CHARTER 5

RESULTS

Part I. Yeast two-hybrid screening

1. Identification of Pm-syntenin binding protein using yeast two-hybrid system

The nucleotide sequence encoding ORF of the Pm-Syntenin region (969 bp) was amplified with specific primers flanked by the *Eco*RI and *Bam*HI restriction sites, respectively. After the PCR products were digested with *Eco*RI and *Bam*HI, the digested fragments were then inserted in-frame into pGBKT7 that has been digested with the same restriction enzyme. The ligation mixture was first transformed into the *E. coli* strain TOP10F′ to screen positive clones. The positive clones were confirmed with a sequencing method before using for the yeast two hybrid screening. The scheme of the recombinant bait vector is shown in Figure 7 and was named BD-syntenin.

A cDNA library was obtained from the haemocytes of WSSV infected shrimps. This cDNA library was carrying *Eco*RI and *Xho*I restriction sites according to the manufacturer's instruction. After fractionation, the cDNA of 800-1,500 bp were pooled and ligated into the pGADT7 prey vector previously that had been digested with the *Eco*RI and *Xho*I. The scheme is shown in Figure 7. The ligated reaction was transformed into *E. coli* strain TOP10F′, 1.5×10^5 independent clones were obtained. All the clones were pooled before plasmid DNA purification with the QIAprep Spin Miniprep Kit. The recombinant plasmid that contained the cDNA library fragments (800-1,500 bp) was digested with the *Eco*RI and *Xho*I for the quality determination of the library. The digested DNA reaction was run on a 1.5% agarose gel electrophoresis and stained with ethidium bromide. The result showed that cDNA size 800-1,500 bp bands were digested (Figure 8). Variable sizes of digested fragments were obtained from randomly selected clones. The results suggested the high quality of the pGADT7-cDNA library was suitable for yeast two hybrid library screening.

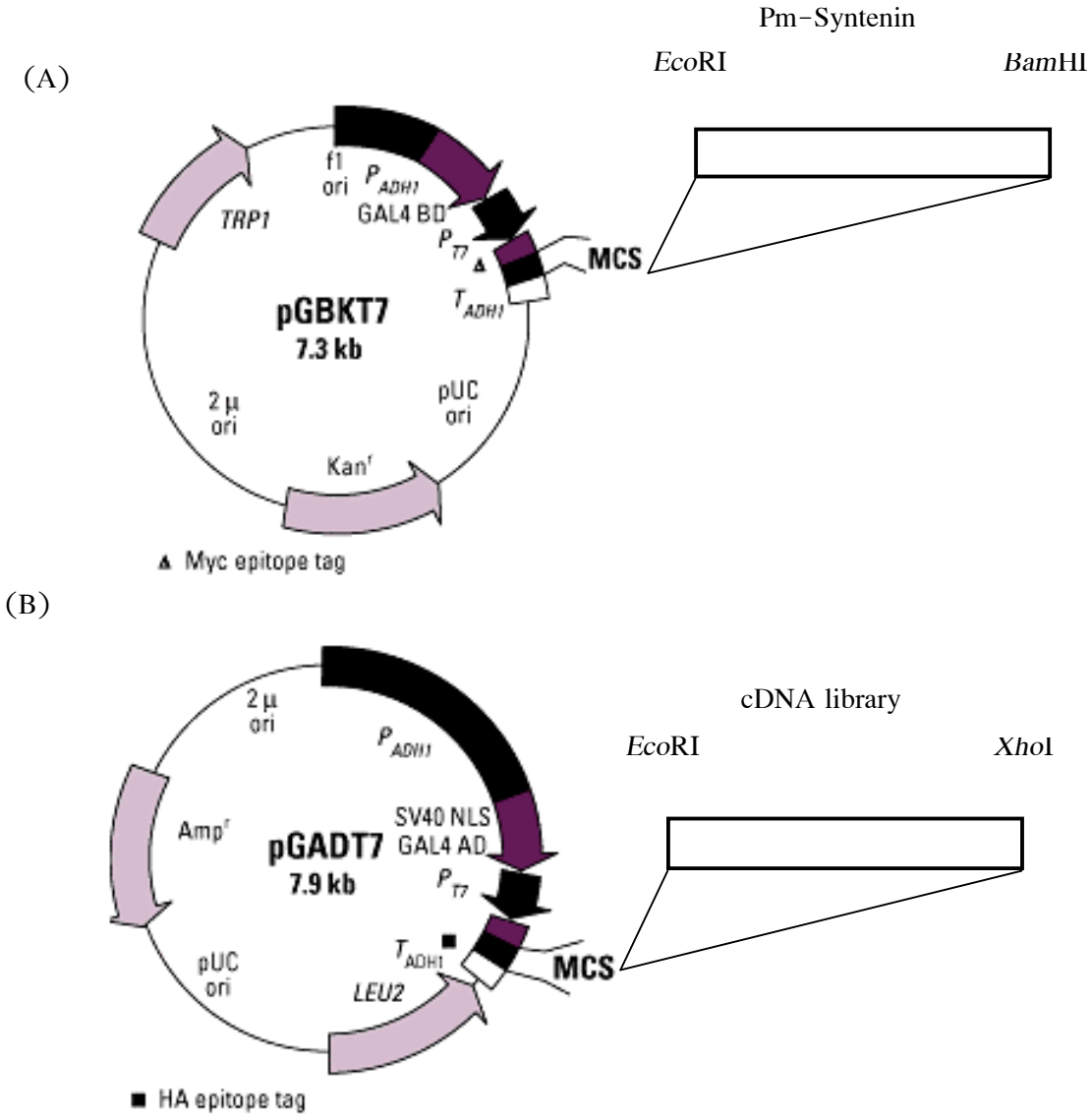

Figure 7. Construction of bait and prey recombinant vectors for yeast two-hybrid

(A) Full length Pm- syntenin was constructed into pGBKT7 that contained the GAL4 DNA binding domain using *Eco*RI and *Bam*HI sites. **(B)** The cDNA library of WSSV infected shrimp (800-1,500 bp) was cloned into pGADT7 at *Eco*RI and *Xho*I restriction enzyme site. This recombinant vector contained the GAL4 activating domain.

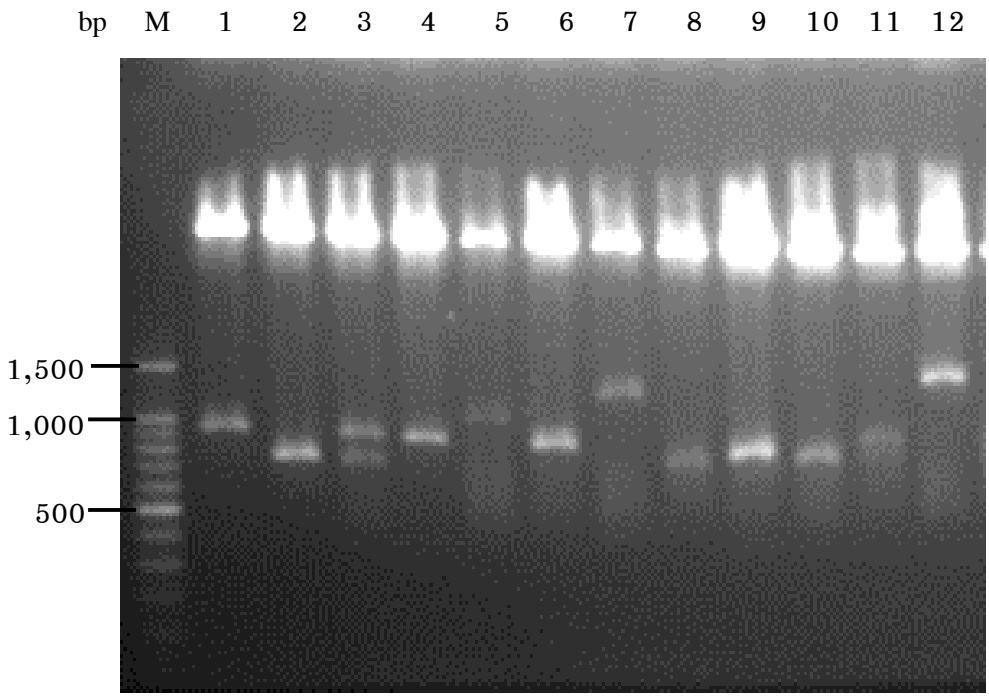

Figure 8. Restriction endonuclease analysis of pGADT7-cDNA

1.5 % (w/v) agarose gel electrophoresis (ethidium bromide staining) of DNA patterns of isolated plasmid transformants with *Eco*RI and *Xho*I digestion.

