



cDNA Cloning and Expression of Cyclophilin A (*LvCypA*) in White Leg Shrimp, *Litopenaeus vannamei*

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ABSTRACT

In present investigation the Cyclophilin A (CypA) of white leg shrimp (*Litopenaeus vannamei*) has been cloned by rapid amplification of cDNA (RACE) and anchored PCR method. The full length of the (*LvCypA*) had 855 bp, containing 495 bp of open reading frame (ORF), encoding 164 amino acids with an estimated molecular mass of 17.6 kDa. The *LvCypA* has four β - strands. Tissue distribution of the *LvCypA* after real time analysis revealed that expression is in order of muscle, gill, lymphoid organ and hepatopancrease.

Article Information

Received 13 June 2016

Revised 20 August 2016

Accepted 18 October 2016

Available online 04 May 2017

Authors' Contributions

FM performed experimental work. ZZF supervised experiment. SMY helped in collection and rearing. MS contributed in manuscript writing and RNA extraction.

Key words

Cloning, Cyclophilin A gene expression, Cyclophilin A, *Litopenaeus vannamei*.

INTRODUCTION

The Cyclophilin A (CypA) is conventional protein reported in bacteria, fungi, plants and mammals (Galat, 1999). The CypA associate with immunophilin family and it systematize into three categories such as cyclophilins (Cyp), FK binding proteins (FKBPs) and parvulins. The CycA proclaimed as cytosolic binding protein for cyclosporine A which is immune inhibitor (Handschumacher *et al.*, 1984), eventually different domains discerned because of its peptidylprolyl cis-trans isomerase and nuclease nature (Takahashi *et al.*, 1989; Fischer *et al.*, 1989; Montague *et al.*, 1994).

The CypA plays momentous task in protein folding, trafficking, assembly, cell signaling, apoptosis, resisting multifarious environmental stress, inflammation, auto-immune disease, rheumatoid arthritis, pathogenesis, vascular disease, hepatitis B and C and atherosclerosis. Furthermore, it also acknowledged to play profound function in the innate immunity system of aquatic animals (Yeh and Klesius, 2008; Qiu *et al.*, 2009; Chen *et al.*, 2011; Kratz *et al.*, 1992; Sherry *et al.*, 1992; Smart *et al.*, 1996; Ivery, 2000; Yurchenko *et al.*, 2002; Cande *et al.*, 2004; Jin *et al.*, 2004; Wang and Heitman 2005; Zhu *et al.*, 2007; Wohlfarth and Efferth, 2008; Yang *et al.*, 2008; Peng *et al.*, 2009; Satoh *et al.*, 2010).

The *Cyclophilin A* has been studied in several organisms including *P. monodon*, *Chlamys farreri*, *Eriocheir sinensis*, *Artemia franciscan*, *Venerupis philippinarum*, *Danio rerio*, *Ictalurus punctatus* and *Clonorchis sinensis*.

Litopenaeus vannamei is a prime aquaculture species and it covers approximately 90% of the farmed shrimps in western hemisphere (Wurmann *et al.*, 2004). The estimated annual production of farmed *L. vannamei* from Asia in 2002 was 316000 mt (Balakrishnan *et al.*, 2011). In China, *L. vannamei* introduced in 1988 for experimental purposes and by 1998 it has been successfully cultured on a commercial scale by then over 1000 hatcheries of *L. vannamei* are in operation (Qing and Hai, 2005). Nevertheless, the steady growth of shrimp farming significantly affected by increasing trends of intensive culture, environmental problems and disease producing microorganisms (Tanticharoen *et al.*, 2008), which has resulted in an enormous economic loss. Like other invertebrates, the absence of acquired immunity in shrimp makes them exclusively to rely on the innate immune system for protection against bacteria which are very abundant in the marine environment (Austin, 1988).

In order to achieve sustainable aquaculture, the understanding of shrimp's immunity is imperative (Qiu *et al.* 2009). In the present study, *LvCypA* of white leg shrimp, *Litopenaeus vannamei* has been cloned and expressed in important tissues. The aim of this study was to have in-depth knowledge of CypA in this commercially important species. The work will provide important contributions for

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further host pathogen relationship.

MATERIALS AND METHODS

Total RNA extraction

To isolate the total RNA, 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 extraction in phenol/chloroform. The extract was precipitated in isopropanol, washed with ethanol and dissolved in DEPC (diethyl pyrocarbonate) water. The concentration were measured with spectrophotomet and the integrity of RNA was checked on 1.2% agarose gel. The RNA was stored at -80 °C until use.

