-exp-S	GCGCCATGGGCTTACCCGTCACTGGAGTAG
ECH-exp-A	CCGCTCGAGTCA*ATGATGATGATGATGATG*TTG GAAGTCACAGCGGACTCC

The primers were designed using Primer Premier 5. The parts underlined are the sites of restriction enzymes *Nco* I and *Xho* I. Italics indicate sequences coding the hexa-histidine tag.

RT-PCR and rapid amplification of the cDNA ends (RACE) were performed to amplify the hepcidin cDNA sequence from *Epinephelus coioides*. The reverse transcription reaction was performed using a PrimeScript™ RT reagent kit (Takara, Dalian, China).

The PCR reaction was performed using a reverse transcription product mixture as cDNA template, *Ex Taq* DNA polymerase (Takara, Dalian, China) and primers EA-S and EA-A (Table 1), designed based on *Epinephelus awoara* hepcidin-like antimicrobial peptide mRNA, and complete coding sequence (GenBank accession no. DQ177321.1), which was earlier identified in our laboratory. The amplification conditions were 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 63 °C (annealing temperature), 60 s at 72 °C, and then 5 min at 72 °C for further extension.

The RACE reactions were performed using primers EA-S for 3′, EA-A for 5′, a SMART™ RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) and an Advantage® 2 PCR kit (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions.

All the expected PCR products were purified from the gel with a Qiaquick Gel Extraction kit (Qiagen, Crawley, UK), and were ligated into T/A cloning vector pMD18-T (Takara, Dalian, China), followed by transformation into *E. coli* DH5*α* competent cells. The positive clones identified by PCR were sequenced at least twice by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China).

Cloning of the EC-hepcidin3 gDNA sequence. Total genomic DNA was prepared from the liver using a MiniBEST Animal Tissue Genomic DNA Extraction kit version 2.0 (Takara, Dalian, China) following the manufacturer's instructions.

PCR reaction was performed using the total genomic DNA as template, primers EA-S and EA-A, and *LA Taq* DNA polymerase (Takara, Dalian, China) to amplify the hepcidin gDNA. The amplification conditions were 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 63 °C, 2 min at 72 °C, and then 10 min at 72 °C for further extension.

A LA PCR™ *in vitro* Cloning kit (Takara, Dalian, China) was used to amplify the upstream region of the hepcidin gDNA. Total gDNA was digested with *EcoR* I following the manufacturer's instructions, and then the digested gDNAs were ligated with the *EcoR* I Cassetter in the kit.

Two PCR reactions were carried out with two different primer pairs. The first was carried out using the purified ligation products as template DNA, mixed with the sterilized water and heated at 94 °C for 10 min, primers EA-A and the Cassette Primers C1 in the kit, and *LA Taq* DNA polymerase. First amplification conditions were 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 2 min at 55 °C, 2 min at 72 °C, and then 10 min at 72 °C for further extension. The second nested and touchdown PCR reaction was performed using the first PCR amplification products diluted 100 times as template, primers EC-A (Table 1) designed based on the sequence of EC-hepcidin3 cDNA, and Cassette Primers C2 in the kit, and *LA Taq* DNA polymerase. The second amplification conditions were 5 min at 94 °C, 5 cycles of 30 s at 94 °C, 4 min at 72 °C, 5 cycles of 30 s at 94 °C, 2 min at 70 °C, 2 min at 72 °C, 30 cycles of 30 s at 94 °C, 2 min at 55 °C, 2 min at 72 °C, and then 10 min at 72 °C for further extension.

Purification, ligation, transformation, identification, and sequencing of amplification products were performed as described above.

Sequence analysis. The gene and predicted protein sequences were analyzed by means of the computer software DNAStar 5.0 (DNA.star Inc., Madison, WI, USA). Homology searches were done using BLASTn and BLASTp by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The molecular mass (MW) and isoelectric point (pI) were predicted using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The signal peptide cleavage site of the deduced EC-hepcidin3 was predicted by means of the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The putative transcriptional factor binding sites were predicted by means of TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). Putative pre-propeptide alignment and phylogenetic analysis of EC-hepcidin3 with other hepcidins were done using Clustalx1.83 and MEGA 4.0.

Prokaryotic expression of EC-proHep3.

Construction of recombinant expression plasmid. A 195-bp sequence coding the EC-proHep3 was inserted into expression plasmid pET-28a (Novagen, Madison, WI, USA) through the restriction enzyme sites of *Nco* I and *Xho* I. The inserted sequence was obtained by PCR reaction using *Pfu* DNA Polymerase (Promega, Madison, WI, USA) and primer ECH-exp-S/ECH-exp-A (Table 1). The PCR product and the expression plasmid were digested with the corresponding restriction enzymes, and ligated with T4 DNA ligase (Takara, Dalian, China). The recombinant expression plasmid was confirmed by PCR and DNA sequencing.

Expression of recombinant EC-proHep3. A single colony of *E. coli* BL21 (DE3) pLysS and Rosetta transformed with pET-28a/EC-proHep3 was inoculated into LB medium containing 50 μg/mL of kanamycin, cultured at 37 °C, and shaken at 200 rpm until OD600 reached 0.3–0.4. Then the cells were induced by the addition of IPTG to a final concentration of 0.4 mM and incubated at 30 °C, and shaken at 180 rpm for an additional 6 h.

