MOLECULAR CLONING AND BIOINFORMATICS ANALYSIS OF SHRIMP LVPROFILIN IMPLICATED IN MUSCLE FORMATION AND MUSCLE SPECIFIC GENE REGULATION

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Abstract

Shrimp is the most important seafood product of Thailand, which bring the large income to serve Thai economic. To promote shrimp agriculture, its growth has to be clearly understood. Shrimp growth is controlled by muscle regulatory genes via their signaling protein. To identify muscle growth related genes and study their functions, we achieved the molecular genetic techniques and bio-informatics tools. In particular, we identified an intriguing gene related to muscle regulation in abdominal muscle of pacific white shrimp, *Litopenaeus vannamei*, named *LvProfilin*. Its cDNA contains a coding sequence of 378 nucleotides. The deduced 125 amino acid exhibit 78% similarity with the shrimp *Fenneropenaeus chinensis* Profilin. The computed chemical formula of LvProfilin is $C_{602}H_{951}N_{159}O_{184}S_6$. The 3 types of posttranslational modifications on LvProfilin were found including Acetylation, ADP-ribosylation and Phosphorylation. The secondary structure of LvProfilin protein was predicted. The seven α-helixs, three β-pleated sheets and eleven coils position were shown on LvDBP23 polypeptide. Additionally, the protein partners of LvProfilin were predicted. The result indicated that LvProfilin could bind with Actin protein as potential regulators of Actin filament dynamics that involving in muscle regulation. Other conjectured LvProfilin binding proteins include Tsr, Rac1, Elongation factor 1 beta, Cappuccino, Abl (Tyrosine-protein kinase) and SUMO. Interestingly, the RNA binding search showed a positive function of RNA binding. These preliminary results indicated that LvProfilin was likely to play an important role in muscle regulation in *L. vannamei* through its protein partners and control groups of RNA expression. Nevertheless, these predicted functions of LvProfilin need to be confirmed by laboratory assay to define its mechanism in the future.

Keywords: Binding protein, Bioinformatics, Muscle regulation, RNA binding, Shrimp profilin

Introduction

Thai shrimp aquaculture is the top supplier of shrimp market worldwide. Breeding programs for developing high quality bloodstock are carried out in many countries while the genetics of growth remains a mystery. Most of the breeding researches point out of the physiology and morphology study with the lack information and understanding of molecular mechanism. To understand the shrimp growth and genetics underlying the desired shrimp traits, significant information about the muscle regulatory gene and their expression profile are needed. Skeletal muscle is a remarkably plastic tissue. It can undergo dramatic changes in size and contractile properties during development, as well as when responding to a variety of physiological conditions in adults. A diverse number of factors such as hormones, passive stretch, use/disuse, and disease can alter the size and fiber type composition of vertebrate skeletal muscles. Similar mechanisms operate in crustacean striated muscles [1, 2]. Differentiation of skeletal muscles begins when the mesodermal

cells in the early embryo become attached to the myogenic lineage, which is then followed by the differentiation of fibers to specific types. This involves the expression in early embryos of skeletal muscle-specific transcription factors (e.g., MyoD, MFY5, Myogenin, MRF4, and MEF2), which regulate the expression of muscle-specific genes with DNA binding property [3, 4]. However, the genes and molecular mechanisms of shrimp growth have not received adequate scientific attention to date. In previous studies, the abdominal muscle cDNA library of shrimp *L.vannamei* was successfully constructed [5] and some muscle regulatory candidate genes were reported such as *LvSUMO-1* [6] and LvDBP23 [7]. Shrimp LvSUMO-1, and its conjugated proteins were detected in cytoplasm and nucleus in several tissues. Interestingly, *LvSUMO-1* mRNA levels showed the high expression in abdominal muscle during the premolt stage, where it has significant activities of protein degradation. It suggests the possible role of LvSUMO-1 in the regulation of shrimp muscle protein degradation through the target protein [6]. Later, the shrimp LvDBP23 protein was identified as the shrimp novel DNA binding protein. The expression of *LvDBP23* mRNA was highly presented in abdominal muscle and swimming leg muscle. *LVDBP23* transcript during the molt cycle was highly expressed in the intermolt stage. *In vitro* nucleic acid binding assays reveal that LvDBP23 protein can bind to both ssDNA and dsDNA, indicating its possible role in gene regulation [7]. Fascinatingly, these two proteins may play important role in regulating the muscle specific gene expression which may relate to the shrimp muscle growth. Additionally, we may obtain these genes as the molecular marker of selective breeding application in the future. For better understanding, the investigation of the muscle regulatory genes has to be carried out as well as the effects of physical and chemical condition to the expression level of those genes.

