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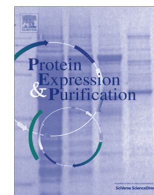

Expression, purification and antibacterial activity of the channel catfish hepcidin mature peptide

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ABSTRACT

Hepcidins are small cysteine-rich cationic antimicrobial peptides. The channel catfish (*Ictalurus punctatus*) hepcidin cDNA has been characterized, but recombinant protein expression and purification was not reported. *I. punctatus* hepcidin is comprised of 96 residues, with eight functionally important conserved cysteine residues located in the C-terminal region of the mature peptide, suggesting that this region is responsible for the antibacterial activity. In this study, a cDNA fragment (*mCH*) encoding the 25 amino acid mature peptide was cloned from channel catfish liver, and inserted into vector pET-32a(+) to produce a construct that expressed a hexahistidine-tagged thioredoxin (trxA) fusion protein that was cleavable using enterokinase. The trxA–mCH fusion protein was expressed in *Escherichia coli* BL21 (DE3) at 25 °C, using 1 mM IPTG for induction. Greater than 80% of the fusion protein was expressed solubly, but was not biologically active. Removal of the trxA fusion partner by enterokinase resulted in mCH that exhibited antibacterial activity against two Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*), and two Gram-negative (*E. coli* and *Pseudomonas aeruginosa*) bacteria.

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Introduction

Antimicrobial peptides (AMPs)[1] are gene-encoded small cationic peptides present in organisms spanning the tree of life. AMPs have a broad spectrum of action against microorganisms including Gram-positive and Gram-negative bacteria, fungi, viruses, and protozoa, and even cancer cells can be susceptible [1–3]. AMPs may serve as ideal substitutes for traditional antibiotics, and research in this area has expanded dramatically in recent years. Hepcidin or liver-expressed antimicrobial peptide 1 (LEAP-1) is an AMP produced predominantly in liver that was first detected in plasma [4] and urine [5] in humans. Homologs have since been identified in other mammals including buffalo [6], sheep [7] and mice [8]. Hepcidin has dual functions; it shows antimicrobial activity against a number of Gram-positive and Gram-negative bacteria [9–11], although in many cases it is not known if the peptides tested were correctly folded. It also acts as an iron regulatory hormone which negatively regulates intestinal iron absorption and macrophage iron release [8,12].

The predicted primary structures of hepcidin from higher and lower vertebrates share six to eight conserved cysteine residues, indicating that the disulfide bridges of hepcidin have been evolutionarily conserved and may relate to biological function [13]. Although the human hepcidin prepropeptide contains 84 residues, only three mature peptide forms comprising 20, 22 and 25 residues were found in urine [5], suggesting these mature peptides are responsible for activity. A number of fish hepcidin sequences have been reported. Three tilapia (*Oreochromis mossambicus*) sequences, and one from large yellow croaker (*Pseudosciaena crocea*) have been determined [9,14]. Recently, three novel hepcidin genes were cloned from the orange-spotted grouper (*Epinephelus coioides*) [11,15], and two genes were identified in medaka (Japanese rice fish; *Oryzias melastigmus*) [16]. Recombinant protein expression has been attempted for some fish hepcidins, but codon preference problems, toxicity of the expressed peptides to *Escherichia coli*, and the reducing conditions of the *E. coli* cytosolic environment prevented the production of correctly folded and biologically active versions of these cysteine-rich, cationic peptides. Some groups have chemically synthesized hepcidin genes to overcome the codon usage issue, and the three hepcidin-like antimicrobial peptides in tilapia (TH1-5, TH2-2, TH2-3) were chemically synthesized in an alternative strategy [9]. Bacteriostatic analysis showed that TH1-5 and TH2-3 possessed antimicrobial activity against several bacteria, while TH2-2 did not. The two mature peptides (AS-hepc2 and AS-hepc6) from Japanese black porgy (Sea Bream) were similarly produced and studied, and AS-hepc6 displayed a wider antimicrobial spectrum than AS-hepc2 [17]. The 3D structures of these peptides were also predicted. Recently, the antibacterial activity of synthetic and recombinant pro-hepcidin from medaka were

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[1] *Abbreviations used:* AMPs, antimicrobial peptides; CI, confidence index; LEAP-1, liver-expressed antimicrobial peptide 1; OD600, optical density; RT, reverse-transcription; TCP, total cell protein.

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determined, and the recombinant form displayed superior antibacterial activity [16]. Fusing AMPs to a variety of tags or charged pro-sequences to mask their toxic effects has also been attempted. The 26-residue hepcidin from Japanese flounder (*Paralichthys olivaceus*) was fused to a hexahistidine tag, but neither three different cleavage sites for removal of the tag, nor cytosolic or periplasmic expression, resulted in soluble protein [18].

A hepcidin gene from channel catfish (*Ictalurus punctatus*) has been characterized, but recombinant protein expression was not reported [19]. Furthermore, reports on expression of small peptides in *E. coli* using native cDNAs are scarce. In this study, we designed an *E. coli* expression system based around the strategy used for Japanese flounder hepcidin [18], but with some modifications. A cDNA fragment encoding the *I. punctatus* hepcidin mature peptide (*mCH*) was cloned from channel catfish liver using RT-PCR, and ligated into the pET-32a(+) expression vector. The resulting thioredoxin fusion protein was expressed solubly in *E. coli* BL21 (DE3), and enterokinase cleavage released free mCH that exhibited robust antimicrobial activity.

