CHARTER 4

MATERIALS AND METHODS

I. Materials

1. Yeast and bacterial strains

Saccharomyces cerevisiae strain AH109 *MATa, try1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2:: GAL*$_{USA}$*-GAL1*$_{TATA}$ *- HIS3, -GAL2*$_{UAS}$ *GAL*$_{TAT\,A}$*-ADE2, URA3:: MEL1* $_{UAS}$ *-MEL1*$_{TATA}$*-lacZ* was purchased from Clontech.

Escherichia coli TOP10F′ strain: [F′{*lac*I^{q}, Tn*10* (Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC), Ø80*lacZ*ΔM15Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R})] *end*A1 *nup*G was purchased from Invitrogen (USA).

Escherichia coli BL21 strain: F^{-}, *omp*T, *hsd*S (r_B^-, m_B^-), *gal* was purchased from Amersham Biosciences.

2. Animal

Penaeus monodon (10-15 g/individual) were obtained from shrimp farm at Songkhla and Satun province. They were maintained in 60 l aquarium with salinity between 20 and 25 ppt at room temperature for two days before starting the experiment. During acclimation shrimps were fed with commercial pellets.

3. WSSV viral stock

WSSV stock for experimental infections was prepared according to the method described by Supamattaya et al. (1998). Infected tissues (mesodermal, eyestalk, gill filament and heart) isolated from WSSV-infected *P. monodon* with prominent white spot were ground into powder in the liquid nitrogen and resuspended in K199 medium. The ratio of tissue to K199 medium was 1:5. After centrifugation at 3,000xg for 10 min

(4°C), the supernatant was filtered through a 0.45 μm sterile membrane. The stock solution of virus was kept in K199 medium at -80°C and thawed at 4°C before use.

4. cDNA library for WSSV infected shrimp

The cDNA library of WSSV infected *P. monodon* was obtained from the project: Genomic Researches for Increasing Culture Efficiency of Black Tiger Shrimp.

The subtractive cDNA library from haemolyph of *P. monodon* experimentally infected with WSSV (Bangrak et al, 2002).

5. Primers

The nucleotide primers for cloning into pGBKT7 and pGADT7, 5′-RACE (Table 5) and RT-PCR (Table 6) were purchased from Life Technologies, USA.

6. Plasmid Vector

- pGEM® - TEasy Vector was purchased from Promega (USA).
- pGBKT7 Vector was purchased from Clontech (USA).
- pGADT7 Vector was purchased from Clontech (USA).
- pGEX-4T-1 Vector was purchased from Amersham Biosciences (USA).

7. Chemicals

All of chemicals and solvents (analytical grade) used were purchased from Life Technologies, USA; Fluka, Switzerland; Sigma, USA, PIERCE, USA, Amersham Biosciences, UK: Clontech, USA and BIO-RAD, USA.

8. Enzymes

All enzymes and other modifying enzymes were purchased from New England Biolabs, USA; Promega, USA; Life Technologies, USA and Sigma; USA.

9. Antibodies

All antibodies were purchased from Amersham Biosciences, USA, Pierce, USA and Roche Molecular Biochemicals, USA.

Table 5. The sequences of the optimal primers used for reverse transcription PCR analysis (RT-PCR)

Primer	PCR fragments (bp)	Sequence	Tm (^{o}C)
5′-β-Actin	500	5′-CAGATCATGTTYGAGACCTTC-3′	46
3′-β-Actin	500	5′-GATGTCCACGTCRCACTTCAT-3′	48
5′-α_2M	546	5′-CGAGATCTACCATTATGAGGATAAC-3′	50
3′-α_2M	546	5′-CTAAGCTTTCAATCGCACCCTTCGA-3′	53
5′-α_2MR	861	5′-CCGGATCCTTGGCTACGCAATCCTG-3′	53
3′-α_2MR	861	5′-CGCTCGAGTCAATCGCACCCTTCGA-3′	59