Lane M: 100 bp marker

Lane 1-12: Plasmid DNA of positive clones (1-12) were digested with *Eco*RI and *Xho*I (800-1,500 bp)

2. Yeast transformation with bait plasmids

BD-syntenin was used as bait in yeast two-hybrid screening. The yeast cells were transformed with a bait vector using lithium acetate method. Tryptophan is a selectable marker of pGBKT7. Therefore, the yeast transformants were grown on SD-Trp medium. The positive clones were maintained on SD-Trp medium. After the cells had been grown on the liquid medium (SD-Trp), DNA plasmid was then purified using the QAIprep Spin Miniprep Kit (QAIGEN) and adding liticase enzyme. PCR was performed using both 5′BD primer and 3′BD primer. The PCR product was analyzed by 1.5% gel electrophoresis. The PCR result was show in Figure 9. The amplification product was observed at the expected size at about 1,200 bp after ethidium bromide staining. In order to eliminate self activator baits or auto-activation that can activate the transcription without activating domain of interaction partners, this clone was checked for auto-activation by using a selective medium containing 3-AT (3-amino-1, 2, 4-triazole) to protect transactivation of the *HIS3* reporter gene in the absence of a protein partner. The concentration of 3-AT was added into the medium at the concentration including 0, 2.5, 5, 7.5, 10, 12.5 and 15 mM 3-AT in the medium to determine the lowest concentration of 3-AT that can prevent growth of yeast cells containing bait plamid. We found that 2.5 mM is the appropriate amount of 3-AT using for yeast two hybrid screening. In addition, the positive clones were tested for the self activation of *LacZ* reporter gene by the β-galactosidase assay using filter-lift method. No self activators baits were found. The last check to eliminate for a false positive clone was to cultures the baits's clones on selective medium absence either leucine or adenine in order to test for the auto-activation of the *LEU2* reporter gene as well. The BD-syntenin was suitable bait for library screening.

3. Identification of syntenin binding protein using library screening

To identify haemocyte proteins that bind Pm-syntenin, we performed a yeast two hybrid screening with the full length of the Pm-syntenin as bait. The Pm-syntenin encodes a 322 amino acid protein containing two PDZ domains in tandem. These two domains span amino acids 135-218 (PDZ1) and 219-299 (PDZ2), respectively. Pm-syntenin was constructed into the DNA binding domain fusion protein vector. The pGADT7-cDNA library was transformed into yeast cells that contained BD-syntenin using

the lithium acetate method. The positive clones were selected on a high stringency medium (SD/-Trp/-Leu/-His/-Ade containing 2.5 mM 3-AT and 5-bromo-4-chloro-3-indolyl-α-D-galactosidase). The positive control for the yeast two hybrid screening was pGBKT7-53 and pGADT7-T according to manufacturer's instruction. The negative of this experiment was performed by co-transformed using BD-syntenin and pGADT7 empty vector. No colony was shown on negative control plate. The efficiency of the co-transformation was $1x10^4$ cfu/μg of DNA. The positive clones were streaked on a selective medium for propagation.

4. Plasmid recovery from yeast cells

Once putative positive clones were obtained then the library plasmid could be recovered through bacterial transformation using the DNA isolated from these clones. Yeast plasmid DNA was purified using the QAIprep Spin Miniprep Kit. PCR was performed using both 5′AD and 3′AD primers to check for the library transformation into yeast with one plasmid or double plasmids to single yeast. The PCR screening result was shown in Figure 10. 5′BD and 3′BD primers were used to determine the baits plasmid from the positive clones. Yeast plasmid DNA was re-transformed into the *E .coli* strain TOP10F′. The transformants were spread into two kinds of selective media. Kanamycin was used as the selectable marker in the medium for pGBKT7 or baits vectors identification and ampicilin was used for identification of pGADT7 vectors. After plasmid DNA was purified from bacteria. The size of cDNA was check by *Eco*RI and *Xho*I digestion.

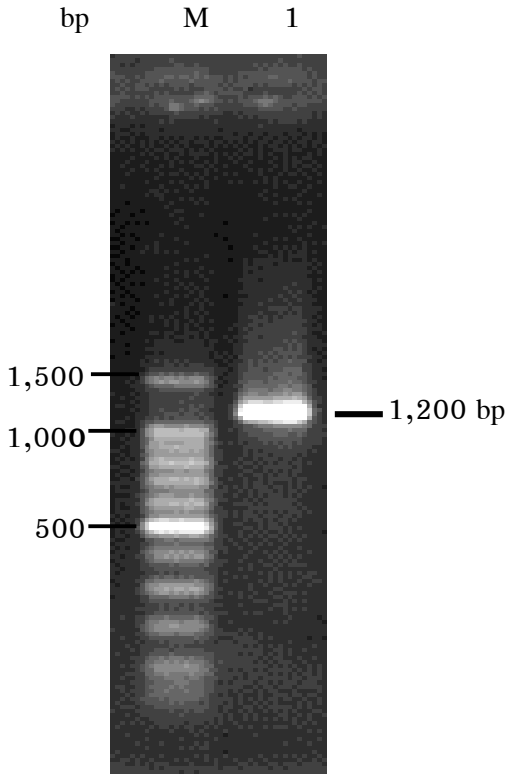

Figure 9. PCR product of positive clones from yeast transformation with BD-syntenin

PCR product was amplified from positive cloned using both 5′BD and 3′ BD primers and run on 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide and visualized with UV light.

Lane M: 100 bp marker

Lane 1: PCR product of positive clone of BD-syntenin

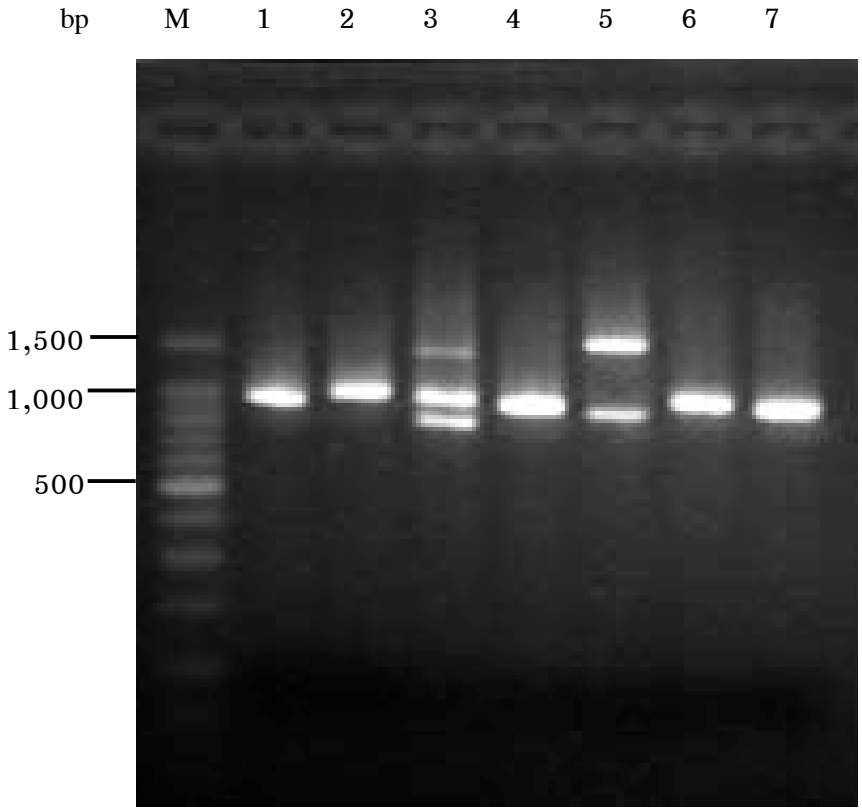

Figure 10. PCR product of positive clones from yeast two-hybrid library screening

PCR products were amplified from positive cloned using both 5′AD and 3′ AD primers and run on 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide and visualized with UV light.

Lane M: 100 bp marker

Lane 1-7: PCR products of positive clones

5. Sequencing analysis

The positive clones of the Pm-syntenin binding proteins that were obtained from the cDNA of the WSSV infected shrimp and the subtracted cDNA library of WSSV infected shrimp were sequenced. The base sequences were analyzed using the BLAST searching tool in NCBI (National Center for Biotechnology Information). The screening of the cDNA library obtained from the WSSV infected shrimps led to the isolation of six genes; α_2M, Proteasome, EF1α, EF2, Lysozyme and β-actin (Table 4). The summary of the putative amino acids from the positive clones were compared with the full length proteins from other organisms as shown in Figure 17.

