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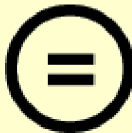
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**Thesis for the Degree of Master of Fisheries Science**

# Molecular characterization and gene silencing using double-strand RNA of adiponectin receptor in white-leg shrimp, *Litopenaeus vannamei*

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

February 2016

# Molecular characterization and gene silencing using double-strand RNA of adiponectin receptor in white-leg shrimp, *Litopenaeus vannamei*

## (흰다리 새우로부터 Adiponectin receptor 의 분자적 특징과 2 중 가닥 RNA 를 이용한 유전자 억제에 관한 연구)

Advisor: Prof. Kim Hyun-Woo

by

Md Jobaidul Alam

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Molecular characterization and gene silencing using double-strand RNA of adiponectin receptor in white-leg shrimp, *Litopenaeus vannamei*

A dissertation

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Molecular characterization and gene silencing using double-strand RNA of adiponectin receptor in white-leg shrimp, *Litopenaeus vannamei.*

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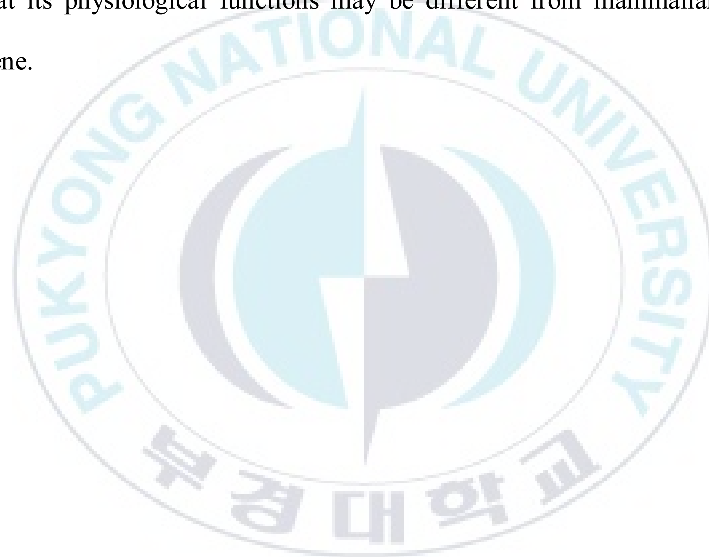
## Abstract

Adiponectin is a polypeptide hormone, secreted exclusively by adipose tissue of the mammals. It is also known as Acrp30, AdipoQ, apM1 or GBP28, was originally identified by Scherer in 1995. It has similar and complementary action to leptin, and may have a role in reducing metabolic derangements that cause diabetes, obesity, and non-alcoholic fatty liver disease. Its mechanism of action, active forms, receptors and signaling pathways are not completely defined but seem to involve AMP-activated kinase (AMPK) and downstream acetyl-CoA carboxylase. We have identified the full length cDNA encoding mammalian AdipoR homolog (Liv-AdipoR) from the white-leg shrimp, *Litopenaeus vannamei,* by the next generation sequencing and bioinformatics analysis. The full length Liv-AdipoR (1245 bp) encoded a protein with 415 amino acid residues. Liv-adipoR has high similarity to AdipoR from insects. Liv-AdipR exhibited the conserved 7 transmembrane domains (7 TMs). Tissue distribution and its expressional responses also measured during molting and feeding cycles by qPCR. The mRNA expression was higher

in hemocyte, hepatopancreas, gonad and muscle, followed by thoracic ganglia and heart tissue. To determine the effects of dsRNA in *L. vannamei*, adiponectin receptor genes, the level of transcripts were measured, after 72 hours of 10pmol dsRNA injection, the transcription level in hepatopancreas, thoracic muscle and flexor muscle showed 74%, 80% and 52% down-regulation respectively. In case of 50pmol dsRNA experiments, transcript was up-regulated in hepatopancreas; whereas in thoracic muscle and flexor muscle, it was highly down-regulated at almost 97% and 93% respectively. We identified that chronic effects of Liv-AdipoR dsRNA injection was considerably different from those in mammals, suggests that its physiological functions may be different from mammalian adiponectin receptors gene.



# 1. Introduction

Adiponectin is a polypeptide hormone secreted exclusively by adipose tissue into the blood of mammals. It has similar and complementary action to leptin, and may have a role in reducing metabolic derangements that cause diabetes, obesity, and non-alcoholic fatty liver disease (Cheeke and Dierenfeld, 2010). Adiponectin, also known as Acrp30, AdipoQ, apM1 or GBP28, was originally identified by Scherer in 1995 (Scherer et al., 1995). It plays a number of beneficial roles in metabolism; it helps to improve insulin sensitivity, glucose tolerance and lipid profile (pattern of lipids in the blood). It also decreases inflammation and atherosclerosis (Zhu et al., 2008).

However, its mechanism of action, active forms, receptors and signaling pathways are not completely defined but seem to involve AMP-activated kinase (AMPK) and downstream acetyl-CoA carboxylase (ACC) (Yamauchi et al., 2002). Adiponectin stimulates glucose utilization and fatty acid oxidation by phosphorylating and activating AMPK (Yamauchi et al., 2002). Activation of AMPK results in phosphorylation of a variety of intracellular

proteins and an increase in ATP generation (Winder and Hardie, 1999). In addition, adiponectin increases fatty acid oxidation through inhibition of acetyl-CoA (Co-A) carboxylase and activation of malonyl-CoA decarboxylase resulting in reduced malonyl-CoA content (Tomas et al., 2002). A decrease in malonyl-CoA concentration increases the transport of long chain fatty acyl-CoA molecules into the mitochondria where they are oxidized (Ruderman et al., 1997).

Two adiponectin receptors designated AdipoR1 and AdipoR2 were discovered by Kadowaki (Yamauchi et al., 2003). AdipoR1 and R2 belong to a new family of membrane receptors predicted to contain seven transmembrane domains with their N termini as internal parts and their C termini as external parts, which is opposite to the topology of all other reported G protein-coupled receptors (GPCRs). AdipoR classified as PAQR (progestin and adipoQ receptors), which shows a unique inversed topology different from the typical GPCRs and has drawn attention for its medical implications. Since it plays key roles in glucose and fatty acid metabolism, AdipoR can be important targets for the various industrial purposes.

AdipoR1 has a higher binding affinity to the globular form of adiponectin; AdipoR2 has a higher binding affinity to the full length adiponectin (Yamauchi et al., 2003). AdipoR1 and AdipoR2 double knockout mice increase the triglyceride level in the liver and exhibit insulin resistance and glucose intolerance, demonstrating that both regulate lipid and glucose homeostasis (Kadowaki et al., 2006). In the skeletal muscle and liver, adiponectin receptors activate AMPK (AMP-activated protein kinase), PPAR alpha, and p38 MAPK to increase the insulin sensitivity (Yamauchi et al., 2003). AdipoR1 and AdipoR2 are expressed in pancreatic beta cells; the function of Adiponectin and AdipoRs in Insulin Producing Cells (IPCs) is less studied than in insulin target tissues such as liver and skeletal muscle (Kadowaki et al., 2006; Matsuzawa et al., 2004).