Table 6. The sequences of the optimal primers for 5′-RACE of α_2M

Primer	Sequence	Tm (°C)
AAP	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′	69
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′	53
UAP	5′-CAUCAUCAUCAUGGCCACGCGTCGACTAGTAC-3′	61
GSP1	5′-GATATGAGGTTCACTTCGAT-3′	43
GSP2	5′-CGTTGTCAGCGTCGACCTTGAG-3′	53
Nested	5′-CTCCATGGTGATCTTCACGTAGGTG-3′	54

II. Methods

1. The construction of bait vector for yeast two hybrid assays

1.1 BD-Syntenin

The cDNA sequence encoding the open reading frame (ORF) of Pm-syntenin was amplified by PCR from a plasmid obtained from previous work (Bangrak, et al., 2000) the forward primer flanked by *Eco*RI site (5′ CGAATTCATGGGTCTGTACCG TC-3′) and reverse primer flanked by the *Bam*HI site (5′GGGATCCTTACAGGTCGGGA ATG-3′). The amplified product was subcloned in frame into pGBKT7 (Clontech), a bait vector that encodes the Gal 4 DNA binding domain and transformed into *E. coli* TOP10F′ (Appendix A, 4). Plasmids containing the inserts were purified with the QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5) and sequenced by using the ABI Prism BigDye Terminator Cycle Sequencing kit with visualization of results on an ABI Prism 377 DNA Sequencer (Appendix A, 6). The plasmid was named as BD-Syntenin.

1.2 BD-NS

BD-NS was constructed by isolating the *Eco*RI- *Xho*I fragment of the N-terminal (amino acid 1-131) of Pm-syntenin and subcloned into pGBKT7. The recombinant vector was transformed into *E. coli* TOP10F′ (Appendix A, 4). Plasmids containing the inserts were purified with the QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5). The plasmid constructs were verified by sequencing (Appendix A, 6).

1.3 BD-PDZ1,2

A plasmid containing PDZ1 and PDZ2 domains of Pm-Syntenin (BD-PDZ1,2) was obtained by using PCR with the forward primer (5□-CCGAATTCATGGTGGCTCCCATCTC-3□) and the reverse primer (5□-CGGGATCCTTACAGGTCGGGAATG-3□). The PCR product was subcloned into *Eco*RI-*Bam*HI of pGBKT7 and transformed into *E. coli* TOP10F′ (Appendix A, 4).

Plasmids containing the inserts were purified with the QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5). The plasmid constructs were verified by sequencing (Appendix A, 6).

1.4 BD-PDZ1

A plasmid of PDZ1 (BD-PDZ1) was constructed by ligating the PCR product that was amplified by the forward primer (5□CCGAATTCATGGTGGCTCCCATCT -3□) and the reverse primer (5□CTTCTCGAGTTATGGCCTGTCTCTAATGGC-3□) into the *Eco*RI-*Sal*I site of a pGBKT7 vector and transformed into *E. coli* TOP10F′ (Appendix A, 4). Plasmids containing the inserts were purified with the QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5) and sequenced by using the ABI Prism BigDye Terminator Cycle Sequencing kit with visualization of results on an ABI Prism 377 DNA Sequencer (Appendix A, 6).

2. Library construction for yeast two hybrid library screening

2.1 WSSV experimental infection

Shrimp were challenged with 100 µl of a $1:10^6$ dilution of the WSSV stock solution (LD_{50} = $4.5x10^6$). Haemolymph was withdrawn from each shrimp for extraction of total RNA.

2.2 Isolation of total RNA from haemolymph of *P. monodon*

Total RNA was prepared from the shrimp haemolymph using Trizol LS® reagent (GIBCO BRL). The detailed protocol used is as follow; after withdrawing the haemolymph from shrimp, 200 µl was vigorously mixed in 200 µl of Trizol LS® reagent and vortex. The vortexed samples were incubated for 5 min at 30 °C to permit the complete dissociation of nucleoprotein complexes and then 0.1 ml of chloroform was added into the tube. The sample tube was vigorously shaken by hand for 15 sec and incubated for 2-3 min at room temperature. The sample was centrifuged at 12,000 rpm for 15 min at 4 °C.

