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cDNA sequence and expression analysis of an antimicrobial peptide, theromacin, in the triangle-shell pearl mussel *Hyriopsis cumingii*

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ABSTRACT

Bivalve molluscs rely on the interaction between cellular and humoral factors for protection against potential pathogens. Antimicrobial peptides (AMPs) have been proven to be one of the most important humoral components that afford resistance to pathogen infection. The AMP gene to be identified was that encoding theromacin in the triangle-shell pearl mussel *Hyriopsis cumingii* (Hc theromacin); this gene was identified from a suppression subtractive hybridization library, and subsequently cloned by 3′ and 5′ rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR). The full-length theromacin cDNA contains 547 bp, with a 294-bp open reading frame that encodes a 97-amino acid peptide, and the deduced peptide sequence contains a 61-amino acid putative mature peptide. The sequence also contains 10 cysteine residues. Reverse transcriptase (RT)-PCR analysis showed that Hc theromacin transcripts were constitutively expressed in the liver, foot, gill, adductor muscle, heart, mantle, intestine, and hemocytes, with the highest level in hemocytes. Theromacin mRNA levels were found to increase after challenge with Gram-positive and Gram-negative bacteria. After injection of the Gram-positive bacteria *Staphylococcus aureus* and *Bifidobacterium bifidum*, Hc theromacin expression showed the highest fold-change at 48 and 36 h after infection, respectively, and its levels decreased gradually thereafter.

1. Introduction

Antimicrobial peptides (AMPs) (also called as host-defense peptides) are evolutionarily conserved components of the innate immune system that play an important role in phagocytosis and local and systemic killing of microbes; these compounds are found in both vertebrates and invertebrates (Bachère et al., 2004; Bulet et al., 2004). Unlike the majority of conventional antibiotics, AMPs may also possess the ability to enhance immunity by functioning as immunomodulators. AMPs are generally composed of 12–50 amino acids. The majority of AMPs have an amphipathic structure, which enables attachment, destabilization, and/or penetration into the microbial cytoplasmic membrane (Brogden, 2005). Most eukaryotic AMPs can be classified into 3 main groups: (i) linear alpha-helical peptides without cysteine (insect cecropins, magainins, etc.); (ii) linear peptides with an extended structure that is characterized by an unusually high proportion of one or more amino acids such as proline or histidine; (iii) peptides with disulfide bonds, which form a looped structure (defensins, protegrin, etc.) (Devine and Hancock, 2002). AMP-encoding genes have been isolated from microorganisms as well as from plants and animals, and the genes isolated from different groups of organisms show a considerable variation in structure and size (Bals and Wilson, 2003). In addition to AMPs, different species often have a reservoir of structurally diverse peptides to combat pathogens (Mitta et al., 2000). Marine invertebrates are solely dependent on physical barriers and the innate immune system for protection against pathogenic agents, and natural antibiotics have been shown to participate in the immune response during experimental bacterial challenges (Munoz et al., 2004). AMPs have been isolated from several marine invertebrates, including chelicerates, molluscs, and crustaceans (Tincu and Taylor, 2004). The first study on AMPs in bivalve molluscs was conducted in the 1990s using reverse genomics. To date, several AMPs have been identified in mollusks such as the gastropod *Dolabella auricularia* (Iijima et al., 2003); marine bivalves *Mytilus edulis* (Charlet et al., 1996), *M. galloprovincialis* (Hubert et al., 1996; Dupuy et al., 2004), *Crassostrea virginica* (Seo et al., 2005) and *C. gigas* (Gonzalez et al., 2007; Gueguen et al., 2006). By using biochemical and molecular cloning techniques, the following 4 groups of cationic cysteine-rich AMPs have been isolated from the hemocytes of unchallenged mussels *M. galloprovincialis* and *M. edulis*: (i) defensin-like peptides MGD-1 and MGD-2 (Mitta et al., 2000; Romestand et al., 2003; Seo et al., 2005; Gueguen et al., 2006); (ii) myticins (Mitta et al., 2000); (iii) mytilins with 8 cysteine residues but different specific cysteine arrays and amino acid sequences (Mitta et al., 2000); and (iv) mytimycin (Charlet et al., 1996). Except for the AMP described above, a novel antimicrobial peptides named

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theromacin was isolated and characterized from annelid *Theromyzon tessulatum* (Tasiemski et al., 2004) and *Hirudo medicinalis* (Tasiemski, 2008). Whereafter theromacin was found in mollusks *Aplysia californica* (GenBank accession no. **ABF21076**) and *Biomphalaria glabrata* (Ittiprasert et al., 2010).

Investigations on AMPs are particularly attractive not only for increasing the basic information on immunity but also for enabling the development of various disease management methods in aquaculture. In the case of freshwater mussels, AMPs have been identified only in the *H. cumingii* cDNA library (Bai et al., 2009). Owing to its numerous advantages such as high productivity, convenient pearl-producing process, high pearl-production efficiency, and high pearl quality, *H. cumingii* is the chief freshwater pearl-producing mussel in China. In the present study, a novel antibacterial peptide named theromacin was identified from the suppression subtractive hybridization library of triangle-shell pearl mussel challenged with *Flavobacterium columnare* G4. Theromacin expression in different *H. cumingii* tissues was investigated, and the effect of infection with two Gram-negative and two Gram-positive bacteria on theromacin expression was studied.

