Fish & Shellfish Immunology 49 (2016) 336–343

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Identification and expression analysis of a new invertebrate lysozyme in Kuruma shrimp (*Marsupenaeus japonicus*)

Hong-tao Liu [a], Jun Wang [a], Yong Mao [a], Min Liu [a], Su-fang Niu [a], Ying Qiao [a], Yong-quan Su [a, *], Chun-zhong Wang [b], Zhi-peng Zheng [c]

[a] State Key Laboratory of Marine Environmental Sciences, Xiamen University, Xiamen, Fujian 361102, China
[b] Putian Fishery Institute, Putian, Fujian 351100, China
[c] Putian Tian-ran-xing Agricultural Development Co. Ltd., Putian, Fujian 351100, China

ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form
16 December 2015
Accepted 21 December 2015
Available online 24 December 2015

Keywords:
Invertebrate lysozyme
Marsupenaeus japonicus
Developmental expression
WSSV
Immune response

ABSTRACT

Lysozyme is an important component of the innate immunity system against invading pathogens. An invertebrate (i-type) lysozyme from the hepatopancreas of Kuruma shrimp *Marsupenaeus japonicus* (Mj-ilys) was identified. The full-length cDNA of Mj-ilys was 580bp with a 429 bp open reading frame encoding a 142 amino acid polypeptide. The encoded polypeptide was predicted to have a 17 amino acid signal peptide, and a 125 amino acid mature protein with a theoretical mass of 14.099 kDa and an isoelectric point (p*I*) of 4.18. A Destabilase conserved domain was predicted in Mj-ilys amino acid sequences which may be stable by 10 cysteine residues forming 5 disulfide bonds. Mj-ilys may loss the muramidase and isopeptidase activities due to the lack of the key catalytic residues. Mj-ilys had high homologous of 80–82% with i-type lysozymes of penaeid shrimps. It was first grouped with other i-type lysozyme of shrimps and crabs in a phylogenetic tree predicted by the Neighbor-Joining method. Mj-ilys mRNA was expressed mainly in hepatopancreas and almost undetectable in other tissues. The mRNA expression of Mj-ilys were all found from fertilized eggs to post-larvae of 17 days (PL17), and its expression exhibited significant differences among each developmental stage. After white spot syndrome virus (WSSV) challenge (3.6×10^8 virions/μl), the time-dependent expression pattern of Mj-ilys in hepatopancreas and gills showed significantly different. These results indicated that Mj-ilys is potentially involved in the ontogenesis and immune defense in Kuruma shrimp.

1. Introduction

Based on the FAO statistics, till 2013 the production of penaeid shrimps globally has been about 4.45 million metric tons and its estimated economic value is greater than 22.6 million dollars [1]. Kuruma shrimp (*M. japonicus*) is recognized as one of the world's three main farming shrimp species, but its farming industry has been limited by severe diseases in recent years, particularly the white spot syndrome virus (WSSV). Shrimps have effective and sophisticated innate immune responses to protect themselves from infection by exogenous pathogens.

Lysozymes make up a large group of enzymes that widely distribute among organisms. They are generally believed to contribute in host immunity via its antimicrobial activities and immunomodulation and in some cases to digestion [2]. Six main lysozyme types occur in the animal kingdom while the invertebrate (i-type) lysozymes are typical for invertebrate organisms since their first discovery in 1975 [3]. Recently numerous studies identified i-type lysozymes among different invertebrate phyla including molluscs, echinoderms, nematodes, annelids, hemichordates, arthropods and porifers [4–14]. In some invertebrates multiple i-type lysozymes occurred differing in their cellular localization and/or biochemical properties [15], and were considered to have crucial roles in host innate immune defense. The i-type lysozymes have been described in some crustaceans such as *Procambarus clarkii*, *Eriocheir sinensis*, *Litopenaeus vannamei* and *Penaeus monodon* [12,13,16]; for Kuruma shrimp *M. japonicus*, however, little information on i-type lysozyme was available. It is worth mentioning that a chicken-type (c-type) lysozyme of Kuruma shrimp had been identified, it displayed lysis activity, but some its characterizations like eight cysteine residue motif and 2 introns

* Corresponding author.
E-mail address: yqsu@xmu.edu.cn (Y.-q. Su).

http://dx.doi.org/10.1016/j.fsi.2015.12.034
1050-4648/

in its sequences are significantly different with i-type lysozymes [17].

In this study, we obtained and characterized both the cDNA and the corresponding genomic sequences of a new i-type lysozyme from Kuruma shrimp *M. japonicus* (Mj-ilys). Tissue-specific mRNA expression of Mj-ilys was performed in eight tissues of healthy individuals. Transcription profiles expressions in early developmental stages of *M. japonicus* as well as temporal expression profiles during WSSV infection were characterized kinetically to preliminarily investigate the potential roles of Mj-ilys in shrimp immune defense.

