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Short communication

Molecular cloning, inducible expression and antibacterial analysis of a novel i-type lysozyme (lyz-i2) in Pacific white shrimp, *Litopenaeus vannamei*

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ABSTRACT

The full-length cDNA coding for a novel invertebrate (i-type) lysozyme was identified in Pacific white shrimp (*Litopenaeus vannamei*). The newly obtained *L. vannamei* lysozyme is similar to the *Penaeus monodon* i-type lysozyme 2, but it is distant from the known *L. vannamei* c-type lysozyme and i-type lysozyme 1 in protein sequence; therefore, it was defined as *L. vannamei* i-type lysozyme 2 (lyz-i2). Expression of *L. vannamei lyz*-i2 transcripts were ubiquitously detected in all tissues we selected, with the highest abundance observed in the hemolymph. Challenge with *Vibrio harveyi* might elicit *L. vannamei lyz*-i2 mRNA expression in the hepatopancreas, intestine, muscle, gill and hemolymph. In the themolymph, specifically, the stimulatory effects of *Vibrio* and lipopolysaccharide (LPS) on *lyz*-i2 transcript levels were durable and transient, respectively; while Polyinosinic:polycytidylic acid [Poly (I:C)] treatment did not affect *lyz*-i2 expression. *L. vannamei* lyz-i2 recombinant protein was generated in an *Escherichia coli* system. By lysoplate and turbidimetric assays, the *L. vannamei* lyz-i2 recombinant protein showed a broad spectrum of antimicrobial properties with high activities against *Micrococcaceae lysodeikticus* and various *Vibrio* species and relatively low activity against *E. coli*. In conclusion, *L. vannamei* lyz-i2 might be a potent antibacterial protein with a role in innate immunity in Penaeid shrimp.

1. Introduction

Lysozymes (E.C. 3.2.1.17), also known as muramidases, are types of antimicrobial peptides (AMPs) that protect organisms by damaging the cell walls of the infectious bacterial pathogens [1,2]. As a part of the innate immune system [3], lysozymes are mainly synthesized in secretary cells in a variety of exocrine glands and are secreted into body fluids to protect against bacterial invasion [4]. Additionally, lysozymes have biological roles in digestion, anti-tumor and anti-virus processes [5]. Lysozymes occur in all major taxa of living organisms. In animals, four major types of lysozymes are distinguished, namely, c- (chicken or conventional), g- (goose), i- (invertebrate) and ch- (chalaropsis) type lysozymes [6,7]. Whereas c- and g-type lysozymes are present in all vertebrates, invertebrates typically produce i-type lysozymes, and sometimes also c-type (e.g., Arthropoda), g-type (e.g., Mollusca) or ch-type (e.g., Nematode) lysozymes [7].

Innate immunity, particularly the AMPs, plays an important role against infection in invertebrates. The antimicrobial activity of crustacean lysozymes was first detected in the Astacidae and Cambaridae species [8], followed by the Artemiidae [9] and Penaeidae species [10]. The first Penaeidae lysozyme cDNA was reported in *Litopenaeus vannamei* [11], which was clarified as a c-type lysozyme. Subsequently, cDNAs coding for lysozymes have

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been reported in *Marsupenaeus japonicus* (c-type [12] and i-type [13]), *Fenneropenaeus merguiensis* (c-type [14]), *Penaeus monodon* (one c-type [15] and two i-type lysozymes [16]), *L. vannamei* (i-type) [17], *Fenneropenaeus indicus* (c-type [18]) and *Litopenaeus stylirostris* (c-type [19]). Lysozyme recombinant proteins for different Penaeidae species were shown to have antibacterial activity [12,14,16,18–20]. In *P. monodon*, the lytic activity of lysozyme showed high against *Vibrio* and *Micrococcus*, and relatively low against other bacteria [15,16]. Additionally, muramidase activity was only found in c-type but not i-type Penaeidae lysozymes [16], and this may be caused by a deficiency of catalytic residues for muramidase in the Penaeidae i-type lysozyme amino acid (a.a.) sequence [17]. Additionally, challenge with bacteria or pathogen-associated molecular patterns (PAMPs) may cause up-regulation of c-type but down-regulation of i-type *lysozyme* mRNA expression levels in different Penaeidae species [14,17,18]. Furthermore, silencing of lysozyme expression by dsRNA led to 100% mortality without any artificial bacterial infection in *M. japonicus* [5].

Pacific white shrimp, *L. vannamei*, is the predominant shrimp species produced in aquaculture. Given that three *lysozyme* genes (*lyz*-c, *lyz*-i1 and *lyz*-i2) have been reported in *P. monodon* [16] but only two *lysozyme* genes (*lyz*-c and *lyz*-i1) were found in *L. vannamei* [11,17], an unknown ortholog gene for the *P. monodon* *lyz*-i2 may occur in *L. vannamei*. The shrimp lyz-i2 is seen distant from lyz-i1 in its a.a. sequence and lytic activity [16]; therefore, we investigated the structural and functional features of lyz-i2 in *L. vannamei* in this study. Based on data from a transcriptome we previously constructed, the full-length cDNA of a novel i-type *lysozyme* gene (*lyz*-i2) was identified from the intestine of *L. vannamei*. The a.a. sequence alignment, phylogenetic relationship and mRNA tissue distribution for three *L. vannamei lysozymes* (*lyz*-c, *lyz*-i1 and *lyz*-i2) were comparatively analyzed. Additionally, the transcriptional expression of *L. vannamei lyz*-i2 was detected in different tissues following exposure to *Vibrio*. Specifically, the inducible expression of *L. vannamei lyz*-i2 mRNA in the hemolymph following bacterial or PAMPs challenge was monitored in a time-course experiment. Furthermore, recombinant *L. vannamei* lyz-i2 protein was generated in an *Escherichia coli* system and its antimicrobial activity was characterized using different bacterial species. Overall, this study may provide the evidence for the third *lysozyme* gene in *L. vannamei* with characterization of its gene expression and protein function.