These proteins are structurally conserved from yeast to man, particularly in the seven transmembrane domains (Yamauchi et al., 2003), and the AdipoR1 yeast homolog YOL002c plays a key role in metabolic pathways that regulate lipid metabolism such as fatty-acid oxidation (Karpichev et al., 2002). In humans and rodents, AdipoR1 and AdipoR2 are expressed ubiquitously; AdipoR1 is abundantly expressed in skeletal muscle, AdipoR2

is predominantly expressed in the liver (Yamauchi et al., 2003). Both AdipoR1 and AdipoR2 genes are expressed in human monocytes and macrophages (Chinetti et al., 2004), and also in a primary human osteoblast cell line (Berner et al., 2004) and arcuate hypothalamus (ARH) (Kadowaki et al., 2008). In addition, this genes are expressed in rat and human pancreatic β cells at levels similar to those expressed in the liver, but greater than that in muscle (Kharroubi et al., 2003). The expression of AdipoR1 and/or AdipoR2 is regulated under physiological and pathophysiological states including fasting/re-feeding, obesity and insulin resistance (Tsuchida et al., 2004).

Although the available sequence information of adiponectin receptors; its biological role is still not clearly established in decapod crustaceans. Due to the lack of genetic tools for introducing or silencing the expression of specific genes and currently several transcriptional studies have been made (Lee et al., 2015). RNA interference (RNAi) has been an effective strategy since 1998 for decreasing the expression of target genes in most metazoan species (Fire et al., 1998). Application of RNAi to the crustaceans may provide an alternative means of increasing the knowledge of their

physiology (Sagi et al., 2013). In fact, many publications already shown that RNAi is an effective method for understanding growth and development (De Santis et al., 2011; Glazer et al., 2010; Soñanez-Organis et al., 2010), immunity (Robalino et al., 2007), and reproduction (Nagaraju et al., 2011; Sathapondecha et al., 2011; Treerattrakool et al., 2011). However, the size, dose, and type of RNA used vary, and the functional mechanism within cells and tissues are not clearly understood (Lee et al., 2015).

We identified the full length cDNA encoding mammalian AdipoR homolog (Liv-AdipoR) from the white-leg shrimp, *Litopenaeus vannamei,* by the Next Generation Sequencing (NGS) and Bioinformatics analysis. The full length Liv-AdipoR (1245 bp) encoded a protein with 415 amino acid residues. Liv-adipoR has high similarity to AdipoR from the insects including *Zootermopsis nevadensis* (67%), *Bombyx mori* (67%) and *Apis dorsata* (62%). Liv-AdipR exhibited the conserved 7 transmembrane domains (7 TMs) and its expression was ubiquitous. Tissue distribution and its expressional responses were also measured during molting and feeding cycles by qPCR. To estimate its function, sequence-specific Liv-AdipoR dsRNA (360 bp) was injected to the shrimp and its effects were studied.

After 72 hours of 10pmol dsRNA injection to the shrimp, the transcription level in hepatopancreas, thoracic muscle and flexor muscle showed 74%, 80% and 52% down-regulation respectively. Finally, we also identified that chronic effects of Liv-AdipoR dsRNA injection was considerably different from those in mammals, suggests its physiological functions may be different from mammalian adiponectin receptors gene.

# 2. Materials and Methods

## 2.1. Experimental animals

Live shrimp *(Litopenaeus vannamei)* of more or less similar size (32.07±7.06 mm in carapace length) and body mass (11.995±3.645g) were purchased from the local seafood market. Before the experiments each shrimp was acclimatized in a circulating aerated seawater tank (10 L) for 7 days at 27°C. The photoperiod was maintained at 12L: 12D and the shrimp were fed diced squid and poly-chaetes (5% of body weight). Salinity (34±2 psu) was maintained by daily adding deionized water and 20% of the total volume was changed weekly with fresh seawater. Octopus Diablo DC 170 skimmer (Reef Octopus, China) was used to eliminate nitrogenous waste from the shrimp culture.

Before dsRNA injection, individual body weight and carapace length were measured. After all experiment, shrimp were sacrificed and dissected. The isolated tissues were directly frozen in liquid nitrogen and stored at -80°C before use for total RNA extraction.

Determination of molt stage was based on the degree of setae development according to the method described by Chan et al. (1988). Instead of pleopods, uropods were used for molt-stage determination, as the uropods gave clear and consistent images in young animals, which can be kept alive after uropod removal (Chan et al., 1988). We have checked their molting period two times, 80 individual shrimp were taken and recorded their molting data. We divided the molting stages by following Cesar et al., (2006). According to Chan et al., (1988) and Cesar et al., (2006), shrimps were anesthetized by being placed in cold seawater (14°C) for approximately 1 min, dried using a paper towel and individually held for dissection of uropods. The endopodites of uropods were observed with X40 magnifications under a microscope. Morphological features of the uropod were taken with a digital camera, and images were analyzed.

The feeding experiments determined the expression level of Liv-adipoR exchange. There were two shrimp groups: feeding group after molting for 5 days, starving group after molting for 5 days. We recorded all the shrimp's molting related information. The starving group was fed any food and the

feeding group was fed as normally. After 5 days, each group was dissected and measured the result.

### 2.2. Double strand RNA synthesis

The target sequence for Liv-adipoR RNAi was determined by the SciTools RNAi design software (http://sg.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx? Source= menu). dsRNA (340 bp) synthesized by the mMESSAGE mMACHINE Kit according to the manufacturer's instructions (Ambion Inc., USA). Briefly, Liv-adipoR sequence-specific forward and reverse primers with a T7 promoter extension at the 5′ end were designed (Table 1). RT-PCR was performed using the primers and the expected PCR product (400 bp) was cloned into the pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α competent cells. After confirmation of the obtained DNA sequence, the template for in vitro transcription was prepared by PCR using the diluted plasmid as a template. The PCR product (20μL) was purified using a PCR purification kit (Bioneer Co, Korea) and dsRNA was synthesized using the mMESSAGE mMACHINE Kit (Ambion Inc., USA). After in vitro transcription, synthesized RNA was purified using

the RNeasy Mini Kit (Qiagen Inc., USA) according to the manufacturer's instructions. For precise annealing of the synthesized dsRNA, RNA samples were subjected to the following conditions: denaturation at 95°C for 5 min and annealing by gradually lowering the temperature (1°C every 30s) from 95°C to 25°C. The integrity and quantity of newly synthesized dsRNA were determined by 1% agarose/EtBr gel electrophoresis and by ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA stock solutions were aliquoted and stored at -80°C before use.

