

Cloning of profilin (FcPFN) from the shrimp *Fenneropenaeus chinensis*, a highly expressed protein in white spot syndrome virus (WSSV)-infected shrimp

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Abstract. We isolated and characterized the profilin (FcPFN) cDNA from hemocytes of *Fenneropenaeus chinensis*, a unique shrimp species from the Yellow Sea. The FcPFN cDNA consists of 830 bp and encodes a polypeptide of 125 amino acids, having a predicted isoelectric point of 5.06. The deduced amino acid sequence of FcPFN shows 36% and 90% amino acid sequence identity to the profilin genes of Pacific white shrimp *Litopenaeus vannamei* and black tiger shrimp *Penaeus monodon*, respectively. The FcPFN mRNA was highly expressed in hemocytes and hepatopancreas and moderately in muscle of normal shrimp. The higher expression of FcPFN mRNA is observed in shrimp infected with the white spot syndrome virus (WSSV), which is a major concern in all shrimp-growing regions of the world. These results suggest a potential role for FcPFN in viral host defense mechanisms.

Keywords: *Fenneropenaeus chinensis*, host defense, profilin, shrimp, white spot syndrome virus.

Introduction

Profilin is a small actin-binding protein found in eukaryotic cells and is critical for cytoskeletal dynamics (Critchley et al. 1999; Pollard 2008). Profilins are potent regulators of actin filament dynamics, by promoting the exchange of ADP to ATP on actin and by the affinity of profilin–actin complexes for actin filament ends (Buss et al. 1992). Profilin can either inhibit or enhance polymerization of actin filaments (Sohn and Goldschmidt-Clermont 1994).

Profilins have a role in cellular processes, such as membrane trafficking, small-GTPase signaling and nuclear activities, neurological diseases, and tumor formation (Birbach 2008; Rawe et al. 2006; Witke 2004). Genetic studies have shown the importance of profilins for cell proliferation and differentiation. Profilin gene disruption leads to grossly impaired growth, motility and cytokinesis,

and embryonic lethality in multicellular organisms, such as insects and mice (Verheyen and Cooley 1994; Haugwitz et al. 1994; Magdolen et al. 1988). Profilin-mutant hemocytes in *Drosophila* exhibited an increased phagocytic activity, showing that profilin is a critical regulator of phagocytosis in *Drosophila* (Pearson et al. 2003). Human breast cancer cell lines contain less profilin than normal breast epithelial cells, and raising the intracellular profilin level by transfection abolishes tumor growth in nude mice, suggesting that profilin plays a role as a tumor suppressor (Janke et al. 2000; Wittenmayer et al. 2004).

A series of studies indicated that profilin, like other actin-binding proteins, might be involved in controlling T-lymphocyte activation and effector functions, through participating in the reorganization of cytoskeleton dynamics at the immune synapse (Billadeau and Burkhardt 2006). There is

Received: February 20, 2009. Accepted: April 4, 2009.

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some indirect evidence of a role of profilin in invertebrate immune response. Smith et al. (1995) reported that sea urchin coelomocytes respond to immune challenge from lipopolysaccharide with significant elevations in profilin transcripts. Transcriptomic analysis of gills from the Pacific white shrimp *Litopenaeus vannamei* infected with white spot syndrome virus (WSSV) revealed an increase in the profilin mRNAs level at 6 h after infection (Clavero-Salas et al. 2007). In plant cells, the actin and actin-binding partners (including profilin) performed reorganization of the cytoskeleton in response to external and internal cues, and thus regulates several vital cellular processes, including reactions to the pathogen attack and wounding (Hussey et al. 2006; Staiger and Blanchon 2006).

In this study, we isolated and characterized the cDNA sequences of a profilin from the shrimp *Fenneropenaeus chinensis*, a unique shrimp species from the Yellow Sea. We suggest a possible role for profilin in the immune response of the shrimp through an increase in profilin expression by white spot syndrome virus (WSSV) infection.

