Research Article

DNA Binding Activity of Marine Shrimp LvProfilin

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Abstract
Shrimp farming is an important business in Thailand and worldwide. The study of molecular biology and biochemical pathway of the key molecules controlling muscle growth is essential to improve shrimp livestock. Profilin is a pivotal protein in muscle formation, especially actin protein. Its nuclear function has been reported in many species for gene regulation. Here in this work, we characterized the function of LvProfilin, marine shrimp profilin from *Litopenaeus vannamei*, both *in silico* and *in vitro*. The phylogenetic tree of LvProfilin among organisms and its 3D protein structure showed that LvProfilin was highly conserved among shrimp and arthropods. The homology modeling of its 3D structure revealed three alpha-helices and six beta-strands similar to most eukaryotic profilins. To interpret its possible function, the gene expression of LvProfilin in various tissues was performed. We found that this gene was expressed in various tissues. This result may imply that LvProfilin could share a common function in all tissues. Nuclear activity has been a promising function of LvProfilin. We performed a DNA/RNA binding prediction analysis using DRNApred. The result indicated that Lysine-90 and Threonine-91 were the putative DNA-binding sites with the probability of 63.12% and 54.16%, respectively. Its binding activity was confirmed *in vitro*, which bound stronger to single-strand DNA than double-strand DNA. To our best knowledge, this is the first report of the DNA binding activity of profilin in invertebrates.

Keywords: Profilin, Marine shrimp, Phylogenetic tree, Tissue distribution, SWISS-MODEL, DNA binding protein

1 Introduction

Profilins, also called chickadees in *Drosophila* sp. are small proteins with molecular mass of 14–17 kDa. They are ubiquitous proteins originally identified as an actin-binding protein over four decades ago in eukaryotes [1], [2]. Recently, profilins have been discovered in *Asgard archaea* [3], [4], and Ectromelia Virus [5]. Profilins play a critical role as a key regulatory factor in actin polymerization dynamics in most cells [6], [7]. Their three-dimensional structures and their binding partners are highly conserved through evolution [8]. Profilins interact with many proteins besides actin through the proline-rich domain [9]. Besides the function of actin regulation, other functions of profilins such as transcription, RNA splicing, or endocytosis were reported as well [6], [10]–[12]. Regarding actin regulation, profilin binds to monomeric actin in a 1:1 complex and prevents the spontaneous formation of microfilaments. It has been shown that

the gene knockdown of *profilins* affects impaired growth, motility, cytokinesis, and embryonic lethality in both invertebrate and vertebrate [13], [14]. Its function in nuclear activities is also interesting since profilins have been found in cytoplasm and nucleus [15]–[18]. A Myb-related transcription factor, $p42^{POP}$, was described as one of the profilin ligands by Lederer *et al.* [19]. They reported that its bonding was mediated by a proline-rich cluster of profilins, and $p42^{POP}$-profilin complexes could be detected in cell lysates. In $p42^{POP}$ transfected cells were shown that nuclear profilin increased remarkably, indicating that profilin was involved in gene regulation [19].

Many nuclear-related proteins show DNA binding activity such as transcription factors, nuclear actin and other related proteins [20], [21]. To determine DNA binding activity, computational methods are widely used for prediction. In general, DNA-binding sites are determined by physicochemical features, local structure, and evolutionary conservation [22]. This insight gave us tools to predict the binding activity of LvProfilin.

In our previous study, we used bioinformatics tools to analyze protein partners of LvProfilin. There are 6 predicted protein partners, including Abl (Tyrosine-protein kinase), Actin, Elongation factor 1 beta, Cappuccino, Rac1 and Tsr. *In silico* prediction demonstrated that the C-terminal and the middle of this protein were conserved with the specific function of Sumoylation at amino acid VKMG (position 17–20) and RNA binding (position 52–98) [23]. This research suggested the possible function of LvProfilin in the nuclear cytoplasm protein transport, gene transcription and stability of protein function. Despite the fact that we have a large amount of biochemical information on profilins function in the crustacean, especially in *Drosophila*, the role of profilin remains uncertain in shrimp. To gain more understanding of the organization, cytoplasmic and nuclear distribution and function of LvProfilin, *in vitro* and *in silico* assays were performed in this study.