### 2.3. Injection of dsRNA and measurement of growth and molting

Injection of each RNA was performed by using a syringe (0.3-mm gauge and 8-mm length) into tail muscle between 2$^{nd}$ pleopods and 3$^{rd}$ pleopods of each shrimp. Ten individuals in the control group were injected with 1X PBS, and the second experiment groups were injected with 10pmol dsRNA (8 individuals) and 50pmol dsRNA (8 individuals). Body weight was measured after 72 hours of injection, and the shrimps were dissected for transcriptional analysis. The mortality and molting frequency were recorded daily.

### 2.4. Total RNA extraction and cDNA synthesis

Total RNA was isolated from dissected tissues using Trizol Reagent (TaKaRa, Japan) following the manufacturer's protocol. RNA purity was verified by measuring the absorbance at 260 and 280nm by ND-1000 spectrophotometer (NanoDrop Technologies, USA), and RNA integrity was detected by electrophoresis on 1.0% agarose gel. Before reverse transcription, total RNA was treated with DNase I (TaKaRa, Japan) to remove the genomic DNA. The cDNA for each sample was synthesized from equal amount of total RNA (1000 ng) by M-MLV reverse transcriptase (Invitrogen Co., USA) following the manufacturer's protocol with random hexamer primer.

### 2.5. Full length cDNA cloning

The full-length cDNA sequence of adiponectin receptor was used as the query to screen the EST database of NCBI adopting the default parameters. The primers used in the cloning were listed in Table 1. PCR reactions were conducted using first-strand cDNA from hepatopancreas tissues as template under the following conditions: 94℃ for 5 min and followed by 30 cycles of

94℃ for 30sec. and 60℃ for 1 min, and extension at 72℃ for 1 min. All the products were then cloned and sequenced. To confirm that the assembled sequence corresponded to a single transcript, a pair of primers (Table. 1) within the predicted 5′ and 3′ untranslated regions were designed to amplify the full-length product. The resulting products were sequenced to verify the obtained contig sequence.

Deduced amino acid sequences were obtained from confirmed sequence using an ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple alignments analysis used the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/) and these results were represented using GenDoc 2.7 (http://www.nrbsc.org/gfx/genedoc/index.html). Also, computer analyses by TopPred 1.10 program (Claros and von Heijne, 1994) were used to the topology prediction of membrane proteins.

### 2.6. Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.07976365 is shown. The percentage of replicate trees in which the

associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 295 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

### 2.7. End-point RT-PCR

The mRNA expression of the Liv-adipoR gene in various tissues, including gill, hemocyte, epidermis, hepatopancreas, gonad, brain, thoracic ganglia, heart and muscle, were determine by end-point RT-PCR. Total RNA from various tissues was extracted and quantified as described previously (Kim et al., 2013). Sequence-specific primers for Liv-adipoR with predicted sizes of 360bp were designed based on its mRNA sequence using the IDTSciTools (http://eu.idtdna.com/analyzer/applications/OligoAnalyzer/). The reference genes 18s rRNA were used as internal controls to calibrate the cDNA template, and a pair of primers for a product of 254 bp (Table 1).

**Table 1. Primers used for Liv-AdipoR**

| Primer name | Sequence | Description |
| --- | --- | --- |
| **Liv-adipoR-F1** | ATGAGTGGCTTCGACGAAATTCTGGGAA | Forward primer for full-length confirm |
| **Liv-adipoR-R1** | GAGATGGCCATGTACCGCCTTACAC | Reverse primer for full-length confirm |
| **Liv-adipoR-RT-F1** | TTCGAGACTGCGGAGGAGTTAG | Forward primer for RT-PCR & Real-time |
| **Liv-adipoR-RT-R1** | GGTTGACATCAAGGAGAAGCTC | Reverse primer for RT-PCR & Real-time PCR |
| **Liv-adipoR-dsRNA-F1** | GAATTTAATACGACTCACTATAGGGCCA CCGTTCTATGGCCCAGAGTGCCTTC | Forward primer for dsRNA synthesis |
| **Liv-adipoR-dsRNA-R1** | GAATTTAATACGACTCACTATAGGGCCA CCGCTGTGAATACGAGCTTCTCC | Reverse primer for dsRNA synthesis |
| **18s rRNA F1** | CTGCGACGCTAGAGGTGAAATTC | Forward primer for RT-PCR & Real-time PCR |
| **18s rRNA R1** | GGTTGCAAAGCTGAAACTTAAAGG | Reverse primer for RT-PCR & Real-time PCR |

### 2.8. Quantitative Real-time PCR

The expression level of Liv-adipoR was measured by quantitative RT-PCR in DNA Engine Choromo4 real-time Detector (Bio-Rad, USA). The cDNAs obtained as described above were stored at -20°C until used as templates for PCR reactions. Pairs of sequence-specific primers (Table 1) were used to amplify products of Liv-adipoR and 18s rRNA as an internal control. Efficiencies of the PCR reactions were calculated from the slope of the standard curve, which was derived from a dilution series (1:10, 1:100, 1:1000, 1:10000, and 1:100000). The real-time PCR reactions were carried out in 20µL reaction systems with 10µL 2×SYBR® Premix Ex Taq™ II (TaKaRa, Japan), 1µL forward primer (10pmol), 1µL reverse primer (10pmol), 5µL cDNA template equivalent to 500ng, and 3µL sterile distilled water. Thermal cycling conditions were 95°C for 30s, followed by 40 cycles of 95°C for 5s, 60°C for 30s, and 72°C for 30s. A melt curve analysis was added (65°C to 95°C, with increment of 0.5°C/s) to demonstrate the specificity of the PCR products, as revealed by a single peak. All data were given in terms of relative mRNA expression as means ± SD (standard deviation). Data were statistically analyzed by one-way analysis of variance

(one-way ANOVA) using the Minitab 16 Statistical software (Minitab Inc., USA) and analyzed the significance of changes in each groups by student T-test using the Excel software (Microsoft, ver. 2013). Difference was considered to be significant at ($P<0.05$).

