Pakistan J. Zool., vol. 44(4), pp. 1029-1034, 2012.

cDNA Cloning and Expression of Proliferating Cell Nuclear Antigen (PCNA) in White Leg Shrimp (*Litopenaeus vannamei*) Challenged With *Vibrio anguillarum*

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Abstract.- In present investigation the proliferating cell nuclear antigene (PCNA) of white leg shrimp (*Litopenaeus vannamei)* (*LvPCNA*) has been cloned by rapid amplification of cDNA (RACE) and anchored PCR method. The full length *LvPCNA* had 1131 bp containing 786 bp of open reading frame (ORF), encoding 261 amino acids with an estimating molecular weight of 28.8 kDa. Tissue distribution of the *LvPCNA* after real time analysis revealed that expression is high in gill and muscle. The expression level after bacterial challenge of *LvPCNA* in hepatopancreas suggested the *LvPCNA* might be involved in defense mechanism of shrimp. The present investigation is a contribution to the existing knowledge of immune responses of shrimp to bacterial challenge.

Key word: cDNA cloning, PCNA, bacterial challenge, *Litopenaeus vannamei*, DNA polymerase δ.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) was referred to as an auxiliary protein for mammalain DNA polymerase-δ (Bravo *et al.*, 1987; Prelich *et al.*, 1987). It has pivotal function in nucleic acid metabolism and is involved in many different cellular processess like repair (Celis and Madsen, 1986), UV- induced DNA damage (Kelman, 1997; Wood *et al.*, 2007), cell- cycle control and chromatin remodeling (Tsuirmot, 1998; Maga and Hubscher, 2003), and catalysis stimulation (Hutton *et al.*, 2008). Besides that, the role of PCNA has also been reported in spermatogenesis and oogensis in vertebrates and invertebrates including *Mursupenaeus japonicus* (Zuber *et al.*, 1989; Goldlewski *et al.*, 1999; Miura *et al.*, 2002; Zhang *et al.*, 2010). PCNA has also been reported to be involved in cell proliferation during zebrafish larval development (Meule *et al.*, 2006).

The PCNA has been isolated from several organisms including mammals (Almendral *et al.*, 1987; Matsumoto *et al.*, 1987; Yamaguchi *et al.*, 1991), insects (Yamaguchi *et al.*, 1990; Tammariello and Denlinger, 1998; Ruike *et al.*, 2006), higher plants (Lopez *et al.*, 1995, 1997; Strzalka and Ziemienowicz, 2007; O'Reilly *et al.*, 1989), marine phytoplankton (Guerini *et al.*, 2000), protozoa (Lin and Carpenter, 1998) and fungi (Bauer and Burgers,1990; Hamada *et al.*, 2002).

Xie *et al.* (2010) have investigated the PCNA in penaeid shrimp *Fenneropenaeus chinensis* and examined the response of immune tissues against bacteria (*V. anguillarum*) and white spot syndrome virus (WSSV). Zhang *et al.* (2010) have studied the molecular mechanism of gonadal development in *Marsupenaesu japonicus.*

In the present study, PCNA of white leg shrimp, *Litopenaeus* challenged with *Vibrio angullarium* has been cloned and expressed in immune related tissues of *L. vannamei.* The aim of this study was to have indepth knowledge of the effect of bacterial-stimulated responses of lymphoid organ, hepatopancreas, gill and muscle. The work will provide important contribtions to the existing knowledge of host pathogen relationship.

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0030-9923/2012/0004-1029 $ 8.00/0

MATERIALS AND METHODS

Experimental shrimps

Shrimps (*L. vannamei*), average size 12.25 cm and average body weight 15.3g, were obtained from Qingdao Fish Market and acclimatized in tanks before the experiment. The seawater was aerated continuously using airstones. The temprature was maintained at 17±1°C throughout the acclimatization and experiment period. Shrimps were fed with polychete twice a day. The sea water was renewed twice a day. The shrimps were divided into two groups (control and treated) in tanks.

Bacterial challenge

The bacterial challenge experiment was initiated by injecting each shrimp with 100 µl of *Vibrio anguillarum* (1.8×10^7 cells per mL). A similar control group of shrimp were injected with 100 µl of sterile PBS (pH 7.2).

The tissues including lymphoid organ, hepatopancreas, gills and muscle were dissected and frozen in liquid nitrogen for total RNA extraction. Four individuals were randomly sampled at each time point of 2, 6, 12, 24, and 48 h post-injection.

