การโคลนและลักษณะสมบัติของ CRUSTIN*Pm5* จากกุ้งกุลาดำ*Penaeus monodon*

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CLONING AND CHARACTERIZATION OF CRUSTIN*Pm*5 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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จากการวิเคราะห์ข้อมูลยีนครัสตินในฐานข้อมูล *Penaeus monodon* Expressed Sequence Tag database (http://pmonodon.biotec.co.th) พบว่ามียีนครัสตินหกไอโซฟอร์ม (crustin*Pm*1-6 และ crustin-like*Pm*) ยีนครัสตินไอโซฟอร์ม 5 ถูกพบในห้องสมุด cDNA ที่เตรียมจากเหงือก อีพิโพไดท์ของกุ้งกุลาดำ ยีนครัสตินไอโซฟอร์ม 5 ประกอบด้วยส่วนที่เป็น open reading frame ขนาด 520 คู่เบส ซึ่งถูกแปลรหัสเป็นโปรตีนขนาด 169 กรดอะมิโน จากการวิเคราะห์เปรียบเทียบลำดับกรดอะมิโนพบว่า ครัสตินไอโซฟอร์ม 5 เหมือนกับครัสตินไอโซฟอร์ม 1 และครัสตินไลค์พีเอ็ม 38% และ 37% ตามลำดับ เมื่อศึกษาการจัดเรียงตัวของยีนครัสตินไอโซฟอร์ม 5 ด้วยวิธี PCR และ genome walking พบว่ายีนครันตินไอโซฟอร์ม 5 ประกอบด้วย 4 exon และ 3 intron ส่วนบริเวณปลาย 5′ ของยีนพบโปรโมเตอร์ และส่วนที่ทำหน้าที่ควบคุมการแสดงออกของยีนคือ บริเวณ NF-KB 2 ตำแหน่ง บริเวณ GATA 5 ตำแหน่ง และ heat-shock regulatory element (HSE) จากการตรวจสอบการแสดงออกของยีนด้วยวิธี RT-PCR พบว่ามีการแสดงออกในอีพิโพไดท์และก้านตา แต่ไม่พบในเม็ดเลือด เมื่อทำการตรวจสอบการแสดงออกของยีนครัสตินไอโซฟอร์ม 1, 5 และ ครัสตินไลค์พีเอ็ม พบว่ามีการแสดงออกมากขึ้นทั้งในกุ้งที่กระตุ้นด้วยความร้อน และ เพิ่มความเค็ม เพื่อศึกษาแอกติวิตีของยีนครัสตินไอโซฟอร์ม 5 โปรตีนจึงถูกผลิตในระบบแบคทีเรีย *E. coli* โปรตีนที่ผลิตมีฤทธิ์ยับยั้งการเจริญของเชื้อแบคทีเรียแกรมบวก แต่ไม่ยับยั้งการเจริญของแบคทีเรียแกรมลบ จากผลแอกติวิตีและแบบแผนการแสดงออกของยีนครัสตินไอโซฟอร์ม5 แสดงให้เห็นว่ายีนนี้น่าจะมีหน้าที่อื่นอีกนอกจากมีความสามารถในการยับยั้งแบคทีเรีย

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต ธิปชัย วัฒนวิจารณ์

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TIPACHAI VATANAVICHARN : CLONING AND CHARACTERIZATION OF CRUSTIN*Pm*5 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*
THESIS ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR PROF. KENNETH SÖDERHÄLL, Ph.D., 106 pp.

From *Penaeus monodon* expressed sequence tag database (http://pmonodon.biotec.or.th), six isoforms of crustin (crustin*Pm*1-6 and crustin-like*Pm*) was identified. Crustin*Pm*5 cDNA identified from a gill-epipodite cDNA library contains 510 bp of open reading frame encoding for a 169 amino acid protein. Sequence comparison showed that the deduced amino acid sequence of crustin*Pm*5 shares identity with crustin*Pm*1 and crustin-like*Pm* 38% and 37%, respectively. The genomic sequence of crustin*Pm*5 was obtained by using PCR and genome walking techniques. The crustin*Pm*5 gene contained four exons interrupted by three introns whilst the 5′upstream sequence contains a putative promoter with two potential binding sites for NF-κB, one complete heat-shock regulatory element (HSE) and five putative GATA factor binding sites. The RT-PCR analysis revealed that the transcripts of crustin*Pm*5 were primarily observed in the epipodite and eyestalk and not in hemocytes. Expression analysis revealed that the transcript levels of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* in epipodite were up-regulated upon heat treatment and hyperosmotic salinity stress. To further characterize the activity of crustin*Pm*5, the recombinant protein was produced in *E. coli* system. The recombinant crustin*Pm*5 exhibited antimicrobial activity against some Gram-positive bacteria in vitro, but did not inhibit the growth of any Gram-negative bacteria tested. These results, together with the transcript expression pattern, indicate a diverse function of the proteins in the crustin family particularly crustin*Pm*5 that might function as a stress mediator in addition to its antibacterial action.

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CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EtBr	ethidium bromide
h	hour
kb	kilobase
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometre
OD	optical density
^{o}C	degree Celcius
ORF	open reading frame
PCR	polymerase chain reaction
ppt	part per thousand
RNA	ribonucleic acid
RT	reverse transcription
sec	second
µg	microgram
µl	microliter
UTR	untranslated region

CHAPTER I
INTRODUCTION

1.1 General introduction

From the world aquaculture production, shrimp has been the species that contributed the highest reported value of the production with more than ten billions US dollars (Source: Yearbooks of Fishery Statistics Summary Tables of Fishery Statistics Capture – Aquaculture Commodities; ftp://ftp.fao.org/fi/stat/summary/a-6.pdf) (Table 1.2). Asia has always led the world production of cultivated shrimp and Thailand is one of the top ten countries in shrimp aquaculture production. The black tiger shrimp farms and hatcheries are dispersed along the coastal areas of the country. Southern provinces such as Nakon Sri Thammarat and Surat Thani yield the majority of harvests, whereas east and central provinces such as Samut Sakhon and Samut Songkhram yield the minority in terms of number. The shrimp cultivation in Thailand has rapidly generated large annual production with several advantages, including appropriate farming areas without serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils and terrain for pond construction. Previously, the black tiger shrimp, *Penaeus monodon*, is the main specie to be cultured therefore, making this penaeid shrimp an economically important species in Thailand (Source: FAO Fishstat 2006). Before the year 2000, Thailand was the world's number one shrimp producer. However, after 2002, the production decreased continuously due to infectious diseases, unfavorable weather, and high salt concentration. Until now, the black tiger shrimp production of Thailand fell down from 83,036 tons in 2002 to 14,283 tons in 2008 (Table 1.1).

To solve these serious problems, the Pacific white shrimp, *Lenaeus vanamei*, was imported and currently, it becomes the main cultured species in Thailand. The advantages of *L. vanamei* are rapid growth rate, low protein requirements, certain disease resistance (pathogen resistant stock), tolerance of high stocking density, tolerance of low salinities, and high survival during larval rearing. However, there are also disadvantages of *L. vanamei* including its ability to act as carrier of various viral

pathogens such as *baculovirus penaei* (BP) and Taura Syndrome virus (TSV). These viruses can be transmitted to the *P. monodon*, the native penaeid shrimp.

To overcome these problems, the native shrimp should be genetically improved to gain resistance to the pathogens and rapid growth rate through the effective genetic selection programs. Domestication will provide captive broodstock while genetic selection will provide high-quality traits with desired properties such as disease resistance, rapid growth rate, tolerance of high stocking density, tolerance of low salinity and temperature, lower protein requirements, high survival during larval rearing as well as some other marketing advantages.

Table 1.1 Thailand's exports of *Penaeus monodon* in 2004-2008.

Year	2004	2005	2006	2007	2008
Quantity (tons)	53,012	33,036	28,987	19,314	14,283

(Source: Office of Agricultural Economics)

Table 1.2 World acquaculture production by principal species in 2006 (Source: FAO Fishstat (2006)

Species Espèce Especie		1998	2001	2002	2003	2004	2005	2006
World total **Total mondial** **Total mundial**	**Q** **V**	**30 485 721** **44 293 849**	**37 954 378** **52 520 916**	**40 391 695** **54 445 590**	**42 673 878** **58 394 567**	**45 921 837** **63 899 802**	**48 489 670** **70 370 882**	**51 653 329** **78 758 387**
Crassostrea gigas	Q	3 433 245	4 107 593	4 234 583	4 341 723	4 436 810	4 516 795	4 592 784
	V	3 255 760	3 383 444	3 511 153	2 600 243	2 724 253	3 062 216	3 072 386
Hypophthalmichthys molitrix	Q	3 329 068	3 918 055	3 849 680	3 832 729	4 017 915	4 157 565	4 358 686
	V	2 907 716	3 232 651	3 215 018	3 197 622	3 369 447	3 538 702	3 686 054
Ctenopharyngodon idellus	Q	2 987 242	3 461 997	3 595 263	3 733 087	3 735 553	3 904 797	4 010 281
	V	2 719 921	2 856 469	2 937 436	3 032 029	3 029 284	3 202 175	3 377 471
Cyprinus carpio	Q	2 383 118	3 067 534	3 139 765	3 307 010	2 923 964	3 046 339	3 172 488
	V	2 404 860	3 025 166	2 768 421	2 945 839	2 816 855	2 821 617	2 965 649
Ruditapes philippinarum	Q	1 474 334	2 091 412	2 357 584	2 603 327	2 859 655	2 946 900	3 095 971
	V	1 969 685	2 490 627	2 814 581	3 126 791	2 218 362	2 619 210	2 820 876
Hypophthalmichthys nobilis	Q	1 585 743	1 660 200	1 722 447	1 928 088	2 101 530	2 207 678	2 394 255
	V	1 441 176	1 430 181	1 482 761	1 658 299	1 807 120	1 916 244	2 126 850
Penaeus vannamei	Q	193 512	280 114	481 298	1 039 576	1 361 200	1 691 154	2 133 381
	V	1 019 747	1 524 005	2 339 603	3 592 104	4 652 638	5 938 521	7 774 098
Carassius carassius	Q	1 036 153	1 526 320	1 701 454	1 793 713	1 949 752	2 086 304	2 097 188
	V	834 114	1 071 556	1 195 559	1 260 436	1 371 765	1 478 141	1 526 257
Oreochromis niloticus	Q	773 195	1 113 737	1 207 818	1 378 305	1 575 715	1 748 461	1 988 726
	V	901 423	1 322 241	1 341 192	1 458 725	1 663 481	1 881 239	2 220 314
Patinopecten yessoensis	Q	856 219	1 196 135	1 207 623	1 156 652	1 126 159	1 239 811	1 361 629
	V	1 179 877	1 555 003	1 577 710	1 511 839	1 418 302	1 677 870	1 883 061
Labeo rohita	Q	660 263	601 233	668 987	711 852	1 199 943	1 209 223	1 332 430
	V	1 411 782	798 967	838 238	952 485	1 576 385	1 371 536	1 562 795
Catla catla	Q	555 265	484 691	554 391	560 940	1 158 452	1 285 344	1 330 633
	V	553 282	461 596	530 753	540 536	1 278 354	1 485 234	1 323 130
Salmo salar	Q	688 227	1 030 005	1 084 934	1 130 784	1 253 047	1 241 188	1 307 684
	V	2 142 602	2 771 299	2 910 542	3 393 110	4 131 581	4 843 832	6 565 857
Sinonovacula constricta	Q	415 032	597 374	635 486	672 402	676 391	713 846	679 010
	V	332 026	477 899	508 389	537 922	507 491	589 836	576 589
Penaeus monodon	Q	503 005	673 012	631 571	730 404	715 447	674 954	658 221
	V	3 215 348	3 935 192	3 631 412	3 382 807	3 387 608	3 106 518	3 122 888
Parabramis pekinensis	Q	449 282	541 115	564 086	524 927	516 869	552 922	594 287
	V	539 138	622 282	648 699	603 666	594 506	642 449	709 634
Chanos chanos	Q	379 650	494 940	527 977	552 043	573 732	594 783	585 375
	V	571 143	718 189	483 455	481 455	707 836	615 913	645 931

1.2 Taxonomy of *Penaeus monodon*

Penaeid shrimp are classified into the largest phylum in the animal kingdom, the Arthropoda. This group of animal is characterized by the presence of pair appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up of 42,000, predominantly aquatic species, which belong to the 10 classes. Within the class Malacostraca; shrimp, crayfish, lobster and crab belong to the order Decapoda. The taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brock et al., 1992):

Phylum Arthropoda
Subphylum Crustacea Brünnich, 1772
Class Malacostraca Latreille, 1802
Subclass Eumalacostraca Grobben, 1892
Order Decapoda Latreille, 1802
Suborder Dendrobranchiata Bate, 1888
Superfamily Penaeoidea Rafinesque, 1815
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Species Penaeus monodon Fabricius, 1798

Scientific name: *Penaeus monodon* (Fabricius), 1798
Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu(Indonesia), Ushi-ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo (Phillipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Tim sa (Vietnam).
F.A.O. Names: Giant tiger prawn, Crevette giante tigre, Camaron tigre gigante.

1.3 Shrimp diseases

Infectious diseases become serious problems in shrimp industry in many countries. In Thailand, the out breaks of infectious disease have become the most serious problem since 1993. The infectious diseases of *P. monodon* are mainly caused by virus and bacteria. The major viruses of concern (in estimated order of pase economic impact for Thailand) are white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV) and monodon baculovirus (MBV). However, with the introduction of shrimp, Taura syndrome virus (TSV) and infectious hypodermal and hematopoeitic virus (IHHNV) have now become important (Flegel, 2006). Vibriosis is the most prevalent bacterial disease causing mass mortalities both in hatcheries and in shrimp growout ponds (Saulnier et al., 2000). The major virulent strains of *Vibrio* in shrimp are *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram*. This is because of an increase in shrimp farming and lack of proper knowledge involving shrimp biology, farm management, and diseases. Moreover, shrimp aquaculture is presently based on wild animals which are not completely acclimatized to the artificial conditions of shrimp hatcheries and farms where water quality, microbiological flora and nutrition are vastly different from those in the natural habitat. These intensive artificial conditions lead to physiological disturbances and immunodeficiencies that increase sensitivity to the pathogens.

1.4 Viral disease

Virus is one of the major pathogens in cultured and wild marine penaeid shrimp. The shrimp farming industry in Thailand encountered a severe problem from viral infectious diseases for over a decade. The first shrimp virus, *Baculovirus penaei* (BP), was isolated from wild penaeid shrimp in the early 1970s. The important virus species that have been reported in *P. monodon* are white spot syndrome virus (WSSV) and yellow-head virus (YHV) which cause white spot syndrome (WSS) and yellow-head disease (YH), respectively (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995). The outbreak of these virus diseases causes great losses in the shrimp producing countries including Thailand.

1.4.1 White spot syndrome (WSS)

White spot syndrome (WSS) is a viral disease affecting most of the commercially globally cultured marine shrimp species (Chou et al., 1995; Wongteerasupaya et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Spann et al., 1997). Original outbreaks were reported from China in 1993 and they spread rapidly thereafter to Japan, Thailand, Korea, India, USA, Central and South America. The disease is possibly spread by contaminated water, decomposing fecal matter or tissue, cannibalism and fluid from infected females. Shrimp may be indirectly infected the disease by expose to previous hatchery or pond growing cycles, contaminated water supplies, contaminated food, equipment surfaces and clothing , or animals which have ingested diseased shrimp.

Lightner (1996) has called this virus white spot syndrome baculovirus (WSSV) because its morphological characteristics are similar to insect baculovirus. However, phylogenetic analysis of ribonucleotide reductase and protein kinase genes revealed that WSSV does not share a common ancestor with baculovirus (Van Hulten et al., 2000; Van Hulten et al., 2001). This virus is an enveloped DNA virus of bacilliform to cylindrical morphology with an average size of 120 x 275 ± 22 nm and has a tail-like projection at one end of the particle (Kasornchandra et al., 1995; Wongteerasupaya et al., 1995). The viral genome contains double-stranded DNA of about 292 to 305 kb in length (Van Hulten et al., 2001; Yang et al., 2001).

The clinical signs of WSS infected shrimps include red or pink body surface and appendages, loose shell and white calcium deposits embedded in shell, white spots 0.5-2.0 mm in diameter on the exoskeleton and epidermis for which the disease is named, lack of appetite and slow movement. However, the disease can occur without the presence of white spots. Major targets of the virons are ecto- and mesodermal origin such as the gills, lymphoid organ, cuticular epithelium. And virions are assembled and replicated in the nucleus. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. If the white spots appear together with lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp around the edges of ponds throughout the day, and a rapid reduction in food consumption, a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of the signs.

Histopathological changes, including prominent intranuclear eosinophilic to basophilic inclusions in the infected cells and cellular degeneration with hypertrophied nuclei and chromatin margination in the cuticular epidermis, gill epithelium, antennal gland, hematopoietic tissue, nervouse tissue and connective tissue and cellular necrosis and detachment of intestinal epithelial tissue can be observed (Wongteerasupaya et al., 1995).

WSS can cause up to 100% mortality, causing a correspondingly devastating economic impact. WSSV is extremely virulent and has a wide host range (Lo et al., 1996). Diseases caused by viruses especially by white spot syndrome virus (WSSV) are the greatest challenge to the worldwide shrimp aquaculture.

The WSSV infection can be detected by several diagnostic methods such as PCR, in situ hybridization, mini array, observation of tissues subjected to fixation or negative staining, and immunological methods using monoclonal and polyclonal antibodies to WSSV or their component proteins (Okumura et al., 2005).

1.4.2 Yellow head disease

Yellow head disease was known to infect and cause mass mortality in shrimp farming operations throughout South East Asian countries. In Thailand, the disease was first reported in 1990. Yellow head disease occurs in the juvenile to sub-adult stages of shrimp, especially at 50-70 days of grow-out (Lightner, 1996). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley et al., 1999). It has been classified in the genus *Okavirus*, family *Roniviridae*, order *Nidovirales* (Walker et al., 2005).

YHV infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and have a generally pale or bleached appearance (Limsuwan, 1991). At the onset of YHD, food consumption of shrimp is at an abnormally high rate for several days, then abruptly ceases feeding. A few moribund shrimp appear swimming slowly near the surface at the pond edges. After infection, mortality may reach as high as 100% of affected populations within 3-5 days from the onset of disease. In the black tiger shrimp, typical signs of YH disease include characteristic yellowing of the hepatopancreas and gill. YHV may occur as latent,

asymptomatic infections in broodstock shrimp and may possibly transfer from these shrimp to their offspring in larval rearing facilities (Chantanachookin et al., 1993).

YHV infection diagnosis could be performed by using immuno-histochemistry, the monoclonal antibody aggregated with a surface glycoprotein and the nucleocapsid protein of virus (Sanchez-Barajas et al., 2009). A conventional RT-PCR or in situ hybridization was also advantage for detection of YHV infection (Wongteerasupaya et al., 1995; Tang et al., 1999).

1.5 Bacterial disease

Vibrio species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). However, the bacteria causing the most serious diseases of the larval and postlarval stages of *P. monodon* are of the genus *Vibrio* which behave more like true pathogens than opportunist (Lightner, 1983; Lightner, 1988; Lightner, 1996).

The luminescent bacterium, *Vibrio harveyi*, frequently related to the outbreaks of luminous vibriosis in cultured *P. monodon* in hatcheries in many countries such as Australia, China, India, Indonesia, Thailand, Philippines, and Taiwan (Vandenberghe et al., 1998). In Thailand, vibriosis is the serious cause of production loss in penaeid shrimp farms (Nash et al., 1992). This bacterial disease causes mortality up to nearly 100% of affected populations: larvae, post-larvae, juveniles, sub-adults and adults (Lightner, 1983). Luminescent vibriosis bacteria, *V. harveyi*, were claimed to be the most causative agent associated with shrimp mortality. *V. harveyi* is a rod shape, Gram-negative bacterium with 0.5-0.8 μm width and 1.4-2.6 μm in length. The bacteria are able to emit a blue-green color light by a reaction catalyzed by luciferase. The substrates are reduced flavin mononucleotide ($FMNH_2$), a long chain aldehyde (RCHO), and oxygen which react according to the following reaction:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + HO + RCOOH + light$$

The disease caused by *V. harveyi* is widely known as luminous disease. The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease. These superficial infections can develop into systemic

infections under some circumstances. These systemic infections can cause mortality. Other gross features of the infected shrimp are milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite, eventually leading to death.

Presumptive diagnosis is made on the basis of clinical signs and culture of the suspensions of hepatopancreas or blood on trytic plate supplemented with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* show strong luminescence in dim light. To avoid drug resistance from use of antibiotic in the control of bacteria, utilization of probiotics such as *Pseudomonas* I-2 and *Bacillus subtilis* BT23 which has been reported to be capable of producing compounds with inhibitory effects on the growth of *V. harveyi* is an interesting alternative strategy to control shrimp pathogenic *Vibrio* in cultured system (Chythanya et al., 2002; Vaseeharan et al., 2003).

1.6 The immune responses in invertebrates

All living organisms have developed immune system for defending themselves against microbial invasion or other foreign substances. Immune system can be evolutionarily classified into two types: adaptive (acquired) and innate (natural) immunity. Vertebrates possess both adaptive and innate immune systems, whereas invertebrates have only innate immune system. The adaptive immune system functions by producing highly specific recognition molecules, known as antibodies, which can memorize foreign molecules after the first exposure. The innate immune system involves a large number of generalized effector molecules.

Innate immune system is a more phylogenetically ancient defense mechanism found in all multicellular microorganisms. This first line of defense helps to limit infection at an early stage and relies on germline-encoded receptors recognizing conserved molecular patterns that are present on the microorganisms (Janeway Jr, 1998). Whereas adaptive immune system is a more sophisticated and complicated mechanism including immunological memory (Lee et al., 2001).