All types of cells were harvested by centrifugation at 4 °C, 6,000 rpm for 10 min, resuspended in phosphate buffered saline (50 mM phosphate buffer, 100 mM NaCl, pH 8.0) and analyzed by 15% (w/v) SDS–PAGE.

The cells expressed successfully were lysed by freezing, thawing, and subsequent sonication. The lysate was centrifuged at 4 °C, 12,000 rpm for 30 min to separate the soluble and insoluble portions. These portions were collected and analyzed by 15% (w/v) SDS–PAGE.

Purification of soluble recombinant protein EC-proHep3. The purification procedure was carried out using an AKTA Purifier 100 (GE Healthcare Life Sciences, Waukesha, WI, USA) and immobilized metal affinity chromatography (IMAC). The collected supernatant containing the soluble component of EC-proHep3 was filtered with a 0.45-μm filter membrane and passed through an affinity chromatography column (GE Healthcare Life Sciences, Waukesha, WI, USA) equilibrated with binding buffer (50 mM PBS, 200 mM NaCl, and 50 mM imidazole, pH 8.0). Then the column was washed in two steps. At the first step it was washed with binding buffer to remove contaminating proteins, and at the second, it was washed by a gradient of 50–225 mM imidazole with binding buffer and eluting buffer (50 mM PBS, 150 mM NaCl, and 400 mM imidazole, pH 8.0) to remove nonspecific binding proteins. Finally the recombinant protein was eluted with eluting buffer.

Both the flow-through and the final eluted fractions were collected and analyzed by 15% (w/v) SDS–PAGE. The collected elution protein of EC-proHep3 was then dialyzed into 20 mM Tris–Cl buffer (20 mM Tris, 20 mM NaCl, pH 8.0) at 4 °C and then its antimicrobial activity was tested.

Characterization of the purified recombinant EC-proHep3 by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Purified recombinant EC-proHep3 was excised from the 15% SDS–PAGE gel. The MALDI-TOF sample preperation procedure was similar to a previous study.[25] Finally, the trypsin protease-digested EC-proHep3 was analyzed using a MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) as described previously.[26,27]

Antibacterial activity assays.

Determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC). The antimicrobial

activity of the purified recombinant EC-proHep3 was tested against several microorganisms including gram-positive and gram-negative bacteria and yeast.

Determination of MIC and MBC was done by a method described previously.[6,28] In brief, dilutions of the EC-proHep3 were made to obtain a series of final concentrations. The cultured microorganism was suspended and diluted in 10 mM NaPB (pH 7.4). Most of the bacteria were grown in Mueller-Hinton broth medium (Difco Laboratories, Detroit, MI, USA), the vibrios were grown in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA), and the yeasts were grown in YPG medium. The assay mixture in one well consisted of 50 μL the diluted purified EC-proHep3, 30 μL the diluted microorganism suspension (1×10^4 CFU bacteria, 1×10^3 CFU yeasts), and 20 μL the corresponding broth. After 24–48 h of incubation at 28 °C, the value of MIC was calculated as the lowest peptide concentration yielding no detectable growth. Aliquots of the mixtures were then plated on corresponding agar plates at 37 °C or 28 °C for 24–48 h, and the value of MBC was calculated as the lowest concentration killing more than 99.9% of the microorganisms. Each assay was performed in triplicate.

Kill-kinetic studies. Bactericidal kinetic studies were done using *Staphylococcus aureus* and *Pseudomonas stutzeri*, as described previously.[6,27] Every bacterium was incubated with EC-proHep3 (12 μM) at 28 °C. At various time points of incubation, 6 μL was taken from the mixture, serially diluted in 10 mM NaPB (pH 7.4), and plated on nutrition broth agar. The plates were incubated at 28 °C for 24 h, and the colonies were counted. The percentage of CFU was defined relative to the CFU obtained from the control (100% CFU at zero min).

Results

Determination of the EC-hepcidin3 cDNA and gDNA sequences

A hepcidin-like gene that included a complete ORF was identified. It was a new hepcidin isoform of the orange-spotted grouper according to sequence analysis, and was named EC-hepcidin3. Furthermore, a full-length cDNA (GenBank accession no. HQ008222) and the gDNA sequence with the upstream region (GenBank accession no. HQ008223) of EC-hepcidin3 were determined. As shown in Fig. 1, the complete cDNA sequence of EC-hepcidin3 was 603 bp, consisting of a 88-bp 5′ untranslated region (UTR) and a 270-bp ORF that encoded a putative peptide of 89 amino acids (AA). The predicted molecular weight of the putative peptide was 9,777.30 Da with a theoretical pI of 4.99. The cDNA sequence also contained a 216-bp 3′ UTR with a polyadenylation signal AATAAA sequence appearing at 195 nt and a poly(A) tail at 217 nt the downstream of the stop codon TGA.

Similarly to other known hepcidins, the putative peptide of EC-hepcidin3 consisted of three domains: a signal peptide (24 AA), a prodomain (40 AA) and the mature peptide (25 AA). The signal peptide cleavage site was predicted between Ala and Leu. The typical motif RX(K/R)R of propeptide convertases was identified. The mature peptide had four cysteine residues, predicted a molecular weight of 2,798.2 Da and a theoretical pI of 7.91. Hence it was a cationic peptide.

