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Cloning and molecular characterization of two complement Bf/C2 genes in large yellow croaker (*Pseudosciaena crocea*)

Wei Wei [a], Haizhen Wu [a], Hongyan Xu [a,*], Tao Xu [a], Xiao Zhang [a], Kangmei Chang [b], Yuanxing Zhang [a,**]

[a] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, PR China*
[b] *College of Ocean Sciences, Zhejiang Ocean University, Zhoushan, Zhejiang Province 316004, PR China*

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A B S T R A C T

Complement components factor B and C2 are two crucial proteases in the alternative pathway (AP) and classical pathway (CP). Two Bf/C2 cDNAs, LycBf/C2A and LycBf/C2B were isolated from the large yellow croaker (*Pseudosciaena crocea*) by suppression subtractive hybridization (SSH) and rapid amplification of cDNA ends (RACE). Through sequence alignment and computer 3D modeling analysis, we found that both of the deduced proteins contain three complement control protein (CCP) modules, a von Willebrand factor A (vWFA) domain, and one serine protease (SP) domain. Both structural analysis and phylogenetic analyses suggested that LycBf/C2A is more like human factor B than human C2 while LycBf/C2B is more human C2-like. After that, RT-PCR assay showed that LycBf/C2A and LycBf/C2B were mostly expressed in liver, albeit detectable in other tissues. Finally, after being infected with attenuated live *Vibrio anguillarum* strain, the expression level of LycBf/C2A and LycBf/C2B were found remarkably up-regulated in liver, spleen and kidney, indicating that the two complement factors play a pivotal role in the immune response to bacterial challenge in large yellow croaker.

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1. Introduction

The complement system plays a crucial role in innate and adaptive host defence against microbial infection. In mammals, its functions include controlling inflammatory reactions, chemotaxis, cellular activation, antimicrobial defence, as well as killing pathogens directly [1]. The complement system can be activated through three different pathways including the classical, lectin, and alternative pathways, which involve the formation of convertases to produce protein fragments by cleaving C3 and C5 [2]. Factor B serves as the catalytic subunit of C3 convertase in the alternative pathway (AP) [3,4], while in the classical pathway (CP), this function is subjected to C2 [5,6]. In fact, Factor B and C2 share similar modular structures including three complement control protein (CCP) modules, one von Willebrand factor A (vWFA) domain, and a serine protease (SP) domain [7], with the CCP and vWFA modules capable to bind to C4b and C3b, respectively [8,9].

In recent years, extensive studies have been focused on complement system in mammals, but little is studied in low vertebrates, particularly in teleost [10]. Although fish appears to possess activation pathways similar to those of mammals, the Bf/C2 identified in fish may contain several isoforms compared with their mammalian counterparts [1]. To date, one kind of factor B or C2-like gene was found in medaka (*Oryzias latipes*) and Japanese dogfish (*Triakis scyllia*) [11,12], while two isoforms of Bf/C2 molecules were reported to exist in zebrafish (*Danio rerio*) and nurse shark (*Ginglymostoma cirratum*) [13–15]. Moreover, the third type of Bf/C2 was identified in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) [16–19]. Importantly, although extensive investigations have been put forward on the gene cloning, structure and evolutionary analysis of Bf/C2 in teleost, only in the rainbow trout it was reported that Bf/C2 was an up-regulated gene in response to inactivated *Vibrio anguillarum* [18].

Large yellow croaker (*Pseudosciaena crocea*) is one of the most important mari-cultured fish in China and Korea because of its delicious meat and economic importance. However, Vibriosis, one of the most serious diseases which affected the large-scale culture of large yellow croaker, has been causing great economic losses [20,21]. And the fish pathogen *V. anguillarum* is the causative agent of this widespread bacterial disease. Therefore, understanding the immune response to attenuated live *V. anguillarum* of large yellow croaker may contribute be useful for developing effective ways to control this disease among farmed fish.

Here, two complement Bf/C2 genes, designed as LycBf/C2A and LycBf/C2B, were cloned and characterized respectively. Furthermore, the spatial and temporal profiles of these two genes expression were

* Corresponding author.
** Corresponding author. Tel.: +86 21 64253065; fax: +86 21 64253025.
E-mail addresses: hyxu@ecust.edu.cn (H. Xu), yxzhang@ecust.edu.cn (Y. Zhang).

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also analyzed in large yellow croaker challenged by attenuated live *V. anguillarum*. Our findings will provide clues for elaborating the role of the complement Bf/C2 in immune response of large yellow croaker against bacterial invasion.

2. Materials and methods

2.1. Bacterial infection and sample preparation

Large yellow croakers of approximately 300 g were collected from DHY Mariculture Company (Zhoushan, Zhejiang Province, China). An attenuated live *V. anguillarum* strain (MVAV6203) was used as stimulator (Chinese patent, No: CN1644680, 2004). 20 fish were injected intraperitoneally with 10^7 CFU/ml MVAV6203 in a total volume of 2 ml as experiment group, and the same amount of fish were injected with sterilized phosphate buffered saline (PBS, pH 7.5) as control. All the fish were cultured in flow-through seawater at 25 °C ± 2. Three fish injected with bacteria were sampled at 0.5, 5, 10, 15, 20 days of post-injection, respectively. And the control fish were sampled in the same way as experiment group. Then the samples from various tissues were dissected and immersed in RNAlater (Qiagen, Germany), subsequently incubated overnight at 4 °C and stored at −20 °C until RNA extraction.