Experimental

Animals and Tissue Sampling

Cultured 3-months-old *Litopenaeus vannamei* from a local shrimp farm in Songkhla, Thailand was transported alive in plastic box with 1/3 water and 2/3 compressed air to the laboratory. Fresh abdominal muscle tissues were collected for RNA extraction.

Total RNA Extraction

Total RNA was extracted from abdominal tissues of 3-months old Pacific white shrimp *L. vannamei* by using TRIZOL Reagent (Invitrogen). Briefly, 200 mg of tissue was homogenized in 500 µl TRIZOL LS reagent. Homogenized tissue was incubated for 15 min at room temperature followed by Chloroform 160 µl added. The tube was vigorously mixed by vortex for 15 sec and incubated at room temperature for 15 minutes. Mixed samples were centrifuged at 12,000 rpm for 15 minutes at 4 ºC, the aqueous phase was transferred to a clean tube following 250 µl of isopropanol added. After 10 min incubation at room temperature, tube was centrifuged at 4 ºC for 15 minutes at 12,000 rpm, and supernatant was withdrawn and remaining RNA pellets were washed with 70% ethanol. Total RNA pellet was air dried for 10–15 minutes at room temperature, dissolved in deionized-distilled water and stored at -80 ºC. Total RNA concentration was calculated by spectrophotometer technique.

RT-PCR and Primer Design

Total RNA (500 ng) of *L. vannamei* abdominal muscle was reverse transcribed for cDNA synthesis by using AMV Reverse Transcriptase (Finnzymes) and specific primers, vPFL-FB (5'GGATCCATGTCTTGGGATCAGTATGTAAGC3') and vPFL_RX (5'CTCGAG

CTAGTAGTTTAGGCCTTTTGGTA3'). Primers used in this experiment, were designed by sequence alignment of shrimp profilin genes from GenBank (NCBI) with the restriction enzyme site. The synthesized cDNA (700 ng) was next amplified by PCR using i-Taq DNA polymerase (Intronbio) with specific primer pairs (10 µM each). The following program ([94 °C, 5 minutes] + [94 °C, 30 seconds; 45 °C, 30 seconds; 72 °C, 1 minute] ^{x} 35 + [72 °C,10 min]) was used for DNA amplification on a GeneAmp® PCR system 9700 (Applied Biosystems). PCR Products were separated on 1.5% agarose gels electrophoresis, stained with ethidium bromide and visualized under ultraviolet light by Gel Doc 1000 genetic Analyzer (*Bio- Rad*).

Cloning and Sequencing

PCR products were cloned into pGEM-T easy cloning vector. Recombinant plasmids were transformed using *Escherichia coli* top 10 F' for plasmid screening and propagation. Plasmid purification was performed using QIAprep spin Miniprep system (Qiagen). Purified plasmids were sequenced by DNA sequencing service (BioDesign).

Sequence Analysis

The expected sequences were searched for nucleotide and protein sequence analysis using online Bioinformatics tools and softwares. The sequence similarity conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi. nlm.nih.gov/BLAST/). The deduced amino acid sequence was analyzed with the Protein Sequence Analysis software (http://www.expasy.org/tools/). Multiple alignment of the LvProfilin protein was performed with the ClustalX Multiple Alignment program (http://www.ncbi. nlm.nih.gov) and Multiple Alignment show by GENEDOC, version 2.6.001 (Nicholas and Nicholas, 1997). ORF finder (http://www.ncbi.nlm.nih.gov/gorf/ gorf.html) was used for prediction novel gene start codon position.

Chemical and Structural Prediction

The physical and chemical parameters of protein were computed by ProtParam tool (http://web.expasy.org/protparam/). To study Post-translational modification of protein, the PeptideMass searched (http://web.expasy.org /peptide_mass/) was performed. The secondary structure of protein was predicted by QuickPhyre program (http://www.sbg. bio.ic.ac. uk/phyre).

Binding Investigation

The protein-protein interaction was predicted by STRING program (http://string-db.org/newstring_cgi/show_input_page.pl?UserId=vdvUwiiQAlRP&sessionId=pRDOux1lwRCV&input_page_type=single_sequence). Additionally, the SUMOplot (http://www.abgent.com//tools/toSumoplot) was searched for SUMO modification site. Following, the protein-RNA binding was predicted by using RNABindR program (http://bindr2.gdcb. iastate.edu/RNABindR/).

