Fish & Shellfish Immunology 34 (2013) 173–182

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Immune gene discovery by expressed sequence tag (EST) analysis of hemocytes in the ridgetail white prawn *Exopalaemon carinicauda*

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ARTICLE INFO

Article history:
Received 11 July 2012
Received in revised form
11 October 2012
Accepted 14 October 2012
Available online 22 October 2012

Keywords:
Exopalaemon carinicauda
Hemocytes
Expressed sequence tags
Immune-related gene

ABSTRACT

The ridgetail white prawn *Exopalaemon carinicauda* is one of the most important commercial species in eastern China. However, little information of immune genes in *E. carinicauda* has been reported. To identify distinctive genes associated with immunity, an expressed sequence tag (EST) library was constructed from hemocytes of *E. carinicauda*. A total of 3411 clones were sequenced, yielding 2853 ESTs and the average sequence length is 436 bp. The cluster and assembly analysis yielded 1053 unique sequences including 329 contigs and 724 singletons. Blast analysis identified 593 (56.3%) of the unique sequences as orthologs of genes from other organisms (*E*-value < 1e-5). Based on the COG and Gene Ontology (GO), 593 unique sequences were classified. Through comparison with previous studies, 153 genes assembled from 367 ESTs have been identified as possibly involved in defense or immune functions. These genes are categorized into seven categories according to their putative functions in shrimp immune system: antimicrobial peptides, prophenoloxidase activating system, antioxidant defense systems, chaperone proteins, clottable proteins, pattern recognition receptors and other immune-related genes. According to EST abundance, the major immune-related genes were thioredoxin (141, 4.94% of all ESTs) and calmodulin (14, 0.49% of all ESTs). The EST sequences of *E. carinicauda* hemocytes provide important information of the immune system and lay the groundwork for development of molecular markers related to disease resistance in prawn species.

1. Introduction

The ridgetail white prawn *Exopalaemon carinicauda* is one of the major economic shrimp species, which naturally distributes in the coasts of Yellow Sea and Bohai Sea, China. The ridgetail white prawn contributes to one third of the gross outcome of the polyculture ponds in eastern China [1]. Given its high commercial interest, studies focus on the diseases causing large mortality and"milky shrimp" disease, with *Hematodinium* confirmed as its main causative agents, has received increasing attention [1]. However, there are no effective measures to take for control of this disease. The basic problem is a lack of enough knowledge of the immune system and defense mechanisms of crustacean [2–4]. Therefore, better understanding of immune system and identification of immune-related genes will be important for the health management in crustacean farming.

Expressed sequence tags (ESTs) are partial sequences of cDNAs typically produced by rapid single-pass sequencing of randomly chosen clones from a cDNA library [5]. ESTs analysis is an efficient approach for gene discovery and exploitation for molecular markers [6–11]. In addition, EST analysis could also be used in investigation of gene expression patterns in different tissues [12], and generation of genetic linkage maps and physical maps [13,14]. The research protocol has been successfully used to discover immune related genes in Pacific white shrimp *Litopenaeus vannamei* and Atlantic white shrimp *Litopenaeus setiferus* [6], kuruma prawn *Marsupenaeus japonicus* [15], black tiger shrimp *Penaeus monodon* [16], Chinese shrimp *Fenneropenaeus chinensis* [17] and Chinese mitten crab *Eriocheir sinensis* [9]. In addition, sex-related genes have also been discovered in ovaries of *P. monodon* by ESTs analysis [18,19]. However, very little is known about immune-related genes in *E. carinicauda*, except conformation of "milky shrimp" disease caused by *Hematodinium* and identification of HSP 70, HSP 90 and Ferritin genes in *E. carinicauda* [1,20–22]. Until now, there were no ESTs of ridgetail white prawn in GenBank. The objective of this study was to discover immune-related genes and understand the immune system of *E. carinicauda* at the molecular

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1050-4648/$ – see front matter
http://dx.doi.org/10.1016/j.fsi.2012.10.026

level through constructing a hemocytes cDNA library and ESTs analysis.

2. Materials and methods

2.1. Animal materials

Thirty healthy adult individuals of *E. carinicauda*, averaging weight 1.18 ± 0.35 g, were collected from a commercial farm in Qingdao, China. They were cultured in filtered aerated seawater (salinity 20‰, pH 8.2) at 18 ± 0.5 °C for 7 days before processing. Hemocytes were collected with syringe which contained an equal volume of anti-coagulant buffer (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) [23], and centrifuged at 800 rpm, 4 °C for 15 min. The hemocytes were preserved in liquid nitrogen immediately, up to RNA extraction.