# 3. Results

## 3.1. Genomic characterization of Liv-adipoR

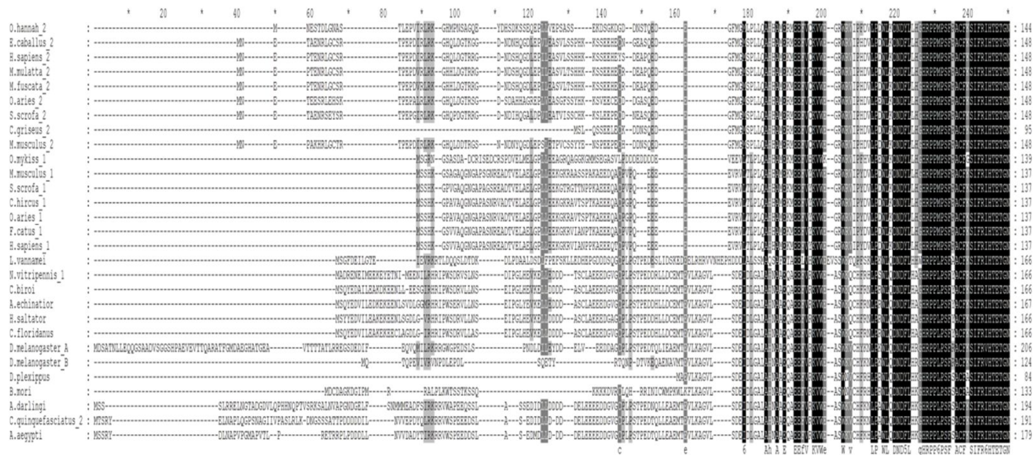
The Next Generation Sequencing (NGS) and PCR-based strategy revealed that, the full-length cDNA of Liv-adipoR were identified with orthologous human adiponectin receptor (Fig. 1). A transcript of Liv-adipoR cDNA (1245bp) encoded 415 amino acids has high similarity to AdipoR from human to insect including *Zootermopsis nevadensis* (67%), *Bombyx mori* (67%) and *Apis dorsata* (62%). Multiple amino acid alignment was performed for comparing structural similarity with other species and it has conserved 7 transmembrane domains (7 TMs) that was a character of typical G-Protein Coupled Receptors (Fig. 2). Topology prediction analysis also showed these characterization using TopPred (Claros and von Heijne, 1994). In addition, alignment result can show the clear pattern of residues ($HGXSX_5RX_6C$) in C-terminal region. The results of the phylogenetic analysis indicated that two major clades of AdipoRs, invertebrate (arthropods) and vertebrates classes. The Liv-AdipoR is classified into invertebrate (arthropods) subgroup (Fig. 3).

```
   1 gctcgctcgctgtcggtgctatatgaatcgttcgaatttgtgagggatttttttaaagtt
  61 cgctgtttgatcgacaccATGAGTGGCTTCGACGAAATTCTGGGAACGGAAATCGAAGTT
                        M  S  G  F  D  E  I  L  G  T  E  I  E  V
 121 CGGAAAAGAACTTTAGATCAGCAATCGCTTGACACAGACAAGGACCTTCCAGATGCCGCG
      R  K  R  T  L  D  Q  Q  S  L  D  T  D  K  D  L  P  D  A  A
 181 TTAGACTCGGGTTTATTCCCCGAATTCAGTAAGCTCCTGGAAGACCACGAACCAGGGGAC
      L  D  S  G  L  F  P  E  F  S  K  L  L  E  D  H  E  P  G  D
 241 GATGACAGCCAGGGATGCCCACTGCCAGGAACGCCGGAGGACGAGTCCCTCATCGACTCA
      D  D  S  Q  G  C  P  L  P  G  T  P  E  D  E  S  L  I  D  S
 301 AAGGAGGATGAAGAATTGCGACACAGAGTAGTCAATCACGAACCACACGATGACGATCTT
      K  E  D  E  E  L  R  H  R  V  V  N  H  E  P  H  D  D  D  L
 361 GCACTCAGTTCTATGGCCCAGAGTGCCTTCGAGACTGCGGAGGAGTTAGTGCACAAGGTC
      A  L  S  S  M  A  Q  S  A  F  E  T  A  E  E  L  V  H  K  V
 421 TGGGAAGAAGTTTCGAGTTGGAAGACGCAACCTTTCTCAAAGCTGCCCAAGTGGCTCCAA
      W  E  E  V  S  S  W  K  T  Q  P  F  S  K  L  P  K  W  L  Q
 481 GACAACGATTTCCTTCATCACTGGCACAGGCCTCCGCTCCCTTCATTCGCTGCCTGCTTC
      D  N  D  F  L  H  H  W  H  R  P  P  L  P  S  F  A  A  C  F
 541 AAATCTATATTTAGGATCCACACTGAAACGGGAAATATTTGGACACATCTTCTAGGATGC
      K  S  I  F  R  I  H  T  E  T  G  N  I  W  T  H  L  L  G  C
 601 ATGGCATTCATCGGAGTAATGGTGTATTTCTTGACGGGATCATCACTTGGTCAGCACTGG
      M  A  F  I  G  V  M  V  Y  F  L  T  G  S  S  L  G  Q  H  W
 661 GTACAGGTTGACATCAAGGAGAAGCTCGTATTCACAGCGTTTTTCATCGGTGCCATCTTC
      V  Q  V  D  I  K  E  K  L  V  F  T  A  F  F  I  G  A  I  F
 721 TGCTTGGGTCTCTCCTTCACCTTCCATACAGTCTCGTGTCACTCGGAGTTTGTAGGAAAA
      C  L  G  L  S  F  T  F  H  T  V  S  C  H  S  E  F  V  G  K
 781 CTCTTTAGCAAATTGGACTATTGTGGGATTGCCTTGCTGATTATGGGATCCTTTGTACCA
      L  F  S  K  L  D  Y  C  G  I  A  L  L  I  M  G  S  F  V  P
 841 TGGTTGTACTATGGGTTTTACTGTGACTTCCAACCAAAGCTTATATACCTTACTGCGGTG
      W  L  Y  Y  G  F  Y  C  D  F  Q  P  K  L  I  Y  L  T  A  V
 901 GTTCTACTTGGAATCACCACGATTGTTGTATCACTATGGGAGAAGTTTAGCACACCAACA
      V  L  L  G  I  T  T  I  V  V  S  L  W  E  K  F  S  T  P  T
 961 TTCAGACCATTGCGAGCAGCTGTGTTCTTGACATTCGGTTTGTCGGGGATTATCCCAGCT
      F  R  P  L  R  A  A  V  F  L  T  F  G  L  S  G  I  I  P  A
1021 GTGCACTACACTCTGATTGAAGGATTTGACTATGCCATCACACGTGCTTCCTTAGGCTGG
      V  H  Y  T  L  I  E  G  F  D  Y  A  I  T  R  A  S  L  G  W
1081 CTTATTCTTATGGGAGCATTGTATGTCTTGGGTGCACTGCTTTATGCTGTACGCGTTCCC
      L  I  L  M  G  A  L  Y  V  L  G  A  L  L  Y  A  V  R  V  P
1141 GAAAGATTCTTCCCAGGCAAATGTGATCTATGGTTCCAGAGTCACCAAATCTTCCACATT
      E  R  F  F  P  G  K  C  D  L  W  F  Q  S  H  Q  I  F  H  I
1201 CTGGTGATTGCTGCAGCATTCGTCCATTATCATGGAATATCCGAGATGGCCATGTACCGC
      L  V  I  A  A  A  F  V  H  Y  H  G  I  S  E  M  A  M  Y  R
1261 CTTACACACGGGGATTGTACAGCAGAAAATCTGGCAGCGATCGAAGCGCAGGACATCCTT
      L  T  H  G  D  C  T  A  E  N  L  A  A  I  E  A  Q  D  I  L
1321 TAGgatttatgaaatgttagtcatttatgcgagtgtgaaagaatctttttcgttctctga
      *
1381 tgattagaaacttgtcttgatcatagtccttgttttatatttttatttattttgattac
1441 agtgtctttattttccattcttagttgccttttcgtgaatgtatgtgaactgtggcaaat
1501 ttgcaggcatcaggttggacttttaaggaggccagtgttagtgttaaaatattttagtgt
1561 ctgctaagattatcacaaactaggcaatctgctaaaatgtgcaagttgtaatttttaaag
1621 tggcatttttttgtgacaatctttaaggtatgttagatacatggactttcaagacttgga
1681 caaaatcatgttttggtgttaaagttttgtgtgtcctgcaatcttatttcttggaaaatg
1741 tgattgaaagaaaacaaggagtttttttgcaggaaattatcattattacttttttgatggtg
1801 gtgttacgttgatatttagcatttatttttttagaatgaagtgtatgtttaatcaaggagc
1861 ctatcaatttttttgtttatattttactgaaaaaatgg 1898
```