1.7 Crustacean immune system

Crustacean immune system is innate immune system based on cellular and humoral components of the circulatory system (Lee et al., 2002). The hard cuticle covering all external surfaces of crustaceans is the first line of defense between them and the environment. Moreover, the innate immune system can respond rapidly if microorganisms invade the animals. Major defense systems are carried out in the hemolymph containing cells called hemocytes. Hemocytes and plasma protein recognize large groups of pathogens by means of common molecular patterns of particular microbes. Hemocytes as effectors of the cellular immune response are also involved in synthesizing the majority of humoral effectors. Cellular response, the actions with direct participation of blood cells, includes phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting (Jiravanichpaisal et al., 2006). On the contrary, humoral factors act in the defense without direct involvement of the cells even though many of the factors are originally synthesized and stored in the blood cells. These factors include enzyme and proteins involved in prophenoloxidase (proPO) system, clotting proteins, agglutinins, hydrolytic enzymes, proteinase inhibitors, and antimicrobial peptides.

1.7.1 Pattern recognition proteins

When foreign substances attack animals, the first immune process is recognition of a broad spectrum of factors that are released or are present on the surface of invading microorganisms. This process is mediated by the hemocytes and by plasmatic protein. There is little knowledge about the molecular mechanisms that mediate the recognition; however, in crustaceans, several types of modulator proteins identified recognize cell wall components of pathogens. The target recognition of innate immunity, so-called “pattern recognition molecules (PRMs)”, is shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as “pattern recognition proteins or receptors (PRPs or PRRs)”. These patterns include the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram positive bacteria, the mannans of yeasts, the β-1,3-glucan of fungi, and double-stranded RNA of viruses (Hoffmann et al., 1999). Most current research has emphasized the possible

roles of non-self recognition molecules in the vertebrate and the invertebrate immune system.

Carbohydrate recognition is important because carbohydrates are common constituents of microbial cell wall, and microbial carbohydrates have distinct structures from those of carbohydrates of eukaryotic cells. Therefore, LPS or/and β-1,3-glucan binding proteins (LBP, βGBP, or LGBP), peptidoglycan recognition protein (PGRP), several kinds of lectins, and hemolin have been identified in a variety of invertebrates with different biological functions proposed following their binding to their targets (Lee et al., 2002).

In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin that, besides its bacterial agglutination ability, increases phagocytic rate (Vargas-Albores, 1995). Recent findings indicate that, in decapods, LGBP and BGBP have retained the crucial components for glucanase activity, and share a common ancestor with glucan receptors, as well as with the glucanase proteins of a wide range of invertebrates; however, these LGBP and BGBP lack glucanase activity, implying that these proteins might have lost their glucanase properties during evolution, but retained their glucan-binding activity as an adaptive process. The cloned cDNA of LGBP and BGBP from hemocytes and hepatopancreas of the white shrimp *Litopenaeus vannamei* indicates the presence of two putative integrin-binding motifs (Arg–Gly–Asp) and a potential recognition motif for β-1,3 linkage of polysaccharides (Padhi et al., 2008). Quantitative real-time RT-PCR analysis revealed that the LGBP transcript in *L. vannamei* hemocytes increases a few hours after *Vibrio alginolyticus* injection (Cheng et al., 2005), confirming that gene expression of this family of proteins is up-regulated in bacterial and fungal infections; similar findings were obtained by injecting the *P. stylirostris* shrimp with the white spot virus (Roux et al., 2002). In some crustaceans, such as the crayfish, the most important mechanism that activates the proPO system involves the participation of PGBP, LGBP, and BGBP; whereas, in the horseshoe crab, these receptors seem to be less important in the proPO activating system (Iwanaga et al., 2005).

1.7.2 Cell-mediated defense reactions

Cellular defense reactions include such processes as phagocytosis and encapsulation (Millar, 1994). Phagocytosis, a common phenomenon in all organisms, includes foreign body attachment, ingestion and destruction. In crustaceans, phagocytes can be found free in the hemocoel or on the surface of arterioles of the hepatopancreas, and/or in the gills (Iwanaga et al., 2005). In the freshwater crab, *Parachaeraps bicarinatus*, and the shore crab, *Carcinus maenas*, phagocytes are the main cells that participate in the elimination of circulating particles in the hemocoel. Phagocytes of *P. bicarinatus* and *Cherax destructor* recognize particularly Gram-negative bacteria, such as *Pseudomonas* spp. and *E. coli* (McKay et al., 1970) In addition, phagocytes of the American lobster, *Homarus americanus*, recognize only Gram-negative but not Gram-positive bacteria (Mori et al., 2006).

Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. This process kills pathogens or, at least, restricts their movement and growth in the hemocoel cavity. Through histochemical analyses, it has been demonstrated that hemocytes, which participate in the encapsulating process, show acid or neutral mucopolysaccharides and glycoproteins (Rather et al., 1983). Destruction of encapsulated organisms occurs due to the decrease in oxygen concentration and the action of hydrolases, or by the toxic action of quinones (Sodörhäll et al., 1984).

1.7.3 Hemocyte adhesion molecules

In invertebrate immunity, cell adhesion is essential for the cellular immune responses of encapsulation and nodule formation. Blood cells of the crayfish, *Pacifastacus leniusculus*, can release a cell-adhesive and opsonic peroxidase called peroxinectin (Lin et al., 2007). A site containing the motif, KGD, appears to be adhesive by binding to a transmembrane receptor of the integrin family on the blood cells (Johansson et al., 1995). Peroxinectin also binds to a peripheral blood cell surface CuZn-superoxide dismutase. The peroxidase-integrin interaction appears to have evolved early and seems conserved; human myeloperoxidase supports cell adhesion via the αMβ2 integrin. There is evidence for peroxinectin-like proteins in other arthropods. Effects by RGD peptides indicate that integrins mediate blood cell

adhesion and cellular immunity in diverse invertebrate species (Johansson et al., 1989). Other blood cell molecules proposed to be involved in cell adhesion in invertebrates include the insect plasmatocyte-spreading peptide, as well as soluble and transmembrane proteins which show some similarity to vertebrate adhesive or extracellular matrix molecules. Proteins such as the Ig family member hemolin or proteins found in insect hosts for parasitic wasps, inhibit cell adhesion and may regulate or block cellular immunity (Johansson, 1999).

1.7.4 The prophenoloxidase (proPO) system

The proPO activating system is composed of several proteins involved in melanin production, cell adhesion, encapsulation, and phagocytosis (Soderhall et al., 1998; Sritunyalucksana et al., 2000).

In vitro studies have shown that phenoloxidase (PO) exists as an inactive precursor prophenoloxidase (proPO) which is activated by a stepwise process involving serine proteases activated by microbial cell wall components such as low quantities of lipopolysaccharides or peptidoglycans from bacteria, and β-1,3-glucans from fungi through pattern-recognition proteins (PRPs) (Kurata et al., 2006). An enzyme capable of activating the proPO in vivo is called prophenoloxidase activating enzyme (factor) (ppA, PPAE, PPAF). In crayfish, ppA is a trypsin-like proteinase present as an inactive form in the hemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA will convert proPO to an active form, phenoloxidase (PO) (Aspan, 1991; Aspan, 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Soderhall et al., 1998; Shiao et al., 2001). It both catalyses o-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can non-enzymatically polymerise to melanin. PO is a sticky protein and can adhere to the surface of parasites leading to melanisation of the pathogens. Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites such as crayfish plague fungus, *Aphanomyces astaci* (Sodërhäll et al., 1982). The production of forming insoluble melanin deposits involving in the process of sclerotisation, wound healing,

and encapsulation of foreign materials (Theopold et al., 2004). To prevent excessive activation of the proPO cascade, it is needed to be regulated by proteinase inhibitors.

The prophenoloxidase activating enzyme (PPA) is a zymogenic protein (proppA). The C-terminal half of the proppA comprises a typical serine proteinase domain with a sequence similar to other invertebrate and vertebrate serine proteinases. The N-terminal half consists of a cationic glycine-rich domain, a cationic proline-rich domain, and a clip-domain, in which the disulfide-bonding pattern is likely to be identical to those of the horseshoe crab big defensin and mammalian β-defensins. The clip-domains in proppAs may function as antibacterial peptides (Wang et al., 2001).

In penaeid shrimp, enzymes in the proPO system are localized in the semigranular and granular cells (Perazzolo et al., 1997). This is in accordance with a study showing that *P. monodon* proPO mRNA is expressed only in the hemocytes (Sritunyalucksana et al., 2000). From the recent report, RNAi-mediated silencing of *P. monodon* PPAE gene significantly decreased the total PO activity (36.7%) in shrimp and additionally increased the mortality of *V. harveyi* infected shrimp, the latter of which correlated with an increase in the number of viable bacteria in the hemolymph. These results indicate that PPAE functions in the proPO system and is an important component in the shrimp immune system (Charoensapsri et al., 2009).

1.7.5 The coagulation system/ the clotting system

Hemolymph coagulation is a defensive response of crustaceans preventing both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al., 1991). It is a proteolytic cascade activated by microbial cell wall components. The coagulation system involves a plasma-clotting protein (CP) and a hemocyte-derived transglutaminase (TG) (Kopacek et al., 1993; Yeh et al., 1998).

Clotting has been most studied in two non-insect arthropod species with significantly different clotting reactions: freshwater crayfish and horseshoe crab. The clotting system in crayfish depends on the direct tranglutaminase (TGase)-mediated cross linking of a specific plasma protein, whereas the process in horseshoe crab is regulated by a proteolytic cascade activated by bacterial elicitors through specific recognition proteins.

In crayfish, clotting occurs through polymerization of a clotting protein in plasma. The crayfish CP is a dimeric protein of which subunit has both free lysine and glutamine. They are recognized and become covalently linked to each other by a calcium ion dependent TGases (Yeh et al., 1998; Hall et al., 1999; Wang et al., 2001).

CPs are synthesized in the hepatopancreas and released to hemolymph. In crustaceans, CPs were found in several species: the freshwater crayfish (Kopacek et al., 1993), *P. monodon* (Yeh et al., 1998), and the lobster *Panulirus interruptus* (Kollman et al., 2005).

1.7.6 Proteinase inhibitors

Proteinase inhibitors, also produced by the hemocytes, are necessary to protect host from microbial proteinases and regulate the proteinase cascades (the proPO and coagulation system). Function of proteinases in many pathogenic fungi is help the fungi in penetrating the cuticle of their arthropod hosts. Proteinases also contribute to the virulence of bacterial pathogens. Some of the proteinase inhibitors in hemolymph may defend the host against such microbial proteinases. For instance, the silk worm (Bombyx mori) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi et al., 1993). Several of Manduca sexta serpin gene-1 variants inhibit bacterial and fungal serine proteinases (Jiang et al., 1998). Proteinase inhibitors in the cuticle or at the surface of the integument might also function in protection against fungal infection. An external secretion from grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski et al., 1996).

In vertebrates, injury and microbial infection lead to activation of the blood coagulation and proPO systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides), resulting in rapid and efficient responses to the threats to health (O'Brien, 1993; Whaley, 1993). Blood clotting and phenoloxidase activation can also be harmful to the host if they are not limited as local and transient reactions. For this reason, the proteinases in these systems are tightly regulated by proteinase inhibitors.

Like blood clotting, phenoloxidase activation is normally regulated in vivo as a local reaction with brief duration. Also comparable to blood clotting, the regulation may be partly due to serine proteinase inhibitors in plasma (Kanost et al., 1996). Pacifastin and, to the lesser degree, α-macroglobulin inhibit crayfish PPO activation (Aspan et al., 1990). Among the low molecular weight inhibitors from insect hemolymph, Kunitz family inhibitors from *M. sexta*, *Sarcophaga bullata*, and *B. mori* (Sugumaran et al., 1985; Saul et al., 1986; Aso et al., 1994) and the 4 kDa locust inhibitors (Boigegrain et al., 1992) can interfere with PPO activation. Serpin-1J from hemolymph of *M. sexta* inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang et al., 1997). Recently, the *M. sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang et al., 2004). In addition, its structure and function were further characterized by cloning and expression in *E. coli* expression system (Zou et al., 2005) The results indicated that serpin-6 plays important roles in the regulation of immune proteinases in the hemolymph. It is likely that each proteinase in the PPO cascade is regulated by one or more specific inhibitors present in plasma or in hemocyte granules.

Moreover, the recently reported function of kazal-type proteinase inhibitors (KPIs) includes the reproductive process in the fresh water prawn *Macrobrachium rosenbergii* required KPI to inhibit the sperm gelatinolytic activity (Li et al., 2009) and bacteriostatic activity of KPI in *P. monodon* (Donpudsa et al., 2009).

1.8 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are effector molecules that play an important role in innate immune system. In all kingdoms, from bacteria to human, a variety of AMPs have been identified and characterized. Most of the AMPs are small in size, generally less than 150-200 amino acid residues, with amphipathic structure and cationic property. However, the anionic peptides also exist. AMPs have a wide variety and diversity in amino acid sequences, structure, and range of activity. AMPs are active against a large spectrum of microorganisms: bacterial and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Murakami et al., 1991;

Hancock et al., 2000; Tharntada et al., 2008) and may also exhibit an anti-tumor property (Cruciani et al., 1991). Moreover, depending on their distribution, antimicrobial peptide expression appears to be regulated by different tissue-specific pathways, and these effectors may consequently participate in either a local or a systemic reaction. Some constitutively expressed within the secretory cells, others are induced upon microbial stimulation (Hancock et al., 2000).

In general, the net positive charge together with the amphipathic structure account for the preferential binding to the negatively charged membrane interface of microorganisms, which is different from the predominantly zwitterionic surface of normal mammalian cells. Thus, they are toxic to the microbes and not to the mammals. The features required for AMPs are the selective toxicity on microbial cells, short bacterial killing time, broad antimicrobial spectra, and no bacterial resistance development (Matsuzaki, 2001). For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane . This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupture of the membrane or that it perturbs the membrane lipid bilayer, allowing for leakage of certain cellular components as well as dissipating the electrical potential of the membrane (Brogden, 2005).

In arthropods, several antimicrobial peptides were isolated and characterized, mainly in insects especially *Drosophila* and chelicerates (horseshoe crabs) (Iwanaga et al., 2005). In horseshoe crabs, these proteins are mainly synthesized in the hemocyte and are stored within the cytoplasmic granules. The cells are highly sensitive to LPS, a major outer membrane component of Gram-negative bacteria, and respond by degranulating the granules after stimulation by LPS. This antimicrobial system in insect differs from those of the crustaceans in that the fat body of the insects is the main site for the antimicrobial peptide synthesis (Engstrom, 1999; Hoffmann et al., 1999).

Crustaceans as well as the shrimp, lacking the adaptive immunity, rely on only the innate immunity. Therefore, the AMPs, exhibiting broad spectra of antimicrobial activity, are important for them to fight the pathogenic invasion (Hancock et al.,

2000). There are many reports on antimicrobial peptides in crustaceans. In 1997, a small peptide named calliectin observed in the hemolymph of blue crab, *Callinectes sapidus* was reported to be responsible for the majority of antimicrobial activity (Khoo et al., 1999). Penaeidins, a antimicrobial peptides acting against Gram positive bacteria and fungi, had been reported in penaeid shrimp *L. vannamei* (Destoumieux et al., 1997). cDNA clones of penaeidin isoform were also isolated from the hemocytes of *L. vannamei*, *P. setiferus* (Gross et al., 2001), and *P. monodon* (Supungul et al., 2004). Crustins, an antimicrobial peptide, were identified from 2 species of *Penaeid shrimp*: *L. vannamei* and *L. setiferus*. Several isoforms of crustins were observed in both shrimp species. The 11.5 kDa antibacterial protein from *Carcinus maenas*, crustins from shrimp showed no homology with other known antibacterial peptides, but possessed sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Peptides derived from the hemocyanin of *L. vannamei*, *P. stylirostris*, and *P. monodon* possessing antiviral activity has also been identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Moreover, histones and histone derived peptides of *L. vannamei* has been also reported as innate immune effectors because they can inhibit growth of Gram-positive bacteria (Patat et al., 2004).

1.9 Crustin

Crustins are defined as cysteine-rich antibacterial polypeptide of ca. 7-14 kDa, with an isoelectric point usually in the range of 7.0-8.7, containing a whey acidic protein (WAP) domain at the carboxyl terminus (Smith et al., 2008). This domain has eight cysteine residues in a conserved arrangement that form a tightly packed structure (4DSC). The term ‘WAP’ is derived from the name given to a family of proteins, originally discovered in the whey fraction of mammalian milk. All crustins described to date possess a leader/signal sequence at the N-terminus and the WAP domain at the carboxyl end (Figure 1.1). The cleavage site, which marks the end of the signal sequence, at least as predicted by software programs, is usually between alanine and glycine, although in some crustins it lies between glycine and glutamine, alanine or threonine. It is unclear if the signal sequence is directing trans-membrane transportation of the protein, as in insects and mammals, or if the mature protein is

released from the haemocytes through regulated exocytosis, as seems to be the case with the penaeidins (Destoumieux et al., 2000; Mun?oz et al., 2002). The WAP domain, in contrast to the signal sequence, is highly conserved between species and in several crustins, especially those from shrimp, aspartic acid and lysine residues are positioned as follows:

-C-XX-D-XX-C-XXXD-K-CC-X-D-

This arrangement, however, is not true in every case. For example, glycine substitutes for aspartic acid after the first cysteine in a *L. vannamei* EST (AAS57715), and serine replaces lysine before the double cysteine in the *Callinectes sapidus* EST (CV462984). The region between the signal sequence and WAP domain is variable but conforms to one of a small number of distinct structural patterns with regard to the presence or absence of other domains. The arrangements of these are largely, but not entirely, conserved within taxonomic sub-groups of arrangements within the decapoda. At least three main subgroups appear to exist and we propose that they should be designated Types I–III (Figure 1.1) at least until future research, especially from non-decapod taxa, renders this classification redundant (Smith et al., 2008). Type I crustins have the region that lies between the signal sequence and the WAP domain is of variable length and cysteine-rich but has more than six of these residues. Type II crustins, on the other hand, possess not only a cys-rich region but also a long gly-rich domain of approximately 40–80 aa adjacent to the signal region (Figure 1.1). A third group of WAP domain-containing proteins from decapods resembles crustins but lack not only the gly-rich domain of the Type II molecules but also the cys-rich region present in both Type I and Type II.

The first crustin, carcinin, was isolated from hemocytes of the shore crab, *Carcinus maenas*, and found to be active against only Gram-positive bacteria (Schnapp et al., 1996; Relf et al., 1999). Subsequently, genomic approaches, such as expressed sequence tag (EST) analysis, have identified several homologs of crustin from a variety of crustacean species including shrimps, crabs, crayfish and lobsters (see review by Smith et al. (2008)). Type I crustins are mainly found in crabs, lobsters and crayfish. Whilst Type II and III crustins are mainly present in shrimps.

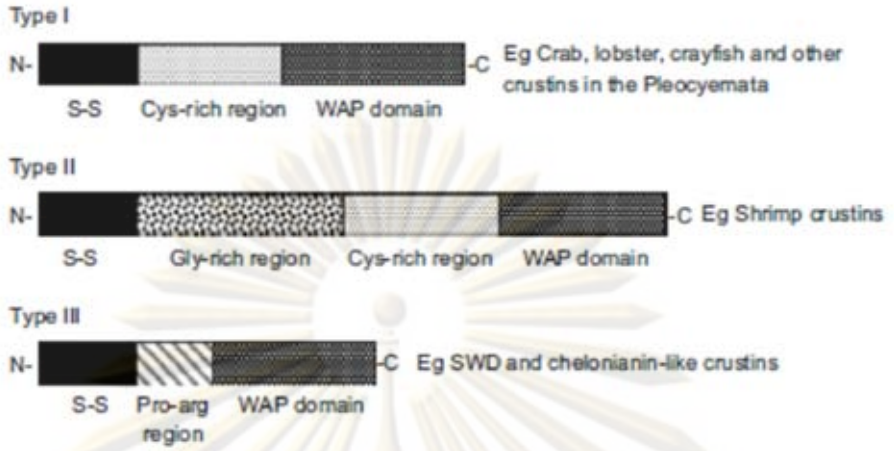


Figure 1.1 Schematic representation (not to scale) of the domain organisation of the three main crustin types from decapods. Signal sequence (ss) (Smith et al., 2008).

Despite the reports of several crustin sequences, only a few studies have described their antimicrobial properties, and these have reported bactericidal or bacteriostatic activity towards mainly or only Gram-positive bacteria (Relf et al., 1999; Zhang et al., 2007; Supungul et al., 2008).

In the black tiger shrimp, *Penaeus monodon*, different isoforms of Type II crustins have been identified by homology screening the sequences of non-redundant EST libraries (Supungul et al., 2004; Tassanakajon et al., 2006). The two major isoforms so found, crustin*Pm*1 and crustin-like*Pm*, were expressed as recombinant proteins in *Escherichia coli* and characterized for their antimicrobial properties (Amparyup et al., 2008; Supungul et al., 2008). Crustin*Pm*1 displays antimicrobial activity against only Gram-positive bacteria whilst crustin-like*Pm* inhibits the growth of both Gram-positive and Gram-negative bacteria, including *Vibrio harveyi*, a shrimp pathogen.

1.10 Objective of the dissertation

In this study, a unique isoform of *P. monodon* crustin, crustin*Pm*5, was characterized. This crustin was identified from the gill-epipodite cDNA library while the other isoforms were mainly expressed in hemocyte. The genomic structure and transcription regulation of crustin*Pm*5 were investigated. The recombinant crustin*Pm*5 protein was produced and further characterized for its antimicrobial activity.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave model # MLS-3750 (SANYO E&E Europe (UK Branch) UK Co.)