Results and Discussion

RT-PCR Amplification and Sequence Analysis

The PCR product showed DNA fragment of about 400 bp (Figure 1) which was cloned into cloning vector and sequenced. The sequencing result represented the 378 bp in length of expected DNA. The highest hit was showed in 78% identity of Profilin protein of shrimp *Fenneropenaeus chinensis.*

The predicted protein size and open reading frame (ORF) were analyzed. The results showed frame 1 translation (Figure 2a) with 125 deduced amino acid with start codon (ATG) and stop codon (TAG) predicted (Figure 2b). From the BLAST search, it is revealed that the similarity of Profilin sequence among crustacean is highly conserved. Due to this reason, the sequence alignment both DNA and protein was performed.

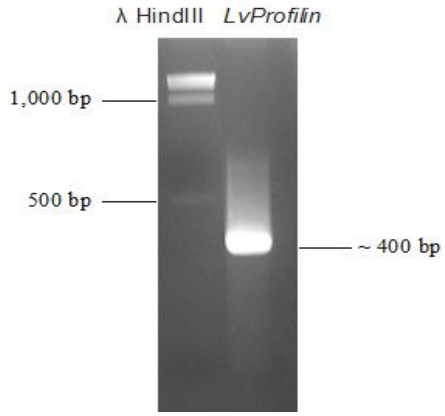
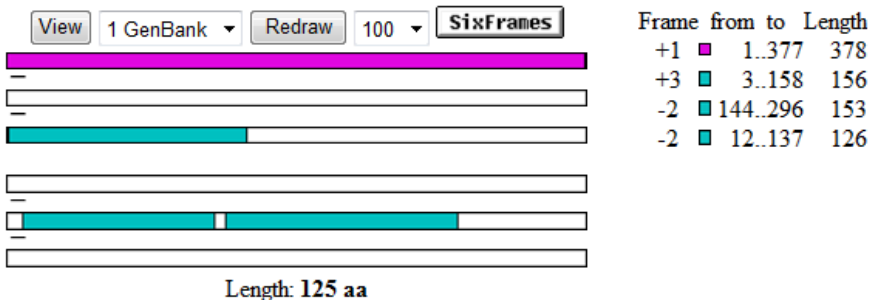


Figure 1. *LvProfilin* gene amplification. The single DNA fragment from RT-PCR amplification was clearly shown in the agarose gel electrophoresis with the approximate size of 400 bp

(a)