Subsequently, the aqueous phase was collected and transferred into a fresh tube and mixed with 0.25 ml of isopropyl alcohol to precipitate RNA. The sample was incubated at -20 ^{o}C for 20 min and then, centrifuged at 12,000 rpm for 15 min at 4 ^{o}C for RNA precipitation. The RNA pellet was washed with 0.5 ml of 75% ethanol followed by centrifugation at 7,500 rpm for 5 min at 4 ^{o}C. The RNA pellet was dried in a vacuum, resuspended in 50 µl of RNase free water and incubated at 60^{o}C for 10 min in order to increase its solubility. The RNA solution was stored at -70^{o}C. The amount of RNA was determined by spectrophotometry at 260 nm and analyzed by formaldehyde agarose gel electrophoresis (Appendix A, 1).

2.3 Construction of cDNA library into pGADT7 vector

Total RNA was isolated from WSSV infected shrimp heamocytic using Trizol reagent (GIBCO BRL). Poly(A)$^{+}$ RNA was purified with Poly(A) Quick mRNA Isolation kit (Stratagene) and used to produce cDNA using the Zap Express cDNA synthesis kit from Stratagene. Briefly, the kit used modified oligo (dT) primer carrying an *Xho*I restriction site and *Eco*RI adapters allow unidirectional cloning in the appropriate vector. The cDNAs were the site fractionated and cDNAs of 800-1,500 bp were pooled and ligated into the pGADT7 prey vector containing activating domain previously digested with the *Eco*RI and *Xho*I restriction enzymes. After transformation in bacteria, 1.5 x10^{5} independent clones were obtained. Several clones were randomly digested with *Eco*RI and *Xho*I, and all of them contained inserts with an average size of 800-1500 bp. Plasmid DNA were prepared using the QIAprep Spin Miniprep Kit (QIAGEN) (Appendix A, 5), and the library was not subsequently amplified. Furthermore, the subtractive cDNA library from haemolyph of the black tiger shrimp infected with WSSV (Bangrak et al., 2002) was subcloned into pGADT7 with *Eco*RI and *Xho*I restriction enzymes and the plasmid prepared for yeast two hybrid assays using the same method as the WSSV infected shrimp cDNA library.

3. Yeast two hybrid screening

3.1 Yeast transformation

A colony of yeast strain AH109 was inoculated into 30 ml of YPDA medium and incubated at 30°C with shaking at 220 rpm. After overnight culture cells were transferred into 300 ml of YPDA medium. Yeast cells were further incubated for several hours until the OD_{600} reached 0.4-0.6. The cell was pallet by centrifugation at 5,000 rpm for 5 min at room temperature. The pellet was washed with sterile deionized water once and resuspended in 1.5 ml of 100 mM lithium acetate pH 7.5. After the solution was incubated at 30 °C for 20 min the cell was transfer into a new micro centrifugation tube 100 µl for each transformation reaction, centrifuged and then the supernatant was removed by pipette. A reaction mixture for yeast transformation consisted of; 240 µl of 50% of PEG3350, 36 µl of 1 M Lithium acetate pH 7.5, 5 µl of denatured herring carrier DNA (10 mg/ml), X µl of plasmid DNA (100 ng to 5 µg) and 79-X µl of sterile distilled water were subsequently added the cell pellet. The mixture was vigorously mixed by vortex for 2 min and then incubated at 30 °C for 30 minutes. The cells were heat shocked at 45°C for 15 min and centrifuged at high speed for 15 seconds. The supernatant was discarded and the pellet gently resuspended with 200- 400 µl sterile distilled water. The cell suspension was spread on selective medium and incubated at 30 °C for 2-4 days.

