Fish & Shellfish Immunology 28 (2010) 490–498

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

# Molecular characterization and expression analysis of a c-type and two novel muramidase-deficient i-type lysozymes from *Penaeus monodon*

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ARTICLE INFO

*Article history:*
Received 9 September 2009
Received in revised form
22 December 2009
Accepted 3 January 2010
Available online 15 January 2010

*Keywords:*
*Penaeus monodon*
Lysozyme
Innate immunity
Antimicrobial activity
Muramidase activity

ABSTRACT

Lysozyme is a widely distributed hydrolase possessing a hydrolytic activity against peptidoglycan in the bacterial cell wall and, hence, causing lysis of the bacteria. Two types of lysozymes; the c-type (*Pm*Lyzc) and the two catalytic residue ablated i-type lysozymes (*Pm*Lyzi1 and 2), were identified from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th). By RT-PCR, *Pm*Lyzc transcript was detected in all tissues: gill, antennal gland, epipodite, heart, hemocyte, hepatopancreas, eyestalk, lymphoid organ and intestine, and highly expressed in hemocyte. The expression of *Pm*Lyzi2 mRNA was highest in heart while undetected in gill, lymphoid organ and intestine. The *Pm*Lyzi1 transcript was expressed only in hepatopancreas. The up-regulation of mRNA transcription after bacterial challenge was observed only with *Pm*Lyzc. To investigate their biological activities, the three mature recombinant proteins were expressed in an *Escherichia coli* system. Although the turbidimetric assay revealed that only recombinant *Pm*Lyzc possessed the muramidase activity, all of them variably exhibited antimicrobial activity against both Gram-positive and -negative bacteria especially the shrimp pathogens, *Vibrio* species. The antimicrobial activities of recombinant *Pm*Lyzc was the most effective one. These results demonstrated that the ability of lysozyme to inhibit the growth of bacteria did not depend only on the muramidase activity. Differences in tissue expression pattern of these gene transcripts and their antimicrobial activities indicated the multifunction of lysozyme as immune defense and digestive enzymes in *P. monodon*.



## 1. Introduction

Lacking an adaptive immune response like all invertebrates, the black tiger shrimp, *Penaeus monodon*, rely on an innate immunity to protect themselves from the surrounding pathogens. This defense system is able to eliminate the invading pathogens efficiently after infection by cellular and humoral defense mechanisms [1]. An important component of humoral mechanism is the antimicrobial peptides (AMPs). The AMPs are the first barriers of the host defense which kill or slow the growth of microbes like bacteria, fungi, viruses and protozoa [2,3]. Among the various AMPs, lysozyme is the most well-known and has been described in numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants and animals.

Lysozyme catalyzes the hydrolysis of β-1,4-glycosidic linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan in the bacterial cell walls [4]. It, thus, plays a role as a bio-defense molecule in the innate immunity against the invasion of bacterial pathogens. Lysozyme is particular important for invertebrate marine animals that constantly contact the microorganisms in the environment [5–7]. In addition to the hydrolytic activity, lysozymes from invertebrates also possess isopeptidase and chitinase activities [8,9]. The finding of lysozyme in the ruminant stomachs of cattle and the digestive organs of marine bivalves also indicates the digestive function of lysozyme [10,11].

Based on their differences in structural, catalytic and immunological characteristics, lysozymes have been traditionally categorized into three major types: chicken-type lysozyme (c-type), goose-type lysozyme (g-type) and invertebrate-type lysozyme (i-type) [12,13]. The c-type lysozyme is the most found group having been identified from several organisms including virus, bacteria, plants, insects, reptiles, avian, fish and mammals [7]. The g-type lysozyme is found in some avian, fish and scallop [8,14].

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doi:10.1016/j.fsi.2010.01.003

The i-type lysozyme is found in the invertebrate. Furthermore, the multiple-typed lysozymes have been reported as c-type plus g-type in bird and brill, and as g-type plus i-type in mollusk [15,16].

The i-type lysozyme was first described for the starfish, *Asterias rubens*, by Jolles and Jolles [17]. The partial N-terminal sequence of the protein was shown to be different from those of other types of lysozyme. Thereafter, similar lysozymes have been identified in other bivalves and categorized as a new type of lysozyme, the so-called i-type lysozyme [8,13]. In 1996, Zavalova et al. [18] identified a novel enzyme from the medicinal leech which hydrolyzed the ε-(γ-Glu)-Lys crosslink between Glu and Lys in the stabilized fibin [19] and hence named it destabilase. The destabilase from leech also possessed the isopeptidase and lytic activity like those i-type lysozymes from bivalves. It was, then, categorized as one of the i-type lysozymes [13,19].

In shrimp, the c-type lysozymes from kuruma shrimp *Marsupenaeus japonicas* [6], white shrimp *Litopenaeus vannamei* [20], black tiger shrimp *P. monodon* [21,22], banana shrimp *Fenneropenaeus merguiensis* [7] and Chinese shrimp, *Fenneropenaeus chinensis* [23] are well characterized. However, the i-type lysozyme from penaeid shrimp has not yet been characterized. Recently, the EST clones with an apparent sequence homologue of the i-type lysozyme were identified from the cDNA libraries of *P. monodon* (http://pmonodon.biotec.or.th) [24]. One of them shows identity to the destabilase of *L. vannamei*. The other one shares 39% identity to the i-type lysozyme from the fly, *Drosophila virilis*. The latter is a novel form of the i-type lysozyme found in shrimp.