(b)

```
1    atgtcttgggatcagtatgtaagcaagcagctggtggaatctgga
1    M  S  W  D  Q  Y  V  S  K  Q  L  V  E  S  G
46   aacgtgaagatgggagccatctgcggtctggatgggtccgtgtgg
16   N  V  K  M  G  A  I  C  G  L  D  G  S  V  W
91   gccgtctcgcccgaactaaagatcacaccagaggaggtgaagacc
31   A  V  S  P  E  L  K  I  T  P  E  E  V  K  T
136  atcaccactcactttggcagtgaccactttagcatcaatggtttg
46   I  T  T  H  F  G  S  D  H  F  S  I  N  G  L
181  atgttgagtggggagaggtatgtatacctgggggggcgacgaaggt
61   M  L  S  G  E  R  Y  V  Y  L  G  G  D  E  G
226  accttgcggggaaagaagggcaaaaagtttgtgcacatgaccaag
76   T  L  R  G  K  K  G  K  K  F  V  H  M  T  K
271  accaacactgccctcatcatcggcattagcgaggaacctggacag
91   T  N  T  A  L  I  I  G  I  S  E  E  P  G  Q
316  cctggcccccgtgcgtgcaccgttgaggccttgggcgactaccta
106  P  G  P  R  A  C  T  V  E  A  L  G  D  Y  L
361  aaaggcctaaactactag
121  K  G  L  N  Y  *
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Figure 2. Prediction of protein size and open reading frame. (a) Open reading frame (ORF) of the longest translation peptide is shown on the frame 1 with the first ATG condon located. (b) The 125 amino acid prediction was shown with the start and stop codon in the bold underline

LvProfilin Sequence Alignment

The alignments of LvProfilin nucleotide and amino acid sequences were made with a selection of Profilin sequences present in GENBANK to identify the extent of conserved and variable domains. Overall, LvProfilin is conserved both DNA (Figure 3a) and protein (Figure 3b) confirming the BLAST result, especially in the N-terminal locating of poly-proline binding site involving in profilin signaling of actin formation [8, 9]. Besides the actin binding site is found in the middle and C-terminal (Figure 3b) [10], several other amino acids are conserved in all species examined thus far (dark shading). These conserved domains were examined for the specific function using several bioinformatics tools.

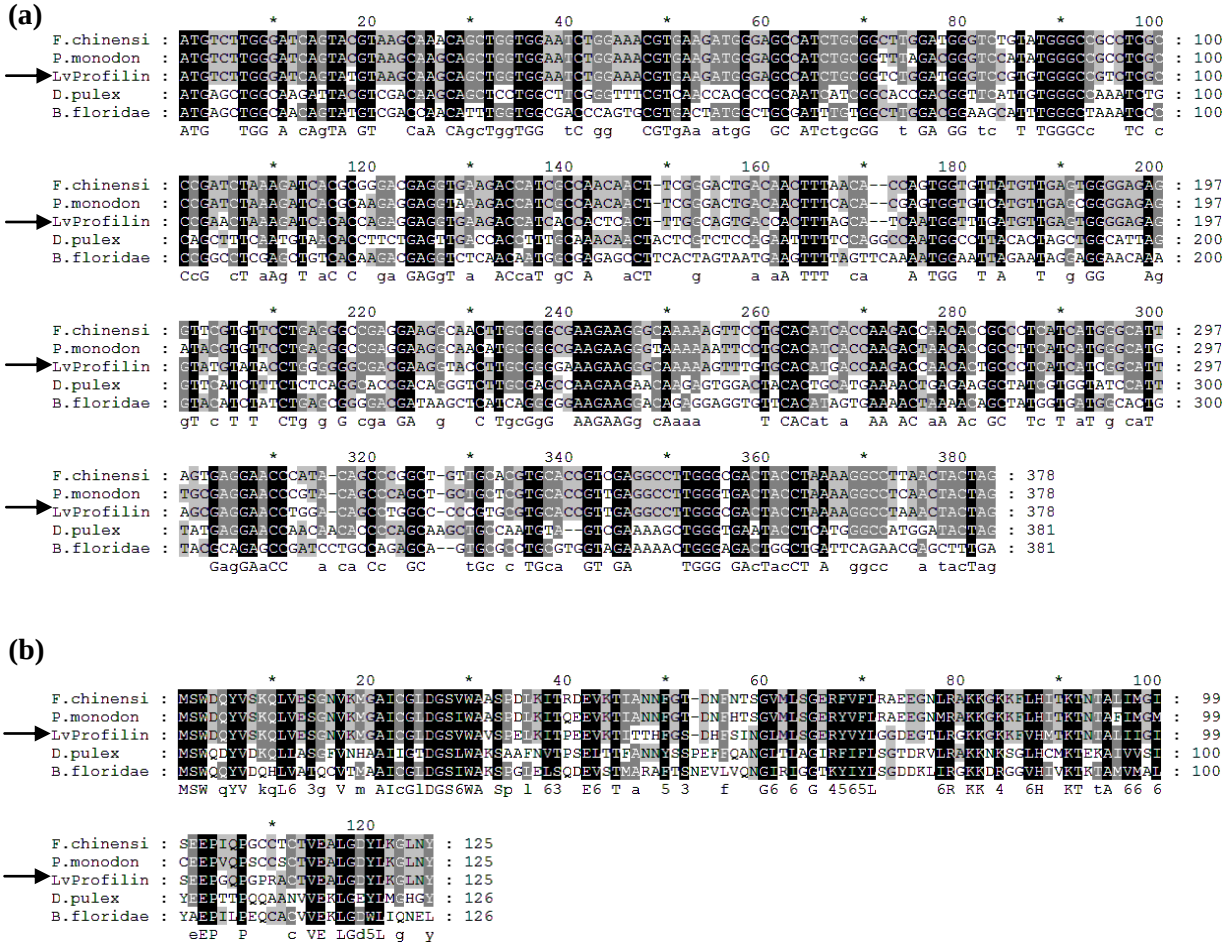

Figure 3. Nucleotide and amino acid sequences alignment of Profilin. Several species of Profilin sequence including *F. chinensis* (FJ480175.1, ACQ90248.1), *P. monodon* (EU106623.1, ABU97474.1), *B. floridae* (XM_002604328.1, XP_002604374.1) and *D. pulex* (GL732587.1, EFX73844.1) from GENBANK) were compared with the *LvProfilin* sequence (the arrow sign)

Bio-informatics Prediction and Analysis

Physical, Chemical and Structure Analysis

ProtParam tool was chosen for the physical and chemical parameters computing. It showed that the chemical formula of LvProfilin is $C_{602}H_{951}N_{159}O_{184}S_6$ with the extinction coefficients of 18,575 M^{-1} cm^{-1} at OD 280 nm, molecular weight 13,552.4 Da and isoelectric point at pH 6.28. The posttranslational modification sites on LvProfilin were searched by PeptideMass. The 3 types of posttranslational modifications were found including Acetylation, ADP-ribosylation and Phosphorylation (data not show). The secondary structure of LvProfilin protein was predicted by QuickPhyre program with the resulted of seven α-helixs (at amino acid position 19-25, 30-34, 60-62, 65-71, 76-80, 85-90 and 93-100), three β-pleated sheets (at amino acid position 3-12, 40-50 and 105-122) and eleven coils position (Figure 4). From Fig 3b, it shows that at the amino acid position of α-helixs and β-pleated sheets have the very high conserve among the species. In contract, the position of coils structure on Profilin protein give the low sequence similarity. Due to these reserve sequence and structure, it reveals that conserve regions are important for the Profilin function.

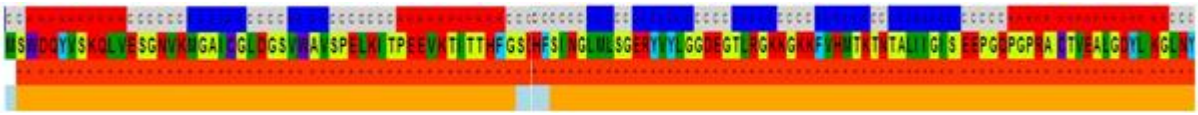

