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Identification and expression analysis of three c-type lysozymes in *Oreochromis aureus*

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ABSTRACT

Lysozyme is an important molecule of innate immune system for the defense against bacterial infections. Three genes encoding chicken-type (c-type) lysozymes, C1-, C2-, C3-type, were obtained from tilapia *Oreochromis aureus* by RT-PCR and the RACE method. Catalytic and other conserved structure residues required for functionality were identified. The amino acid sequence identities between C1- and C2-type, C1- and C3-type, C2- and C3-type were 67.8%, 65.7% and 63.9%, respectively. Phylogenetic tree analyze indicated the three genes were firstly grouped to those of higher teleosteans, Pleuronectiformes and Tetraodontiformes fishes, and then clustered to those of lower teleosteans, Cypriniformes fishes. Bioinformatic analysis of mature peptide showed that the three genes possess typical sequence characteristics, secondary and tertiary structure of c-type lysozymes. The three tilapia c-type lysozymes mRNAs were mainly expressed in liver and muscle, and C1-type lysozyme also highly expressed in intestine. C1-type lysozyme mRNA was weakly expressed in stomach, C2- and C3-type mRNAs were weakly expressed in intestine. After bacterial challenge, up-regulation was obvious in kidney and spleen for C1-type lysozyme mRNA, while for C2- and C3-type lysozyme obvious increase were observed in stomach and liver, suggesting that C1-type lysozyme may mainly play roles in defense, while C2- and C3-type lysozyme mainly conduct digestive function against bacteria infection. All the three c-type recombinant lysozymes displayed lytic activity against Gram-negative and Gram-positive bacteria. These results indicated that three c-type lysozymes play important roles in the defense of *O. aureus* against bacteria infections.

1. Introduction

Lysozyme (GenBank ID: EC3.2.1.1.17) is one of the most important non-specific immune factors in living organisms. The biological function of the lysozyme is exerted by hydrolyzing β-1, 4 linked glycosidic bond of the peptidoglycan on the bacterial cell wall [1]. Six types of lysozymes including chicken-type lysozyme (c-type), goose-type lysozyme (g-type), invertebrate-type lysozyme (i-type), T4 phage lysozyme, bacterial lysozyme and plant-type lysozyme have been reported [2].

C-type lysozyme is most abundant, being distributed in many of vertebrates from fish species to mammals and also found in invertebrates [3]. C-type lysozyme were isolated from fishes, including rainbow trout (*Oncorhynchus mykiss*), Japanese flounder (*Paralichthys olivaceus*), gibel carp (*Carassius auratu*), grass carp (*Ctenopharyngodon idellus*) [3–6], and also found in prawns and shrimps, including tiger shrimp (*Penaeus monodon*), green tiger prawn (*Penaeus semisulcatus*) and kuruma shrimp (*Marsupenaeus japonicus*) [7–9]; G-type and i-type lysozymes had been reported in some aquatic species, including g-type lysozyme in Japanese flounder, orange-spotted grouper (*Epinephelus coioides*), Atlantic salmon (*Salmo salar*) and bay scallop (*Argopecten irradians*) [10–13], and i-type lysozyme in sea cucumber (*Stichopus japonicus*) [14]. C-type lysozyme hasn't been reported in fish species of Perciformes up to date. In this study, three c-type lysozymes were firstly found in *Oreochromis aureus*.

Several studies showed that expression of c-type lysozyme gene was significantly induced after bacterial challenge, suggesting that c-type lysozymes might play a role in the defense of fish. For

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example, when Japanese flounders were experimentally infected with *Edwardsiella tarda*, the mRNA level of c-type lysozyme was increased in the head kidney, spleen and ovary [15]. A clear up-regulation mostly in hematopoietic organs (spleen, head kidney, liver) of Senegalese sole (*Solea senegalensis*) was observed after stimulation with LPS or infection with *Photobacterium damselae* [16]. Expression level of c-type lysozyme was up-regulated in liver, head kidney, spleen and liver of grass carp (*C. idellus*) challenged with *Aeromonas hydrophila* [6]. But no significant change was observed in brill after injection of LPS or bacteria [17]. Whether c-type lysozymes certainly have defense function remains unknown, and more research are needed.

Tilapia is one of the most important freshwater species in aquaculture in the world. However, recently culture of tilapia has been severely threatened by bacteria infection. Better understanding of the mechanism of the innate immune response in tilapia will facilitate the development of effective approaches for disease prevention. In the present study, we isolated three c-type lysozyme cDNAs from tilapia (*O. aureus*) and investigated the expression patterns in normal individuals as well as in the individuals challenged with *A. hydrophila*. Lytic activities of three recombinant lysozymes produced from a prokaryotic system were also determined.

2. Materials and methods

2.1. Fish, bacteria, and infections

Tilapia (*O. aureus*) with body weight of 60–80 g and body length of 16–20 cm were collected from Gao Yao Aquaculture Base of Pearl River Fisheries Research Institute (Guangzhou, China). A total of 40 individuals acclimated for 7 days were divided into two groups, treated group ($n = 20$) and control group ($n = 20$). The treated group was intraperitoneally injected with *A. hydrophila* (5×10^5 CFU for each individual), while the control group was intraperitoneally injected with the same volume of saline.