2 Materials and Methods

2.1 *Animals and tissue sampling*

The animal experiment was carried out following Ethical Principles and Guidelines for the Use of Animals (Thai) adapted from the International Committee on Laboratory Animal Science, ICLAS, which had been done in previous research [23]. The RNA samples of shrimp organs of white shrimp (*Litopenaeus vannamei*), 3 months old, from a local shrimp farm in Eastern Thailand which were preserved and stored at –80 °C from previous research including total RNA of muscle, stomach, nerve, leg, eyestalk, heart, hepatopancreas, lymphoid, intestine and gill were used for this study.

2.2 *Sequence and structure analysis*

The DNA sequence of LvProfilin has been reported with the accession no. KU695585. Multiple alignments of amino acid sequences were performed using Clustal W. The aligned Profilin amino acid sequences were used to construct phylogenetic trees in the Molecular Evolutionary Genetic Analysis X (MEGAX) program using the neighbor-joining algorithm with the Poisson model. The constructed phylogenetic tree was tested by bootstrap analysis with 1000 replicates to determine the statistical significance of the clustering of Profilin proteins. The protein sequences in the phylogenetic tree are indicated by their accession number at NCBI.

2.3 *RT-PCR of LvProfilin in different tissues*

Total RNA concentration was calculated by spectrophotometer technique at a wavelength of 260 nm. Total RNA was reverse transcribed for cDNA synthesis by using AMV Reverse Transcriptase (Finnzymes) with specific primers, vPFL_F (5'-GGATCCATGTCTTGG GATCAGTATGTAAGC-3') and vPFL_R (5'-CTCGAG CTAGTAGTTTAGGCCTTTTGGTA-3'). The synthesized cDNA was next amplified by PCR using Platinum Taq DNA polymerase (Invitrogen) with the same specific pairs of primer (10 μM each). The DNA amplification program was performed following Laoong-u-thai *et al.* [23]. PCR products were separated and detected on 1.5% agarose gels electrophoresis, stained with *SYBR® Gold* (Invitrogen) and visualized under ultraviolet light by Gel documentation Felix 2050 (Biostep). The amplification results were converted to intensity using ImageJ software (National Institutes of Health, United States) to analyze the gene expression quantity. All experiments were performed at least in triplicate. The data were reported as mean ± standard

deviation (SD). The significance of the results was analyzed by One Way ANOVA at p-value < 0.05 and Tukey HSD test (https://www.statskingdom.com/).

2.4 *Homology modeling*

The amino acid sequence of Lvprofilin (GenBank accession number KU695585.1) from the GenBank database was used as a query sequence for homology modeling using the SWISS-MODEL server [24], [25].

Then target-template sequence alignment was performed using the putative X-ray protein template in PDB database to create the 3D model of LvProfilin protein [26]. The most successful homology model was selected based on Global Model Quality Estimation (GMQE) and QMEAN (Qualitative Model Energy Analysis) statistical parameters [25], [27]. The algorithm provides structural accuracy with no error above 5 Å [27]. GMQE is a quality estimation which combines properties from the target–template alignment and the template search method. The value ranges between 0 and 1. The high values indicate more putative models. The QMEAN4 scoring function comprises a linear combination of structural descriptors employing statistical potentials [28].

2.5 *Site of DNA-binding prediction*

DNA-binding sites of LvProfilin were predicted by submitting the sequence to web-based tools namely DRNApred. The putative propensities for DNA-binding was shown in the range of 0 to 1. The value displays likelihood that the protein residues binding to double-stranded DNA sequence with the propensities >0.4727 are defined as putative DNA-binding residues [28].

2.6 *Expression of GST-LvProfilin protein*

The LvProfilin gene was inserted into the pGEX-4T-1 expression vector (Amersham Biosciences). The recombinant plasmid pGEX4T-1-LvProfilin was purified using QIAprep spin Miniprep system (Qiagen) and transformed into *E. coli* BL21 (Amersham Biosciences). LvProfilin sequence was confirmed by DNA sequencing service (BioDesign) before expression. The recombinant cell was cultivated in the media containing 100 µg/mL of ampicillin and the expression of a recombinant protein was induced by the addition of 0.1 mM IPTG. Cells were harvested, then lysed, and the GST-LvProfilin fusion protein was detected on a 12% SDS-PAGE.

