รายงานวิจัยฉบับสมบูรณ์

โครงการ การ Cloning การแสดงออก การศึกษาคุณสมบัติของ pro-apoptotic gene, caspase-3 ในกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาว และการศึกษา apoptosis ในปูทะเลที่ติดเชื้อไวรัสตัวแดงดวงขาว

Cloning, expression and characterization of pro-apoptotic gene, caspase-3 in the white-spot syndrome virus infected black tiger shrimp *Penaeus monodon* and the study of apoptosis in white-spot syndrome virus infected mud crab *Scylla serrata*

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Cloning, expression and characterization of pro-apoptotic gene, caspase-3 in the white-spot syndrome virus infected black tiger shrimp *Penaeus monodon* and the study of apoptosis in white-spot syndrome virus infected mud crab *Scylla serrata*

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ.และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

ตอนที่ 1: การ Cloning การแสดงออก การศึกษาคุณสมบัติของ pro-apoptotic gene, caspase-3 ในกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาว

ที่มาของงานวิจัยและวัตถุประสงค์ อะพอพโทซิส (apoptosis) พบในกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาว (WSSV) และพบมากขึ้นตามเวลาที่มีการติดเชื้อ ยังไม่มีการพิสูจน์ว่าอะพอพโทซิสเป็นสาเหตุการตายของกุ้งที่ติดไวรัสหรือไม่ และ caspase-3 ยีนที่แสดงออกเมื่อเกิดขบวนการ apoptosis ก็ยังไม่มีการศึกษามาก่อน การศึกษานี้มุ่งที่จะคัดแยก โคลน และศึกษาคุณสมบัติของ caspase และศึกษาการแสดงออกของ caspase ในกุ้งที่ทำให้ติดเชื้อไวรัส WSSV โดยศึกษาตามเวลา

วิธีการวิจัย ทำการคัดหายีน caspase จากยีนห้องสมุดเม็ดเลือดของกุ้งกุลาดำ โดยใช้ชิ้นส่วน DNA ของ caspase ของกุ้ง *P.merguiensis* เป็น probe ทำการ clone และหาลำดับนิวคลีโอไทด์ของยีน caspase ทำการกระตุ้นให้เกิดการแสดงออกของยีนใน E. coli และนำโปรตีนที่ได้มาสกัดบริสุทธิ์นำไปทดสอบคุณสมบัติการทำงานของ caspase-3 ผลิตแอนติบอดีต่อโปรตีน caspase-3 ในกระต่าย นอกจากนี้ยังศึกษาการแสดงออกของ caspase-3 ที่ระดับ mRNA โดยวิธี semi-quantitative RT-PCR และระดับโปรตีนโดยวิธี Western blot ในกุ้งกุลาดำที่ทำให้ติดเชื้อ ทำการเก็บตัวอย่างเหงือกของกุ้งที่เวลา 24 ชั่วโมง 36 ชั่วโมง และ 48 ชั่วโมงหลังจากการฉีดเชื้อ โดยเทียบผลการทดลองกับกุ้งกลุ่มควบคุม คือไม่ได้ฉีดเชื้อ WSSV หาตำแหน่งของการแสดงออกของ caspase-3 ในเนื้อเยื่อเหงือกของกุ้งกุลาดำโดยวิธี immunohistochemistry

ผลการทดลอง นิวคลีโอไทด์ของยีน caspase ของกุ้งกุลาดำ ประกอบด้วย 1202 คู่เบส โดยมี 954 คู่เบสที่เป็น open reading frame ที่ให้ 317 อะมิโนเอสิด โปรตีนที่ได้มีตำแหน่ง QAC RG เป็ปไทด์ 5 ตัว ที่เป็นลักษณะเฉพาะของโปรตีน caspase ลำดับอะมิโนเอสิดของ caspase ของกุ้งกุลาดำ มีความเหมือนกับ caspase ของกุ้ง *P. merguiensis* อยู่ 83% และเหมือนกับ ICE ของ drosophila melanogaster 30% โปรตีนที่ได้เมื่อทดสอบคุณสมบัติพบว่ามีคุณสมบัติเป็น caspase-3 การศึกษาโดยวิธี Western blot แสดงให้เห็นว่าแอนติบอดีมีปฏิกิริยากับ caspase ที่สกัดบริสุทธิ์ และส่วนของ lysate ของ E.coli ที่มี plasmid ที่ถูกกระตุ้นให้แสดงออก และโปรตีนสกัดจากเนื้อเยื่อกุ้งที่ขนาด 36 kDa และ 26 kDa ซึ่งเท่ากับขนาดของ procaspase และ proteolytic intermediate ของ caspase การแสดงออกของระดับ mRNA ของ caspase ในกุ้งที่ติดเชื้อ WSSV มากขึ้นที่เวลา 48 ชั่วโมงหลังการฉีดเชื้อ และเวลากุ้งใกล้ตาย (moribund) ซึ่งสอดคล้องกับระดับของโปรตีนของ caspase ที่เพิ่มขึ้นที่เวลา 24 ชั่วโมง และ 48 ชั่วโมง การศึกษาโดยวิธี immunohistochemistry โดยใช้แอนติบอดีต่อ caspase-3 ในเหงือกของกุ้งที่ติดเชื้อไวรัส WSSV พบผลบวกในซัยโตพลาสซึมของเซลล์ที่เกิดอะพอพโทซิสและเซลล์ที่รูปร่างปกติ

สรุป การศึกษานี้พบยีนที่มีลักษณะคล้าย caspase-3 ในกุ้งกุลาดำและยีนนี้มีการแสดงออกมากขึ้น ตามเวลาของการติดเชื้อไวรัสตัวแดงดวงขาว (WSSV) ไวรัสนี้กระตุ้นการเกิดอะพอพโทซิสโดยผ่านเส้นทาง caspase-3 และเป็นสาเหตุของการตายของกุ้ง

Abstract

Part I: Cloning, expression and characterization of pro-apoptotic gene, caspase-3 in the white-spot syndrome virus infected black tiger shrimp *Penaeus monodon*

Rationale and objectives: It was reported that apoptosis occurred progressively in the time course study of white spot syndrome virus (WSSV) infected shrimp, *Penaeus monodon (P. monodon)*. Whether apoptosis cause death in the WSSV-infected shrimp has not been elucidated as well as there is little report about apoptotic gene, caspase-3 in shrimp. Therefore, the recent study aimed to isolate, clone and characterize caspase-3, the executioner of apoptosis, in shrimp *P. monodon* and also studied the time course expression of caspase 3 of the shrimp upon WSSV-infection.

Methods: Caspase *(Pm*Casp*)* had been screened from *P. monodon* hemocyte library using *P. merguiensis* caspase as a probe. The caspase containing clones were sequenced and 5' RACE was performed to obtain full-length cDNA of *Pm*Casp. *Pm*Casp was constructed and expressed in *E. coli*, and the recombinant protein was purified and determined the caspase-3 activity. Recombinant protein was used to produce polyclonal antibody against *Pm*Casp in rabbit. To determine the expression of *Pm*Casp in WSSV infected shrimp, the black tiger shrimp, *P. monodon* were divided into two experimental groups, the vehicle control and the WSSV-injected groups. Gills were collected at 24, 36, 48 h post injection (pi). Semiquantitative RT-PCR was performed by using specific primers for caspase3. Rabbit antiserum against *Pm*Casp protein was used in western blot analysis and immunohistochemistry.

Results: Caspase cDNA has been identified from *P. monodon* hemocyte library. The full-length of *Pm*Casp consists of 1,202 bp with a 954-bp open reading frame, encoding 317 amino acids. The deduced protein contains a potential active site QACRG pentapeptide that found in most caspases. The deduced PmCasp protein sequence shows significant (83%) identity with that of P. erguiensis and 30% identity with ICE protein of Drosophila melanogaster, and xhibits caspase-3 activity in vitro. By western blot analysis, the antiserum reacted with purified recombinant *Pm*Casp, lysates of *E. coli* containing the expressed plasmid, and shrimp crude proteins at 36-kDa and 26-kDa bands likely to correspond to the deduced inactive procaspase and proteolytic intermediate form, respectively. The study of expression of *Pm*Casp-3 in WSSV-infected shrimp by semi-quantitative RT-PCR revealed that *Pm*Casp-3 was up-regulated at 48 h p.i. and at moribund. It was supported by western blot analysis, the levels of *Pm*Casp-3 protein were relative increased at 24 h and 48 h p.i. when compared to the normal control. Immunohistochemical analysis in gills from WSSV-infected shrimp demonstrated that the immunoreactivity was localized in the cytoplasm of normal-looking cell as well as apoptotic cells.

Conclusion: The present study demonstrates that caspase-3 like gene is conserved in shrimp *P. monodon* and expressed with the progressively after WSSV infection. WSSV induced apoptosis in *P. monodon* might cause death of the shrimp and mediated via caspase-3 pathway.

บทคัดย่อ

ตอนที่ 2: การศึกษา apoptosis ในปูทะเลที่ติดเชื้อไวรัสตัวแดงดวงขาว

ที่มาของงานวิจัยและวัตถุประสงค์ ไวรัสตัวแดงดวงขาว (Whit spot syndrome virus, WSSV) ก่อให้เกิดโรคติดต่อระบาดในอุตสาหกรรมการเพาะเลี้ยงกุ้งในประเทศไทย และอีกหลายประเทศในเอเชีย กุ้งที่ติดเชื้อไวรัสชนิดนี้จะตายทั้งบ่อภายใน 3-7 วัน มีสัตว์ในกลุ่ม crustacean และ arthropod หลายชนิดที่อาศัยอยู่ร่วมกันในบ่อกุ้ง และบริเวณใกล้บ่อกุ้ง เป็นพาหะและเป็นที่สะสมของไวรัสชนิดนี้ได้ เป็นที่น่าสนใจว่า ปูทะเล (mud crab, *Scylla serrata*) ติดเชื้อไวรัสชนิดนี้ได้โดยไม่แสดงอาการของโรคและสามารถมีชีวิตอยู่ได้ มีรายงานว่ากุ้งที่ติดเชื้อไวรัสจะเกิดการกระตุ้นการฆ่าตัวตายของเซลล์ (apoptosis) และ apoptosis อาจเป็นสาเหตุการตายของกุ้ง การวิจัยนี้มุ่งศึกษาปูทะเลที่ได้รับเชื้อไวรัสจะเกิดภาวะ apoptosis และการที่ปูทะเลทนต่อการติดเชื้อไวรัสมีความเกี่ยวข้องกับ apoptosis หรือไม่

วิธีการทดลอง ฉีดเชื้อไวรัส WSSV ให้กับปูทะเลแล้วสังเกตอัตราการตายของปูเป็นเวลา 8 วัน เก็บเหงือกของปูมาศึกษาที่เวลา 24 ชั่วโมง 68 ชั่วโมง และ 96 ชั่วโมง หลังจากฉีดเชื้อ นำเหงือกไปผ่านขั้นตอนการศึกษาโดยกล้องจุลทรรศน์ธรรมดาและ RT-PCR ทำการย้อมชิ้นเนื้อด้วยสี H & E เพื่อศึกษาพยาธิสภาพของเนื้อเยื่อและศึกษาการเกิด apoptosis โดยวิธี TUNEL การศึกษาการแสดงออกของ caspase 3 gene และ caspase โปรตีนโดยวิธี RT-PCR และ western blot โดยใช้ไพร์เมอร์ และแอนติบอดีที่ได้จากกุ้งกุลาดำ

ผลการทดลอง จากการศึกษาอัตราการตายของปูทะเลหลังรับเชื้อ WSSV พบการตายของปูเพียง 10% ในช่วงเวลาทำการทดลอง 8 วัน ซึ่งมีค่าไม่แตกต่างจากกลุ่มควบคุมเซลล์ที่มีการติดเชื้อ จากการศึกษาพยาธิวิทยาของเนื้อเยื่อเหงือกปูที่ฉีดเชื้อไวรัส WSSV พบลักษณะของเซลล์ที่มีการติดเชื้อ คือมีนิวเคลียสที่ขยายขนาดใหญ่ขึ้นและมีอินคลูชั่นอยู่ภายในพบจำนวนมากขึ้นตามเวลา ไม่พบลักษณะของเซลล์ที่มีนิวเคลียสขดตัวแน่นหรือเซลล์ที่มีการแตกของนิวเคลียสซึ่งเป็นลักษณะของเซลล์ที่เกิด apoptosis แต่เมื่อศึกษาด้วยวิธี TUNEL พบเซลล์ที่ให้ผลบวกในเซลล์ที่ติดเชื้อไวรัส คือเซลล์ที่มีลักษณะนิวเคลียสขนาดใหญ่ขึ้น การศึกษาด้วยวิธี RT-PCR และ western blot ที่ใช้ไพร์เมอร์ และแอนติบอดีที่ได้จากกุ้งกุลาดำ ไม่พบแถบของ DNA หรือโปรตีนที่ตำแหน่งใดกับ RNA หรือโปรตีนที่สกัดจากปู แต่เมื่อใช้แอนติบอดีต่อ cleaved caspase-3 ที่ได้จากมนุษย์ สามารถทำปฏิกิริยากับแถบโปรตีนที่มีขนาดที่คาดว่าเป็น cleaved caspase

สรุป ปูที่ติดเชื้อ WSSV กระตุ้นให้เกิดภาวะ apoptosis ในเซลล์ที่ติดเชื้อไวรัส แต่ไม่เกิดการขดตัวแน่นของนิวเคลียส หรือการแตกของนิวเคลียสเป็นไปได้ว่า apoptosis อาจมีผลต่อการที่ปูทนต่อการติดเชื้อไวรัส WSSV

Abstract

Part II: The study of apoptosis in white-spot syndrome virus infected mud crab *Scylla serrata*

Rationale and objectives: White spot syndrome virus (WSSV) is the cause of a widespread epizootic in cultured shrimp in Thailand and many other countries in Asia. A number of crustacean and other arthropod species have been proposed as reservoirs for the virus including the mud crab, *Scylla serrata*. The mud crab infected with WSSV showed tolerance to the virus and show no mortality. It has been reported that apoptosis occurs in WSSV-infected shrimp and may cause death of the shrimp. This study was carried out to determine whether apoptosis occur in the mud crab *Scylla serrata* experimentally infected with WSSV and study the if it related to the tolerance of the crab upon virus infection.

Methods: WSSV were experimentally injected to mud crab, *Scylla serrata* and the animals were observed for the mortality rate. Gills of the WSSV-infected crab were collected at 24, 65 and 96 h post injection (pi) and processed for histological study and RT-PCR. Histopathology of the crab after WSSV injection was investigated by light microscopy with H&E staining and apoptosis was determined by the DeadEnd colorimetric TUNEL system. The transcript level of caspase in the crab was performed by RT-PCR using the *Pm*Casp primers and the expression of protein level of caspase was investigated using anti *Pm*Casp antibody and anti-human cleaved caspase-3 as primary antibody.

Results: By histological study, gills of WSSV-infected crab showed the sign of WSSV infection and progressively increase in infection was observed at 65h and 96 h pi. However, WSSV infection in the crab did not lead to mortality even though the sign of heavy infection found. WSSV-infected mud crab showed cumulative mortality only 10% at 8 pi which was not significantly from that of uninfected control. The condensed and fragmented nuclei, the apoptotic morphology, were not observed in gills of WSSV-infected crab. However, by the specific methods to detect apoptosis TUNEL assay, the TUNEL positive cells were observed in WSSV-infected cells or in normal looking cells. The specific primers of *Pm*Casp did not give expected band with crab RNA extract by RT-PCR and anti *Pm*Casp antibody did not react with any band in western blot analysis. However a positive band at expected size of cleaved caspase-3 was detected when anti-human cleaved caspase-3 was used to immunoblot with the crab protein extract.

Conclusion: Based on the results of this study, the mud crab, *Scylla serrata* as the WSSV reservoirs are able to carry the infection and WSSV induce apoptosis but not show morphological sign of apoptosis, condensed and fragmented nuclei. The tolerance of this crab to the virus infection may be correlated with apoptosis.

หน้าสรุปโครงการ (Executive Summary)

ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่

1. ชื่อโครงการ (ภาษาไทย) การศึกษาการแสดงออกของ apoptotic และ antiapoptotic genes ในกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาวและกุ้งที่ทนต่อการติดเชื้อไวรัสตัวแดงดวงขาว

(ภาษาอังกฤษ) Expression of apoptotic and antiapoptotic genes in the black tiger shrimp *Penaeus monodon* that are susceptible and tolerate to white-spot syndrome virus

2. **ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail**

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3. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Shrimp is one of the most important species in aquaculture. During the last decade, the worldwide shrimp culture has suffered from diseases caused by viruses particularly by white-spot syndrome virus (WSSV), resulting in significant economic losses (Flegel, 1997). Due to the extreme virulence of WSSV and a wide host range covering almost all crustaceans (Flegel, 1997), it is difficult to prevent and inhibit the spread of the virus.

It was noticed that although most of the WSSV-infected shrimp die, a few of them still survive (Flegel, 1997). These survivors may have special defense mechanism that either destroy the virus completely or tolerate the presence of the virus. Like other invertebrates, shrimp lacks specific immunity, and its disease resistance relies on its innate defense system including a series of humoral and cellular immune factors (Soderhall, 1999).

Apoptosis is one of the innate immune response against viral infection and has been found in the WSSV-infected shrimp. Wongprasert et al. (2003) revealed that the number of apoptotic cells in WSSV infected shrimp seemed to rapidly increase in the beginning and slightly plateau until the shrimp died. Cells displaying nuclear condensation and fragmentation characteristic of apoptosis did not contain WSSV virions while those containing WSSV virions were not apoptotic. They concluded that apoptosis is the shrimp innate immune response to prevent virus infection in bystander cells and WSSV infected cell

has an ability to prevent apoptosis. It has been recently reported that apoptosis rates were lower in immune *P. japonicus* than in naive shrimp after WSSV challenge (Wu and Muroga, 2004) so the enhanced level of protection in the immune response is not the result of the greater level of apoptosis. On the contrary, the study of differential gene expression in hepatopancreas of WSSV-resistant shrimp *P. japonicus* showed the number of genes encoding apoptotic-related proteins expressed at a higher level in the virus resistant shrimp (Pan et al., 2005). It therefore comes to a controversy that apoptosis plays an important role in the resistance of the shrimp against WSSV or causes death of the shrimp.

Many carrier species of WSSV such as mud crab *Scylla serrata* has active viral infection but show no mortality (Kanchanapum et al., 1998). This phenomenon indicates that high levels of viral particles in the body do not quarantine death. So far it has not been known whether apoptosis occurs in host reservoir like the mud crab and how they tolerate WSSV infection.

Terminal deoxynucleotidyl transferase (TdT) -mediated dUTP-nick end labelling (TUNEL) and transmission electron microscopy (TEM) are usually used for studying apoptosis in viral infected shrimp and the study has not yet extended to molecular level. This study aims to studying the role of apoptosis in WSSV-infected shrimp in molecular level particularly on the expression of apoptotic and antiapoptotic genes in WSSV- susceptible and WSSV-tolerated *P. monodon*; and to compare these with those in WSSV-infected mud crab *Scylla serrata*. The study would help to explain the role of apoptosis or the innate immune response and the development of disease tolerance in virus-infected shrimp. Knowledge on apoptosis in virus-tolerated shrimp may help to develop strategies to prevent shrimp death after viral infections. Moreover the primers developed might be useful for screening domesticated *P. monodon* broodstock that express WSSV-tolerated genes in selective breeding program.

4. วัตถุประสงค์

Overall objective

To study whether apoptosis is involved in the tolerance or death of the WSSV-infected *Penaeus monodon*

Specific objectives

1. To determine the expression of apoptotic and antiapoptotic genes in WSSV-susceptible *P. monodon*, WSSV-tolerated *P. monodon* and WSSV-susceptible mud crab *Scylla serrata*, by using RT-PCR, and to quantify the transcription levels by using realtime RT-PCR
2. To localize the apoptotic and antiapoptotic mRNA in WSSV-infected shrimp by using *in-situ* hybridization under electron microscopy.

5. ระเบียบวิธีวิจัย

Experiment 1: Time-course study of expression of apoptotic- and antiapoptotic-related genes in WSSV susceptible *P. monodon*

Experimental animals

Healthy juvenile black tiger shrimp *P. monodon* are purchased from commercial ponds and screened for viruses by PCR. The viruses screened include WSSV, YHV, MBV, HPV and IHHNV. The shrimp are stocked in six circular concrete tanks containing 1.8 tons of brackish water at 10 ppt and 25°C with adequate aeration to ensure a level of dissolved oxygen above 5 ppm. The shrimp are allowed to acclimate for 5 days before experiments and they are fed with commercial feed pellets at 3 % BW daily. They are divided into two groups; vehicle-injected control and WSSV-injected groups. Each group is composed of three replicates, each tank contains 70 shrimp.

