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Short communication

Cloning and expression analysis of c-type lysozyme gene in golden pompano, *Trachinotus ovatus*

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ABSTRACT

It is well known that lysozymes are key proteins to teleosts in the innate immune system and possess high bactericidal properties. In the present study, a c-type lysozyme gene (To-lysC) was cloned from golden pompano, *Trachinotus ovatus*. The To-lysC cDNA is composed of 743 bp with a 36 bp of 5′-UTR, 432 bp open reading frame (ORF) and 275 bp 3′-UTR, encoding a polypeptide of 144 amino acids (GenBank accession no: KT935522). Phylogenetic analysis revealed that To-lysC showed highest similarity to *Perca flavescens* lysC. Quantitative real-time PCR (qRT-PCR) analysis showed that To-lysC had relatively high expression level in the head kidney, gill and brain. After *Vibrio harveyi* infection, transcripts of To-lysC increased and reached its peak at 12 h p.i. These results indicated that To-lysC may play an important role in innate immune response to bacteria.

1. Introduction

Lysozyme (EC 3.2.1.17) is considered to be an integral component of innate immune system protecting against microbial infections, which is widely distributed in eukaryotes and prokaryotes [1]. The mechanism of lysozyme to kill bacteria is through hydrolyzing β-1, 4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer in the bacterial cell wall [2,3]. Lysozymes are classified into six types: chicken-type lysozyme (c-type) [4], goose-type lysozyme (g-type) [5], invertebrate lysozyme (i-type) [6], plant lysozyme [7], bacteria lysozyme [8] and T4 phage lysozyme (phage-type) [9]. The c-type lysozyme has been found in many organisms, such as viruses, bacteria, plants, insects, fishes, reptiles, birds and mammals [10].

In fish, lysozyme has been classified as an important component of the immune system [3,11,12]. Lysozymes have been characterized in a number of fish species such as Japanese flounder (*Paralichthys olivaceus*) [13], Turbot (*Scophthalmus maximus*) [14], brill (*Scophthalmus rhombus*) [15], grass carp (*Ctenopharyngodon idellus*) [16], orange-spotted grouper (*Epinephelus coioides*) [3], kelp grouper (*Epinephelus bruneus*) [17] and red-spotted grouper (*Epinephelus akaara*) [12]. Fish lysozymes are reported to defend against bacteria [11,12,14] and viruses [3]. Besides their antimicrobial activity, lysozymes have many other functions, including immune modulatory, anti-inflammatory and antitumor activities [18–20].

Golden pompano, *Trachinotus ovatus* is widely cultured in China, Japan, Australia and other countries [21] because of its excellent seafood quality and its high market value [22–26]. As intensive aquaculture expanded and culture density increased, diseases occurred more frequently, which led to considerable economic losses [27,28]. However, the information about the innate immune system of golden pompano is largely unknown. In this study, a lysC gene (To-lysC) was cloned from golden pompano, *Trachinotus ovatus*, and its tissue distribution and expression profile in response to *Vibrio harveyi* infection were investigated. The present results contribute to better understanding of innate immune response to bacteria in golden pompano.

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2. Materials methods

2.1. Fish

Golden pompano (weight 46.3 ± 0.6 g) were obtained from Shenzhen Experimental Station of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. The fish were maintained in aerated sea water tank at 28 °C and fed for 1 week prior to experimental manipulation. The fish were anesthetized with diluted eugenol (1: 10000; Shanghai Reagent Corp., China) before killing. The muscle, spleen, head kidney, gill, liver, intestine, heart and brain were sampled from the killed fish and immediately frozen by liquid nitrogen, followed by storage at −80 °C until used.

2.2. Amplification of T. ovatus c-type lysozyme (To-lysC) cDNA

Total RNA was extracted from the head kidney of golden pompano using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, and the quality of total RNA was detected by electrophoresis on 1% agarose gel. In order to obtain the full length cDNA, the first-strand cDNA was synthesized from total head kidney RNA with the SMART™ RACE cDNA amplification kit (Clontech, USA) following the manufacturer's protocol for 5′/3′ RACE. All the primers (Table 1) were designed based on the conserve sequences obtained from Large yellow croaker (KKF29954.1), starry flounder (BAL44624.1), yellow perch (ACO34809.1) and so on. In detail, the gene specific primer CLys5′NGSP1,CLys3′GSP1 (Table 1) and UPM (supplied by the kit) were used for the first round PCR. The PCR was conducted at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 50 s. The product of the first round PCR was diluted 10 times and then used as the template for the nested PCR. The nested PCR was performed with the gene specific primers CLys5′NGSP2, CLys3′GSP2 (Table 1) and NUP (supplied by the kit). The PCR conditions were 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 50 s with a final elongation step of 10 min at 72 °C.