Figure 4. Three dimentional study of LvProfilin protein. E (blue color), H (red color) and C (gray) represent the α-helix, β-pleated sheet and coil respectively

Protein Interaction Analysis

LvProfilin was searched for the interaction partners. The STRING program was used for prediction of protein-protein interaction. The result came out with 6 interacting protein partners including Actin, Tsr, Rac1, Elongation factor 1 beta, Cappuccino and Abl (Tyrosine-protein kinase) (Figure 5). The binding activity with Actin protein is well known activity of vertebrate Profilin proteins as the 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 [11, 12, 13]. In this case, the binding may involve in muscle regulation and formation in shrimp. Another interacting protein, Tsr or Cofilin, is an actin severing and depolymerizing protein. The mutation of this protein can cause a syndrome of phenotypic effects in Drosophila [14]. The interaction of LvProfilin and Cofilin may function in muscle rearrangement and degradation. Rac1, one of Rho-family small G-proteins, is essential regulators in the rapid actin reorganization leading to the formation of filopodia, lamellipodia and stress fibers [15, 16, 17, 18]. The binding with Rac1 of LvProfilin may control the muscle fiber formation. In term of LvProfilin interacting with Cappuccino protein, there is the research supported this evident. It is reported that a two-hybrid screen for Drosophila Profilin protein binds to Cappuccino. This, together with the similarity of mutant phenotypes, suggests that Profilin and Cappuccino may interact during development [19]. One more interacting protein is Abl suggested that Profilin might link Abl function to the cytoskeleton [20].

Interestingly, the interaction of LvProfilin with Elongation factor 1 beta (Ef1 beta) may bring the important function of specific gene regulation involving in muscle growth. Elongation factor is basic protein in translational elongation, the steps in protein synthesis. It is supported by Skare and team reporting the possible function of Profilin in pre-mRNA splicing [21].

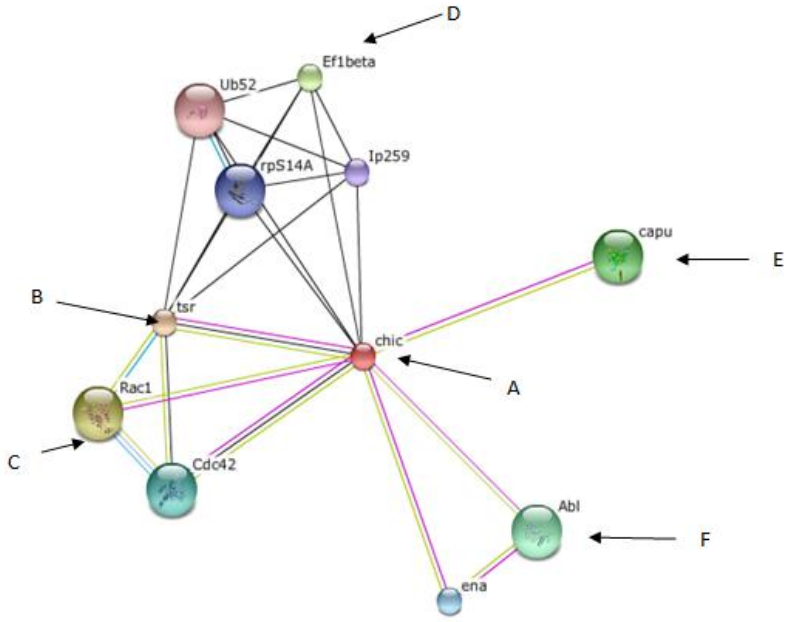

Figure 5. Protein-protein interaction network of LvProfilin. The picture shows protein network of LvProfilin binding protein, A (chic; Actin-profilin complex, B (tsr; Cofilin), C (Rac1; Ras-related), D (Ef1beta; Elongation factor 1 beta), E (Capu; Cappuccino) and F (Abl; Tyrosine-protein kinase)

Furthermore, we searched for the RNA binding possibility of LvProfilin. position 52-98 (Figure 6). These results interpret the possible function of gene regulation by LvProfilin. Additionally, the SUMOplot search was performed for the SUMO modification of LvProfilin. SUMO modification is the post-translational modification process of protein by SUMO protein known as Sumoylation. This process has been indicated to regulate the stability of protein function, nuclear cytoplasm protein transport and gene transcription [22]. We found that LvProfilin has the sumolational site with the high probability of 0.76 at amino acid position 18 (Figure 7). In our previous work, we identified LvSUMO-1 protein [6]. We expect that LvProfilin may bind with our LvSUMO-1. These preliminary results indicated that LvProfilin may play important role in muscle regulation of shrimp *L.vannamei*.

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1          11         21         31         41         51
MSWDQYVSKQ LVESGNVKMG AICGLDGSVW AVSPELKITP EEVKTITTHF GSDHFSINGL
++++++++++ --+++-+-+- --+++++++- ---+------ --------++ -+++++++--

    61         71         81         91         101        111     121
MLSGERYVYL GGDEGTLRGK KGKKFVHMTK TNTALIIGIS EEPGQPGPCA CTVEALGDYL KGLNY
+++++++++ +++++++++ +++++++++ +++++++-+- --+-+----- ---------- +++++
```