Materials and methods

Sample collection

Fleshy prawn, also called Chinese white shrimp or oriental shrimp (*Fenneropenaeus chinensis*), were caught at Chaeseokpo in Taean-gun, Chungnam, Republic of Korea. The shrimp weighed between 54.94 and 63.70 g. Hemolymph was withdrawn from the ventral sinus of each shrimp into a 1-mL sterile syringe (25-gauge) containing anticoagulant solution (30 mM trisodium citrate, 340 mM sodium chloride, 10 mM EDTA, 115 mM glucose, pH 7.55). Hemocytes were collected by centrifugation (800 × g, 10 min, 10°C), and total RNA was isolated with TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions. Hepatopancreatic and muscle tissues were dissected from 5 shrimp and dispersed in TRIzol® reagent using a homogenizer.

Detection of WSSV infection

WSSV-free or WSSV-infected shrimp were obtained from a farm in Pyoungtaek-si, Gyeonggi-do, Republic of Korea. All Shrimple® tests were performed according to the manufacturer's protocol (EnBioTec Laboratories; Tokyo, Japan). In short, the pleopods removed from the

shrimp were placed in a microcentrifuge tube filled with grinding buffer and processed using a tissue grinder. After applying 3 drops of the supernatant to the test strip, the development of pink bands in both zones indicates a valid test and the presence of white spot syndrome virus (WSSV) (Powell et al. 2006). For the detection of WSSV infection using the polymerase chain reaction (PCR), genomic DNA was prepared from pleopods by using 8M TNEs-urea buffer and purified with phenol/chloroform (Asahida et al. 1996). The primers WSSV-P3 (5'-TCT TCA TCA GAT GCT ACT GC-3') and WSSV-P4 (5'-TAA CGC TAT CCA GTA TCA CG-3') were used to amplify the WSSV genome fragment (Kimura et al. 1996).

Sequencing and phylogenetic analysis

The plasmids from the cDNA library of *F. chinensis* hemocytes were isolated using a Plasmid Miniprep Kit (Qiagen, USA) and subjected to sequence analysis using both the T7 forward and SP6 reverse primers (Promega, USA) in an automatic sequencer (ABI3730xl, Applied Biosystems Inc., USA). The sequence was subjected to homology analysis with sequences from the NCBI database using the BlastX software (<http://www.ncbi.nlm.nih.gov/BLAST/>) of GenBank. Multiple sequence alignments generated using ClustalW (<http://align.genome.jp/>). A phylogenetic tree was performed with the MEGA3.1 (<http://www.megasoftware.net>) and neighbor-joining (NJ) algorithm. The reliability of the branching was tested by using bootstrap re-sampling (1000 pseudo-replicates).

Real-time RT-PCR

Total RNA was extracted from hemocytes, hepatopancreas, and muscle of shrimp by using the TRIzol® reagent. The first-strand cDNA synthesis was carried out using the Advantage RT-for-PCR Kit (BD Biosciences, USA). Real-time PCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Roche, Germany) and the following forward and reverse primers: FcPFN-F (5'-GTT GAG TGG GGA GAG G-3'), FcPFN-R (5'-GTA AGA GAG GGT CAG AGG A-3'), β -actin-F (5'-CAG ACT ACC TGA TGA AGA TCC-3'), and β -actin-R (5'-ATT CCA TGC CCA AGA ATG A-3'). Following an initial 10-minute Taq activation step at 95°C, LightCycler PCR was conducted under the following cycling conditions: 95°C for 15 s, 55°C for 5 s, 72°C for 15 s, and fluorescent reading.

Results and discussion

Identification of shrimp FcPFN cDNA

We isolated the EST clone, 23-32-p, from a cDNA library of *F. chinensis* hemocytes, which shares 90% amino acid identity with the profilin of black tiger shrimp *Penaeus monodon* (GenBank accession no. ABU97474). The shrimp EST clone was named *Fenneropenaeus chinensis* FcPFN (GenBank accession no. FJ480175). The FcPFN cDNA is composed of 830 bp, consisting of a 5'-UTR of 68 bp, an open reading frame of 378 bp, and a 3'-UTR of 384 bp with a poly(A) tail (Figure 1). The deduced amino acid sequence of the FcPFN cDNA contains 125 amino acids with a theoretical mass of 13.75 kDa and an expected pI value of 5.06. Analysis using NetPhos2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>) revealed that Tyr6, Thr39, and Ser63 residues are the possible phosphorylation sites. Bovine profilin is phosphorylated on its C-terminal Ser by protein kinase C (PKC)-zeta, resulting in downregulation of PI3-kinase (Vemuri and Singh 2001).

Phosphorylation of profilin modulates its activity, affecting the cytoskeletal dynamics.