The gDNA sequence of EC-hepcidin3 consisted of two introns and three exons (Fig. 1). The exon 1 (175 bp) contained 5′ UTR, a DNA sequence that encoded the signal peptide, and 5 AA residues of the prodomain. Intron 1 (82 bp) and intron 2 (127 bp) were located within the DNA sequence encodes the prodomain, and exon 2 (78 bp) encoded 26 AA residues of the prodomain, and exon 3 (321 bp) consisted of 9 AA residues of the prodomain and the mature peptide plus 3′ UTR.

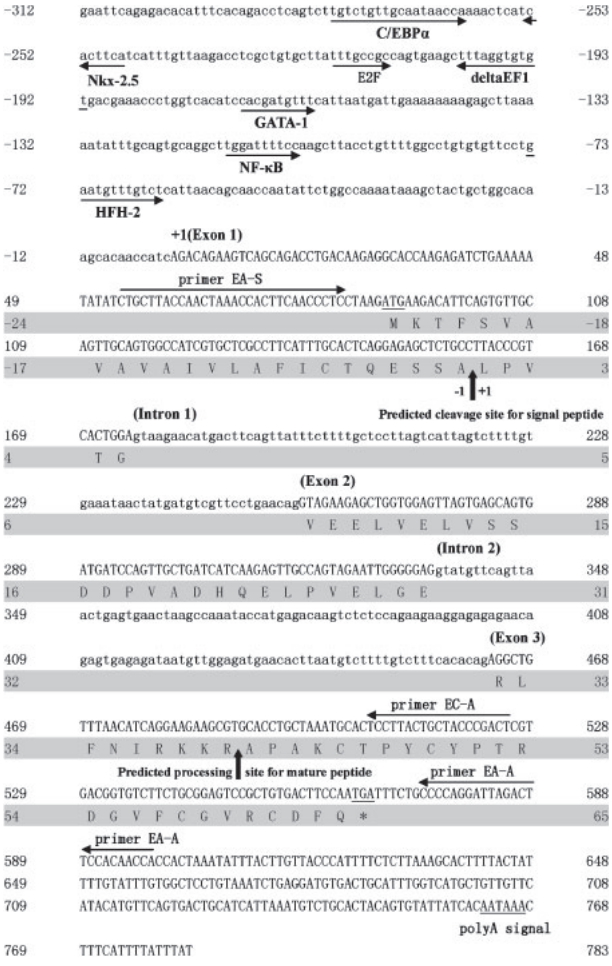

Fig. 1. Genomic DNA, cDNA, and the Predicted Amino Acid Sequence of EC-hepcidin3.

Numbering of the genomic sequence is relative to the transcription start site. Locations of putative transcription factor binding sites and primer binding sites are indicated by arrows. Exons are shown in capital letters and introns in small letters. The start codon (ATG), stop codon (TGA), and polyadenylation signal (AATAAA) are underlined. Deduced amino acid sequences are translated and shadowed. Numbering of the deduced amino acid sequences is relative to the first amino acid residue of propeptide. Vertical arrows show the predicted positions of cleavage sites for the signal peptide and the mature peptide.

In addition, a 312-bp upstream region sequence of the EC-hepcidin3 gene was identified, and the putative transcriptional factor binding sites were predicted by TFSEARCH. The putative transcription factor binding sites included C/EBPα (−279 nt), Nkx-2.5 (−253 nt), E2F (−219 nt), deltaEF1 (−202 nt), GATA-1 (−171 nt), NF-κB (−113 nt), and HFH-2 (−73 nt) in the upstream region of the EC-hepcidin3 gene, but there was no TATA box consensus sequence.

Comparison and phylogenetic analysis of the putative peptide of EC-hepcidin3

A putative pre-propeptide alignment (Fig. 2) and phylogenetic analysis (Fig. 3) of EC-hepcidin3 with other hepcidins were done. The other hepcidins obtained from GeneBank included, as follows: 31 hepcidins of fish, containing 4, 5, 6, 7, 8 cysteine residues in the mature peptide, 2 hepcidins of amphibians containing 8 cysteine residues in the mature peptide, and 7 hepcidins of mammals containing 8 cysteine residues in the mature peptide.