5.1 Alpha-2-macroglobulin (α_2M)

The sequence analysis showed that the clone consists of 546 bp sequences inserted into the pGADT7 vector. The amino acid sequence was compared with the sequence of previously known α_2M. Pm-α_2M shows a similarity to the C-terminal of the α_2M of the Kuruma shrimp, *Marsupenaeus japonicus* (78% identity, 87% similarity, GenBank accession number AB108542), Horseshoe crab, *Limulus polyphemus* (32% identity, 51% similarity, GenBank accession number T18544), Honey bee *Apis mellifera* (33% identity, 56% similarity, GenBank accession number XM392454) and Soft tick, *Ornithodoros moubata* (34% identity, 51% similarity, GenBank accession number AF538967). The comparison of amino acid residues was shown in Figure 11.

5.2 Proteasome subunit alpha type 6

The sequencing data showed the similarity of library cDNA with that of the C-terminal of proteasome including stop codons and 3′UTR (untranslated region). The amino acid residues comparison of proteasome from *P.monodon* (Pm-proteasome) shows the similarity to the C-termanal of the proteasome subunit alpha type 6 of the Red flour beetle, *Tribolium castaneum* (66% identity, 80% homology, GenBank accession number XM963562), Mosquito, *Aedes aegypti* (63% identity, 80% similarity, GenBank accession number CH477622), Japanese gecko, *Gekko japonicus* (63% identity, 80% homology, GenBank accession number AY880395) and Humans, *Homo sapiens* (63% identity, 80%

homology, GenBank accession number BC023659) as shown in Figure 12. The Pm-prteasome contained 64 amino acid residues (Figure 17).

5.3 Elongation factor-1-α (EF1α)

Several clones of sequencing data of the cDNA library of the WSSV infected shrimp revealed a similarity to the C-terminal of elongation factor-1-α of the Pill bug, *Armadillium vulgare* (95% identity, 96% homology, GenBank accession number U90046), Spider crab, *Libinia emarginata* (93% identity, 96% homology, GenBank accession number U90050), *Heteromysis formosa* (91% identity, 96% homology, GenBank accession number AF063413) and Humans, *Homo sapiens* (80% identity, 84% homology, GenBank accession number AF397403). The amino acids comparisons of EF1α with other organisms were shown in Figure 13. These cDNA fragments included stop codons and the poly A tail and 3′UTR (untranslated region). The C-terminal of Pm- EF1α was obtained from yeast two hybrid screening that composed of 207 amino acids (Figure 17).

5.4 Elongation factor-2 (EF2)

The BLAST results showed the similarity of positive clones from yeast two hybrid screening to the N-terminal part of elongation factor-2 from the Spider crab, *Libinia emarginata* (87% identity, 94% homology, GenBank accession number AY305506), the Pill bug, *Armadillium vulgare* (83% identity, 88% homology, GenBank accession number AF240816), Horseshoe crab, *Limulus polyphemus* (77% identity, 85% homology, GenBank accession number AF240821) and Silkworm, *Bombyx mori* (76% identity, 87% homology, GenBank accession number DQ515926). The amino acid sequences of Pm-EF2 were aligned with other organisms in Figure 14. The C-terminal of Pm-EF2 composed of 82 amino acids as show in Figure 17.

5.5 Lysozyme

Lysozyme was identified from the cDNA library screening of *P. monodon*. This clones composed of 132 amino acids of the C-terminal of lysozyme (Figure 17.).

The sequencing analysis showed the similarity to lysozyme of the Green tiger prawn, *Penaeus semisulcatus* (91% identity, 96% homology, GenBank accession number AY169675), the Pacific white shrimp, *Litopenaeus vannamei* (89% identity, 94% homology, GenBank accession number AF425673), the Kuruma shrimp, *Masupenaeus japonicus* (84% identity, 90% homology, AB080238) and Mouse, *Mus musculus* (52% identity, 70% homology, GenBank accession number NM017372) as shown in Figure 15.

5.6 β-actin

The amino acid sequences of the positive clones showed a similarity to those of the full length of β-actin of the Lobster, *Homarus gammarus* (100% Identity, 100% homology, GenBank accession number AJ581663), the Pacific white shrimp, *Litopenaeus vannamei* (100% identity, 100% homology, AF300705), the Kuruma prawn, *Masupenaeus japonicus* (100% identity, 100% homology, GenBank accession number AB055975) and Pool frog, *Rana lessonae* (98% identity, 99% homology, GenBank accession number AY272628) as shown in Figure 16. The Pm-β-actin encodes 193 amino acids and lacks the N-terminal part of lysozyme (Figure 17).

Table 7. BLAST results from library screening

Gene	Species	% Identity	Functions
1. Alpha-2- macroglobulin	*Marsupenaeus japonicus*	78	Protease inhibitor and immune effectors
2. Proteasome subunit alpha type 6	*Tribolium castaneum*	66	Proteolytic system and biodefense peptide
3. Elongation factor-1-α	*Armadillium vulgare*	94	Translation factor
4. Elongation factor-2	*Libinia emarginata*	87	Translation factor
5. Lysozyme	*Penaeus semisulcatus*	91	Bacterial hydrolysis
6. β-actin	*Litopenaeus vannamei*	100	Cytoskeleton component

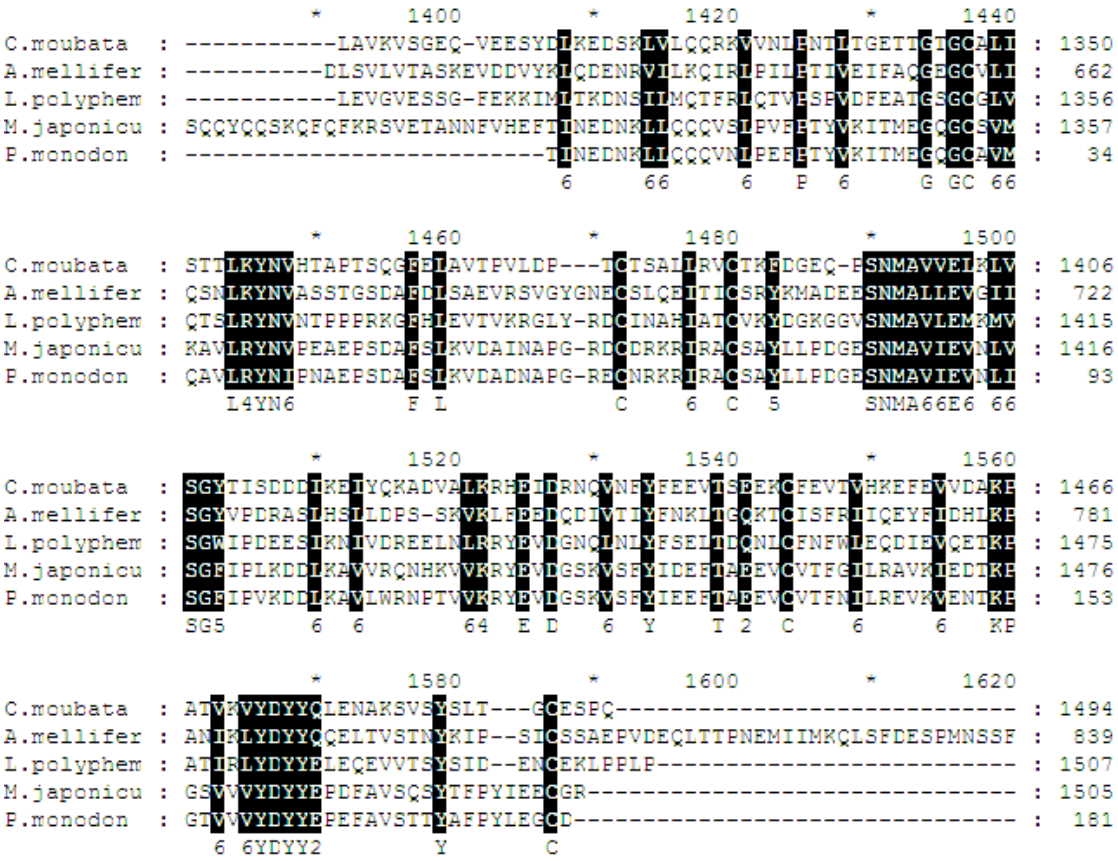

Figure 11. Comparison of the amino acid sequences of Pm-α_2M and other organisms

The sequences were aligned using a Clustal X algorithm. Similar residues are shown in white on a black background. A dash represents a gap in the indicated proteins. The Kuruma shrimp, *Marsupenaeus japonicus* (GenBank accession number AB108542), Horseshoe crab, *Limulus polyphemus* (GenBank accession number T18544), Honey bee *Apis mellifera* (GenBank accession number XM392454) and Soft tick *Ornithodoros moubata* (GenBank accession number AF538967).