First strand cDNA synthesis

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

Cloning of full length LvCypA and sequencing

The short fragment of *Cyp A* was cloned using degenerated primers, Forward 5'ACNGGHGARAARGGHTTYGG 3' and reverse primer 5'GTG WTGGGKCCRGCRITDGC 3' from conserved regions of available sequences in GenBank database such as *P. monodon* (ABV90639.1), *Chlamys farreri* (Ay363759.1), *Homo sapiens* (BC007104.1), *Ixodes scapularis* (DQ066345) and *Danio rerio* (AY 391451.1). The obtained PCR product was separated on 1.2% agarose gel, and purified by PCR purification kit. The product was ligated with PMD18-T vector (Takara) and transferred into the competent cells (*E. coli* DH5 α). 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 CypA* (*LvCypA*) with other known sequences was done using Blast programs (www.ncbi.nlm.nih.gov/). After isolation of partial sequence, the RACE technique was used to clone the left part of sequence namely, 5' and 3' ends of *LvCypA* cDNA. The gene specific primers (GSP1 5' TCTACAAGGGCTCGTGCTTCCAC 3' and GSP2 5'GCTTCAGAGCGAAGTTCTCGTCCTC 3') were used. The PCR condition for fragment sequence was 94 °C for 4 min, 94 °C for 30 s, 54 °C for 30 s, 72 °C 30 s, 30 cycles; 72 °C 5 min. The RACE PCR 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.

Similarities of nucleotide and amino acid sequences of *LvCypA* were analyzed using BLAST programs at NCBI website (www.blast.ncbi.nlm.nih.gov/Blast) and

(<http://web.expasy.org/protparam/>). The protein domain features were determined by (<http://www.smart.embl-heidelberg.de/>). The glycosylation were performed with the (www.comp.chem.nottingham.ac.uk/cgi-bin/glyco/bin/getparams.cgi). The signal peptide was predicted with the Signal P-4.0. (www.cbs.dtu.dk/services/services/SignalP-4.0/output.php). Using Clustal X software, the *LvCypA* deduced amino acid sequence was compared with other known sequences of *CypA* available in GenBank database. The phylogenetic tree constructed with MEGA 4.0 software by the neighbour- joining (NJ) method.

RT-PCR analysis of LvCypA mRNA expression

The real time 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 primer, RT1 5' TCGCAGTTCTTCATCTGCAC 3' and RT2 5'AGTTGGCGATCACCCTTTC 3'. The total volume of 20 μ l containing 10 μ l of 2 X SYBR Green master mix, 1 μ l of diluted cDNA, 1 μ l of each primer, 0.4 μ l 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 for 15 s, 60 °C for 1 min. Each plate was run with the internal control (β -actin) gene as reference gene (primer for β -actin F 5' GCTAACCGCGAGAAGATGAC 3' R 5' CAGGGCATATCCCTCGTAGA 3'). 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 $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were presented as the mean \pm 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 $p < 0.05$.

RESULTS

Full length and phylogenetic analysis of LvCypA

LvCypA had total of 855 bp nucleotide sequence which was deposited in GenBank (accession No: JN546074.1). The sequence analysis showed that there is an open reading frame (ORF) of 495 bp which encodes 164 amino acids with an estimated molecular mass of 17.62012 kDa and predicted isoelectric point (pI) 8.253. It has 17 strong basic amino acids, 14 strongly acidic amino acids, 52 hydrophobic amino acids and 43 polar

amino acids. In 3' UTR (untranslated region), the sequence has polyadenylation signal (AATAAA), with a poly (A) tail (Fig. 1). The software analysis showed that there is no putative signal peptide. The glycosylation prediction showed that LvCypA is characterized by presence of six N-glycosylation sites (N³, N⁷¹, N¹⁰⁶, N¹⁰⁸, N¹⁴⁹ and N¹⁶⁰). The deduced amino acid sequence revealed the LvCypA has four β - strands at position of 49-56, 60-64, 96-103 and 111-116 respectively (Fig. 1). The peptidylprolyl cis-trans isomerase signature existed in the LvCypA, which is located in 48-56 sites, respectively (Figs. 1, 2).

The protein blast (blastp) search of the NCBI showed that LvCypA has high homology with other animals such as, *P. monodon* (100% similarity), *Eriocheir sinensis* (100% similarity), *Chlamys farreri* (100% similarity), *Artemia franciscan* (100% similarity), *Aedes aegypti* (100% similarity), *Argopecten irradians* (100% similarity), *Ixodes scapularis* (100% similarity), *Xenopus laevis* (98% similarity) (Table I).

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

Table I.- Homology cyclophilin A between the white shrimp *L. vannamei* and others.