Automatic micropipette P10, P20, P100, P200, and P1000 (Gilson Medical Electrical)

Balance PB303-s (Mettler Teledo)

Biological safty cabinets (Nuaire)

Biophotometer (Eppendrof)

Centrifuge 5804R (Eppendrof)

Centrifuge Avanti^{TM} J-301 (Beckman Coulter)

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Thermo Electron Corporation)

Gel document (Syngene)

GelMate2000 (Toyobo)

Gene pulser (Bio-RAD)

Hoefer^{TM} miniVE (Amersham Biosciences)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Microplate reader: FLUOstar OPTIMA (BMG Labtech)

Pipette tips (10, 20, 200, and 1000 µl (Axygen)

Power supply: Power PAC 300 (Bio-RAD Laboratories)

Refigerated incubator shaker (New Brunswick Scientific)

Thermal cycler mastercycler gradient (Eppendrof)

Touch mixer model # 232 (Fisher Scientific)

96 well cell culture cluster, flat bottom with lid (Costar)

2.1.2 Chemicals and regents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Ampicillin (Biobasic)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, X-gal (Fermentus)

Bromophenol blue (BDH)

Calcium chloride, $CaCl_2$ (Merck)

Chloramphenicol (Biobasic)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue R-250, $C_{45}H_{44}N_3O_7S_2Na$ (Sigma)

100 mM dATP, dCTP, dGTP, and dTTP (Promega)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA), disodium salt dehydrate (Fluka)

Ficoll™

Formaldehyde, CH_2O (BDH)

GeneRuler™ 100 bp DNA Ladder

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine, NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (Merck)

Isopropanol, C_3H_7OH (Merck)

Isopropyl-beta-D-thiogalactopyranoside, IPTG (Fermentas)

Methanol, CH_3OH (Merck)

N,N'-methylene-bisacrylamide, $C_7H_{10}N_2O_2$ (USB)

2-mercaptoethanol, C_2H_6OS (Fluka)

0.22 μm millipore membrane filter (Millipore)

Prestained protein molecular weight marker (Fermentus)

Potassium chloride, KCl (Ajax)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajax)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate, $C_{12}H_{25}O_4SNa$ (Sigma)

Sodium dihydrogen orthophosphate, $NaH_2PO_4.H_2O$ (Carlo Erba)

di-Sodium hydrogen orthophosphate, Na_2HPO_4 (Carlo Erba)

Sodium hydroxide, NaOH (Eka Nobel)

TRIreagent (Molecular biology)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

2.1.3 Enzymes

DNaseI (Promega)

DyNazyme II DNA^{TM} polymerase (Finnzymes, Finland)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (Promega)

Taq DNA polymerase (Fermentas)

Pfu polymerase (Promega)

2.1.4 Microorganisms

Escherichia coli BL21 (DE3)

Escherichia coli strain XL-I blue

Escherichia coli strain 363

Salmonella typhimurium

Klebsiella pneumoniae

Enterobacter cloacae

Erwinia carotovora

Aerococcus viridans

Micrococcus luteus

Bacillus megaterium

Staphylococcus aureus

Staphylococcus haemolyticus

Vibrio harveyi 639

2.1.5 Kits

GenomeWalker™ Universal Kit (Clontech)

HiYield™ Gel/PCR Fragments Extraction Kit (Real Genomics)

ImProm-II™ Reverse Transcription System (Promega)

Qiaprep® Spin Miniprep Kit (QIAGEN)

2.1.6 Vectors

pGEM®-T Easy vector (Promega)

pET-19b (Novagen)

2.2 Softwares

BlastX (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

ClustalX (Thompson et al., 1997a)

GENETYX (Software Development Inc.)

PHYLIP (Felsenstein, 1993a)

SECentral (Scientific & Education software)

SignalP (http://www.cbs.dtu.dk/services/SignalP/)

SMART (http://smart.embl-heidelberg.de/)

Genetools (SYNGENE)

2.3 Samples

Black tiger shrimp *Penaeus monodon* 16 to 20 g body weight, purchased from local market, were used for heat and osmotic stress experiment and 3 to 5 g body weight, purchased from shrimp farms in Surat Thani province, were used for gene-silencing experiment.

2.4 cDNA sequence analysis

DNA sequences were edited and translated using the GENETYX software program (Software Development Inc.). Putative motifs and domains were investigated using SMART program. Related sequences were aligned using ClustalX program (Thompson et al., 1997b). The potential cleavage site of the signal peptide was predicted by SignalP software (http://cbs.dtu.dk/services/SignalP/). Aligned

sequences were bootstrapped 1000 times using Seqboot. Boostrapped neighbour-joining trees were constructed using Neighbour and Consense. All phylogenetic reconstruction programs are routine in PHYLIP (Felsenstein, 1993b). Trees were appropriately illustrated using TreeView (http://taxonomy.zoology.gla.ac.uk/rod.html).

2.5 Tissue expression analysis of Crustin*Pm*5 by semi-quantitative RT-PCR

2.5.1 Tissue collection

Gill, epipodite, antennal gland, heart, hepatopancreas, intestine, lymphoid organ, and eyestalk were isolated separately from an individual normal shrimp and immediately frozen in liquid nitrogen (-176ºC). Hemocytes were prepared from hemolymph. Hemolymph was collected from the ventral sinus of shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 µl of anticoagulant (10% (w/v) sodium citrate). Hemolymph was immediately centrifuged at 800 ×g for 10 min at 4ºC to separate hemocytes from the plasma. The tissue and hemocyte samples were briefly homogenized by a pestle in 1 ml of ice-cold Trizol reagent (Gibco BRL) and kept in -70ºC for total RNA extraction.

2.5.2 Total RNA extraction

Total RNA from tissues or hemocytes of black tiger shrimp were extracted using Trizol reagent as described by (Chomczynski et al., 1987). Tissues or hemocyte samples were homogenized in 1 ml of Trizol reagent. The homogenized samples were incubated at room temperature for 5 min to completely dissociate of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, the mixture was shaked vigorously by hand for 15 seconds and incubated at room temperature for two to three minutes. The samples were centrifuged at 12000 ×g for 10 min at 4°C. The mixture was separated into a lower red, phenol chloroform phase and colorless aqueous phase. The RNA remains in the aqueous phase. The aqueous phase was transferred into a new 1.5 ml centrifuge tube and the same volume of isopropanol was added to precipitate RNA. The samples were incubated for 10 min at room temperature and centrifuged at 12000 ×g for 15 minutes at 4°C. The RNA pellet was washed with 1 ml of cold 75% ethanol and centrifuged at 12000 ×g for 15 minutes at

4°C. The ethanol solution was discarded and the RNA pellets were air-dried for 5-10 min. RNA samples were dissolved with DEPC-treated water and stored at -70°C.

The total RNA was further treated with 1 µl of RQ1 RNase-free DNase (Promega, 1 unit/µl) at 37°C for 30 min to remove the contaminated DNA. The total RNA was extracted again with Trizol reagent as mention above. The RNA pellet was preserved in 75% ethanol at -20°C until use. Before use, the RNA pellet was air-dried and resuspended in appropriate volume of DEPC-treated water.

2.5.3 Determination of the quantity and quality of RNA samples

The quantity and quality of total RNA was spectrophotometrically measured at 260 nm and analyzed by formaldehyde-agarose gel electrophoresis, respectively. The concentration of total RNA could be determined in ng/µl using the following formular:

$$[\text{RNA}] = A_{260} \text{ x dilution factor x } 40$$

An absorbant unit at 260 nm corresponds to approximately 40 ng/µl of RNA (Sambrook et al., 1989). The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at the wavelength of 280 nm. The good quality of RNA sample should have an $A_{260/280}$ ratio above 1.7.

The quality of the extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis as following described. A 1.2% (w/v) formaldehyde agarose gel was prepared in 1x MOPS buffer (final concentration of 0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0). The gel slurry was heated until completely dissolving and placed to cool down at room temp before formaldehyde (0.66 M final concentration) was added. Then, the melted formaldehyde-agarose gel was poured into a chamber set and applied the comb. The RNA marker and RNA samples were prepared under the denaturing condition. The RNA sample in DECP-treated water, 7.4 M of formamide, 1.64 M of formaldehyde, 1x MOPS and DECP-treated water to a final volume of 12 µl were heated at 70 °C for 10 min and the mixtures were immediately chilled on ice. After that, three microliters of the 5x RNA loading dye buffer containing 50% (v/v) glycerol, 1mM EDTA, pH8.0, 0.25% (w/v) bromophenol

blue, 0.25% (w/v) xylene cyanol FF and 0.025% (w/v) ethidium bromide was added to each sample and loaded to formaldehyde-agarose gel. Electrophoresis was run in 1x MOPS buffer at 100 volts for 50 min. Sizes of RNA were visualized under a UV transilluminator by comparing with a standard RNA marker (Promega).

2.5.4 First stranded cDNA synthesis

The 1st standed cDNA was generated using 1 µg of total RNA and 0.5 µg of oligo (dT_{20}) primer with an ImProm-II™ Reverse Transcription system kit (Promega). The reaction was incubated at 70°C for 5 min and immediately placed on ice for 5 min. After that, 4 µl of 5x reaction buffer, 2.6 µl of 25 mM $MgCl_2$, 1 µl of dNTP Mix (10 mM each), 20 units of Ribonuclease inhibitor and 1 µl of ImProm-II reverse transcriptase were added and the reaction mixture was gently mixed. The reaction was incubated at 25°C for 5 minutes and at 42°C for 60 min. Then, the reaction was incubated at 70°C for 15 min to terminate reverse transcriptase actively.

2.5.5 Semi-quantification of mRNA expression by RT-PCR

Semi-quantitative RT-PCR was used to examine tissue specific expression and expression pattern of crustin*Pm*5 mRNA. Total RNA from shrimp tissues was extracted and then subjected to cDNA synthesis as described above. The β-actin gene expression was used as the internal control. The PCR conditions were set up as described below.

2.5.5.1 PCR conditions

Each PCR reactions were carried out in a total volume of 25 µl containing 10 mM Tris–HCl, pH8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, optimal concentration of $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, or 0.2 mM of each primer for β-actin, 1.5 unit of DyNAzyme II DNA Polymerase (Finnzymes) and an optimal concentration of template cDNA or 3 µl of 10-fold diluted template cDNA for β-actin using a Thermal cycler mastercycler gradient (Eppendrof). The reactions were predenatured at 94ºC for 2 min followed by optimal cycles or 25 cycles for β-actin of denaturation at 94 ºC for 30 sec, annealing at an

optimal temperature or 52ºC for β-actin for 30 sec and extension at 72°C for 1 min. The final extension was at 72ºC for 5 min. The PCR products were analyzed using a TBE 2% agarose gel electrophoresis. The PCR parameters were optimized as follows: 1) The optimal $MgCl_2$ concentration between 0.5-3.0 mM $MgCl_2$ was examined using the standard PCR conditions. The concentration that gave the highest yield and specificity was chosen. 2) The PCR amplifications were carried out at different cycle numbers: 20, 24, 28, 32, 36 and 40. After amplifications, PCR products were run on 1.2% agarose gel. The number of cycles that amplified the PCR product in the exponential range and did not reach a plateau level was chosen. 3) The optimal amount of cDNA template was examined using the standard PCR condition. The concentration that gave the highest yield and specificity was chosen. 4) The optimal primer annealing temperature was adjusted to increase amplification specificity of each primer pair. Annealing temperatures that gave the best results was used.

The primer name, annealing temperatures and cycle numbers for RT-PCR are shown in Table 2.2. The PCR amplification was carried out in a Thermal cycler mastercycler gradient (Eppendrof). The reactions were performed in triplicate. The PCR products were analyzed by electrophoresis on 1.2% agarose gel.

2.5.5.2 Agarose gel electrophoresis and quantitative analysis

The 1.2% (w/v) agarose gel was prepared using 1× TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH8.0). The slurry of agarose in 1× TBE buffer was melted in a microwave oven until completely dissolved. The solution was allowed to cool at room temperature to 60ºC before pouring into a casting tray with a well-forming comb. The gel was submerged in a chamber containing enough amount of 1× TBE buffer for covering the gel.

The PCR products were mixed with one-sixth volume of 6× loading dye (0.25% bromophenol blue and 25% Ficoll in water) before loading into the well. A DNA ladder (100 bp marker) was used as standard DNA markers. Electrophoresis was carried out in 1× TBE buffer at 100 volts for 25 min. The gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 1 min and destained to remove excess EtBr by washing with distilled water for 15 min. The PCR product was visualized under a UV transilluminator and photographed.

Table 2.1 Primer sequence using for cloning strategies

Primer name	Sequence
	Primers for RT-PCR
RT-crus1F	5' TGTTCTGCGTCCTGGCGATG 3'
RT-crus1R	5' TGGCTTGCACACGTGTTGTC 3'
RT-crus5F	5' GCTGTGACCGATGGACAGTA 3'
RT-crus5R	5' CGTCATGCAAGTGACAAGAG 3'
RT-crus7F	5' GTTCATCGCACAGCCGAGAG 3'
RT-crus7R	5' TGCAGACGGTGTCGTTCAAG 3'
Actin-F	5' GCTTGCTGATCCACATCTGCT 3'
Actin-R	5' ATCACCATCGGCAACGAGA 3'
	Primers for Genome walk
GSP-crus5F	5' ATGCGAGTGGCGGGATATCT 3'
GSP-crus5R	5' CTACCGGCCGTTGTAGTAGG 3'
GSP2	5' CACGGACGCCACGGCCACTACTAGATA 3'
GSP3	5' ACCGGCCCCATCGTGCCAATACATAC 3'
GSP2-1	5' ACCTGGTTGCTGAACGTCTGTATGTCTA 3'
GSP2-2	5' ACAACAACGGTATCCTCATACACCTCAT 3'
Full-crus5F	5' ATCCTACATACCGTCACATGAA 3'
Full-crus5R	5' CTACCGGCCGTTGTAGTAGG 3'
	Primers for recombinant protein expression
0671pET19+HisF	5' **CCATGG**GCCATCATCATCATCATCACCAGTACATTGGATT CGGCGTG 3'
0671pETR	5' G**CTCGAG**GATCCCTACCGGCCGTTGTAGTAGG 3'

* Restriction site are shown as bold text.

Table 2.2 PCR condition for RT-PCR

Gene	Primer name	Annealing temperature (ºC)	Cycle number
β-actin	Actin-F Actin-R	55	25
crustin*Pm*1	RT-crus1F RT-crus1R	58	25
crustin*Pm*5	RT-crus5F RT-crus5R	58	40
crustin-like*Pm*	RT-crus7F RT-crus7R	58	25

The intensity of bands was measured and normalized relative to that of β-actin using the commercial image analysis software package (GeneSnap and GeneTools, SynGene). The data were then subjected to statistical analysis for comparison between groups. Significantly different expression levels were treated using One Way Analysis of Variance (ANOVA) following by a post t hoc test (Duncan's new multiple range test). Significant differences were indicated at $p< 0.05$.

2.6 Determination of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA expression levels after stress treatment

2.6.1 Heat-shock treatment

The experimental *P. monodon* shrimp (~20 g wet body mass) were acclimatized at 28±1 °C for one week before heat treatment. Nine shrimp were transferred to the tank at 33±1 °C for 2 h and allowed to recover at 28±1 °C for 6 h. For the control group, nine shrimp were maintained in the tank at 28±1 °C. After heat treatment, shrimp tissues were collected. Total RNA was extracted and cDNA was synthesized as describe above.

2.6.2 Salinity changes

P. monodon (about 20 g of body mass) were maintained in a 500-liter tank with aeration at 25 ppt salinity for two weeks. They were then separated into 3 groups; the control group (25 ppt), the groups reared in low salinity (3 ppt) and in high salinity (40 ppt). Epipodites were excised and collected from three individual shrimps per treatment group. The samples from the control group were collected at zero time point whereas the samples from the low salinity and the high salinity groups were collected at 6 h, 24 h and 2 weeks. Total RNA was extracted and cDNA was synthesized as mention above.

2.6.3 Semi-quantification of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* expression by RT-PCR

The expression level of crustin*Pm*5 transcripts was determined by RT-PCR analysis as described above. Transcript levels of the two other *P. monodon* crustin homologs, crustin*Pm*1 and crustin-like*Pm*, were assayed by RT-PCR as described before (Amparyup et al., 2008; Supungul et al., 2008) using specific primers of crustin*Pm*1 and crustin-like*Pm* (Table 2.1). The crustin gene transcripts from control and heat-induced shrimp were normalized against the expression of β-actin. The relative expression levels of heat-induced shrimp were also normalized against the expression of the control shrimp and tested using one-way analysis of variance (ANOVA) (Steel et al., 1980) followed by Duncan's new multiple range test (Duncan, 1955) using SPSS software.

2.7 Genomic organization of crustin*Pm*5 gene

2.7.1 Preparation of *P. monodon* gemonic DNA

Genomic DNA was prepared from the pleopods of *P. monodon* using phenol-chloroform extraction. The pleopods were homogenized in 700 µl of extraction buffer (100 mM Tris buffer pH8.0, 100 mM EDTA pH8.0, 250 mM NaCl, 1% (w/v) SDS, and 100 µg/ml Proteinase K) and incubated overnight at 65ºC. Then, 5 µg of RNase A were added into the lysate. Then, the samples were incubated for 30 min at 37ºC, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) by inverting and mixing for 20 min and centrifuged for 10 min at 5,000 rpm. The upper phase was

transferred to a new tube, extracted with chloroform:isoamyl alcohol (24:1) by inverting and mixing for 20 min and centrifuged for 10 min at 5,000 rpm. The upper phase was transferred to a new tube. The genomic DNA was precipitated with two volumes of cold absolute ethanol. The mixture was centrifuged at 5,000 rpm for 1 min. The genomic DNA was washed with 70% ethanol, air-dried, and then dissolved in 50 µl of TE buffer (10 mM Tris-HCl pH8.0 and 1 mM EDTA pH8.0).

2.7.2 Quality of genomic DNA

The quality of genomic DNA was checked by 0.6% agarose gel electrophoresis. One microliter of experimental genomic DNA (0.1 µg/µl) and 1 µl of control genomic DNA (0.1 µg/µl) were loaded and run on 0.6% agarose gel in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under the UV light to estimate the size of product. A good genomic DNA preparation should contain DNA larger than 50 kb with minimum smearing.

2.7.3 Amplification and cloning of genomic crustin*Pm*5 genes

The primers, GSP-crus5F and GSP-crus5R (Table 2.1), designed from the cDNA sequences were used for the amplification of the corresponding genomic DNA sequences. The genomic DNA (50 ng) was used for the PCR amplification in 50 µl reaction containing 1 unit Advantage 2 Polymerase Mix (Clonetech), 1× Advantage 2 PCR buffer, 200 µM of dNTP, 0.2 µM each of the appropriate forward and reverse primers. The initial denaturation was at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 58ºC for 45 sec, elongation at 72ºC for 3 min; and the final extension at 72ºC for 10 min. The 5 µl of PCR products were analyzed on 1.2% agarose gel in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under the UV light to determine the size of PCR product. The expected PCR product was purified using NucleoSpin® Extract II Kits (MACHEREY-NAGEL). The expected DNA band was excised and purified from agaorse gel.

2.7.4 Agarose gel electrophoresis

The PCR products were electrophoretically analysed by agarose gel which was prepared by melting the slurry 1.2% (W/V) agarose gel in 1x TBE (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH8.0) using the microwave oven. After the gel solution was cool down at 55°C pouring into a tray and applied the well comb. Before loading into the gel, the PCR products were combined with 1/10 volumes of the 10x loading dye (0.25% bromophenol blue, 0.25% Xylene cyanol FF, and 25% Ficoll in water). The GeneRuler™ 100 bp and 1 kb DNA Ladder plus (fementas) were used for identifying of the DNA size. The agarose gel electrophoresis was performed in 1x TBE buffer at 100 volts until the lower bromophenol blue dye migrated about 3/4 of the gel length. After that, the gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 30 sec and destained by submerged in distilled water for 10 min. Ethidium bromide-stained PCR products were visualized under the UV transilluminator.

2.7.5 Purification of PCR product from agarose gel

The expected bands were purified from agarose gel by NucleoSpin® Extract II Kits (MACHEREY-NAGEL) as described below: The expected product was excised from the gel using a clean sharp scalpel, and then the weight of the gel slice was determined. The gel slice was completely dissolved in three volumes of NT buffer containing chaotropic salt at 60°C. The sample was then loaded into the column and centrifuged at 12,000x g for 1 min to remove the supernatant. The column was washed with 500 μl of NT2 buffer and centrifuged as described above. Six hundred microliters of NT3 buffer were added into the column and centrifuged. The additional centrifugation was used for completely removal of the NT3 buffer containing ethanol. The column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 40 μl of elution NE buffer (5 mM Tris-Cl, pH8.5) and stood at room temperature for 1 min before centrifugation. The eluted DNA was then stored at -20°C until used.

2.7.6 Cloning of DNA fragment into pGEM-T Easy

The DNA fragment was ligated into pGEM-T Easy vector (Figure 2.1). The pGEM-T Easy vector was 3,015 bp in length and had unique restriction sites in the multiple cloning region flanked by T7 and SP6 RNA promoters, therefore T7 and SP6 primers can be used to identify the recombinant clone. The reaction was composed of 5 µl of 2x Rapid ligation buffer, 1 µl of pGEM®-T Easy Vector (50 ng), proper amount of PCR product, 1 µl of T4 DNA ligase (3 Weiss units/µl), and deionized water to a final volume of 10 µl. The reactions were mixed by pipetting, briefly spun and incubated at 4°C overnight. The appropriate amount of insert in the ligation reaction was calculated following equation:

$$\text{ng of insert} = \left(\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} \right)$$

2.7.7 Competent cells preparation

The starter of *E. coli* strain XL-1 blue was prepared from a single colony cultured in 10 ml of LB broth (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) and cultured at 37°C with shaking at 250 rpm overnight. One percent of starter was inoculated into 1 L of LB broth and incubated at 37°C with vigorous shaking for 3-5 h until OD_{600} of the cells reached 0.5-0.7. Cells were then chilled on ice for 15-30 min and harvested by centrifugation at 5,000x g for 10 min at 4°C. The supernatant was removed as much as possible. The cell pellet was washed twice time with cold sterilized water and followed by cold sterilized 10% (v/v) glycerol with gently mixing and centrifugation. The pellet was suspended in cold sterilized 10% (v/v) glycerol to a final volume of 2-3 ml. This cell suspension was divided into 40 µl aliquots and stored at -80°C until used.