Total RNA extraction

The tissues were homogenised in D solution (Guanidine thiocyanate 48g, sodium lauryl sarcosinate 0.5g and 0.75mol/L sodium citrate 3.33ml pH 7) followed by phenol/chloroform extraction. Total RNA was precipitated in isopropanol washed with ethanol and dissolved in DEPC (diethyl pyrocarbonate) water. The concentration were measured with spectrophotometry and the integrety of RNA was checked on 1.2% agorase gel. The RNA was stored at -80°C until use (Qiu *et al.*, 2009).

First strand cDNA synthesis

The cDNA prepared from total RNA by Moloney Murine Leukemia virus transcriptase at 37°C for 15 min followed by 85°C for 5s with oligo-dT adaptor primer following the protocol of manufacture (a reverse transcription system (Promega) (Qiu *et al.*, 2009).

Cloning of full length LvPCNA and sequencing

The short fragment of *LvPCNA* was cloned using degenerated primers from conserved regions of availabe sequences and designed a pair of primers PCNA-F2 (TTGCCATCTTGTGAGTTTGC) and PCNA-R2 (TGCCTCCTCCTCCTTGTCTA). The obtained PCR product was separated by 1.2% agarose gel, and purified by PCR purification kit. The product was ligated with PMD18-T vector (Takara) and transfered into the competent cells (*E.coli* DH5α). The selected clones were screened with M13 forward and reverse primers, and the positive clones were sequenced by Huada Institute for Gene Research Center. The similarity analysis of *L. vannamei* PCNA (*LvPCNA*) with other known sequences was done using Blast programs (www.ncbi.nim.nih.gov/). The RACE amplification was conducted using the specific primers (CCTGCACAAAGGAAGGAGTC for 5' end and CAGATACGTGCGAACTCCCCAGAAG for 3' end) of *LvPCNA*. The PCR condition for RACE profile was 94°C, 4 min; 94°C, 30 s; 68°C, 30 s; 72°C, 1 min; 35 cycles; 72°C, 5 min. The target RACE product was purified, subcloned, sequenced, and assembled.

Statistical analysis

Data were presented as the mean ± standard error. Significant differences between means were tested using one-way analysis of variance followed by least significant difference tests, using the SPSS statistical package (version 13.0) at a significance level of $p<0.05$.

RT-PCR analysis of LvPCNA mRNA expression

The real time (RT) PCR analysis was performed on ABI 7500 real time detection system in the presence of SYBR-green. 152 bp fragment of PCR product was amplified using forward and reverse primers of *LvPCNA* (forward: TGTCGCTCGTGTCCCTCA and reverse: ACGGTGTCTGCGTTATCCTG). The total volume of 20 µl, containing 10 µl of 2 X SYBR Green Master Mix, 1 µl of diluted cDNA, 1 µl of each primer, 0.4 µl of ROX reference dye

(50X) and the total volume was adjusted with PCR graded water. The PCR profile was 95°C for 10 min; followed by 40 cycles of 95°C for15 s; 60°C, for 1 min. Each plate was run with the internal control (*β-actin*) gene as reference gene In the end of PCR analysis of amplification products was taken based on the dissociation curve. Data were analyzed using the 7500 System Sequence Detection Software Version 1.4.0.25 (PE Applied Biosystems, Foster City, CA, USA). The results were presented as fold transcription relative to that of the *β-actin* gene with the what is this $2^{-\Delta\Delta Ct}$ method.

RESULTS

Full length and phylogenetic analysis of Lv PCNA

LvPCNA had a total of 1131 bp nucleotide sequence which was deposited in GenBank (accession No: JN546075.1). The sequence analysis showed that there is a 112 bp 5'UTR, a 233 bp of 3'UTR and ORF of 786 bp which encodes 261 amino acids. Its molecular mass is 28.8 KDa and predicted isoelectric point (pl) 4.470. It has 24 strong basic amino acids, 40 strongly acidic amino acids, 93 hydrophobic amino acids and 67 polar amino acids. The protein blast (blastp) search of the NCBI showed that *LvPCNA* had high homology with other animals such as 98% with *M. japonicas*, 98% with *F. chinensis*, 84% with *Eriocheir sinensis*, 82% with S*podoptera frugiperda* and 80% with *Drosophila melanogaster*. Multiple alignment of *LvPCNA* showed that it was well conserved among other examined organisms; and the eukaryotic conserved domains of PCNA were also noted in the multiple amino acid sequence (Figs. 1, 2).

The constructed phylogenetic tree based on the amino acid sequence of PCNA indicated that *LvPCNA* has close evolutionary line with crustacean, followed by insects and comparatively less related with human (Fig. 3).

Tissue distribution

The real-time RT-PCR revealed that expression *LvPCNA* is present in all the investigated tissues and noted transcripts level is significantly high in muscle and gill than in lymphoid organ and hepatopancreas (Fig. 4). The expression level of muscle is 1.7 times higher than the hepatopancreas, while the comparison with gill showed no difference; similarly it is 1.3 times higher than lymphoid organ.