**Fig. 1. The full-length of Liv-adipoR.**

The capital letters are indicated open read frame (ORF) region.

The stop codon (TAG) is indicated by an asterisk.



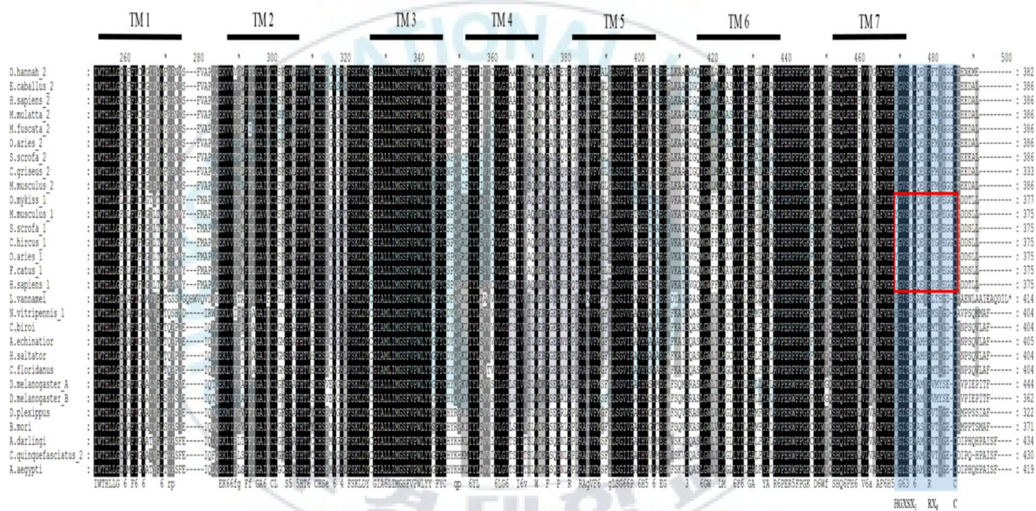



**Fig. 2. Multiple alignment of Adiponectin Receptors with those from various specieses.**

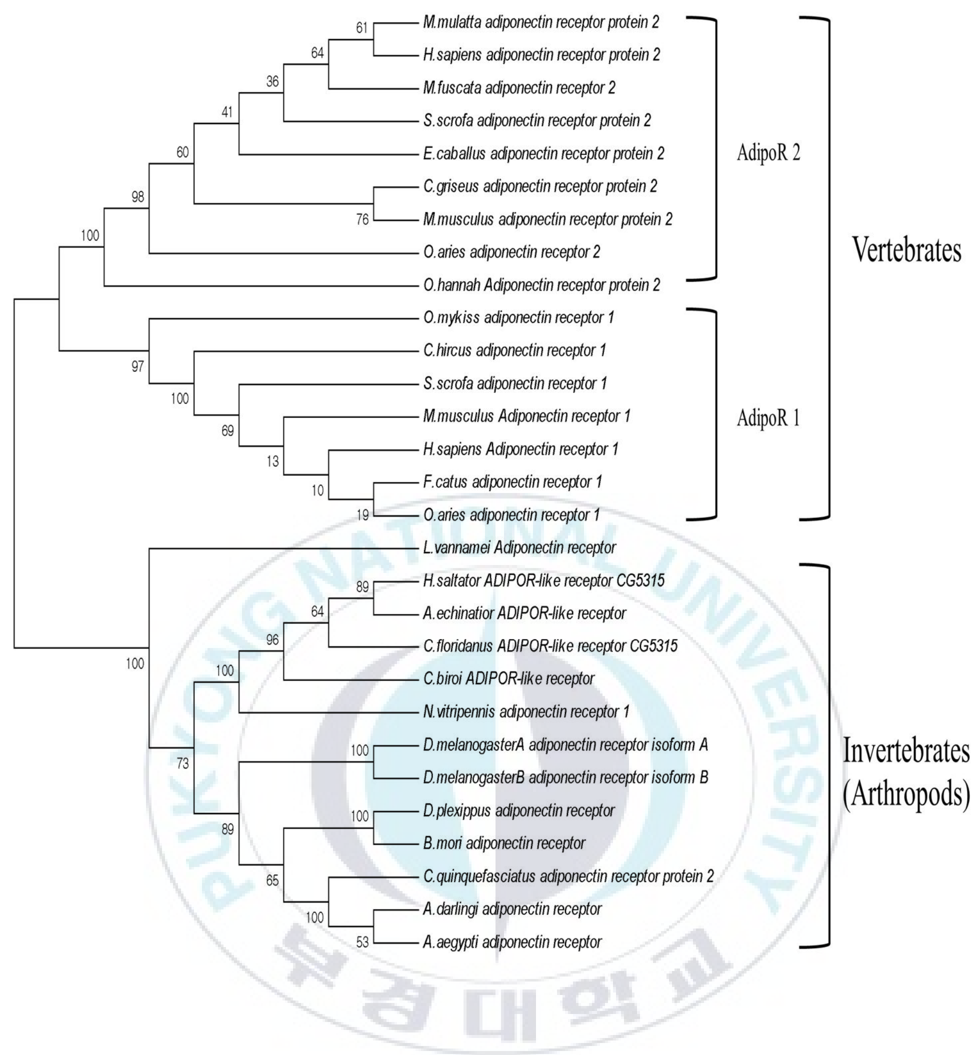



**Fig. 3. Phylogenetic analysis of Liv-AdipoR with other AdipoR family members.**

The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA6, which shows the two indicated clades of AdipoR.

