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Molecular characterization and expressing analysis of the c-type and g-type lysozymes in Qihe crucian carp *Carassius auratus*

Meijuan Wang [a, b], Xianliang Zhao [a], Xianghui Kong [a, *], Li Wang [a, b], Dan Jiao [b], Hongxu Zhang [b]

[a] College of Fisheries, Henan Normal University, Henan Province, PR China
[b] College of Life Science, Henan Normal University, Henan Province, PR China

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ABSTRACT

Lysozyme as an important nonspecific immune factor, can kill bacteria by hydrolyzing β-1,4-glycosidic linkages of peptidoglycan layer, and plays an important role in innate immune response against pathogen infection. In the present study, we report molecular cloning, tissue distribution and functional characterization of the c-type and g-type lysozymes in Qihe crucian carp *Carassius auratus* (designated as *Ca-clys* and *Ca-glys*, respectively). The full-length of *Ca-clys* and *Ca-glys* cDNA were cloned using RT-PCR and RACE methods. Catalytic and other conserved residues, required for functionality, were identified by multiple sequence alignment and structure predicted. The findings indicating the Ca-clys with signal peptide sequence, while the Ca-glys without, imply that the two isozymes function in different sites of cell. Phylogenetic analysis revealed that *Ca-clys* and *Ca-glys* genes evolve at different rates. Moreover, spatial expression analysis showed that *Ca-clys* transcript was most abundant in kidney and least in gill. However, the expression level of *Ca-glys* was significantly lower compared with *Ca-clys* in various tissues, which was the most abundant in spleen and least in brain. After intraperitoneal injection with *A. hydrophila* and lipopolysaccharide (LPS), the mRNA levels of *Ca-clys* and *Ca-glys* were generally up-regulated in liver and gill, but indicated the different expression changes in spleen, kidney and head kidney. With regard to the lysozyme activity, it was showed that the total enzyme activities generally increased in liver, gill, spleen, and head kidney after stimulation. These results confirmed that both *Ca-clys* and *Ca-glys* play an important role in non-specific immunity after *A. hydrophila* invasion. In this study, it was speculated that expressions of Ca-clys and Ca-glys were regulated in different patterns against pathogens.

1. Introduction

Lysozyme, as an important immune factor in the innate immune system, can catalyze the hydrolysis of β-(1,4)-glycosidic between the N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of bacterial cell wall, causing bacterial cell lysis and protecting organisms against the invasion of bacterial pathogens [1]. Based on their structural, catalytic, and immunological difference, lysozymes can be categorized into six types: chicken-type (c-type), goose-type (g-type), invertebrate-type (i-type), plant-type, bacterial-type and T4 phage-type [2–4]. The c-type and g-type of lysozymes exist in the fish and other vertebrate [2]. It has been demonstrated that lysozyme functions mainly in innate immunity against the invasion of bacterial pathogen [5,6]. Apart from their antimicrobial activity, lysozymes have also been reported to perform many other functions, such as digestion [7], complement together [8], antiviral [9], antitumor [10] and anti-inflammatory [11]. Therefore, lysozyme plays an important role in innate immunity and physiological activities.

The c-type lysozyme in fish was first reported in *rainbow trout* [12], and then founded in *Scophthalmus rhombus* [13], *Solea senegalensis* [14], *Epinephelus coioides* [15] and so on. Generally, the c-type lysozyme contains two conserved catalytic sites (Glu and Asp), conserved cysteine residues, forming disulfide bonds, and a N-terminal signal peptide, indicating to be a secret protein. In natural, most organisms only have one c-type lysozyme type. However, during the evolution, various c-type lysozymes had differentiated

* Corresponding author. No. 46, Jianshe Road, College of Fisheries, Henan Normal University, Xinxiang 453007, PR China. Tel.: +86 0373 3328928.
E-mail address: xhkong@htu.cn (X. Kong).

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after gene replication and chromosome doubling in some organisms. There were three kinds of c-type lysozymes in *Oreochromis aureus*, and their three-dimensional structures were similar, but the expression and function were different [16]. In rainbow trout, two kinds of c-type lysozymes (I and II type) were reported, with only one amino acid difference between them, but indicated the significant difference in the biological functions [12]. The two c-type lysozymes exist in the abalone (abLysC1 and abLysC2) with the homology of 81.5% [17].

The g-type lysozyme was discovered in various species of fish, such as *Oplegnathus fasciatus* [18], *Scophthalmus maximus* [19], *Epinephelus coioides* [20], and *Dicentrarchus labrax* [21], the molecule of which, without the signal peptide, was usually bigger than that of c-type [2]. It possessed three conserved catalytic sites (Glu, Asp, and Asp), lacked the disulfide bond and N-terminal signal peptide, which was supposed to be an intracellular protein. In *Gadus morhua*, there were two kinds of g-type lysozymes (codg1 and codg2), indicating the different transcription initiation sites [22]. In Ictalurus punctatus, three kinds of g-type lysozymes (Lyg, LygI1 and LygI2) were found, with the homology of 71%–83% among them, and indicated the different expression levels in various tissues. Therefore, it was predicted that the cooperation among them could enhance the immune ability [23]. Currently, in most fish, such as *Scophthalmus rhombus* [13], *Ictalurus punctatus* [23], and *Ctenopharyngodon idellus* [24], the lysozyme was found to exist in two forms: c-type and g-type.

