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Molecular cloning, expression and antibacterial activity of goose-type lysozyme gene in *Microptenus salmoides*

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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 g-type lysozyme gene was cloned from *Microptenus salmoides*. The g-type sequence consisted of 582 bp, which translated into a 193 amino acid (AA) protein (GenBank accession no: MH087462). The predicted molecular weight and theoretical isoelectric point were 21.36 kDa and 6.91 respectively and no signal peptide was observed. The qRT-PCR analysis showed that the g-type lysozyme gene was differentially expressed in various tissues under normal conditions and the highest g-type lysozyme level was observed in liver, gill and spleen while there seemed to be low expression in the muscle, heart and head-kidney. The expression of g-type lysozyme was differentially upregulated in the spleen, gill and intestine after stimulation with heat stress and *Aeromonas hydrophila* (*A. hydrophila*). Under heat stress and *A. hydrophila* injection, the g-type lysozyme mRNA levels all in spleens, gill and intestine tissues increased significantly ($P < 0.05$), with the maximum levels attained at 12 h, 24 h (or 12 h) and 24 h. Thereafter, they all decreased significantly ($P < 0.01$) and the expression in gill returned to nearly the basal value within 72 h. Those results suggested that g-type lysozyme was involved in the immune response to heat stress and bacterial challenge. The cloning and expression analysis of the g-type lysozyme provide theoretical basis to further study the mechanism of anti-adverseness in *Microptenus salmoides*. The g-type lysozyme gene perhaps also played an important role in the immune responses against bacterial invasion.

1. Introduction

Lysozyme is an important component of the innate immune response against pathogen infection. It is widely distributed among eukaryotes and prokaryotes and protects from microbial infections. Lysozyme kills bacteria by hydrolyzing β-1,4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan layer in the bacterial cell wall [1]. It has been categorized into six types: chicken-type (c-type) lysozyme, goose-type (g-type) lysozyme, invertebrate-type (i-type) lysozyme, phage lysozyme, bacterial lysozyme and plant lysozyme, the classification were based on their differences in structural, catalytic and immunological characters [2]. Lysozymes have been characterized in a number of fish species such as red-spotted grouper, *Epinephelus akaara* [1], Turbot (*Scophthalmus maximus*) [3], orange-spotted grouper (*Epinephelus coioides*) [4], kelp grouper (*Epinephelus bruneus*) [5], grass carp (*Ctenopharyngodon idellus*) [6] and golden pompano, *Trachinotus ovatus* [7]. In addition, The g-type lysozyme has been found in many organisms such as *Epinephelus coioides* [8], *Dicentrarchus labrax* [9], *Silurus asotus* [10], *S. maximus* [11] and two bivalve scallops, bay scallop (*Argopecten irradians*) [12] and Zhikong scallop (*Chlamys farreri*) [13]. However, to date, little information is available on the regulation and genomic structure of the g-type lysozyme in *Microptenus salmoides*. In the present investigation, the molecular cloning and sequencing of lysozyme gene in *Microptenus salmoides* were reported.

The expression of lysozyme genes typically shows a distinct spatial, temporal pattern and species dependent [2]. Lysozyme transcripts have been detected in various tissues such as gills, heart, hemocytes, hepatopancreas and muscle [14,15]. In fish, lysozyme has been considered as an important component of the immune system [16]. They were reported to defend against bacteria [3,17] and viruses [18]. Besides their antimicrobial activity, lysozymes have many other functions, including anti-inflammatory, immune modulatory and antitumor activities [19–21]. The expression of g-type lysozymes were significantly upregulated in different tissues after infection with *Aeromonas hydrophila* in grass carp [6]. In addition, the recombinant proteins of g-type lysozymes purified from *Escherichia coli* show antimicrobial activity against various Gram-positive and Gram-negative bacteria in different

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Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Application
Lysozyme -F1	AACWKCTCAGCAGGACARNCT	Conserved region cloning
Lysozyme -R1	TKRTACCACTGAGCTCTGGC	Conserved region cloning
Lysozyme-GSP1	GTCACAAGCACCATCATGGGGACACAA	3′ RACE
Lysozyme-GSP1	CCCTGGATGACCCCGACCTGCTCTTTGT	3′ RACE
Lysozyme-NGSP1	ACATGTAGCACGGACCGGAGGACATTT	5′ RACE
Lysozyme-NGSP1	TCCAAGAATGGAAGTTACCAGAGCCTCATA	5′ RACE
UMP	GGATCCGAAAAAAGATTTTTGAACAACTTTG AAGCTTAACGGCTCTCATTCTTGCAA dT	Oligo (dT)-adaptor
NUP	GGCCACGCGTCGACTAGTACT17	
β-actin-F1	TGGCATCACACCTTCTACAA	β-actin
β-actin-F2	AGGATCTTCATGAGGTAGTC	β-actin
RT-Lysozyme-F1	GCAAAGACTGACGCGGGCAGAATGG	Real-time PCR
RT-Lysozyme-F2	CCCAGGCGTTATACGCTCCTCTCTT	Real-time PCR

species [17,22]. With the multiple functions, lysozymes are regarded to play important roles in the innate immunity and physiological activities. The expressions of g-type lysozymes to heat stress and bacteria were also investigated in the present study.