3.2 Library screening

The yeast two-hybrid screening was carried out with the MATCHMAKER Gal4 Two-Hybrid System 3 (Clontech). The bait BD-syntenin was used to screen 10^5 independent recombinant clones of the haemocyte cDNA library in *Saccharomyces cerevisiae* strain AH109 using the lithium acetate transformation method. The protein interactions between pGBKT7-53 and pGADT7-T were used as a positive control. A negative control also was used transformed with a transformed BD-Syntenin with the AD vector only. Positive clones were selected from colonies on a synthetic dropout (SD) plate lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp) containing 5-bromo-4-chloro-3-indolyl- α -galactopyranoside. Colonies were visible after 2-4 days at 30 °C.

3.3 β- galactosidase assay: Colony lift method

The positive clones were streaked on the selective medium at 30 °C for 2-4 days. The fresh colony was lifted by sterile dried Whatman filter paper. Another sterile filter paper was presoaked with fresh Z-buffer/X-gal solutions and placed onto a new clean plate. The filter, with colony side up was frozen in liquid nitrogen for 10 seconds and thawed at room temperature 10 times before careful placing it on the soaked filter paper without air bubbles. The plate was incubated at 30 °C and periodically checked for the appearance of a blue colony.

3.4 DNA recovery from the positive clones and characterization

The plasmid DNA was then isolated from those positive clones that activated all four yeast reporter genes (*HIS3*, *ADE2*, *LacZ* and *MEL1*) using the QIAprep Spin Miniprep Kit (QIAGEN) (Appendix A, 5) adding liticase enzyme. The positive clones were first screened by PCR amplification with the 5′BD primer (5′-TCATCGGAAGAGAG TAG-3′) and the 3′BD primer (5′-GTCACTTTAAAATTTGTATACA-3′) and the 5′AD primer (5′-CTATTCGATGAAACCCCACCAAACC-3′) and the 3′AD primer (5′GTGAA CTTGCGGGGTTTTTCAGTATCTACGATT-3′). The PCR product was analyzed by 1.5% gel electrophoresis (Appendix A, 2). The yeast plasmid DNA was transformed into *Escherichia coli* Top10F′ (Appendix A, 4) to recover the library plasmid using antibiotic selection; kanamycin for the BD vector selection and ampicilin for the AD vector selection. The plasmid was purified using QIAprep spin miniprep kit (QIAGEN) (Appendix A, 5) and analyzed by restriction digestion. The library plasmid inserts with a confirmed interaction (blue yeast colonies) were sequenced using the ABI Prism 377 DNA Sequencer (Appendix A, 6). Gene database searches were performed through the National Center for Biotechnology Information using the BLAST network service. To confirm the screening result, recovered plasmids were retransformed into the host strain, as recommended by manufacturer

4. *In vitro* interaction assay

4.1 Expression and purification of recombinant Pm-Syntenin

A *Bam*HI-*Pst*I fragment containing the entire coding region of Pm-syntenin was obtained from previous work (Bangrak et al., 2002). These recombinant plasmids as well as an insertless pQE40 were transformed into *E. coli* M15 and the proteins were also prepared according to Bangrak et al. (2002).

4.2 Expression and purification of GST-fusion protein

The receptor binding domain sequence of Alpha-2-macroglobulin (181 amino acids) was cloned into the pGEX-4T-1 vector (Amercham Biosciences), to create a fusion protein with the GST protein at *Eco*RI and *Xho*I restriction enzyme sites. The vector with insert was transformed into *E.coli* TOP10F′ for maintenance and the insert was sequenced to confirm that the sequence was in-frame. The protein was expressed in the *E.coli* BL21 (Amercham Biosciences). Protein production was accomplished by standard methods of bacterial growth in the presence of ampicillin, followed by induction with 0.5 mM IPTG. The bacterial pellet was resuspended in lysis buffer (50 mM NH_2PO_4, pH 8.0, 300 mM NaCl and 10 mM imidazole) containing 1 mM PMSF and centrifuged at 4 ^{o}C for 20 min at 6000xg. The supernatant was incubated with Glutathione Sepharose 4B resin (Amersham Biosciences) for 1 h at room temperature, washed three times with phosphate-buffered saline, pH 7.4, and eluted with 10 mM reduced glutathione elution buffer. The presence of protein was confirmed by 12 % SDS-PAGE.