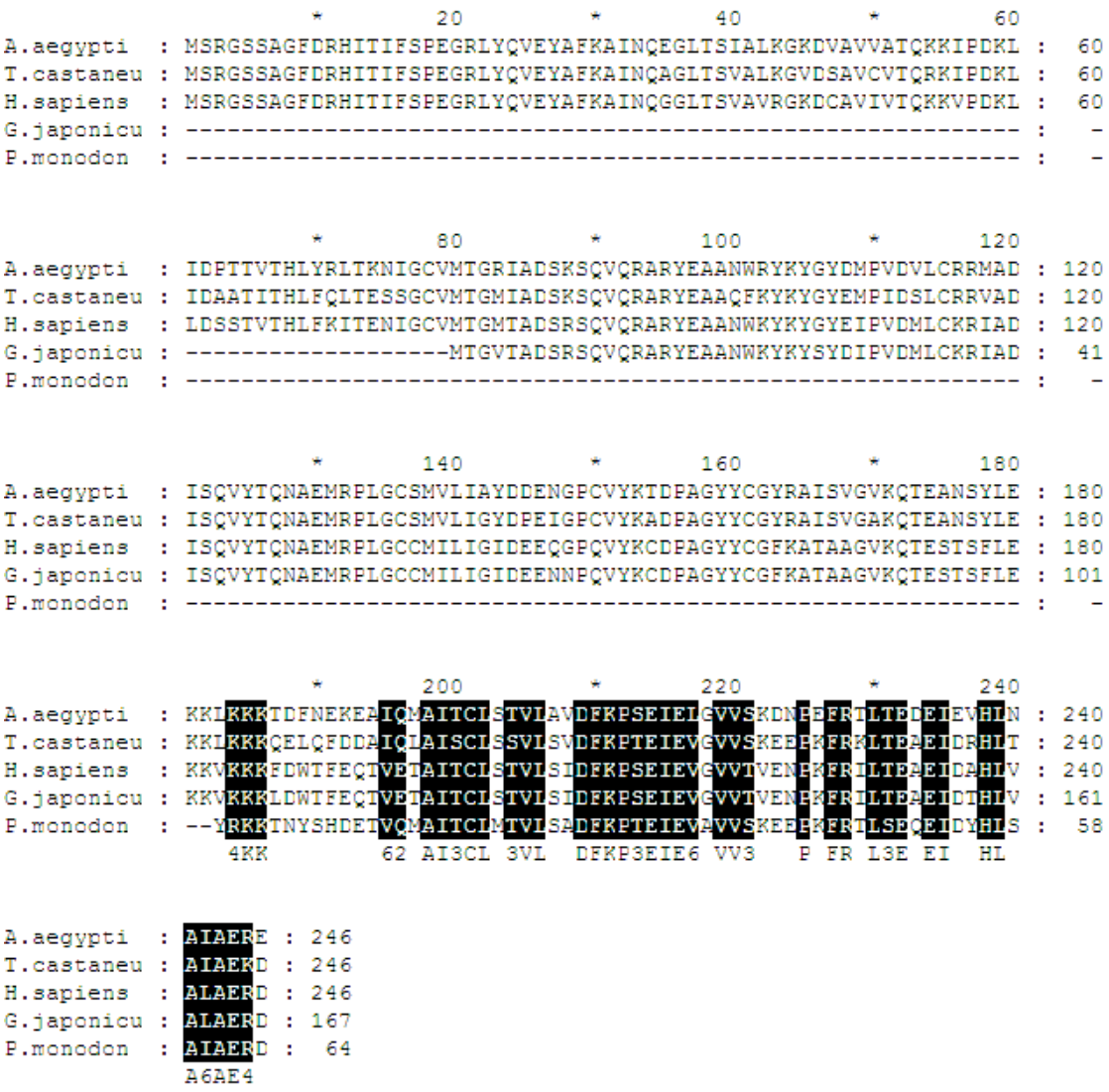

Figure 12. Comparison of the amino acid sequences of Pm-proteasome with other organisms

The sequences were aligned using a Clustal X algorithm. Similar residues are shown in white on a black background. A dash represents a gap in the indicated proteins. The proteasome subunit alpha type 6 of Red flour beetle, *Tribolium castaneum* (GenBank accession number XM963562), Mosquito, *Aedes aegypti* (GenBank accession number CH477622), Japanese gecko, *Gekko japonicus* (GenBank accession number AY880395) and Humans, *Homo sapiens* (GenBank accession number BC023659).

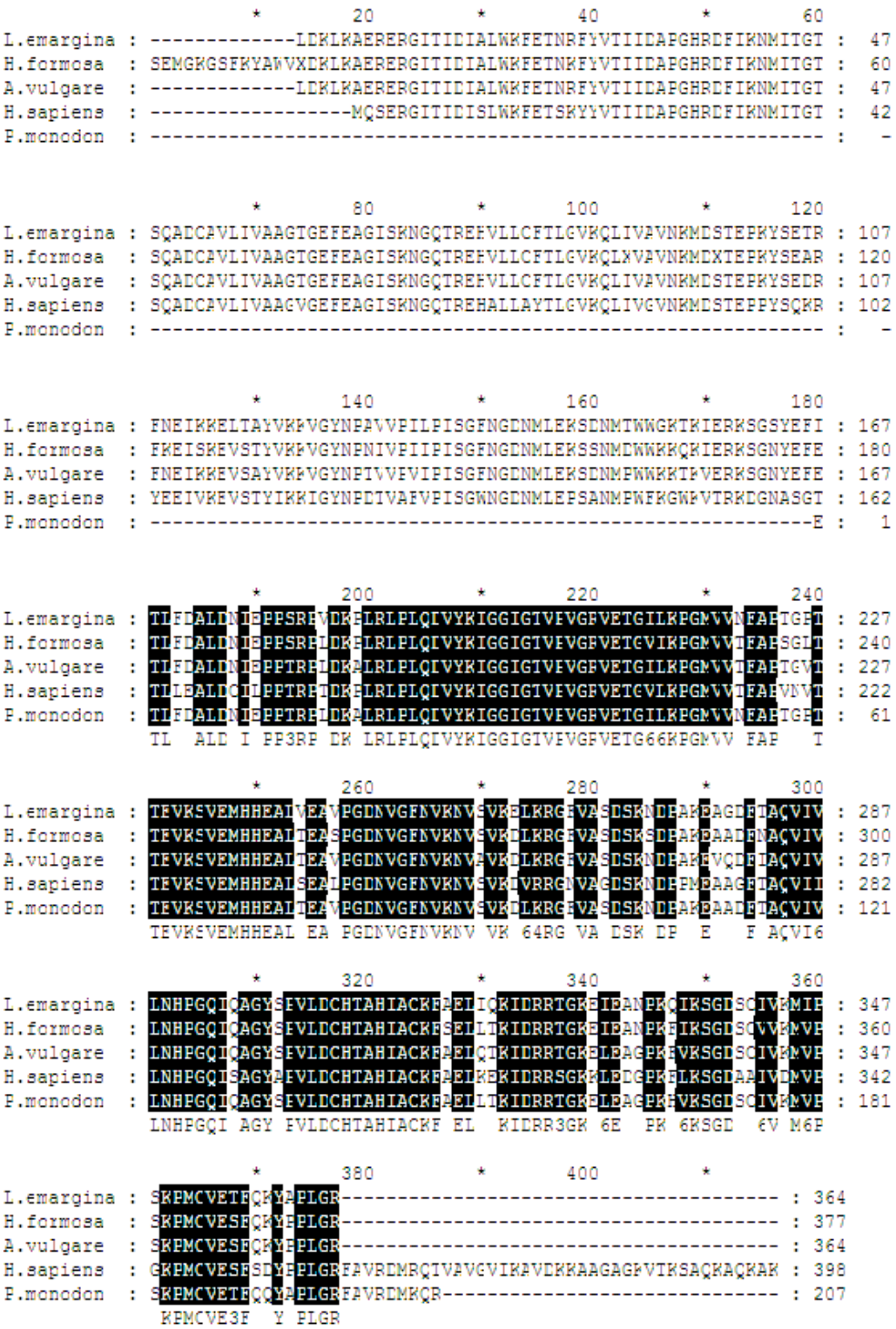

Figure 13. Comparison of the amino acid sequences of Pm-EF1α with other organisms

Figure 13. Continued

The sequences were aligned using a Clustal X algorithm. Similar residues are shown in white on a black background. A dash represents a gap in the indicated proteins. The amino acid residues of elongation factor-1-α of the Pill bug, *Armadillium vulgare* (GenBank accession number U90046), Spider crab, *Libinia emarginata* (GenBank accession number U90050), *Heteromysis formosa* (GenBank accession number AF063413) and Humans, *Homo sapiens* (GenBank accession number AF397403).