2. Materials and methods

2.1. Animals

Healthy Pacific white shrimp (*Litopenaeus vannamei*) with body weights of 7.7 ± 0.8 g and body lengths of 8.1 ± 0.7 cm were collected from the Dongfang shrimp culture center, Zhanjiang, China, and maintained in artificial seawater at 28 °C under a 12D:12L photoperiod. Shrimp were anaesthetized on ice and sacrificed by decapitation. All animal experiments were conducted in accordance with the guidelines and approval of the Ethics Committees of South China Sea Institute of Oceanology, Chinese Academy of Sciences.

2.2. Molecular cloning of a novel i-type lysozyme cDNA in Pacific white shrimp

Total RNA from the intestine of Pacific white shrimp was extracted by using TRIzol (Invitrogen) and reverse transcribed into first-strand cDNA with SuperScript II (Invitrogen). By BLAST analysis of a *L. vannamei* Illumina transcriptome that was previously constructed by our lab, a unigene of 1441 bp (Suppl. 1) was found to share high sequence homology with the *P. monodon* i-type *lysozyme* 2 cDNA (GenBank: GQ478704). Based on this, gene specific primers were designed to amplify the partial sequence of a novel i-type *lysozyme* (*lyz*-i2) in *L. vannamei*. The corresponding full-length sequence was obtained by 3'- and 5'-rapid amplification of cDNA ends (RACE). The cloning strategy for the full-length *L. vannamei* *lyz*-i2 cDNA is shown in Fig. 1A. The signal peptide of *L. vannamei* lyz-i2 was predicated by using the SignalP 3.0 program. The a.a sequence alignment of different lysozyme types was performed by using Clustalx1.8 and presented with GeneDoc. Phylogenetic analysis was constructed based on nucleotide differences (*p*-distance) by the Neighbor-Joining method with Mega 6.0.

2.3. Tissue distribution of different L. vannamei lysozyme isoforms

Transcriptional tissue distributions of different *lysozyme* isoforms [*lyz*-c (GenBank: AF425673), *lyz*-i1 (GenBank: JN039375) and *lyz*-i2] in Pacific white shrimp were examined by using RT-PCR in selected tissues. The tissue samples, including the brain, eyestalk, hepatopancreas, heart, stomach, intestine, gill, muscle and hemolymph, were collected as described previously [21]. Total RNA was isolated by using TRIzol, digested with RNase-free DNase I (Invitrogen) and reverse-transcribed with Superscript II. The obtained RT samples were used as templates in PCR with primers (Suppl. 2) specific for *L. vannamei lyz*-c, *lyz*-i1 and *lyz*-i2 transcripts, respectively, and the amplification cycle was 33 for each *lysozyme* isoform after semi-quantitative validation. In this case, RT-PCR of *ß-actin* mRNA was used as an internal control.

2.4. In vivo challenge of Pacific white shrimp with Vibrio harveyi

V. harveyi was cultured in 2216e broth at 30 °C until OD_{600} reached 0.6, and it was harvested by centrifugation at 3000*g* for 5 min. The collected bacterial cell pellet was washed three times in phosphate buffered solution (PBS) with 33‰ salinity. The *V. harveyi* was heat-inactivated at 95 °C for 5 min and diluted in sterile artificial seawater with a density of 1.0×10^8 CFU/mL as described previously [22]. The pericardial cavity of each shrimp was injected with 100 μL solution of *V. harveyi* solution using a 23-gauge needle attached to a 1 mL syringe, and an injection of artificial seawater alone was used as a control. Different tissue samples (brain, eyestalk, hepatopancreas, heart, stomach, intestine, gill, muscle and hemolymph) from six individuals were harvested at 24 h after injection. The tissue samples were frozen in liquid nitrogen and stored at −80 °C for RNA extraction and reverse transcription.

Given that hemolymph is the tissue with the maximum *lyz*-i2 expression response to *V. harveyi* challenge, a more delicate time-course experiment was performed with heat-killed *V. harveyi* (1.0×10^8 CFU/mL), lipopolysaccharide (LPS, 2 μg/μL, Sigma) or Polyinosinic:polycytidylic acid [Poly (I:C), 2 μg/μL, Sigma] injections. The operation of injection was performed as described above, and an injection of artificial seawater alone was used as a control. At 0, 6, 12, 24, 36, 48, 60 and 72 h post-injection (hpi), six shrimp from each group were randomly killed and sampled for hemolymph collection. The hemolymph samples were dissolved in TRIzol for RNA extraction and reverse transcription.

2.5. Measurement of L. vannamei lyz-i2 mRNA by real-time PCR

For *L. vannamei* *lyz*-i2 mRNA measurements, total RNA from tissue samples were isolated by using TRIzol, digested with DNase I, and reverse transcribed with SuperScript II. The real-time PCR was carried out on a RotorGene RG-3000 (Qiagen) by using SYBR Premix Ex Taq™ II (TaKaRa) with specific primers and PCR conditions (Suppl. 3). In this case, *ß-actin* mRNA was used as the internal