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An i-type lysozyme from the Asiatic hard clam *Meretrix meretrix* potentially functioning in host immunity

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ABSTRACT

Lysozymes function in animal immunity. Three types of lysozyme have been identified in animal kingdom and most lysozymes identified from bivalve molluscs belong to the invertebrate (i) type. In this research, we cloned and sequenced a new i-type lysozyme, named MmeLys, from the Asiatic hard clam *Meretrix meretrix*. MmeLys cDNA was constituted of 552 bp, with a 441 bp open reading frame encoding a 146 amino acid polypeptide. The encoded polypeptide was predicted to have a 15 amino acid signal peptide, and a 131 amino acid mature protein with a theoretical mass of 14601.44 Da and an isoelectric point (p*I*) of 7.14. MmeLys amino acid sequence bore 64% identity with the Manila clam (*Venerupis philippinarum*) i-type lysozyme and was grouped with other veneroid i-type lysozymes in a bivalve lysozyme phylogenetic tree predicted using Neighbor-Jointing method. Recombinantly expressed MmeLys showed lysozyme activity and strong antibacterial activity against Gram positive and Gram negative bacteria. MmeLys mRNA and protein were detected to be mainly produced in hepatopancreas and gill by the methods of semi-quantitative RT-PCR and western blotting. In addition, MmeLys gene expression increased following *Vibrio parahaemolyticus* challenge. Results of this research indicated that MmeLys represents a new i-type lysozyme that likely functions in *M. meretrix* immunity.

1. Introduction

Lysozymes make up a large group of proteins that distribute ubiquitously among organisms ranging from virus to human [1]. Lysozymes share an enzymatic activity to cleave the β-(1, 4) linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan in bacterial cell walls [2–4], but differ in amino acid sequence, molecular mass, and biochemical properties. Depending on the amino acid sequence, lysozymes are classified into six types (i.e., phage type, bacterial type, plant type, chicken type, goose type, and invertebrate type); among them the chicken (c) type, goose (g) type and invertebrate (i) type are present in the animal kingdom [5]. The function of animal lysozymes is generally considered to be in host immunity via bacterial cell lysis [6], non-enzymatic antimicrobial activities [7–11], and immunomodulation [12,13]. For some species, lysozymes also function in digestion after adaptive evolution [14–18].

The presence of an i-type lysozyme in animals was proposed by Jollès and Jollès [19] based on the finding of differences in N-terminal sequence between the Starfish, *Asterias rubens*, lysozyme and c-type and g-type lysozymes. This hypothesis has been confirmed later by the identification of lysozymes from bivalve molluscs [18,20–23,26–28] and annelids [21,24,25] that share a similar N-terminus or conserved sequence region with the *A. rubens* lysozyme. In addition, multiple forms of i-type lysozyme have been found in some invertebrate species [18,23,26,27,29].

I-type lysozyme is considered to play an important role in the immunity and digestion in invertebrates. I-type lysozymes have been reported to have strong antimicrobial activity against Gram positive and Gram negative bacteria [22,26,30,31]. At the same time, they are found to express in multiple tissues and organs [28], and the expression level can be induced to increase by bacterial challenges [32,33]. On the other hand, high lysozyme activity has been detected in bivalve digestive tissues [14] and the associated species have the ability to use bacteria as nutrient source [34,35], suggesting that lysozyme may function as a digestive enzyme. Findings in the studies about eastern oyster (*Crassostrea virgincia*) lysozyme have supported the digestion function of i-type lysozymes [18]. Intriguingly, invertebrates could produce different lysozymes for immunity [28] and digestion [18], and digestive i-type lysozymes be evolved from immune lysozymes via positive selection [27].

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During the annotation of an Asiatic hard clam, *Meretrix meretrix*, cDNA library [36], we identified an EST that potentially encodes a protein homologous to the Manila clam, *Venerupis philippinarum* (synonyms *Tapes japonica*), lysozyme and named it MmeLys. The objectives of this research were: (1) to clone and sequence the full-length cDNA of MmeLys, (2) to determine the lysozyme activity and antibacterial activity of the encoded protein, (3) to analyze the tissue origin of the gene expression, and (4) to observe the changes in gene expression pattern in response to bacterial challenge.

2. Material and methods

2.1. Clams, Vibrio challenge and sample collection

The Asiatic hard clams, *M. meretrix* (3.5–4.5 cm in shell length, 2–3 years old), used for the challenge were bought from a market in Qingdao, China and acclimated for 1–2 week (25 °C, 30‰ salinity and under continuous aeration) in the laboratory. Before challenge, the clams were fed with the algae *Isochrysis galbana*. The *Vibrio parahaemolyticus* strain MM21 isolated from the moribund clams and characterized to pathogenic to *M. meretrix* [37] was used to challenge the clams. The challenge experiments were done according to the procedure reported by Yue et al. [37]. Briefly, the acclimated clams were split randomly into two groups; one group was injected with 100 μl per clam of MM21 suspension at the concentration of $\sim 5 \times 10^6$ CFU ml^{-1} and the other group (control) received 100 μl per clam of phosphate buffered saline (PBS, 0.01 M, pH 7.2). Twenty four hours after challenge, tissues were sampled from hepatopancreas, gill and mantle of 4 clams per group. The dissected tissue blocks were reserved in liquid nitrogen before processing for RNA and protein extraction.