The 5′ and 3′RACE PCR products were analyzed on 1% agarosegels electrophoresis and purified by AxyPrep™ DNA gel extractionkit (Axygen, USA). The purified products were cloned into the pMD-18T vector (Takara, Japan) and sequenced, respectively. Assembly ofthe full-length gene was done with DNAMAN 5.2 software.

2.3. Bioinformatics analysis of To-lysC

The nucleotide and predicted amino acid sequences of To-lysC were analyzed using Genetyx7.0 software. The similarity of To-lysC with other To-lysC was analyzed using the BLASTp search program at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple-sequence alignment of the reported To-lysC amino acid sequences was performed using ClustalX2.0 and a phylogenetic tree was constructed using the MEGA 5.0 software.

Table 1
Sequences of primers used in this study.

Primers	Sequence (5′-3′)
NUP	AAGCAGTGGTATCAACGCAGAGT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
CLys3′GSP1	GGAGTCTGGTGTTTCTGCTCTTTGTG
CLys3′GSP2	TCTGGTGTTTCTGCTCTTTGTGGCTG
CLys5′NGSP1	CCATTTGAAGTGGGCGTTTGTCCGTT
CLys5′NGSP2	GTTGCCACGATAACCATCCATCCCAT
RT-Actin-F	CCATTTGAAGTGGGCGTTTGTCCGTT
RT-Actin-R	GTTGCCACGATAACCATCCATCCCAT
RT-CLys-F	GGAGTCTGGTGTTTCTGCTCTTTG
RT-CLys-R	GGTGGCTCTAGTGTTGTAGTTCG

Table 2
GenBank accession numbers of lysC used in this study.

Protein	Accession no.
Trachinotus ovatus	KT935522
Larimichthys crocea	KKF29954.1
Platichthys stellatus	BAL44624.1
Perca flavescens	ACO34809.1
Solea senegalensis	ABC49680.1
Oplegnathus fasciatus	ADZ44620.1
Scophthalmus rhombus	BAF75844.1
Epinephelus coioides	AFK78901.1
Maylandia zebra	XP_012777516.1
Homo sapiens	AAA36188.1
Gorilla gorilla	NP_001266591.1

2.4. V. harveyi challenge of the fish

V. harveyi used for immune stimulus was obtained from South China Sea Fisheries Research Institute (Guangzhou, China) was activated twice following the methods described by Shen et al. [29]. The challenge experiment was performed as follows: each fish of the experimental group was injected intraperitoneally (i.p.) with 200 μl live *V. harveyi* which was resuspended in PBS (2×10^7 CFU/ml) and the control group was injected with 200 μl PBS. Head kidney of six fish in each group was collected at 0, 6, 12, 24, 48 and 72 h post-injection for further analysis.

2.5. Quantitative analysis of To-lysCmRNA expression profiles

For the tissue distribution analysis, total RNA was extracted from the muscle, spleen, head kidney, gill, liver, intestine, heart and brain using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was also extracted from fish head kidney at different time points after challenged with *V. harveyi*. Each sample contained 3 independent individuals respectively to eliminate the individual differences. The first-strand cDNA was synthesized using the PrimeScript Reverse Transcriptase (Takara, Japan) following DNaseI (NEB, USA) treatment. The To-lysCspecific primers used for qRT-PCR were listed in Table 1. Amplification of β-actin mRNA was used as internal controls. Primers used for β-actin amplification were listed in Table 1.

The qRT-PCR assay was carried out through an IQ5 Real-time PCR System (Bio-Rad Laboratories). Dissociation curve analysis of amplification products was performed at the end of each PCR reaction. The amplification was performed in a 25 μl reaction volume containing 12.5 μl of 2 × SYBR Premix Ex Taq (Takara, Japan), 1 μl sense primer and 1 μl antisense primer (10 μm), 2 μl of 1:5 diluted cDNA and 8.5 μl of PCR-grade water. The thermal profile for qRT-PCR was 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s and 55 °C for 30 s. After the PCR program, qRT-PCR data were analyzed with the IQ5 software. The C_t values of the target gene (To-lysC) and a chosen reference gene (β-actin) were obtained from each sample. The standard equation and correlation coefficient were determined by constructing a standard curve using a serial dilution of cDNA; To-lysC: $Y = -0.316x + 10.98$, $R^2 = 0.996$; β-actin: $Y = -0.309x + 9.786$, $R^2 = 0.991$; Y is the logarithm of the starting template to base 10 and x is the C_t values. The baseline was set automatically by the software. PCR efficiency was calculated according to the protocol by Schmittgen and Livak (2008) [30]. The relative expression level of To-lysC was analyzed by $2^{-\triangle\triangle Ct}$ method [31].