2.7 *In vitro ssDNA and dsDNA binding assay*

DNA binding activity was carried out following the method of Laoong-u-thai *et al.* [29]. Briefly, the total secreted recombinant proteins (100, 200, and 400 mg) of GST-LvProfilin and GST were incubated with ssDNA-cellulose and dsDNA-cellulose beads (USB) at a concentration of 10 mg/mL in binding buffer (10 mM TRIS-HCl, pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100, and 125 mM NaCl and 10% Glycerol) supplement with Leupeptinand and Dithiothreitol (DTT). The mixture was incubated at 4°C overnight with shaking. The beads were washed to remove the unbound protein with the 100 mL of binding buffer (without Leupeptin and DTT). After the last wash, the samples were resuspended with the binding buffer followed by boiling in the SDS loading buffer. The amount of GST-LvProfilin and GST recombinant protein, which was bound with nucleic acid on the beads, was determined by 12% SDS-PAGE.

3 Results and Discussion

LvProfilin sequence has been reported in our previous research [23]. Here, sequence and structure analysis along with tissue-specific expression and protein interaction assay of LvProfilin have been performed to understand its possible function, especially nuclear function activity.

3.1 *LvProfilin sequence and structure for functional analysis*

Multiple aligments and phylogenetic tree (Figure1) of LvProfilin showed a high conserved domain of actin binding site among Penaeus shrimp and crustacean. We retrieved profilin sequences from species with full length sequencing, and that we considered them as representatives of the evolution of Insecta, Hexanauplia, Maxillopoda and Malacostraca. The protein sequences were aligned using MEGAX. The phylogenetic tree already suggests different clades of profilins exist. LvProfilin clusters in the same group with shrimp