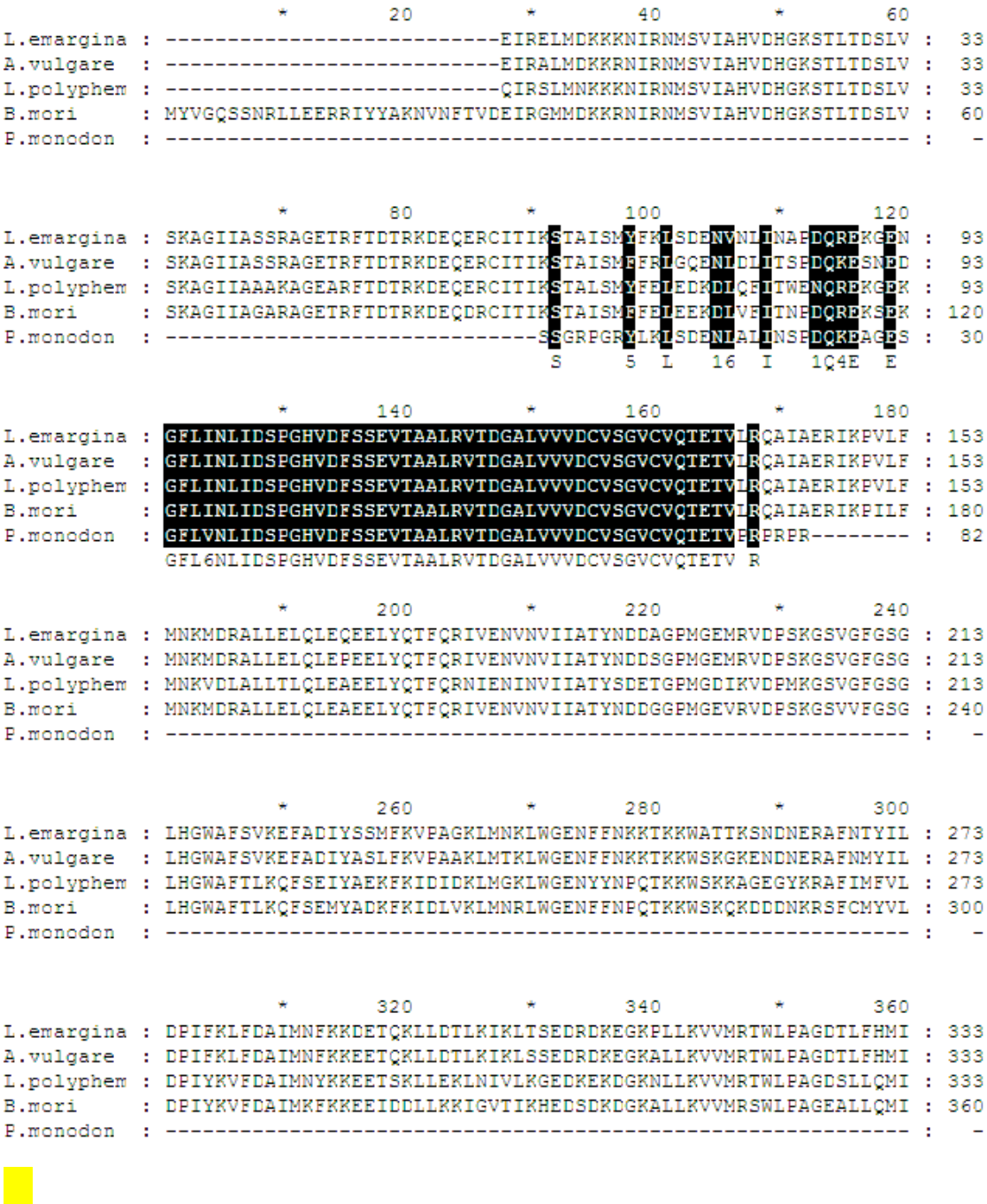

Figure 14. Comparison of the amino acid sequences of Pm-EF2 with other organisms

The sequences were aligned using a Clustal X algorithm. Similar residues are shown in white on a black background. A dash represents a gap in the indicated proteins. The amino acid of elongation factor-2 from the Spider crab, *Libinia emarginata* (GenBank accession number AY305506), the Pill bug, *Armadillium vulgare*

Figure 14. Continued

(GenBank accession number AF240816), the Horseshoe crab, *Limulus polyphemus* (GenBank accession number AF240821) and the Silkworm, *Bombyx mori* (GenBank accession number DQ515926).

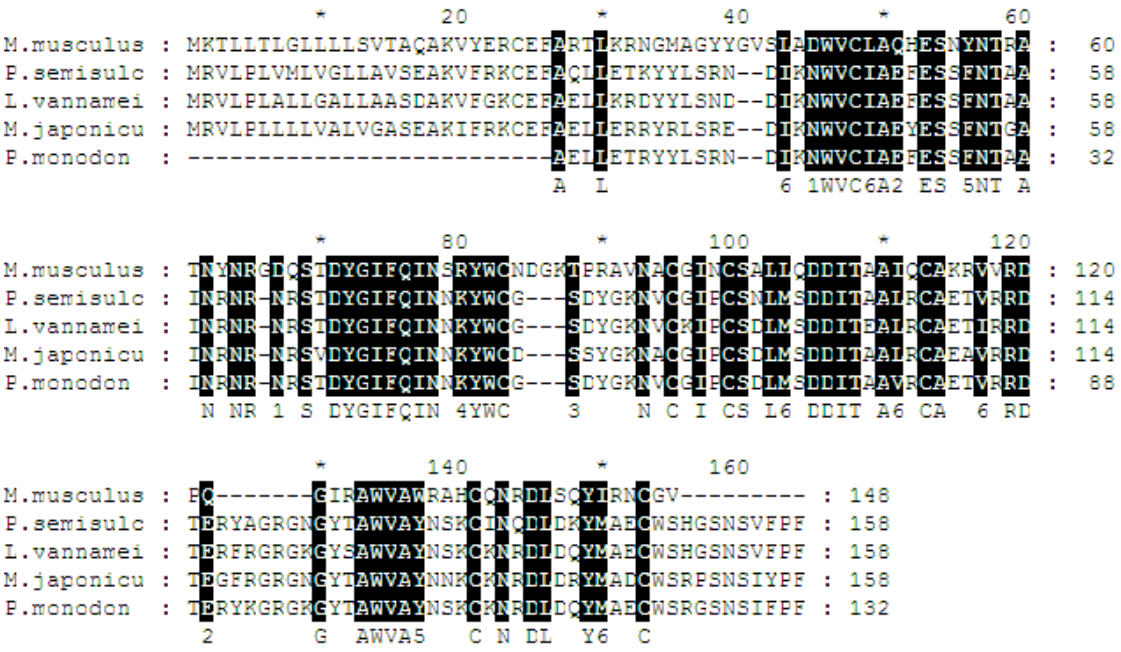

Figure 15. Comparison of the amino acid sequences of Pm-lysozyme with other organisms

The sequences were aligned using a Clustal X algorithm. Similar residues are shown in white on a black background. A dash represents a gap in the indicated proteins. The amino acid sequencing of lysozyme from the Green tiger prawn, *Penaeus semisulcatus* (GenBank accession number AY169675), the Pacific white shrimp, *Litopenaeus vannamei* (GenBank accession number AF425673), the Kuruma prawn, *Masupenaeus japonicus* (AB080238) and Mouse, *Mus musculus* (accession number NM017372).

