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Gene identification and recombinant protein of a lysozyme from freshwater mussel *Cristaria plicata*

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ABSTRACT

Lysozymes are important proteins to bivalve in the innate immune responses against bacterial infections, and provide nutrition as digestion enzymes. A new LYZ1 from the freshwater mussel *Cristaria plicata* was cloned by rapid amplification of cDNA ends (RACE) and nested PCR method. The full-length cDNA sequence of CpLYZ1 was 763 bp. The cDNA contained a 5′-terminal untranslated region (UTR) of 21 bp, a 3′- terminal UTR of 259 bp with a 29 bp poly(A) tail, a tailing signal (AATAAA) and the open reading frame of 483 bp. The CpLYZ1 cDNA encoded a polypeptide of 160 amino acids with a predicted molecular mass of 17.8 kDa, and a theoretical isoelectric point of 6.07. The comparison of the deduced amino acid sequences with LYZs from other species showed that the enzyme belonged to i-type lysozyme. The mRNA transcript of CpLYZ1 could be detected in all the examined tissues with the highest expression level in hepatopancreas. The expression levels of CpLYZ1 in hemocytes, hepatopancreas and gill significantly increased after *Aeromonas hydrophila* challenge. The expression level of CpLYZ1 in hemocytes sharply decreased from 6 h to 24 h and significantly increased at 48 h, and was the highest level in hepatopancreas at 24 h, and was the maximum level in gill at 48 h. Furthermore, the recombinant CpLYZ1 was induced to be expressed as an inclusion body form by IPTG at 37 °C for 4 h, and then was purified by using the Ni^{2+} affinity chromatography. The relative enzyme activity of the recombinant CpLYZ1 was influenced on pH and temperature. The optimal pH and temperature was 5.5 and 50 °C, respectively. Against *Escherichia coli*, *A. hydrophila*, *Staphyloccocus aureus*, *Bacillus subtilis*, *Streptococcus* sp. and *Staphylococcus epidermidis*, the recombinant CpLYZ1 had bacteriolytic activity.

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1. Introduction

Lysozyme (muramidase, EC3.2.1.17) is a ubiquitous enzyme in numerous phylogenetically diverse organisms [1]. The enzyme can cleave the glycosidic bond between two amino sugars, N-acetylmuramic acid and N-acetylglucosamine, of the peptidoglycans forming bacterial cell walls, and cause cell lysis [2,3]. The major biological role of lysozymes is host defense, the enzyme can act as antibacterial and immune-modulating agent [1,4]. At the same time, lysozyme is regarded as important digestive enzymes in some animals, especially for filter-feeding organisms [5–8].

Several types of known lysozymes have been identified, including chicken (c), goose (g), phage, bacteria, plant, and invertebrate (i) types. i-type lysozyme is a novel family ascertained solely in invertebrate. The first i-type lysozyme is purified from the starfish *Asterias rubens* [9], and recently developed by Ito et al. [10] and Nilsen et al. [7]. At present, about 20 i-type lysozymes have been found from nematoda, mollusca, arthropoda and echinodermata, respectively [11–13]. However, little is known about the molecular characterization of Lysozyme from freshwater mussels.

Cristaria plicata, which is a species of freshwater bivalve of the mollusca phylum, is widely distributed throughout eastern Asia. In China, it is often utilized in the cultured pearl industry. However, it has been suffering serious problems due to the outbreak of diseases in recent years, which causes significant economic loss to the pearl culture [14]. Therefore, it is very necessary to further research the innate immune mechanisms of the bivalves. Previously, an intracellular Cu–Zn SOD, CAT and Cp-piGST gene have been reported and described from *C. plicata* [15–17].

In this study, an i-type lysozyme gene was cloned from *C. plicata* (CpLYZ1). The expression patterns of CpLYZ1 mRNA transcript in different tissues of normal bivalve and the transcription regulation pattern of hemocytes, hepatopancreas and gill of bivalve by *Aeromonas hydrophila* and PBS challenge with quantitative real-time PCR. The CpLYZ1 cDNA sequence was subcloned into the pET30a vector and was expressed in *Escherichia coli* BL21 (DE3). The

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enzyme activity under the different temperature, pH and bacteriolytic activity against Gram-positive and Gram-negative bacteria of recombinant protein were examined.

2. Materials and methods

2.1. Experimental animal and bacteria challenge

The freshwater mussel *C. plicata*, which were 15–22 cm in shell length, were collected from Poyang Lake in Jiangxi province, China. They were cultured in freshwater tanks at room temperature with continuous oxygenation and changing the water every day for one week before processing.

To the bacterial challenge experiment, fifty bivalves were selected for expression patterns experiment. The bivalves were randomly divided into 2 groups and each group included 25 animals. The groups were injected into the adductor muscle 0.1 mL of phosphate buffer solution (PBS, pH 7.0) as control group and 0.1 mL bacterial suspension (*A. hydrophila*, 10^9 cell mL^{-1}) as challenged group. Hemocytes, gill and hepatopancreas from five animals of each experiment group and control group were collected at 0, 6, 12, 24 and 48 h post-injection and immediately stored in liquid nitrogen until used.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacture's instructions. The integrity of RNA was checked by electrophoresis on 1% agarose gel in TAE (Tris 40 mM, Acetic acid 1 mM, EDTA 40 mM) buffer. The gel was stained with ethidium bromide and RNAs were visualized under UV light. The Smart cDNA was synthesized and amplified using a Clontech SMART PCR cDNA Synthesis Kit (Clontech) by following the supplier's protocol. The synthesis reactions were performed at 65 °C for 5 min, 42 °C for 1 h, and terminated by heating at 70 °C for 15 min, the cDNA mix was stored at −80 °C for PCR and quantitative real-time PCR.