Sequence analysis of the shrimp FcPFN

The deduced amino acid sequence of the shrimp FcPFN was compared to profilins isolated from other known species (Figure 2). Sequence alignment showed that FcPFN had 36, 36.8, 39.2, 40, and 40.8% amino acid identity with profilins isolated from the Pacific white shrimp *Litopenaeus vannamei* (GenBank accession no. ABI93174), honey bee *Apis mellifera* (AAS50159), yellow fever mosquito *Stegomyia aegypti* (EAT34395), Japanese lancelet *Branchiostoma belcheri* (AAL75808), and domestic silkworm *Bombyx mori* (AAT99314), respectively. In addition, FcPFN showed 29.6% and 32% amino acid identity to plant profilins of the common tobacco *Nicotiana tabacum* (CAA57632) and soybean *Glycine max* (ABG88188), respectively. A phylogenetic relationship of FcPFN with profilins of other species was investigated using MEGA3.1 software (Figure 3). FcPFN was most closely related to that of black tiger shrimp.

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      10      20      30      40      50      60
CAGTGTGAAGCGCCAGCACGTCACACATTCTCTACCTCGACTTTTGGACCTGTAATTC
      70      80      90     100     110     120
TTACAAAGATGTCTTTGGGATCAGTACGTAAGCAAACAGCTGGTGGAACTCTGGAAACGTGA
      M S W D Q Y V S K Q L V E S G N V K
     130     140     150     160     170     180
AGATGGGAGCCATCTGCGGCTTGGATGGGTCTGTATGGGCCGCTCGCCCGATCTAAAGA
      M G A I C G L D G S V W A A S P D L K I
     190     200     210     220     230     240
TCACGGGGACGAGGTGAAGACCATCGCCAACAACCTCGGGACTGACAACTTTAACACCA
      T R D E V K T I A N N F G T D N F N T S
     250     260     270     280     290     300
GTGGTGTATGTTGAGTGGGGAGAGGTTCTGTTCCTGAGGGCCGAGGAAGGCAACTTGC
      G V M L S G E R F V F L R A E E G N L R
     310     320     330     340     350     360
GGGCGAAGAAGGGCAAAAAGTTCCTGCACATCACCAGACCAACACCGCCCTCATCATGG
      A K K G K K F L H I T K T N T A L I M G
     370     380     390     400     410     420
GCATTAGTGAGGAACCCATACAGCCCGGCTGTTGCACGTCACCGTCGAGGCCCTGGGCG
      I S E E P I Q P G C C T C T V E A L G D
     430     440     450     460     470     480
ACTACCTAAAAGGCCTTAACTACTAGTTTCCCTGGTTCAAATTTCGTCTCTCTGACCC
      Y L K G L N Y *
     490     500     510     520     530     540
TCTCTTACATCTTCCAGCTTCTCTTTTCATCTTCTTCTCCCTTCATCATCTTCTTCTGT
     550     560     570     580     590     600
GCTATGGGTCGATTTTAAACCATTTTCTTTATTTCCGTTTCCATATAATTTTGTTGTG
     610     620     630     640     650     660
ACTGATCAATTGTGGCACTTTAAAAAGATAAAACACAGGATTAATAATTTCCAGCTTGAAT
     670     680     690     700     710     720
TTCCTTTTGAATTAAAGTAAAGTGGTAAATTGCACTGGCAAAAGGTC TACTGATCATTTTA
     730     740     750     760     770     780
CTGCCACGCAATGTAAACAAATTATTAAGCTTGTGATATTATCTATTTCCTACCGATTAC
     790     800     810     820     830
ATTCAATAAAAAATGAATTTCCAAAAA

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Figure 1. cDNA and predicted amino acid sequences of fleshy prawn *Fenneropenaeus chinensis* FcPFN (GenBank accession no. FJ480175). Nucleotide sequence is shown in the upper line and the deduced amino acid sequence is in the lower line. Start and stop codons are shown in bold.