Figure 6. RNA binding activity of LvProfilin. The RNA binding possibility of LvProfilin is shown in – (negative activity) and + (positive activity).
The RNA binding site is located in the box

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                *
  1 MSWDQYVSKQ LVESGNVKMG AICGLDGSVW AVSPELKITP EEVKTITTHF
 51 GSDHFSINGL MLSGERYVYL GGDEGTLRGK KGKKFVHMTK TNTALIIGIS
101 EEPGQPGPCA CTVEALGDYL KGLNY
```

No.	Pos.	Group	Score
1	K18	VESGN **VKMG** AICGL	0.76
2	K80	EGTLR **GKKG** KKFVH	0.50

No.	Pos.	Group	Score
3	K83	LRGKK **GKKF** VHMTK	0.32

Figure 7. SUMO modification site on LvProfilin. The SUMO modification site with the highest possibility of 0.76 is showed on Lysine (K) position 18 (star) located on the motive region of VKMG (Valine-Lysine- Methionine- Glycine; amino acid position 17-20 with bold underline)

Conclusions

The molecular characterization and bioinformatics analysis of shrimp muscle regulatory gene and protein are the most important for better understanding the mechanism of muscle growth in shrimp. Here, we identified another important gene called *LvProfilin*. This gene encodes the 125 amino acids with the computed chemical formula of $C_{602}H_{951}N_{159}O_{184}S_6$. The secondary structure predicts seven α-helixs, three β-pleated sheets and eleven coils. The six protein partners of LvProfilin were classified including Actin, Tsr, Rac1, Elongation factor 1 beta, Cappuccino and Abl with the muscle regulatory function related and RNA binding activity. Finally, the sumoylation search showed the high possibility of modification site of SUMO protein on LvProfilin. However, these predicted functions of LvProfilin have to be confirmed by laboratory assay in near future.

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