Fish & Shellfish Immunology 27 (2009) 73–77

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Cloning of penaeidin gene promoter in tiger shrimp (*Penaeus monodon*)

Shih-Hu Ho [a,1], Yen-Ling Song [a,b,*]

[a] Institute of Zoology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan, ROC
[b] Department of Life Science, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan, ROC

ARTICLE INFO

Article history:
Received 20 November 2008
Received in revised form
21 April 2009
Accepted 4 May 2009
Available online 10 May 2009

Keywords:
Tiger shrimp
Penaeus monodon
Penaeidin
Promoter
Promoter activity

ABSTRACT

Penaeidins belong to a family of antimicrobial peptides that are expressed in the hemocytes of penaeid shrimps. Using an extender PCR method and a nested PCR, we cloned two types of genomic fragment flanking the 5′ end of penaeidin gene in tiger shrimp (*Penaeus monodon*): Type536 and Type411 sequences. Both fragments contained TATA box, GATA, dorsal and AP-1 motifs and were ligated to an expression vector with a luciferase reporter gene. The constructs were then delivered into *Drosophila* S2 cell line. The promoter functions of the two fragments were determined using a luciferase expression assay. The study demonstrated that Type411 sequence performed higher transcriptional activity than Type536. Alignment of the upstream sequences of penaeidin genes in *P. monodon* and *Litopenaeus vannamei* showed that the promoter regions were obviously more diverse than the 5′UTRs. Phylogenetic analysis indicated the presence of two types of promoters that are not species-specific in the two shrimps.

1. Introduction

Penaeidins belong to a family of antimicrobial peptides that exhibit both Gram-positive antibacterial and antifungal activities [1]. They have been found in *Litopenaeus* [2], *Marsupenaeus* [3], *Fenneropenaeus* [4], *Penaeus* [5] and *Farfantepenaeus* [6] shrimps. In crustaceans, penaeidins have been found exclusively in penaeids and different from other antimicrobial peptides such as crustins [7] and anti-lipopolysaccharide factors [8]. Penaeidins are constitutively synthesized and stored in the shrimp hemocytes, localized in granulocyte–cytoplasmic granules, and released in response to appropriate stimuli such as infections [9,10]. Three classes of penaeidins such as PEN2, PEN3 and PEN4 were identified in the Pacific white shrimp *Litopenaeus vannamei* [2,11,12] and each class is encoded by a unique gene [13]. Recently, a novel class PEN5 in *F. chinensis* has been identified and reported to be up-regulated after bacterial challenge [14]. These results differed from the PEN3 in *L. vannamei*, in which transcripts decreased 6–12 h after microbial challenge then increased to normal levels after 48 h post-challenge [10].

Penaeidin was also identified from another economically important aquaculture shrimp species, the tiger shrimp *Penaeus monodon*, in previous studies [5,15,16] in which higher mRNA expression was detected during nauplius I and intermoult stage [17]. In the cDNA sequence, an open reading frame that coded for a peptide composed of 74 amino acids was found. A cleavage site of secretory signal peptide was predicted between amino acids 19 and 20. The calculated molecular mass of mature penaeidin was about 6.1 kDa and the estimated pI of this peptide was 9.1. The peptide is composed of a proline-rich N-terminus and a C-terminus containing six cysteine residues engaged in three disulfide bridges.

The names PEN2, PEN3 and PEN5 in tiger shrimp have been used in previous studies. An EST homologue PEN2 precursor was reported by Supungul et al. [18]. In addition, one study reported the identification of a cDNA for PEN5 in the tiger shrimp [16] and another studied the molecular characterization of PEN5 [19]. Also, a mo-penaeidin was identified in *P. monodon* by Chiou et al. [17]. However, through phylogenetic cluster tree analysis, it appeared that only one penaeidin class exists in *P. monodon* [5,17]. These penaeidins, however, could not be reliably clustered in *Litopenaeus* PEN3 group [5] or PEN2 group [17] due to low bootstrap values. It indicates that penaeidins from different penaeid shrimps are diverse.