Virus infection

The virus isolate used in this study originates from WSSV-infected *P. monodon* in Thailand. The stocked shrimp are injected intramuscularly with purified WSSV using a 26-gauge needle. After the injection, twenty shrimp from each group were sampled at 0, 6, 12, and then every 12 hours until all of the shrimp were sampled out. Tissue samples from the shrimp are subjected to study as in subsequent sections.

1. Total RNA extraction

The tissues of the shrimp include hemocytes, haematopoitic organ, lymphoid organ, hepatopancreas and gill are dissected out of the body and rapidly kept in liquid nitrogen until

ready for extraction. The RNA extraction is performed using TRIzol reagent (Gibco-BRL, Life Technologies, Tokyo, Japan). Tissue is homgenizedand subjected to chloroform extraction, isopropanol precipitation and ethanol washing according to the manufacturer's recommendations. The precipitated RNA is dissolved in 50 ul RNase-free water, and the RNA is quantified by measuring the absorbance at a wavelength of 260 nm.

2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Primers of apoptotic and antiapoptotic genes

The sequence of genes encoding for apoptosis- and antiapoptosis- related proteins are searched from GenBank. The candidate sequences of genes encoding apoptosis related proteins include caspases, p53, cellular apoptosis susceptibility gene, and others. The interested antiapoptotic sequences are genes in Bcl2-family, IAP family, CED 9, p35 and others. Primers corresponding to sequences of apoptotic and antiapoptotic genes are then designed and are prepared by a DNA synthesizer from BSU (BKK, Thailand). The PCR primers are used in PCR to amplify the partial fragment of cDNA encoding the apoptosis- or antiapoptosis-related genes of *P. monodon*. From our colleques studies, Alix gene, a member of IAP family, was successfully cloned in *P. monodon* tissue. The sequence of Alix and the primers are shown in the appendix. Some certain sequences searched from GenBank are aligned and also shown in the appendix.

First strand cDNA synthesis is performed using SuperScript™ III reverse transcriptase (Invirogen Life Technologies) according to the manufacturer's protocol. PCR of the cDNA obtained is performed using *Taq* DNA polymerase (Promega) with specific primer sets for the different genes. The primers that give the specific amplification are then used in realtime RT-PCR in order to quantify the amount of gene expression.

3. Realtime quantitative reverse-transcription polymerase chain reaction (Realtime RT-PCR)

Transcription levels are measured by RT-PCR using ABI 7000 SDS (PE Applied Biosystems, USA). The realtime RT-PCR primers are identical to those used in RT-PCR but the TaqMan probes that span the region between the forward and reverse primers are selected from apoptotic or antiapoptotic sequences through the use of Primer Express 2.0 software (Applied Biosystems, CA). The TaqMan probes are synthesized and labeled with fluorescent dyes, 6-carboxyfluoroscein (FAM) on the 5' end and *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA) the 3'-end. The methods follow the manufacturer's instruction. Realtime RT-PCR assays are performed in triplicate. The data acquisition and analysis are carried out by ABI Prism 7700 sequence detector software (User Bulletin, Perkin Elmer).

Experiment 2: Time-course study of expression of apoptotic and antiapoptotic genes in WSSV-susceptible mud crab *Scylla serrata*

Experimental animal

The mud crab *Scylla serrata* are purchased from a commercial farm and stocked in the tanks with 5 cm seawater (30 ppt) and bricks to create land structure for the animals. The molecular biology methods approached to the crab are the same as those performed with *P. monodon*.

Experiment 3: Study of the expression of apoptosis- and antiapoptosis- related genes in WSSV tolerated shrimp

WSSV-tolerated shrimp are shrimp that have WSSV infection (PCR positive) but survive. Groups of the WSSV-tolerated shrimp include 1) shrimp that receive heat inactivated virus 2) shrimp that receive immunostimulant (low dose of WSSV) 3) shrimp from the WSSV-tolerant family.

Shrimp from these groups are either induced (e.g. by exposure to formalin-killed WSSV or low-dosed WSSV or from the Shrimp Genetic Improvement Center, BIOTEC, NSTDA. The shrimp are studied for the expression of apoptosis- and anti-apoptosis-related genes by RT-PCR and real time RT-PCR, using the same sets of primers developed in experiment 1.

Experiment 4. Localization of apoptotic and anti-apoptotic mRNA in WSSV-infected shrimp by *in-situ* hybridization, under electron microscopy

In this study, using *In-situ* hybridization under electron microscopy could reveal cells that express apoptosis and antiapoptosis-related genes in WSSV infected shrimp. Shrimp tissues including lymphoid organ, haematopoitic tissue and gill are dissected from WSSV infected shrimp in experiment 1. Five shrimp are collected from each time point.

Riboprobes preparation A plasmid pGEM-T Easy (Promega) containing nucleotide region of expected sequences of apoptosis or antiapoptosis cDNA is used as the template for the preparation of probes. Digoxigenin (DIG)-UTP-labeled sense and antisense riboprobes are generated from linearized cDNA plasmids by in vitro transcription RNA labeling kits T7 and SP6 RNApolymerase (Boehringer Mannheim Co. IN, USA), respectively. Labeled RNA probe is visualized with alkaline phosphatase-conjugated anti-digoxigenin antibody and the substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP). *In-*

situ hybridization electron microscopy is performed following the protocol previously described by Pantoja and Lightner, 2001. (see detail in the full proposal)

แผนการดำเนินงานตลอดโครงการ Workplan

Activities	Year 1				Year 2			
	Month 1-3	Month 4-6	Month 7-9	Month 10-12	Month 1-3	Month 4-6	Month 7-9	Month 10-12
1. Apoptotic and antiapoptotic genes are searched from GenBank and primers are designed								
2. Primers designed are tested in WSSV infected shrimp tissues.	◄——►							
3. Primers that give the successful amplification are employed in experiment 1: Expression of apoptotic and antiapoptotic related genes in WSSV susceptible shrimp by RT-PCR	◄——►							
4. Quantification of the expression of apoptotic and antiapoptotic related genes by real time RT- PCR		◄——►						
5. Expression of apoptotic and antiapoptotic related genes in WSSV infected mud crab by RT-PCR and determine transcript levels by real time RT-PCR.			◄——►					
6.Expression of apoptotic and antiapoptotic related genes in WSSV tolerated shrimp by RT-PCR and determine transcript levels by real time RT-PCR.				◄——►				
7. Localization apoptotic and antiapoptotic related mRNA in WSSV infected shrimp by *in situ* hybridization electron - microscopy.						◄——►		◄——►

ผลงานที่คาดว่าจะตีพิมพ์ได้

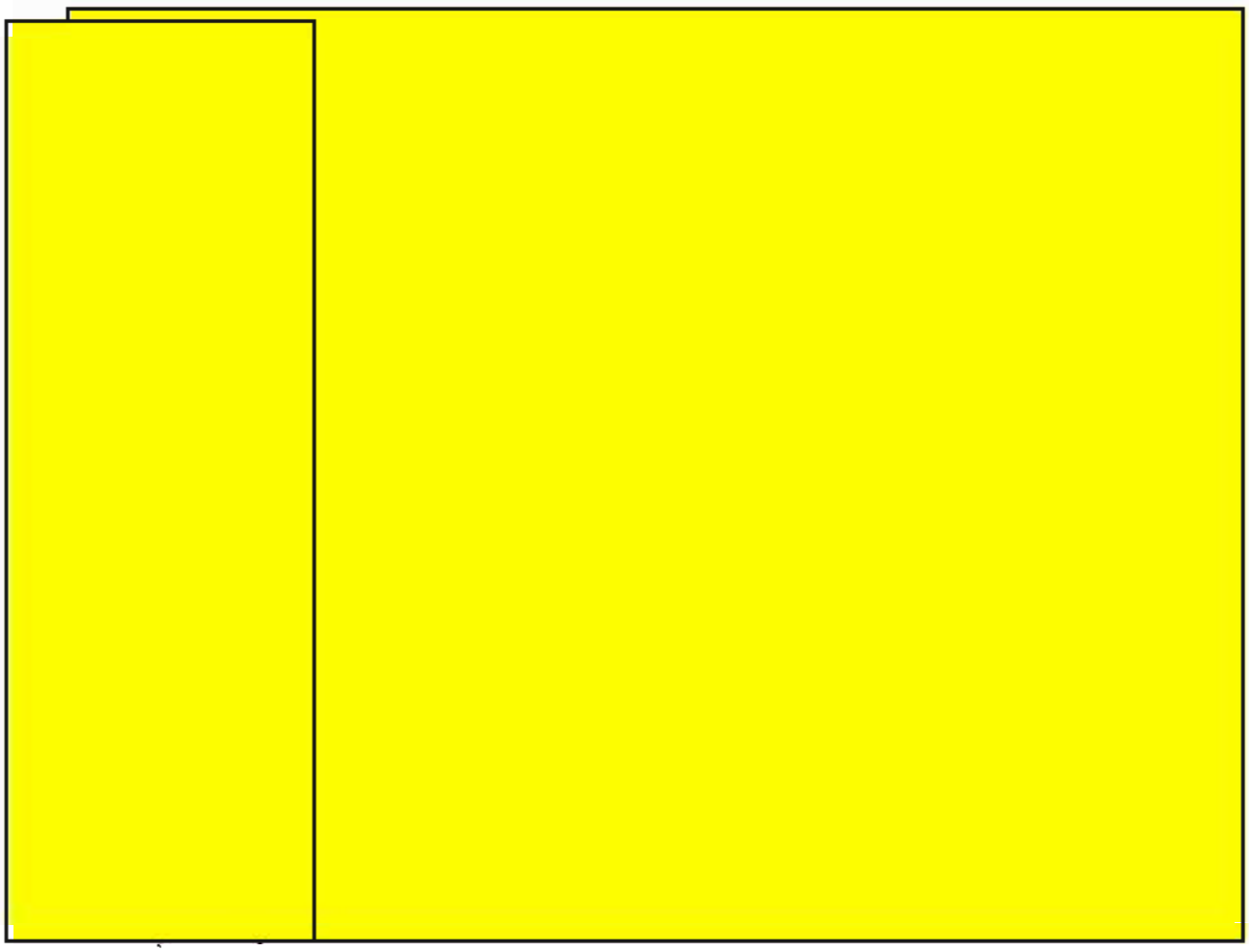
First year

1. Timecourse expression of apoptotic and antiapoptotic genes in white spot syndrome virus infected shrimp, *P. monodon*. Aquaculture

Second year

1. Expression of apoptotic and antiapoptotic genes play roles in white spot syndrome virus tolerated shrimp, *P.monodon*. Journal of General Virology

2. Localization of apoptotic and antiapoptotic mRNA in *P. monodon* tissues infected with white spot syndrome virus by *in situ* hybridization electron microscopy. Diseases of Aquatic Organisms.



หมายเหตุ

Note!!!

The reasons why some methods were not performed as proposed in the proposal.

In situ hybridization was cancelled.

In situ hybridization was cancelled because the specific antibody has been developed and was used for immunolocalization of caspase -3 in the tissue.

Real time RT-PCR was cancelled.

Semi-quantitative RT-PCR was replaced the real time RT-PCR because it also could explain the information and I could not afford the cost of real time RT-PCR with my grant.

Study of expression of caspase-3 in the WSSV-tolerated shrimp was terminated.

The animals were not available for study. Almost all of the farmers have turned to culture white shrimp *L. Vannamei*.

เนื้อหางานวิจัย

We started with searching the known sequences of other species caspase-3, Bax, Bcl2 and p35 from the data bases and designed the degenerated primers to amplify the RNA extracted from the shrimp tissues. Unfortunately, the primers designed did not give the expected products when RT-PCR and sequencing were performed. Fortunately, sequences of proapoptotic gene, caspase of *Penaeus merguiensis* had been lately identified so we have been concentrated on studying caspase-3 in *P. monodon* upon White spot syndrome virus (WSSV) infection. In order to scope the work that has been done, the title of the research is changed as follow.

การ Cloning การแสดงออก การศึกษาคุณสมบัติของ pro-apoptotic gene, caspase-3 ในกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาว และการศึกษา apoptosis ในปูทะเลที่ติดเชื้อไวรัสตัวแดงดวงขาว

Cloning, expression and characterization of pro-apoptotic gene, caspase-3 in the white-spot syndrome virus infected black tiger shrimp *Penaeus monodon* and the study of apoptosis in white-spot syndrome virus infected mud crab *Scylla serrata*

วัตถุประสงค์

Overall objective

To study whether apoptosis is involved in death of the WSSV-infected *Penaeus monodon* and in tolerance of WSSV-infected mud crab *Scylla serrata*

Specific objectives

1. To isolate, clone, express and characterize caspase-3 gene of *P. monodon*
2. To determine the time-course expression of apoptotic caspase-3 gene in WSSV-infected *P. monodon* by using RT-PCR, and to quantify the transcription levels by using semi-quantitative RT-PCR. Study was also performed with WSSV-susceptible mud crab *Scylla serrata*.
3. To determine the time-course expression of caspase-3 protein in WSSV-infected *P. monodon* by Western blot analysis and localize the apoptotic protein caspase-3 in the tissue of WSSV-infected shrimp by immunohistochemistry. Study was also performed with WSSV-susceptible mud crab *Scylla serrata*.
4. To study the apoptosis in WSSV-infected mud crab *Scylla serrata* and to determine the time-course expression of caspase-3 gene and caspase-3 protein in WSSV-infected mud crab using primers and anti-capase-3 antibody obtained from *P.monodon*

การดำเนินงานวิจัย

Since the objectives has been changed so some of the methods approached had been modified.

Part I: Studying of caspase-3 in *P.monodon* upon WSSV infection

Experiment 1: Isolation, expression and characterization of caspase-3 gene of *Penaeus monodon*

Isolation of P. monodon Caspase cDNA: Plaque hybridization assay

Plaque hybridization assay was performed to screen for *PmCasp* from a *P. monodon* hemocyte cDNA library (Tonganunt et al., 2005) using a putative caspase-3 cDNA from *P. merguiensis* (*PmeCasp*) as a probe. The sequence of the probe was amplified and DIG-labeled by PCR using a plasmid containing *PmeCasp* as template (Phongdara et al., 2006). Primers are PmeF (5'-TGT CTA TGT TGG CGG AAC CAA-3') and PmeR (5'-TCA CCT GCT GAC TTT GAG AAG-3') designed from the reported cDNA sequence (GenBank accession

no.**AY839873**). Plaques (2 x 104) of *P. monodon* cDNA library were transferred onto nitrocellulose membranes and subjected to hybridization using the DIG-labeled *PmeCasp* probe. Hybridization was performed overnight at 60°C followed by a series of washes according to Roche's protocol. The membrane was then incubated with an anti-DIG antibody conjugated to alkaline phosphatase (Roche) at room temperature for 1 h. Hybidized signals were visualized using a chromogenic solution containing NBT/BCIP (Roche). Positive clones were excised *in vivo* and cDNA library plasmids were subjected for sequencing by Macrogen Co. Ltd. (South Korea).

Organisms

Juvenile *P. monodon* were purchased from local farms in Chachoengsao province, Thailand.

RNA preparation

Shrimp hemolymph was collected in an equal volume of anticoagulant AC1 solution (Soderhall and Smith, 1983) and the hemocytes enriched fraction attained by centrifugation at 800xg for 15 min. Total RNA was then prepared from this fraction using Trizol reagent (Invitrogen). RNA concentration and quality were measured by spectrophotometric analysis using absorbance at 260 and 280 nm.

Purification of PmCasp fusion protein in E. coli

PmCasp was amplified by PCR using the identified cDNA plasmid as a template and *PmCasp* specific primers (PmF; 5'-**GGA ATT CCA T**AT GAG CGA CGC CGA CGA CTC-3' and PmR; 5'-**CGC GGA TCC** TCA GAA GTA AAT TTC ACG AAG-3', where underlines represent restriction sites and bold represents flanking non-complimentary sequences). PCR reactions of 50 µl consisted of 100 ng of DNA template, 0.2 µM of each primer, 5 unit of Taq DNA polymerase (Invitrogen), and 1X PCR DIG labelling Mix (Roche) in 1X PCR reaction buffer. The mixture was denatured at 94°C for 5 min, and amplification was performed using a cycling scheme of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, for 30 cycles. To generate a pET15b-*Pm*Casp plasmid, this 960-bp *Nde*I-*Bam*HI amplicon containing the complete *PmCasp* cDNA was inserted in-frame with the same enzymes that digested pET15b (Novagen) so that a coding region for an N-terminal His6 tag was fused to the *PmCasp* open reading frame. Correct sequence and in-frame insertion of the insert was verified by DNA sequencing. After transformation and cloning into *E. coli* BL21

pLysS (DE3) cells, His6-fusion *Pm*Casp protein was expressed by induction with 1 mM IPTG. The fusion protein in the bacterial lysates was purified using Ni-NTA beads (Qiagen) and analyzed by SDS-PAGE (12% (w/v) resolution and western blotting discrimination and visualization with anti-His antibody (Zymed).

Antibody production

A polyclonal antiserum against *Pm*Casp was raised in rabbits by injection of 100 μg of purified His6-*Pm*Casp fusion protein in complete Freund's adjuvant followed by four booster injections in incomplete adjuvant at one-weekly intervals. Serum was collected from the rabbits 1 week after the last boost.

Caspase activity assay

Caspase activity of His6-*Pm*Casp fusion protein was detected using Caspase-Glo 3/7 Assay (Promega) essentially as described (Phongdara et al., 2006), except for minor modifications. His6-*Pm*Casp, was plated (350 ng, 200 μl per well) into 96-well plates for assays. Luminescent signals were measured every 30 min within a total period of 3.5 h by Wallac Victor Multi-label Counter (PerkinElmer). Inhibition of caspase-3 activity was achieved using caspase-3 inhibitor (ApoAlert Caspase-3 inhibitor, DEVD-CHO, Clontech) at a final concentration of 5 μM. The results are expressed as the mean ± SD of three replicates.

Tissue distribution of *Pm*Casp protein by western blotting

Shrimp hemolymph and hemocytes, heart, muscle, and gill tissues were collected from ten normal shrimp and homogenized in 100 mM NaCl, 50 mM Tris buffer, pH 8.0 containing 1mM phenylmethanesulfonyl fluoride. The homogenates were centrifuged at 3000 xg for 5 min and the supernatants were collected. Total protein concentrations in each shrimp tissue extract and the hemolymph fraction were determined using a Bio-Rad protein assay kit. Approximately equal amounts of proteins (25-30 μg) were subject to SDS-PAGE (15% (w/v)) resolution and western blotting based discriminatory quantification using rabbit anti-*Pm*Casp antibody at a dilution of 1:1000 (detected by AP-conjugated goat anti-rabbit IgGs (Zymed) with NBT/BCIP substrate solution (Roche). The detection procedure was performed according to the supplier's protocol.

Sequence data analysis

DNA and protein analysis were carried out using the EXPASY web server (http://au.expasy.org/). To identify related sequences, a protein-protein basic local alignment search tool (blastp) search was carried out using the NCBI protein database (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of protein sequences were performed and a phenogram was generated by ClustalW [(Thompson et al., 1994), http://www.es.embnet.org/ Doc/ phylodendron/ clustal-form.html], using the default settings. An analysis for protein domains was accomplished by Conserved Domain Database (Marchler-Bauer et al., 2005).

Experiment 2: Time-course study of expression of apoptotic genes in WSSV susceptible *P. monodon*

Experimental challenge

Healthy juvenile *P. monodon* shrimp of 8 to 12 g body weight (BW), were collected from a commercial farm and confirmed to be WSSV-, YHV-, MBV-, HPV- and IHHNV-negative using respective diagnostic PCR or RT-PCR kits (Farming IntelliGene Biotechnology Corporation, Taiwan or Shrimp Biotechnology Business Unit, Thailand). Shrimp were maintained at 25°C in tanks containing 50 L of brackish water (10 ppt) with adequate aeration to ensure a level of dissolved oxygen above 5 ppm. The shrimp were fed with commercial feed pellets at 3% BW daily and allowed to acclimate for 5 days before experiments begun. The shrimp were divided into 2 groups. In the first (control) group, each shrimp was injected with 0.1ml lobster hemolymph buffer (LHB) (Paterson and Stewart, 1974) while each shrimp in the second (infected) group was injected intramuscularly with 0.1 ml crude WSSV extract in LHB. A crude extract of WSSV was prepared from hemolymph of moribund shrimp obtained from a commercial rearing pond experiencing white spot virus outbreak. After the inoculation, shrimp from each group were randomly sampled at 24 and 48 h post-injection (pi). Gills were dissected from the live shrimp and kept in liquid nitrogen for semi-quantitative RT-PCR and western blot analysis. Some shrimp were perfused and fixed in Davidson's fixative for paraffin sectioning according to the methods described by Bell and Lightner (Bell and Lightner, 1988).