Species name	Identity	Similarity	E-value	GenBank accession No
<i>Penaeus monodon</i>	96%	100%	9e-89	ABV90639.1
<i>Eriocheir sinensis</i>	87%	100%	5e-80	AEC48729.1
<i>Chlamys farreri</i>	76%	100%	2e72	AAR11779.1
<i>Artemia franciscan</i>	79%	100%	2e-72	ABN13586.1
<i>Aedes aegypti</i>	76%	100%	3e-72	ABF18058.1
<i>Argopecten irradians</i>	77%	100%	5e-72	ABM92916.1
<i>irradians</i>	80%	100%	1e-71	AAV66982.1
<i>Ixodes scapularis</i>	76%	98%	2e-70	AAI53776.1
<i>Xenopus laevis</i>				

1	1	tacatggggagtgccgcctcacgtactcttgcgagcagacccacaccaacttagccaccA	M
2	G	N	P K V F F D I A A D N Q P V G R I V
61	TGGGCAATCCCAAAGTCTTTTTCGACATTGCCGCTGACAACCAGCCCGTTGGCAGGATCG		
22	M E L R A D V V P K T A E N F R S L C T		
121	TCATGGAGCTCCGCGCCGACGTGGTCCCAAGACGGCCGAGAACTTCCGGTCGCTGTGCA		
42	G E K G F G	Y K G S C F H R V I P N F M	
181	CGGGCGAGAAGGGCTTCGGCTACAAGGGCTCGTGCTTCCACCGCGTGATCCCCAACTTCA		
62	C Q G G	D F T A G N	G T G G K S I Y G N
241	TGTGCCAGGGCGGCGACTTCACCGCCGGCAACGGCACGGGCGGCAAGTCCATCTACGGCA		
82	K F E D E N F A L K H T G P G I L S M A		
301	ACAAATTCGAGGACGAGAACTTCGCTCTGAAGCACACCGGCCCCGGCATCCTGTCCATGG		
102	N A G P	N T N	G S Q F F I C T V K T S W
361	CCAACGCCGGCCCCAACCAACGGGTGCGAGTTCTTCATCTGCACCGTCAAAACCTCCT		
122	L D N K H V V F G T V V E G M D V V R Q		
421	GGCTGGACAACAAGCACGTGGTCTTCGGCACCGTGGTGGAGGGCATGGACGTCGTGCGCC		
142	V E G F G T P	N	G S C K R K V V I A N C
481	AGGTCGAGGGCTTCGGCACGCCAACGGCTCGTGCAAGCGGAAAGTGGTGATCGCCAACT		
162	G Q L *		
541	GCGGCCAGCTGTAAagtctcagaacattccgccttagccgcccacagttttttttttctg		
601	atgtaattgaggatccaggatataatctttgctgtattggcacttcagtggttaaatttcg		
661	gcttgaaaatttaatgctatataacgtaaagggtggtgaaacaggataggtgttcttccat		
721	tttttttgttttattagtttcataagtgggtcatgttctggaatggtgacgcattatgct		
781	gatattcagcatttctgtcttctcacttcataataataacccaacaaccccccccccaaaa		
841	aaaaaaaaaaaaaaaa		

Fig. 1. Sequences of nucleic acid and deduced amino acid from the white leg shrimp *L. vannamei* Cyclophilin A. The polyadenylation signal sequence is underlined, the asterisk shows the stop codon. The red box is showing the Cyp- type peptidylprolyl cis-trans isomerase signature. The black boxes are showing the glycosylation sites.