Escherichia coli, Micrococcus lysodeikticus, Streptococcus agalactiae, A. hydrophila, Pseudomonas aeruginosa, *Vibrio cholerae*, *Vibrio vulnificus, Bacillus subtilis* were kindly provided by the Laboratory of Fish Disease Prevention and Treatment in Pearl River Fisheries Research Institute.

2.2. RNA extraction and reverse transcription

Total RNA was isolated from 30 mg of tilapia tissues using Trizol Reagent (Invitrogen, Carlsbard, CA) according to the manufacturer's instructions. RNA samples were treated with DNaseI (Promega, Madison, WI) to eliminate the contamination of genomic DNA. Reverse transcription of the total RNA was performed using RNA PCR Kit (AMV) Ver.2.1 (TaKaRa, Dalian, China) to create RACE-ready first-strand cDNA for cDNA cloning. The reaction system contained 2.75 μl H_2O, 2 μl $MgCl_2$, 1 μl 10 × RT Buffer, 1 μl dNTP, 0.5 μl Oligo dT-Adapter primer, 0.25 μl RNase Inhibitor, 0.5 μl AMV Reverse Transcriptase and 2 μl total RNA (1 μg) in a final volume of 10 μl. The reaction condition was 42 °C for 30 min, 99 °C for 5 min and 4 °C for 5 min. For gene expression analysis, total RNA from each sample was reverse-transcribed using PrimeScript II 1st-strand cDNA Synthesis Kit (TaKaRa).

2.3. Molecular cloning of three c-type lysozymes

Primers were designed according to the conserved sequences of c-type lysozymes from *Takifugu rubripes* (GenBank ID: AB126243), *S. senegalensis* (GenBank ID: DQ293993), *P. olivaceus* (GenBank ID: AB050469). Primer sequences are shown in Table 1. To clone the 3′ end of C1-type lysozyme, 3′ RACE PCR was applied using forward

Table 1
Primers used for lysozyme cloning, real-time PCR and engineering expression.

Primer	Sequence (5′–3′)	Primer usage
M13 Primer M4 (Rev)	GTTTTCCCAGTCACGAC	3′RACE PCR
LyoCSP3 (Fwd)	GACTATGGCATCTTTCAGATCAAT	3′RACE for C1-type; RT-PCR of C2- and C3-type lysozyme
LyoCSR3 (Rev)	ACCACATCCTGCCANGTAGG	RT-PCR for C2-type lysozyme
LyoCSR4 (Rev)	CCAGGCWACCCAGGCVCVGA	RT-PCR for C3-type lysozyme
UpM (Fwd)	CTAATACGACTCACTATAGGGGCAAGCAGT GGTATCAACGCAGAGT	5′RACE PCR
LyoC-1R1 (Rev)	ACAGAAACAGAAGAAGACGGGGACATC	5′RACE PCR for C1-type lysozyme
LyoC-2R1 (Rev)	CTACGCCATCCATACCAGGCTGCG	5′RACE PCR for C2-type lysozyme
LyoC-3R1 (Rev)	CAACCCAGGCTGTGATGCCTTGTTC	5′RACE PCR for C3-type lysozyme
M13Primer M4	GTTTTCCCAGTCACGAC	3′RACE
LyoC-2P6	CAAATCAATTGCAAGGACC	3′RACE PCR for C2-type lysozyme
LyoC-3P4	CAATGACCGTCGCATTAATTCAG	3′RACE PCR for C3-type lysozyme
LyoC-1F	ACGCGGGGACATTCGGACAAGGAAAAAT	RT-PCR
lyoC-1 R	CAGAAACAGAAGAAGACGGGGACATC	RT-PCR
LyoC-2F	ACGCGGGGACAGTTACAA	RT-PCR
lyoC-2 R	TATGGAAACAGTTTGAATG	RT-PCR
LyoC-3F	AGCAGCAACTGTGAAACAGCAC	RT-PCR
LyoC-3R	GTAGAAACAGAGGAGGATGGAG	RT-PCR
C1-sf2	ACATCATCAACTCGCATAACG	Real-time PCR
C1-sr2	TAGAACGCCAGCCATACCAGG	Real-time PCR
C2-sf2	AGACCCACCACAACAACGATG	Real-time PCR
C2-sr2	CGACCTTGACAGTGACTACGC	Real-time PCR
C3-sf2	CAGATAAATAGCCGCTGGTGG	Real-time PCR
C3-sr2	GCCTTGTTCCCTAACGATACG	Real-time PCR
β-actin-R1	TGTGGTGTGTGGTTGTTTTG	Real-time PCR
β-actin-F1	CAGCAAGCAGGAGTACGATGAG	Real-time PCR
LyoC1ORF3	TCGTGA**GCATGC**AAAATCTTCGAGCG	Engineering expression
LyoC1ORF4	CCAGGT**AAGCTT**TTAAACACCACATCCA	Engineering expression
LyoC2ORF1	GTAATA**GCATGC**AAACGCTTCCAGCGCT	Engineering expression
LyoC2ORF2	GGTGAT**AAGCTT**TTATGCCGATGACACC	Engineering expression
LyoC3ORF7	CGG**GGTACC**AAAGTCTTTGAGCGCTGTG	Engineering expression
LyoC3ORF8	TGACCC**AAGCTT**TTAAAGACGACATCCGG	Engineering expression