In the present study, the *P. monodon* c-type and the two forms of i-type lysozymes were presented. The phylogenetic analysis and tissue distribution of all three lysozyme transcripts were carried out. The recombinant proteins of the three lysozymes were over-expressed in an *Escherichia coli* system and their antimicrobial activities characterized.

## 2. Materials and methods

### 2.1. Bacterial strains

*E. coli* strain Rosetta(DE3)pLysS was used for protein expression. For the antibacterial assays, the Gram-positive bacteria: *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, and the Gram-negative bacteria: *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Vibrio cholera*, *Vibrio mimicus*, *E. coli*, were used.

### 2.2. Animals and sample preparation

Juvenile *P. monodon* shrimp approximately 3 months old, 20 g of body weight, were acclimatized in aquaria at an ambient temperature of 28 °C and a salinity of 15 ppt for a week before use in the experiments. Shrimp tissues (eyestalk, antennal gland, epipodite, gill, lymphoid organ, hepatopancreas, intestine and heart) were dissected and snap-frozen in liquid nitrogen. Hemocytes were isolated by centrifugation at 800 g for 10 min at 4 °C. The hemocyte pellet was resuspended in 1 ml of TRI Reagent® (Molecular Research Center).

### 2.3. Total RNA extraction and cDNA synthesis

The samples were homogenized in TRI Reagent® and total RNA was extracted according to the manufacturer's instruction. The total RNA was treated with DNase (Promega) to remove the DNA contamination. After determining the RNA concentration, 1 μg of total RNA was reverse transcribed into cDNA using oligo$(dT)_{18}$ primers with ImProm-II™ Reverse Transcriptase System kit (Promega) according to the manufacturer's protocol. The synthesized cDNA was stored at −20 °C until use.

### 2.4. Sequence and phylogenetic analysis

The cDNA sequences of the c-type lysozyme (*Pm*Lyzc, accession no. GQ478702), i-type lysozyme-like protein 1 (*Pm*Lyzi1, accession no. GQ478703) and i-type lysozyme-like protein 2 (*Pm*Lyzi2, accession no. GQ478704) were retrieved from the *P. monodon* EST database (http://pmonodon.biotec.or.th). They were analyzed for the identity and similarity by BLAST online [25] and the domain identification was analyzed by PROSITE and SmartTM databases (http://smart.embl-heidelberg.de/). The molecular weight and p*I* were predicted using the Expasy software (http://www.expasy.org/). The signal peptide was predicted using the online SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) [26].

The amino acid sequences deprived of signal peptides of c-, i- and g-type lysozymes from SwissProt and GenBank databases were aligned using the ClustalX [27]. Based on the alignment, a phylogenetic tree was constructed using the Phylip program. Bootstrap analysis was performed for values representing 1000 replicates using the SeqBoot. The human and rat α-lactalbumins that were similar to the c-type lysozyme were used as the outgroups.

### 2.5. Tissue specific expression of lysozymes

Expression of *Pm*Lyzc, *Pm*Lyzi1 and *Pm*Lyzi2 in various shrimp tissues was determined by semi-quantitative RT-PCR. Total RNAs were extracted from the hemocyte, hepatopancreas, lymphoid organ, gill, intestine, heart, epipodite, eyestalk and antennal gland using TRI Reagent® according to the manufacturer's instruction for the syntheses of single-strand cDNAs. The semi-quantitative PCR was, then, performed to determine the level of each lysozyme transcript using gene specific primers designed using the SECentral program (Scientific & Educational Software) as listed in Table 1.

**Table 1**
The sequences of primers used in RT-PCR and cloning into the expression vector. The NcoI and XhoI sites are underlined.

| Primer | Sequence | Usage |
|---|---|---|
| PmLyzc_F | 5′ ATACCATGGGCCATCATCATCATCATCACAAGGTCTTCAGGAAGTGCG 3′ | Cloning |
| PmLyzc_R | 5′ CTCGAGCACTTAGAATGGGAATATCGAGTTG 3′ | Cloning |
| PmLyzi1_F | 5′ CCATGGGCCATCATCATCATCATCACGCAGAATTGGAGGATAGTTGC 3′ | Cloning |
| PmLyzi1_R | 5′ CTCGAGTCACTCTACACTCGGAGGATTTGTG 3′ | Cloning |
| PmLyzi2_F | 5′ CCATGGGCCATCATCATCATCATCACGAAGGGGTTGACCCCAACT 3′ | Cloning |
| PmLyzi2_R | 5′ CTCGAGCTACGTAGCAGTTGGAGAAGAAG 3′ | Cloning |
| Lyzc _F | 5′ TCCTCTGGTGCTGCTGGTTG 3′ | RT-PCR |
| Lyzc_R | 5′ GGTTGCGGTTGCGGTTGATG 3′ | RT-PCR |
| Lyzi1_F | 5′ CCGTTACCAAGCCTTACTG 3′ | RT-PCR |
| Lyzi1_R | 5′ TAATCGGTCGCGTAGTCCTC 3′ | RT-PCR |
| Lyzi2_F | 5′ CGACAACGCGGAACAGAAGG 3′ | RT-PCR |
| Lyzi2_R | 5′ CGGTGGTGCTGACAGATGGA 3′ | RT-PCR |