2.2. Total RNA extraction and mRNA purification

The hemocytes were treated with Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol. The RNA samples were determined with 1% agarose gel electrophoresis and spectrophotometer, all OD_{260}/OD_{280} were between 1.8 and 2.0. The mRNA were isolated and purified with PolyATract mRNA Isolation System (Promega, USA), stored in −70 °C for the next step.

2.3. cDNA library construction

The cDNA library was constructed by using SMART cDNA Library Construction Kit (Clontech, USA), according to the protocol of the manufacturer. The first strand cDNA was synthesized using SMART Scribe™ MMLV reverse transcriptase with SMART IV oligonucleotide, CDS III/3′ PCR primer, DTT and dNTPs provided in the kits. The double-stranded cDNA (ds-cDNA) was synthesized by LD-PCR (long distance PCR), using 5′PCR primer, CDS III/3′ PCR primer and Advantage 2 PCR kit (Clontech, USA). The ds-cDNA was digested by proteinase K (20 μg/μL) at 45 °C for 20 min immediately, and then was digested by *SfiI* enzyme at 50 °C for 2 h. Following, the cDNA size fractionated by CHROMA SPIN-400 (Clontech, USA), and the first three fractions containing cDNA were collected and pooled in a clean tube for packaging. The ligation of cDNA to λTriplEx2 vector had parallel ligation reactions which had different vector-to-cDNA ratios 1:0.5, 1:1 and 1:1.5, ensuring to obtain a high volume library. Each ligation was packaged with the Packagene Lambda DNA Packaging Extracts (Promega, USA). The titre of unamplified library was detected with *Escherichia coli* XL1-Blue on LB/$MgSO_4$ plates; the percentage of recombinant clones was determined by blue/white screening approach. The unamplified library was amplified and the titre of amplified library was detected as above.

2.4. Sequencing

A small quantity of *E. carinicauda* cDNA library was converted to *E. coli* BM 25.8 strain. Single colonies were picked randomly; bacterial plasmids were extracted by conventional method and sequenced at the 5′ end of each cDNA using the 5′pTriPLEx2 primer (5′-CTC CGA GAT CTG GAC GAG C-3′) and 3730 XL automatic sequencing machine (ABI).

2.5. Data analysis and annotation

Vector sequences and primer sequences were removed by using Cross-match software [24,25]. Short sequences (<100bp in length) or poor quality sequences were eliminated. High quality ESTs were analyzed with Phrap program [26]. They were divided into contiguous sequences (contigs) and singlets. All of the unique sequences were subjected to the non-redundant (nr) protein and nucleotide database with BLASTx and BLASTn program searching for functional annotation [27], and sequence homology was accepted as *E*-value < 1e-5. Unique sequences were clustered into categories using Cluster of Orthologous Groups of proteins (COG). Gene Ontology (GO) annotions were assigned using program Gopipe [28] according to its uniprot accession number [29]. In addition, the unique sequences associated with the innate immune response were identified by sequence alignment according to the previously public report.

3. Results

3.1. mRNA and cDNA library quality

The A260/A280 ratio of the RNA was 1.91. The integrity of the total RNA was verified by agarose gel electrophoresis (Fig. 1). The total amount of RNA (5 mg) was used for mRNA isolation. Purified mRNA of the hemocytes for cDNA library construction was 0.5 μg. The cDNA synthesis produced cDNA fragments in a range of sizes, but only the fragments from 500 bp to 1000 bp were extracted and used for the cDNA library construction. The cDNA library was of high quality and contained about 2.8×10^6 pfu/mL and the recombinant efficiency of cDNA library was over 99% by blue/white screening.

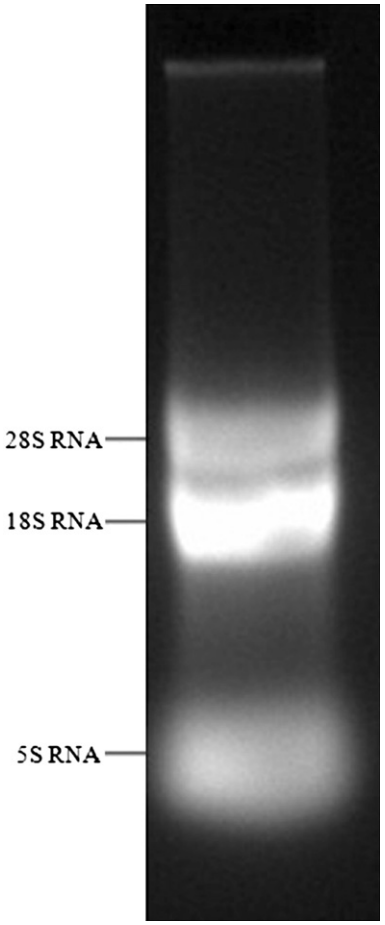

Fig. 1. Total RNA from the hemocytes of *E. carinicauda* for the cDNA library construction.