Expression of *Pm*Casp transcript in WSSV-infected shrimp by semi-quantitative RT-PCR

Total RNA was extracted from gills of WSSV-infected and control shrimp and processed for RT-PCR to determine *Pm*Casp level, using β-actin gene as an internal

control. A partial sequence of 825 bp of *Pm*Casp was amplified using specific primers (see Fig. 1) to *Pm*Casp (PmCaspF:- 5'-GGA GGA ACC TGC GAA GAA C-3' and PmCaspR:- 5'-AGC GTC 1 GAG TGG ATG TAA GG-3'). A partial sequence of 377 bp of β-actin gene was amplified as a control using primers actin-F (5'-TGA CGG CCA GGT GAT CAC CA-3') and actin-R (5'-GAA GCA CTT CCT GTG AAC GA-3'). RT-PCR was performed using conditions as follows: 50oC for 30 min, and 94oC for 2 min, followed by 25 cycles of 94oC for 30 s, 55oC for 30 s, and 68oC for 1min and a final extension at 68°C for 5 min. Confirmation of PCR-amplicons to the assumed corresponding cDNA (PCR fidelity) was attained by sequencing of the PCR products. Amplified cDNA was resolved by TAE-(1.2% w/v) agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide (2 g/ml). The fluorescent images were captured and analyzed by densitometer using the Scion Image, release Alpha 4.0.3.2. Transcript level of *Pm*Casp was normalized with that of βactin obtained from the corresponding total RNA sample. The data were analyzed from three individuals at each of three time points post infection (24 h, 48 h and moribund) by one way analysis of variance (ANOVA) using SPSS for Windows and an alpha level of 0.05.

Expression of *Pm*Casp protein in WSSV-infected shrimp by western blot analysis

Total protein was also extracted from aliquots of the same gills as used for RNA analysis above, by homogenization into 100 mM NaCl, 50mM Tris pH 8.0, 1mM PMSF as detailed above. For each sample, 100 µg of the total protein extract was resolved by SDS–PAGE (15% (w/v) acrylamide) and *Pm*Casp protein identified and quantified by western blotting using rabbit anti-*Pm*Casp antisera at a dilution of 1:1000 or with anti-*Pm*Casp antisera that has been preabsorbed with *Pm*Casp fusion protein (1:1000 dilution). The blot of recombinant *Pm*Casp protein was also incubated with anti-human caspase-3 (Cell signaling technologies) at a dilution of 1:1000. Subsequently, AP-conjugated goat anti-rabbit IgGs (Zymed) at a dilution 1:2000 were used and detection was performed with a substrate solution (NBT/BCIP, Roche). The detection procedure was performed according to the supplier's protocol.

Localization of *Pm*Casp in WSSV-infected tissues

Shrimp were fixed in the Davidson fixative for 24 h and processed for paraffin sectioning. The sections of 7 µm thickness were immunohistochemically stained to localize *Pm*Casp protein in the tissue, following the protocol described in the Histostain-SP kit (Zymed) with slightly modifications. Briefly, the sections were firstly immersed in 3% (v/v)

H_2O_2 in absolute methanol for 15 min to block endogenous peroxidase activity, and then washed three times with 0.1 M PBS, pH 7.4 (5 min each). The sections were covered with blocking solution for 1 h and subsequently incubated with primary antisera, rabbit anti-*Pm*Casp at a concentration of 1:1000, at 4°C, overnight, and washed three times with PBS-T (0.1 M PBS, pH 7.4 containing 0.1% (v/v) Tween 20). The sections were incubated with second antibody, HRP conjugated goat anti-rabbit IgG, at concentration 1:2000 for 2 h. After the sections were washed three times with PBS-T, 5 min each, the staining was developed by covering the sections with the substrate AEC to produce red color. The reaction was terminated in water and the sections were counterstained with Meyer hematoxylin for nuclei staining. To verify specificity of the substance detected, two control staining procedures were carried out: one without primary antisera and another with *Pm*Casp preabsorbed primary antisera. Hematoxylin and eosin (H&E) staining was carried out to determine histopathological features of the WSSV-infected gills according to that described by (Wongteerasupaya et al., 1995) and (Wongprasert et al., 2003).

ผลการวิจัย

Results

Sequence analysis of *Pm*Casp

Using a putative caspase-3 from *P. merguiensis* (Phongdara et al., 2006) as a probe to screen a *P. monodon* hemocyte cDNA library by plaque hybridization yielded two positive clones (0.01% of clones screened). DNA sequence analysis revealed that they were identical, yielding a single putative caspase homologue. The inserted cDNA of 1,202 bp contained 41 nucleotides of 5'-untranslated region (UTR), a 954-nucleotide open reading frame, and a 207-nucleotide 3'-UTR including a potential poly (A) tail and a putative polyadenylation signal (AATAAA) (Fig. 1). The entire 954 bp-open reading frame encodes a deduced protein of 317 amino acids with a predicted molecular weight of 36 kDa and an isoelectric point of 5.6. A typical caspase family protease active-site QACRG pentapeptide was located from amino acids 190-194. Putative cleavage sites that generate large and small subunits were proposed to be at TAVDG, between Asp55 and Gly56, and VQTDS, between Asp215 and Ser216 (Fig. 1). BLASTp homology search (Altschul et al., 1990) revealed high similarity to putative and actual genes encoding caspase-1, -3, -6, and -7 enzymes of various animals such as insects, fish, birds, and mammals. In addition, a conserved domain characteristic of typical caspases was detected by a Conserved Domain Database (Marchler-Bauer et al., 2005). *Pm*Casp sequence was compared to the first

caspase sequence reported in shrimp, *P. merguiensis* and to the 7 identified caspases from a fruit fly *D. melanogaster*, phylum arthropoda. In addition, since *Pm*Casp exhibited a caspase-3 activity (see below), caspase-3 enzymes from the relatively well-studied human and rat were also included in the comparison (Fig. 2A). The alignment unsurprisingly revealed that *Pm*Casp showed highest identity (83%) to the already cloned putative caspase from white shrimp *P. merguiensis* (*Pme*Casp), which was used as the probe to detect *PmCasp*. With other organisms, the sequence showed 13-30% identity with *D. melanogaster* caspases and revealed 28% and 26% identity with human and rat caspase-3, respectively. Among *D. melanogaster* caspases, *Pm*Casp revealed relatively high similarity to ICE (30% identity) and to DCP-1 (29% identity). The comparison using the default parameter generated phenogram (Fig. 2A) also demonstrated that *Pm*Casp is relatively more closely related to mammalian Casp-3, and fly ICE and DCP-1 proteins. In this regard, the putative domain structure of *Pm*Casp was therefore compared to human Casp-3, fly ICE and DCP-1 (Fig. 2B). The active site of shrimp *Pm*Casp is the same as human Casp-3 with a pentapeptide motif of QACRG, whilst that of the two fly caspases is slightly modified to QACQG. The cleavage sites of shrimp sequence are not conserved with any organisms. In addition, the reported conserved consensus sequence motif for cleavage sites reported in mammalian and insect caspases are not present in these putative shrimp caspases. Although the sequence and activity of *Pme*Casp have been studied, its potential cleavage sites have not yet been predicted (Phongdara et al., 2006). Therefore, the potential aspartate cleavage sites generating large and small subunits of *Pm*Casp were proposed to be at the nearest aspartate residues to the cleavage sites of the three compared sequences (human Casp-3, and fly ICE and DCP-1) (Fig. 2B). With this respect, putative proteolytic cleavage sites of *Pm*Casp are Asp55 and Asp215, and the predicted molecular weights of the large and small subunits of *Pm*Casp would therefore be 17.9 kDa (160 amino acid residues) and 11.8 kDa (102 amino acid residues), respectively (Fig. 2B). Hence, it is noted that *Pm*Casp appears to have a relatively short N-terminal prodomain of 55 amino acid residues when compared to other known caspases with long prodomains which are of 130-220 amino acids (Villa et al., 1997).

_Pm_Casp exhibit caspase-3 activity and is inhibited by a caspase-3 inhibitor

The proluminescent substrate of caspase-3 and -7 enzymes (caspase-Glo 3/7) was used to test for activity of the purified recombinant *Pm*Casp protein (see below for protein expression). Recombinant *Pm*Casp protein exhibited significantly higher luminescence levels

compared to the no enzyme control, and this was totally negated by addition of 5 μM DEVD-CHO, a synthetic (specific) peptide inhibitor of caspase-3 (Fig. 3). The data thus suggested *Pm*Casp confers caspase-3 like activity.

Detection of *Pm*Casp in shrimp tissues

The *PmCasp* was cloned in frame into the pET15b plasmid and overexpressed as a His6-tagged fusion protein. A band of approximately 36 kDa corresponding to the His6-*Pm*Casp fusion protein was observed after IPTG induction and found to react with both anti-His and anti-*Pm*Casp antibodies (Fig. 4A, lane 4). No bands were found at the same positions in both non induced and induced transformed *E. coli* with empty pET15b, and non-induced pET15b-*Pm*Casp transformed cells (Fig. 4A, lanes 1 to 3). Recombinant His6-*Pm*Casp was purified using Ni-NTA beads and shown to react with both anti-His and anti-*Pm*Casp antibodies (Fig. 4A, lane 5).Interestingly, an immunoreactive band of approximately 26 kDa was found in induced *E. coli* lysates expressing pET15b-*Pm*Casp when detected with both anti-His and anti-*Pm*Casp antibodies (Fig. 4A, lane 4). It is likely that this band was a proteolytic intermediate form of *Pm*Casp which contained His-tag moiety. To determine the distribution of *Pm*Casp, 25-30 g of total protein extract of hemolymph, hemocytes, hearts, muscle, and gills of shrimp were analyzed. The result of western blot analysis using anti-*Pm*Casp antibody indicated the presence of *Pm*Casp in all tested tissues whereas no positive signal was detected for the hemolymph fraction (Fig. 4B). Signal from hemocyte lysate was shown to be relatively stronger than from other tissues. Detected bands of 36 kDa were in accordance with the predicted molecular mass from the amino acid sequence deduced from the *PmCasp* cDNA, although a reacted band size from the hemocyte homogenate was slightly larger. It was also noted that a band of 26 kDa expected to represent a partial proteolytic intermediate form was observed from only the hemocyte homogenate.

Expression of *Pm*Casp in WSSV-infected shrimp

The transcript levels of *Pm*Casp were investigated in gills, which are one of WSSV target organs, of WSSV-infected shrimp by semi-quantitative RT-PCR. The intensity of the signal of the transcript levels of *Pm*Casp were compared to those of β-actin levels. The results demonstrated that the transcript levels of *Pm*Casp were relatively high in the gills of WSSV infected shrimp at 48 h pi and in the moribund shrimp when compared to the control shrimp. Although, the level detected at moribund was not significantly different from that of

the normal control (Figs. 5A-B). At the protein levels, the anti-*Pm*Casp antibody was used to immunoblot with proteins extracted from gills of the control and WSSV-infected shrimp. The antibody detected a relatively strong signal at approximately 26 kDa, along with very faint bands at 36 kDa of all tested samples (Fig. 5C). The density of the 26-kDa bands of the 48 h pi and moribund samples showed a relatively increase compared to that of normal control and 24 h pi shrimp. Two immunoreactive bands at 36 kDa and 26 kDa were also detectable from the purified *Pm*Casp protein, however, strong reactivity was seen with the 36-kDa protein (Fig. 5C-1). The same result was obtained when anti-human caspase-3 antibody was used (Fig. 5C-2). The bands of 26 kDa were completely disappeared when the anti-*Pm*Casp antibody was pre-absorbed with the *Pm*Casp fusion protein (Fig. 5D-2), indicating the specificity of the anti-*Pm*Casp antibody.

Localization of *Pm*Casp protein by immunohistochemistry

H&E stained sections of gills from WSSV-infected shrimp revealed histopathological changes, including cells with enlarged nuclei and nuclear inclusion (Fig. 6A), typical characteristics of WSSV infection. Some cells showed condensed and fragmented nuclei especially at the later stages of infection. Localization of *Pm*Casp protein in the gills of control and WSSV-infected shrimp was investigated by immunoperoxidase based immunohistochemistry using rabbit anti-*Pm*Casp antisera. In gills of WSSV-infected shrimp, the immunoreactivity was observed in the cytoplasm of both apoptotic cells (cells with condensed nuclei (Fig. 6C) and fragmented nuclei (Fig. 6E), and normal-looking cells (Fig. 6D). Gills of control shrimp also showed positive immunoreactivity in some normal cells (Fig. 6B). However, no cells with clear signs of apoptosis were observed in the gills of control shrimp. Negative controls (primary antibody omitted) all showed no immunoreactive staining (Fig. 6F).

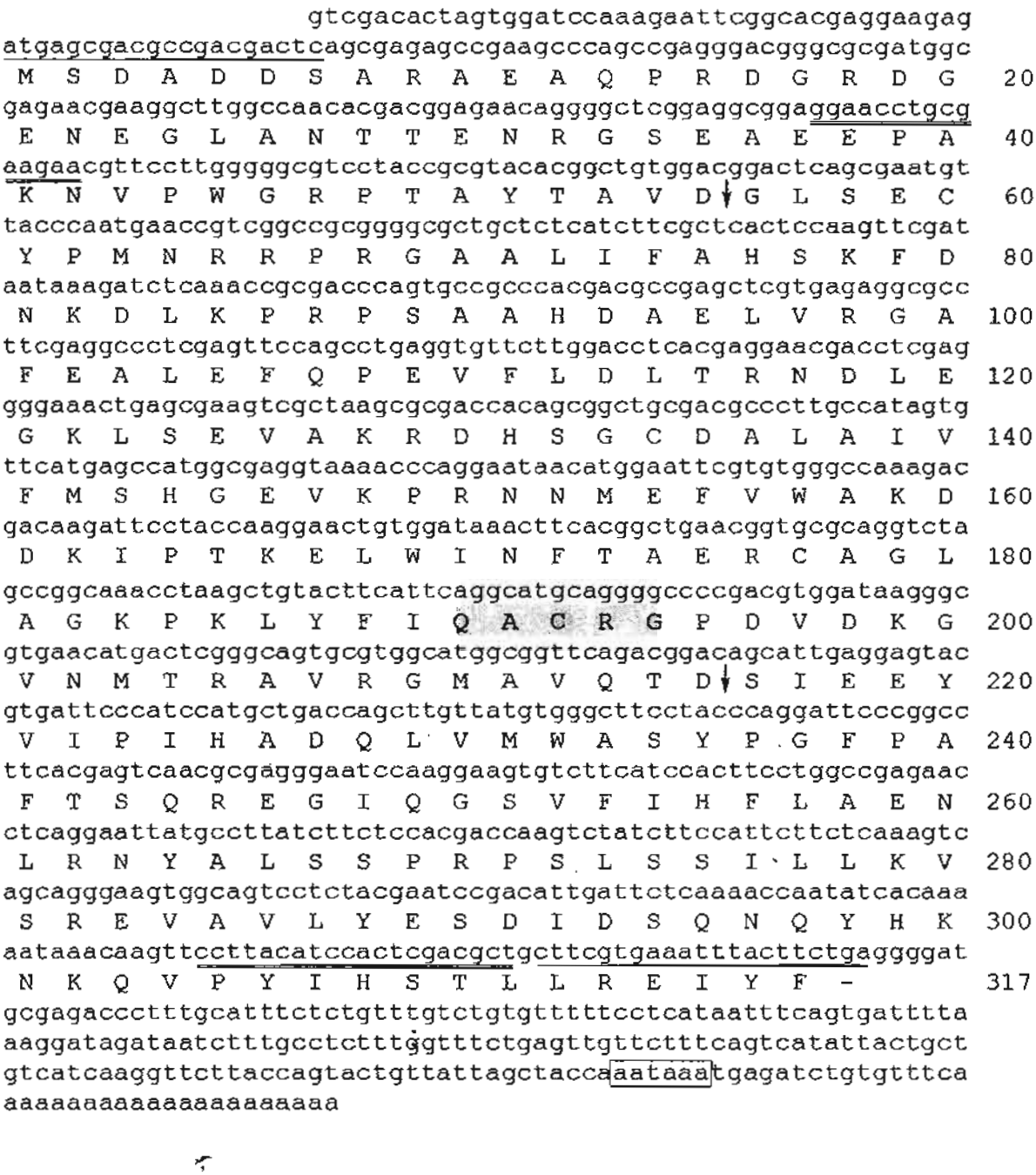


Figure 1. Nucleotide and deduced amino acids sequence of the full-length *PmCasp*.

An open reading frame of 954 nucleotides encoding 317 amino acids contains a putative active-site pentapeptide (shaded) and a potential polyadenylation signal (underlined). The potential cleavage sites (Asp55 and Asp215) are indicated by arrows. Primers used for *Pm*Casp expression and for RT-PCR are underlined and double underlined, respectively.

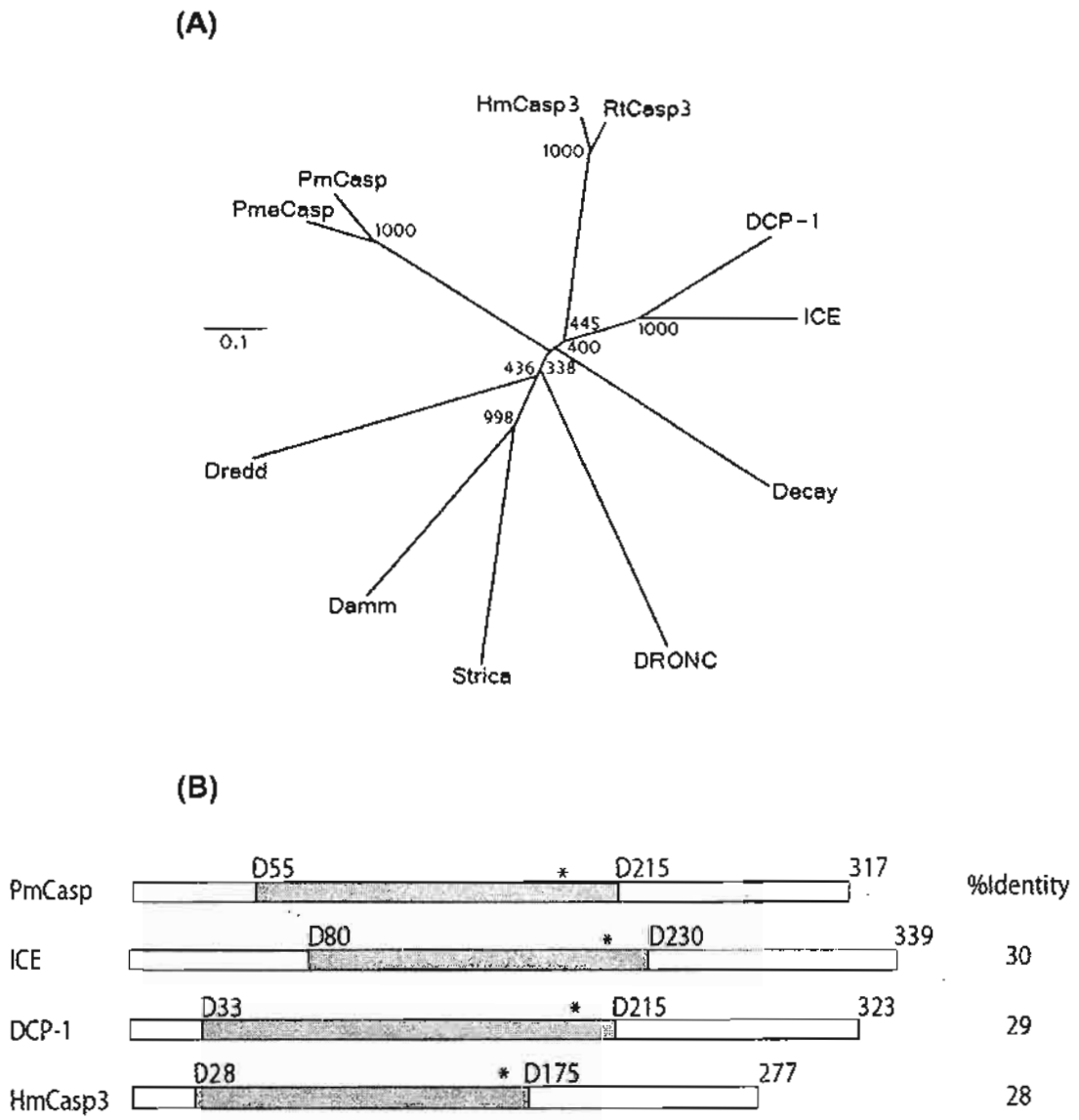


Figure 2

(A) Phylogenetic tree showing the relationship amongst caspase amino acid sequences aligned using ClustalW. Protein sequences from the GenBank database were PmCasp, *Penaeus monodon* (**DQ846887**), PmeCasp, *P. merguiensis* (**AY839873**), HmCasp3, *Homo sapiens* (**U26943**), RtCasp3, *Rattus norvegicus* (NM_012922), DCP-1, ICE, Decay, DRONC, Strica, Damm and Dredd, all from *Drosophila melanogaster* (**AAB58237**, **O01382**, **AF130469**, **AF104357**, **AF242735**, **AF240763** and **Q8IRY7**, respectively). The bootstrap values for 1000 independent comparisons are added at each branch point

(B) Putative structure of *Pm*Casp compared to known caspase sequences. Potential aspartate cleavage sites between large and small units are indicated, where numbers represent positions in the protein. Length of amino acid sequence is indicated on the right end of boxes. The position of pentapeptide sequence QACR/QG is shown by an asterisk. % identity of *Pm*Casp to other sequences is indicated on the right margin.