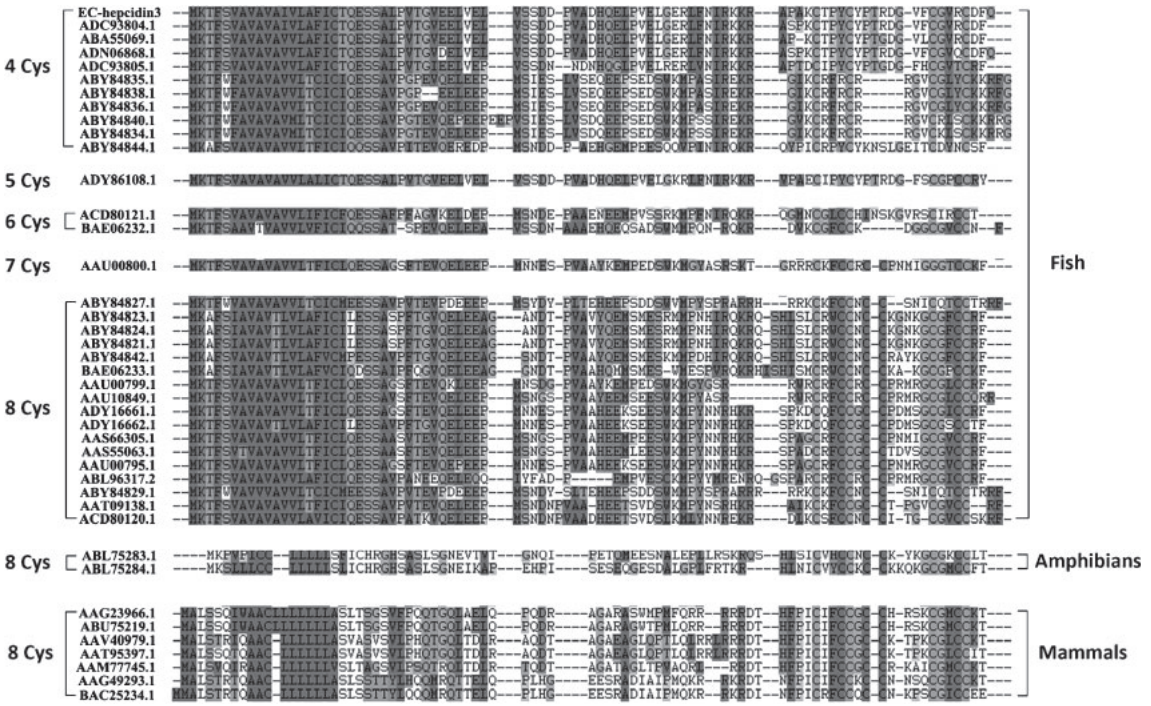

Fig. 2. ClustalX Alignment and Comparison of the Putative Pre-Propeptide of EC-hepcidin3 with Other Hepcidins.

The other hepcidins obtained and predicted from GeneBank were as follows: 31 hepcidins of fish, *Acanthopagrus schlegelii* (AAU00795.1, AAU00799.1, AAU00800.1); *Dissostichus mawsoni* (ABY84836.1, ABY84838.1, ABY84824.1, ABY84829.1); *Epinephelus awoara* (ABA55069.1); *Epinephelus bleekeri* (ADY86108.1); *Epinephelus coioides* (ADC93804.1, ADC93805.1); *Epinephelus moara* (ADY16661.1, ADY16662.1); *Epinephelus malabaricus* (ADN06868.1); *Gymnodraco acuticeps* (ABY84834.1); *Lateolabrax japonicus* (AAS55063.1, AAT09138.1); *Lycodichthys dearborni* (ABY84844.1, ABY84842.1); *Notothenia angustata* (ABY84835.1, ABY84823.1, ABY84827.1); *Pagrus major* (AAS66305.1, AAU10849.1); *Paralichthys olivaceus* (BAE06232.1, BAE06233.1); *Pogonophryne scotti* (ABY84840.1, ABY84821.1); *Pseudosciaena crocea* (ABL96317.2); *Sebastes schlegelii* (ACD80120.1, ACD80121.1), two hepcidins of amphibians, *Xenopus tropicalis* (ABL75283.1, ABL75284.1), and seven hepcidins of mammals, *Canis lupus familiaris* (AAV40979.1, AAT95397.1); *Gorilla gorilla* (ABU75219.1); *Homo sapiens* (AAG23966.1); *Mus musculus* (AAG49293.1, BAC25234.1); *Sus scrofa* (AAM77745.1).

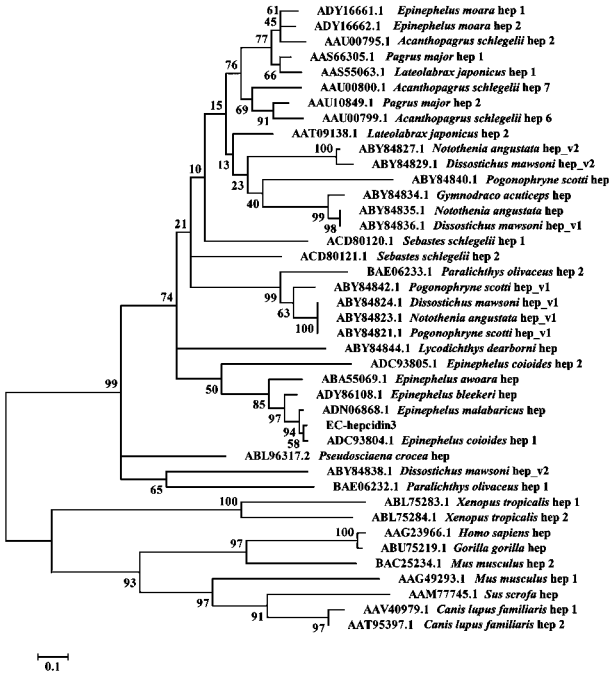

Fig. 3. Phylogenetic Analysis of the Putative Pre-Propeptide of EC-hepcidin3 and Other Hepcidins.

Numbers next to branches indicate bootstrap values from 1,000 replicates.

Alignment indicated that in all the hepcidins listed above, the predicted signal peptide cleavage sites of the fish hepcidins were highly conserved and different from those of amphibians and mammals. All the signal peptides of fish hepcidins listed above were 24 AA in length, and the signal peptide cleavage site of the fish hepcidins listed above was between Ser-Ser-Ala^{24} and X^{25}. X was Leu in the hepcidins of *Epinephelus* sp., containing four and five cysteine residues in the mature peptide, and was replaced by other amino acids (Val, Phe, Thr, Gly, Ser, Ile, or Ala) in the other fish hepcidins listed above.