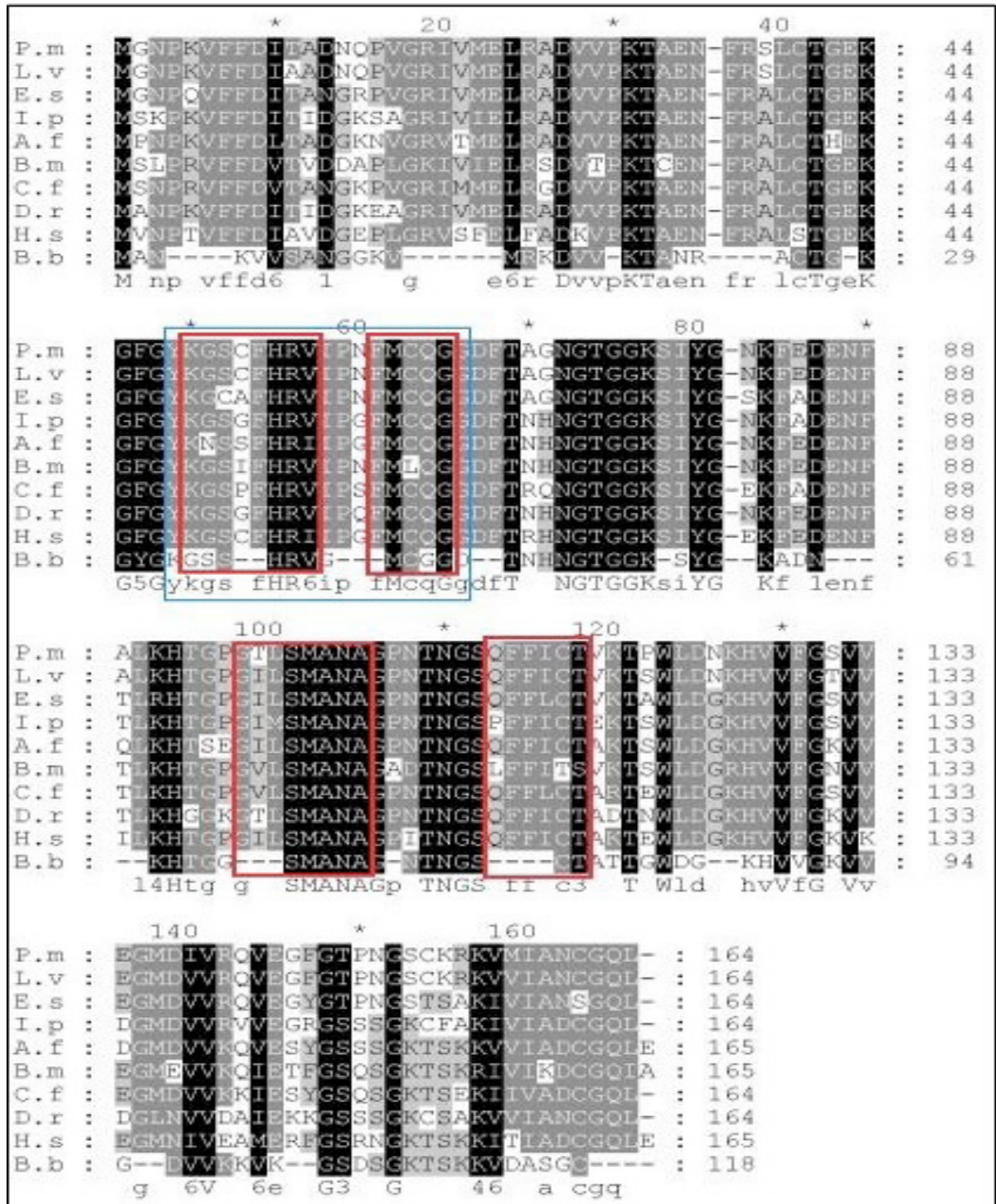


Fig. 2. ClustalX alignment and comparison of the amino acid sequences deduced from *Lv-CypA* cDNA with some similar protein sequences of cyclophilin A. The other similar proteins are predicted and obtained from GenBank as follows: *Penaes monodon* (ABV90639.1), *Eriocheir sinensis* (AEC48729.1), *Chlamys farreri* (AAR11779.1), *Artemia franciscana* (ABN13586.1), *Bombyx mori* (NP_001037301.1), *Ictalurus punctatus* (NP_001187167.1), *Beauveria bassiana* (AAN39296.1), *Danio rerio* (CAX13413.1), *Homo sapiens* (AAU13906.1). Residues in black background indicate higher level of amino acid similarity. The four red boxes are showing the beta strands while the blue box is showing the existence of cis-trans isomerase.

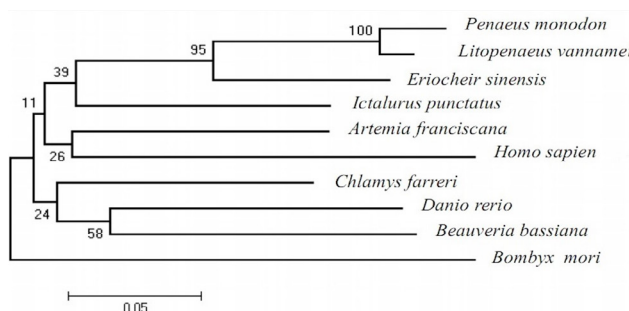


Fig. 3. A phylogenetic tree constructed with the neighbor-joining method. The tree is based on an alignment corresponding to full length amino acid sequences, using ClustalX and megAlign. The numbers shown at the branches denote bootstrap majority consensus values of 1000 replicates. The GenBank accession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