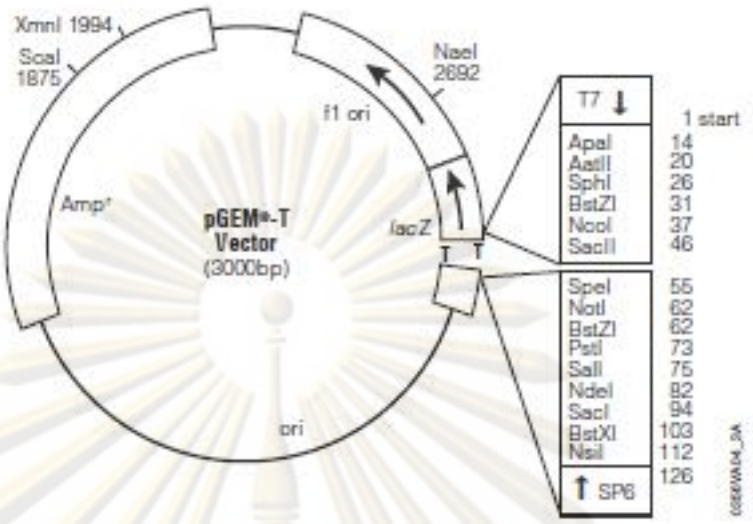


Figure 2.1 pGEM® -T easy vector map and multiple cloning site sequences (Promega)

2.7.8 Electrotransformation

The ligation reaction was transformed to *Escherichia coli* XL-1 Blue. The competent cells were gently thawed on ice, mixed with 1 µl of ligation mixture and then placed on ice for 1 min. The mixture was transformed by electroporation in a cold 0.2 cm cuvette with setting the apparatus as follows: 25 µF of the Gene pulser, 200 Ω of the pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After electroporation, SOC medium (2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was immediately added to the cuvette and quickly resuspended cells. The cell suspension was transferred to a new tube and incubated at 37 °C with shaking at 250 rpm for an hour. One hundred microliters of transformant was then spread onto a LB agar plate containing 100 µg/ml of amplicillin, 20 µg/ml of X-gal and 30 µg/ml of IPTG and then incubated at 37°C for overnight. After incubation, the recombinant clone was identified by colony PCR using universal T7 and SP6 primers.

2.7.9 Screening of transformant by colony PCR

White colonies were picked and screened for the inserts by colony PCR. The amplification was carried out in a 20 µl reaction volume containing 1x PCR

buffer, 200 µM of dNTP mix, 0.25 µM of T7 and SP6 primers, 2.5 units of DyNAzyme™ II DNA polymerase. The single colony was diluted in 10 µl sterilized water. One microliter of colony suspension was employed as the template in the PCR reaction. The PCR profile was performed at 94 °C for 3 min, 30 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis.

2.7.10 Plasmid DNA extraction using QIAprep® Miniprep kit

The plasmid was isolated from the positive clones by a QIAprep® Miniprep kits described in Qiagen's handbook. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane under high salt condition. Firstly, bacterial cells were harvested by centrifugation and resuspend in 250 µl P1 buffer containing RNase A. Next, the 250 µl P2 buffer was added and the reaction was mixed thoroughly by inverting the tube 4–6 times for cell lyses. The cell lysate was neutralized by adding 350 µl N3 buffer. After maximum speed centrifugation for 10 min, the supernatant containing the plasmid was applied to column by pipetting. The column was centrifuged for 30–60 s, and then the flow-through was discarded. The QIAprep spin column was washed twice by adding 0.5 ml Buffer PB and 0.75 ml Buffer PE, respectively, and then centrifuged to remove residual ethanol from PE Buffer. Finally, the QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl EB buffer (10 mM Tris-HCl, pH8.5) to the center of each column. After incubation at room temperature for 1 min, the eluted fraction was collected by centrifugation for 1 min.

2.7.11 Detection of the recombinant plasmid

The recombinant plasmid contained interested gene was examined with restriction enzyme digestion using *Eco*RI. The digested plasmid was analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp and 1kb ladder marker). The recombinant plasmid was sequenced by an automatic DNA sequencer at the Macrogen Inc.

2.7.12 Genomic DNA library construction

Four genomic DNA libraries were constructed by digesting the genomic DNA separately with four blunt-end restriction enzymes (Figure 2.2). In each reaction, 2.5 µg of genomic DNA was digested in 100-µl reaction with 80 units of restriction enzyme (*Dra*I, *Eco*RV, *Pvu*II or *Stu*I) and 1× restriction enzyme buffer. The digestion mixtures were incubated for 2 h at 37ºC. The reactions were then vortexed at slow speed for 5-10 min and incubated further at 37ºC overnight. Each digestion reaction was analyzed by running 5 µl on 0.6% agarose gel electrophoresis to determine whether the digestion was completed.

Then, each reaction tube was added an equal volume (95 µl) of phenol and vortexed at slow speed for 5-10 sec. The mixture was briefly spun at room temperature to separate the aqueous and organic phases. The upper aqueous phase was transferred into a new tube. The upper phase was then added an equal volume (95 µl) of chloroform and vortexed at slow speed for 5-10 sec. The mixture was briefly spun at room temperature to separate the aqueous and organic phases. The upper phase was transferred into a new tube. The upper phase was added two volumes (190 µl) of ice cold 95% ethanol, 1/10 volume (9.5 µl) of 3 M NaOAc (pH4.5) and 20 µg of glycogen. The mixture was vortex at slow speed for 5-10 sec and centrifuged at 14,000 rpm for 15 min at 4ºC. The supernatant was decanted and the pellet was washed in 100 µl of ice cold 80% ethanol. The supernatant was decanted and the pellet was air-dried . The pellet was dissolved in 20 µl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH7.5) and vortexed at slow speed for 5-10 sec. To determine the approximate quantity of DNA after purification, 1 µl of each reaction was run on 0.6% agarose gel electrophoresis.

The genomic DNA fragments from the four digestion reactions were ligated with GenomieWalker™ adaptors. For each ligation reaction, the digest was added to 1.9 µl of 25 µM GenomeWalker adaptor, 1.6 µl of 10× ligation buffer and 0.5 µl of T4 DNA ligase (6 units/µl). The reaction mixture was incubated overnight at 16ºC. The reactions were stopped by incubating at 70ºC for 5 min. Each reaction was added 72 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and vortexed at slow speed for 10-15 sec. The four libraries were named *Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries.

2.7.13 Determination of the 5' upstream sequences of crustin*Pm*5 gene

To “walk” along the genomic DNA, two PCR amplifications were done for each library. The first or primary PCR uses the outer adaptor primer (AP1) provided in the kit and an outer, gene-specific primer (GSP1) designed from the known sequences. The primary PCR products were, then, used as template for the secondary or nested PCR using the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). The secondary PCR product begins from the known sequence at the 5' end of GSP2 and extends into the unknown adjacent genomic DNA (Figure 2.2).

The gene-specific primers (GSP2, GSP3, GSP2-1 and GSP2-2 (Table 2.1) was designed from the sequences close to the 5' end of the known genomic sequence. For primary PCR amplification, 0.4 µl of each library was used in a 20 µl reaction volume containing 1 unit DyNAzyme II DNA polymerase (Finnzyms), 1× buffer, 0.2 mM of dNTP, 0.2 µM of AP1 and GSP2 or GSP2-1. The initial denaturation was at 94ºC for 2 min, followed by 40 cycles at 94ºC for 30 sec, and 72ºC for 5 min and the final extension at 72ºC for 5 min. The primary PCR product was diluted 50 folds (1 µl into 49 µl) with water. The secondary PCR amplification used 0.4 µl of the diluted primary PCR product in a 20 µl reaction volume containing 1 unit DyNAzyme II DNA polymerase (Finnzyms), 1× buffer, 0.2 mM of dNTP, 0.2 µM of AP2 and GSP3 or GSP2-2. The initial denaturation was at 94ºC for 2 min, followed by 5 cycles at 94ºC for 45 sec, 68ºC for 45 sec, and 72ºC for 5 min; 30 cycles at 94ºC for 45 sec, 65ºC for 45 sec, and 72ºC for 5 min; and final extension at 72ºC for 10 min. The secondary PCR products were cloned into the pGEM®-T easy vector (Promega) and subsequently sequenced.

The primers Full-crus5F and Full-crus5R were designed to amplify the whole crustin*Pm*5 genomic DNA segments containing both the 5' regulatory sequences and the genes. The PCR products were cloned, sequenced and analyzed in order to confirm the contiguous nature of the DNA fragments obtained from the genome walking technique.

The putative promoters, 5' *cis*-regulatory elements in the 5' upstream sequences and the putative start sites of the crustin*Pm*5 gene were predicted by using

the MATCH™/TRANFAC program (http://www.gene-regulation.com) (Biobase GmbH) and the Neural Network Promoter Prediction (http://www.fruitfly.org /seq_tools/promoter.html) (Reese, 2001), respectively.

2.8 Recombinant protein expression for crustin*Pm*5

2.8.1 Construction of the protein expression clones

To express the recombinant crustin*Pm*5, gene specific primers containing restriction site, 0671pET19+HisF and 0671pETR were designed. The primers were designed for the amplification of gene fragment encoding the mature proteins without signal peptides. A plasmid containing crustin*Pm*5 gene was used as template. The included *Xho*I and *Not*I sites at the 5' and 3' ends of the gene fragments respectively were for cloning into the expression vector. The crustin*Pm*5 gene fragments were PCR amplified in a final reaction volume of 50 µl containing 0.02 ng of plasmid template, 0.4 M of each primer, 0.2 mM of each dNTP and 3 units of *Pfu* polymerase (Promega). The PCR amplification was carried out at 94ºC for 2 min, followed by 30 cycles of 94ºC for 30 sec, 58ºC for 30 sec and 72ºC for 1 min, and final extension at 72ºC for 7 min. The amplified products were analyzed using 1.2% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The *Xho*I-*Not*I fragments containing the crustin*Pm*5 was prepared from the T&A clones and subcloned into the *Nco*I–*Xho*I digested pET-19b expression vector (Novagen) (Figure 2.3). The expression clone was named RecCrus*Pm*5.

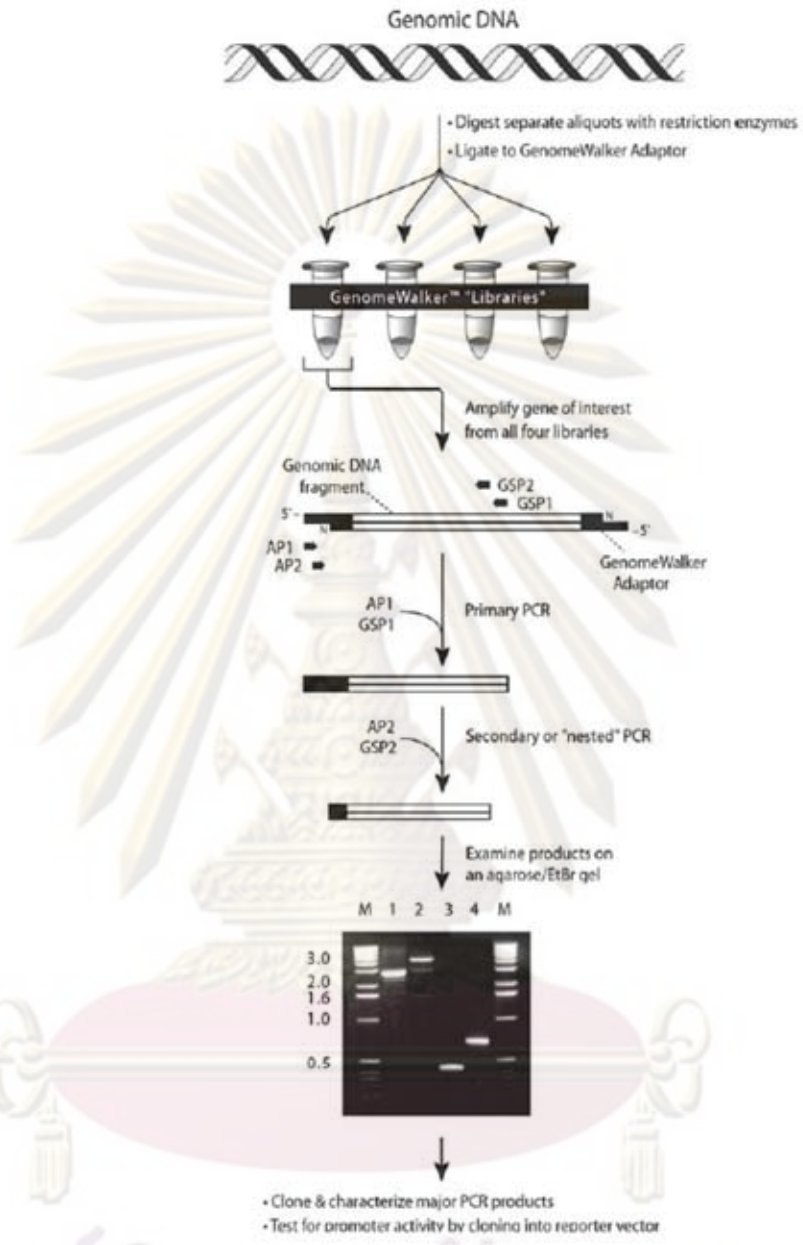


Figure 2.2 Flow chart of the BD GenomeWalker™ protocol. The genomic libraries were constructed for use as templates for nested PCR. (Source: www.bdbiosciences.com)

2.8.2 A-tailing procedure for blunt-ended PCR fragments

Thermostable DNA polymerases with proofreading activity, *Pfu* DNA polymerase, generate blunt-ended fragments during PCR amplification. Nevertheless, the PCR fragment can be modified using the A-tailing procedure for cloning into the T&A cloning vector. The purified DNA fragment was tailed with an adenine nucleotide. The 10 µl reaction consisted of 6 µl of purified PCR fragment generated by a proofreading polymerase, 1 µl of 10 mM Tris–HCl, pH8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 1 mM $MgCl_2$, 0.2 mM of dATP, 5 unit of DyNAzyme II DNA polymerase (Finnzymes) and water. The reaction was incubated at 70ºC for 30 min.

2.8.3 Recombinant protein expression

The expression plasmid RecCrusPm5 were transformed into the expression host, *Escherichia coli* BL21 (DE3). The starter culture was prepared by inoculating a single colony from a freshly streaked plate into a 2 ml LB medium containing 100 µg/ml of ampicillin and incubating at 37ºC overnight with shaking at 250 rpm.

The starter was diluted 1:100 into two cultures, namely uninduced culture and induced culture, supplemented with antibiotics and incubated at 37ºC with shaking at 250 rpm until the OD600 reached 0.6. Protein expression was induced by the addition of 1 M isopropyl-β-D-thiogalactopyranoside to a 1 mM final concentration. The two cultures were incubated at 37ºC further with shaking for 0, 1, 2, 3 and 4 h, respectively. The 2 ml cultures were aliquot at each time point and the pellets collected by centrifugation at 10,000 ×g for 10 min at 4ºC. The cell pellets were stored at -80ºC for further analysis.

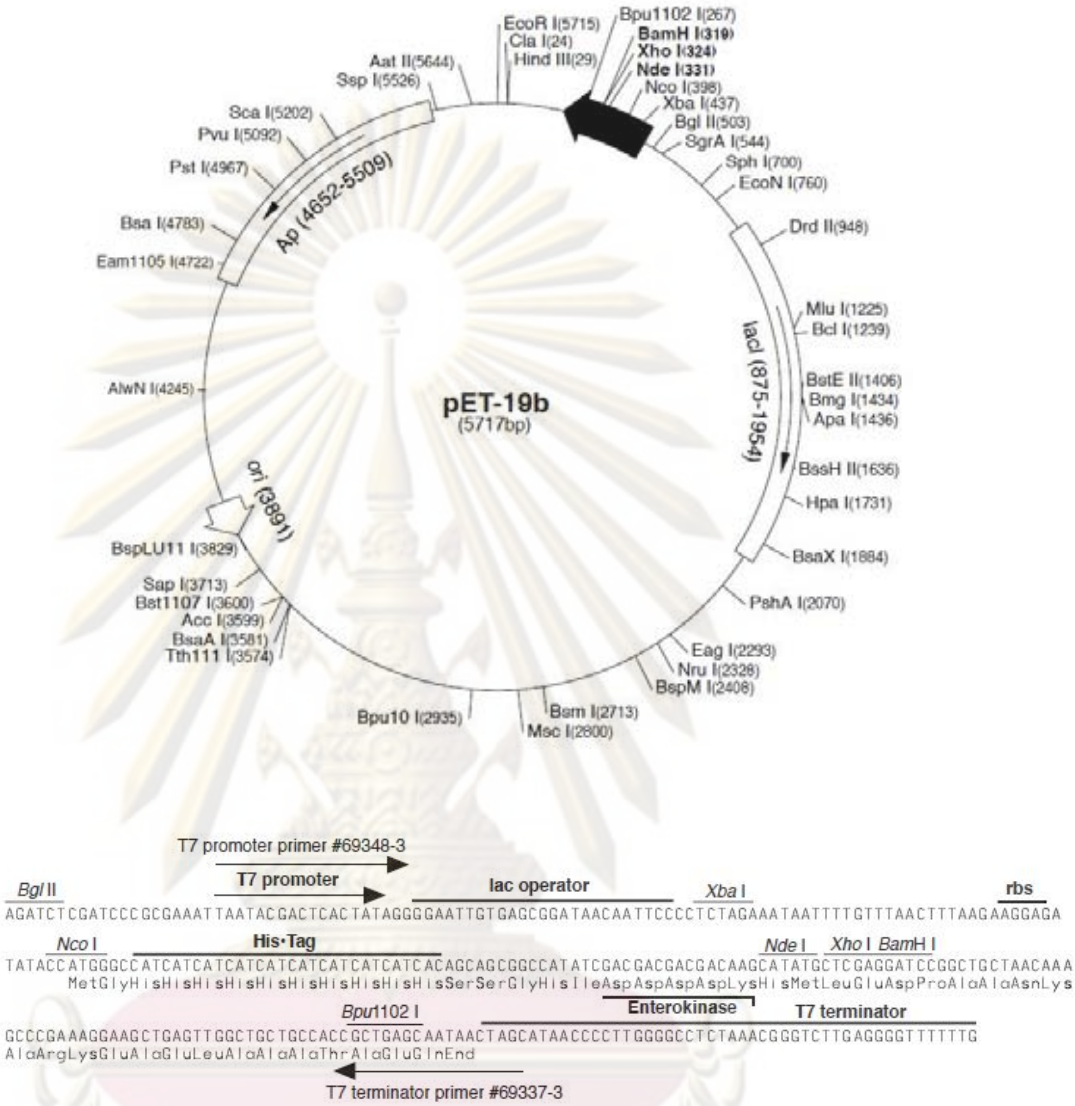


Figure 2.3 pET-19b vector map and sequences in and around the multiple cloning sites (Novagen)

2.8.4 Purification of recombinant proteins

Cells were harvested at 3 h after IPTG induction by centrifugation at 10,000 ×g for 10 min at 4ºC. The cell pellet was collected and resuspended in 1× PBS buffer, pH7.4 by pipetting up and down. The cell suspension was disrupted by French Press at 9000 psi (Jorgensen et al.,1995). The cell lysate was centrifuged at 12,000 × g for 15 min at 4°C to collect the inclusion bodies. The inclusion bodies were wash one time with 20 mM phosphate buffer, pH7.4 containing 1% TritonX-100 following with phosphate buffer, pH7.4 without TritonX-100. The washed inclusion bodies were solubilized in denaturing solution (20 mM phosphate buffer, pH7.4, 8 M urea, 0.5 M NaCl and 20 mM imidazol). The recombinant protein solution was purified under denaturing condition using nickel affinity chromatography (GE Healthcare).

The Ni-NTA agarose was packed into the PD-10 column and washed 3 times with distilled water and binding buffer (20 mM phosphate buffer, pH7.4, 8 M urea and containing 20 mM imidazole), respectively. The denatured protein solution was loaded into the PD-10 column at room temperature. The flow through was collected by a gravity flow. The column was washed with binding buffer to remove unbound proteins. After washing, the protein was eluted with an elution buffer (20 mM phosphate buffer, pH7.4, containing 100 mM imidazole). The presence and purity of the purified protein was evaluated by 18% SDS-PAGE. The imidazole was removed by dialysis for at least 10 h at 4ºC against 50 mM phosphate buffer pH12 twice.

2.8.5 Protein analysis

2.8.5.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A discontinuous system of SDS-PAGE was used. The gel solutions were prepared as shown in the Appendices. After the glass plates and spacers were assembled, the components of the separation gel solution were mixed thoroughly and pipette into the gel plate setting. Then, a small amount of distilled water was careful layered over the top of the separation gel solution to ensure that a flat surface of gel be obtained. When the polymerization was complete, water was poured off. The stacking gel solution was prepared, mixed thoroughly and poured on

top of the separating gel. A comb was placed in position with excess gel solution overflowing the front glass plate. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with distilled water to remove excess unpolymerized acrylamide.

Protein samples were prepared by resuspending the proteins in 1× sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2.88 mM 0.02% bromophenol blue, 2-mercaptoethanol). The samples were then boiled for 10 min and either held at room temperature or kept on ice until loaded into the gel.

After boiling, the protein samples and the prestained protein marker were loaded into the wells. Electrophoresis was conducted in 1× running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) at a constant current of 20 mA until the tracking dye (bromophenol blue) reached the bottom of the separating gel.

The gel was placed in Coomassie blue staining solution (0.1% (w/v) Coomassie brillianr blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol) at room temperature with gentle shaking for 1 h, immersed in destaining solution (10% (v/v) acetic acid, 10% (v/v) methanol) and incubated at room temperature with agitation for 1-3 h. Destaining solution was replaced regularly to assist the removal of stain.