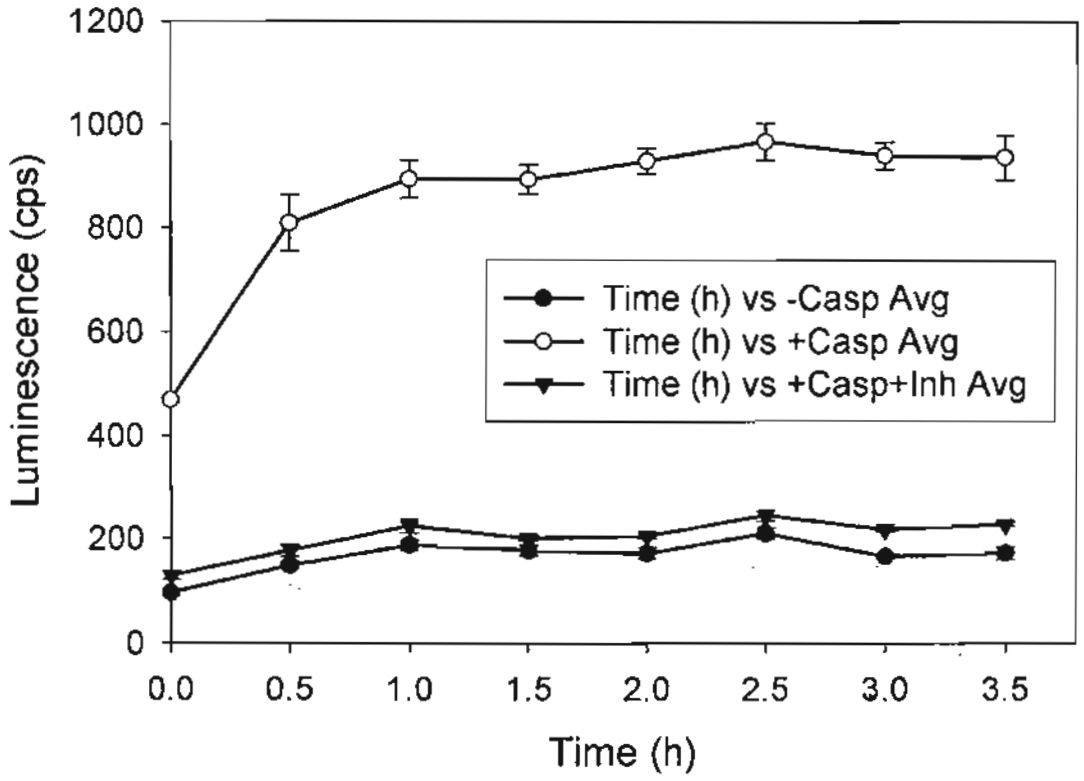


Figure 3. Caspase activity assay.

The substrate for caspase-3 and -7 (Caspase Glo3/7) was incubated in a reaction in the presence (+Casp) or absence (-Casp) of 350 ng purified recombinant *Pm*Casp-3 protein with (+ Inh) or without 5 uM of the specific caspase -3 inhibitor, DEVD-CHO. Caspase activity was measured every 30 min as luminescence (CPS, count per second), and are displayed as mean ± SD of three replicates.

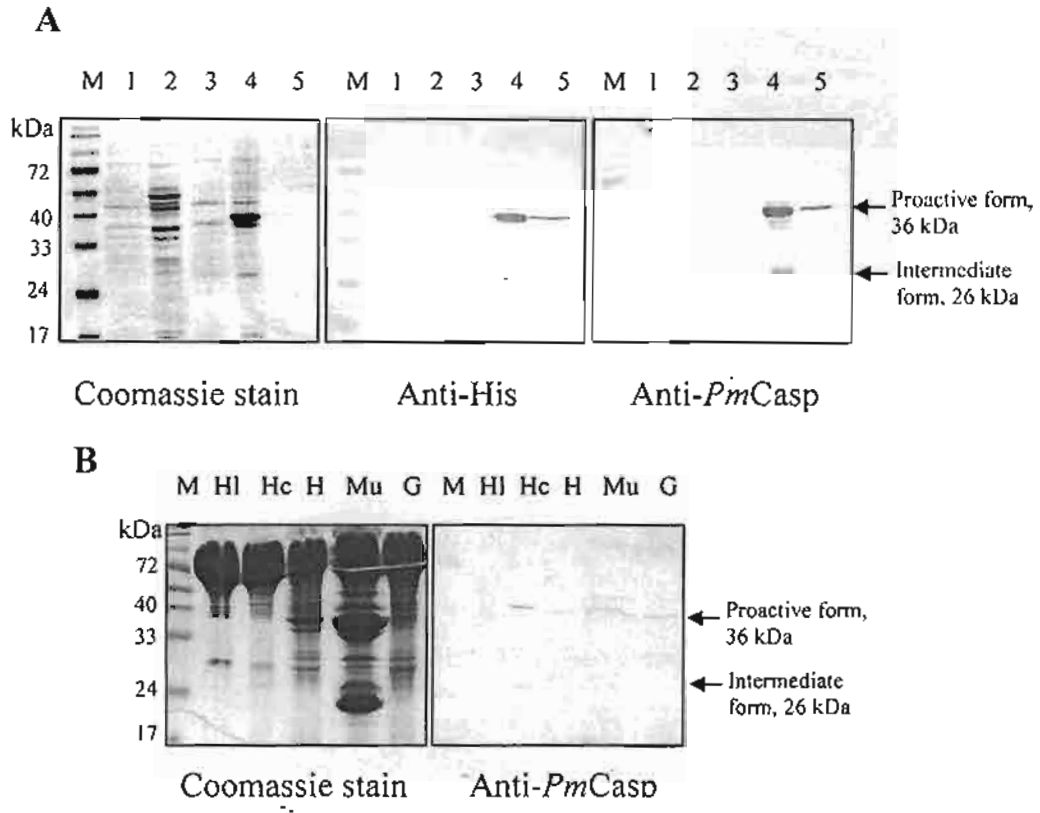
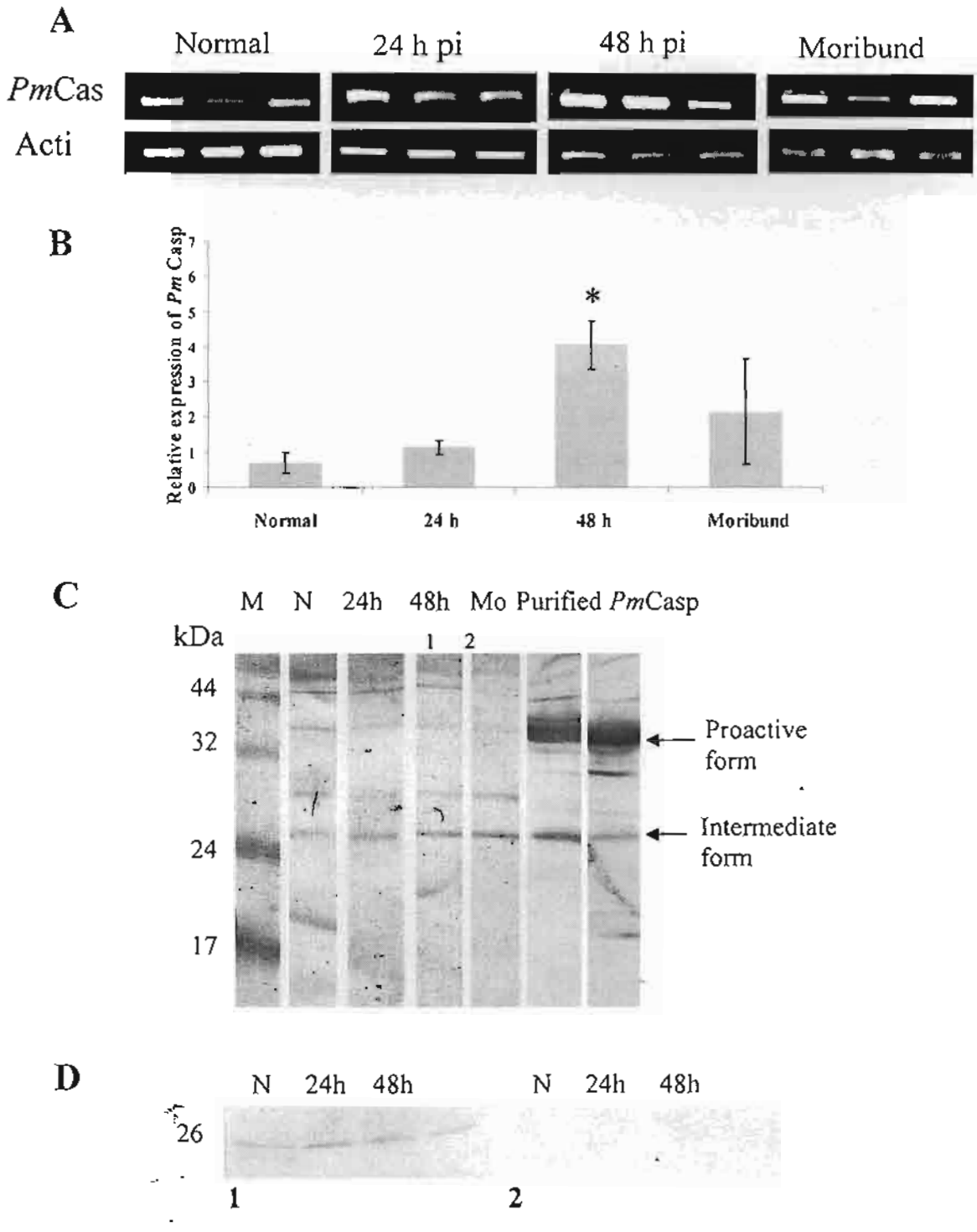


Figure 4. Expression of *Pm*Casp.

(A) Expression and purification of recombinant His6-*Pm*Casp protein from *E. coli*. Lanes: M, protein molecular weight markers; 1, cell lysate of the transformed *E. coli* with pET15b not induced with IPTG; 2, cell lysate of the transformed *E. coli* with pET15b induced with IPTG; 3, pET15b-*Pm*Casp transformed cells not induced with IPTG;pET15b-*Pm*Casp transformed cells induced with IPTG; 5, purified His6-*Pm*Casp protein. An expected band size of recombinant *Pm*Casp is indicated. Samples were subjected to SDS-PAGE and visualized by Coomassie brilliant blue staining and analysed by immunoblotting using anti-His or anti-*Pm*Casp antibody.

(B) Detection of *Pm*Casp protein in 25-30 µg total protein derived from extracts of different tissues of *P. monodon*. Immunoblot of a SDS-PAGE was performed using anti-*Pm*Casp antibody. Lanes: Hl, hemolymph; Hc, hemocytes; H, heart; Mu, muscle; G, gills. Expected band sizes of pro-active form and partially proteolytic intermediate of *Pm*Casp protein are indicated.


A
Normal
24 h pi
48 h pi
Moribund
PmCas
Acti
B
Relative expression of Pm Casp
0
1
2
3
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7
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Normal
24 h
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Moribund
C
M N 24h 48h Mo Purified PmCasp
kDa
1 2
44
32
24
17
Proactive form
Intermediate form
D
N 24h 48h N 24h 48h
26
1
2

Figure 5

Figure 5. Transcriptional and translational expression of *Pm*Casp from gills of WSSV-infected shrimp.

(A) Representative gel of RT-PCR analysis of the *Pm*Casp and β-actin mRNA of the control and WSSV-infected shrimp at 24, 48 h pi and when moribund.

(B) The ratios between the densitometry values of transcript levels of *Pm*Casp and β-actin genes derived from RT-PCR for control and WSSV-infected shrimp at 24, 48 h pi and when moribund are expressed as the mean ± S.D., N=3 each. * indicates the value statistically different from the control at $p<0.05$.

(C) The expression of *Pm*Casp protein in gills of control (N), and WSSV-infected shrimp at 24, 48 h pi and when moribund (Mo), displayed as western blots probed with anti-*Pm*Casp. Purified *Pm*Casp protein was probed with (1) anti-*Pm*Casp and (2) anti-human caspase-3. Expected band sizes of pro-active form and partially proteolytic intermediate of *Pm*Casp protein are indicated.

(D) The expression of *Pm*Casp protein in gills of control and WSSV infected shrimp at 24, 48 h pi and when moribund, analyzed on western blot and probed with (1) anti-*Pm*Casp and (2) preabsorbed anti-*Pm*Casp antibody (1:1000 dilution).

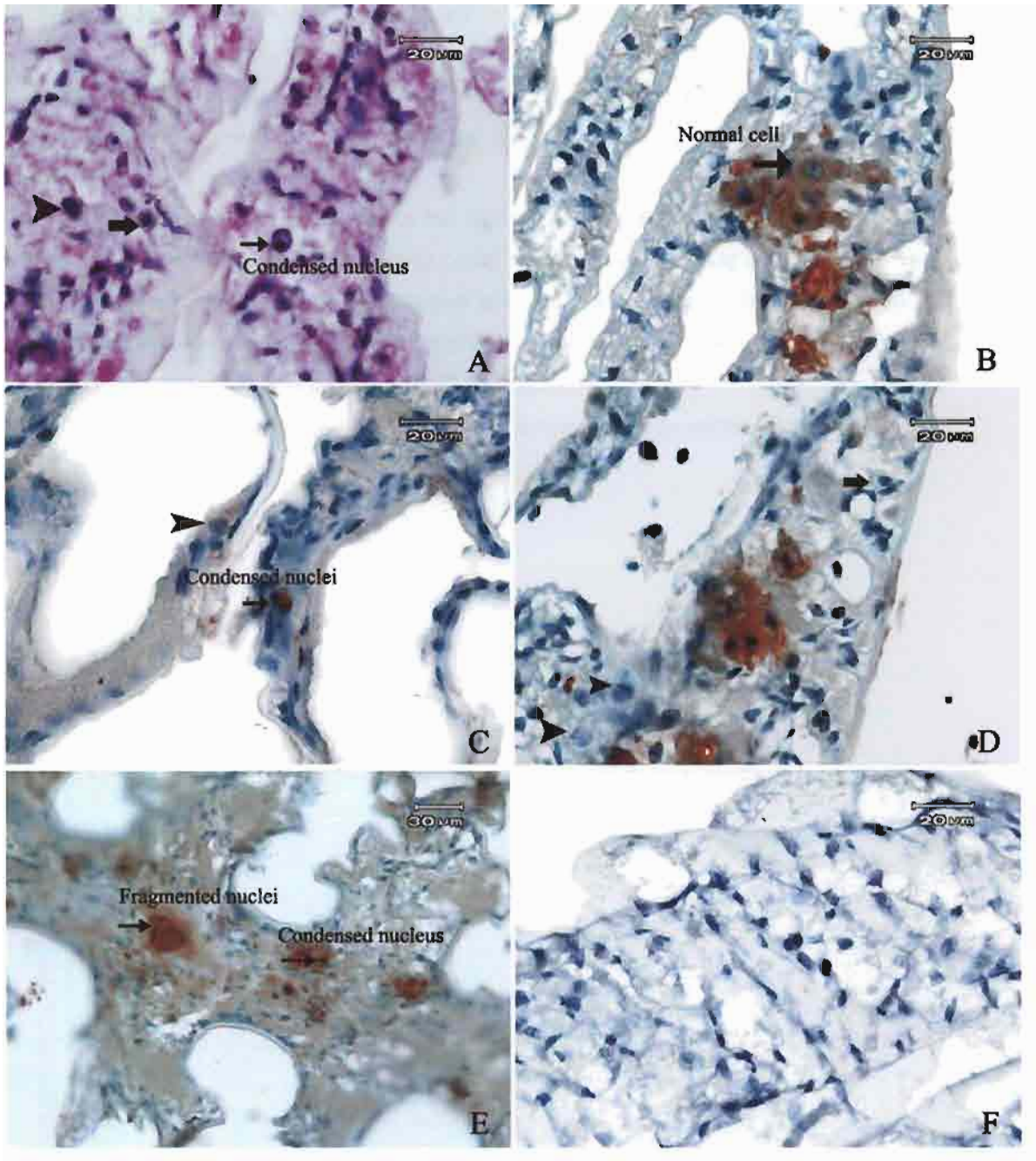
20 µm
Condensed nucleus
A
20 µm
Normal cell
B
20 µm
Condensed nuclei
C
20 µm
D
30 µm
Fragmented nuclei
Condensed nucleus
E
20 µm
F

Figure 6.

Figure 6. Localization of *Pm*Casp in gills of WSSV-infected shrimp.

(A) A typical H&E stained section from gills of WSSV-infected shrimp at 24 h pi. showing cells with nuclear enlargement (arrow head) and cells with intranuclear inclusion (arrow), characteristic phenotypes of WSSV infection. Cell with condensed nucleus is also shown. (B-E) Shrimp gill sections immunohistologically stained for *Pm*Casp with HRP-AEC (Zymed) development (red color).

(B) *Pm*Casp immunoreactivity in gills of normal shrimp at 24 h pi. The imunoreactivity is localized in cytoplasm of normal cells.

(C-D) *Pm*Casp immunoreactivity in gills of the WSSV-infected shrimp at 24 h pi. The immunoreactivity is localized in apoptotic cells with condensed nuclei (C) and normal-looking cells (D). The virus infected cells, the cells with enlarged nuclei (arrow head) and intranuclear inclusion (arrow) are shown.

(E) *Pm*Casp immunoreactivity in gills of the WSSV-infected shrimp at 48 h pi. The immunoreactivity is localized in apoptotic cells with condensed and fragmented nuclei.

(F) No positive immunoreactvity was detected in the primary antibody-omitted (control) sections.

