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Short communication

Molecular characterization and expression analysis of the large yellow croaker (*Larimichthys crocea*) complement component C6 after bacteria challenge

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ABSTRACT

The complement system is an indispensable component in innate immunity. As one of them, complement component C6 plays an important role in the complement system, mediating membrane attack complex (MAC) formation and bacterial lysis. In this study, full-length complement component C6 (LycC6) cDNA (GenBank accession no. KT962992) was cloned from the large yellow croaker (*Larimichthys crocea*) and an ORF of 3156 bp was identified. The deduced amino acid sequence predicted that LycC6 contained conserved residues and domains known to be critical for C6 function. The quantitative real-time PCR analysis revealed that the LycC6 transcript was expressed in all the examined tissues. Complement component C3 is another central molecule in the complement system, converging the upstream complement signals and mediating the MAC assembly pathway formation. LycC6 was indispensable for active MAC formation after *Vibrio anguillarum* (*V. anguillarum*) challenge. Complement component C3 of large yellow croaker (LycC3) and LycC6 expression increased in the liver, spleen and kidney, and was the highest in the liver after *V. anguillarum* and polyinosinic:polycytidylic acid (Poly I:C) challenge. The results suggested that LycC6 might be an important immune-related gene, which is playing an important role in the immune defenses against bacterial infection.

Statement of relevance: The complement component C6 is an important component that plays a major role in the complement system. This study provides the first investigation of the cloning and characterization of the complement component C6 gene from *Larimichthys crocea*, and analysis of the expression of complement component C6 and complement component C3 of *Larimichthys crocea* after *Vibrio anguillarum* and acid Poly I:C challenge. The work described has not been published or is under consideration for publication elsewhere. The study was reviewed and approved by the Ethics Committee of Animal Experiments of Zhejiang Ocean University.

1. Introduction

The immune system plays a important role and essential in protecting man and other animals against pathogens. Innate and adaptive immune responses are recognized. Adaptive immunity traces back to the early period of vertebrate evolution, between the divergence of cyclostomes and cartilaginous fish (Fujita et al., 2004). The innate immune response is an older defense mechanism, and becomes activated before the adaptive immune response (Hoffmann, 1999). The complement system is very important in innate immunity responses. In general, the complement was found to recognize and eliminate pathogens through direct killing and/or stimulation of the innate immunity (Moffitt and Frank, 1994; Boackle, 2003; Gasque, 2004). In mammals, the complement system can enhance phagocytosis through macrophage active cytokine modulation (Collins and Bancroft, 1992). Previous research revealed that the complement system can be efficiently activated by the bacterial lipopolysaccharide (LPS) as a defense against bacterial infection in mice, and consequently, the bacterial survival rate was increased when the complement system was deficient (Collard et al., 2000). This suggested that the complement was playing an important role in the host defenses against bacterial infection after activation. Complement deficiency also impacted the complex immunity of pyogenic infection (Morgan and Walport, 1991).

The complement component C3 is the core of the entire innate immunity. It activates both the classical and alternative complement activation pathways, and is necessary for the downstream production of opsonins and direct killing of pathogens (Stahl et al., 2012). The complement components C5b, C6, C7, C8 and C9 aggregate to form the MAC (Muller-Eberhard, 1986; Tegla et al., 2011). First, a soluble C5b–6 complex is formed after C5b binds to C6 through a metastable binding site in the vicinity of the activating cell. The subsequent C7 binding to the C5b–6 complex facilitates C7 insertion into the lipid bilayer of the target cell membrane. C8 binds to the C5–7 complex and further anchors this trimeric complex into the membrane surface. Finally, the

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C5b–8 complex and C9 polymerize to form the pore-like structure called the MAC in the lipid bilayer (Würzner, 2000; Gasque, 2004; Thai and Ogata, 2004). C6 was first reported in humans (Discipio and Gagnon, 1982). In previous animal model studies, C6-deficiency in poly vinyl benzyl glucose (PVG) rat and Peru-Coppock mouse strains resulted in MAC deficiency (Leenaerts et al., 1994). This suggested that the terminal complement complex of the MAC required for cell lysis might not be assembled without C6, and it was also studied in various other species (Chondrou et al., 2006; Wang et al., 2008; Kimura and Nonaka, 2009; Wang et al., 2009; Mikrou and Zarkadis, 2010; Shen et al., 2011; Shen et al., 2013).

The *Larimichthys crocea* (*L. crocea*) was one of the most economically important marine fish in China, but various environmental stressors severely affected *L. crocea* immunity, and the increasing environmental pollution resulted in considerable economic losses (Martinez-Urtaza et al., 2004; Zheng et al., 2006). In particular, *L. crocea* aquaculture suffered from various pathogenic diseases (Harikrishnan et al., 2011), resulting in great economic losses. In this study, we aimed to characterize the open reading frame (ORF) of *L. crocea* LycC6 gene, the LycC6 transcript tissue distribution, and LycC6 domain architecture. Furthermore, we assessed the expression levels of LycC3 and LycC6 transcripts in various tissues (liver, kidney and spleen) from *L. crocea* after infection with *V. anguillarum* and Poly I:C challenges.