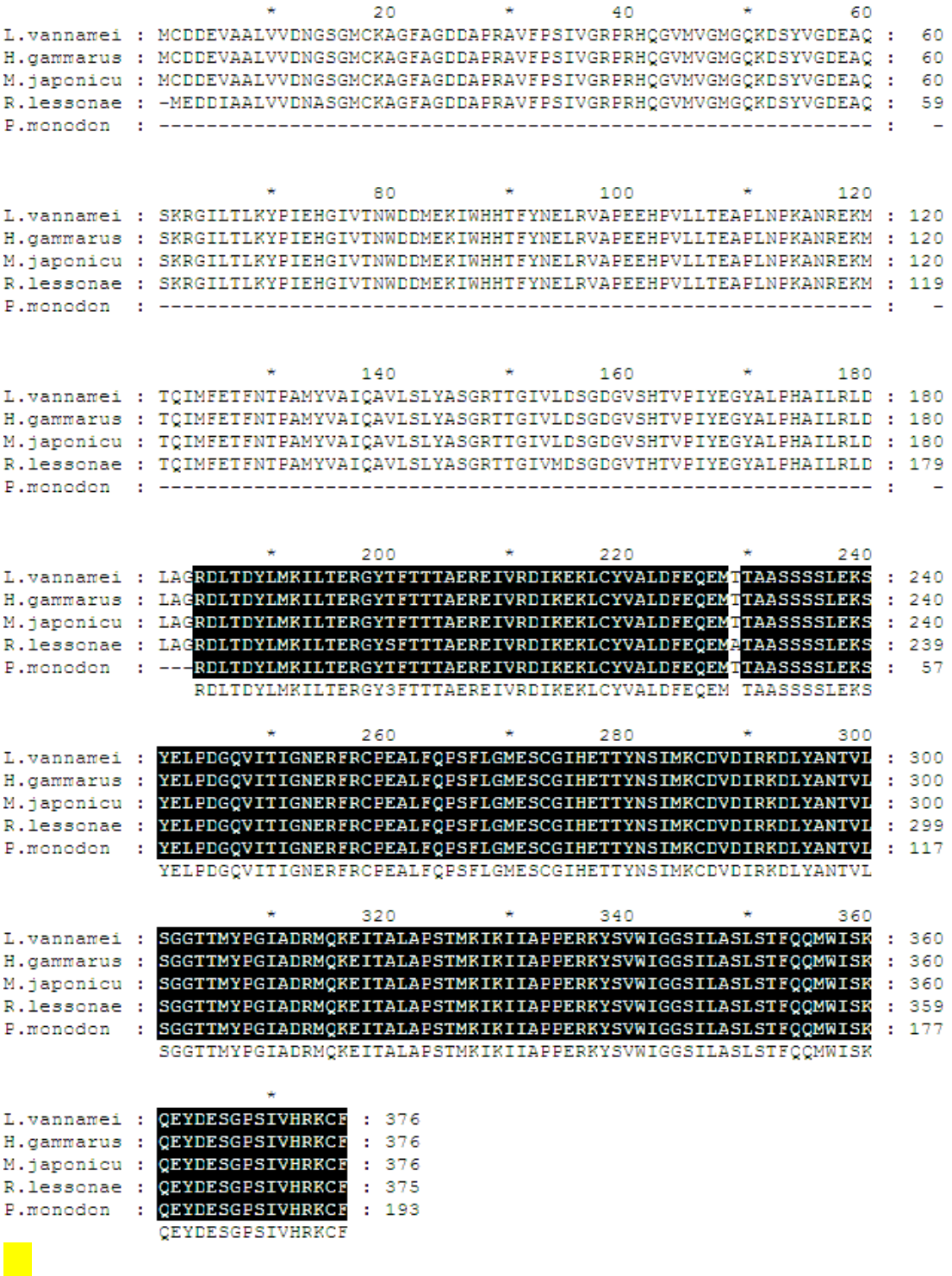

Figure 16. Comparison of the amino acid sequences of Pm- β-actin with other organisms

Figure 16. Continued

The sequences were aligned using a Clustal X algorithm. Similar residues are shown in white on a black background. A dash represents a gap in the indicated proteins. The β-actin amino acid sequences from the Lobster, *Homarus gammarus* (GenBank accession number AJ581663), the Pacific white shrimp, *Litopenaeus vannamei* (GenBank accession number AF300705), the Kuruma prawn, *Masupenaeus japonicus* (GenBank accession number AB055975) and Pool frog, *Rana lessonae* (accession number AY272628).

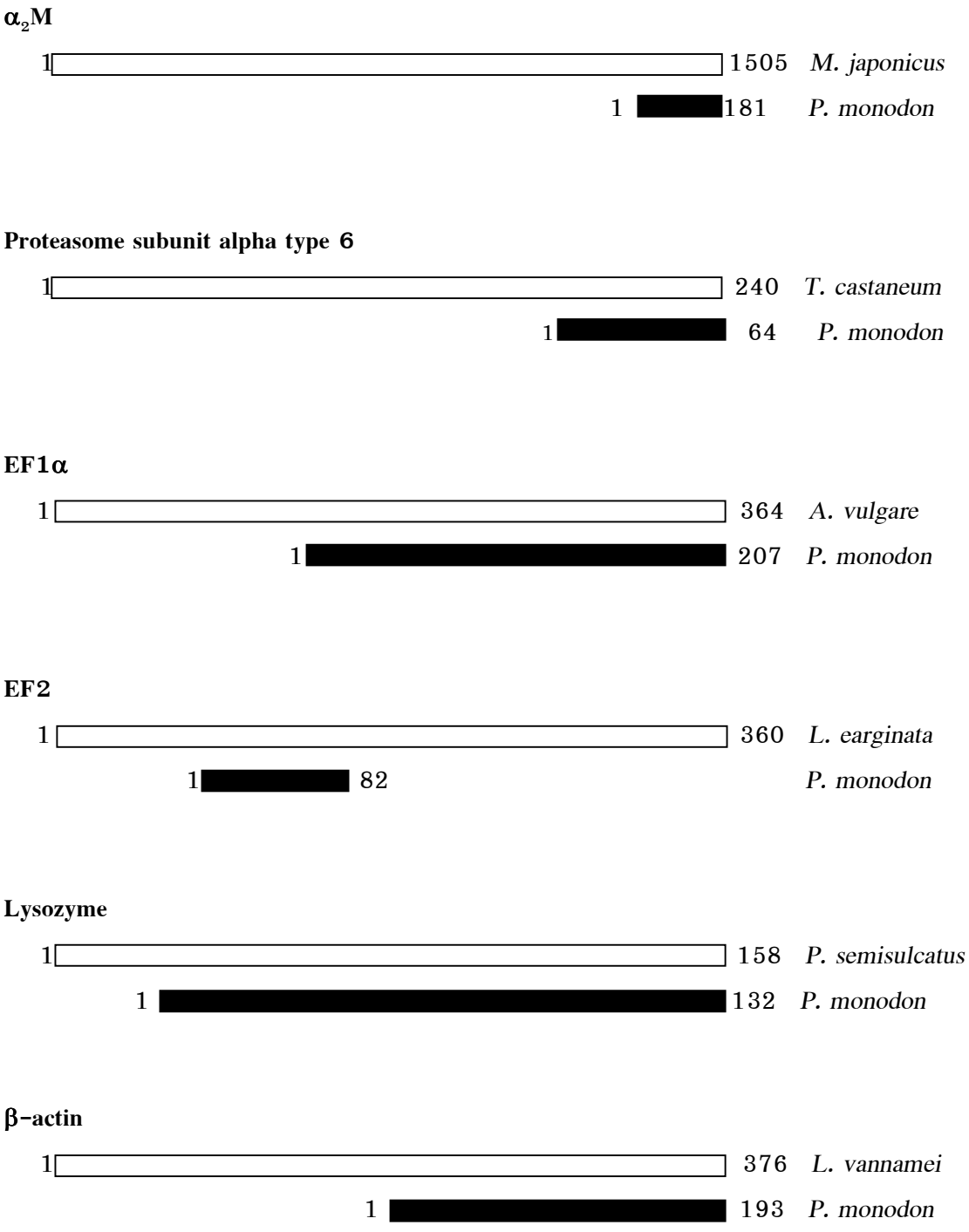

Figure 17. The summary of the syntenin binding protein amino acid residues

▬ : The black bars that show the position of Pm-syntenin binding proteins were obtained from yeast two-hybrid library screening.

Figure 17. Continued

□: The white bars represented the full length amino acid residues of proteins from other organisms; α_2M: the Kuruma prawn, *Marsupenaeus japonicus* (GenBank accession number AB108542), Proteasome subunit alpha 6: the Red flour beetle, *Tribolium castaneum* (GenBank accession number XM963562), EF1α: the Pill bug, *Armadillium vulgare* (GenBank accession number U90046), EF2: the Pacific white shrimp, *Libinia emarginata* (GenBank accession number AY305506), Lysozyme: the Ggreen tiger prawn, *Penaeus semisulcatus* (GenBank accession number AY169675), β-actin: the Pacific white shrimp, *Litopenaeus vannamei* (GenBank accession number AF300705).

6. Retransformation of Pm-syntenin and Pm-α_2M into yeast cells

In order to confirm the *in vivo* interaction of Pm-syntenin and Pm-α_2M using yeast two hybrid systems the plasmids of BD-syntenin and AD-α_2M (SA1) were purified from *E. coli* strain TOP10F′. Then we performed a co-transformation of both plasmids into yeast cells using the lithium acetate method. The positive clones were grown on high stringency medium (SD/-Trp/-Leu/-His/-Ade containing 2.5 mM 3-AT and 5-bromo-4 chloro-3-indolyl-α-D-galactosidase). The positive control was pGBKT7-53 and pGADT7-T according to the manufacturer's instruction. The negative controls were performed by co-transformed BD-syntenin and pGADT7 empty vector and pGBKT7 and AD-α_2M into yeast cells. No colony was grown on negative control plate. In addition, 286 amino acid residues of α_2M was obtained from 5′ RACE and cloned into pGADT7 vector (SA2). The yeast two hybrid assays of BD-syntenin and SA2 were determined. The positive result of α-galactosidase activity was shown in Figure 18. These 286 residues of SA2 also bind to full length Pm-syntenin.