Discussion

Many studies have been devoted to the cloning, expression, purification, and characterization of recombinant human and *Drosophila* caspases, whilst their central conserved role in apoptosis, is driving research in other organisms. In contrast, in shrimp there is little understanding of the cellular, molecular or genetic basis of the apoptotic process. Indeed, to date only one caspase gene in *P. merguiensis* has been identified (Phongdara et al., 2006). A high level of caspase-3 expression was correlated with shrimp mortality in *P. merguiensis*, supporting the link between caspases and apoptosis in shrimp. However, prior to this report no caspases have been established or made available for further work in any other shrimp species, including *P. monodon*, a commercially important species and model host for viral pathogens. Herein we report the cloning, and development of diagnostic antisera and PCR primers to allow further studies, to a caspase-3 homologue in *P. monodon*. This represents the first identification and characterization of a member of the caspase family of proteases, together with the success in development of anti-caspase for detection of the caspase associated with the initiation of apoptosis in *P. monodon*. The *P. monodon* caspase (*Pm*Casp) cDNA derived sequence shows a predicted ORF of 317 amino acid residues, molecular weight of approximately 36 kDa and a pI of 5.6 and includes the typical well conserved caspase family signature and caspase family cysteine active site motifs. Importantly, the pentapeptide active-site motif QACRG is also present. Sequence comparison to caspases from a well-studied arthropod model, *Drosophila melanogaster* revealed similarity to ICE and DCP-1 proteins. They are short N-terminal prodomain-containing caspases which have been suggested to act as effector caspases such as mammalian caspase-3 and -7 (Fraser and Evan, 1997; Song et al., 1997). Interestingly, this study demonstrated that *Pm*Casp has substrate specificity and inhibition characteristics similar to caspase-3. Therefore, the sequence similarity together with the activity assay suggest that *Pm*Casp maybe a member of the caspase-3 subfamily. Potential cleavage sites of *Pm*Casp were proposed to be at Asp55 and Asp215, yielding an N-terminal prodomain, large and small subunits of 55, 160, and 102 amino acids, respectively. It has been proposed that caspase-3 can be cleaved at several alternative sites to become activated (Wang et al., 1996), and therefore both experimental confirmation of he indicated putative cleavage sites as well as evaluation of other potential sites is required and in particular with reference to the active form(s) and specific roles of *Pm*Casp. Certainly, alternative aspartate residues that, with respect to other caspase sequences, are more conserved as well as other less conserved aspartic residues are present in the *Pm*Casp sequence. When

analyzed by western blot, anti-*Pm*Casp antibody reacted principally to bands with molecular weight of ca 36 kDa and 26 kDa of the protein lysates of i) induced *E. coli* expressing pET15b-*Pm*Casp, ii) shrimp hemocyte and gill extracts, and iii) purified fusion *Pm*Casp protein. The 36 kDa band corresponded to the expected molecular weight for *Pm*Casp whereas the 26 kDa protein band potentially represented its proteolytic intermediate form. In most cases, proactive form of caspases are activated by two successive proteolytic cleavages; first between the large and small subunits, and then between the large subunit and the prodomain (Cohen, 1997; Wolf and Green, 1999; Shi, 2002). Therefore, it is likely that the 26 kDa protein fragment was a partially processed *Pm*Casp intermediate and composed of the prodomain and large subunit. This evidence is supported by the finding that anti-His antibody reacted with the 26 kDa band in the lysate of *E. coli* expressing the recombinant *Pm*Casp, since a His moiety was tagged at the N-terminal end upstream of the prodomain. Even though an active caspase requires a heterotetramer consisting of each two small and two large subunits, the prodomain of caspase is frequently not removed during the activation process (Denault and Salvesen, 2002). Like in this study, the partially processed intermediate of *Pm*Casp also functions as shown in activity assay, although that notion would require as a condition that our anti-*Pm*Casp antisera cannot detect the small subunit under these conditions to explain the absence of the 12 kd fragment. The occasional weaker detection of smaller bands, around 30 kDa (Fig. 4, lane 4 and Fig. 5C-1 and may represent partial non-specific proteolytic fragments or non-specific binding but in any case when present were always minor components. Although *Pm*Casp protein showed less than 30% amino acid sequence identity to human caspase-3 sequence, it can cross react with the anti-human caspase-3 anti-sera. This finding suggests that some parts of the amino acids sequences of *Pm*Casp are conserved with those of human and are reacted with the anti-human caspase-3 antibody. The distribution of procaspase in various tissues of the normal shrimp included hemocyte, heart, muscle and gills suggesting that this enzyme is present at basal level in most cells as latent proenzyme and the expression might correlate with maturation and apoptosis in some cell tissues (Krajewska et al., 1997). A strong 26 kDa band was observed in hemocyte and the lightly higher expression of *Pm*Casp in hemocytes suggested that caspase-3 like *Pm*Casp might play an important role in the regulation of apoptosis in the immune system since hemocytes are responsible for shrimp defense mechanisms (Johansson et al., 2000; van de Braak, 2002). The result that the signals of 26 kDa bands at 48 h pi and at moribund were relatively high when compare to that of the control suggested that the *Pm*Casp protein was more active at the later stage of infection.

The localization of positive immunoreactivity of anti-*Pm*Casp antibody within apoptotic cells of gills of WSSV-infected shrimp supported the reactivity of the antibody to the active *Pm*Casp enzyme.The level of *Pm*Casp transcript in gills of WSSV-infected shrimp at 24 h pi was not significantly different from that of the control. In this regard, it is possible that induction of apoptosis in WSSV-infected shrimp in the early infection period (0-24 h post infection) is the result from the cleavage of pro-caspase zymogen granules that stored in the cytoplasm. Therefore in early infection period, only a fraction of the active enzyme is needed to be function to effect cell death. The *Pm*Casp transcript levels are gradually increased and the significant level was detected at 48 h pi indicating that proportionately more caspase may be required to enable effective execution of the cells, coinciding with an increased level of proteolytic intermediate caspase as shown in western blot analysis. Furthermore, the increase in transcript level of *Pm*Casp was accompanied by the high numbers of apoptotic cells in WSSV-infected shrimp as time passed (Wongprasert et al., 2003). The expression of caspase declined at moribund may be due to the progressive break-down of DNA (Nagata et al., 2003) and RNA (Bushell et al., 2004) during the degrading phase of apoptosis or necrosis. The occurrence of an increase in expression of caspase gene and protein in WSSV-infected shrimp suggested that WSSV induces activation of caspase which involved in apoptotic cell death of the shrimp. The relationship between apoptosis and viral disease in shrimp has remained largely unknown until now. If apoptosis causes death in viral infected shrimp, then strategies to protect cells from apoptosis or early induction upon infection prior to viral replication may be very useful to prevent either the loss of shrimp when infected with the viruses in the cultures, or to prevent or restrict infections.

Part II:

The study of the apoptosis and expression caspase-3 in WSSV-infected mud crab *Scylla serrata*

Experimental animal

The mud crab *Scylla serrata were* purchased from a commercial farm and reared at Samutsongkram province, Thailand. The crabs were stocked in the tanks with 5 cm seawater (30 ppt) with adequate aeration to ensure a level of dissolved oxygen above 5 ppm and bricks to create land structure for the animals. The crabs were fed with commercial feed pellets at 3% BW daily and allowed to acclimate for 7 days before experiments begun. The crabs were divided into 2 groups, 70 each. In the first (control) group, each crab was injected with 0.1ml lobster hemolymph buffer (LHB) while each crab in the second (infected) group was injected intramuscularly with 0.1 ml crude WSSV extract in LHB (the same lot as used with the shrimp). After the inoculation, 5 crabs from each group were randomly sampled at 24, 48 and 65 h post-injection (pi). Gills were dissected from the live crab and kept in liquid nitrogen for semi-quantitative RT-PCR and western blot analysis. Some crab were perfused and fixed in Davidson's fixative for paraffin sectioning. Forty animals in each group were reared in separate tanks for determination of mortality.

Since it has not been reported that WSSV induce apoptosis in mud crab, therefore TUNEL method was the first approach used for apoptosis detection in this crustacean.

Light microscopic study of tissues specially stained for fragmented DNA of apoptotic cells in mud crab (TUNEL ASSAY)

The TdT-mediated dUTP nicked-end labeling assay or TUNEL assay is a specific staining technique for such DNA fragments that can be done with paraffin-embedded tissue sections. The DeadEnd Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown visualized under light microscopy.

Live crabs are immediately fixed by injecting Davidson's fixative into the body parts, followed by immersion in the fixative for 72 h. Then the gills were dissected out and placed

in embedding cassettes. The tissues are processed through an automatic tissue processor and were sectioned at 3-5 um thickness, in serial sections, on poly-L-lysine coated microscope slides. TUNEL assay kit (Promega) is used and the processes are performed following the manufacturer's protocol. Positive and negative control sections are included to avoid false-positive and false-negative results. Tissue treated with 100 ul of DNase I (20ug/ml) to digest nuclear DNA into fragments was used as the positive control. Negative controls consist of identically treated tissue without the enzyme for the TdT reaction. The hallmark of apoptosis, condensed and fragmented nuclei were observed under light microscope

Expression of *PmCasp* in WSSV-infected mud crab *Scylla serrata* by semi-quantitative RT-PCR

Because we have primers and antibody of the *Pmcasp* so we wanted to check whether the primers could amplify the caspase-3 and the anti-*Pmcasp* antibody could cross react with those of the mud crab.

RNA extraction

Total RNA was extracted from gills of WSSV-infected and control crab and processed for RT-PCR to determine *Pm*Casp level, using β-actin gene as an internal control. Primers used were identical with those used to determine the expression of Casp in shrimp. The method of semi-quantitative RT-PCR were performed as the same as those done with the shrimp. The data were analyzed from three individuals at each of three time points post infection (24, 65, and 96 h pi).

Expression of *PmCasp* protein in WSSV-infected shrimp by western blot analysis

Total protein was extracted from aliquots of the same gills as used for RNA analysis above, by homogenization into 100 mM NaCl, 50mM Tris pH 8.0, 1mM PMSF as detailed above. For each sample, 100 µg of the total protein extract was resolved by SDS–PAGE (15% (w/v) acrylamide) and *Pm*Casp protein identified and quantified by western blotting using rabbit anti-*Pm*Casp antibody at a dilution of 1:1000. The blot of crab protein extract was also incubated with anti-cleaved human caspase-3 (Cell signaling technologies) at a dilution of 1:1000. Subsequently, AP-conjugated goat anti-rabbit IgGs (Zymed) at a dilution 1:2000 were used and detection was performed with a substrate solution (NBT/BCIP, Roche). The detection procedure was performed according to the supplier's protocol.

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Results

Mortality of the mud crab after WSSV injection

Observation of the mortality of the crab upon WSSV infection revealed that the crab started to die, under our experimental condition, at 6 day after injection and the mortality rate reached only 10 % at day 8 that was not different from the control (Table 1)

Histopathology and apoptosis detection in gills of the mud crab infected with WSSV

By H&E staining, the gills of the mud crab infected with WSSV showed specific signs of the disease, the inclusion body and enlarged nuclei at 24 h pi (Fig 7C, D) and the number of WSSV infected cells increased progressively at 65 h and 96 h pi (Fig 7E, F). The hallmark features of apoptosis, condensed and fragmented nuclei were not observed in gills of WSSV-infected crab. When the specific tool to detected apoptosis, the DeadEnd colorimetric TUNEL assay was used, the result showed the positive cells in WSSV-infected cells (Fig. 8C, D) and some normal looking cells at 24 h pi and the number of positive cell progressively increased at 65 h (Fig. 8E, F) and 96 h pi (Fig. 8G, H).

Expression of *Pm*Casp in WSSV-infected mud crab

The transcript level of caspase in gills of WSSV-infected crab was determined by semi-quantitative RT-PCR using *Pm*Casp primers. The results demonstrated no expected bands at any time points. By Western blot analysis, the anti-*Pm*Casp antibody was used to immunoblot with the protein extracted from gills of the control and WSSV-infected mud crab. The results showed that the antibody did not react with any protein bands at any time points. However, when anti-human cleaved caspase-3 antibody was used in immunoblotting, the antibody detected the faint positive bands at the expected size of cleaved caspase-3 at every time points (Fig.9).

Table 1: Percentage of cumulative mortality of mud crab after infected with WSSV, N=40 each

Experimental group	% cumulative mortality of WSSV-infected mud crab (day after injection)			
	1-5	6	7	8
Control	-	-	2.5	7.5
WSSV-infected	-	2.5	7.5	10

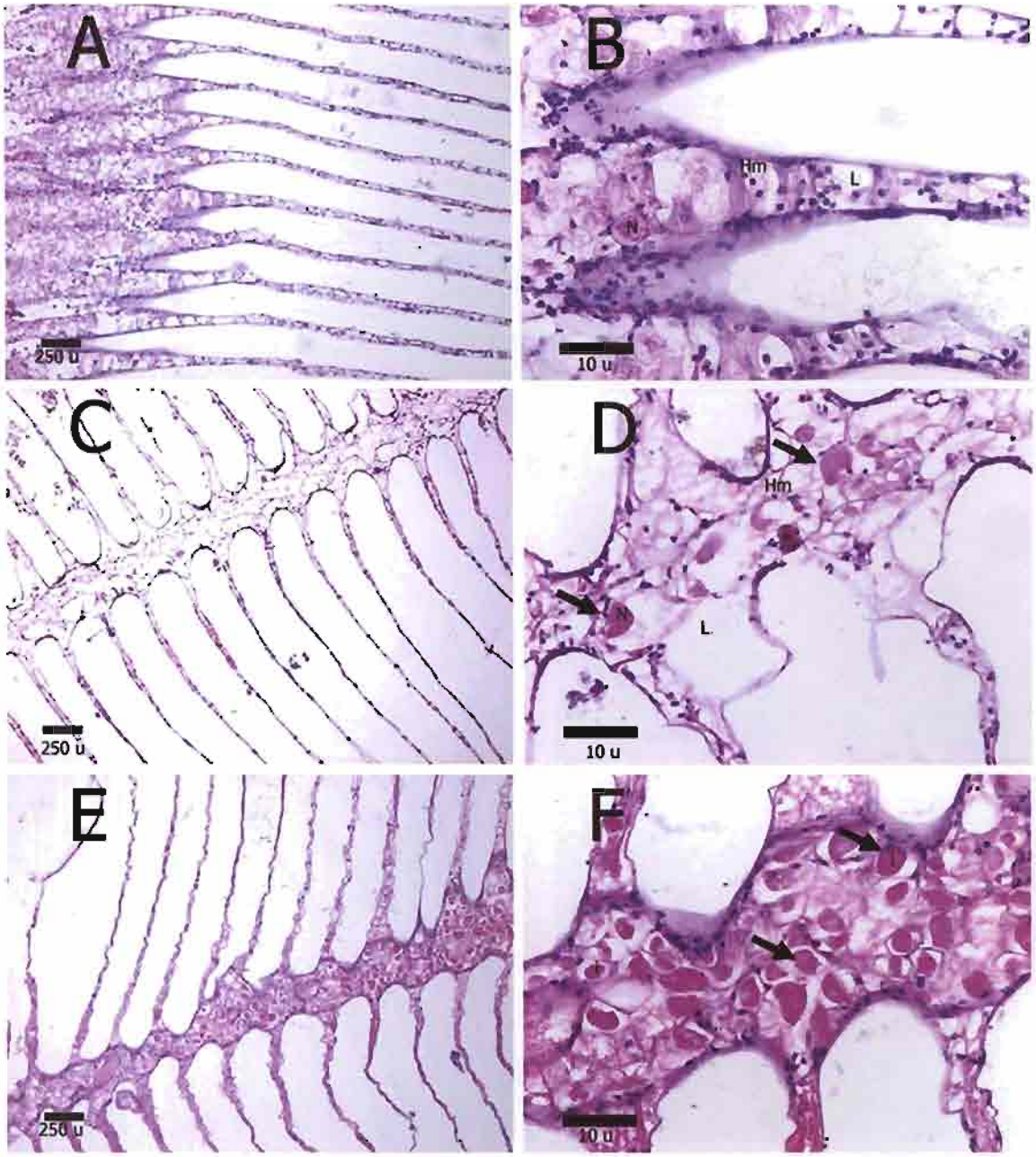

Figure 7. Light micrographs showing the histopathology of gills of WSSV-infected mud crab, stained with H&E

(A), (B) Normal gill showing normal pilar cell (N) and hemocytes (Hm) in lacunae (L)

(C), (E) Low magnification of gills of WSSV-infected mud crab showing sign of WSSV infection, cells with intranuclear inclusion at 24 h, 65 h pi, respectively

(D) Gill of WSSV-infected crab at 24 h pi showing intranuclear inclusion (arrow)

(F) Gill of WSSV-infected crab at 65 h pi showing multiple enlarged nuclei with intranuclear inclusion (arrow)

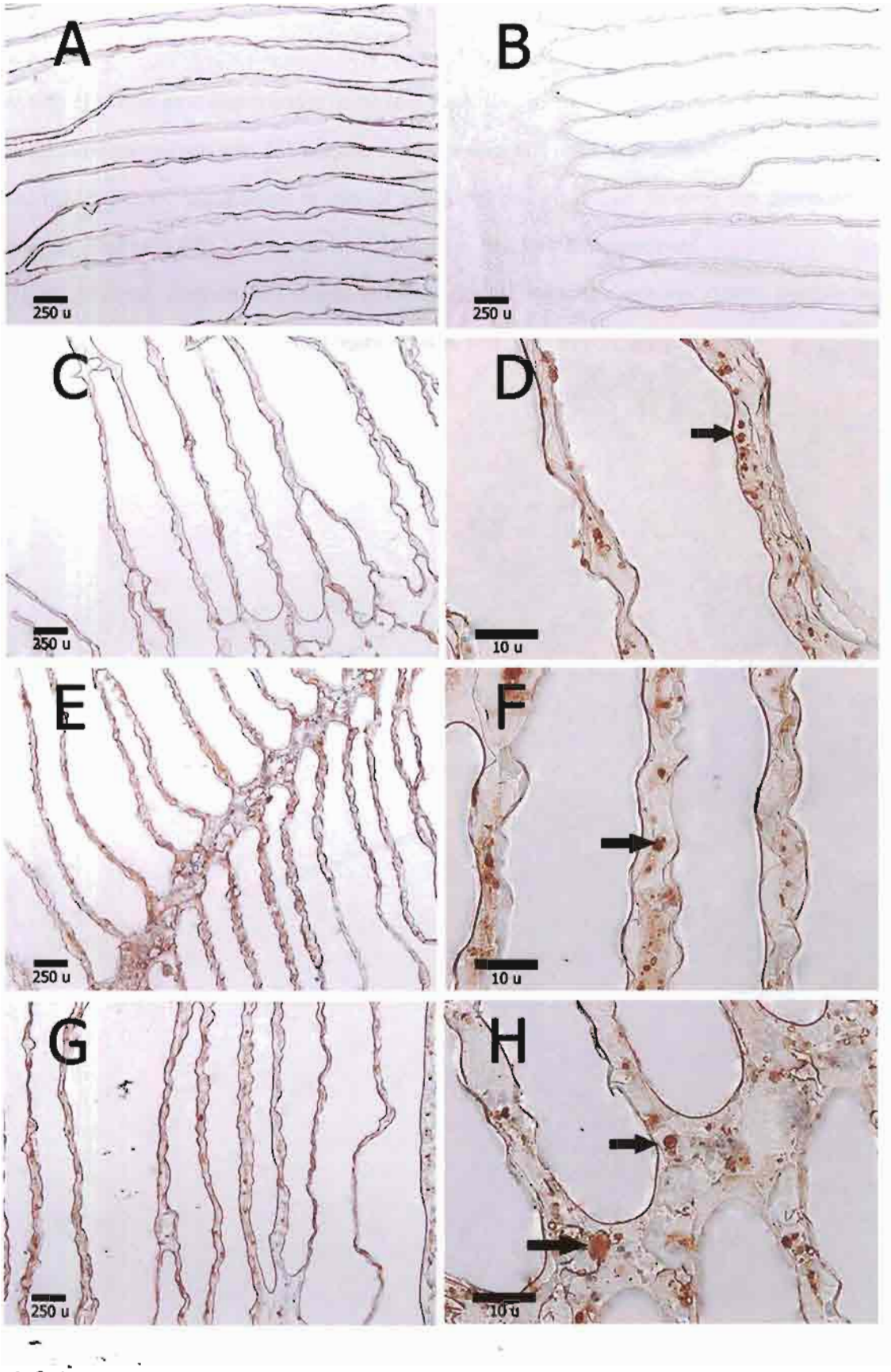

Figure 8.

Figure 8. Light micrographs of TUNEL positive nuclei in gills of WSSV-infected mud crab

(A) Gills of normal mud crab showing no positive nucleus

(B) Negative control gills with TdT enzyme omitted showing no positive nucleus

(C), (E), (G) Low magnification of gills of WSSV-infected mud crab showing the distribution of brownish color of TUNEL positive nuclei at 24 h, 65 h, and 96 h pi, respectively.

(D), (F), (H) High magnification of gills of WSSV-infected mud crab showing TUNEL positive stain (arrow) in WSSV-infected or enlarged nuclei at 24 h, 65 h, and 96 h pi, respectively.