Phylogenetic analysis of the hepcidin family indicated that the fish hepcidins listed above were in a cluster different from the hepcidins of amphibians and mammals, and that EC-hepcidin3 was in the same branch position as published *Epinephelus coioides* hep 1 and near the other hepcidins of *Epinephelus* sp. containing 4 and 5 cysteine residues in the mature peptide.

Recombinant expression and purification of EC-proHep3

Construction of the recombinant expression plasmid

Recombinant expression plasmid pET-28a/EC-pro-Hep3 was constructed. Its coding sequence included two AA residues (Met and Gly) from the site of restriction enzyme *Nco* I and primer ECH-exp-S at the N-terminus, EC-proHep3, and a C-terminal His-tag from primer ECH-exp-A (Fig. 4). The amino acid sequence of recombinant EC-proHep3 was MGLPVTGVEELVEL-

VSSDDPVADHQELPVELGERLFNIRKKRAPAKCTP-YCYPTRDGVFCGVRCDFQHHHHHH. The estimated molecular mass was 8.3 KDa.

Recombinant production of EC-proHep3

Each of two host *E. coli* strains that contained the recombinant expression plasmid was cultured and induced to express under conditions as described in section above, and was analyzed by 15% (w/v) SDS–PAGE (Fig. 5). Recombinant expression plasmid pET-28a/EC-proHep3 did not expresse in *E. coli* BL21 (DE3) pLysS, but expressed in *E. coli* Rosetta. The SDS–PAGE gel indicated that recombinant protein EC-proHep3 was soluble, and that its molecular weight was about 12 KDa (Fig. 5).

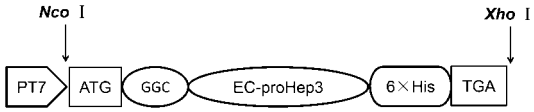

Fig. 4. Construction of Expression Vector pET-28a/EC-proHep3.

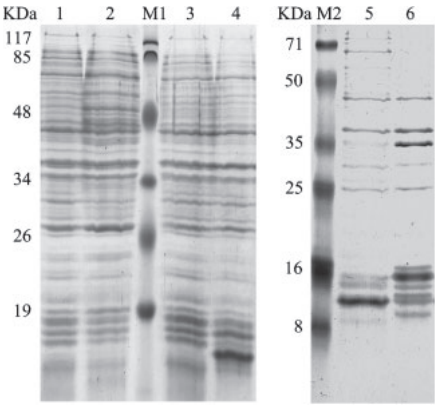

Fig. 5. SDS–PAGE Analysis of pET-28a/EC-proHep3 Expression in Two *E. coli* Strains and the Dissolubility of Recombinant EC-proHep3.

The gel was stained with Coomassie Brilliant Blue R-250. Lane 1 and lane 3, induced with IPTG for 0 h as control in BL21 (DE3) pLysS and Rosetta; lane 2 and lane 4, induced with IPTG for 6 h in BL21 (DE3) pLysS and Rosetta; lane 5, soluble portion of induced Rosetta cells; lane 6, insoluble portion of induced Rosetta cells. Lane M1 and lane M2, protein markers SM0441 and RM006.

Purification of soluble recombinant protein EC-proHep3

Recombinant protein EC-proHep3, expressed in *E. coli* Rosetta, was purified by IMAC. The supernatant was collected from the sonicated crude extract of fusion protein after centrifugation, and was purified through a Ni^{2+}-chelating-Sepharose column, as shown in Fig. 6A. Both the flow-through and the final eluted fractions were collected and analyzed by 15% (w/v) SDS–PAGE (Fig. 6B). Approximately 32.4 mg of pure recombinant EC-proHep3 was obtained in 1 L of bacteria culture.

Because the recombinant protein EC-proHep3 collected contained a higher concentration of imidazole, which was used in eluting the protein conjugated with Ni^{2+} in the affinity chromatography column, and could be toxic to bacterial cells, it was necessary to remove imidazole completely by dialysis, as described in section above.

Characterization of the recombinant proteins

The approximately 12 KDa purified recombinant product was collected for MS fingerprinting. The results indicated that the purified recombinant protein was the expected EC-proHep3 (Fig. 7).

Antibacterial activity assays

Determination of MIC and MBC

The antimicrobial activity of the recombinant EC-proHep3 was measured against a panel of microorganisms by MIC and MBC assays. The MIC and MBC values obtained are reported in Table 2. The purified recombinant EC-proHep3 showed activities against three gram-positive bacteria, *Corynebacterium glutamicum* (MIC 48–96 μM, MBC 48–96 μM), *Micrococcus lysodeikticus* Fleming (MIC 24–48 μM, MBC 48–96 μM), and *Staphylococcus aureus* (MIC 1.5–3 μM, MBC 1.5–3 μM), and two gram-negative bacteria *Pseudomonas fluorescens* (MIC 24–48 μM, MBC 24–48 μM), and *Pseudomonas stutzeri* (MIC < 1.5 μM MBC < 1.5 μM).