2.3. Amplification and cloning of an internal CpLYZ1 cDNA fragment

PCR amplification was done by using Smart cDNA as template, and two degenerated primers LYZ1F and LYZ1R (Table 1) were designed on the basis of the cDNA conserved regions of LYZ coding sequences from different species, *Ostrea edulis* (AB179776), *Crassostrea gigas* (AB179775). The PCR reaction volume contained H_2O 18.5 μL, 10× PCR buffer 2.5 μL, dNTPs 2.0 μL, LYZ1F 0.6 μL, LYZ1R 0.6 μL, cDNA 0.6 μL, ExTaq (Takara, Japan) 0.2 μL and the cycling parameters included a 5 min denaturation at 94 °C, 35 cycles of heat denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, polymerization at 72 °C for 1 min, and a 10 min final extension at 72 °C. The PCR product was then cloned into the PMD18-T vector (Takara) and was sequenced.

Table 1
Primers used in this present study.

Primer	Usage	Sequence (5′ → 3′)
LYZ1F	Initial PCR	GGAYGTIGGIWSIYTIWSITGYGG
LYZ1R	Initial PCR	CIYTIGGICCICCRTTRTG
LYZ1F1	3′-RACE	TATTATGAAGACTGCGGAAGCCCGGG
LYZ1F2	3′-RACE	TATGGCAAGGTACGGTCCCAATTCC
LYZ1R1	5′-RACE	GGCTTCCGCAGTCTTCATAATATGGCTC
LYZ1R2	5′-RACE	CGGAATTGGGACCGTACCTTGCCATATA
Long	RACE	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT
Short	RACE	CTAATACGAC TCACTATAGGGC
YGLYZ1F	Real-time PCR	CCAGATCAAAGAGCCATATTATG
YGLYZ1R	Real-time PCR	GTAGGTTCTTCTTCACGTCCG
YGβ-actinF	Real-time PCR	TGTGCTGTCTGGCGGTTCA
YGβ-actinR	Real-time PCR	TCCTCTCTGGTGGAGCGATG
BDLYZ1F	ORF cloning	AAGGGTACCATGGCAGTCCAAA CATTTTTCTC
BDLYZ1R	ORF cloning	GGAGAATTC CTAACATTCGAAA CTCTGAACCCTT

Y = C/T, W = A/T, R = A/G, S C/G. I: Hypoxanthine.
The restriction enzyme sites are underlined.

2.4. Rapid amplification of cDNA ends (RACE)

The 5-end and 3-end of the CpLYZ1 were cloned by using SMART-RACE and nested PCR Method. Four gene-specific primers LYZ1F1, LYZ1F2, LYZ1R1 and LYZ1R2 (Table 1) were designed based on partial sequence of CpLYZ1 fragment. For 5′-RACE, the first round reaction was performed with primers LYZ1R2 and 10× Universal Primer A Mix (UPM) under the following conditions: dH_2O 33.7 μL, 10× Ex Taq Buffer 5.0 μL, dNTP mixture (2.5 Mm each) 4.0 μL, UPM 5 μL, LYZ1R2 1 μL, cDNA 1 μL, Ex Taq polymerase (5 U/μL) (Takara, Japan) 0.3 μL, and the cycling parameters were as follows: conducted at 95 °C for 5 min followed by 5 cycles of 94 °C for 30 s and 72 °C for 2 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 90 s, 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 90 s, and then an additional extension at 72 °C for 10 min. The second round reaction was performed using the LYZ1R1 and UPM primer under the same PCR conditions. For 3′ RACE, primer LYZ1F1 and UPM in the first round PCR and LYZ1F2 and UPM in the second round were used under the same PCR conditions. All of the PCR products were cloned into the PMD18-T vector (Takara), respectively, and were sequenced again. The full-length cDNA of CpLYZ1 was obtained by combining the sequences of 5-RACE, middle fragment and 3-RACE fragments.

2.5. Sequence analysis

The CpLYZ1 cDNA sequence was analyzed using the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Protein prediction was performed using software at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). Multiple alignments were generated at the web site (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The signal peptide and cleavage site of CpLYZ1 were determined using the software SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). A phylogenetic tree was constructed based on the deduced amino acid sequences using the Neighbor-Joining (NJ) algorithm within MEGA version 4.0. Reliability of the tree was assessed by 1000 bootstrap repetitions. The amino acid sequences of selected species' LYZ for the phylogenetic tree were shown in Table 2.

2.6. Tissues distribution and temporal expression of CpLYZ1 mRNA after A. hydrophila challenge

The mRNA expression of CpLYZ1 in different tissues, including hemocytes, hepatopancreas, gill, adductor muscle and mantle of unchallenged mussels, and the temporal expression of CpLYZ1 in hemocytes, hepatopancreas and gill of mussels challenged with *A. hydrophila* were determined by quantitative real-time PCR. Total RNA extraction and cDNA synthesis were described above. The cDNAs were diluted 100 times with RNA-free water for next step. The quantitative real-time PCR was performed on an Eppendorf Mastercycler ep Realplex2 PCR. Gene-specific primers YGLYZ1F, YGLYZ1R for CpLYZ1 (Table 1) were used to amplify a product of