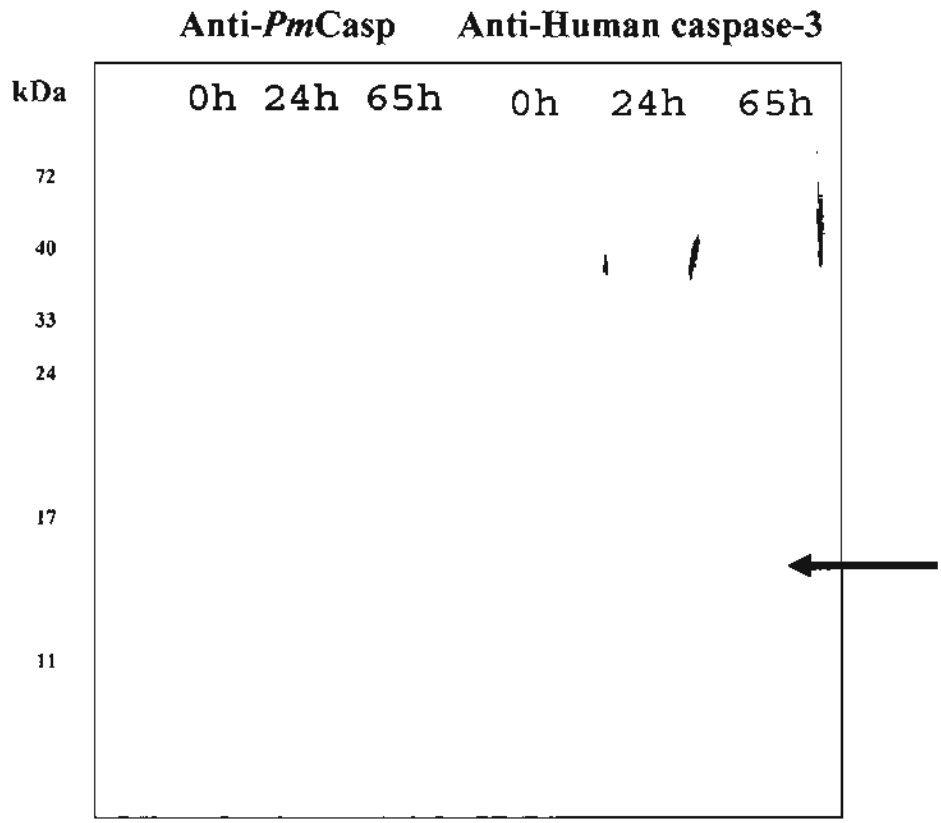

Figure 9. Western blot membrane showing the expression of caspase-3 protein in gills of control (0), and WSSV-infected mud crab at 24, 65 h pi. The blot probed with anti-*Pm*Casp and anti-human cleaved caspase-3 (1:1000 dilutions). No band was detected when anti-*Pm*Casp was used as a primary antibody. Blot probed with anti-human caspase-3 showed expected band at the sizes of cleaved caspase-3 (arrow).

Discussion

Apoptosis is one of the innate immune response against viral infection and has been found in the WSSV- and yellow head virus-infected shrimp. Khanobdee et al. (2002) regarded the widespread and progressive occurrence of apoptosis in *P. monodon* infected with yellow head virus (YHV) as a major cause of dysfunction and death of the host. Wu and Muroga (2004) revealed that high mortality of WSSV infection in *P. japonicus* was accompanied by a high incidence of apoptosis. Wongprasert et al. (2003) revealed that the number of apoptotic cells in WSSV infected shrimp seemed to rapidly increase in the beginning and slightly plateau until the shrimp died. Cells displaying nuclear condensation and fragmentation characteristic of apoptosis did not contain WSSV virions while those containing WSSV virions were not apoptotic. All of these report suggested that apoptosis cause death of shrimp. Therefore, the assumption that apoptosis might be implicated in shrimp death during viral infection seems possible, as previously proposed by Flegel and Pasharawipas (Flegel and Pasharawipas, 1998).

Interestingly, the mud crab (*Scylla serata*), one of the reservoir of the virus, can live with the virus with no mortality (Supamattaya et al., 1998). This phenomenon indicates that high levels of viral particles in the body do not quarantine death. Therefore the viral-host interaction between WSSV and the mud crab *S. serata* is deserved for an investigation. So far it has not been known whether apoptosis occurs in host reservoir like the mud crab and how they tolerate WSSV infection. This study aimed to investigate if apoptosis occur in crab experimentally infected with WSSV and whether it is related with the tolerance to the WSSV infection of the crab.

This study demonstrated that the gill tissue of mud crab histologically showed the sign of the virus infection that increased in severity with the time progressed. Interestingly, even though the hallmark features of apoptosis, condensed and fragmented nuclei were not found under light microscopy and H&E stain but the positive apoptotic cells were observed when the gills were processed for TUNEL assay. Moreover, it was shown that the TUNEL positive stained the cells with enlarged nuclei or cell with intranuclear inclusion and also some normal looking cells. With the sign of heavy infection the crab were still alive during the period of study 65 h and only 10% of the animals died at 8 day pi. The results suggested that WSSV induce apoptosis in crab and apoptosis mostly occurred in WSSV-infected cells but apoptosis did not cause death of the crab during 65 h pi observation. This phenomenon is contrast with those in WSSV-infected shrimp in which the apoptosis occur in the bystander cell not in the infected cell and apoptosis is the way the shrimp protect the

normal cell from the WSSV infection (Wongprasert et al., 2003). For the crab, it might be possible that apoptosis is induced by the animal to eliminate virus infected cells but there are some signals to inhibit the cells to undergo condensed or fragmented nuclei. For example, virus may express proteins that block the death response or apoptosis (Everette, 1999).

The study of caspase expression in WSSV-infected crab using *Pm*Casp primers and anti- *Pm*Casp antibody were not successful that may suggest the primers and the antibody used were not specific to the crab caspase. And the result that the anti-cleaved human caspase-3 could react with the crab protein extract at an expected band of cleaved caspase suggested that some parts of the sequence of crab caspase are conserved with those of human.

This study is the preliminary study of the study of apoptosis in WSSV-infected mud crab *Scylla serrata*. The study suggested apoptosis occurred in WSSV-infected crab and not cause death of the crab. Apoptosis play different role in different species of crustacean particularly the shrimp and the crab infected with WSSV. Whether apoptosis play role in the virus persistant in the mud crab *Scylla serrata* has to be further investigated.

Output ที่ได้จากโครงการ

The recent study suggests the different role of apoptosis in the different species of crustacean particularly shrimp *P.monodon* and mud crab *Scylla serrata*. The full length sequence of caspase-3 of *P.monodon* has been identified and the primers and the antibody developed are very useful as markers of the study of apoptosis in shrimp. Knowledge that caspase-3 is up-regulated progressively with the time after injection correlated with the death of shrimp help to develop strategies to prevent shrimp death after viral infections; for example using caspase inhibitor or silencing of caspase-3. Moreover, the study on apoptosis in WSSV-tolerated mud crab will help to understand the host-virus interaction and also the way to prevent death in virus persistant species.

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ภาคผนวก

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Cloning and characterization of a caspase gene from black tiger shrimp (*Penaeus monodon*) infected with white spot syndrome virus (WSSV)

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Abstract

A black tiger shrimp (*Penaeus monodon*) caspase cDNA homologue (*Pm*Casp) has been identified from a hemocyte library using a previously identified caspase homologue from the banana shrimp (*Penaeus merguiensis*) as a probe. The full-length *Pm*Casp was 1202 bp with a 954-bp open reading frame, encoding 317 amino acids. The deduced protein contained a potential active site (QACRG pentapeptide) conserved in most caspases. It had 83% identity with caspase of *P. merguiensis* and 30% identity with drICE protein of *Drosophila melanogaster*, and it exhibited caspase-3 activity in vitro. *Pm*Casp was cloned and expressed in *Escherichia coli* and a rabbit polyclonal antiserum was produced. In Western blots, the antiserum reacted with purified recombinant *Pm*Casp and with lysates of *E. coli* containing the expressed plasmid. In crude protein extracts from normal shrimp, the anti-serum reacted with 36-kDa and 26-kDa bands likely to correspond to inactive procaspase and its proteolytic

intermediate form, respectively. *Pm*Casp expression was measured in normal shrimp and in white spot syndrome virus (WSSV)-infected shrimp at 24 and 48 h post injection (pi) by semi-quantitative RT-PCR, Western blot analysis, and immunohistochemistry. Semi-quantitative RT-PCR analysis revealed up-regulation of *Pm*Casp at 48 h pi and expression remained high up to the moribund state. These results were supported by Western blot analysis showing increased *Pm*Casp protein levels at 24 h and 48 h pi when compared to normal control shrimp. Immunohistochemical analysis of gills from the WSSV-infected shrimp revealed immunoreactivity localized in the cytoplasm of both normal and apparently apoptotic cells. In summary, a caspase-3 like gene is conserved in *P. monodon* and is up-regulated after WSSV infection.

Key words: apoptosis, caspase, *Penaeus monodon*, shrimp, WSSV, ICE

Footnote: The GenBank accession no. for the *Penaeus monodon* caspase (*Pm*Casp) cDNA sequence reported in this paper is **DQ846887**.

1. Introduction

Apoptosis or programmed cell death is involved both in embryonic development and in defense against viral pathogens. It is also responsible for inducing cell death from a wide variety of signals such as UV, toxins, hormones, serum growth factor deprivation, chemotherapeutic agents, ionizing radiation and autoimmune disorders (Tomei et al., 1993). The process is evolutionarily conserved across animal taxa. Most of the morphological changes resulting from apoptosis are caused by activation of enzymes in the caspase family. Currently, more than a dozen caspase family members have been discovered and classified relative to their involvement in inflammation or programmed cell death (see reviews by Cohen, 1997; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). Some upstream caspases hierarchically activate others in a proteolytic cascade that creates an amplification circuit. Activation of downstream executioner caspases (caspase-3, -6, and -7) leads to proteolytic cleavage and concomitant inactivation or activation of their specific cellular protein targets to yield the physiological and morphological changes characteristic of apoptosis (Grutter, 2000). For example, cleavage of nuclear lamin is required for nuclear shrinking and budding, and cleavage of the cytoskeleton proteins fodrin and gelsolin causes the loss of overall cell shape (Hengartner, 2000).

Based on morphological and biochemical analysis, it has been suggested that viral infections may induce apoptosis in shrimp. Khanobdee et al. (2002) regarded the widespread and progressive occurrence of apoptosis in *P. monodon* infected with yellow head virus (YHV) as a major cause of dysfunction and death of the host. Wu and Muroga (2004) revealed that high mortality of WSSV infection in *P. japonicus* was accompanied by a high incidence of apoptosis. These results supported the earlier proposal by Flegel and Pasharawipas (1998) that apoptosis might be implicated in shrimp death as a result of viral infection. On the other hand, Wongprasert et al. (2003) reported that cells displaying nuclear condensation and fragmentation characteristic of apoptosis did not contain WSSV virions while those containing WSSV virions were not apoptotic. They also presented preliminary results suggesting that caspase-3 activity increased in WSSV-infected shrimp.

To confirm whether or not caspase-3 activity increases in WSSV-infected shrimp, we decided to follow its time-course expression in laboratory challenge tests with the black tiger shrimp *Penaeus monodon*. To do this, we first identified the caspase-3 gene in shrimp using the sequence of a recently described caspase-3 from the banana shrimp *Penaeus merguiensis* (Phongdara et al., 2006) as a probe. We then cloned, sequenced and expressed the gene and confirmed its activity before developing immunochemical and molecular methods to follow its expression during the course of WSSV infection.

2. Materials and methods

2.1. General techniques

Escherichia coli XL1-Blue and BL21 pLysS (DE3) were used for general cloning and protein expression, respectively. DNA manipulation and microbiological methods were performed according to established protocols (Sambrook and Russell, 2001).

2.2. Plaque hybridization assay

Plaque hybridization assay was performed to screen for *PmCasp* from a *P. monodon* hemocyte cDNA library (Tonganunt et al., 2005) using a putative caspase-3 cDNA from *P. merguiensis* (*PmeCasp*) as a probe. Using a DIG DNA Labeling Kit (Roche), *PmeCasp* was amplified and PCR-labeled with primers PmeF (5'-TGT CTA TGT TGG CGG AAC CAA-3') and PmeR (5'-TCA CCT GCT GAC TTT GAG AAG-3') designed from the reported cDNA sequence (GenBank accession no. AY839873) and with a plasmid containing *PmeCasp* as template (Phongdara et al., 2006). Plaques (2×10^4) from the *P. monodon* cDNA library were transferred onto nitrocellulose membranes and subjected to hybridization using the DIG-labeled

PmeCasp probe. Hybridization was performed overnight at 60°C followed by a series of washes according to the Roche protocol. The membrane was then incubated with an anti-DIG antibody conjugated to alkaline phosphatase (Roche) at room temperature for 1 h. Hybridization signals were visualized using a chromogenic solution containing NBT/BCIP (Roche). Positive clones were excised *in vivo* and cDNA library plasmids were sequenced by Macrogen Co. Ltd. (South Korea).

2.3. RNA preparation

Total RNA was prepared from shrimp gills using Trizol reagent (Invitrogen) as described by the manufacturer. Briefly, 50-100 mg of gills was homogenized in 1 ml of Trizol reagent. The homogenate was treated with 200 µl of chloroform and centrifuged for 15 min at 10000 xg to recover the aqueous phase. The RNA was precipitated by adding 1 volume of chilled isopropanol, before being washed with 75% (v/v) ethanol, dried and resuspended in diethyl pyrocarbonate (DEPC)-treated water. RNA quality and quantity were measured by spectrophotometric analysis using absorbance at 260 and 280 nm.

2. 4. Purification of PmCasp fusion protein in E. coli and antibody production

PmCasp was amplified by PCR using the identified cDNA plasmid as a template and *PmCasp* specific primers (see Fig.1) (PmF; 5'-**GGA ATT C**<u>**CA TAT G**</u>AG CGA CGC CGA CGA CTC-3' and PmR; 5'-**CGC** <u>**GGA TCC**</u> TCA GAA GTA AAT **TTC ACG AAG-3'** containing flanking non-complementary sequences (bold type) so that desired restriction sites (underlined) would be included in amplicons. PCR reactions of 50 µl consisted of 100 ng of DNA template, 0.2 µM of each primer, 5 unit of Taq DNA polymerase (Invitrogen), and 1X PCR DIG labeling Mix (Roche) in 1X PCR reaction buffer. The mixture was denatured at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. To generate a pET15b-*Pm*Casp plasmid, this 960-bp *Nde*I-*Bam*HI amplicon containing the complete *PmCasp* cDNA was inserted in-frame with the same enzymes that digested pET15b (Novagen), so that a coding region for an N-terminal His_6 tag was fused to the *PmCasp* open reading frame. Correct sequence and in-frame insertion of the insert were verified by DNA sequencing. After transformation and cloning into *E. coli* BL21 pLysS (DE3) cells, His_6-fusion ***Pm*Casp protein** was expressed by induction with 1 mM IPTG. The fusion protein in the bacterial lysates was purified using Ni-NTA beads (Qiagen) and analyzed by SDS-PAGE (12% w/v) plus Western blotting using anti-His antibody (Zymed). A polyclonal antiserum against *Pm*Casp was subsequently raised in rabbits by injection of 100 µg of the purified His_6-*Pm*Casp fusion protein

in complete Freund's adjuvant followed by four booster injections in incomplete adjuvant at one-week intervals. Serum was collected from the rabbits 1 week after the last boost.

2.5. Caspase activity assay

Caspase activity of the His_6-*Pm*Casp fusion protein was detected using Caspase-Glo 3/7 Assay (Promega) essentially as previously described (Phongdara et al., 2006), except for minor modifications. Briefly, His_6-*Pm*Casp was plated into 96-well plates for assays (350 ng in 200 µl per well) and luminescent signals were measured every 30 min within a total period of 3.5 h using a Wallac Victor 2 Multi-label Counter (PerkinElmer). Inhibition of caspase-3 activity was achieved using caspase-3 inhibitor (ApoAlert Caspase-3 inhibitor, DEVD-CHO, Clontech) at a final concentration of 5 µM. The results are expressed as the mean ± SD of three replicates.

2.6. Tissue distribution of PmCasp protein by Western blotting

Protein extracts were prepared from ten normal shrimp. Shrimp hemolymph was collected in an equal volume of anticoagulant ACI solution (Soderhall and Smith, 1983) and centrifuged at 800 xg for 15 min to obtain the hemocyte pellet. The hemocyte pellet, heart, muscle, and gill tissues were homogenized in 100 mM NaCl, 50 mM Tris buffer, pH 8.0 containing 1 mM phenylmethanesulfonyl fluoride. The homogenates were centrifuged at 3000 xg for 5 min and the supernatants were collected. Total protein concentrations in each shrimp tissue extract and hemolymph fraction were determined using a Bio-Rad protein assay kit. Approximately equal amounts of proteins (25-30 µg) were subject to SDS-PAGE (15% w/v) and Western blotting using rabbit anti-*Pm*Casp antibody at a dilution of 1:1000. Positive binding was detected using AP-conjugated goat anti-rabbit IgGs (Zymed) with NBT/BCIP substrate solution (Roche) according to the supplier's protocol.

2.7. Sequence analysis

Analysis of DNA and protein sequences was carried out using the EXPASY web server (http://au.expasy.org/). To identify related sequences, a protein-protein basic local alignment search tool (blastp) search was carried out using the NCBI protein database (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of protein sequences were performed and a phenogram was generated by ClustalW [(Thompson et al., 1994), http://www.es.embnet. org/ Doc/ phylodendron/ clustal-form.html], using default settings. An analysis for protein domains was carried out using Conserved Domain Database software (Marchler-Bauer et al., 2005).

168

169 *2.8. Experimental challenge*

170 Juvenile *P. monodon* (Crustacea, Decapoda) of 8 to 12 g body weight (BW) were
171 purchased from local shrimp farms in Chachoengsao province, Thailand and confirmed to be
172 WSSV-, YHV-, MBV-, HPV- and IHHNV-negative using respective diagnostic PCR or RT-
173 PCR kits (Farming IntelliGene Biotechnology Corporation, Taiwan or Shrimp Biotechnology
174 Business Unit, Thailand). Shrimp were maintained at 25°C in tanks containing 50 L of brackish
175 water (10 ppt) with adequate aeration to ensure a level of dissolved oxygen above 5 ppm. The
176 shrimp were fed with commercial feed pellets at 3% BW daily and allowed to acclimate for 5
177 days before experiments began. The shrimp were divided into 2 groups. In the first (control)
178 group, each shrimp was injected with 0.1 ml lobster hemolymph buffer (LHB) Paterson and
179 Stewart, 1974 while each shrimp in the second (infected) group was injected intramuscularly
180 with 0.1 ml crude WSSV extract in LHB. A crude extract of WSSV was prepared from
181 hemolymph of moribund shrimp obtained from a commercial rearing pond experiencing a white
182 spot syndrome virus outbreak. After inoculation, shrimp from each group were randomly
183 sampled at 24 and 48 h post-injection (pi). Gills were dissected from the live shrimp and kept in
184 liquid nitrogen for semi-quantitative RT-PCR and Western blot analysis. Some shrimp were
185 perfused and fixed in Davidson's fixative for paraffin sectioning according to the methods
186 described by Bell and Lightner (1988).

187

188 *2.9. Analysis of PmCasp transcript in WSSV-infected shrimp by semi-quantitative RT-PCR*

189 Total RNA was extracted from gills of WSSV-infected and control shrimp and processed
190 for RT-PCR to determine *Pm*Casp level, using β-actin gene as an internal control. A partial
191 sequence of 825 bp of *Pm*Casp was amplified using specific primers (see Fig. 1) to *Pm*Casp
192 (PmCaspF:- 5'-GGA GGA ACC TGC GAA GAA C-3' and PmCaspR:- 5'-AGC GTC GAG
193 TGG ATG TAA GG-3'). A partial sequence of 377 bp of β-actin gene was amplified as a control
194 using primers actin-F (5'-TGA CGG CCA GGT GAT CAC CA-3') and actin-R (5'-GAA GCA
195 CTT CCT GTG AAC GA-3'). RT-PCR reactions were carried out in a 25 µl reaction solution
196 containing 100 ng of total RNA, 10 mM of each forward and reverse primer, 1 µl of SuperScript
197 One-Step RT/Platinum Taq mix (Invitrogen), and 1X reaction buffer.

198 RT-PCR was performed using the following conditions: 50°C for 30 min and 94°C for 2
199 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min with a final
200 extension at 68°C for 5 min. Confirmation of PCR-amplicons to the assumed corresponding

cDNA (PCR fidelity) was attained by sequencing of the PCR products. Amplified cDNA was resolved by TAE-(1.2% w/v) agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide (2 µg/ml). The fluorescent images were captured and analyzed by densitometer using the Scion Image, release Alpha 4.0.3.2. Transcript level of *Pm*Casp was normalized with that of β-actin obtained from the corresponding total RNA sample. The data were analyzed from three individuals at each of three time points post infection (24 h, 48 h and moribund) by one way analysis of variance (ANOVA) using SPSS for Windows and an alpha level of 0.05.