Kill-kinetic studies

In the kinetic study, a highly sensitive gram-positive bacterial strain, *S. Aureus*, and a highly sensitive gram-negative bacterial strain, *P. stutzeri*, were used to evaluate the bactericidal activity of the purified recombinant EC-proHep3 (Fig. 8). The purified recombinant EC-proHep3 killed approximately 45% and 100%

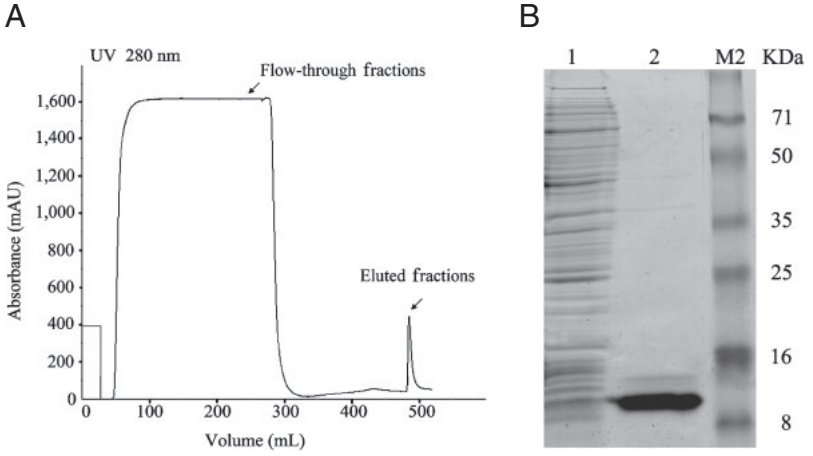

Fig. 6. Purification of Recombinant Protein EC-proHep3 by IMAC (A) and Purified EC-proHep3 Showed on 15% (w/v) SDS–PAGE Gel (B). Lane 1, flow-through fractions; lane 2, eluted fractions.

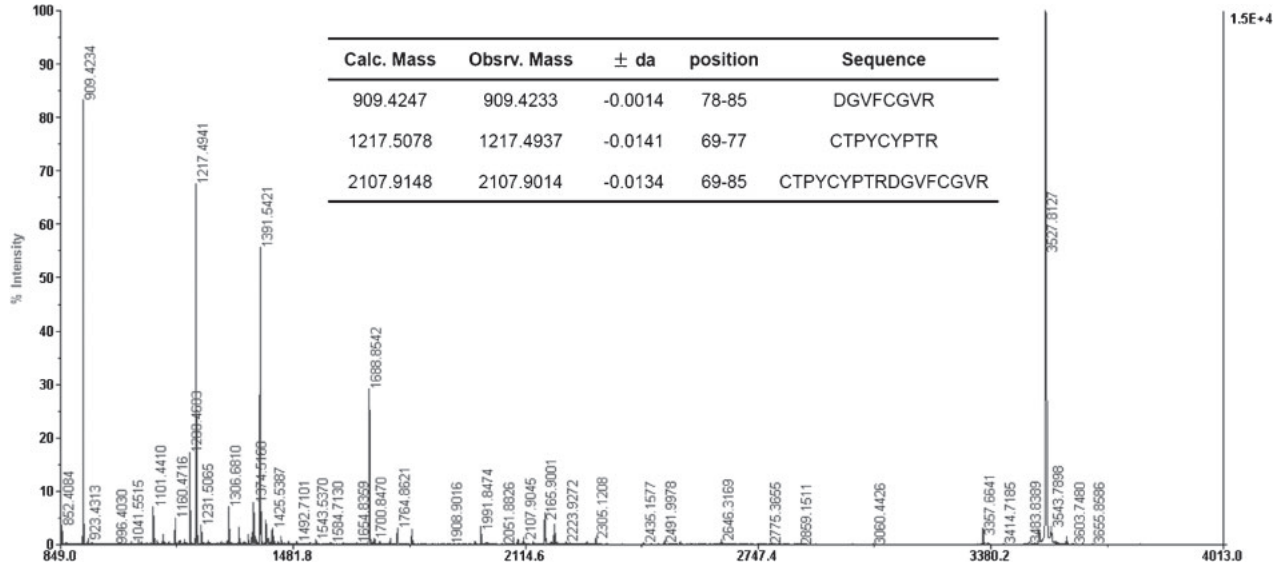

Fig. 7. MS Fingerprint of EC-proHep3.

The mass of each peptide fragment was determined by MALDI-TOF–TOF MS, and was displayed in the graph. In the table, ±da means the difference between the mass observed and the calculated mass.

Table 2. Antimicrobial Activity of Recombinant EC-proHep3

Microorganisms	CGMCC no.[a]	MIC (b–c μM)	MBC (d–e μM)
Gram-positive bacteria			
Bacillus cereus	1.447	>96	NT[f]
Bacillus subtilis	1.3343	>96	NT
Corynebacterium glutamicum	1.1886	48–96	48–96
Micrococcus luteus	1.0290	>96	NT
Micrococcus lysodeikticus Fleming	1.0634	24–48	48–96
Staphylococcus aureus	1.0363	1.5–3	1.5–3
Staphylococcus epidermidis	1.2429	>96	NT
Gram-negative bacteria			
Aeromonas hydrophila	1.2017	>96	NT
Escherichia coli	1.2389	>96	NT
Escherichia coli MC1061	g	>96	NT
Pseudomonas aeruginosa	1.0205	>96	NT
Pseudomonas fluorescens	1.0032	24–48	24–48
Pseudomonas stutzeri	1.1803	<1.5	<1.5
Shigella flexneri	1.1868	>96	NT
Vibrio alginolyticus	1.1833	>96	NT
Vibrio fluvialis	1.1609	>96	NT
Vibrio harvryi	1.1593	>96	NT
Vibrio prahaemloyticus	1.1615	>96	NT
Yeast			
Candida albicans	2.2411	>96	NT
Pichia pastoris GS115	h	>96	NT

a, CGMCC no. indicates China General Microbiological Culture Collection number.

b, c, MIC values are expressed as the interval of concentration b–c μM, where b is the highest concentration tested at which microbial growth was observed, and c is the lowest concentration yielding no detectable microbial growth (n = 3).

d, e, MBC values are expressed as the interval of concentration d–e μM, where d is the same means as the b, and e is the lowest concentration tested that inhibited microorganism growth or that killed more than 99.9% of the microorganisms (n = 3).

f, Not tested.

g, ATCC (American Type Culture Collection) no. 25922.

h, *Pichia pastoris* GS115 was purchased from Invitrogen.