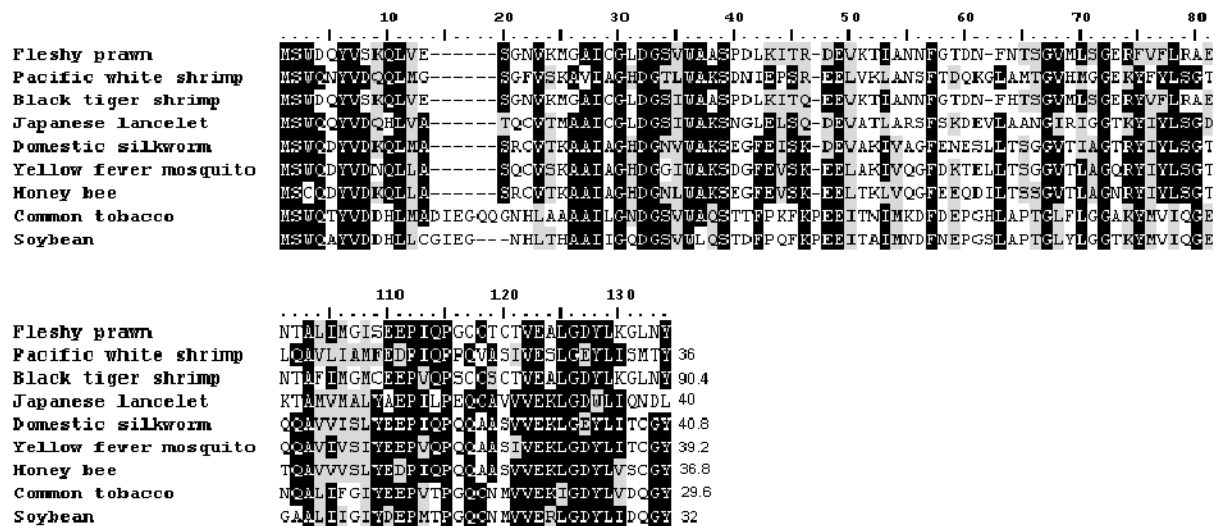


Figure 2. Multiple alignment of amino acid sequence of FcPFN from *F. chinensis* with those of other profilins from the black tiger shrimp (GenBank accession no. ABU97474), Pacific white shrimp (ABI93174), Japanese lancelet (AAL75808), domestic silkworm (AAT99314), yellow fever mosquito (EAT34395), honey bee (AAS50159), common tobacco (CAA57632), and soybean (ABG88188). Identical amino acids are highlighted by black boxes.

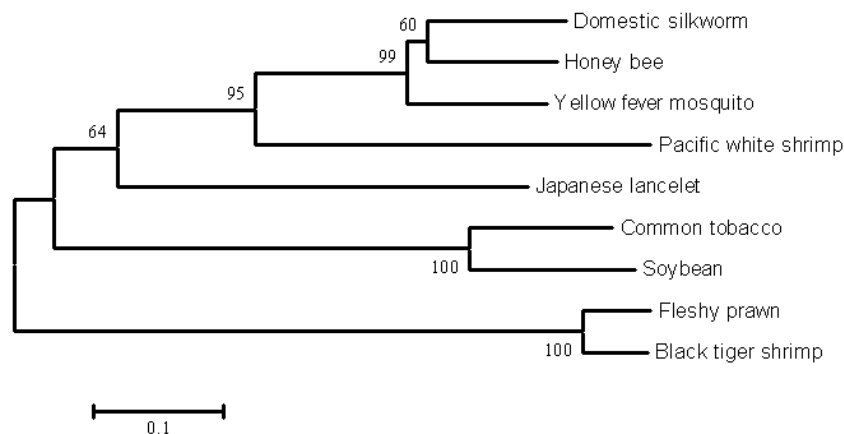


Figure 3. Phylogenetic relationship between the FcPFN of *F. chinensis* and profilins of other known species. A phylogenetic tree of the aligned sequences was constructed using the neighbor-joining (NJ) algorithm within MEGA3.1. The degree of confidence for each branch point was determined by bootstrap analysis (1000 repetitions).

Expression analysis of shrimp FcPFN

To examine the tissue distribution of FcPFN transcript, quantitative real-time RT-PCR was performed using total RNA from hemocytes, hepatopancreas, and muscle of normal shrimp. FcPFN mRNA was expressed highly in hemocytes and hepatopancreas, and moderately in muscle (Figure 4A). This is consistent with previous results that mouse profilin I is expressed at high levels in the spleen, thymus, brain, heart, uterus, and platelets, but not in skeletal muscle (Witke et al. 1998).