Qihe crucian carp *Carassius auratus*, as the important commercial fish, are widely cultured in the northern region of Henan province. In recent years, the intensive aquaculture with the high density always results in the decrease of immune level and evenly incurs the occurrence of fish disease. Up to now, the immune defense is thought as one of the most efficient ways in preventing from the disease. The lysozyme, as an important innate immunity factor, was generally founded in skin mucus of fish. However, molecular structure and expressing analysis of the c-type and g-type lysozymes have been scarcely studied in freshwater fish. In this study, the c-type and g-type lysozymes in *C. auratus* (designated as *Ca-clys* and *Ca-glys*, respectively) were cloned by reverse transcript PCR (RT-PCR) and the rapid amplification of cDNA ends (RACE) methods, the expression changes was determined using the quantitative real time - PCR (qRT-PCR) method, and the lysozyme activity was measured using the turbidimetey method. In this study, the aim is to find the differences by the comparison of gene sequence and molecular structure between *Ca-clys* and *Ca-glys*, as well as the expression profile after stimulation with *A. hydrophila* and LPS respectively. Based on the expression responses of *Ca-clys* and *Ca-glys to pathogen*, the new insights were shed on the prevention from pathogen infection.

2. Material and methods

2.1. Fish and bacteria

Qihe crucian carp *Carassius auratus*, with the body weight of 50 ± 3 g, were obtained from the breeding farm in Hebi city Henan province, which were acclimated in aerated water at 23 ± 1.5 °C for two weeks before experiments.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from the liver of *C. auratus* using RNAiso Plus (Takara, Dalian, China) according to the instruction of manufacture. The quality of RNA was evaluated by 1.5% agarose gel electrophoresis. The first-strand cDNA was synthesized using the liver total RNA with the HiFi-MMLV cDNA Kit (Takara, Dalian, China). The reaction system contains 2 μL total RNA, 4 μL dNTP Mix, 2 μL Primer Mix, 4 μL 5 × RT Buffer, 2 μL DTT, 1 μL HiFi-MMLV, and 5 μL RNase-Free Water in a final volume of 20 μL. The reaction condition was at 42 °C for 45 min and 85 °C for 5 min.

2.3. Full-length cDNA cloning and sequencing of Ca-clys and Ca-glys

The primers to amplify the intermediate fragment were designed according to the conserved sequences of c-type lysozymes from *Cyprinus carp* (Gene Bank ID: AB027305), *Ctenopharyngodon idellus* (Gene Bank ID: EU835654), *Danio rerio* (Gene Bank ID: BC114260), and *C. auratus* (Gene Bank ID: KJ703111), and g-type lysozyme from *C. carp* (Gene Bank ID: AB084624), *C. idellus* (Gene Bank ID: EU835653), and *D. rerio* (Gene Bank ID: NM_001002706). The forward and reverse primers for c-type lysozyme were named as the primer CF and CR respectively, and for the g-type lysozyme were the primer GF and GR (Table 1). The amplification was performed by the 2 × Taq Master Mix with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, a final extension for 3 min at 72 °C. The products were analyzed on 1.5% agarose electrophoresis and purified by DNA gel extraction kit (Takara, Dalian, China). The purified DNA was cloned into the pMD-19T vector (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5a for sequencing.

The 3′ and 5′ RACE primers were designed based on the intermediate sequence. The 3′ RACE primers, combining with the forward gene-specific primer C3′ F, G3′ F and the 3′ RACE kit reverse primer 3′ Oligo (T) (Table 1), were used to clone the 3′ end of c-type lysozyme (*Ca-clys*) and g-type lysozyme (*Ca-glys*), respectively. The PCR reaction was performed at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 3 min. The PCR product was used as the template for the nested PCR. The *Ca-clys* and *Ca-glys* nested PCR were performed respectively with the gene-specific primers C3′ NF, G3′ NF and 3′ Olig (supplied by the kit). The PCR reacting conditions were same as the first round PCR. The expected DNA fragment was purified and cloned into pMD-19T vector, which was transferred into the bacteria DH5α, being cultured for sequencing. The 5′ RACE for *Ca-clys* and *Ca-glys* were performed using the designed 5′ RACE primers, and the gene-specific primer C5′ R, G5′ R and the 5′ RACE

Table 1
The primers used in this study.

Primer	Sequence (5′–3′)
CF	CGCTKTGATGTTGTYCGTAT
CR	CCAGGTRTCCCATGMTTTCA
GF	AAACTRGCTGAGCATGATCTGG
GR	TGGGCTGKGGCMACAACATC
C3′ F	GGGACTTGATGGCTTTGAGGGATTC
C3′ NF	CTCATTGTGAAAACCGAAGGACTG
G3′ F	GAAATGTCCTGAATAATGGATGGTC
G3′ NF	CAGGACCACTGGAAAAGACTACTC
3′ Oligo(T)	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTT
3′ Olig	CTGATCTAGAGGTACCGGATCC
C5′ R	GATTTCAGTCCTTCGGTTTTCACAA
C5′ NR	CCTGTGGGTCTTATACTTACTCTCC
G5′ R	CTGGCGACAACACCATTGGAGTAGT
G5′ NR	CTCTGGTCTCTCTGGATATGATGGC
5′ Oligo(T)-Adaptor	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT
5′ -Adaptor	GACTCGAGTCGACATCG
qPCR-CF	GAGGGATTCTCACTTGGCAACTAT
qPCR-CR	TTCAGTCCTTCGGTTTTCACAATG
qPCR-GF	TGTGGTCCCAAGAGCAATG
qPCR-GR	TGGCAACAACATCATTGGAGTAGTC
β-actin F	TCACACCTTCTACAACGAGCTGCG
β-actin R	GAAGCTGTAGCTCTCTCGGTCAG