### 3.2. Expression analysis of Liv-adipoR

To determine the expression profile of various tissues, end point RT-PCR was performed (Fig. 4). As shown in the Fig. 4, end-point RT-PCR analysis revealed that Liv-AdipoR mRNAs were distributed ubiquitously in a wide range of tissues. The mRNA expression was higher in hemocyte, hepatopancreas, gonad and muscle, followed by thoracic ganglia and heart tissue. No mRNA expression was found in epidermis, brain and gill tissue. The expression level of Liv-AdipoR was regulated in hepatopancreas of *L. vannamei* during the molt cycle. From the early post-molt phase (A) to inter-molt (C) phase the expression was increased slowly. After inter-molt phase the expression was increased dramatically and the maximal expression was detected in the onset pre-molt phase. After that the expression was followed by a sharp decline in the next (early pre-molt and late pre-molt) phases (Fig. 5). But there was no significant difference between the molting stages.

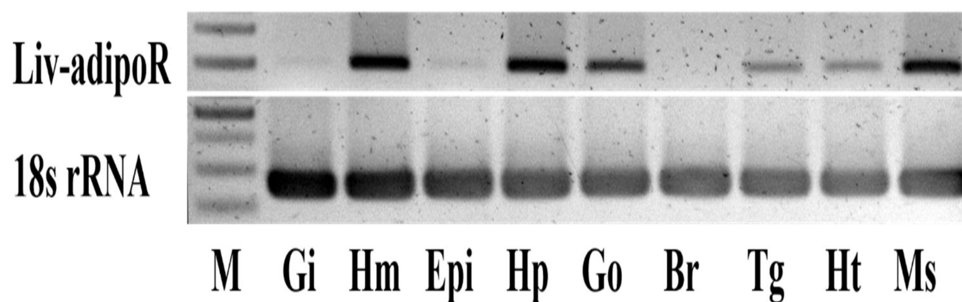



**Fig. 4. Expression level of Liv-adipoR in various tissues of *Litopenaeus vannamei.***

End-point RT-PCR was carried out for 30 cycles and products were separated on 1.5% agarose gel. The 18S rRNA was used as a control. M, size marker; Gi, gill; Hm, hemocyte; Epi, epidermis; Hp, hepatopancreas; Go, Gonad; Br, brain; Tg, thoracic ganglia; Ht, heart; Ms, muscle.

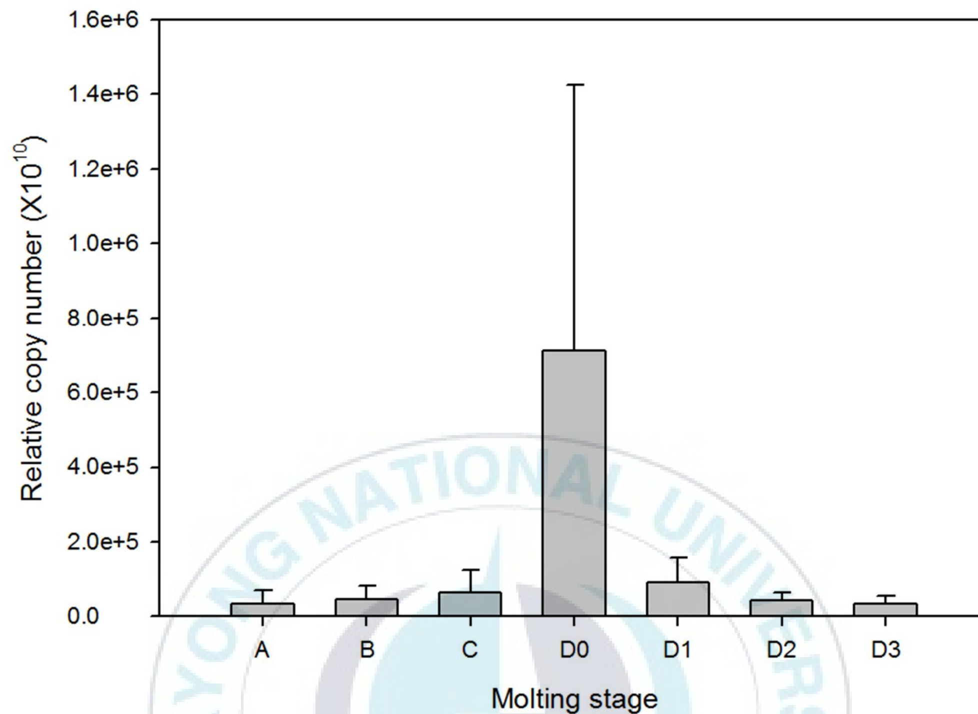


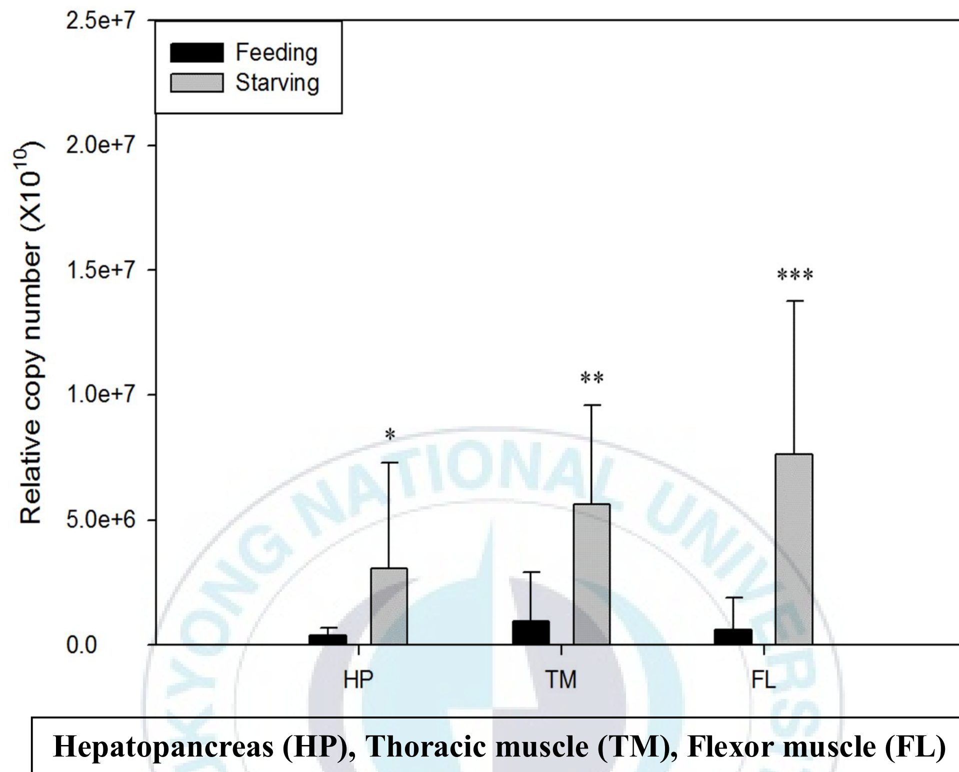

**Fig. 5. Relative copy number of Liv-AdipoR transcript in *Litopenaeus vannamei* during the molting cycles.**

Copy numbers were normalized by the number of 18S rRNA. There were no significant difference in each groups. From A to D3: Stage A (early-post molt), Stage B (late post-molt), Stage C (inter-molt), Stage D0 (onset of pre-molt), Stage D1 (early pre-molt) Stage D2 (intermediate pre-molt), Stage D3 (late pre-molt).