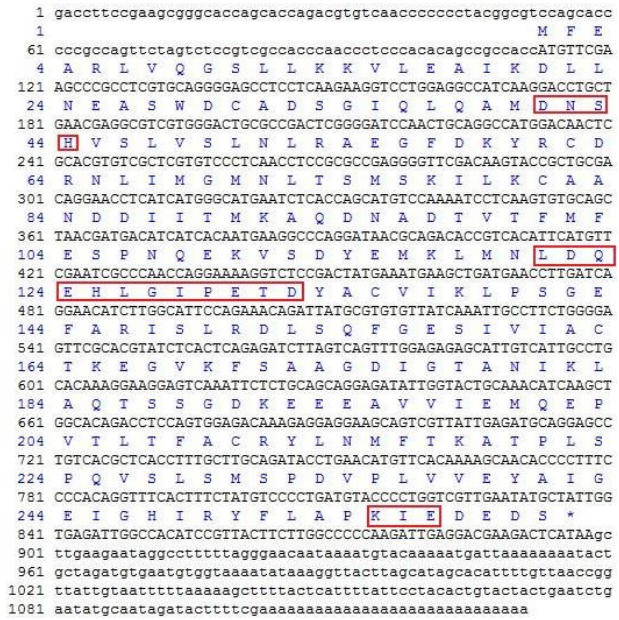

Fig. 1. Complete nucleotide and amino acid sequence for shrimp *LvPCNA*. The boxed areas show centre loop, interdomain connecting loop and C-terminal, respectively.

Expression level of LvPCNA in tissues challenged with bacteria

The expression of *LvPCNA* in hepatopancreas of *L. vannamei* after *Vibrio* challenge is shown in Figure 4. In hepatopancreas the highest expression was noted at 12 h and 48 h, while at other time points it showed inhibitory response.

DISCUSSION

PCNA has been cloned in several species including crustaceans, such as *F. chinensis*, *M. japonicus* and *Eriocheir japonica sinensis* (Chines mitten crab). We have successfully cloned PCNA in *L. vannamei* an important aquaculture species. The multiple alignment of *LvPCNA* showed that it has certain conserved domains of eukaryotic PCNA like interdomain connecting loop, a C terminal tail and

Center loop. These conserved domains were also discussed in *F. chinensis* (Xie *et al.*, 2008), *M. japonicas* (Zhang *et al.*, 2010) and *Eriocheir japonica sinensis* (Zha *et al.*, 2010). For replication and other nucleic acid metabolisms the domains are important (Tsurimot, 1998; Maga and Hubscher, 2003). The homology of *LvPCNA* and deduced amino acid analysis showed that it is closest to arthropods and it denoted high conservation among

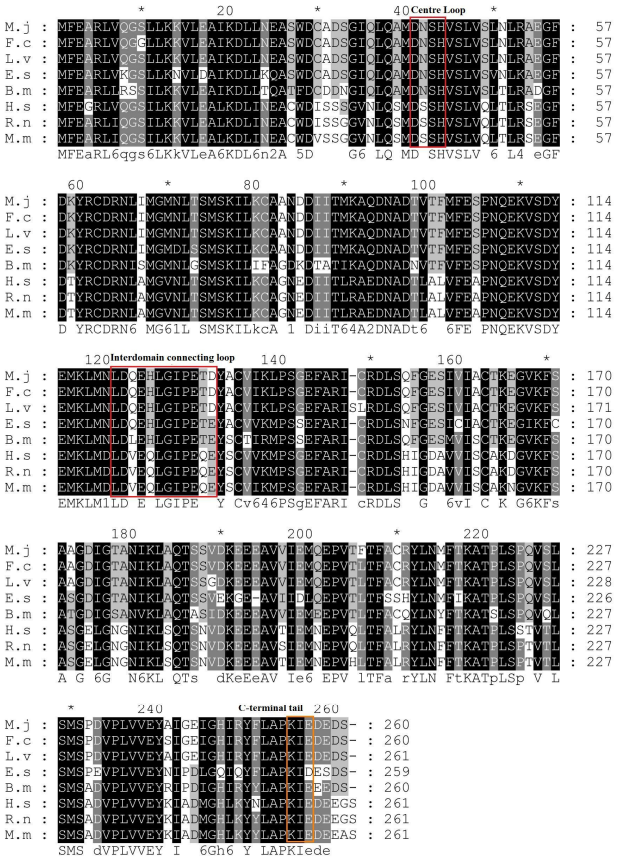

Fig. 2. Multiple alignment of the deduced amino acid sequence of the *LvPCNA* with other PCNAs. The three important domains are boxed. The other similar protein sequences of PCNA has obtained from GenBank data base. *Fenneropenaeus chinensis* (ABM668151.1), *Litopenaesus vannamei* (AEP83535.1), *Marsupenaeus japonicas* (ACA097181.1) *Eriocheir sinensis* (ACK58408.1), *Bombyx mori* (NP_001036825), *Homo sapiens* (NP_872590.1), *Rattus norvegicus*, (NP_071776.1) *Mus musculus* (NP_0351751). Residue in black background indicate higher levels of amino acid similarity.