Microptenus salmoides is one of the largest fish species in the world. It is widely cultured in China and other countries because of its excellent quality as a food and high market value. However, as intensive aquaculture expanded and culture density increased, diseases occurred more frequently, which led to considerable economic losses. In fish, the innate immunity plays a critical role against different pathogens. Lysozymes were reported as immune effectors and participated in the host immune response. However, no researches have demonstrated the presence of lysozymes in this species. Therefore, characterization of the lysozyme is urgently needed to elucidate the immune defense mechanism of this fish, which will ultimately contribute to disease control and health management of aquaculture. Studying the structure and function of a gene is the key to know its function mechanism. So in the present study, we cloned and characterized a g-type lysozyme gene. The tissue expression and temporal expression patterns after heat stress and *Aeromonas hydrophila* infection were analyzed. The present results elucidate the functions of the g-type lysozyme, and provide important information for better understanding of innate immunity against pathogen infection in *Aeromonas hydrophila*.

2. Materials and methods

2.1. Fish

Microptenus salmoides were purchased from a bazaar in Luo yang, Henan Province, China. The initial weight of the fish is 100 ± 3 g and they were acclimatized in a laboratory recirculating seawater system at 25 ± 0.2 °C, dissolved oxygen above 6.0 mg L^{-1} and pH from 7.5 to 8.7. The fish were fed twice daily for 2 weeks before experimental manipulation. Six fish were anesthetized with MS-222 (tricaine methanesulfonate, Sigma, USA) at the concentration of 100 mg L^{-1} for sampling. Then the head-kidneys, spleens, gills, intestines, livers, adipose tissues, brains, hearts, muscles and eyes were dissected, and immediately frozen under liquid nitrogen for RNA preparation. Each tissue was operated with three replicates.

2.2. Heat stress

24 fish in every tank (three tanks as one group) were subjected to heat stress. Fish were transferred from the tank at 25 ± 0.2 °C to 34 ± 0.2 °C, and adequate dissolved oxygen was provided. Three fish were randomly sampled from each tank (9 fish each group) at 0, 6, 12, 24, 48 and 72 h after stress and the untreated fish were used as the control (0 h). Individual spleens, gills and intestines were dissected on an ice bed and washed thoroughly with chilled saline (0.89 g NaCl L^{-1}), dried quickly over a piece of filter paper and stored at −80 °C for total RNA extraction.

2.3. A. hydrophila challenge

For the bacterial challenge experiment, *A. hydrophila* were obtained from Nanjing Agricultural University (Nanjing, China). The bacteria were activated twice following the methods described by Zhang et al. [23]. For the bacterial challenge experiment, 90 fish were kept in three tanks (30 individuals in each tank). Then the fish were injected with 1 mL live *A. hydrophila* in PBS (10^6 CFU/mL) and the uninfected fish were used as the control (0 h), respectively. The injected groupers were placed in separate treatment tanks. Samples sampled and stored as described above.

2.4. Cloning the full-length and sequencing of g-type lysozyme

Total RNA was extracted from liver tissues of *M. salmoides* using Trizol Reagent (Invitrogen, USA), according to the manufacturer's protocol. The quality of the total RNA was confirmed by electrophoresis on 1% agarose gel. The RNA transcribed into cDNA using the Powerscript II reverse transcriptase with CDS primers (SMART RACE cDNA Amplification kit, Clontech, USA). For the isolation of *M. salmoides* lysozyme cDNA fragment, multiple alignment of the amino acid sequences of other species g-type lysozyme were performed using the DNAMAN multiple sequence alignments program. Degenerate primers (Lysozyme-F1, Lysozyme-R1) were designed based on the highly conserved sequences of g-type lysozyme (Table 1). The PCR reaction was performed in a total volume of 20 mL using Gradient Mastercycler (Eppendorf, Germany). The PCR temperature profiles were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min and an additional extension at 72 °C for 10 min. The resulting PCR products were purified using agarose gel DNA purification kit (TaKaRa, Dalian, China) and ligated into a PMD18-T vector (TaKaRa, Japan) for DNA sequencing.

The corresponding full-length sequence of *Microptenus salmoides* was obtained by 3′- and 5′- rapid amplification of cDNA ends (RACE). The cloning strategy for the full-length *Microptenus salmoides* g-type lyz cDNA was followed our previous method [24]. The 5′-GSP (gene specific primer) and NUP (nested universal primer) pair and the 3′-GSP and UMP pair were used for amplifying the 5′ and 3′ DNA fragments, respectively. The primer of oligo (dT) used as the reverse primer (Table 1). The PCR cycle was as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and then a final elongation step at 72 °C for 10 min. The 5′ and 3′ RACE PCR products were analyzed with 1% agarose gel electrophoresis and purified with the AxyPrep™ DNA Gel Extraction Kit (Axygen, USA). The purified products were cloned into the pMD-18T vector (Takara, Japan) and sequenced. The full length gene was assembled with the DNAMAN