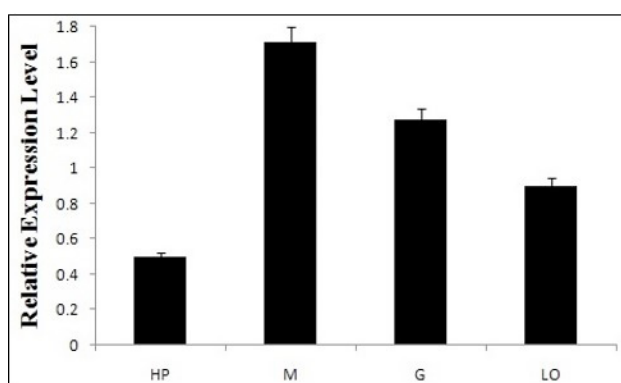


Fig. 4. Expression of *LvCypA* in different tissues of shrimp. The abundance of *LvCypA* mRNA was normalized with β -actin. Vertical bars representing the mean value error. HP, hepatopancreas; M, muscle; G, gill; LO, lymphoid organ.

Tissue distribution of *LvCypA* mRNA

The real time RT-PCR revealed that expression *LvCypA* is ubiquitous in all the investigated tissues and noted transcripts level is significantly higher in muscle than in lymphoid organ and hepatopancreas ($P < 0.05$). The expression level of muscle is 1.2 fold higher than the hepatopancreas, 0.4 times higher than the gill and 0.8 times higher than lymphoid organ (Fig. 4).

DISCUSSION

In present investigation a Cyclophilin A gene cloned from white leg shrimp (*Litopenaeus vannamei*) by rapid amplification of cDNA (RACE) and anchored PCR method. The sequence has the similar characters for

basic structural function of Cyclophilin A, the cyclophilin type peptidyl-prolylcis-trans isomerases signature and conserved amino acid residues are similar with already investigated CypA in different species (Howard *et al.*, 2003; Piotukh *et al.*, 2005; Eisenmesser *et al.*, 2002; Qiu *et al.*, 2009; Chen *et al.*, 2011). The *LvCypA* has polyadenylation signal (AATAAA) which is in accordance with the *P. monodon* (Qiu *et al.*, 2009) and *V. philippinarum* (Chen *et al.*, 2011), *Dasyatis akajei* (Tu *et al.*, 2003) and *Griffithsia japonica* (Lee *et al.*, 2002). However, in some species, the polyadenylation signal is not present such as CypA of *Blatella* (Martinez-Gonzalez and Hegardt, 1995) and CypA of *Xenopus laevis* (Masse *et al.*, 2004). It has been demonstrated that it is often related with alternative of tissue specific polyadenylation (Zhao *et al.*, 1999). The Cyclophilin A is a receptor for immunosuppressor agent cyclosporin A. The Cyclosporin A is a cis-trans peptidyl-prolyl isomerase (PPIase) which carry on the functions of different proteins by getting in contact with target proteins, actually PPIase catalyzes the cis-trans peptidyl-prolyl isomerization of prolyl-peptide bonds (Sherry *et al.*, 1992; Walsh *et al.*, 1992; Fruman *et al.*, 1994).

The results of aligned sequences indicated that deduced amino acid sequence of *LvCypA* was homologous with reported cyclophilin A genes available in GenBank database. Glycosylation predication revealed six N-glycosylation sites in *LvCypA*, which were neither discussed in *P. monodon* (Qiu *et al.*, 2009) nor in any other literature on cyclophilin A. The protein predication sites revealed that it has no signal peptide region which is consistent with the analysis in *P. monodon* (Qiu *et al.*, 2009).

The distribution of *LvCypA* in normal tissues of hepatopancreas, gill, lymphoid organ, and muscle were measured. The results suggested that *LvCypA* is ubiquitously expressed. The highest level was in muscle followed by gill and lymphoid organ while in hepatopancreas the expression was detected in lower level. The expression level of normal tissues of *LvCypA* is in disagreement with that of *P. monodon* (Qiu *et al.*, 2009), such variation of *cyclophilin A* expression were also reported in other species such as in *D. akajei* (Tu *et al.*, 2003) and in *X. laevis* (Masse *et al.*, 2004).

CONCLUSION

In conclusion, the full-length cDNA of *LvCypA* were cloned from *L. vannamei*, and the deduced amino acid sequence was highly similar to amino acid residues reported from other species. *LvCypA* is ubiquitous. The present result provides the valuable information about the Cyclophilin A protein in *L. vannamei* and suggested to be

challenged with different pathogens in order to know its immune function.

ACKNOWLEDGEMENT

Thanks to China Government Scholarship council for providing PhD Scholarship.

Conflict of interest statement

We declare that we have no conflict of interest.

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