2. Materials and methods

2.1. Larimichthys crocea

Healthy large yellow croakers (weight 800 ± 15 g, estimated age ca. 8 months) were obtained from Zhejiang Dahaiyang Technology Co., Ltd. (Zhoushan, Zhejiang Province, China). The fishes were maintained at 25 °C in an aerated seawater tank and fed the commercial diet for two weeks prior to the beginning of the experiment. Water in the tank was changed daily. After acclimation, three groups of 100 individuals were randomly chosen for challenge experiments. *L. crocea* were then intraperitoneally infected with *V. anguillarum* (1×10^8 CFU/mL, resuspended in PBS, pH 7.4), Poly I:C (0.5 mg/100 g) (Mu et al., 2014), and PBS (as control; 300 μL/200 g). The animals of all the groups were anesthetized by immersion in MS222 before tissue sampling, as required. The liver, kidney, and spleen tissues were harvested from five fish per group, at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h after injection, respectively. All procedures were in accordance with the guidelines of the Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People's Republic of China, November 14, 1988), and were approved by the Animal Ethics Committee of Zhejiang Ocean University (Zhoushan, China).

2.2. Cloning of the complete L. crocea LycC6 cDNA ORF

The complete cDNA sequence of LycC6 was obtained after amplification with specific primers (Table 1), which were designed based on the *L. crocea* whole-genome data (Wu et al., 2014). Total RNA was isolated from the liver, kidney and spleen using the Trizol Total RNA Kit (Invitrogen, USA), and cDNA synthesis was performed with the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan). The reactions were performed according to the manufacturer's instructions. The PCR amplification was conducted using a Thermal Cycler (Bio-Rad, USA), with the following amplification conditions: 4 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 30 s at 60–65 °C, and 2 min at 72 °C, with the final extension of 5 min at 72 °C. The PCR products were gel-purified with the NucleoTrap Gel Extraction Kit (TIANGEN, China) after 2% agarose gel electrophoresis, and sequenced at Shanghai Invitrogen Biological Technology Company (China).

Table 1
PCR primers used in this study.

Primer	Sequences(5′–3′)
For the complete cDNA ORF	
LycC6–1-F	ATGACTGTCCAGGAATTCAAC
LycC6–1-R	CGGTTCCTCTGCCAGAGCATCG
LycC6–2-F	AGTGCAAGTTACCACCCA
LycC6–2-R	AGGAGGCAGCTCGTCTAT
LycC6–3-F	GAGGCCAACCCTGTAATGG
LycC6–3-R	TCATGTGGACGGACAGGCGCC
For qRT-PCR	
RT-LycC6-F	GGGGAATCACTGGGTTTG
RT-LycC6-R	GGAAGGAGCACAGGGACA
RT-LycC3-F	CACCTTGTGTAAAATTCTACCATCC
RT-LycC3-R	CCCTGAGGACCCACATCATAA
β-Lyc actin-F	TCGTCGGTCGTCCCAGGCATCAG
β-Lyc actin-R	ATGGCGTGGGGCAGAGCGTAACC

2.3. Sequence analysis

The amino acid sequences of LycC6 were predicted with the Expert Protein Analysis System (http://www.expasy.org/). The nucleotide homology search was conducted with the BLASTn program of NCBI (http://www.ncbi.nlm.gov/BLAST/). Multiple sequence alignments were performed with ClustalW2 (http://pbil.ibcp.fr/htm/index.php). The theoretical MW and isoelectric point (pI) were determined by the Expasy-ProtParam online tool (http://www.expasy.org/tools/protparam.html). Protein structure was predicted with the SMART online tool (http://smart.embl-heidelberg.de/). The functional sites in proteins were predicted with the Eukaryotic Linear Motif resource online tool (http://elm.eu.org/). The genome data of the organism was obtained from the UCSC online tool (http://genome.ucsc.edu/).

2.4. LycC6 mRNA distribution in tissues

Total RNA was extracted from the heart, gill, liver, intestine, muscle, spleen, brain, and kidney. Each tissue was obtained from at least 5 healthy animals. Two μg of total RNA were reverse transcribed in a final volume of 20 μL using the PrimeScriptTM RT Reagent Kit (Tli RNaseH Plus, TaKaRa, China) following the manufacturer's instructions. For each sample, the test and control reactions were run in triplicate. qRT-PCR was performed in a reaction mixture of 20 μL, containing 0.8 μL primer-F (10 μmol/L), 0.8 μL primer-R (10 μmol/L), 10 μL 2 × SYBR® Premix Ex Taq™ II, 0.8 μL cDNA sample (100 ng/μL), 0.4 μL ROX II, and 7.2 μL ddH_2O (the reagent concentration refer to its manufacturer's instructions). The standard cycling conditions were: 95 °C for 1 min (initial polymerase activation), followed by 40 cycles of 10 s at 95 °C, and 30 s at 60 °C. Specificity of the PCR reaction was verified by dissociation curve analysis performed from 55 to 95 °C, and *L. crocea* β-actin gene was used as the internal standard. All qRT-PCR primers are given in Table 1.

2.5. Temporal expression profiles of LycC3 and LycC6

After the assessment of the relative expression of LycC3 and LycC6 genes in different tissues, the liver, kidney and spleen were selected as candidate tissues for the investigation of the temporal expression levels of these genes after *V. anguillarum* and Poly I:C challenges. We randomly sampled five animals from each group at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h after each challenge, respectively. The extracted tissues were stored at −80 °C until use. The total RNA extraction, cDNA synthesis and qRT-PCR analyses were performed as described above.