4.3 *In vitro* binding assays (GST pull-down assays)

For pull-down experiments, two methods were used. First, the interaction between 6xHis-syntenin and GST-α_2M was examined by incubating the purified GST-α_2M fusion protein with a Glutathione Sepharose 4B resin (50 µl of 50% bed slurry) for 1 h. Purified recombinant 6xHis-syntenin protein was added, and incubation was continued for another 2 h at room temperature. After incubation, the beads were washed ten times with pH 7.4 phosphate-buffered saline. The fusion protein was eluted by the addition of buffer containing reduced glutathione. The proteins were resolved by 12 % SDS-PAGE and transferred onto nitrocellulose membrane. Blots were incubated with Anti-His Tag antibody (conjugated His-Probe horseradish peroxidase conjugated, Pierce; diluted 1:20,000) and the blots were visualized using the ECL detection reagent (Pierce). To confirm the presence of the GST-fusion protein, the blots were tested with Anti-GST (Amercham Biosciences;

1:2000) and conjugated goat anti-mouse IgG-alkaline phosphatase (Pierce; diluted 1:20,000). Lumi-Phos was used as a substrate for detecting chemiluminescence.

In the second method, the full length cDNA of Pm-syntenin was cloned into pGEX-4T-1 (Amersham Biosciences) and the recombinant protein was prepared according to the above protocol. A DNA template of pGADT7-α_2M that already contains the T7 RNA polymerase promoter was used in an *in vitro* transcription/translation reaction by the TNT quick-coupled transcription/translation system. The α_2M protein was radio labeled with ^{35}S-methionine (MP Biomedical Inc.). The in vitro binding reaction was generated by adding ^{35}S-α_2M and GST-syntenin into buffer A, containing 50 mM HEPES, pH 7.5, 70 mM KCl, 0.5mM ATP, 5 mM $MgSO_4$, 1 mM dithiothreitol (DTT), 0.05% nondidet P-40, 2 μg/ml BSA, 0.5 mM PMSF, and protease inhibitor mixture (Roche Molecular Biochemicals) and allowed to form complexes at 4 °C for 90 min. The Glutathione 4B beads were then added and incubated for 30 min. The complex was washed with buffer A four times and twice with buffer B (buffer A supplemented with 0.1% Nondidet P-40). Binding proteins were eluted into SDS-loading buffer (50mM Tris-HCl, pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), boiled for 5 min and loaded onto 12 % SDS-PAGE gels. The result was visualized by fluorography and imaged with a phosphorimager system (Bio-Rad).

5. Analysis of expression of Pm-α_2M transcript

To determine the expression of the Pm-α_2M in shrimp, RT-PCR analysis was performed. Primer were designed for each of the detected sequences and synthesized by Life Technologies, USA. The sequences of primers for α_2M were 5′-CGAGATCTACCAT TAATGAGGATAAC-3′(sense primer) and 5′-CTAAGCTTTCAATCGCACCCTTCGA-3′ (anti-sense primer). Degenerate β-Actin primers was designed according to the conserved amino acid sequences QLMFETF and MKCDVDI (Yeh et al., 1999) and used to amplify a PCR product of 500 bp in the RT-PCR experiments as an internal control of gene expression. One microgram each of total RNA from haemocytes of uninfected samples and infected samples were used as template in 50 μl of RT-PCR according to the One-Step RT-PCR Kit (QIAGEN). The linear range was at cycle 25. The reaction was started at 50 °C for 30 min followed by an initial PCR activation step at 95 °C for 15 min followed by 25 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min. The RT-PCR

products of each sample were examined by 1.5% agarose gel electrophoresis and quantified by computerized gel documentary (Bio-Rad). The identity of PCR products was confirmed by cloning into pGEM® -TEasy and sequence analysis using the ABI prism 377 apparatus.