2.10. Expression of PmCasp protein in WSSV-infected shrimp by Western blot analysis

Total protein was also extracted from aliquots of the same gills as used for the RNA analysis above, by homogenization in 100 mM NaCl, 50mM Tris pH 8.0, 1 mM PMSF as detailed above. For each sample, 100 µg of the total protein extract was resolved by SDS–PAGE (15% (w/v) acrylamide) and *Pm*Casp protein was identified and quantified by Western blotting using rabbit anti-*Pm*Casp antisera at a dilution of 1:1000 or with anti-*Pm*Casp antisera that had been preabsorbed with *Pm*Casp fusion protein (1:1000 dilution). The blot of recombinant *Pm*Casp protein was also incubated with anti-human caspase-3 (Cell signaling technologies) at a dilution of 1:1000. Subsequently, AP-conjugated goat anti-rabbit IgGs (Zymed) at a dilution 1:2000 was used and detection was performed with the substrate solution NBT/BCIP (Roche) according to the supplier's protocol.

2.11. Localization of PmCasp in WSSV-infected tissues

Shrimp were fixed in the Davidson fixative for 24 h and processed for paraffin sectioning. Sections of 7 µm thickness were immunohistochemically stained to localize *Pm*Casp protein following the protocol described in the Histostain-SP kit (Zymed) with slight modifications. Briefly, the sections were first immersed in 3% (v/v) H_2O_2 in absolute methanol for 15 min to block endogenous peroxidase activity, and then washed three times with 0.1 M PBS, pH 7.4 (5 min each). The sections were covered with blocking solution for 1 h and subsequently incubated with the primary antiserum (rabbit anti-*Pm*Casp) at a concentration of 1:1000, at 4°C, overnight, and washed three times with PBS-T (0.1 M PBS, pH 7.4 containing 0.1% (v/v) Tween 20). The sections were incubated with the second antibody (HRP conjugated goat anti-rabbit IgG) at a dilution of 1:2000 for 2 h. The sections were washed three times with PBS-T for 5 min each, and red staining was developed by covering the sections with the

234 substrate AEC. The reaction was terminated in water and the sections were counterstained with
235 Meyer hematoxylin for staining nuclei. To verify specificity of the substance detected, two
236 control staining procedures were carried out: one without primary antiserum and another with
237 *Pm*Casp preabsorbed primary antisera. Hematoxylin and eosin (H&E) staining was carried out to
238 determine histopathological features of the WSSV-infected gills as described by
239 Wongteerasupaya et al. (1995) and Wongprasert et al. (2003).
240
241 **3. Results**
242 *3.1. Sequence analysis of PmCasp*
243 Using a putative caspase-3 from *P. merguiensis* (Phongdara et al., 2006) as a probe to
244 screen a *P. monodon* hemocyte cDNA library by plaque hybridization yielded two positive
245 clones (0.01% of clones screened). DNA sequence analysis revealed that they were identical,
246 yielding a single putative caspase homologue. The inserted cDNA of 1202 bp contained 41
247 nucleotides of 5'-untranslated region (UTR), a 954-nucleotide open reading frame, and a 207-
248 nucleotide 3'-UTR including a potential poly (A) tail and a putative polyadenylation signal
249 (AATAAA) (Fig. 1). The entire 954 bp-open reading frame encoded a deduced protein of 317
250 amino acids with a predicted molecular weight of 36 kDa and an isoelectric point of 5.6. A
251 typical caspase family protease active-site QACRG pentapeptide was located from amino acids
252 190-194. Putative cleavage sites that generate large and small subunits were proposed to be at
253 TAVDG, between Asp55 and Gly56, and VQTDS, between Asp215 and Ser216 (Fig. 1).
254 A BLASTp homology search (Altschul et al., 1990) revealed high similarity to putative
255 and actual genes encoding caspase-1, -3, -6, and -7 enzymes of various animals such as insects,
256 fish, birds, and mammals. In addition, a conserved domain characteristic of typical caspases was
257 detected by a Conserved Domain Database (Marchler-Bauer et al., 2005). *Pm*Casp sequence was
258 compared to the first caspase sequence reported in shrimp, *P. merguiensis* and to the 7 identified
259 caspases from a fruit fly *D. melanogaster*, phylum arthropoda. In addition, since *Pm*Casp
260 exhibited caspase-3 activity (see below), caspase-3 enzymes from relatively well-studied humans
261 and rats were also included in the comparison (Fig. 2A). The alignment revealed that *Pm*Casp
262 showed highest identity (83%) to the already cloned putative caspase from banana shrimp *P.*
263 *merguiensis* (*Pme*Casp) that had been used as a probe for its detection. Compared with other
264 organisms, the *Pm*Casp sequence showed 13-30% identity with *D. melanogaster* caspases and
265 28% and 26% identity with human and rat caspase-3, respectively. Among *D. melanogaster*
266 caspases, *Pm*Casp exhibited relatively high similarity to drICE (30% identity) and to DCP-1
267 (29% identity). The comparison using the default parameter generated phenogram (Fig. 2A) also

268 demonstrated that *Pm*Casp is relatively more closely related to mammalian Casp-3, and fly ICE
269 and DCP-1 proteins. In this regard, the putative domain structure of *Pm*Casp was therefore
270 compared to human Casp-3, fly ICE and DCP-1 (Fig. 2B). The active site of shrimp *Pm*Casp is
271 the same as human Casp-3 with a pentapeptide motif of QACRG, whilst that of the two fly
272 caspases is a slightly modified QACQG. Cleavage sites in the shrimp sequence were not
273 conserved when compared to other organisms. In addition, conserved consensus sequence motifs
274 for cleavage sites reported in mammalian and insect caspases were not present in the putative
275 shrimp caspases. Although the sequence and activity of *Pme*Casp have been studied, its potential
276 cleavage sites have not yet been predicted (Phongdara et al., 2006). Therefore, the potential
277 aspartate cleavage sites generating large and small subunits of *Pm*Casp were proposed to be
278 those nearest aspartate cleavage residues of the three compared sequences (human Casp-3, and
279 fly ICE and DCP-1) (Fig. 2B). Using this reasoning, putative proteolytic cleavage sites of
280 *Pm*Casp are Asp55 and Asp215, and this would yield predicted molecular weights of the large
281 and small subunits of *Pm*Casp at 17.9 kDa (160 amino acid residues) and 11.8 kDa (102 amino
282 acid residues), respectively (Fig. 2B). If so, *Pm*Casp would appear to have a relatively short N-
283 terminal prodomain of 55 amino acid residues when compared to other known caspases with
284 longer prodomains of 130-220 amino acids (Villa et al., 1997).
285
286 *3.2. Caspase-3 activity of recombinant PmCasp*
287 The proluminescent substrate of caspase-3 and -7 enzymes (caspase-Glo 3/7, Promega)
288 was used to test for activity of the purified recombinant *Pm*Casp protein (see below for protein
289 expression). Recombinant *Pm*Casp protein exhibited significantly higher luminescence levels
290 than the no-enzyme control and this was totally negated by addition of 5 μM DEVD-CHO, a
291 synthetic (specific) peptide inhibitor of caspase-3 (Fig. 3). Thus, the data suggested that
292 recombinant *Pm*Casp had caspase-3 activity.
293
294 *3.3. Detection of PmCasp in shrimp tissues*
295 *PmCasp* was cloned in-frame into plasmid pET15b and overexpressed as a His_6-tagged
296 fusion protein. A band of approximately 36 kDa corresponding to the His_6-*Pm*Casp fusion
297 protein was observed after IPTG induction and it was found to react with both anti-His and anti-
298 *Pm*Casp antibodies (Fig. 4A, lane 4). No bands were found at the same position in either non-
299 induced or induced transformed *E. coli* with empty pET15b, or in non-induced pET15b-*Pm*Casp
300 transformed cells (Fig. 4A, lanes 1 to 3). Recombinant His_6-*Pm*Casp was purified using Ni-NTA
301 beads and shown to react with both anti-His and anti-*Pm*Casp antibodies (Fig. 4A, lane 5).

Interestingly, an immunoreactive band of approximately 26 kDa was found in induced *E. coli* lysates expressing pET15b-*Pm*Casp when detected with both anti-His and anti-*Pm*Casp antibodies (Fig. 4A, lane 4). It is likely that this band corresponded to a proteolytic intermediate form of *Pm*Casp that contained the His-tag moiety.

To determine the distribution of *Pm*Casp, 25-30 µg of total protein extract of hemolymph, hemocytes, the heart, muscle and gills of shrimp were analyzed. Western blot analysis using anti-*Pm*Casp antibody indicated the presence of *Pm*Casp in all tested tissues, whereas no positive signal was detected from the hemolymph fraction (Fig. 4B). The signal from hemocyte lysate was stronger than that from other tissues. Detected bands of 36 kDa were in accordance with the molecular mass predicted from the deduced amino acid sequence of *PmCasp* cDNA, although the reactive band from the hemocyte homogenate was slightly larger. In addition, a band of 26 kDa expected to represent a partial proteolytic intermediate form was observed only from the hemocyte homogenate.

3.4. Expression of PmCasp in WSSV-infected shrimp

In WSSV-infected shrimp, the transcript levels of *Pm*Casp were investigated in gills (a major target organ of WSSV) by semi-quantitative RT-PCR referenced to transcript levels of shrimp β-actin. This revealed significantly higher *Pm*Casp transcript levels in the gills of WSSV infected shrimp at 48 h pi when compared to uninfected control shrimp. However, at the moribund stage of infection, the level was not significantly different from the control (Figs. 5A-B).

Western blot assay using anti-*Pm*Casp antibody with proteins extracted from the gills of WSSV-infected shrimp revealed relatively strong signals at approximately 26 kDa together with very faint bands at 36 kDa (Fig. 5C). The density of the 26-kDa bands for WSSV-infected shrimp at 48 h pi and at the moribund stage were more intense when compared to the 26 kDa bands from shrimp at 24 h pi and from normal control shrimp. Two immunoreactive bands at 36 kDa and 26 kDa were also detected with purified *Pm*Casp protein but in that case the strongest reactivity was seen with the 36-kDa band (Fig. 5C-1). The same result was obtained when anti-human caspase-3 antibody was used (Fig. 5C-2). The bands at 26 kDa completely disappeared when the anti-*Pm*Casp polyclonal antibody was pre-absorbed with the *Pm*Casp fusion protein (Fig. 5D-2), indicating that the proteins shared common reactive epitopes.

3.5. Immunohistochemistry

335 H&E stained sections of gills from WSSV-infected shrimp revealed histopathological
336 changes, including cells with enlarged nuclei containing basophilic inclusions typical for WSSV
337 infection (Fig. 6A). Some cells showed condensed and fragmented nuclei especially at the later
338 stages of infection. Peroxidase-based immunohistochemistry for localization of *Pm*Casp protein
339 in the gills of control and WSSV-infected shrimp using rabbit anti-*Pm*Casp antiserum revealed
340 positive reactions in the cytoplasm of both apoptotic cells (cells with condensed nuclei as in Fig.
341 6C and cells with fragmented nuclei as in Fig. 6E) and in cells of normal appearance (Fig. 6D).
342 Gills of normal control shrimp showed no cells with clear signs of apoptosis but did show
343 positive immunoreactivity in some cells of normal appearance (Fig. 6B). Negative control slides
344 with the primary antibody omitted gave no immunoreactive staining (Fig. 6F).
345
346 **4. Discussion**
347 Many studies have been devoted to the cloning, expression, purification, and
348 characterization of recombinant human and *Drosophila* caspases. Due to their conservation and
349 central role in apoptosis, they are driving research in several organisms. In shrimp there is
350 currently little understanding of the cellular, molecular or genetic basis of apoptosis and to date,
351 only one caspase gene from *Penaeus merguiensis* has been identified (Phongdara et al., 2006). In
352 that study, elevated caspase-3 expression was associated with shrimp mortality from WSSV
353 infection, supporting a link between caspase-induced apoptosis and death. This opened the
354 possibility for similar work on other commercially important shrimp species such as *P. monodon*
355 that has frequently been used as a model to study host-viral interactions. Characterization of a
356 caspase-3 like protease from *P. monodon* (*Pm*Casp) and demonstration of it association with
357 death from WSSV infection parallels the earlier work on *P. merguiensis* (Phongdara et al.,
358 2006). In addition, binding to the specific anti-*Pm*Casp polyclonal antibody confirmed the earlier
359 work indicating that a standard antibody to human caspase-3 and standard reagents to measure
360 and inhibit caspase-3 activity can be used to study caspase-3 in at least two penaeid shrimp. This
361 should facilitate further investigations into the role of apoptosis in shrimp viral infections.
362 Comparison of *Pm*Casp to the well-studied arthropod model, *Drosophila melanogaster*,
363 revealed similarity to drICE and DCP-1 proteins. These are short N-terminal prodomain-
364 containing caspases that have been suggested to act as effector caspases similar to mammalian
365 caspase-3 and -7 (Fraser and Evan, 1997; Song et al., 1997). The sequence similarities are
366 reflected in the ability of *Pm*Casp to react with reagents designed for the study of caspase-3 in
367 vertebrates and support the contention that *Pm*Casp may be a member of the caspase-3
368 subfamily.

Potential cleavage sites of *Pm*Casp were proposed to be at Asp55 and Asp215, yielding an N-terminal prodomain of 55 amino acids, a large subunit of 160 and a small subunit of 102. It has been proposed that caspase-3 can be cleaved at several alternative sites to become activated Wang et al., 1996. Therefore, experimental confirmation of the putative cleavage sites and evaluation of other potential sites particularly with reference to active form(s) and their specific roles are needed before *Pm*Casp can be fully characterized. For example, aspartic residues other than those we proposed for cleavage sites are present in the *Pm*Casp sequence and the reasoning behind our choice may eventually turn out to be flawed.

When analyzed by Western blot, anti-*Pm*Casp antibody reacted principally to bands with molecular weights of ca 36 kDa and 26 kDa in protein extracts from shrimp. The 36 kDa band corresponded to the expected molecular weight for *Pm*Casp whereas the 26 kDa protein band potentially represented its proteolytic intermediate form. In most cases, proactive forms of caspases are activated by two successive proteolytic cleavages. The first occurs between the large and small subunits, and the second between the large subunit and the prodomain (Cohen, 1997; Shi, 2002; Wolf and Green, 1999). Thus, we consider it is likely that the 26 kDa protein fragment is a partially processed *Pm*Casp intermediate composed of the prodomain and large subunit. This evidence is supported by our finding that the anti-His antibody reacted with the 26 kDa band in the lysate of *E. coli* expressing the recombinant *Pm*Casp and by the fact that the His moiety was tagged at the N-terminal end upstream of the prodomain. Even though active caspase is composed of a heterotetramer consisting two small and two large subunits, the prodomain is frequently not removed during the activation process (Denault and Salvesen, 2002), and this may explain why the partially processed intermediate of *Pm*Casp showed caspase activity assay. This notion requires that our polyclonal anti-*Pm*Casp does not react with the small subunit in order to explain absence of a reactive 12 kDa fragment in our gels. The occasional detection weak bands around 30 kDa (Fig. 4, lane 4 and Fig. 5C-1 and 2) may represent partial non-specific proteolytic fragments or non-specific binding. In any case, these were always minor components when present.

It is perhaps surprising that *Pm*Casp protein could cross react with the anti-human caspase-3 anti-sera despite the fact that it showed less than 30% amino acid sequence identity to the human caspase-3 sequence. The finding suggests that the overall form of the two proteins may be highly conserved and that they possess sufficient amino acids sequences similarity to give the cross reaction.

Our immunohistochemical and immunoblot analyses revealed that *Pm*Casp (procaspase) was present at basal levels in most cells as a latent proenzyme (Krajewska et al., 1997). The strong 26 kDa band observed in hemocyte protein extracts and the somewhat higher expression of *Pm*Casp in them suggested that *Pm*Casp like caspase-3 might play a role in apoptosis related to immunity (Johansson et al., 2000; van de Braak, 2002). This was supported by the fact that intensity of the 26 kDa band at 48 h pi with WSSV was relatively more dense than that of the control in Western blots of gills that contain large populations of hemocytes. Thus, a good portion of the positive immunohistochemical reaction within apoptotic cells of gills of WSSV-infected shrimp may have originated from active *Pm*Casp enzyme.

The level of *Pm*Casp transcript in gills of WSSV-infected shrimp at 24 h pi was not significantly different from that of the control. In this regard, it is possible that induction of apoptosis in WSSV-infected shrimp in the early infection period (0-24 h post infection) could be the result of cleavage of pro-caspase zymogen granules stored in the cytoplasm. If so, a feedback response to declining levels of zymogen or to increased active caspase may have resulted in the significant increase in *Pm*Casp transcript detected at 48 h pi and coinciding with an increased level of the proteolytic intermediate seen in Western blots. This proposal would be consistent with a previous report describing an increase of apoptotic cells as WSSV infections progress in shrimp (Wongprasert et al., 2003). The subsequent decline in *Pm*Casp expression as shrimp approached the moribund state may have resulted from the progressive break-down of DNA (Nagata et al., 2003) and RNA (Bushell et al., 2004) associated with the increasing level of apoptosis and/or associated necrosis.

The increase in caspase gene expression and caspase activity in WSSV-infected shrimp suggests that WSSV induces activation of caspase leading to apoptotic cell death in shrimp. If so, the phenomenon would support a proposal of the viral accommodation concept (Flegel, 2007) that induced apoptosis is a major cause of mortality in shrimp viral infections. If this proves to be correct, then strategies to prevent apoptosis induction after viral infection may be useful in reducing production losses either by preventing shrimp death or delaying it until they can be harvested.

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519
520
521 **Figure legends**
522 **Fig. 1. Nucleotide and deduced amino acid sequences of the full-length *PmCasp*.** An open
523 reading frame of 954 nucleotides encoding 317 amino acids contains a putative active-site
524 pentapeptide (shaded) and a potential polyadenylation signal (boxed). The potential cleavage
525 sites (Asp55 and Asp215) are indicated by arrows. Primers used for *Pm*Casp expression and for
526 RT-PCR are underlined and double underlined, respectively.
527

Fig. 2. Sequence alignment of caspase sequences. (A) Phylogenetic tree showing the relationship amongst caspase amino acid sequences aligned using ClustalW. Protein sequences from the GenBank database were PmCasp, *Penaeus monodon* (DQ846887), PmeCasp, *P. merguiensis* (AY839873), HmCasp3, *Homo sapiens* (U26943), RtCasp3, *Rattus norvegicus* (NM_012922), DCP-1, drICE, Decay, DRONC, Strica, Damm and Dredd, all from *Drosophila melanogaster* (AAB58237, O01382, AF130469, AF104357, AF242735, AF240763 and Q8IRY7, respectively). The bootstrap values for 1000 independent comparisons are added at each branch point. (B) Putative structure of *Pm*Casp compared to known caspase sequences. Potential aspartate cleavage sites between large and small units are indicated, where numbers represent positions in the protein. Length of amino acid sequence is indicated on the right end of boxes. The position of the pentapeptide sequence QACR/QG is shown by an asterisk. The % identity of *Pm*Casp to other sequences is indicated on the right margin.

Fig. 3. Caspase activity assay. The substrate for caspase-3 and -7 (Caspase Glo3/7) was incubated in a reaction in the presence (+Casp) or absence (-Casp) of *Pm*Casp-3 in the form of 350 ng of the purified recombinant protein with (+ Inh) or without 5 μM of the specific caspase-3 inhibitor DEVD-CHO. Caspase activity was measured every 30 min as luminescence (CPS, count per second) and is displayed as mean ± SD of three replicates.

Fig. 4. Expression of *Pm*Casp. (A) Expression and purification of recombinant His_6-*Pm*Casp protein from *E. coli*. Lane M, protein molecular weight markers; Lane 1, cell lysate of the transformed *E. coli* with pET15b not induced with IPTG; Lane 2, cell lysate of the transformed *E. coli* with pET15b induced with IPTG; Lane 3, pET15b-*Pm*Casp transformed cells not induced with IPTG; Lane 4, pET15b-*Pm*Casp transformed cells induced with IPTG; Lane 5, purified His_6-*Pm*Casp protein with the expected band size of recombinant *Pm*Casp indicated. Samples were subjected to SDS-PAGE and visualized by Coomassie brilliant blue staining and analysed by immunoblotting using anti-His or anti-*Pm*Casp antibody. (B) Detection of *Pm*Casp protein in 25-30 μg total protein derived from extracts of different tissues of *P. monodon*. Immunoblot of an SDS-PAGE gel was performed using anti-*Pm*Casp antibody. Lane Hl, hemolymph; Lane Hc, hemocytes; Lane H, heart; Lane Mu, muscle; Lane G, gills. Expected band sizes of the pro-active *Pm*Casp form and its partial proteolytic intermediate are indicated.