2.8.5.2 Amount of protein determination

The protein content was measured according to Bradford method (Bradford, 1976) using bovine serum albumin (Fluka) as the standard (Appendix C). This method is base on the binding of coomassie brilliant blue G 250 dye to proteins. When the dye binds to proteins, the red form of dye is converted to the blue color. One hundread microliters of diluted sample solution was mixed with Bradford working buffer and left for 2 min before the absorbance at 595 nm was measured. The Bradford working buffer (100 ml) was a mixture of 6 ml Bradford stock solution (350 g Coomassie blue G250, 100 ml 95% ethanol and 200 ml 85% phosphoric acid), 3 ml 95% ethanol, 6 ml 85% phosphoric acid and 85 ml distilled water.

2.8.6 Immunoblotting analysis

For immunoblotting analysis, separated proteins on SDS-PAGE were electrotransferred to a nitrocellulose membrane in a transfer buffer containing 48 mM Tris-HCl pH 9.2, 39 mM glycine, 20% methanol using Trans-Blot® SD (Bio-Rad) (Figure 2.4) at 100 mA for 1 h. The membrane, gel and filter papers were soaked in transfer buffer before they were consequently laid on Trans-Blot® SD. The filter paper was placed on platform, followed by membrane, gel and filter paper, respectively as the model shown in Figure 2.7. The membrane was blocked by immersion in 5% skimmed milk in phosphate buffer saline pH 7.4 containing 0.05% Tween20 (PBS-Tween buffer) at room temperature overnight with orbital shaking, washed in PBS-Tween buffer, then incubated with the mouse anti-His antiserum (GE Healthcare) (1:3000 dilution in 1% skimmed milk in PBS-Tween buffer) at 37 °C for an hour. After washing in the same buffer, bound primary antibody was amplified by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:5000 dilution, Jackson Immuno Research Laboratories, Inc.) for an hour and after washing (as above) the bound antibody was detected by color development using NBT/BCIP (Fermentas) as substrate dissolving in 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$ pH 9.5.

2.9 Characterization of recombinant protein

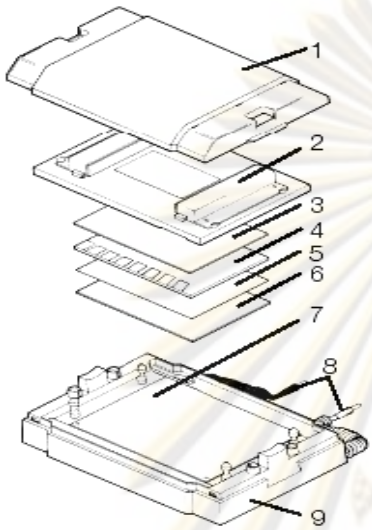
2.9.1 MALDI-TOF mass spectrometry

The molecular mass of the purified recombinant crustin*Pm*5 protein was analyzed by Matrix Assisted Laser Desorption-Ionization Time of Flight (MALDI-TOF) mass spectrometry by the commercial service of the Proteomic Service Center, Bioservice Unit (BIOTEC, Thailand).

2.9.2 Antimicrobial activity assay

The bacterial strains used in this study included Gram-negative bacteria, *Escherichia coli* 363, *Salmonella thyphimurium*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Erwinia carotovora*; Gram-positive bacteria, *Aerococcus viridans*, *Micrococcus luteus*, *Bacillus megaterium*, *Staphylococcus aureus*.

Moreover, marine pathogenic organisms for shrimps were also considered including *V. harveyi* 639.



1. Safety lid
2. Cathode assembly with latches
3. Filter paper
4. Gel
5. Membrane
6. Filter paper
7. Spring-loaded anode platform mounted on 4 guideposts
8. Power cables
9. Base

Figure 2.4 Exploded view of the Trans-Blot® SD (Bio-Rad).

Antimicrobial activities and determination of minimum inhibitory concentration (MIC) values was determined by liquid growth inhibition assays as previously described (Destoumieux et al., 1999). Ten microliters of each diluted peptide, or sterile deionised water as a control, were incubated in a sterile micro titration plates with 100 µl of a suspension of mid-logarithmic growth phase culture of bacteria diluted in culture medium to OD_{600} = 0.001. Poor-broth nutrient medium (1% bactotryptone, 0.5% NaCl, pH 7.5) was used for standard bacterial strains cultures. For marine bacteria strains, they were grown in saline peptone water (1.5% peptone, 1.5% NaCl, pH 7.2). Bacteria were grown overnight under vigorous shaking at 30°C or 37°C according to the strains and measured at 600 nm. The MIC value was recorded as the range between the highest concentration of the peptide where bacterial growth was observed and the lowest concentration that caused 100% of inhibition bacterial growth.

CHAPTER III
RESULTS

3.1 Sequence analysis of crustin*Pm*5 cDNA from *Penaeus monodon*

Six types of crustin genes (crustin*Pm*1, crustin*Pm*2, crustin*Pm*3-4, crustin*Pm*5, crustin*Pm*6 and crustin-like*Pm*) were identified from *Penaeus monodon* EST database (http://pmonodon.biotec.or.th). These crustin cDNAs were mainly found in the hemocytes cDNA library, except crustin*Pm*5 which was identified from gill-epipodite cDNA library of heat-induced shrimp. The full-length cDNA of crustin*Pm*5 had a predicted open reading frame of 510 bp in length encoding a polypeptide of 169 amino acids (Figure 3.1A). Sequence analysis using the signalP program revealed the presence of a signal peptide with 19 amino acids, resulting in a 150 residue mature protein with a calculated molecular mass of 15.8 kDa and a predicted pI of 7.82. The deduced amino acid sequence of crustin*Pm*5 showed typical characters of the shrimp TypeII crustin (Smith et al., 2008) that contain a long gly-rich domain adjacent to the signal region followed by a cys-rich region and a C-terminus whey acidic protein (WAP) domain and exhibited 38% and 37% overall sequence identity with crustin*Pm*1 (Supungul et al., 2008) and crustin-like*Pm* (Amparyup et al., 2008), respectively. Sequence alignment using the ClustalX program (Thompson et al., 1997) revealed a relatively large variation in the Gly-rich and the Cys-rich regions at the amino terminus and a more conserved carboxyl terminus containing eight cysteine residues which form a four disulfide core (4DSC) or a WAP domain (Figure 3.1B). However, the WAP domain of each crustin isoform is quite distinct sharing about 57% to 58% identity. To determine the relationship of these *P. monodon* crustins and other crustacean crustins, a phylogenetic tree was constructed using the boostrapped neighbour-joining method to compare the amino acid sequence of the WAP domain. The result showed three separate groups of crustin: TypeI, TypeII and TypeIII (single WAP, SWD) (Figure 3.2). *P. monodon* crustins are grouped with other shrimp TypeII crustin but can be separated into 4 different subgroups. The first subgroup consists of crustin*Pm*1 and crustin*Pm*2. The

second one consists of crustin*Pm*3-4 and crustin*Pm*6. The third and the last one consist of crustin*Pm*5 and crustin-like*Pm* (crustin*Pm*7).

(A)

```
ATGCGAGTGGCGGGATATCTAGTAGTGGCCGTGGCGTCCGTGGCTGTGACCGATGGACAG
 M  R  V  A  G  Y  L  V  V  A  V  A  S  V  A  V  T  D  G  Q
TACATTGGATTCGGCGTGCCGGGCCAAGGGTTAGTGGACAGCCTGAACGGTCTCATCAGT
 Y  I  G  F  G  V  P  G  Q  G  L  V  D  S  L  N  G  L  I  S
GGAGGGGGTTTCCCAGGAGGCCATTTCCCAGGCCAAGGAGGCCATTTCCCAGGCCAGGGA
 G  G  G  F  P  G  G  H  F  P  G  Q  G  G  H  F  P  G  Q  G
GGCCATTTCCCAGGCCAAGGAGGAAATTTCCCTGGCCAAGGAGGCAACTATCCAGGCCAA
 G  H  F  P  G  Q  G  G  N  F  P  G  Q  G  G  N  Y  P  G  Q
GGAAGCTGCAAATACTGGTGCAGGTCACCCGAGAATCAGTATTACTGCTGTGACCGGGGC
 G  S  C  K  Y  W  C  R  S  P  E  N  Q  Y  Y  C  C  D  R  G
AACAACCAGGGCCAAGGAAACTATCCGGGAAGCAAACCAGGTTTCTGCCCCGCTGTGCGG
 N  N  Q  G  Q  G  N  Y  P  G  S  K  P  G  F  C  P  A  V  R
GACGTCTGCCCCCCGACCCGCTTCGGTGTCGGCCGCCCCATCCAGTGCGCCCACGACGGC
 D  V  C  P  P  T  R  F  G  V  G  R  P  I  Q  C  A  H  D  G
CAGTGCTACGCCTCAAACGACAAATGCTGCTTCGACAGATGCCTCGGGGAACACGTGTGC
 Q  C  Y  A  S  N  D  K  C  C  F  D  R  C  L  G  E  H  V  C
AAGCCCGCTACCTACTACAACGGCCGGTAG
 K  P  A  T  Y  Y  N  G  R  *
```

(B)

```
crustinPm5    MRVAGYLVVAVASVAVTDGQYIGFGVPGQGLVDSLNGLISGGGFPGGHFPGQGG-HFPGQ
Crus-likePm   --MLKFVVLSVVAVAVVHAQ-------DKGNADTR--FLGGLGVPGGGVPGVGGGFLPG-
crustinPm1    --MKGLGVILFCVLAMASAQ---------SWHGGR-----PGGFPGGGRP--GG--FPG-
                :    *: .  :*:. .*         .  .          *.***  *  **  :**

crustinPm5    GGHFPGQGGNFPGQGGNYPG-QGSCKYWCRSPENQYYCCDRGNNQGQGNYPGSKPGFCPA
Crus-likePm   ---VPGHGGVVPGGGGLLPGGQFECNY-CRTRYG-YVCC-------------KPGRCPQ
crustinPm1    ----GGRPGGRPGGFPSVTAPPASCRRWCETPENAFYCCESRYEPEAP--VGTKILDCPK
                  *: *  **     ..   .*.  *.:  . : **              *   **

crustinPm5    VRDVCPPTR-FGVGRPIQCAHDGQCYASNDKCCFDRCLGEHVCKPATYYNGR-
Crus-likePm   IRDTCPGLR-KGVP---ICRQDTDCPGS-DKCCFDTCLNDTVCKPIVAGSQG-
crustinPm1    VRDTCPPVRFLAVEQPVPCSSDYKCGGL-DKCCFDRCLGQHVCKPPSFYEFFA
               :**.**  *  .*    *  * .* .  ****** **.: ****    .
```

Figure 3.1 (A) Nucleotide and deduced amino acid sequences of crustin*Pm*5. An asterisk indicates the stop codon. The putative signal peptides are underlined. The shaded amino acids are the predicted WAP domain. The conserved 12 Cys residues are in boxes. (B) Multiple alignments of deduced amino acid sequences of crustin*Pm*1, 5 and crus-like*Pm* were performed using the Clustal X program. The putative signal peptides are underlined. The Gly-rich region, Cys-rich region and the WAP domain are indicated in shaded amino acid, box and black box, respectively. An asterisk indicates amino acid identity, and (.) and (:) indicate amino acid similarity.

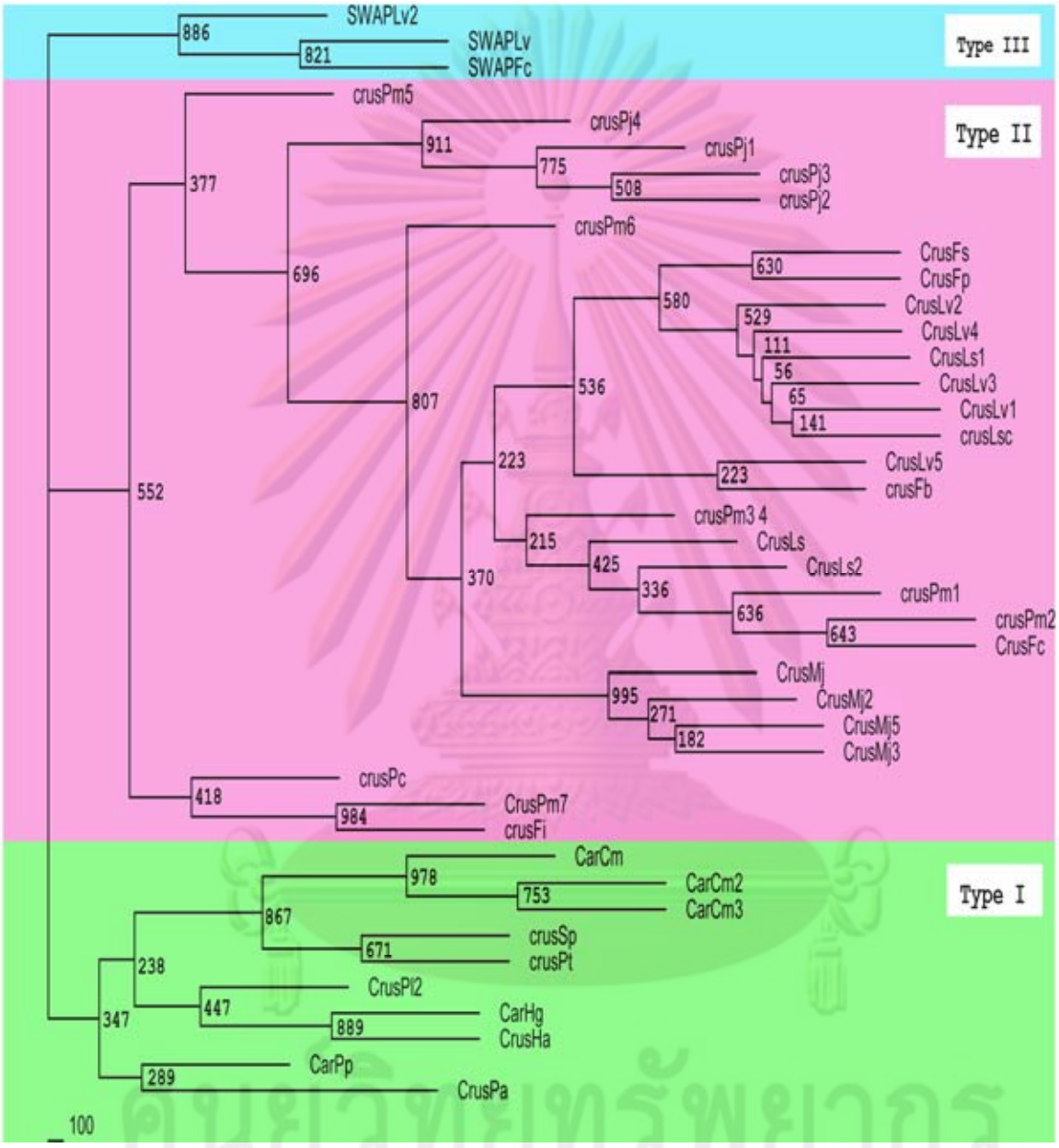


Figure 3.2 Phylogenetic tree of WAP domain sequences. The number at the nodes corresponds to bootstrap proportions. *Lv*, *Litopenaeus vannamei*; *Pm*, *Penaeus monodon*; *Fc*, *Fenneropenaeus chinensis*; *Fs*, *Fenneropenaeus setiferus*; *Mj*, *Marsupenaeus japonicus*; *Ls*, *Litopenaeus setiferus*; *Ha*, *Hyas araneus*; *Hg*, *Homarus gammarus*; *Pa*, *Panulirus argus* ; *Pl*, *Pacifastacus leniusculus*; *Pp*, *Portunus pelagicus*; *Cm*, *Carcinus maenas*; *Pt*, *Portunus trituberculatus*; *Pc*, *Paralithodes camtschaticus*; *Fb*, *Farfantepenaeus brasiliensis*.

3.2 Organization of crustin*Pm*5 gene

3.2.1 Determination of introns and exons of crustin*Pm*5 gene

To determine introns and exons within the ORF of crustin*Pm*5 gene, the genomic DNA was amplified using gene specific primers, GSP-crus5F and GSP-crus5R (Table 2.1), designed from the 5′ and 3′ end of the crustin*Pm*5 ORF. The 773 bp PCR fragment was acquired and sequenced (Figure 3.3). The genomic sequence was then analyzed by comparing to the cDNA sequence. The result revealed that curstin*Pm*5 contained three exons (75, 242 and 193 bp) and two introns (148 and 116 bp) within the coding region (Figure 3.7).

Figure 3.3 Agarose gel electrophoresis of PCR product of crustin*Pm*5 amplified from the shrimp genomic DNA analyzed on a 1.5% agarose gel.

Lane M: GeneRuler™ 1 kb DNA Ladders (Fermentas)
Lanes 1: Negative control (without DNA template)
Lanes 2-4: PCR product from amplification of shrimp genomic DNA

The genome walking technique was used to identify further into the untranslated region of crustin*Pm*5. Two gene specific primers, GSP2 and GSP3 (Table 2.1), designed from the first exon of the ORF region (Figure 3.7) were used to amplify the four genomic libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). Four PCR fragments were obtained from the secondary PCR using GSP2 primer. As shown in Figure 3.4, the PCR products of 600, 400, 800 and 850 bp were amplified from *Eco*RV, *Pvu*II and *Stu*I libraries. The PCR products were cloned and sequenced. The result revealed that only PCR product of 600 bp amplified from *Eco*RV library contained the correct sequence composing of one intron (606 bp) inserted between the 5′ untranslated region (UTR).

Totally, the genomic sequence of crustin*Pm*5 gene of *P. monodon* contained four exons (31, 75, 242 and 194 bp, respectively) separated by three introns (606, 148 and 116 bp, respectively) (Figure 3.7), conforming with the canonical GT/AG splicing recognition rule at the extreme ends of each intron. The 5′-UTR was located in the first exon and the first nucleotide of the second exon, whereas the protein-coding region including the stop codon is contained in the remaining exons (Figure 3.8). Interestingly, two long stretches of pure dinucleotide (CT) repeats, $(CT)_{15}$ and $(CT)_{20}$, amongst a CT and $(GT)_2(CT)_n$ rich region were found in the first intron, but the degree of polymorphism of this compound microsatellite region remains to be established.

3.2.2 Upstream regulatory region of crustin*Pm*5 gene

After the exon-intron finding, the 5′ upstream sequence was determined to localize the promoter and regulatory elements. The crustin*Pm*5 gene-specific primers, GSP2-1 and GSP2-2 were designed from the known gene sequences close to the 5′ end (Table 2.1). The nested PCR technique was used for the amplification of DNA libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). The primary and secondary PCR products of crustin*Pm*5 were analyzed by agarose gel electrophoresis (Figure 3.5). The secondary PCR of crustin*Pm*5 genome walking showed the PCR product of 700 bp in *Dra*I library. The PCR product was then cloned and subsequently sequenced. The genomic sequence was confirmed by amplification of the upstream region and the whole genomic segments. Analysis of the obtained genomic provided information of the regulatory sequences as shown in Figure 3.6.

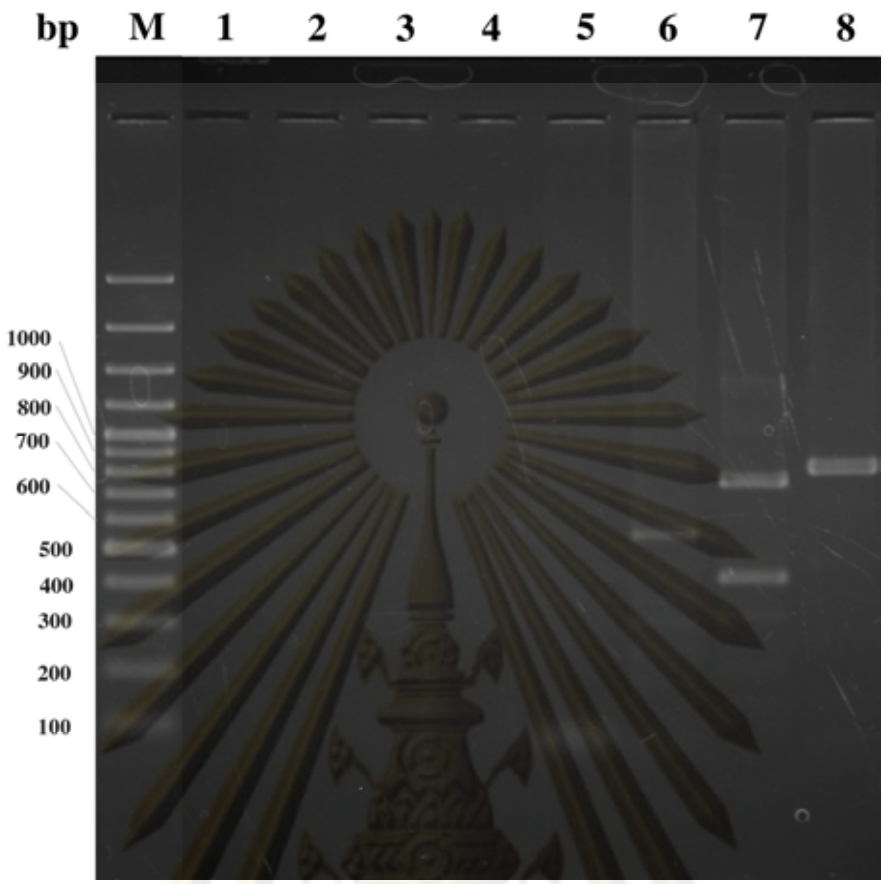


Figure 3.4 Agarose gel electrophoresis of the primary and secondary PCR product of the first genome walking of crustin*Pm*5 gene amplified from the four genomic libraries. The products were run on 1.2% agarose gel.

Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas)
Lane 1: The primary PCR product from *Dra*I library
Lane 2: The primary PCR product from *Eco*RV library
Lane 3: The primary PCR product from *Pvu*II library
Lane 4: The primary PCR product from *Stu*I library
Lane 5: The secondary PCR product from *Dra*I library
Lane 6: The secondary PCR product from *Eco*RV library
Lane 7: The secondary PCR product from *Pvu*II library
Lane 8: The secondary PCR product from *Stu*I library

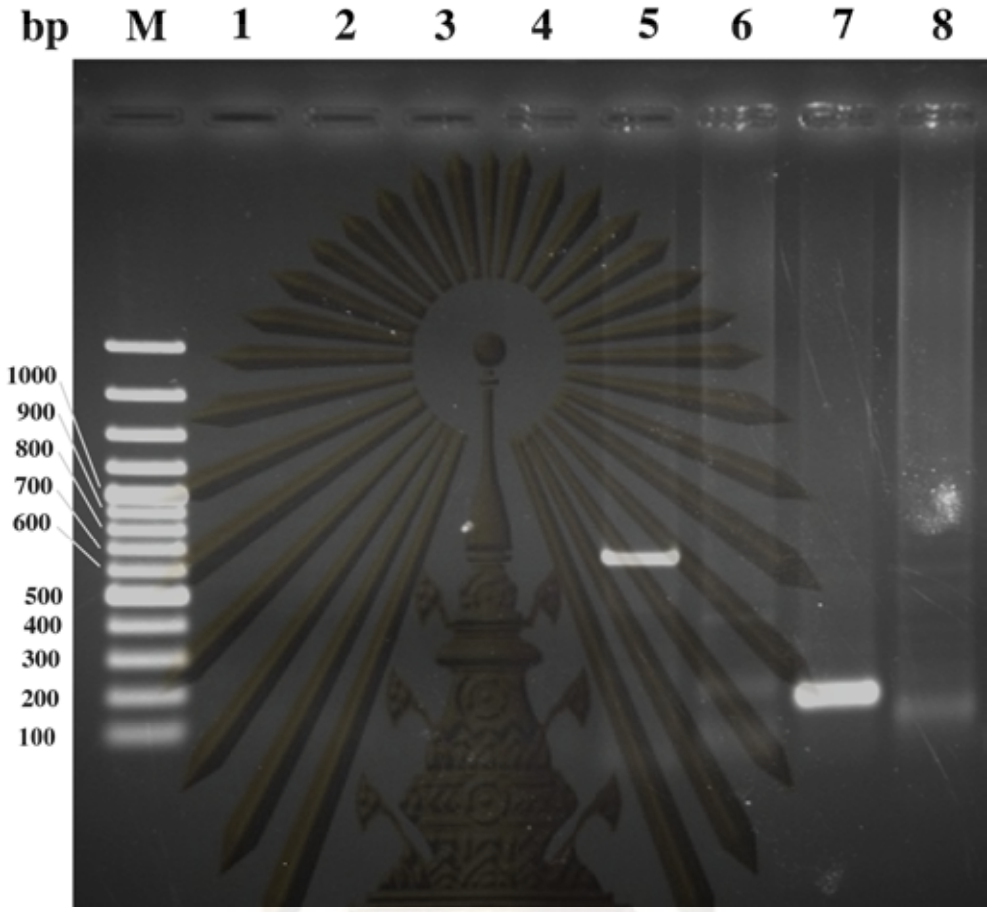


Figure 3.5 Agarose gel electrophoresis of the primary and secondary PCR product of the second genome walking of crustin*Pm*5 gene amplified from the four genomic libraries. The products were run on 1.2% agarose gel.

Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas)
Lane 1: The primary PCR product from *Dra*I library
Lane 2: The primary PCR product from *Eco*RV library
Lane 3: The primary PCR product from *Pvu*II library
Lane 4: The primary PCR product from *Stu*I library
Lane 5: The secondary PCR product from *Dra*I library
Lane 6: The secondary PCR product from *Eco*RV library
Lane 7: The secondary PCR product from *Pvu*II library
Lane 8: The secondary PCR product from *Stu*I library

The gene organization of *P. monodon* crustin*Pm*5 gene starting from the putative pre-mRNA start sites was predicted using the Neural Network Promoter Prediction (Reese, 2001). The upstream sequence analysis revealed a putative core promoter region at about 39 base pairs upstream (−39 region) from the putative transcriptional start site. The 5′ upstream sequences were analyzed for the putative *cis*-regulatory elements by using the MATCH™ analysis program searching the sequences against the TRANSFAC database (Heinemeyer et al., 1998) with the cut-offs for both core and matrix similarities of 0.9. Some potential binding sites of important transcription factors were predicted including two NF-κB, one complete heat-shock regulatory element (HSE), and five GATA factor binding sites (Figure 3.8).

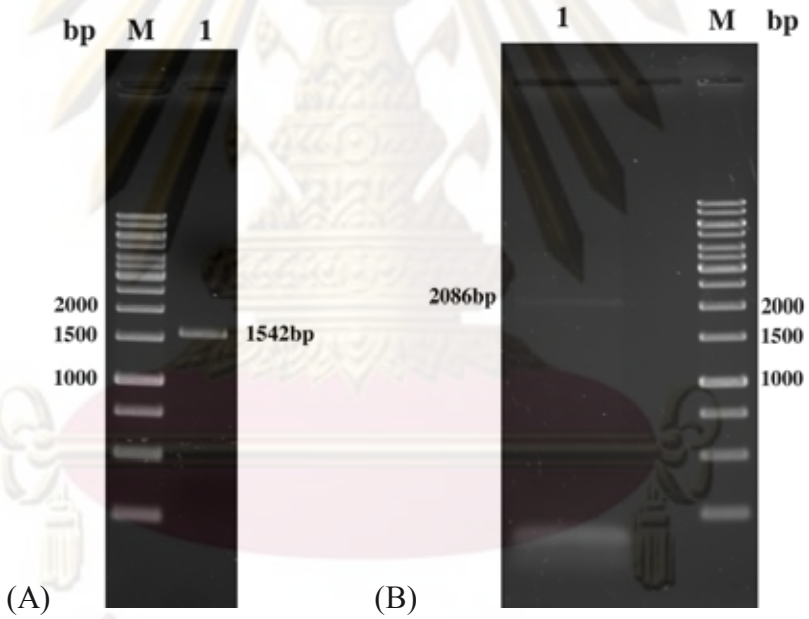


Figure 3.6 Agarose gel electrophoresis of PCR product of upstream region of crustin*Pm*5 amplified from the genomic DNA. The PCR product was run on 1.2% agarose gel.

(A) Lane M: GeneRuler™ 1 kb DNA Ladders (Fermentas)
Lane 1: The PCR product of upstream region of crustin*Pm*5 gene
(B) Lane M: GeneRuler™ 1 kb DNA Ladders (Fermentas)
Lane 1: The PCR product of whole genomic segment of crustin*Pm*5 gene

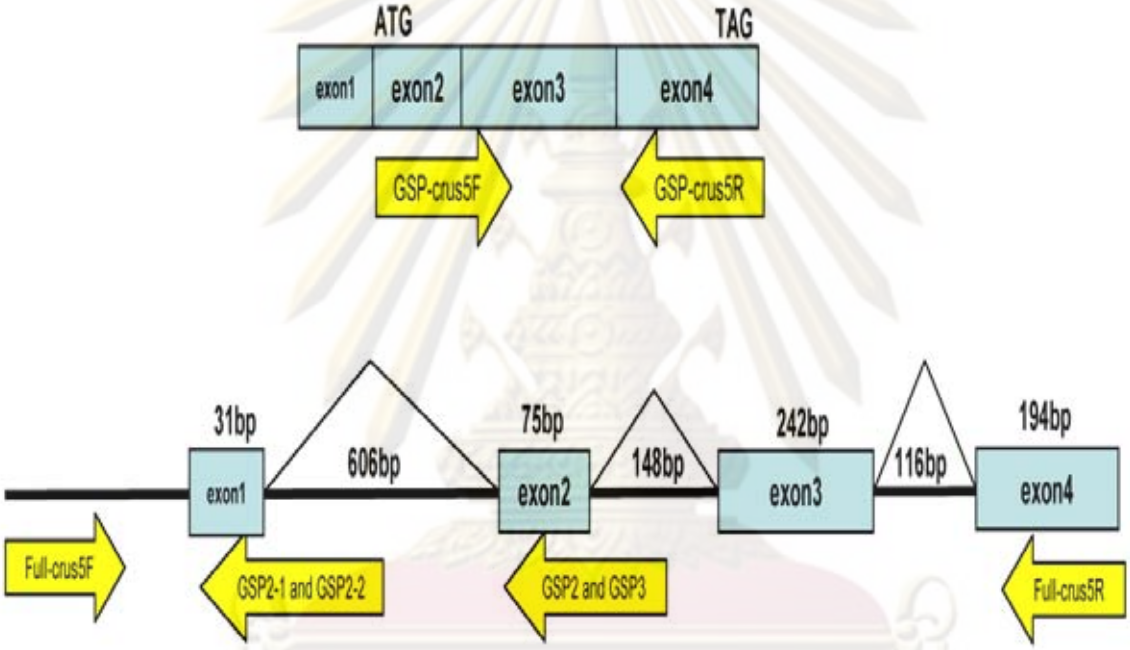
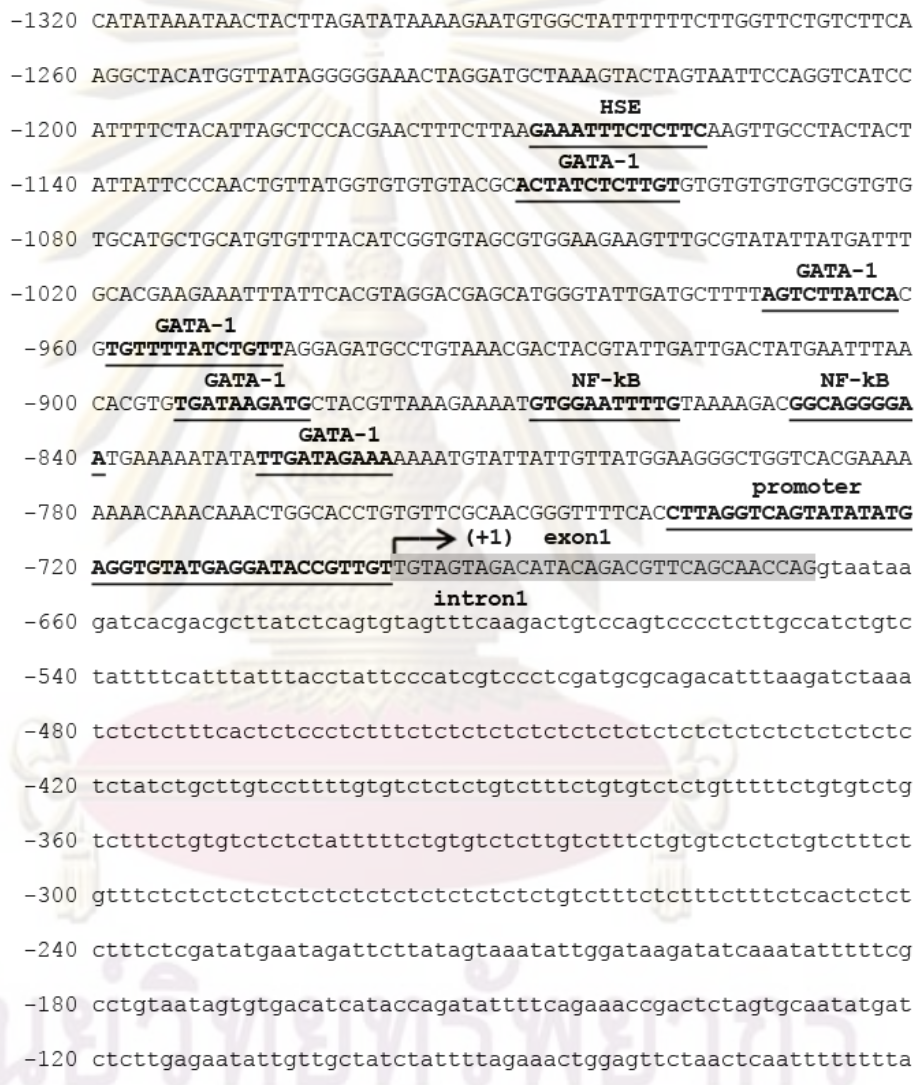


Figure 3.7 Schematic diagram showing of intron-exon organization of crustin*Pm*5 gene.



Continue

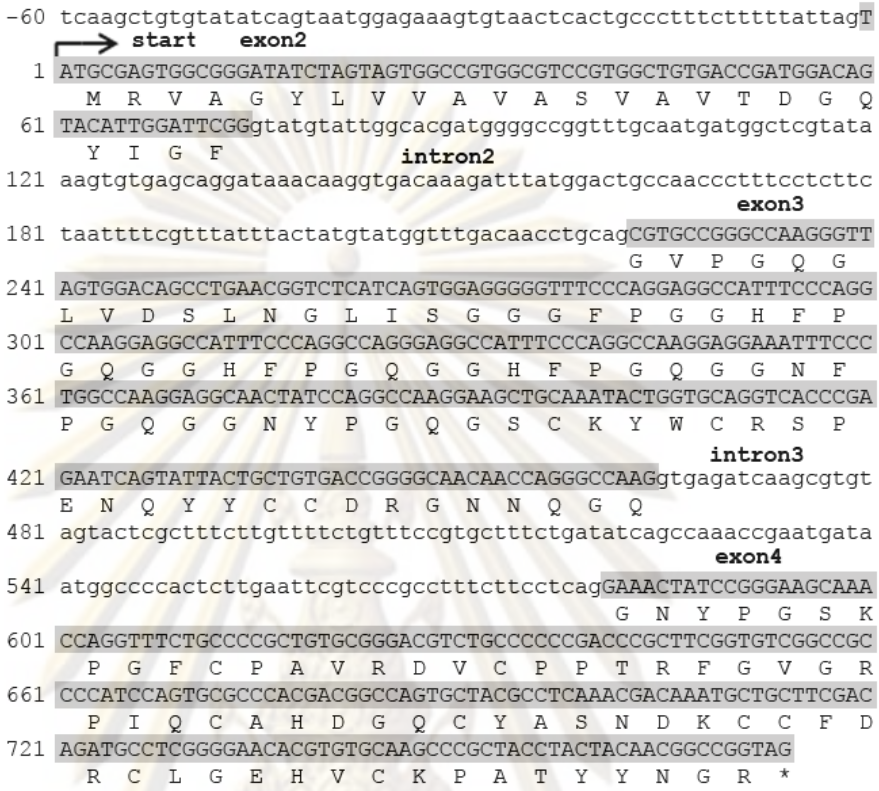


Figure 3.8 The nucleotide sequence of crustin*Pm*5 gene showing the 5' upstream genomic sequence, four exons interrupted by three introns. The 5′- upstream and exon sequences are shown in upper case and shade upper case, respectively. While intron sequences are in lower case. The transcription start site is shown by (+1) in exon1 and, starting from the transcription start site (marked by an arrow). The putative binding sequence motifs for transcription factors and promoter are underlined and bolded with the name of the corresponding factor shown. The translation initiation codon begins from the first methionine in the exon2.

3.3 Tissue distribution of crustin*Pm*5 transcripts in healthy *P. monodon* shrimp

3.3.1 Tissue and total RNA preparation

Gill, epipodite, antennal gland, heart, haemocyte, hepatopancreas, intestine, lymphoid organ and eyestalk of shrimp were collected for total RNA isolation. Then, total RNA was treated with RQ1 RNase-free DNase to remove the genomic DNA. The A_{260}/A_{280} ratio of total RNA samples were 1.5 to 1.8 indicating the acceptable quality of total RNA. The quality of total RNA was analyzed using 1.2% formaldehyde-agarose gel electrophoresis. Each tissue revealed a predominant band of 18S rRNA (1.9 kb) as shown in Figure 3.9.

3.3.2 Tissue distribution

To test the differential expression of crustin*Pm*5 in the hemocytes, hepatopancreas, lymphoid organ, gills, epipodite, eyestalk and antennal glands of *P. monodon*, RT-PCR was performed using gene specific primers, RT-crus5F and RT-crus5R, designed at the non-conserved region to avoid non-specific amplification of other isoforms of crustin. Beta-actin gene was used as the internal reference control for standardization between PCR reactions and templates. The sizes of PCR products are 534 bp and 317 bp for crustin*Pm*5 and β-actin, respectively. From these tissues, crustin*Pm*5 transcript was expressed at a low level in the epipodite and eyestalk only, and at very low or undetectable expression levels in the remaining tissues including hemocytes (Figure 3.10).

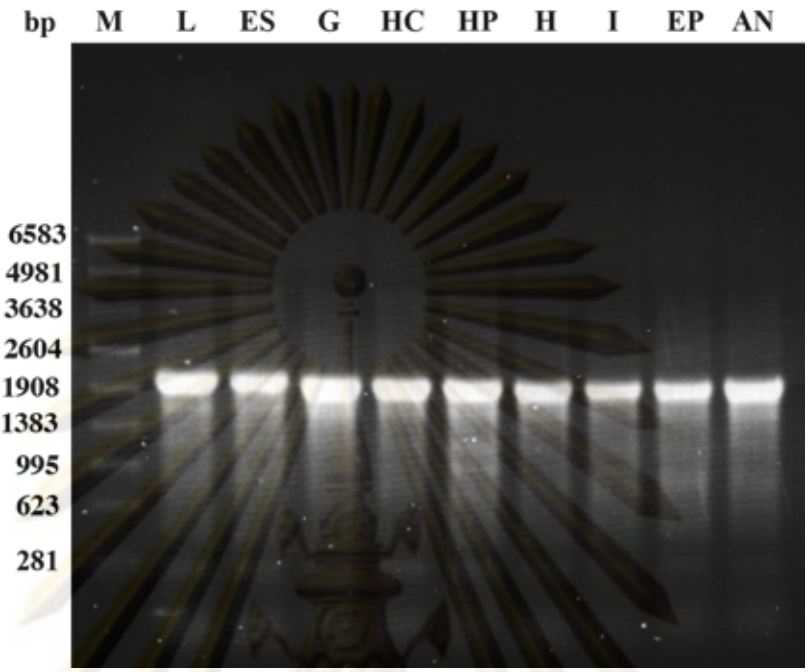


Figure 3.9 Total RNAs isolated from various tissues of *P. monodon* run on a 1.2% formaldehyde agarose gel.

Lane M: RNA marker

Lane L: Total RNA from lymphoid

Lane ES: Total RNA from eyestalk

Lane G: Total RNA from gill

Lane HC: Total RNA from haemocyte

Lane HP: Total RNA from hepatopancreas

Lane H: Total RNA from heart

Lane I: Total RNA from intestine

Lane EP: Total RNA from epipodite

Lane AN: Total RNA from antennal gland

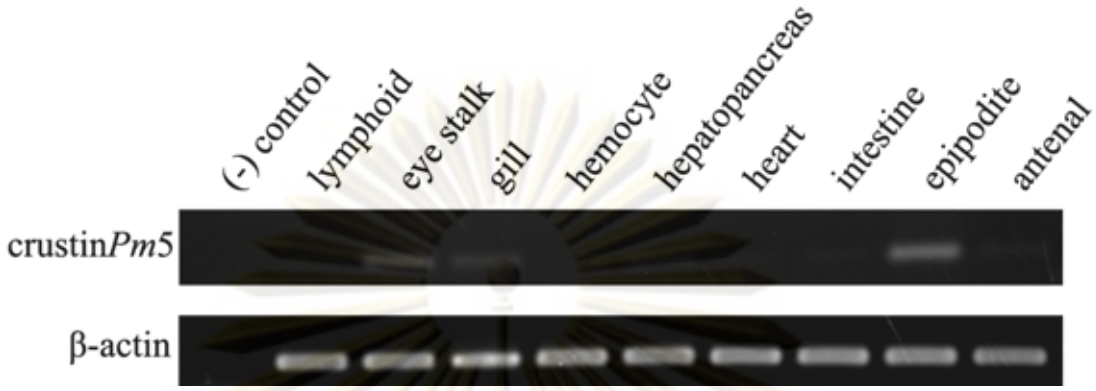


Figure 3.10 Expression of crustin*Pm*5 gene in healthy shrimp *Penaeus monodon* by RT-PCR analysis. The β-actin gene was used as an internal control gene.

3.4 Relative expression levels of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA after heat treatment

The presence of a putative heat-shock regulatory element (HSE) within the 5′ upstream region of the crustin*Pm*5 gene, identified from heat-shocked shrimp cDNA library, suggested that this gene is probably heat inducible. Thus, the mRNA transcript level of crustin*Pm*5 was examined in various shrimp tissues by RT-PCR following a transient increase in temperature (heat shock). The heat treatment was performed by transferring the shrimps from a tank at 28±1°C to 5°C above (33±1°C) for 2 h and then back to 28±1°C for 6 h for recovery. Total RNA was isolated from the nine tissues of the normal shrimp and heat induced shrimp. RT-PCR was performed using gene specific primer, RT-crus5F and RT-crus5R, with PCR condition in Table 2.2 and PCR products were run on 1.2% agaorse gel electorphoresis. From Figure 3.11, an induction of crustin*Pm*5 gene transcript expression was observed in immune tissues (lymphoid organ, hemocytes and hepatopancreas), as well as up-regulated expression in the epipodite after heat treatment. However, the effect of heat on the expression of crustin*Pm*5 gene in eyestalk, gill and antennal gland was inconclusive (Figure 3.11).

In addition, we further investigated whether heat also affects the expression of

the other two isoforms of *P. monodon* crustin, crustin*Pm*1 and crustin-like*Pm* using gene specific primers, RT-crus1F and RT-crus1R for crustin*Pm*1 and RT-crus7F and RT-crus7R for crustin-like*Pm* (Table 2.1). RT-PCR images were analyzed using Genetools (SYNGENE) to determine DNA band intensity. RT-PCR analysis showed significant up-regulation ($p<0.05$) of crustin*Pm*1, crustin*Pm*5 and crustin-like*Pm* transcripts in the epipodite of the heat-treated shrimp by about 2.7, 2.4 and 1.3 fold, respectively, as illustrated in Figure 3.12.