The quantitative RT-PCR was performed to determine the differences in the feeding and starving group. In the experiment of nutritional status, the expression level of Liv-AdipoR in hepatopancreas ($P<0.05$), thoracic muscle ($P< 0.01$) and flexor muscle ($P< 0.001$) was shown higher in starving group than the feeding group (Fig. 6).

### 3.3. Silencing of Liv-adipoR with dsRNA

To determine the effects of dsRNA in *L. vannamei* adiponectin receptor genes, the level of transcripts were measured after dsRNA injection in hepatopancreas, thoracic muscle and flexor muscle. After 72 hours of 10pmol dsRNA injection, the transcription level in hepatopancreas, thoracic muscle and flexor muscle showed 74%, 80% and 52% down-regulation respectively (Fig. 7). In case of 50pmol dsRNA experiments, transcript was up-regulated in hepatopancreas; whereas in thoracic muscle and flexor muscle, it was highly down-regulated at almost 97% and 93% respectively. However, in case of 50pmol dsRNA, the decrease level was statistically significant only in the thoracic muscle and flexor muscle.




Hepatopancreas (HP), Thoracic muscle (TM), Flexor muscle (FL)

**Fig. 6. Relative copy number of Liv-AdipoR transcript in the feeding and starving group of *Litopenaeus vannamei.***

Copy numbers were normalized by the number of 18S rRNA. Statistical significance was accepted only when P <0.05 (*), <0.01 (**), <0.001 (***).

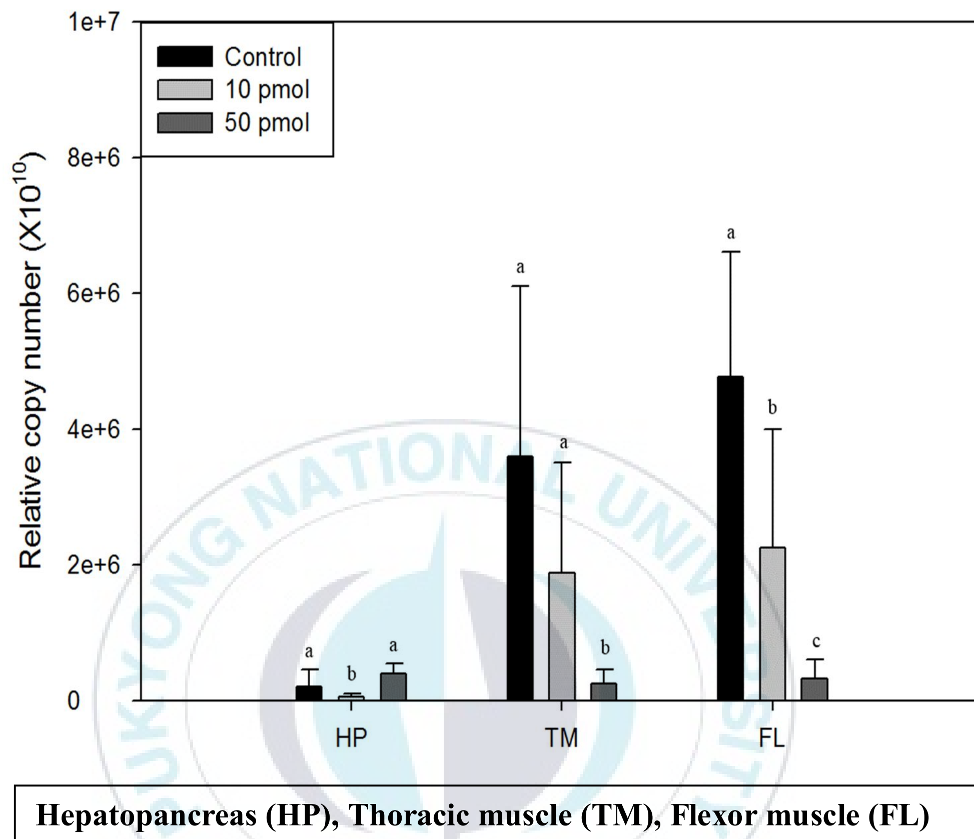



**Fig. 7. Relative copy number of Liv-AdipoR transcript in different tissues at various concentration of dsRNA injection.**

Copy numbers were normalized by the number of 18S rRNA. Means with differnet letters are significantly different (P <0.05).

# 4. Discussion

We have cloned and determined molecular characterizations of an adiponectin receptor-like in *Litopenaeus vannamei* that shares significant sequence identity with human AdipoR. Adiponectin receptors following reported studies in mammal has directed interaction with adiponection and it can mediate adiponectin effects; important effects in stimulating glucose utilization, fatty acid oxidation and improving insulin sensitivity (Kadowaki et al., 2006). By comparing Liv-AdipoR with AdipoRs in other animals, we found that Liv-AdipoR has highly conserved 7 transmembrane regions. This protein structure is similar to those of mammalian AdipoR1 and AdipoR2 which are opposite to the topology of all other reported GPCRs. Structure of AdipoR showed that the residues are found predominantly in the intracellular loops or the intracellular face of the TM domains, indicating that conserved features may be required for signal transduction and that the situation may be similar to that observed in GPCRs (Zhu et al., 2008). For example, the rhodopsin-type GPCRs have relatively low sequence similarities, yet they maintain 7 TM architecture with invariant residues observed within

the intracellular loops and much less conservation in the extracellular loops (Gether, 2000). Computer analysis using a program that predicts protein structure (TopPred) also indicates that the deduced protein is located in the plasma membrane and typically has seven transmembrane domains as well as intracellular N-terminal and extracellular C-terminal domains. The conserved sequences of N-terminal regions and the invariant residues ($HGXSX_5RX_6C$) within the C-terminal regions may be the ligand binding sites (Zhu et al., 2008).

Most recently, a 2-hybrid study revealed that the C-terminal extracellular domain of AdipoR1 interacted with adiponectin, whereas the N-terminal cytoplasmic domain of AdipoR1 interacted with APPL (adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif) (Mao et al., 2006). Moreover, interaction of APPL with AdipoR1 in mammalian cells was stimulated by adiponectin binding, and this interaction played important roles in adiponectin signaling and adiponectin-mediated downstream events such as lipid oxidation and glucose uptake (Kadowaki et al., 2006). From this information it clearly indicated that

adiponectin receptors directly interacted with adiponectin and mediated adiponectin effects (Mao et al., 2006).