7. Identify the binding domain of Pm-syntenin and α_2M

To further define which domain of Pm-syntenin is required for the interaction with α_2M, the AD-α_2M (SA1) was co-transformed with individual plasmids that contained the complete sequence of Pm-syntenin (BD-syntenin), i.e. the 131 amino acids of the N-terminal (BD-NS), PDZ1-PDZ2 (BD-PDZ1,2; amino acids 117-322) and PDZ1 (BD-PDZ1; amino acids 117-219). Table 8 shows the results of the growth of yeast cells with a blue colony (+) in the selective medium when the interaction of the two proteins occurred and no growth of yeast cells (-) when there is no interaction of the two proteins tested. Yeast co-transformed with AD-α_2M and BD-NS can grow on SD medium producing a blue colony, and also give a strong β-galactosidase reporter expression (Figure 18.), but no interaction was observed with any of the PDZ domains (Table 8, No. 5 and 6). These data suggest that Pm-syntenin binds α_2M through its N-terminal 131 amino acids in vivo, in the yeast two-hybrid system.

Table 8. Detail of the binding results obtained from different binding and activating domain fusions

Binding domain fusion	Activating domain fusion	SD+X-α-Gal
1.  BD-Syntenin	-	(-)
2. -	 AD-α_2M	(-)
3.  BD-Syntenin	 AD-α_2M	(+)
4.  BD-NS	 AD-α_2M	(+)
5.  BD-PDZ1,2	 AD-α_2M	(-)
6.  BD-PDZ1	 AD-α_2M	(-)

Results are indicated as;

(+) when the interaction occurs (Blue colony on selective medium [SD/-Trp/-Leu/-His/-Ade containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside(X-α-Gal)])

(-) when there was no interaction (no growth on selective medium)

Diagrams illustrate the structure of Pm-syntenin and α_2M deduced from their amino acids sequences. The distinct domains within each protein are shown by different annotations as follows:

 Full length DNA of Pm-syntenin

 N-terminal of Pm-syntenin

 PDZ1 of Pm-syntenin

 PDZ2 of Pm-syntenin

 C-terminal of shrimp α_2M (SA1)

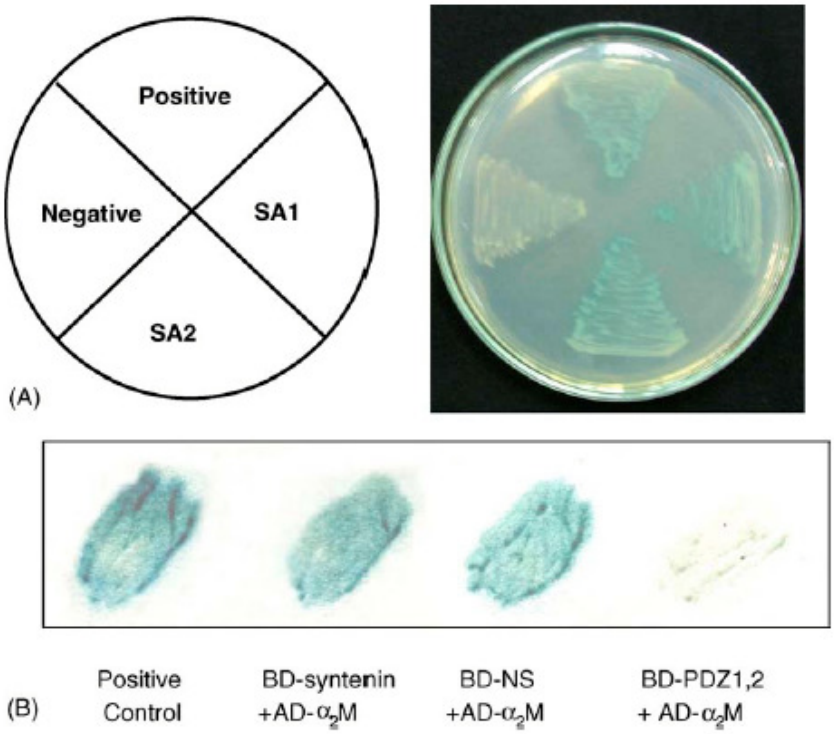

Figure 18. Yeast two-hybrid assay

(A) *S. cerevisiae* AH109 cells were co-transformed with full length of syntenin in the pGBKT7 vector, and the C-terminal 181 amino acids residues of α_2M in pGADT7 (SA1) and 286 amino acids residues of α_2M in pGADT7 (SA2). Transformed cells were selected on SD selective medium. The positive control is yeast cells that were co-transformed with pGBKT7-53 and pGAD7-T (Clontech) and the negative control for α-galactosidase activity is yeast cell that did not activate the *MEL1* reporter gene. **(B)** The filter containing the selected lysed yeast cells and liquid X-Gal was used to verify the activation of *LacZ* by interaction between two known proteins. *S. cerevisiae* AH109 cells were co-transformed with (1) pGBKT7-53 and pGADT7-T (positive control); (2) BD-syntenin and AD- α_2M; (3) N-terminal of Pm-syntenin in pGBKT7 (BD-NS) and AD-α_2M; (4) PDZ1-PDZ2 of Pm-syntenin in pGBKT7 (BD-PDZ1, 2) and AD-α_2M. Transformed cells were selected on medium (SD/-Trp/- Leu/-His/-Ade) except for BD-PDZ1, 2 was obtained from SD/-Trp/-Leu.

Part II. *In vitro* protein binding assay

1. GST pull down assay

1.1 Binding of GST-α_2M and 6xHis-syntenin

To confirm the interaction between syntenin and α_2M, we carried out a GST pull-down experiment. GST-α_2M was coupled to Glutathione Sepharose 4B beads and then incubated in the presence of the 6xHis-syntenin. An Anti-His Tag antibody and Anti-GST antibody were used to visualize the specific protein in a western blot analysis. As shown in Figure 19, Glutathione beads effectively pulled down both GST and GST-α_2M, although the pull-down efficiency was higher with GST than with GST-α_2M as is shown in lanes 3 and 4 of Figure 19., respectively. When the same membrane was probed with an anti-His-Tag antibody, 6xHis-syntenin was only present in the pull-down product of GST-α_2M (lane 2), being absent in the GST pull-down product (lane 1). These data suggest that GST-α_2M, not GST alone, was capable of co-precipitating Pm-syntenin.

1.2 Binding of GST-syntenin and ^{35}S-Met labeled α_2M

In order to further validate the presence of a specific interaction between α_2M and syntenin, we performed a reverse pull-down assay where *in vitro* translated and radioactive-labeled α_2M was incubated with either GST-syntenin or GST alone. As is shown in Figure 20, the amounts of α_2M added to the reaction mixtures were equal (lanes 1 and 2, input). In addition, GST-syntenin and GST proteins were equally pulled down by glutathione beads (lanes 1 and 2, Coomassie). In this system, GST-syntenin, not GST alone, was capable of co-precipitating α_2M (lanes 1 and 2, pull-down). Taken together with the data shown in Figure 19 and 20 and Table 8, it is now clear that Pm-syntenin specifically binds the C-terminus receptor binding domain of α_2M, through its N-terminus portion.