2.2. Full-length cDNA cloning of LycBf/C2A and LycBf/C2B

The partial LycBf/C2A and LycBf/C2B sequences were obtained from our SSH library of the large yellow croaker [22]. To isolate the full-length cDNA of LycBf/C2A and LycBf/C2B, RACE PCR was carried out using a SMART RACE cDNA Amplification Kit (Clontech, USA). Two pairs of gene-specific primers (LycBf/C2A-F1 and LycBf/C2A-R1, LycBf/C2B-F1 and LycBf/C2B-R1) were designed according to the known EST sequence of LycBf/C2A and LycBf/C2B, respectively (Table 1). The primer set of LycBf/C2A-F1 and UPM primer (supplied by Clontech) or LycBf/C2B-F1 and UPM primer were used for 3′ RACE, and 5′ RACE was performed with a primer set of LycBf/C2A-R1 and UPM primer or LycBf/C2B-R1 and UPM primer. The PCR products were purified, cloned into pMD19-T vector (Takara, Japan) and subsequently sequenced. Finally, the full-length cDNAs of Bf/C2 gene were assembled by combining the 5′ and 3′ RACEs' fragments, which were confirmed by RT-PCR with two pairs of terminal primers (LycBf/C2A-F2 and LycBf/C2A-R2, LycBf/C2B-F2 and LycBf/C2B-R2) (Table 1).

Table 1
Primers used for analysis of the LycBf/C2A and LycBf/C2B genes from *Pseudosciaena crocea*.

Selected gene	Nucleotide sequence of primer (5′ → 3′)	Purpose
LycBf/C2A-F1	CAGACTGGTGAAAAAGAGGT	LycBf/C2 cDNA cloning by RACE
LycBf/C2A-R1	TGAAGCAGTGAGCAGCAGTC	
LycBf/C2B-F1	GTCTTGACTGCTGCTCACTG	
LycBf/C2B-R1	CATAGTCGTAGAACTCCTTCAC	
LycBf/C2A-F2	ATGGTCTTTTATTTTTCAGGAGG	RACE confirmation
LycBf/C2A-R2	TTAGTTTTGAAAAAACTCCATTG	
LycBf/C2B-F2	ATGAGTTCGGCTGAAGTAATTTT	
LycBf/C2B-R2	TTAAATGATATCAGGCAGGAACT	
LycBf/C2A-F3	CAGATGGTGCTTATAATATGGGC	LycBf/C2 expression analysis by
LycBf/C2A-R3	GCACCCCATGGATGCATTTTCCTC	RT-PCR and real-time PCR
LycBf/C2B-F3	AGAATCCCACCAGGGGCCCAGAG	
LycBf/C2B-R3	CCACTCCACTCCCTGTTCGACATG	
β-Actin-F	GACCTGACAGACTACCTCATG	β-Actin expression
β-Actin-R	AGTTGAAGGTGGTCTCGTGGA	

2.3. DNA sequence analysis

DNA sequences could be analyzed for similarity with other known sequences using the BLAST program [23]. Multiple sequence alignment was conducted using the ClustalW 1.8 program (http://searchlauncher.bcm.tmc.edu). Phylogenetic tree was constructed based on the deduced amino acid sequences using the neighbor-joining (NJ) program of MEGA version 4.0 [24]. Protein structure predictions were performed using software at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). The putative ORFs were analyzed for the presence of N-linked glycosylation sites with the NetNGlyc 1.0 Server [25].

2.4. 3D Modelling of LycBf/C2A and LycBf/C2B

Multiple sequence alignments were carried out with the program Homology (InsightII-2005 version) basing on the following template human complement factor sequences: human complement Bf (PDB code: 2OK5 [26]), Bb segment of human Bf (PDB code: 1RRK [27]), and two human C2a fragment (PDB code: 2I6Q [28] and 2ODP [29]). The alignment was obtained using the PAM120 matrix and automatic multiple alignment parameters. The atomic coordinates of all the above templates were from the Brookhaven Protein Data Bank.

Three-dimensional models of the LycBf/C2A and LycBf/C2B were built according to the following procedure: (a) basing on both sequence and structure alignments, homology identifies 18 "blocks" which are likely to contain structurally conserved regions (SCR); (b) the atomic coordinates of the backbone atoms inside the SCR were transferred from the reference X-ray structure to the model; (c) fragments connecting the scaffold elements (usually loops) were generated *de novo* from initially random loop conformation using constrained minimization algorithm that adjusts the conformation to make the flanking residues fit the flanking conserved residues in the model. Initial models of LycBf/C2A and LycBf/C2B were then optimized by an "up-grading" method [30], the consecutive steps were as below:

(1) a 95 Å × 85 Å × 75 Å periodic bounded water box was added to the protein
(2) the positions of the water were optimized by using a consecutive series of short (1.5 ps) MD runs and energy minimizations
(3) heavy atoms of the enzyme were kept fixed, and the whole system was minimized
(4) backbone atoms of the enzyme were constrained, and the whole system was minimized
(5) α-carbons of the enzyme were constrained, and the whole system was minimized
(6) whole system was minimized without any constraints

The optimized structures were subjected to the program PROCHECK [31], ERRAT [32], and VERIFY-3D [33] in order to confirm its geometrical reliability. The program PROCHECK, ERRAT, and VERIFY-3D could be executed on-line (http://nihserver.mbi.ucla.edu/SAVS/).

2.5. Tissue expression profile analysis of LycBf/C2A and LycBf/C2B

Various tissues, including spleen, kidney, liver, gills, heart, brain, skin, and muscle, were collected from three normal fish. Total RNA was isolated using RNAprep pure Tissue Kit (Tiagen, China) followed by the treatment with DNase I (Promega, USA) and converted to cDNA using M-MuLV reverse transcriptase (Toyobo, Japan) according to the manufacturer's instructions. The primers of LycBf/C2A-F3 and LycBf/C2A-R3 were used for amplifying LycBf/

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