The molecular characterization and expression pattern of tiger shrimp penaeidin have been studied previous. For more understanding of the penaeidin gene regulation and further possible

Abbreviations: PCR, polymerase chain reaction; S2 cell, *Drosophila* S2 cell line; FCS, foetal calf serum; ANOVA, analysis of variance.

* Corresponding author. Institute of Zoology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan, ROC. Tel.: +886 2 3366 2455; fax: +886 2 2366 0243.
E-mail address: song@ntu.edu.tw (Y.-L. Song).
[1] Present address: Taiwan Ocean Research Institute, National Applied Research Laboratories, 3F No. 106, Ho-Ping E. Road, Sec. 2, Taipei 106, Taiwan, ROC.

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.05.001

application, the 5′-upstream regions of the gene from *P. monodon* were cloned in this study and the promoter activity was assessed in heterogeneous cell culture system. The *penaeidin* promoter sequences from tiger shrimp and Pacific white shrimp were compared and analyzed phylogenetically.

2. Materials and methods

2.1. Extraction of genomic DNA from hemocytes

The isolation method of genomic DNA was modified from the mammalian protocol [20]. Hemolymph from tiger shrimp (*P. monodon*) was collected using a syringe containing anticoagulant (0.1 M sodium citrate, 0.4 M sucrose, 0.01 M Tris–HCl, pH 7.6, osmolarity was adjusted to 780 mOsm kg^{-1} by adding sucrose) and centrifuged at 700*g* for 15 min at 4 °C. The resulting hemocyte pellet was lysed in lysis buffer (10 mM Tris–HCl, 0.1 M EDTA, 0.5% (w/v) SDS, 20 μg mL^{-1} RNase A, pH 8.0) and incubated for 1 h at 37 °C. Proteinase K (final conc. 100 μg mL^{-1}) was then added into the lysate and incubated for 3 h at 50 °C. The lysate was purified by phenol:chloroform:isoamyl alcohol ratio of 25:24:1 (Amresco) extractions. Aqueous phases were pooled and DNA was precipitated by adding 0.2 volume of 10 M ammonium acetate and 2 volume of ethanol. After washing with 70% ethanol, DNA was dried and dissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.

2.2. Extender PCR

The cloning of penaeidin gene upstream sequence was modified from extender polymerase chain reaction (PCR) method [21]. Genomic DNA was partially digested with RsaI. Digested DNA was ethanol precipitated, washed, dried, dissolved and then ligated with adapters which were prepared by annealing two oligonucleotides 5′-TGCGAGTAAGGATCCTCACGCAAGGAATTCCGACCAGACACCCTAG-3′ and 5′-/5Phos/-CTAGGGTGTCTGGTCGC-3′. Unligated adapters were eliminated by Sephadex G-200 or Montage™ PCR Centrifugal Filter Devices (Millipore). Using the ligated DNA as template, a first round PCR (25 cycles run for 1 min at 94 °C, 30 s at 64 °C and 1 min 30 s at 72 °C) was run using 1 pmol forward adapter P1 primer (5′-TGCGAGTAAGGATCCTCACGCA-3′) and 10 pmol reverse penaeidin specific primer 5′-CAGGAAGACCAGGCAGACCACGAGA-3′ (designed according to GenBank accession no. AF475082 penaeidin cDNA sequence [5]) and the amplicon was subjected to a nested second round PCR using 10 pmol adapter P2 primer (5′-CGCAAGGAATTCC GACCAGACA-3′) and 10 pmol reverse penaeidin specific primer 5′-CCATGGCGGACACAGGAAGGAACCC-3′. The thermal cycling parameters were 1 min at 94 °C, and 30 cycles run for 20 s at 94 °C, 20 s at 66 °C and 1 min at 72 °C. The band of the PCR product on electrophoresis gel was eluted then ligated to pGEM-T Easy vector (Promega). The ligated vector was subsequently introduced to *E. coli* DH-5α competent cells to amplify the vector. Clones were sent to the commercial company (Mission Biotech, Taiwan) for sequencing.