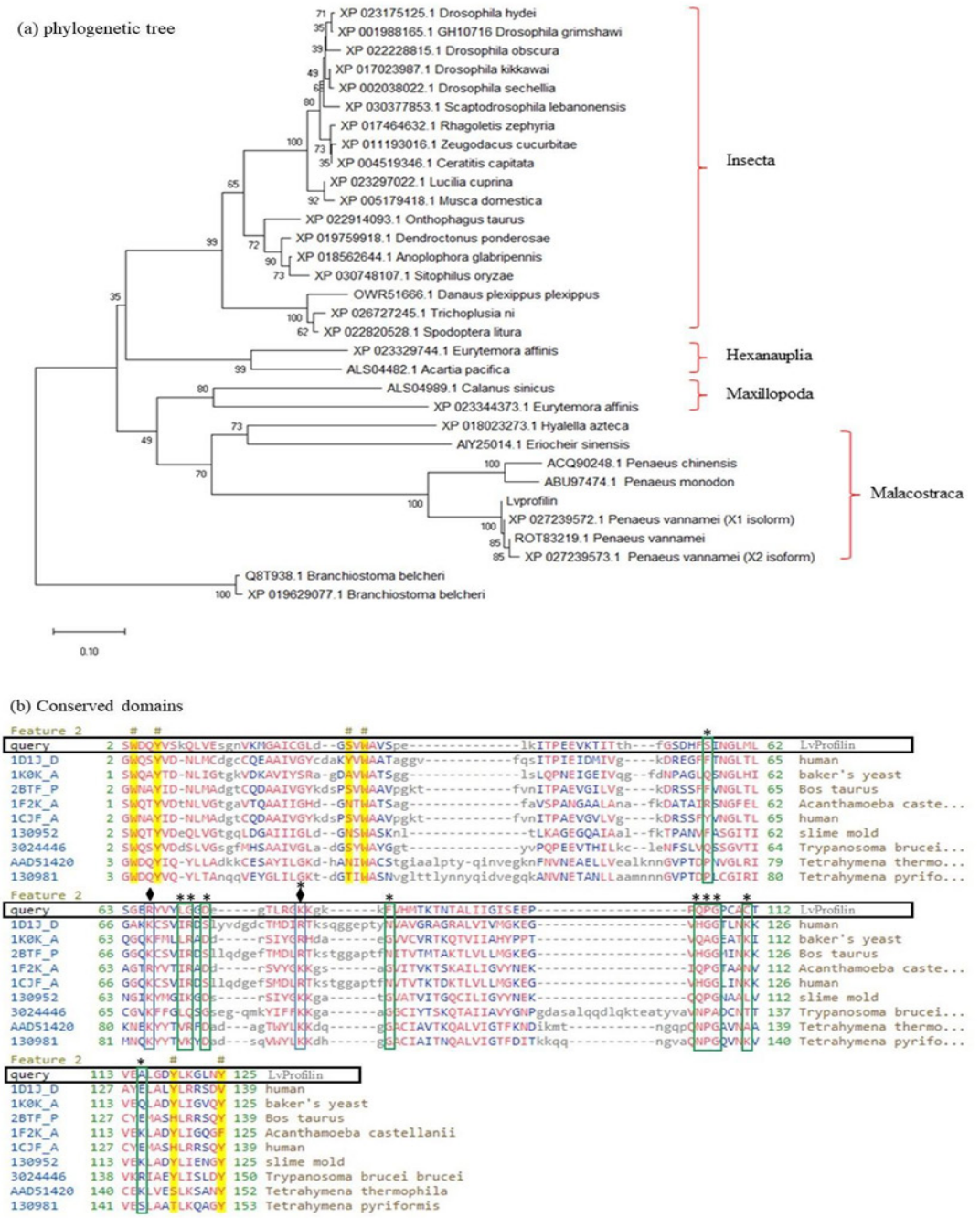


Figure 1: Neighbor-joining phylogenetic tree of the *L. vannamei* profilin protein (LvProfilin) and its conserved domains. The phylogenetic tree was constructed by using the neighbor-joining method (a). Bootstrap percentage value was performed using 1000 replicates to test the relative support for particular clades. Numbers represent GenBank accession numbers. Three conserved domains of LvProfin was predicted using BLASTP and ClustalW (NCBI web search) (b) including actin binding site (*green box), poly-proline binding site (# yellow label) and putative PIP2-interaction site (⧫ blue box).

profilins [Figure 1(a)]. The LvProfilin sequences showed the similarity among the profilin family, which may be considered as related functions through evolution.

However, BLASTP search (NCBI-conserved domain) showed 11 actin binding site residues on LvProfilin, including S-56, L-70, G-71, D-73, K-80, F-85, Q-105, P-106, G-107, C-111 and A-115 (Figure 1(b) *green box). This region shows the main function of profilin in all eukaryotes involved in the dynamic turnover and restructuring of actin filament, especially in muscle [30], by increasing the rate of nucleotide exchange on the actin monomer, thereby charging the monomer with ATP and possibly enhancing actin filament dynamics [31]. Besides, the other two main domains of profilin, poly-proline binding site (Figure 1(b) # yellow box) on C- and N- terminal and putative PIP2-interaction site (Figure 1(b) ♦ blue box) were identified on LvProfilin.

In all mammals, at least two isoforms (profilin I and II) of profilin have been reported [32], while in mice, four isoforms of this protein have been characterized (*pfn1*, *pfn2*, *pfn3* and *pfn4*) [33]. At present, one isoform of profilin has been found in Penaeus shrimp [34] along with crustacean [35]. However, there are reports of profilins involved in nucleus activities such as mRNA splicing, nuclear cytoplasm protein transport and gene transcription [30], [32], as well as the prediction result of our previous study [23].

As mentioned earlier, LvProfilin has been predicted to have Sumoylation modification which may explain its function in nuclear activity such as protein stability, subcellular localization, transcriptional regulation, DNA repair, signal transduction, and chromatin remodeling [36]–[38]. However, Sumoylation on profilin has not yet been reported.