2.2. Full-length cDNA sequence determination

The full-length cDNA sequence of MmeLys was determined by 3′ and 5′ rapid-amplification of cDNA ends (RACEs) using gene-specific primers designed from the MmeLys EST sequence and adapter primers (T3 or T7) (Table 1). A plasmid DNA mixture that contained cDNAs of the entire *M. meretrix* cDNA library [36] was used as the template. The 5′ RACE was performed in a total reaction volume of 20 μl containing 14.4 μl of PCR-grade water, 2 μl of 10×PCR Buffer, 1.2 μl of $MgCl_2$ (25 mM), 0.4 μl of dNTP mix (10 μM), 0.4 μl of each of the T3 primer and MmeLysR1 primer (Table 1) (10 μM), 0.2 μl (1U) of Taq polymerase (Promega) and 1 μl of plasmid suspension. The PCR parameters were as followings: 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 40 s, followed by the final extension at 72 °C for 10 min. The same PCR procedure was followed for the 3′ RACE using the primers of T7 and MmeLysF1 (Table 1) except that an annealing temperature of 60 °C instead of 57 °C was used in the 35 cycles of amplification. PCR products were gel-purified and cloned into the pMD19-T simple vector (TaKaRa) and then sequenced at Shanghai Sangon Company (Shanghai, China). The predicted full-length MmeLys cDNA sequence was verified by PCR amplification using MmeLysF2 and MmeLysR2 (Table 1) and sequencing of the PCR product.

Table 1
Primers used in this study.

Primer	Usage	Sequence
T7	3′-RACE PCR	5′-GTAATACGACTCACTATAGGGC-3′
T3	5′-RACE PCR	5′-AATTAACCCTCACTAAAGGG-3′
MmeLysF1	3′-RACE PCR	5′-CCATACTGGACTGACTGTGGGAGA-3′
MmeLysR1	5′-RACE PCR	5′-TGGCAACCACCTGATTCTAGC-3′
MmeLysF2	Sequence verify	5′-ACCGGCATGATCAGTTTAATTG-3′
MmeLysR2	Sequence verify	5′-ATGGTATAAACATAACATTCCT-3′
LYBamHI-F	PCR for plasmid construction	5′-AGCGGATCCGCCAGCGTAGAGAAGAGAG-3′
LYSal I- R	PCR for plasmid construction	5′-CGCGTCGACTTAATGAACATTACTGCATC-3′
lys-RT-F	semi-quantitative RT-PCR	5′-AGAATCAGGTGGTTGCCATC-3′
lys-RT-R	semi-quantitative RT-PCR	5′-TCGGGCAGTGGTAGTAGGAG-3′
actin-F	semi-quantitative RT-PCR	5′-TTGTCTGGTGGTTCAACTATG-3′
actin-R	semi-quantitative RT-PCR	5′-GACTGATTTCTTACGGATG-3′

2.3. Computational sequence analysis

MmeLys amino acid sequence was deduced from the cDNA sequence using the software BioEdit. Signal peptide was predicted by both neural networks and hidden Markov models on a Signal IP 3.0 Server [38]. Isoelectric points and molecular weight of the deduced protein were determined using the "Compute pI/MW" tool on the ExPASY Server (http://www.expasy.org/tools) [39]. Sequence similarity search was conducted using BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment was performed using the ClustalX [40].

2.4. Phylogenetic analysis

Phylogenetic analysis was done with amino acid sequence using the program Mega 3.0 [41]. Amino acid sequence of MmeLys and other i-type lysozymes identified from bivalve molluscs were aligned using ClustalX and a consensus tree was then constructed by Neighbor-Jointing (NJ) method. The robustness of each topology was checked by 1000 bootstrap replications. The i-type lysozymes included in this analysis were MmeLys, 3 sequences from *V. philippinarum* (ACU83237, BAC15553, BAB33389), 2 sequences from *Calyptogena* sp. (AAN16212, AAN16211), 2 sequences from *Crassostrea gigas* (BAF48045, Q6L6Q6), 2 sequences from *Crassostrea virginica* (B3A003, P83673), 2 sequences from *Mytilus galloprovincialis* (BAF63423, AAN16210), 1 sequence from *Chlamys islandica* (CAC34834), 1 sequence from *Mytilus edulis* (AAN16207), and 1 sequence from *Ostrea edulis* (Q6L6Q5). In addition, the lysozymes of *Eisenia andrei* (ABC68610) and *Hirudo medicinalis* (AAA96144) were included as outgroup.

2.5. MmeLys recombinant production

A DNA fragment containing the predicted MmeLys coding region excluding signal peptide plus a BamH I restriction site at the 5′ end and a Sal I restriction site at 3′ end was generated by PCR using the primers of lysBamH I–F and lysSal I-R (Table 1). The PCR was done under the same conditions as that for verifying the predicted full-length MmeLys cDNA. After BamH I and Sal I cleavage, the DNA fragment was inserted into a pGEX-4T-1 plasmid (GE healthcare, containing N-terminal GST affinity tag) to construct a pGEX-MmeLys recombinant plasmid. The recombinant plasmid was then introduced into competent *E. coli* BL21 cells. The transformed *E. coli* was selected on the LB plate containing 100 mg/ml ampicillin. Plasmids were then extracted from selected cells and sequenced to confirm the in-frame insertion of the MmeLys coding sequence. After the confirmation, one colony of transformed *E. coli* was grown in 5 ml of LB broth containing 100 mg/ml ampicillin at 37 °C overnight with shaking. An aliquot of 200 μl of this culture was inoculated into 200 ml of LB broth containing 100 mg/ml ampicillin and cultured at 37 °C with shaking until the OD600 of