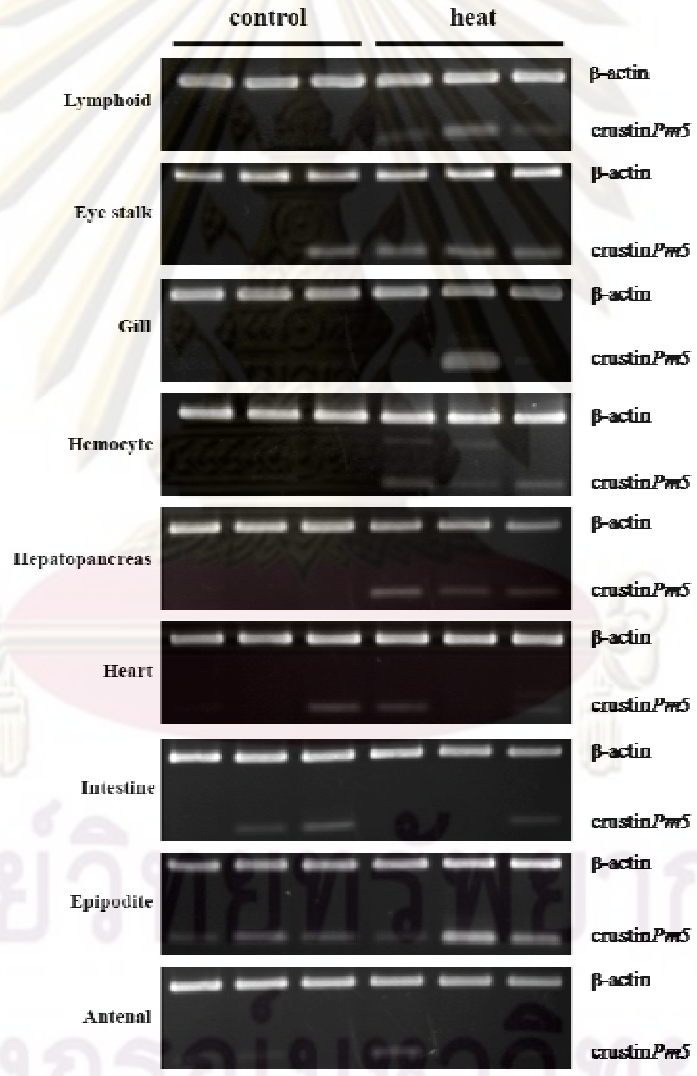


Figure. 3.11 Tissue distribution analysis of crustinPm5 by RT-PCR analysis. Comparison of crustin*Pm*5 mRNA in normal (control) and heat-induced shrimp (three individuals each). Beta-actin was used as an internal control gene.

(A)

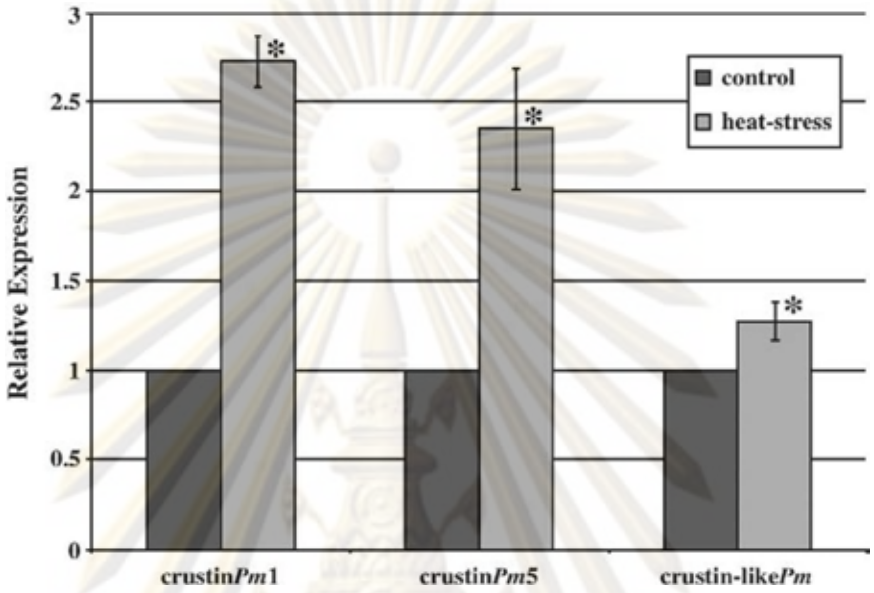


Figure 3.12 Expression of crustin genes in heat-induced shrimp *P. monodon*. The crustin transcripts from normal and heat-induced shrimp were normalized against the expression of β-actin (n=3). The expression of crustin*Pm*5 was examined in epipodite whereas the expression of crustin*Pm*1 and crustin-like*Pm* was examined in hemocytes. Significant differences at $p<0.05$, are marked with an asterisk.

3.5 Relative expression levels of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA after salinity changes

Observing the crustin*Pm*5 transcript in the epipodite, it was interesting to study the influence of salinity on its expression. The expression of the crustin*Pm*5 transcript as well as those of the other two crustin isoforms was then examined. The shrimp following salinity induced stress from 25 ppt to low salinity (3 ppt) and to high salinity (40 ppt) at 6 h, 24 h and 2 weeks. Total RNA was isolated from the epipodite of the normal shrimp (25 ppt), hyperosmotic stressed shrimp (40 ppt) and hypoosmotic stressed shrimp (3 ppt). RT-PCR was performed using gene specific primer for the three crustin genes with PCR condition in Table 2.2 and PCR products

were run on 1.2% agaorse gel electorphoresis. RT-PCR images were analyzed using Genetools (SYNGENE) to determine DNA band intensity. Shrimp showed a

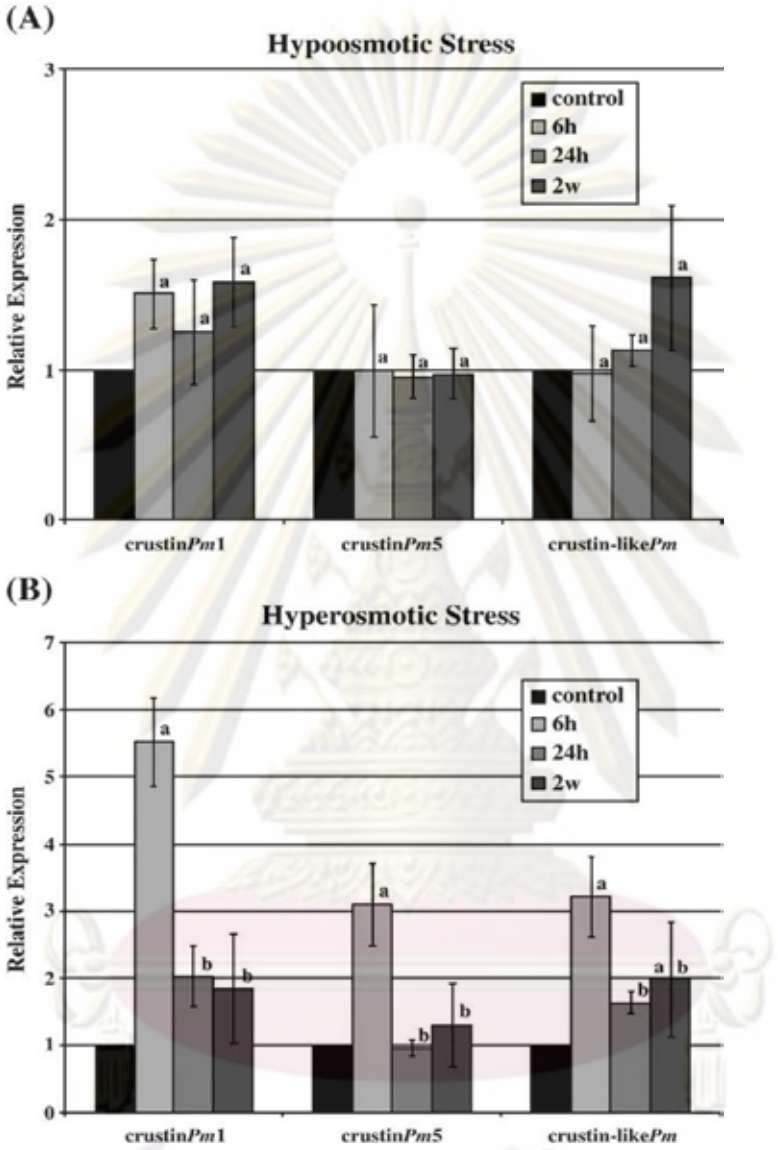


Figure 3.13 The relative expression ratio of crustin genes in the epipodite of shrimps that were transferred from 25 ppt to 3 ppt (A, hypoosmotic) and 40 ppt (B, hyperosmotic) for 6 h, 24 h and 2 weeks prior to assay. The crustin transcripts were analyzed by RT-PCR and normalized against the expression of β-actin (n=3). Means with the same lower case letters (above each bar) are not significantly different at the pb0.05 level.

significant up-regulated expression level of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNAs by 3.1, 5.5 and 3.2 fold, respectively, at 6 h after transfer from 25 ppt

to 40 ppt (hyperosmotic stress) and returned to normal after 24 h (Figure 3.13B) whereas no significantly change was observed when transfer to 3 ppt (hypoosmotic stress) (Figure 3.13A).

3.6 Recombinant protein expression of crustin*Pm*5

Since the recombinant protein of crustin*Pm*1 showed anti-bacterial activity from the previous report (Supungul et al., 2008), the recombinant crustin*Pm*5 protein (rcrustin*Pm*5) was subjected to over-producing in *E. coli* expression system to compare their activities.

3.6.1 Preparation of a DNA fragment encoded mature crustin*Pm*5

The crustin*Pm*5 clone from heat stress gill-epipodite library was amplified using the primers designed from the cDNA sequence that amplified the encoded mature peptide of crustin*Pm*5. The *Xho*I site with DNA sequence encoded for 6 ×His and *Not*I sites were included at the 5' end of the primers (Table 2.1). The PCR product was run on 1.2% agarose gel electrophoresis to isolate the specific fragment of 491 bp. The purified PCR product and expression vector pET-19b digested with *Xho*I and *Not*I were run on 1.2% agarose gel electrophoresis (Figure 3.14A) and elute the DNA fragments.

3.6.2 Construction and transformation of expression vector

The rcrustin*Pm*5 were over-expressed in an *E. coli* system using pET-19b as an expression vector. The purified *Xho*I-*Not*I crustin*Pm*5 fragment (Figure 3.14A) was ligated into the *Xho*I/*Not*I-digested pET-19b. Then, the ligation mixture was transformed into the *E.coli* JM109. The colony PCR technique was used for screening of the desired recombinant expression plasmid. The plasmid was extracted from the positive colony and digested with *Xho*I and *Not*I (Figure 3.14B) before subjected to sequence to confirm the correct construction. Figure 3.14B showed the correct DNA fragment of 491 bp was observed. The correct expression plasmid was transformed into an expression-strain *E. coli*, *E. coli* BL21 (DE3). The colony PCR technique indicated that the positive clones posses crustin*Pm*5 fragment as expected (Figure 3.14C).

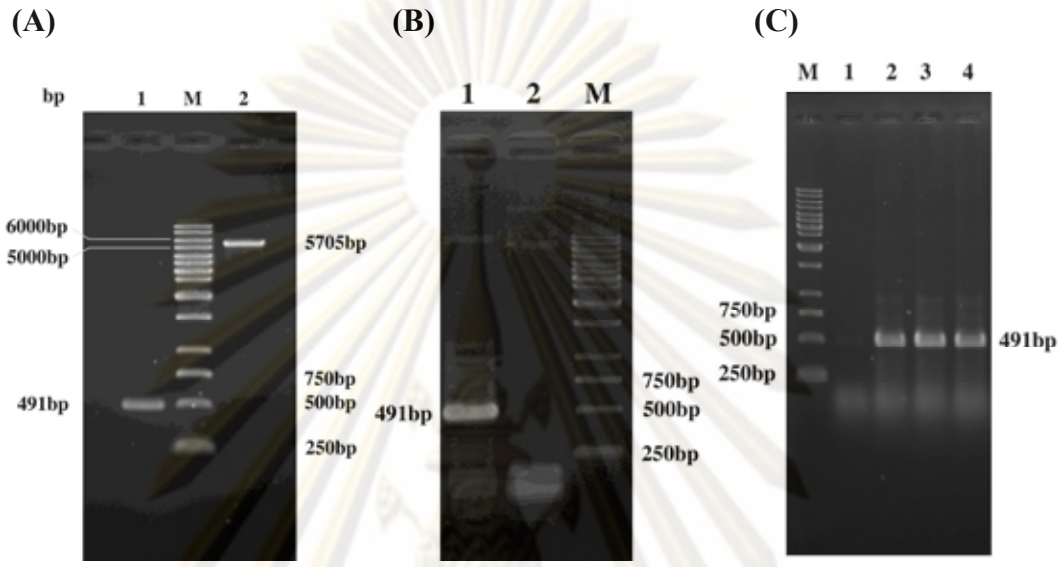
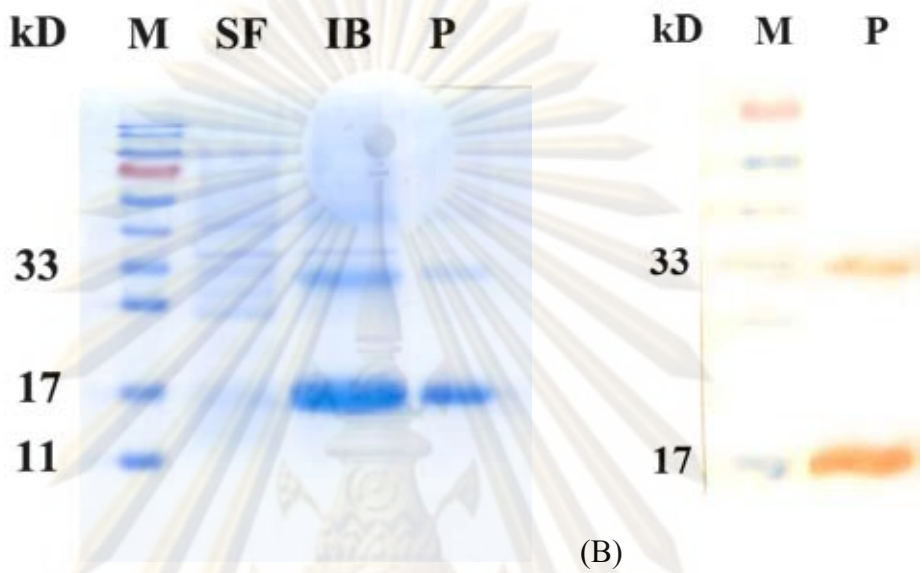


Figure 3.14 Agarose gel electrophoresis of crustin*Pm*5 fragment and pET-19b digested with *Xho*I and *Not*I. The samples were run on a 1.2% agarose gel (A). Agarose gel electrophoresis of recombinant expression plasmid (rcrustin*Pm*5 plasmid) digested with *Xho*I and *Not*I (B) and the direct PCR screening of *E. coli* BL21 (DE3) containg the recombinant plasmid (C).

(A)Lane M: GeneRuler™ 1 kb DNA Ladders (Fermentas)
Lane 1: Purified crustin*Pm*5 digested with *Xho*I and *Not*I
Lane 2: Purified pET-19b digeted with *Xho*I and *Not*I
(B) Lane M: GeneRuler™ 1 kb DNA Ladders (Fermentas)
Lane 1: The recombinant plasmid digested with *Xho*I and *Not*I
Lane 2: pET-19b plasmid digested with *Xho*I and *Not*I
(C) Lane M: GeneRuler™ 1 kb DNA Ladders (Fermentas)
Lane 1: PCR product of *E. coli* BL21 (DE3) clone transformed with pET-19b
Lane 2-4: PCR product of *E. coli* BL21 (DE3) clone transformed with the recombinant plasmid (rcrustin*Pm*5 plasmid)

3.6.3 Expression of recombinant clone

The transformant was cultured in LB medium containing 100 μg/ml ampicillin, at 37°C for overnight. The starter was inoculated in LB medium containing 100 μg/ml ampicillin to increase the number of cells. Then, the IPTG was added for the over-expression of the rcrustin*Pm*5. The rcrustin*Pm*5 was expressed as an inclusion fusion protein of about 17 kDa as shown in Figure 3.15A. Since rcrustin*Pm*5 contained the His-tag and was expressed as the inclusion bodies, it was purified with the nickel-NTA column in the denaturing condition. The inclusion bodies were solubilized with denaturing solution containing 20 mM imidazole (binding buffer). The solubilized protein was loaded to the nickel-NTA column and the column was washed with binding buffer. The purified recombinant protein, rcrustin*Pm*5 protein, was eluted with denaturing solution containing 100 mM imidazole (elution buffer). The protein samples, soluble fraction, inclusion fraction and purified fraction, were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.15A). Small amount of rcrustin*Pm*5 dimer (about 34 kDa) could be seen as usual for cysteine-rich proteins. Western blot analysis was used to confirm the identity of the rcrustin*Pm*5. Because the recombinant protein contained 6×His-tag, a monoclonal antibody, mouse anti-His, was used as a primary antibody. The bound primary antibody was amplified by alkaline phosphatase-conjugated rabbit anti-mouse IgG and detected by colorimetric method using NBT/BCIP as substrate (Figure 3.15B). The purified fraction showed two bands of 17 kDa and 34 kDa. The purified protein was dialyzed against 50 mM phosphate buffer pH12. The recombinant protein was identified using MALDI-TOF mass spectrometry (Figure 3.16). The result revealed that the recombinant protein had a molecular mass of 16605.038 Da.



(A) (B)

Figure 3.15 The expression and purification of the recombinant crustin*Pm*5 protein. SDS-15% (w/v) PAGE analysis showing the expressed proteins in *E. coli* BL21 (DE3) host cells containing rcrustin*Pm*5 in the soluble fraction, inclusion fraction and the purified rcrustin*Pm*5 protein (A). Western blot analysis of the expressed rcrustin*Pm*5 protein (B).

(A) Lane M: Protein marker
Lane SF: The soluble fraction of rcrustin*Pm*5 expression
Lane IB: The inclusion fraction of rcrustin*Pm*5 expression
Lane P: The purified rcrustin*Pm*5 protein
(B) Lane M: Protein marker
Lane P: The purified rcrustin*Pm*5 protein

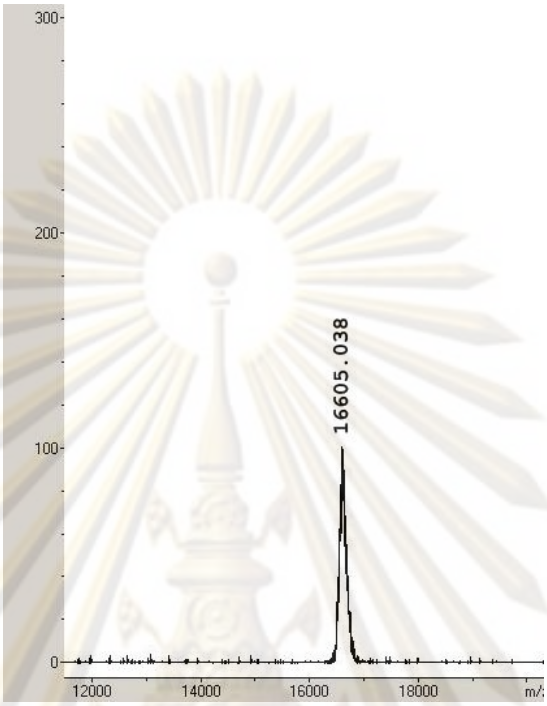


Figure 3.16 MALDI-TOF mass spectrometric determination of the molecular weight of the rcrustin*Pm*5.

3.7 Antimicrobial activity of crustin*Pm*5

The antimicrobial activity of the purified rcrustin*Pm*5 protein against several strains of Gram-positive and Gram-negative bacteria was determined by a liquid growth inhibition assay (Table 2). Purified rcrustin*Pm*5 was found to be highly active against *S. aureus* (MIC value of 0.78–1.56 μM) and had a lower activity against *S. haemolyticus* (MIC value of 12.5–25 μM) and *M. luteus* (MIC value of 25–50 μM). No significant activity was observed against the other Gram-positive bacteria, or all six species of Gram-negative bacteria tested, including the shrimp pathogen *V. harveyi* 639.

Table 3.1 Concentration range of rcrustin*Pm*5 against various strains of microorganisms in liquid growth inhibition assay.

Microorganisms	MIC value (µM)*
Gram (+) bacteria	
Aerococcus viridans	>100
Staphylococus aureus	0.78-1.56
Staphylococus haemolyticus	12.5-25
Micrococcus luteus	25-50
Bacillus megaterium	>100
Gram (-) bacteria	
Enterobacter cloacae	>100
Klebsiella pneumoniae	>100
Salmonella thyphimurium	>100
Escherichia coli 363	>100
Erwinia carotovora	>100
Vibrio harveyi 639	>100

* MIC are expressed as the interval *a–b*, where *a* is the highest concentration tested at which microorganisms are growing and *b* the lowest concentration that causes 100% growth inhibition.

CHAPTER IV
DISCUSSION

The sustainability and development of shrimp aquaculture are largely at stake as significant ecological and pathological problems are increasing in the vast majority of the shrimp producing countries. Prevention and control of diseases are now the priority for the durability of this industry. Within the past decade, intensification of the shrimp production has increased but with little corresponding increase in scientific knowledge of shrimp physiology. Within this field, shrimp immunology is a key element in establishing strategies for the control of diseases in shrimp aquaculture.

Antimicrobial peptides (AMPs) are important components of the innate immune system (Hancock et al., 2000). Crustins are antibacterial proteins containing a four-disulphide core (4DSC) or a whey acidic protein (WAP) domain, and are found in a variety of crustaceans. The cDNA sequences of crustins have been reported in *Litopenaeus vannamei* (Bartlett et al., 2002) (Vargas-Albores et al., 2004), *Litopenaeus setiferus* (Bartlett et al., 2002), *Penaeus monodon* (Supungul et al., 2004), *Marsupenaeus japonicus* (Rattanachai et al., 2004), *Fenneropenaeus chinensis* (Zhang et al., 2007), *Panulirus argus* (Stoss et al., 2004), *Homarus gammarus* (Hauton et al., 2006), *Carcinus maenas* (Brockton et al., 2007) and *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2006). In *P. monodon*, the analysis of crustin cDNA sequences revealed the presence of different isoforms, several of which are abundantly expressed in hemocytes and two isoforms have previously been characterized (Amparyup et al., 2008; Supungul et al., 2008). In their study, the recombianant protein of mature crustin*Pm*1 and crustin-like*Pm* were expressed in the *E. coli* expression system. Both recombinant protein exhibited antimicrobial activity against Gram-positive bacteria but crustin-like*Pm* also displayed activity against Gram-negative bacteria.