3.2 *Expression of LvProfilin gene in shrimp organs*

Profilin has been reported in various organs, including the brain, kidneys, testes [33] and hepatopancreas [35]. To verify the expression of LvProfilin transcripts in shrimp, we analyzed the mRNA levels of LvProfilin in various tissues of juvenile shrimp by reverse transcriptase-PCR. As shown in Figure 2, the mRNA expression of LvProfilin was detected in various tissues, which were enriched in the leg, heart, as well as in muscle (abdominal muscle) and smooth muscle. The expression level was significantly different in all tissues ($p = 0.016$), especially the leg compared with

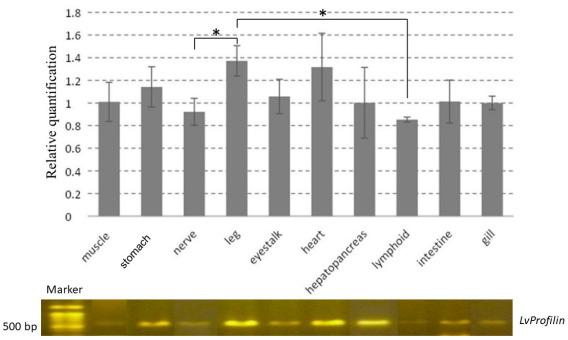


Figure 2: The expression profile of LvProfilin in various tissues of *Litopenaeus vannamei* using RT-PCR. The relative quantification of LvProfilin cDNA was normalized by the 16s rRNA gene. 1.5% Agarose gel electrophoresis was used for DNA detection and quantification stained with a fluorescent dye (SYBR™ Gold, Theremofisher). The significance of the results was analyzed by One Way ANOVA at p-value < 0.05 and Tukey HSD test.

nerve and lymphoid. This result suggested that the LvProfilin is not a tissue specific protein that was expressed in several tissues in shrimp as well as in vertebrates [39]. However, those tissues were high muscle activity which was related to the main activity of profilin in actin filament dynamics [14], [40]–[43]. Besides, vertebrate profilins are also involved in nuclear activities and gene regulation, especially in humans, as we mentioned earlier. Interestingly, these functions of profilin may be similar in shrimp and crustacean (invertebrates). As we know, all crustaceans have molting processes [44], [45] which are related to high protein turnover, and profilin may be one of the key molecules that regulate this process.

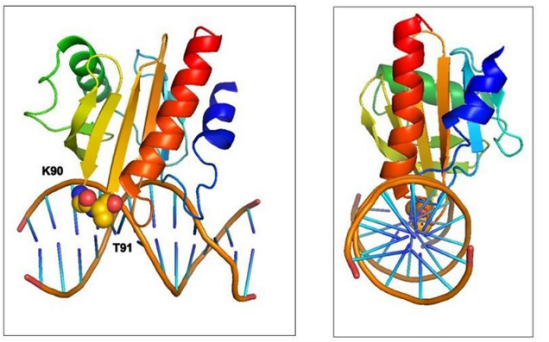
According to a previous study, we reported that LvProfilin was predicted to be modified by SUMO protein [23]. Additionally, LvSUMO-1 in shrimp has been reported that its mRNA is up-regulated during the molting process (premolt stage) in abdominal muscle [46]. These evidences may reveal that LvProfilin may play a role in gene regulation or nuclear activity in shrimp, especially in muscles, as its mRNA was detected in many muscle-related tissues (both smooth and abdominal muscle). Furthermore, some researchers reported that the function of profilin in shrimp be involved in the immune response. For example, Kong *et al.* [47], the high expression of profilin mRNA of

Litopenaeus vannamei is observed in the white spot syndrome virus (WSSV) infected shrimp. In addition, transcriptomic analysis of gills from *Litopenaeus vannamei* infected WSSV showed an increasing mRNAs level of the profilin at 6 h after infection [48].

There is an evidence that tries to explain the function of profilin in nuclear activity, as mentioned earlier, but to perform their binding activity to DNA/RNA, remains unexplored. In this research, we continued to predict and confirm the nucleic acid-protein binding activity of LvProfilin to be more understanding of its function.