Fig. 5. Transcriptional and translational expression of *Pm*Casp from gills of WSSV-infected shrimp.

562 (A) Representative gel of RT-PCR analysis of the *Pm*Casp and β-actin mRNA of the control
563 and WSSV-infected shrimp at 24, 48 h pi and when moribund.
564 (B) The ratios between the densitometry values of transcript levels of *Pm*Casp and β-actin
565 genes derived from RT-PCR for control and WSSV-infected shrimp at 24, 48 h pi and
566 when moribund are expressed as the mean ± SD of three replicates. * indicates values
567 significantly different from the control at $p<0.05$.
568 (C) The expression of *Pm*Casp protein in gills of control (N), and WSSV-infected shrimp at
569 24, 48 h pi and when moribund (Mo), displayed as Western blots probed with anti-
570 *Pm*Casp. Purified *Pm*Casp protein was probed with (1) anti-*Pm*Casp and (2) anti-human
571 caspase-3. Expected band sizes of pro-active form and partially proteolytic intermediate
572 of *Pm*Casp protein are indicated.
573 (D) The expression of *Pm*Casp protein in gills of control and WSSV-infected shrimp at 24,
574 48 h pi and when moribund, analyzed by Western blot probed with (1) anti-*Pm*Casp and
575 (2) preabsorbed anti-*Pm*Casp antibody (1:1000 dilution).
576
577 **Fig. 6. Localization of *Pm*Casp in gills of WSSV-infected shrimp.**
578 (A) A typical H&E stained section from gills of WSSV-infected shrimp at 24 h pi showing
579 cells with nuclear enlargement (arrow head) and intranuclear inclusions (arrow)
580 characteristic of WSSV infection. A cell with a condensed nucleus is also shown.
581 (B-E) Shrimp gill sections immunohistologically stained for *Pm*Casp with HRP-AEC
582 (Zymed) development (red color).
583 (B) *Pm*Casp immunoreactivity in gills of normal shrimp at 24 h pi. The imunoreactivity is
584 localized in the cytoplasm of normal cells.
585 (C-D) *Pm*Casp immunoreactivity in gills of WSSV-infected shrimp at 24 h pi. Virus infected
586 cells with enlarged nuclei (arrow head) and intranuclear inclusions (arrow) are shown.
587 The immunoreactivity is localized in apoptotic cells with condensed nuclei (C) and
588 normal-looking cells (D).
589 (E) *Pm*Casp immunoreactivity in gills of the WSSV-infected shrimp **at 48 h pi. The**
590 immunoreactivity is localized in apoptotic cells with condensed and fragmented nuclei.
591 (F) No positive immunoreactvity detected when the primary antibody was omitted in control
592 sections.
593

Fig. 1

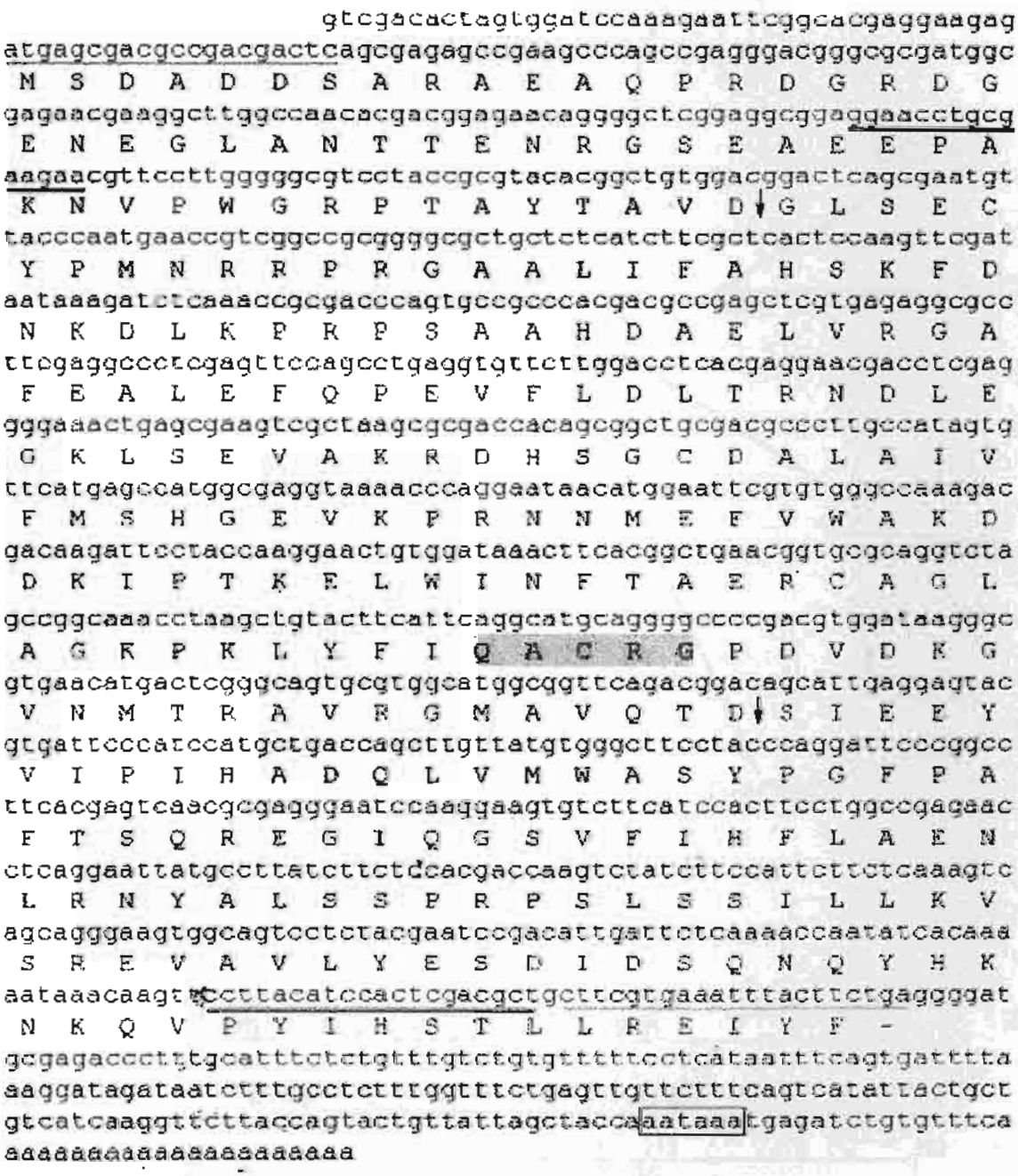
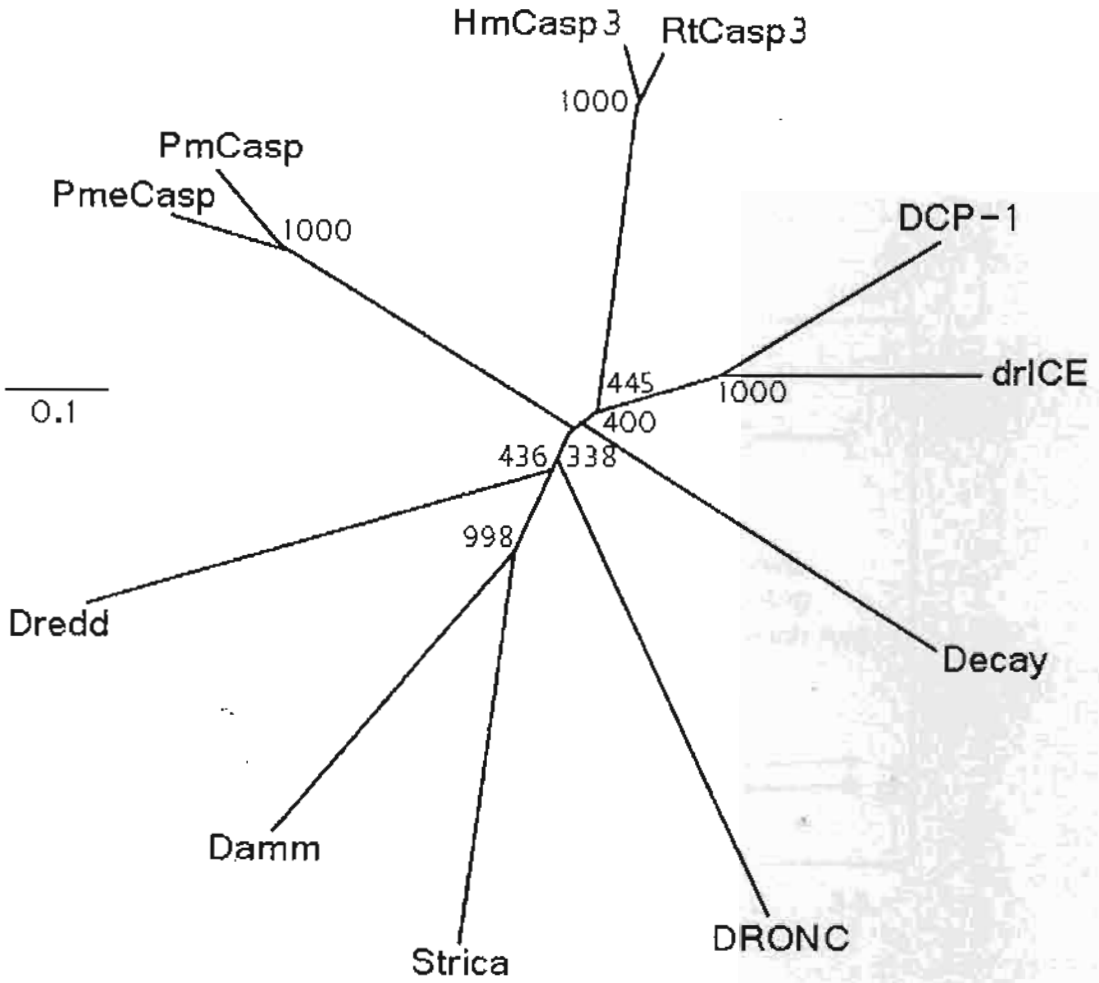


Fig. 2

A



B

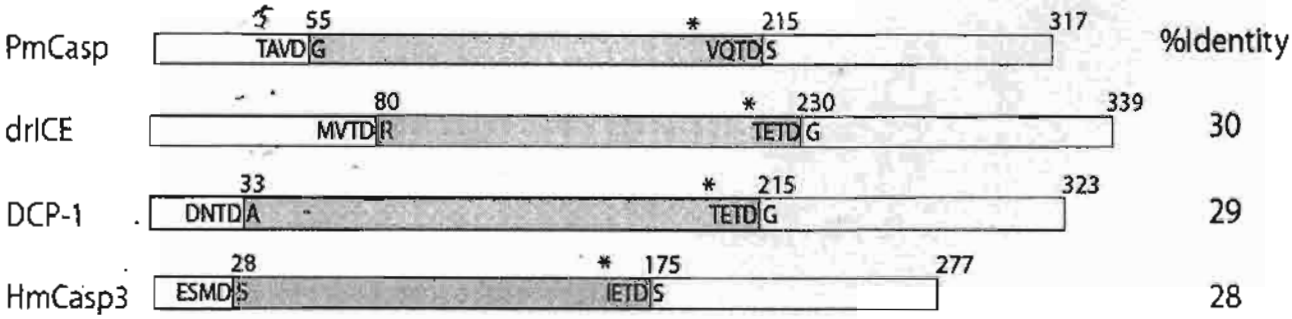


Fig. 3

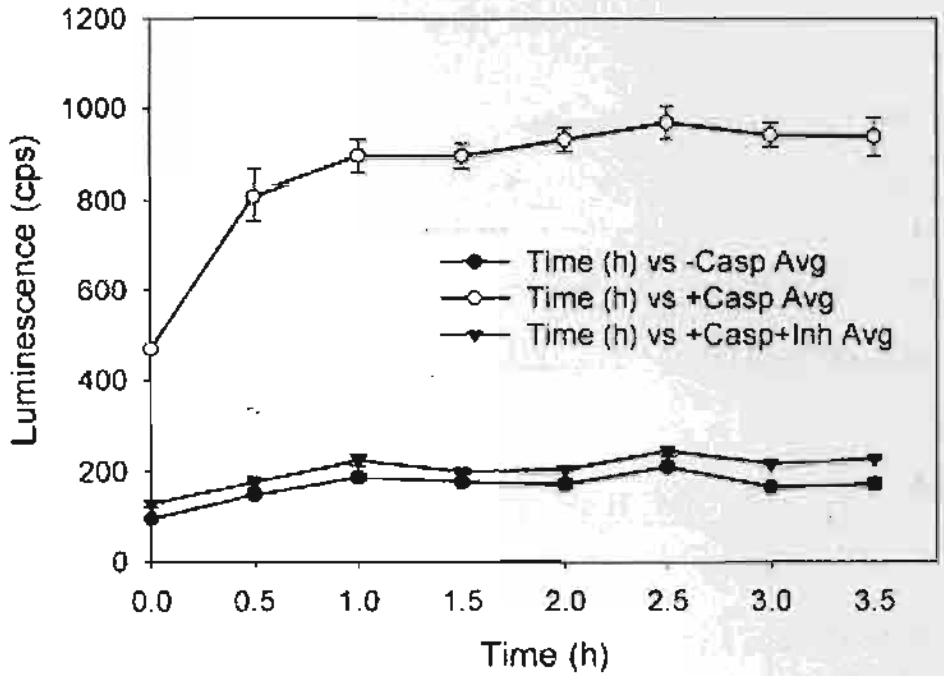
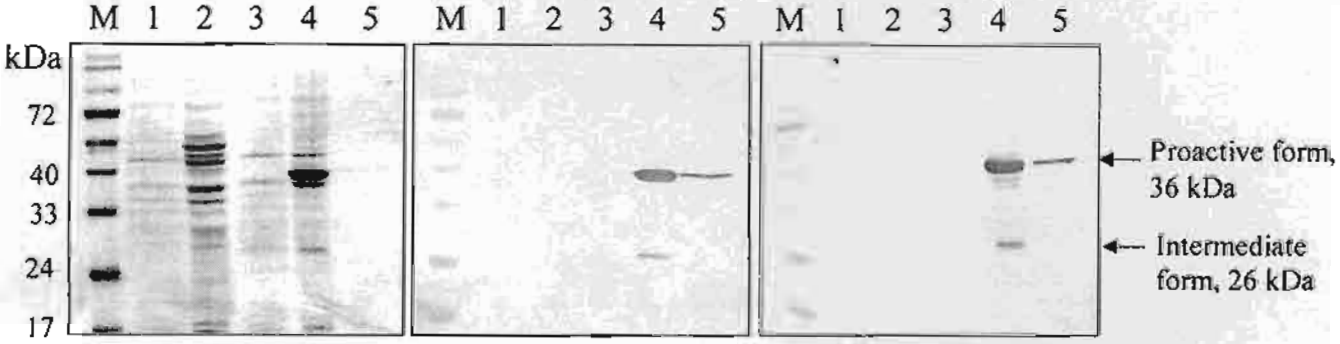


Fig. 4

A

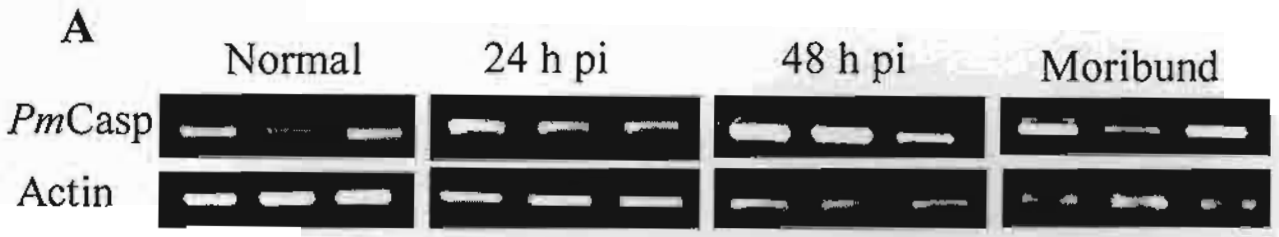


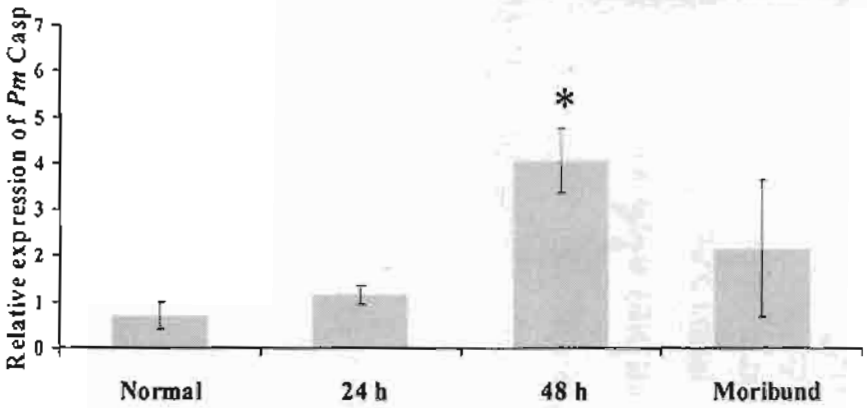
Coomassie stain Anti-His Anti-*Pm*Casp

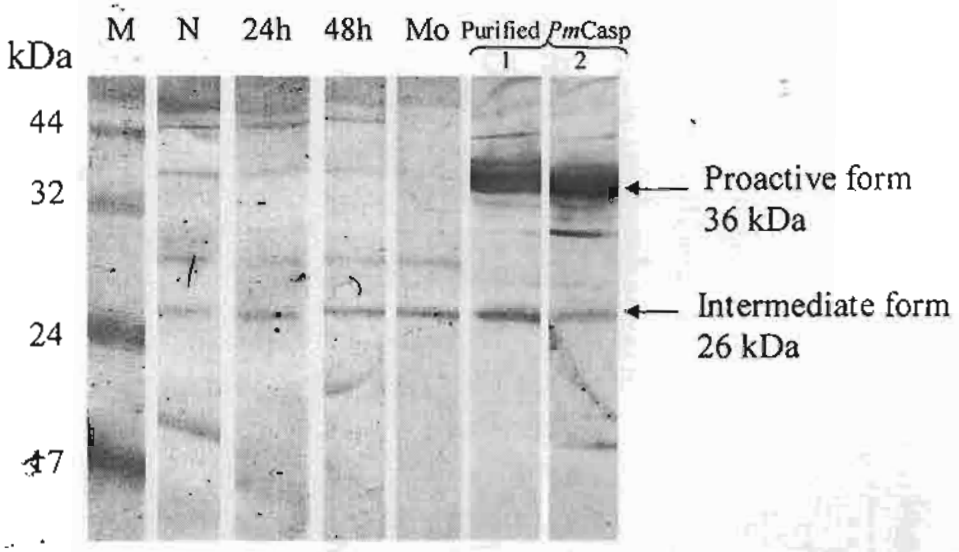
B

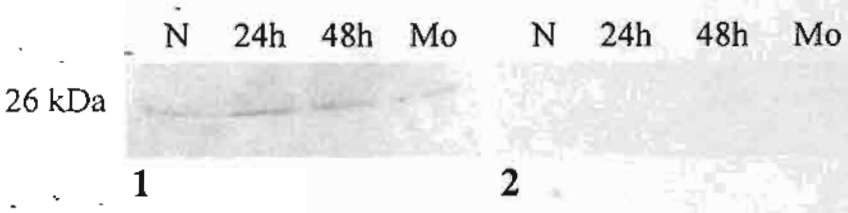
Coomassie stain Anti-*Pm*Casp

Fig 5.









สมาคมกายวิภาคศาสตร์ (ประเทศไทย)

มอบเกียรติบัตรฉบับนี้เพื่อประกาศเกียรติคุณของ

ดร.กนกพรรณ วงศ์ประเสริฐ และคณะ

เรื่อง "Expression and Characterization of Caspase 3 Gene in White Spot Syndrome Virus Infected Shrimp, P. monodon"

ในฐานะได้รับ **รางวัล ศาสตราจารย์ นายแพทย์ วิเชียร ดิลกสัมพันธ์**

แก่ผลงานวิชาการแบบบรรยาย ด้านอณูวิทยาและเทคโนโลยีชีวภาพ

การประชุมกายวิภาคศาสตร์ (ประเทศไทย) ครั้งที่ ๒๙

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