Up to now, no study has been reported about the expression patterns of AdipoR in shrimp/decapod crustaceans. In mammals, AdipoR1 is ubiquitously expressed and most abundantly expressed in skeletal muscle, whereas AdipoR2 is most abundantly expressed in mouse liver (Kadowaki and Yamauchi, 2005). Unlike mammals, in which AdipoR is expressed primarily in specific tissues, the expression level of Liv-AdipoR was found in the different tissues with apparently varying levels (Fig. 4); similar results was found in other arthropods, such as *Bombyx mori*, which suggested that AdipoR was not a tissue-specific protein, and it may have diverse roles in crustaceans (Zhu et al., 2008).

In crustaceans, the hepatopancreas is an important organ for the absorption and storage of nutrients, and can synthesize digestive enzymes for food digestion (Vogt et al., 1989). The stored nutrients are transported to the muscle, gonads and other tissues during the growth and reproductive stages (Jiang et al., 2009). Furthermore, the hepatopancreas is an important site for the synthesis of vitellogenin and

sex steroid hormones, and for some biosynthetic steps in these pathways (Swevers et al., 1991). It revealed that hepatoncreas of shrimp functions as a key center of metabolism and biochemistry (Fig. 4).

The expression level of Liv-AdipoR was found in gonad, which may suggest a role of Liv-AdipoR in reproduction; in hemocyte, it may play an important role in immune response, hemocyte is also involved in the regulation of different physiological functions i.e., exoskeleton hardening, cuticle damage healing, coagulation, carbohydrate metabolism, and protein/amino acid transportation and storage (Jiravanichpaisal et al., 2006). As mammalian studies also showed that AdipoR has the function of regulation of inflammation (Yamauchi and Kadowaki, 2013). But in crustaceans, we did not find any connection about AdipoR and immune function.

The expression level of Liv-AdipoR was regulated in hepatopancreas of *L. vannamei* during the molt cycle. After inter-molt phase the expression was increased dramatically and the maximal expression was detected in the onset premolt phase (D0). After that the expression was followed by a sharp decline in the next (early premolt and late premolt) phases

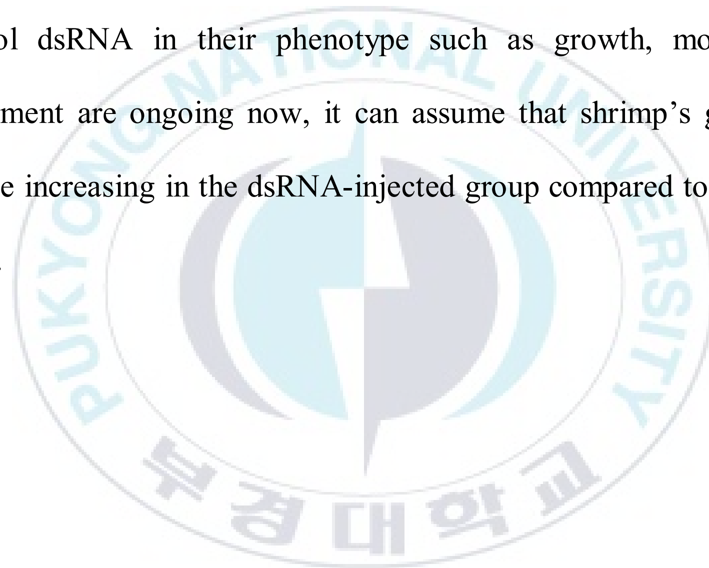
(Figure 5). So, we can assume that at the onset premolt stage (D0), suggesting increased muscle structural protein expressions during this stage, the glucose and fat utilization is excessively high and the duration of this stage is very short, so at this stage of molting the physiological functions is not clear.

In mammals, a fat-derived protein known for its effects on the liver and skeletal muscle might also serve as an energy-conserving signal to the brain during periods of starvation. In case of the increases in adiponectin concentrations in the serum and the increases in the AdipoR1 expression level under fasting conditions (i.e. starving condition) (Kadowaki et al., 2008). It has been suggested that adiponectin may stimulate the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC), downstream of AMPK, in the hypothalamus (Andersson et al., 2004; Carling, 2005). Phosphorylation of AMPK and ACC was suppressed after re-feeding (Andersson et al., 2004). Administration of adiponectin increased the phosphorylation of AMPK and ACC. In our research of Liv-adipoR transcription level of nutrient condition could explain that high expression level was showed in starving condition. According to

research in mammalian, Liv-adipoR might be active increasing AMPK activation in the hepatopancreas, it has been reported to decrease the expression of genes encoding hepatic gluconeogenic enzymes such as glucose-6-phosphate and phosphoenolpyruvate carboxykinase 1 (Lochhead et al., 2000). Reduction of these gluconeogenic enzymes by AdipoR, which may be among mechanisms by which restoration of AdipoR that could lead to reduce endogenous glucose production, apparently increased glucose infusion rate (Kadowaki et al., 2008). Briefly, starving condition in shrimps (increasing AdipoR expression level) might be stimulating energy expenditure, promotes food intake centrally, and stimulates free fatty-acid utilization, that is, it could be using their energy (Jeffares et al., 2006).

Sequence-specific dsRNA injection of was demonstrated to be the most successful strategy for gene-specific RNA knockdown in most decapod crustaceans (Sagi et al., 2013). Both thoracic muscle and flexor muscle were shown the high knockdown levels according to concentration of dsRNA. Although the amounts of injected dsRNA increased, knockdown levels of hepatopancreas unaffected by concentration of

dsRNA. Recently, other studies also have identified that various dsRNA concentration of Lv-MSTN/GDF11 gene (1pmol, 10pmol and 100pmol) unaffected in skeletal muscle (Lee et al., 2015). The mechanisms of RNAi in shrimp remains unclear, it may be appeared that have low expression level in tissues. As we mentioned above, at the high level of AdipoR transcript exhibited increasing energy expenditure. From this, we performed that long-term experiments for determination of effects of 50pmol dsRNA in their phenotype such as growth, molting. This experiment are ongoing now, it can assume that shrimp's growth rate may be increasing in the dsRNA-injected group compared to the control group.



# 5. Conclusion

In summary, our results demonstrate that adiponectin receptor is expressed in hepatopancreas and muscles of *Litopenaeus vannamei*. Molecular characteristics indicated that well conserved 7 transmembrane domain as homolog of mammalian AdipoR. Liv-adipoR has associated with their molting process especially at the pre-molt stage (D0). We also found that nutrient condition has an effect on transcript of Liv-adipoR that have the energy regulation such as glucose uptake or expenditure, fatty acid oxidation. Down-regulation of Liv-adipoR result from dsRNA in muscles may enhance the energy storage follow their growth rate at high-fat conditions. We identified that chronic effects of Liv-AdipoR dsRNA injection was considerably different from those in mammals, suggests that its physiological functions may be different from mammalian adiponectin receptors gene. Nevertheless, the evidences for relation with growth or molting is not clear, this research can give the basic knowledge for arthropod of adiponectin receptor.

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