3.3 *Homology modeling and nucleotide-binding prediction of LvProfilin*

The SWISS-MODEL server was used to generate a homology model of LvProfilin. Firstly, the selected suitable template protein structures in PDB were selected according to the following criteria that the selective template should display a high coverage >65% sequence similarity. Then the GMQE and QMEAN4 values were investigated as previously described to define the most putative model. The high scoring of GMQE and QMEAN4 were statistically acceptable for the homology model results. Moreover, the putative X-ray template proteins from the PDB database were defined according to relatively high GMQE scoring. Resulted homology models for LvProfilin protein showed a clear active region responsible for binding to nucleotides (Figure 3). DNAPred [49] was employed for the prediction of protein-DNA binding sites of LvProfilin. The accurate identification of binding between LvProfilin and DNA was calculated by this machine-learning-based method, widely used to predict protein-nucleotide binding sites. DNA-binding sites of LvProfilin were predicted by submitting the sequence to web-based tools, namely DRNApred. The putative propensities for DNA-binding were shown in the range of 0 to 1. The value displays the likelihood that the protein residues binding to double-stranded DNA sequence with the propensities >0.4727 are defined as putative DNA-binding residues [49]. The results showed that LvProfilin provided two surface-exposed loop-residues, Lysine-90 and Threonine-91, for binding to double-stranded-DNA with the propensities of 0.6475 and 0.4561, respectively (Figure 3). Side-chain polarity of both lysine and threonine residues,



(a) (b)

Figure 3: Homology model identified Lvprofilin binding to double-stranded DNA side view (a) and top view (b). Using sequence alignment, the crytallized protein structure of *Hevea brasiliensis* (PDB: 5FDS) displayed the highest structural similarity and was utilized as a model template. The structure contains 3 alpha-helices and 6 beta-strands. Prediction from DRNApred indicated that a couple of residues, Lysine-90 and Threonine-91, located at the β4-β5 surface-exposed loop of LvProfilin, were the putative DNA-binding sites with the probability of 63.12% and 54.16%, respectively.

located in a protruding loop-structure may facilitate protein-DNA interactions. Additionally, LvProfilin is a DNA-binding protein containing DNA-binding domains and has a specific or general interaction with single- or double-stranded DNA. In general, DNA-binding proteins bind to the major groove of DNA because the putative surface-exposed epitope of the proteins exposes more functional groups that identify a base pair. However, is some known minor grooves responsible for DNA binding [50]. It is believed that LvProfilin is involved in the transcriptional process, which modulates the process of transcription. Thus interactions between Lvprofilin and DNA activate functional gene expression.

3.4 *In vitro nucleic acid-binding analysis of LvProfilin*

Based on the previous nucleotide-binding prediction of LvProfilin, the nucleic acid-binding assay was performed to establish the DNA-binding activity of LvProfilin.

GST and GST-LvProfilin were expressed in *E.coli* BL21. Protein extraction showed that both proteins were soluble proteins [Figure 4(a)]. Total

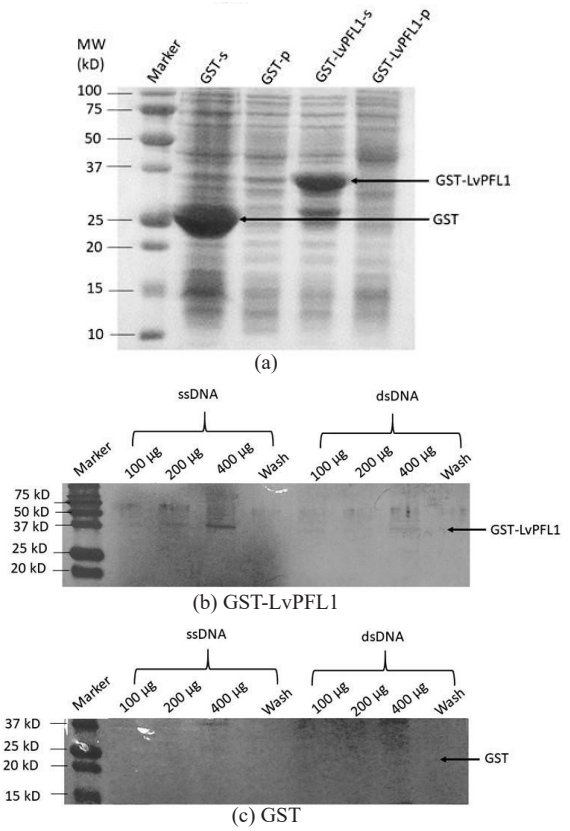


Figure 4: GST and GST-LvProfilin recombinant proteins expression and *in vitro* nucleic acid binding assay. GST and GST-LvProfilin recombinants protein lysates (a) *s* stands for supernatant, and *p* stands for pellet after cell extraction. *In vitro* nucleic acid binding assay showed the different amounts of recombinant GST-LvProfilin (b) and GST (c) of 100, 200, 400 microgram protein which was used for binding with ssDNS (single-strand DNA) and dsDNA (double-strand DNA), respectively.