Materials and methods

Reverse-transcription (RT)-PCR and sequence analysis

Channel catfish (0.7 kg in body weight) was supplied from a fish culture farm in Shanghai, China. Total RNA was extracted from liver using Trizol reagent (Invitrogen, USA). First strand cDNA was synthesized using the 3′-RACE system (Invitrogen, USA). To obtain the full-length hepcidin cDNA, PCR amplification was performed using forward primer (5′-ATGAGGGCAATGAGCATC GCG-3′) and reverse primer (5′-TTAGAACCTGCAGCAGAACCC-3′) which were designed with reference to the database nucleotide sequence (GenBank ID: AY834209). PCR using *Ex Taq* DNA polymerase (Takara, Japan) was performed as follows; 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. The reaction mixture was as follows; forward and reverse primers (0.4 μM of each), 2.5 μl first strand cDNA as a template, 800 μM dNTPs, 10 μl 10 × *Ex Taq* buffer, and 1 μl *Ex Taq* DNA polymerase (5 U/μl). The final volume was adjusted to 100 μl with sterilized water. Subsequently, a cDNA fragment (*mCH*) encoding the mature peptide was amplified by PCR using forward primer (5′-CGGAATTCCAGAGTCACCTCTCTCTG TGC-3′) and reverse primer (5′-CCCAAGCTTGAACCTGCAGCAGA ACCCACA-3′) including *Eco*RI and *Hind*III sites, respectively. PCR was carried out as described above, except full-length cDNA was used as template, and the annealing temperature was changed to 60 °C. Amplified DNA fragments were subcloned into the pSURE-T simple vector (Aidlab Biotech, China) using *E. coli* strain DH5α as a host. DNA sequencing was carried out by Shanghai Sangon Biological Engineering Technology Corporation (China) using the BigDye terminator v3.1 sequencing kit and the ABI PRISM 3730 DNA sequencer.

Engineering the fusion protein expression construct

The recombinant plasmid pSURE-*mCH* was digested with restriction enzymes *Eco*RI and *Hind*III (Takara, Japan), and the digested fragment was resolved on an agarose gel, extracted, ligated into pET-32a(+) (Novagen, USA) pre-digested with *Eco*RI and *Hind*III, and transformed into competent *E. coli* DH5α cells. Positive clones were identified by PCR, digestion with *Eco*RI and *Hind*III, and DNA sequencing.

Expression and purification of the fusion protein

The confirmed recombinant expression construct (pET-32a-*mCH*) was transformed into competent *E. coli* BL21 (DE3) cells. Colonies were used to inoculate 5 ml LB medium containing 100 μg/ml ampicillin, and cultured with shaking at 37 °C. When the optical density (OD_{600}) reached 0.6, cells were collected and resuspended in 1 L LB medium containing 100 μg/ml ampicillin and 1% glucose to reduce expression prior to induction, and cultured with shaking at 37 °C until the OD_{600} reached 0.6. Expression was induced by adding 1 mM IPTG. Cells were cultured at 25 °C for a further 8–12 h, and harvested by centrifugation at 8000*g*, 4 °C, for 10 min. The pellet was resuspended in pre-cooled PBS (pH 7.3). Cells were lysed by sonication, and the extract was clarified by centrifugation at 12,000*g* for 10 min. Proteins were separated by Tricine–SDS–PAGE, stained with Coomassie Brilliant Blue R-250, images scanned using a 2000 GEL-DOC system (Bio-Rad Laboratories, USA), and analyzed using Quantity One software version 4.4.0 (Bio-Rad Laboratories, USA).

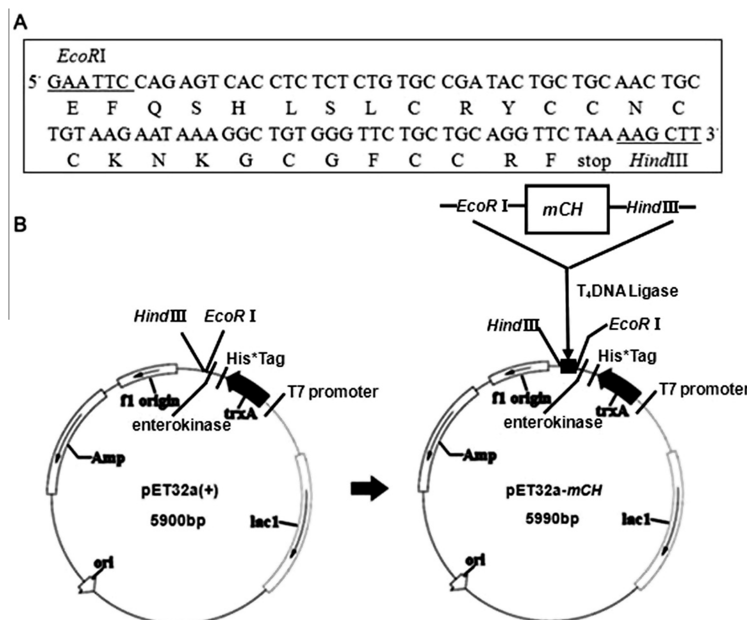

Fig. 1. cDNA and amino acid sequences of channel catfish hepcidin. (A) Gene and protein sequence corresponding to the mature peptide. (B) Schematic representation of the pET-32a-*mCH* fusion protein expression plasmid. Restriction endonucleases *Eco*RI and *Hind*III are underlined.

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