2.3. Nested PCR for cloning Type411 upstream sequence

Two forward primers in this nested PCR were designed from the more upstream sequence (data not shown) which was obtained by a further extender PCR. The first round PCR with the forward primer 5′-CATTTAAATTAGTTAATGGCTTCTGC-3′ and reverse primer 5′-ACTATCACCTGATTAATACATGACCTT-3′ designed from penaeidin gene intron (GenBank accession no. FJ227936) and the genomic DNA as template was carried out. The amplicon was subjected to the nested second round PCR using the forward primer 5′-CATT-TACATGAAATTGAAAAGAACTG-3′ and reverse penaeidin intron primer 5′-TTGGTAAGACAGTAGACTCATGGTT-3′. The amplicon was cloned to pGEM-T Easy vector and sequenced.

2.4. Analysis of nucleotide sequences

The sequences were analyzed for potential transcription factor binding sites with Match™ – 1.0 Public/TRANSFAC® 6.0 program (BIOBASE, Wolfenbüttel, Germany) (http://www.gene-regulation.com/pub/programs.html#match) using high quality matrices and 0.85 as matrix and core similarity cut-off.

The promoter sequences of shrimp penaeidin gene were collected from this study and the NCBI Entrez. The multiple sequence alignment was created with BioEdit [22], ClustalW [23] and GeneDoc (http://www.psc.edu/biomed/genedoc) [24]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [25] and the tree was constructed based on the neighbour-joining method.

2.5. Construction, transfection and activity assay of luciferase plasmids

Two types of the 5′-upstream regions of the tiger shrimp penaeidin gene, 5′-end to +62 bp of Type536 and Type411 (Fig. 1), were respectively subcloned into the SacI/*Xma*I site of pGL3-Basic firefly luciferase reporter vector (Promega). These two plasmids were named pGL3-536 and pGL3-411 respectively.

Drosophila S2 cells were routinely cultured in Schneider's Drosophila Medium (Invitrogen) supplemented with 10% foetal calf serum (FCS) and grown at 26 °C.

The luciferase assay was performed using Dual-Glo luciferase assay system (Promega) with *Renilla* luciferase gene vector as an internal control for normalization of transfection efficiency. Transfection experiments were performed in 24-well cell culture plates. Briefly, recipient S2 cells were seeded at a density of 80–90% confluence. After removal of culture medium and single wash using fresh medium without FCS, the cells were cotransfected with 1 μg of firefly luciferase reporter construct DNA and 0.1 μg of control plasmid (*Renilla* luciferase gene) per well using 2 μL Cellfectin Transfection Reagent (Invitrogen) in 200 μL medium without FCS according to the manufacturer's recommendations. At 20 h post transfection, 200 μL medium with 20% FCS was added. Cells were harvested at 28 h post transfection, firefly and *Renilla* luciferase activity was measured by the Dual-Glo luciferase assay system according to the manufacturer's instructions and chemiluminescence was read by a Luoroskan Ascent FL (Labsystems) reader. The co-expressed *Renilla* luminescence was used to normalize the firefly luminescence.

3. Results and discussion

3.1. Two types of penaeidin gene upstream sequences

An upstream sequence (Type536 in Fig. 1, GenBank accession no. FJ418753) was obtained using the extender PCR method. Except the primer sequence, there were 59 overlapping nucleotides that were identified (58/59 bp) from a penaedin cDNA sequence (GenBank accession no. AF475082). Using the nested PCR, we obtained an amplicon that revealed a genomic sequence (GenBank accession no. FJ418752) containing another type of upstream sequence (Type411 in Fig. 1) followed by the penaeidin gene.

The transcription start sites were identified from previous cDNA sequencing [15,26] (GenBank accession no. AF475082) and found consistently at 80 bp upstream of the ATG. Using the Match/TRANSFAC program, TATA box motifs were identified in position 17–31 bp ahead of transcription start sites in Type536