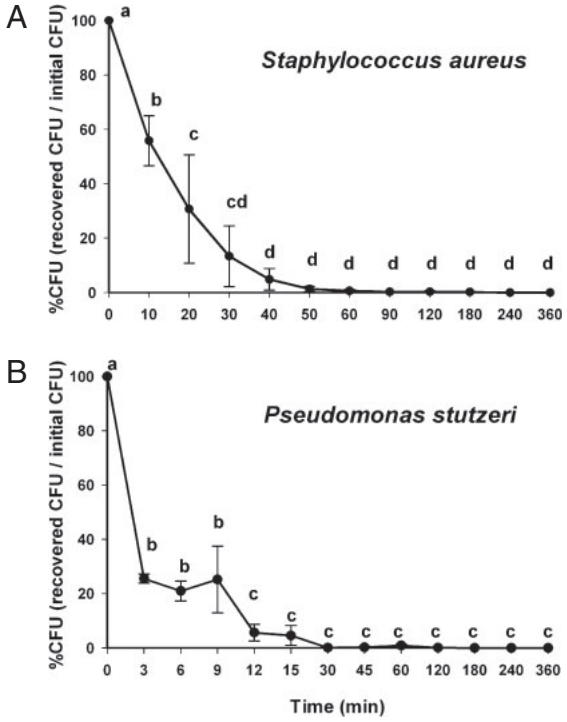

Fig. 8. Kinetics of Killing of *Staphylococcus aureus* (A) and *Pseudomonas stutzeri* (B) by Purified Recombinant EC-proHep3.

The percentage of CFU was defined relative to the CFU obtained in the control (100% at 0 min). Each point represents the average of three independent experiments, and vertical bars represent mean ± SD. These were analyzed by one way ANOVA, followed by Tukey's *post hoc* test. Identical letters (a, b, c, d) indicate no significant difference between different time points, and different letters indicate statistically significant differences ($p \leq 0.05$) between time points.

S. aureus after incubation of the bacteria with the peptide for 10 min and 60 min respectively (Fig. 8A), while the purified recombinant EC-proHep3 immediately killed approximately 75% and 100% *P. stutzeri* after 3 min and 30 min respectively (Fig. 8B).

Discussion

In this study, a new hepcidin isoform gene, EC-hepcidin3, was cloned from the orange-spotted grouper. Its cDNA sequence and genomic organization were determined. The cDNA sequence of EC-hepcidin3 consisted of an ORF of 270 bases, and possessed four

cysteine residues in the mature region. The predicted signal peptide cleavage site of the putative EC-hepcidin3 peptide and the typical motif RX(K/R)R of propeptide convertases were conserved, as most other fish hepcidins.

The genetic structure and organization, with three exons and two introns of the EC-hepcidin3 gene, were highly conserved, as in the hepcidins of human, mouse, and fish.[1,5,11,29,30] Analysis of the promoter region of EC-hepcidin3 revealed multiple putative transcription factor binding sites, among which C/EBPα and NF-κB were important transcription factors. C/EBPα, enriched in the liver, is an important transcription factor in hepatic gene expression,[31] is likely to be a key regulator of hepcidin transcription, and provides a novel mechanism for cross-talk between the C/EBP pathway and iron metabolism.[32] Rel/NF-κB transcription factors are conserved from *Drosophila* to humans, and they play important roles in the Toll signalling pathway and the defense system.[33] EC-hepcidin3 also had a putative binding site of transcription factor E2F. E2F1 is a transcription factor recruited by NF-κB following LPS activation of TLR4, and Barnes *et al.* concluded that the presence of E2F1 might explain the different magnitudes of induction responding to LPS between the two hepcidin isoforms in Barramundi.[13] There is no TATA box consensus sequence in the upstream region of the EC-hepcidin3 gene, as there is for the 6-cysteine hepcidin of *Paralichthys olivaceus*.[10] The TATA-box is a highly conserved element, strictly located between -35 and -25 relative to the transcription start site in most eukaryotic RNAP II genes, which presents fraction between 20–46% in yeast genes, about 30% of *Drosophila* genes, and 35% of human genes. Little is known about the structure and function of the core promoter in most non-TATA genes. It is likely that some mechanisms left to be elucidated.[34]

Since 2000, when the first hepcidin was discovered, almost all of the hepcidins identified in mammals or fish are characterized by eight cysteine residues in the mature peptide. However, the homologous hepcidin isoforms with four- or six-cysteine residues fewer than eight in the mature region have been identified in certain fish species in recent years.[8–10,17] Various hepcidin variants exist widely in fish more than in mammals are thought to be associated with adaptive differentiation to the complexity of the aquatic environment.[6] To date reported hepcidins with four cysteine residues have been identified only in *Epinephelus* sp. and the Antarctic notothenioid. In the Antarctic notothenioid, the reduced cysteine variant of hepcidin is thought to be evolutionally adapted to the polar environment.[17] But *Epinephelus coioides* inhabits subtropical and tropical regions, and a phylogenetic analysis indicated that 4- and 5-cysteine hepcidins of *Epinephelus* sp. are in the same branch (Fig. 3) and far from the hepcidins of the Antarctic notothenioid. Thus there might be different evolutionary patterns of hepcidin genes among fish species, but the elements driving this adaptive evolution of reduced cysteine are not clear in detail now.