2. Materials and methods

2.1. Construction and analysis of a triangle shell pearl mussel suppression subtractive hybridization library induced by F. columnare G4

The triangle shell pearl mussels used to construct subtracted library were obtained in May 2008 from pearl station, Gong'an, Hubei province of China. Three shells (about 14 cm in shell length) infected with *F. columnare* G4 were selected, with the hepatopancreas dissected out for the construction of infected group subtracted library. Another three shells from the same pearl station were used for the subtracted library with the above-mentioned organs dissected out. All samples were separately washed three times in PBS (pH 7.2), and snap-frozen in liquid nitrogen before being stored at −70 °C for further use.

The total RNA was prepared for RT-PCR detection from hepatopancreas of infected and uninfected groups using Trizol Reagent (Invitrogen, USA). About 1600 mg tissues containing approximately equal amount of hepatopancreas from two infected and two uninfected shell were used as tester and driver samples, respectively. The mRNA was then isolated from the total RNA using the PolyA Tract Isolation System (Promega). The concentration of mRNA was determined by a spectrophotometer.

cDNA was synthesized and amplified using a Clontech SMART PCR cDNA Synthesis Kit (Clontech) by following the manufacturer's instruction. The PCR products were used for RACE PCR. The mRNA from tester and driver samples was subjected to SSH and selective PCR amplification using the PCR-Select cDNA Substraction Kit (Clontech) by following the manufacturer's instruction.

Forward- and reverse-subtracted cDNA were digested with RsaI and labelled as probes with digoxigenin using a DIG High Prime system (Boehringer Mannheim). The differential screening was performed following the methods of the PCR-Select Differential Screening Kit User Manual (Clontech). Hybridization, rinsing of the membrane, and the detection reaction were performed using the DIG High Prime Labeling and DetectionStarter Kit I (Boehringer Mannheim) by following the manufacturer's instruction. The positive clones were sequenced using the dideoxy chain-termination method on an automatic DNA sequencer (ABI Applied Biosystems Model 377). Homology searches were performed using the BLASTN, BLASTX and TBLASTX programs at web servers of the National Center for Biotechnology Information. One clone obtained was found to be homologous to invertebrate antibacterial peptide theromacin and was further analyzed. Another clone found was homologous to vertebrate β-actin.

2.2. Cloning the theromacin cDNA sequence

To obtain the full-length cDNA sequence of Hc theromacin, 3′ RACE were performed by using the gene-specific primers 3-F1, 3-F2 and adaptor primers (Table 1). The universal primers mix (UPM) was the mixture of the long form (UPM Long and short form (UPM Short). For the 3′-RACE, the PCR was initially performed with primers UPM/3-F1 followed by a nested PCR with primers UPM/3-F2. The annealing temperature of first and second PCR was 62 °C and 65 °C, respectively. 5′ sequence was amplified with a specific primer 5-F1 and 5-R1. The resultant products were isolated using the agarose purification Kit (Omega), and cloned into pMD18-T vector by following the manufacturer's instruction. Putative clones were screened by PCR using the above primers, and the selected clones were sequenced. Homology searches were performed by BLAST at web servers of the National Center for Biotechnology Information.

2.3. Sequence analysis

Protein prediction was performed using software at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn/). The putative ORFs were analyzed for the presence of signal peptides using the algorithms Signal P 3.0. The transmembrane regions were identified by the TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). A multiple alignment was generated using the CLUSTAL W program. Sequence identities were calculated using the MEGALIGN program within DNASTAR. Phylogenetic analysis was performed using the neighbor-joining method within the Mega 3.0 molecular evolutionary genetic analysis software package. Data were analyzed using Poisson correction, and gaps were removed by pairwise deletion. The degree of confidence for each branch point was determined by bootstrap analysis (1000 times). All the sequences used for the phylogenetic analysis were listed in Table 2.

Table 1
Oligonucleotide primers used to amplify the Hc theromacin gene.

Name	Sequence (5′–3′)	Usage
UPM Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Race-PCR Universal primers
UPM Short	CTAATACGACTCACTATAGGGC	
3-F1	TATGGAACACCTGTAGCGAGCGAT	3′ RACE 1st round PCR
3-F2	AAACCAACTGTGGCGAAGCCTACC	3′ RACE 2nd round PCR
5-F1	ATATCCCAATGCTATAACTA	5′ RACE 1st round PCR
5-F2	TGCCATTTTCACTGGTGTTCT	5′ RACE 2nd round PCR
β-actin F	CCGTGTTTCCATCCATCGT	Semi-quantitative/Real-time quantitative control
β-actin R	CAGGACTGGGTGCTCTTCA	
RT-F	GATTCGTTCTCAGTGATTGCT	Semi-quantitative/Real-time quantitative PCR amplification
RT-R	GCTCGCTACAGGTGTTCCATA	