2. Materials and methods

2.1. Sampling for cloning

Healthy individuals of *M. japonicus* (approximately 10–12 g each) were obtained from a shrimp farm in Dongshan County (Fujian Province, China) and acclimated for two weeks (25 °C, 30 salinity and under continuous aeration) in laboratory. Hepatopancreas were sampled and immediately preserved in RNAfixer (Bio-Teck, China) at −20 °C. Muscle tissues were preserved using absolute ethyl alcohol at −20 °C. Total RNA of hepatopancreas was isolated using RNAiso Plus (TaKaRa, Japan), After quantification with the ND-1000 NanoDrop spectrophotometer (Thermo Scientific, USA) at the A260/A280 ratio, the first-strand cDNA was synthesized using a SMARTer® PCR cDNA synthesis Kit (Clontech, USA) according to the manufacturer's protocol. Total genomic DNA was extracted from muscle tissues and used as template for PCR.

2.2. Cloning of cDNA and genomic DNA sequences of Mj-ilys

The full-length cDNA sequence of Mj-ilys was obtained by RACE based on the sequenced segment which annotated an i-type lysozyme-like protein 1 (*L. vannamei*) from the cDNA library of the hepatopancreas in our laboratory. The 5′RACE was performed in a total reaction volume of 50 μl containing 37.5 μl of PCR-grade water, 5 μl of 10* PCR buffer, 4 μl of dNTP mix (2.5 mM), 1 μl of each primer (10 mM), 0.5 μl of Taq polymerase (TaKaRa, Japan) and 1 μl of cDNA mix (Table 1). The PCR parameters were as followings: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 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 in Table 1 except that an annealing temperature of 53 °C instead of 54 °C was used in the 30 cycles of amplification. PCR products were gel-purified, cloned into the pMD19-T simple vector (TaKaRa, Japan) and sequenced at the Beijing Genomics Institute (BGI) genomic Center (Shenzhen, China). The genomic DNA sequences of Mj-ilys were cloned by the same ways above except using different pairs of designed primers in Table 1.

2.3. Molecular characterization and phylogenetic analysis

The full-length cDNA and corresponding genomic DNA sequences of Mj-ilys were assembled and edited using the Lasergene DNASTAR software 8.0. Expert protein analysis system (http://expasy.org/) was used to analyze the deduced amino acid sequence to conduct molecular weight (kDa) and isoelectric point (pI), and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was employed to predict the signal peptide of the Mj-ilys protein [18,19]. To predict the protein domains the SMART software (http://smart.embl.de/) was used [20]. The presumed tertiary structure was established using the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/) [21]. The homologous sequences of Mj-ilys were searched using BLAST program available at the NCBI website with default settings on the GenBank database. Amino acid sequences of Mj-ilys and other invertebrate lysozymes identified from other species were aligned using ClustalX. The phylogenetic analysis was conducted using the program Mega 6.06 and a consensus tree was then constructed by Neighbour-Jointing (NJ) method [22]. The robustness of each topology was checked by 1000 bootstrap replications.

2.4. Tissue distribution of Mj-ilys transcripts

After two weeks acclimation, healthy individuals (n = 6) were sampled randomly for eight tissues including hepatopancreas, gills, heart, stomach, muscle, eyestalk, intestine and hemocytes for transcript expression. Total RNA from each sample was extracted as described above and the cDNA was synthesized using a PrimeScript®RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instruction. The quantitative real-time PCR (qRT-PCR) was conducted on an ABI 7500 fast real-time PCR System (Applied Biosystems, USA) using a SYBR®Premix Dimer Eraser™ Kit (TaKaRa, Japan). A pair of Mj-ilys gene-specific primers, RTF and RTR, was used to qRT-PCR. The amplification was performed in a total volume of 20 μl containing 10 μl of SYBR Green I real-time PCR master mix, 2 μl of cDNA, 0.6 μl of each primer, 0.4 μl ROX Reference Dye Dye II and 6.4 μl of DEPC water. The real-time PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s and 72 °C for 30 s. Dissociation was performed and melting curve of amplification products was produced at the end of each PCR to

Table 1
List of primers used in this study.

Primers ID	Primer sequences	Applications
F1	ACTATTCTTCCTGGTGGT	cds PCR
R1	ATAGTCCAGACAGTCCTTG	cds PCR
FF1	TGGACAGACGCTGATAAA	3′RACE
FF2	GGAATCCTCACGCCTCAT	3′RACE
RR1	TCAGGCACAAAGACGAAG	5′RACE
RR2	GCATGAGGCGTGAGGATT	5′RACE
RA	AAGCAGTGGTATCAACGCAGAGTAC	RACE
Oligo dT-RA	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN	RT/RACE
IN-F1	GACAGTTACCGCCAAGCA	Genomic DNA cloning
IN-R1	GCCTGGTTTATCAGCGTC	Genomic DNA cloning
IN-F2	TGGACAGACGCTGATAAA	Genomic DNA cloning
IN-R2	TCAGGCACAAAGACGAAG	Genomic DNA cloning
RTF	GACAGTTACCGCCAAGCA	qRT-PCR
RTR	TGTTCCAGCACACCGAGT	qRT-PCR
EF1-α-RT-F	AAGGAACTGGAGGCAGGACC	qRT-PCR
EF1-α-RT-R	ACACCCACAGCCACCGTTTG	qRT-PCR