A previous study of human hepcidin indicated that a mature hepcidin molecule containing eight cysteine residues exists as a simple hairpin structure with disulfide bridges linking the two arms in a ladder-like configuration.[35] By structure calculations and dynamic signatures in NMR spectra, Hunter *et al.* confirmed two disulfide bonds between Cys^1–Cys^8, Cys^3–Cys^6. A subsequent study of bass hepcidin determined a similar structure.[36] In 2009, Jordan *et al.* confirmed two further disulfide connectivities of Cys^2–Cys^4 and Cys^5–Cys^7 by proton NMR spectroscopy and X-ray crystallography.[37] In our study, the positions of the four cysteine residues in the putative mature peptide of EC-hepcidin3 corresponded to the positions at Cys^1, Cys^3, Cys^6, and Cys^8 of human mature hepcidin respectively (Fig. 2). Hence it can be assumed that the two disulfide connectivities of the EC-hepcidin3 mature peptide are likely to be Cys^1–Cys^4 and Cys^2–Cys^3.

Most antimicrobial activities of mammal and fish hepcidins are determined using synthetic mature hepcidin peptides.[6,12,18,29,36] For research purposes, as well as industrial production, recombinant expression of small and highly disulphide-bonded peptides, is expected to yield properly refolded, active products.[24] To date, eight-cysteine hepcidins of mammals and fish have successfully been expressed in the *E. coli* prokaryotic expression system and the *P. pastoris* eukaryotic expression system.[19–23,38,39] However, no effective expression system to obtain recombinant four-cysteine hepcidin has yet been reported.

In this study, recombinant expression plasmid pET-28a/EC-proHep3 was constructed, and the soluble recombinant product was successfully obtained in *E. coli* Rosetta. We found that EC-proHep3 failed to express in *E. coli* BL21(DE3) pLysS. This result was similar to that for the expression of EBNA-1.[40] There are some low-usage codons in *E. coli*,[41] which can affect the translation rate of foreign genes in *E. coli*.[42] Three low-usage codons (AGG, CCC, GGA) were found through analysis of the sequence encoding EC-proHep3. *E. coli* Rosetta cells provide a plasmid that contains the rare tRNA genes for seven low-usage codons (AGA, AGG, AUA, CUA, GGA, CCC, CGG) of *E. coli*, and hence *E. coli* Rosetta can dramatically improve expression level.[40] Differently from human mature hepcidin peptide and Pro-Omhep1 expressed as inclusion bodies,[23,39] recombinant EC-proHep3 was expressed in soluble form in pET-28a vector. This should facilitate the purification process and future applications of recombinant EC-proHep3 in research and aquaculture. In comparison with the eight-cysteine version, whether four-cysteine hepcidin is prone to be solubly expressed needs further elucidation.

In this study, recombinant EC-proHep3 was 73 AA with an estimated MW 8.3 KDa, but the apparent MW on SDS–PAGE was 12 KDa. A similar gel shift was found in our previous study of recombinant expression of medaka hepcidin.[23] The expression of recombinant EC-proHep3, including the prodomain and the mature peptide besides the mature peptide, was based on our consideration of future application and the potential effects of prodomain on mature peptide activity. The prodomain plays an essential role in the correct folding of many proteins, including some disulfide-coupled proteins,[43–46] and the recently reported recombinant product of Pro-Omhep1 showed more potent activities against bacteria, viruses, and cancer cells than the synthetic mature peptide, Om-hep1.[23]

Purified recombinant EC-proHep3 showed activity against three gram-positive bacteria, *C. glutamicum*,

M. lysodeikticus Fleming, and *S. Aureus*, and two gram-negative bacteria, *P. Fluorescens* and *P. stutzeri*. As reported, the synthetic mature peptide of EC-hepcidin1 significantly inhibits the growth of *S. aureus*,[18] and *Pseudomonas* sp. is pathogenic to groupers,[47] and thus recombinant EC-proHep3 is a potential antimicrobial agent for aquaculture and veterinary medicine.

In summary, a new four-cysteine hepcidin isoform gene, EC-hepcidin3, was identified in the orange-spotted grouper. Its cDNA and gDNA sequences were determined. In addition, an effective prokaryotic expression plasmid, pET-28a/EC-proHep3, was constructed, and a soluble recombinant product, EC-proHep3, was obtained in large quantities from *E. coli* Rosetta. The purified recombinant EC-proHep3 showed potent antimicrobial activity against certain gram-positive and gram-negative bacteria *in vitro*. The data obtained in this study should improve our knowledge of hepcidin in fish. Four-cysteine hepcidin may exist in some fish species such as groupers, and might possess antimicrobial activity similar to eight-cysteine hepcidin. EC-hepcidin3 is likely to play a role in the innate immune system of the orange-spotted grouper, and the recombinant EC-proHep3 might be an alternative potential therapeutic for the future.

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