Increase in shrimp FcPFN expression by WSSV infection in shrimp hemocytes

White spot syndrome virus (WSSV) is the causative agent of a disease that has led to severe mortalities of cultured shrimp in many countries (Flegel 1997; Lightner 1996; Lo et al. 1999; Park et al. 1998). WSSV infection is a major factor in all shrimp-growing regions of the world, and the existence of the virus in wild shrimp populations has raised serious concern about the impacts of WSSV on the environment and native aquatic species. To investigate whether the expression of FcPFN mRNA is affected by WSSV infection,

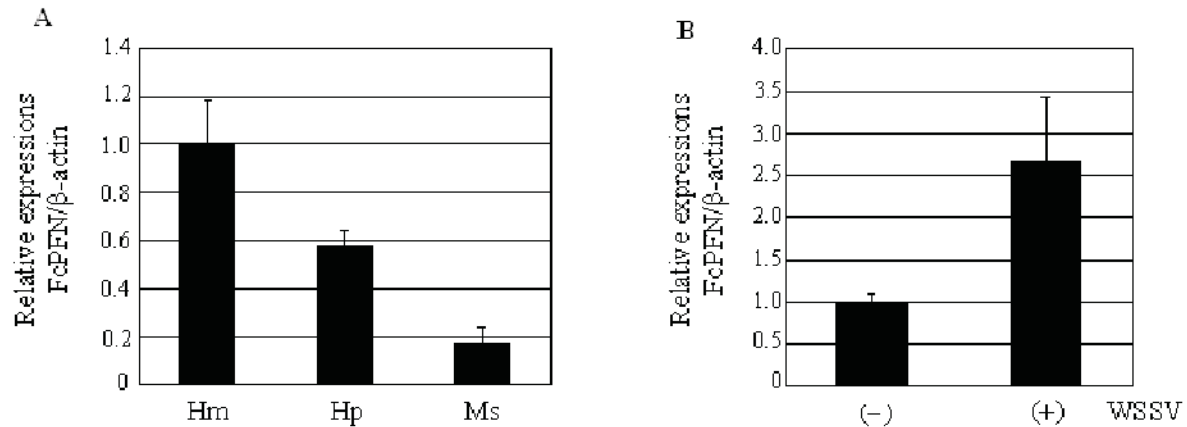


Figure 4. Real-time RT-PCR analysis of the expression of FcPFN mRNA. (A) Expression of FcPFN mRNA in shrimp tissues. The levels of FcPFN transcript were expressed relative to the β -actin transcript level. Expression levels were calculated relative to the level of FcPFN in hemocytes (arbitrarily defined as 1). Hm = hemocytes; Hp = hepatopancreas; Ms = muscle. (B) Increase in FcPFN mRNA by the WSSV infection in shrimp hemocytes. The levels of FcPFN transcript were expressed relative to the β -actin transcript level. Expression levels were calculated relative to the level of FcPFN in WSSV-free sample (arbitrarily defined as 1).

quantitative real-time RT-PCR was done using hemocytes from WSSV-free or WSSV-infected shrimp *F. chinensis*. Hemocytes of WSSV-infected shrimp expressed higher levels of the FcPFN mRNA when compared to those of WSSV-free shrimp (Figure 4B), which is consistent with the increase in profilin mRNAs level in the Pacific white shrimp *L. vannamei* infected with WSSV (Clavero-Salas et al. 2007). Profilin is involved in microglial activation associated with morphological change, migration, and phagocytic behavior of microglial cells, which acts as the first and main form of active immune defense in the central nervous system (CNS) (Dong et al. 2004). These results suggest FcPFN in hemocytes might be involved in viral host defense mechanisms in shrimp.

Although we suggest the possible role of FcPFN in viral host defense system, there are different concerns regarding the role of profilins. Plant profilins are regarded as pan-allergens because they exhibit significant cross-reactivity to IgE (Scheurer et al. 2001). In the innate recognition of the pathogen by the host, the protozoan *Toxoplasma gondii* profilin activates dendritic cells through TLR11 and is the first chemically defined ligand for this TLR (Yarovinsky et al. 2005). The biological activity of FcPFN from *F. chinensis* should be examined to define the role of FcPFN.

Acknowledgments. We thank Drs. H.-S. Han and I.-K. Jang for their help in obtaining shrimp samples. This work was supported by a grant from the National Fisheries Research and Development Institute (NFRDI), Republic of Korea.

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