recombinant GST and GST-LvProfilin with different amounts were incubated with calf thymus ssDNA-cellulose and dsDNA-cellulose beads. It showed that only GST-LvProfilin protein is bound with both ssDNA and dsDNA [Figure 4(b)]. Increasing the protein amount is highly affected by binding efficiency. However, GST-LvProfilin interacted more tightly with ssDNA than dsDNA [Figure 4(b)]. This result confirmed the possible function of LvProfilin in ssDNA and dsDNA interaction.

In vitro nucleic acid-binding assay had been used to identify DNA/RNA binding proteins, both prokaryote and eukaryote, such as GR-RBP4, DdrB, ribonucleoprotein, SSB, nucleolin, human Rcd-1 and LvDBP23 [29], [51]–[56]. There are many motifs that were reported as DNA/RNA binding sites such as 5-stranded β-barrel structure, known as an oligonucleotide-binding (OB) fold of Ddrb [37], Gly/Arg-rich (GAR) of nucleolin [39], Arg on the positively-charged cleft of Rcd-1 [40], zinc finger [42] and KTS (Lysine, Threonine, and Serine) residues [43] etc. It is reported that this KTS site inserted into zinc finger domains of Wilms' tumor suppressor *WT1* showed the binding ability to LRRC15 gene leading to dramatic transcriptional activation. The optimal binding site of the (+KTS) isoform of EWS–WT1 is 5′-GGAGG(A/G)-3′ [57]. Interestingly, LvProfilin predicted DNA binding sites are Lysine-90 and Threonine-91. The following amino acid-92 of this protein is Asparagine (N) [23] which is grouped in polar amino acid as well as Serine. These KTN residues on LvProfilin could have the same function of nucleotide binding to specific genes leading to transcriptional regulation [57]. Another possible evidence of DNA binding activity of LvProfilin in shrimp (invertebrate) is that we found 6 beta-strands with Lysine-90 and Threonine-91 predicted DNA binding sites at the surface-exposed loop of β4-β5. This structure is slightly similar to the 5-stranded β-barrel structure of the OB-fold domain in vertebrates which has been recognized as an important motif of single-stranded DNA binding proteins. This type of protein plays an important role in DNA replication, DNA recombination, DNA repair, transcription, translation, cold shock response, and telomere maintenance [52], [58]. This evidence revealed that LvProfilin may act as ssDNA and dsDNA binding protein controlling nuclear activity in marine shrimp responding to filamentous cell function such as molting cycle or immune system. However, LvProfilin activity may play a more important role in gene regulation than DNA replication as its binding activity is stronger in ssDNA than dsDNA [Figure 4(b)]. In normal cells, ssDNA regions are mainly involved in DNA replication and transcription activity. These regions are less stable which are easy to damage by chemical and nucleolytic cleavage enzymes. Single strand DNA binding (SSB) proteins play an important role in protecting and stabilizing ssDNA regions until the correct cellular processes occur [59]. Additionally, after SSB protein binding to

ssDNA, they show the ability to recruit other partner proteins to bind at ssDNA as substrate [60]. These protein partners could predict by using the Molecular Docking analysis to recruit the important interacting proteins [61] to explore LvProfilin specific function in the future. Moreover, some studies show the binding activity of SSB to ssDNA for DNA repair, including telomere [62], [63]. As LvProfilin was identified as ssDNA binding protein. Its specific functions need to be explored in the future for the possible application in shrimp farming benefit.

4 Conclusions

Profilins have been characterized originally as actin-binding protein. Later, other related functions of profilins have been revealed in many functions, including nuclear function. In this research, LvProfilin showed DNA binding activity in both single and double strands. It is the first report of profilin's function in nucleic acid binding activity in invertebrates and eukaryotes. However, the specific binding sites predicted in this research have to be confirmed in the future study along with the mechanism of protein-DNA binding activity.

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