6. Amplification of α_2M cDNA using 5′RACE

6.1 First strand cDNA synthesis

In first step, five micrograms of sample total RNA was used as template by mixing with 2.5 pmole GSP1 (gene specific primer) and DEPC-treated water for a final volume of 15.5 μl. The mixture was incubated at 70°C for 10 min and then chilled on ice for 5 min to denature RNA. This content was mixed with the reaction buffer (2.5 μl of 10x PCR buffer, 2.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix and 2.5 μl of 0.1 M DTT). After incubation at 42°C for 5 min, 1 μl of Superscript ™ II RT was added, gently mixed and further incubated at 42°C for 2 h, the reaction was then terminated by incubation at 70°C for 15 min. To remove the RNA template from the first strand cDNA product, 1 μl of RNase mix was added and incubated at 37°C for 30 min. The first strand cDNA was stored on ice or at −20°C until the purification step was performed.

6.2 Purification of cDNA by GlassMax DNA isolation spin cartridge

To remove the excess nucleotides and GSP1, the first strand cDNA product was mixed with 120 μl of binding solution (6 M NaI) and then the solution was transferred to a GlassMax spin cartridge and centrifuged at 13,000x*g* for 20 sec. The cartridge was washed three times with 0.4 ml of cold 1x wash buffer and 0.4 ml of 70% ethanol, respectively. After that the cartridge was centrifuged additionally for 1 min to remove remain ethanol. The cDNA was eluted from spin cartridge by adding 50 μl of distilled water (65°C) and centrifugation at 13,000xg for 20 sec.

6.3 TdT tailing of cDNA

In order to generate the abridged anchor primer-binding site on the 3′ end of the cDNA, TdT tailing was performed according this condition. The 10.0 µl of purified cDNA was mixed with reaction mixture (6.5 µl of DEPC-treated water, 5.0 µl of 5x tailing buffer, 2.5 µl of 2 mM dCTP) and incubated at 94°C for 3 min and then chilled on ice for 1 min. Next, 1.0 µl of TdT was added to this mixture and subsequently incubated at 37°C for 30 min. The reaction was terminated by heat inactivation at 65°C for 10 min and then dC-tailed cDNA was placed on ice for further analysis.

6.4 PCR of dC-tailed cDNA

The PCR reaction was performed in 50 µl mixture containing 1xPCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM of dNTP, 400 pmole of each primers (GSP2 and AAP), 5 µl of dC-tailed cDNA and 2.5 units of Taq DNA polymerase (Life Technologies). Amplification was started with an initial PCR activation step at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, specific annealing temperature for 1 min, 72°C for 1 min and final extension step at 72°C for 10 min. The first PCR product was subjected to nested PCR performed with GSP3 and AUAP primer (200 pmole of each primer). The 5′-RACE products were analyzed by 1.8% agarose gel electrophoresis and visualized by ethedium bromide staining under UV-light (Appendix A, 2). The PCR amplification products were purified using QIAquick PCR purification (QIAGEN) and ligated to pGEM® -TEasy (Appendix A, 3). The ligation products were transformed into *E. coli* TOP10F′ (Appendix A, 4). The recombinant plasmids were isolated as described in Appendix A, 5 and the recombinants were screened by restriction enzyme digestion. The nucleotide sequence of the recombinants was determined by using automated DNA sequencing (ABI Prism 377 DNA Sequencer) (Appendix A, 6).