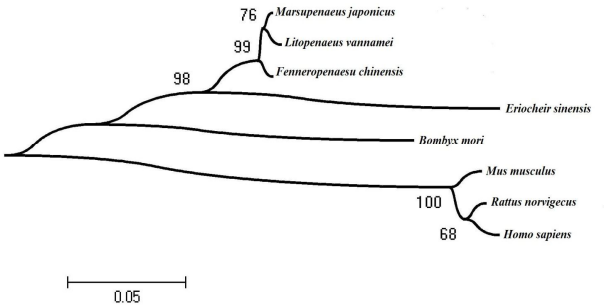

Fig. 3. A phylogenetic tree constructed with the neighbor- joining method. The tree is based on an alignment corresponding to full – length amino acid sequences, using ClustalX and megAlign. The numbers shown at the branches denote bootstrap majority consensus values of 1000 replicates. The Gene Bank accession numbers are same as given in Fig. 2.

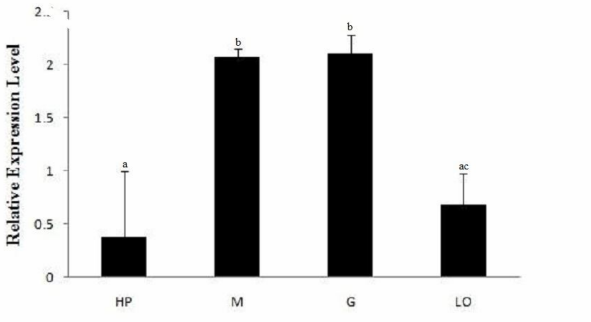

Fig. 4. Relative expression of *LvPCNA* in different tissues. HP, hepatopancreas; M, muscle; G, gill; LO, lymphoid organ.

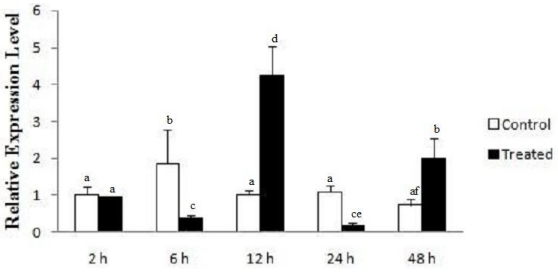

Fig. 5. Relative expression of *LvPCNA* after bacterial challenge in hepatopancreas at different time points.

other counterpart species. Earlier it has been reported (Xie *et al.*, 2008, Zhang *et al.*, 2010) that PCNA expression level in nonproliferating cells is either absent or is at low level (Xie e*t al.*, 2008).

Our analysis in *LvPCNA* suggest that the PCNA is well expressed in proliferating tissues. The present study support the existing knowledge of expression pattern in tissues of examined crustacean organism. In human the PCNA has role for prognosis of tumor and cancer development (Lee *et al.*, 1995) because of active role in proliferation. Besides crustacean and human, the proliferation of PCNA has also been examined by Liu *et al.* (2005) in dinoflagellate (*Prorocentrum donghaiens*) and in green algae (*Dunaliella salina*) and considered it as a marker of cell proliferation.

The present study suggests that PCNA is well expressed in gill, muscle, lymphoid organ and hepatopancrease respectively and these expression patterns are in accordance with *F. chinensis* (Xie *et al.*, 2008).

It is the first study of hepatopancreas after bacterial challenge. Earlier in *F. chinensis* (Xie *et al.*, 2008) the HPT investigated after *Vibrio anguillarum* challenge and reported up regulation at 6, 24, 72 and 96 h respective. In hepatopancreas the up regulation was observed after 12 and 48 h. The present experiment revealed that *LvPCNA* is involved in immune responses, however, in depth immune related experiments are still needed, which will be more important to know the immune role of PCNA in crustaceans.

ACKNOWLEDGEMENT

The first author is grateful to China Government Scholarship Council for Ph.D. scholarship. The present study is part of Ph.D. dissertation.

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(Received 7 April 2012, revised 23 May 2012)