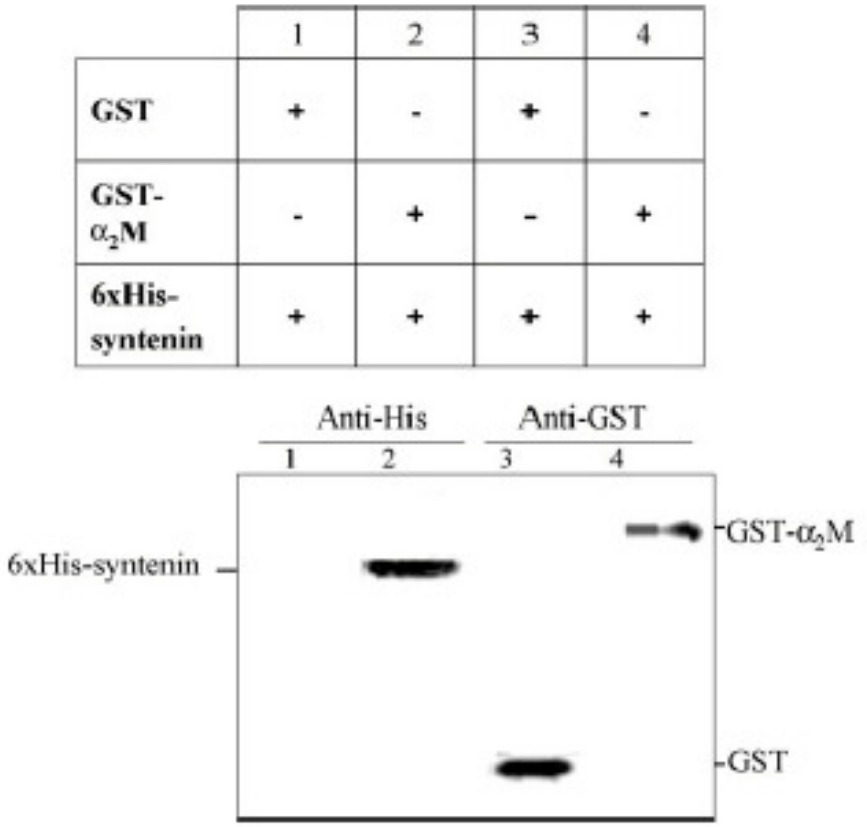

Figure 19. *In vitro* binding assay I

Purified GST-α_2M and GST-protein were detected by specific antibodies in the presence of 6xHis-syntenin. A Glutathione sepharose bead pull-down was performed on the combined proteins. The eluted material was loaded on SDS-PAGE gels, transferred and detected using specific antibodies. When not pull downed, there was no band detected with anti-His tag antibody (lane 1) but a GST band was detected with anti-GST antibody (lane 3). In the case where the combined proteins were pull downed (lane 2, 4), syntenin was detected with anti-His Tag antibody and α_2M was detected with anti-GST antibody

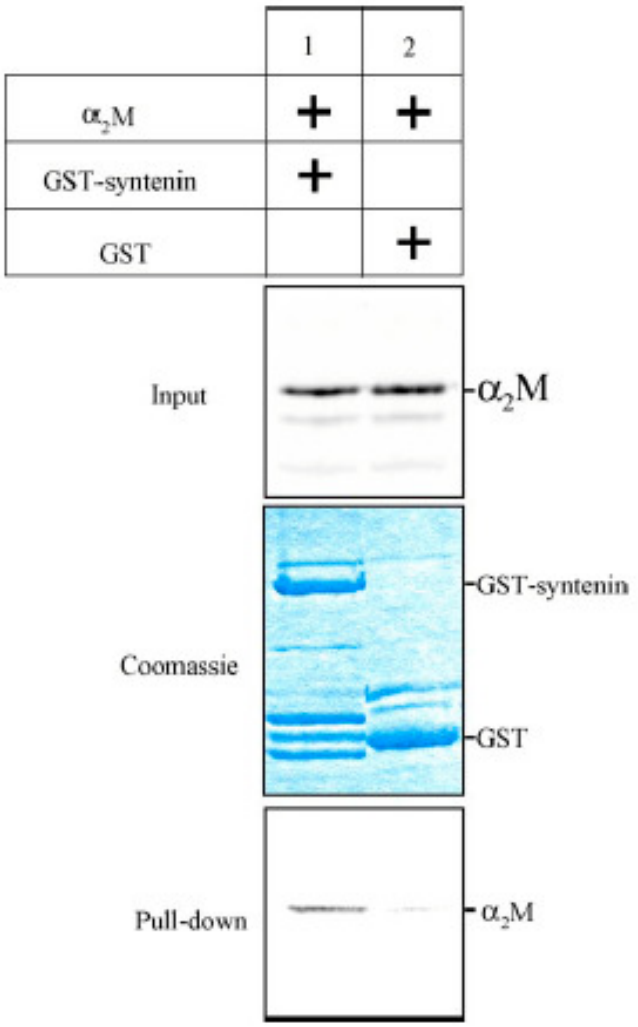

Figure 20. *In vitro* binding assay II

α_2M protein was obtained from an *in vitro* transcription/translation of plasmid pGADT7-SA1 in the presence of ^{35}S-Met labeled protein combined with GST-syntenin. A Glutathione sepharose bead pull-down assay was performed on the combined proteins. The material was loaded onto SDS-PAGE gels and detected by using fluorography.

Part III Gene expression of Pm-α_2M in haemocytes of shrimp

To address whether α_2M functions as an immune effectors in *P. monodon*, we performed RT-PCR analyses and a semi-quantitative assay on RNA extracts from normal and WSSV-injected shrimps using specific primers designed from the SA1 sequences in the receptor binding part of Pm-α_2M (546 bp). The PCR product was directly sequenced and found to have the same sequence as the Pm-α_2M. RT-PCR of β-actin was used as an internal control in the semi-quantitative analysis to insure that the RT-PCR for each sample contained the same amount of total RNA and that the extracted RNA was intact. The Pm-α_2M was observed in five individual of normal shrimps (N) and WSSV-infected shrimps (I) as shown in Figure 21. Interestingly, the level of Pm-α_2M expression increased when the shrimp was infected with WSSV.

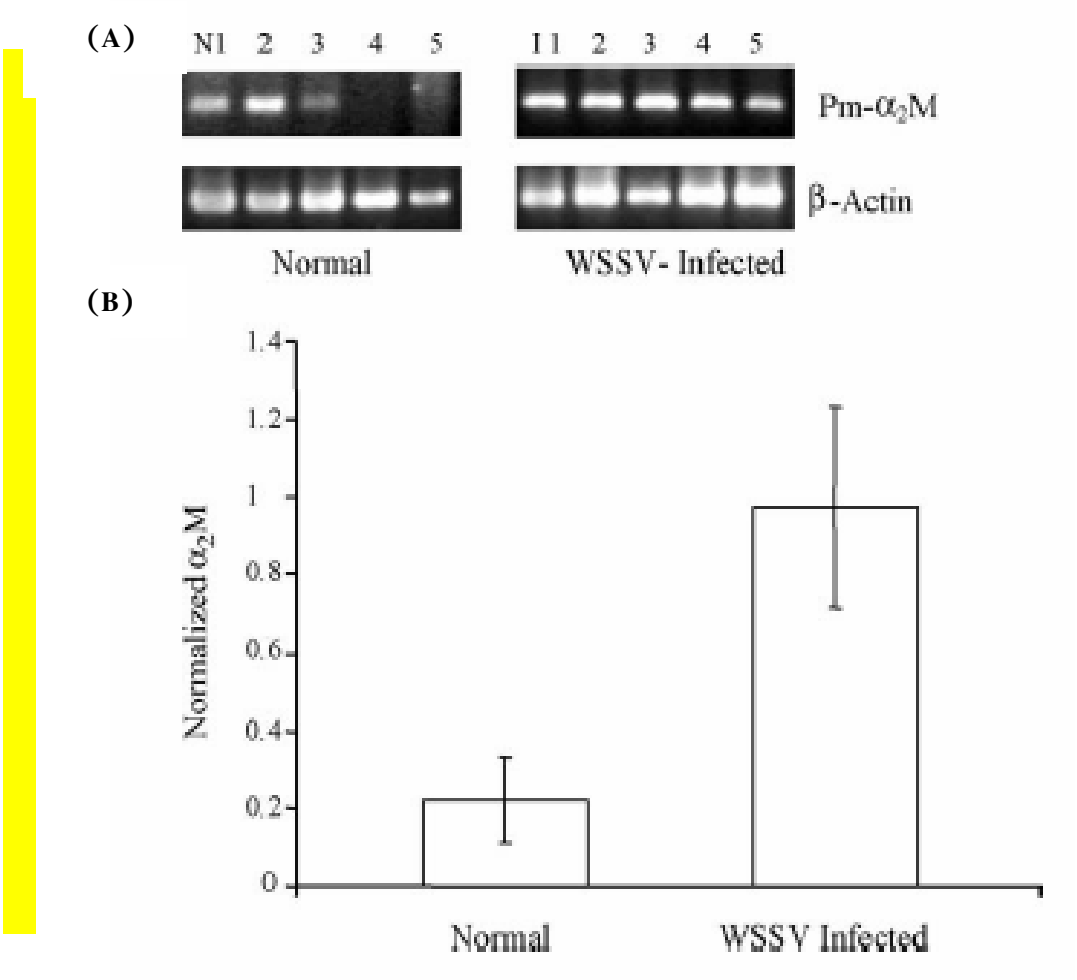

Figure 21. Gene expression of α_2M in haemocytes of shrimp

(A) Total RNA isolated from haemocytes of the five uninfected individuals of *P. monodon* and the five individuals infected with WSSV were used in reverse transcription PCR experiments. Amplification with β-actin was performed in parallel (bottom 2 panels) as a control. Images are ethidium bromide staining of the RT-PCR product after 25 cycles of amplification, N.1-N.5: normal (uninfected samples); I.1-I.5: infected samples (48 h WSSV post injection samples). **(B)** The normalized α_2M was calculated from the images using Scion images software. The data represent the average results obtained from individuals of the normal and infected shrimp.