Here, the third and unique isoform, crustin*Pm*5, was chosen for further study because of its difference in tissue-specific expression and variation in WAP domain. Unlike the other two isoforms (crustin*Pm*1 and crustin-like*Pm*), crustin*Pm*5 was identified from the gill-epipodite of heat-induced shrimp and not hemocytes. The

predicted amino acid sequence of crustin*Pm*5 revealed that it has characteristics of the Type II crustins, as described by Smith et al. (2008). Specifically, crustin*Pm*5 is predicted to contain a signal peptide, a Gly-rich region, a Cys-rich region and a WAP domain at the C-terminus. The WAP domain has been described in proteins with diverse functions (Moreau et al., 2008) including antiproteinase and antimicrobial activities (Sallenave, 2000; Hagiwara et al., 2003). So far no proteinase inhibitory activity has been reported for Type I and Type II crustins. On the other hand, it has recently been reported that the shrimp single WAP domain proteins (SWD), categorized as Type III crustins, possess both antibacterial and antiproteinase activities (Amparyup et al., 2008; Jia et al., 2008). Although the WAP domain of the three crustin isoforms in *P. monodon* shared reasonable overall amino acid sequence similarity (~57% to 58%), they are nevertheless quite distinct suggesting that they might possess different functional properties.

Tissue distribution analysis revealed a unique characteristic of crustin*Pm*5 in that, whilst most crustins seem to be constitutively expressed in hemocytes (Smith et al., 2008), no detectable expression of the crustin*Pm*5 transcript was observed in this tissue. Rather constitutive crustin*Pm*5 transcript expression was only found in the epipodite and eyestalks, but it was inducible in most tissues after heat shock. The finding that the crustin*Pm*5 transcript is heat inducible is in accordance with the presence of a likely heat-shock element (HSE) in the 5′ upstream region of the crustin*Pm*5 gene.

Most crustins reported so far have been found mainly in the hemocytes but with transcript expression levels that were also observed in the intestine, gills and heart (Supungul et al., 2004; Zhang et al., 2007). Two crustin-like transcripts from the spiny lobster, *P. argus Latreille* (Decapoda: Palinuridae), and the fiddle crab, Uca pugilator Bosc. (Decapoda: Ocypodidae), have been found to be expressed in regenerating epithelial tissue in the olfactory organ and regenerating limbs, respectively (Durica et al., 2006; Brockton et al., 2007). Although it appeared that crustins are typically constitutively expressed in multiple tissues, the expression of crustin*Pm*5 in the epipodite is quite distinct. Moreover, it was found that the expression of crustin*Pm*5 as well as the other two crustin isoforms transcripts in

shrimp epipodite was up-regulated in the epipodite of *P. monodon* upon hyperosmotic stress (transfer from 25 ppt to 40 ppt).

Epipodites of crustaceans are known to play an important role in ionic exchange are involved in osmoregulatory function (Flik et al., 2000; Cieluch et al., 2004). The synthesis of crustin*Pm*5 in this organ and its induction upon salinity stress may indicate a diverse function or co-opted functions of AMPs in the crustin family. Previously, it has been shown that crustin expression in the crab, *C. maenas*, was affected by temperature stress suggesting that it might be involved in processes to maintain homeostatic integrity against injury, trauma or environmental stresses, such as heat shock (Brockton et al., 2007; Smith et al., 2008).

To study the genome arrangement of the crustin*Pm*5 gene, genome walking technique was used for approaching the unknown DNA sequences from a known DNA sequences (Siebert et al., 1995). Amplification of the genomic crustin*Pm*5 gene was also performed to reveal the intron-exon structures. In the genome walking technique, the four DNA libraries of shrimp genomic DNA were constructed and used for the nested PCR reaction using the gene specific primers. Genomic DNA sequence analysis revealed that the crustin*Pm*5 gene is encoded by four exons interrupted by three introns, in contrast to crustin-like*Pm* gene that contains only two exons and one intron (Amparyup et al., 2008). The data clearly demonstrated that the two isoforms of *P. monodon* crustins are encoded by different genes and the isoform diversity is not derived from alternative splicing of the same gene. It should be noted that introns 2 and 3 of the crustin*Pm*5 gene are inserted within the coding sequence of exons 3 and 4, whereas the coding sequence of the crustin-like*Pm* gene is contained within one exon and thus is not interrupted by an intron. The splicing of interrupted intron(s) on the protein coding sequence probably resulted in a low level of gene expression. Previously, the carcinin gene has been reported to compose of three exons and four introns (Brockton et al., 2007).

The regulation of gene expression of crustin*Pm*5 was examined by determining the promoter and regulatory sequences located 5′ upstream of the gene. By sequence searching against the TRANSFAC regulatory sequence database, the 5′ upstream sequence of crustin*Pm*5 was marked with a variety of putative *cis*-regulatory elements. The *cis*-regulatory elements, known to be involved in immune

response and/or regulate the expression of antimicrobial peptides including nuclear factor (NF)-kappaB, GATA and heat-shock element (HSE), were particularly interesting as they were identified in the upstream regions of the *P. monodon* crustin genes.

HSEs are important components in the promoter region of any heat-shock protein regulated gene. When cells receive heat or other related stress stimulation, activated heat-shock factors will bind to the HSEs leading to the rapid initiation of transcription of those HSE containing genes (Chuang et al., 2007).

NF-κB plays an important role in the Toll signaling pathway and defense system (Anderson, 2000). It is used to induce production of antimicrobial peptide in mammals, amphibian and insect (Engstrom, 1999; Miele et al., 2001; Tsutsumi-Ishii et al., 2002; Beinke et al., 2004; Mineshiba et al., 2005). NF-κB was previously identified in the upstream sequence of crustin-like*Pm* and anti-lipopolysaccharide factors (ALFs) gene. Both of these genes were previously reported that their transcription level were increased by *V. harveyi* challenge (Amparyup et al., 2008; Tharntada et al., 2008). These suggested that crustin*Pm*5 gene has a potential to be a LPS inducible gene.

GATA factor binding sites were reported to be involved in the interactions with NF-κB transcription factor in fat body-specific expression in insects (Senger et al., 2006). The GATA site is required for the activity of penaeidin 2 promoter of *Litopenaeus vannamei* (O'Leary et al., 2006). Seven and five putative GATA sites were also found in the ALF genes.

Both potential GATA factor and NF-κB binding sites, but not a HSE, were also identified in the crustin-like*Pm* gene (Amparyup et al., 2008). In accordance to the absence of a HSE, a lower level of heat induction of crustin-like*Pm* gene (1.3 fold) was observed as compared to those of crustin*Pm*5 (2.4) and crustin*Pm*1 (2.7). The highest up-regulation of crustin*Pm*1 transcript by heat induction suggests that the crustin*Pm*1 gene is likely to contain heat-shock regulatory element(s) in the 5′ upstream region and this notion required further investigation.

The crustin*Pm*5 was over-produced using the *E. coli* expression system. This system was chosen because it is easy to manipulate and provides a high protein production in a few days. Although *E. coli* lacks posttranslational modification such

as glycosylation or digestion of signal peptide, this experiment expressed just mature peptide without signal peptide and the crustin*Pm*5 contains no a putative glycosylation site. The recombinant protein of crustin*Pm*1 and crustin-like*Pm* were successfully expressed using *E. coli* expression system and exhibited the antimicrobial activity (Amparyup et al., 2008; Supungul et al., 2008).

The rcrustin*Pm*5 gene was cloned into a pET-19b expression vector. Although, the vector provided start codon and 10×His tag following by cloning region, DNA sequence encoding for 6×His tag was directly added after start codon of gene specific primer to decrease the excess amino acid residues between His-tag and the rcrustin*Pm*5. The rcrustin*Pm*5 protein was expressed as the inclusion form so it was purified under denaturing conditions. Although inclusion-body protein may contain a mixture of non-native conformations, the polypeptide chains themselves are usually complete and intact. In fact, purification of protein from inclusion bodies can offer certain advantages, since the bodies are often readily separated from other cell components (Marston, 1986) and can thus be isolated in a relatively pure state.

Antimicrobial assays against various strains of bacteria revealed that the recombinant crustin*Pm*5 displayed only significant detectable activity against some Gram-positive bacteria. This finding is in some accordance with the previous reports that most crustins, including carcinin of *C. maenas* (Relf et al., 1999), the crustin-like*Fc*1 proteins of *F. chinensis* (Zhang et al., 2007), crustin*Pm*1 from *P. monodon* (Supungul et al., 2008) and carcinin-like of *Scylla partamamosan* (Imjongjirak et al., 2009), exhibit bactericidal activity against Gram-positive and not Gram-negative bacteria. The current clear exception is that of the crustin-like*Pm* product which also exhibited activity against Gram-negative bacteria (Amparyup et al., 2008). However, these crustins display variation in both species specificity, and perhaps amongst different strains of the same broad species, and effectiveness for killing or preventing the growth of the bacteria, which may be due to sequence variation in the WAP domain of crustins. In crustaceans, antimicrobial peptides (AMPs), including penaeidin (Destoumieux et al., 1997) and carcinin (Chisholm et al., 1992; Schnapp et al., 1996), are synthesized and stored in the granular hemocytes and released by exocytosis upon microbial invasion. Surprisingly, crustin*Pm*5 transcripts were found to be mainly expressed in the epipodite of *P. monodon* whilst the expression in

hemocytes, as well as other tissues, was only found to be induced after heat induction. Generally, antimicrobial molecules are widely distributed in cells of the immune system such as leukocytes/hemocytes and in the tissues that encounter bacterial infections such as the intestine and gills.

A direct antimicrobial action is a typical function of AMPs. However, there are some reports that showed the alternative function of AMPs. The expression of two Type I crustins in regenerating tissues (Stoss et al., 2004; Durica et al., 2006) is suggestive of a possible role for crustins in recovery from trauma or response to physiological stress or as negative regulators of other host-defence factors. For the reason that this protein only exhibited antimicrobial activity against a few Gram-positive bacteria in vitro and not against Gram-negative bacteria including this isolate of *V. harveyi* thus, the effect of crustin*Pm*5 may involve its function as a modulator of immune responses or tissue damage and injury related stresses following bacterial pathogen infection. Certainly, it has become evident that several cationic antimicrobial peptides have diverse functions in modulating immunity and having an impact on infections and inflammation (Brown et al., 2006), whilst any role in viral immunity remains unclear.

CHAPTER V
CONCLUSIONS

Crustin*Pm*5 was identified from gill-epipodite cDNA library of heat-induced shrimp. The full-length cDNA of crustin*Pm*5 had a predicted open reading frame of 510 bp in length encoding a polypeptide of 169 amino acids containing a 19 amino acid signal peptide and a 150 amino acid mature protein.

The genomic sequence of crustin*Pm*5 contained four exons separated by three introns. The putative promoter was identified at 39 base pairs upstream. Several putative *cis*-regulatory elements including NF-κB, GATA and complete heat-shock regulatory element (HSE) were predicted in the 5′ upstream sequences of crustin*Pm*5 gene.

The crustin*Pm*5 transcript was predominantly expressed in the epipodite and eyestalk. The transcription level of crustin*Pm*5 was up-regulated in lymphoid organ, hemocytes, hepatopancreas and epipodite of heat-treated shrimp. In addition, crustin*Pm*1 and crustin-like*Pm* transcription levels were increased in epipodite of heat-treated shrimp. Moreover, transcripts of the three crustin isoforms were induced upon hyperosmotic stress.

A recombinant protein of crustin*Pm*5 was successfully produced in the *E. coli* system. The purified recombinant crustin*Pm*5 protein has the molecular mass of 16605.038 Da. The recombinant protein had antimicrobial effect against the *S. aureus, S. haemolyticus* and *M. luteus*, representatives of Gram-positive bacteria but did not display acitivity against Gram-negative bacteria.

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APPENDICES

Appendix A

1. Preparation for SDS-PAGE electrophoresis

Stock reagents

30 % Acrylamide, 0.8% bis-acrylamide, 100 ml

Acrylamide 29.2 g

N,N′-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8 75 ml

10% SDS 4 ml

Distilled water 21 ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8 50 ml

10% SDS 4 ml

Distilled water 46 ml 118

SDS-PAGE

15 % Seperating gel

30 % Acrylamideml solution 5.0 ml

Solution B 2.5 ml

Distilled water 2.5 ml

10% (NH4)2S2O8 50 µl

TEMED 10 µl

5.0 % Stacking gel

30 % Acrylamideml solution 0.67 ml

Solution C 1.0 ml

Distilled water 2.3 ml

10 % (NH4)2S2O8 30 µl

TEMED 5.0 µl

5X Sample buffer

1 M Tris-HCl pH 6.8 0.6 ml

50% Glycerol 5.0 ml

10% SDS 2.0 ml

2-Mercaptoethanol 0.5 ml

1 % Bromophenol blue 1.0 ml

Distilled water 0.9 ml

One part of sample buffer was added to four parts of sample. The mixture was heated 5 min. in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane 3.03 g

Glycine 14.40 g

SDS 1.0 g

Dissolve in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3).

Appendix B

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Genomic structure, expression pattern and functional characterization of crustin*Pm*5, a unique isoform of crustin from *Penaeus monodon*

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ABSTRACT

A unique isoform of crustin, crustin*Pm*5, was identified from a gill-epipodite cDNA library of the tiger shrimp, *Penaeus monodon*. The crustin*Pm*5 cDNA contains an open reading frame (ORF) of 510 bp encoding a 169 amino acid protein. The deduced amino acid sequence of crustin*Pm*5 showed 38% and 37% overall sequence identity with those of crustin*Pm*1 and crustin-like*Pm*, respectively, two crustin isoforms previously reported. The crustin*Pm*5 gene contained four exons interrupted by three introns whilst the upstream sequence contains a putative promoter with two potential binding sites for NF-κB, one complete heat-shock regulatory element (HSE) and five putative GATA factor binding sites. The transcripts of crustin*Pm*5 were primarily observed in the epipodite and eyestalk and not in hemocytes. Expression analysis revealed that the transcript levels of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* in epipodite were up-regulated upon heat treatment and hyperosmotic salinity stress. The recombinant crustin*Pm*5 exhibited antimicrobial activity against some Gram-positive bacteria *in vitro*, but did not inhibit the growth of any Gram-negative bacteria tested. These results, together with the transcript expression pattern, indicate a diverse function of the proteins in the crustin family particularly crustin*Pm*5 that might function as a stress mediator in addition to its antibacterial action.

1. Introduction

Crustins are cysteine-rich antimicrobial proteins containing a whey acidic protein (WAP) domain. The first crustin, carcinin, was isolated from hemocytes of the shore crab, *Carcinus maenas*, and found to be active against only Gram-positive bacteria (Schnapp et al., 1996; Relf et al., 1999). Subsequently, genomic approaches, such as expressed sequence tag (EST) analysis, have identified several homologs of crustin from a variety of crustacean species including shrimps, crabs, crayfish and lobsters (see review by Smith et al. (2008)). All known crustins possess a signal sequence at the amino terminus and a WAP domain at the carboxyl terminus. The WAP domain generally consists of 50 amino acid residues with eight cysteine residues at defined positions. They form four intracellular disulfide bonds creating a tightly packed structure (Grütter et al., 1988), described on PROSITE as a four-disulphide core (4DSC).

Three types of crustin (Types I–III) have been designated, according to the distinct domain structure in the central region between the signal sequence and the WAP domain (Smith et al., 2008). Type I crustins are mainly found in crabs, lobsters and crayfish, whilst Type II and III crustins are mainly present in shrimps. Despite the reports of several crustin sequences, only a few studies have described their antimicrobial properties, and these have reported bactericidal or bacteristatic activity towards mainly or only Gram-positive bacteria (Relf et al., 1999; Zhang et al., 2007; Supungul et al., 2008).

In the black tiger shrimp, *Penaeus monodon*, different isoforms of Type II crustins have been identified by homology screening the sequences of non-redundant EST libraries (Supungul et al., 2004; Tassanakajon et al., 2006). The two major isoforms so found, crustin*Pm*1 and crustin-like*Pm*, were expressed as recombinant proteins in *Escherichia coli* and characterized for their antimicrobial properties (Amparyup et al., 2008b; Supungul et al., 2008). Crustin*Pm*1 displays antimicrobial activity against only Gram-positive bacteria whilst crustin-like*Pm* inhibits the growth of both Gram-positive and Gram-negative bacteria, including *Vibrio harveyi*, a shrimp pathogen. In this study, we report the characterization of a unique isoform of crustin, crustin*Pm*5, which was identified from the gill-epipodite cDNA library of *P. monodon*. Unlike the other crustins, crustin*Pm*5 is mainly expressed in the epipodite and eyestalk but not in the hemocytes. Moreover, here we report its expression was induced upon heat shock and hyperosmotic salinity stresses. The genomic structure and transcription regulation of crustin*Pm*5 were investigated. The recombinant crustin*Pm*5 protein was produced and further characterized.

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2. Materials and methods

2.1. Animals and sample preparation

Juvenile *P. monodon* shrimp of about 20 g wet mass were obtained from local farms and were used for tissue specific expression. Shrimps were acclimatized in laboratory aquaria at a temperature of 28 + 1 °C and a salinity of 15 ppt NaCl for one week before use in the experiments. Shrimp tissues (eyestalk, antennal gland, epipodite, gills, lymphoid organ, hepatopancreas, intestine and heart) were dissected and snap-frozen in liquid nitrogen. Hemocytes were not stored as above but rather, after isolation by centrifugation at 800 g for 10 min at 4 °C, the hemocyte pellet was resuspended immediately in 200 μL of TRI Reagent® (Molecular Research Center, USA) for RNA extraction as detailed below.

2.2. Total RNA extraction and cDNA synthesis

The samples were homogenized in TRI REAGENT® (MRC) and total RNA was extracted according to the manufacturer's instruction, and then incubated with RNase-free DNase I (Promega, USA) to remove contaminating genomic DNA before being reverse transcribed into cDNA using oligo $(dT)_{18}$ primers with ImProm-II™ Reverse Transcriptase System kit (Promega, USA) according to the manufacturer's protocol. The synthesized cDNA was stored at −80 °C.

2.3. Genomic DNA isolation

Genomic DNA was prepared from muscle tissues by a standard phenol–chloroform based method as described previously (Tassanakajon et al., 1998). The DNA concentration was measured spectrophotometrically by monitoring the absorbance at 260 and 280 nm.

2.4. Characterization of genomic structure and promoter region of crustin*Pm*5 gene

The sequence of the crustin*Pm*5 gene was obtained by a PCR-based strategy using genomic DNA as a template and the Full1F and Full1R primers (Table 1), designed from the non-redundant crustin*Pm*5 cDNA (EST) so as to amplify from the start to stop codons of the crustin*Pm*5 gene. Approximately 20 ng of template DNA was used for the PCR amplification in 50 μL reaction containing one unit Advantage 2 Polymerase Mix (Clonetech), 1× Advantage 2 buffer, 200 mM each dNTP, 0.2 mM each primer with an initial denaturation step at 94 °C for 2 min, followed by: 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min. The expected DNA fragment was cloned into the pGEM-T easy vector and sequenced.

To determine 5′ upstream sequence, the BD GenomeWalker™ Universal Kit (Clonetech, USA) was performed following the manufacturer's instruction. Briefly, genomic DNA was separately digested with four restriction enzymes, *Eco*RV, *Dra*I, *Pvu*II and *Stu*I for the construction of four genomic pool libraries. The genomic fragments from each pool were ligated to the adaptor provided in the kit. Primary PCR was performed using AP1 primer and the gene specific primer, GSP1 (Table 1) and using four genomic DNA libraries as templates. The amplifications were performed in the 50 μL reaction containing one unit Advantage 2 Polymerase Mix (Clonetech), 1× Advantage 2 buffer, 200 mM each dNTP, 0.2 mM each primer with an initial denaturation step at 94 °C for 2 min, followed by: (a) 7 cycles at 94 °C for 25 s, 72 °C for 3 min and (b) 32 cycles at 94 °C for 25 s, 67 °C for 3 min. The PCR was finally polymerized at 67 °C for 7 min after the final cycle. Nested PCR was then performed upon the primary PCR products as templates using the AP2 primer and the nested gene specific primer, GSP2 (Table 1). The cycling conditions were as follows: 94 °C for 5 min, 5 cycles of 94 °C for 25 s, 72 °C for 3 min, 20 cycles of 94 °C for 25 s, 67 °C for 3 min and the final polymerization step was at 67 °C for 7 min. The resulting PCR products were cloned into the pGEM-T easy vector and subsequently sequenced. The whole fragment of crustin*Pm*5 gene was amplified by PCR using GMF and GMR primers (Table 1). The PCR product was cloned and sequenced in both directions.

The putative promoter and transcription start site of crustin*Pm*5 were predicted by the neural network promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001). Transcription factor binding sites were predicted by Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/tess) (Schug, 2003).

Table 1
Sequence of various primers used for different experiments.

Primer	Sequences (5′-3′)[a]	Source of sequence information
Full1F	ATGCGAGTGGCGGGATATCT	The current study
Full1R	CCTACTACAACGGCCGGTAG	The current study
GSP1	ACCGGCCCCATCGTGCCAATACATAC	The current study
GSP2	CACGGACGCCACGGCCACTACTAGATA	The current study
GMF	ATCCTACATACCGTCACATGAA	The current study
GMR	CTACCGGCCGTTGTAGTAGG	The current study
CrusF	CCATGGGCCATCATCATCATCATCACCGAGTGGCGGGATAT	FJ380049
CrusR	CATATG-CTACCGGCCGTTGTAGTAGG	FJ380049
RTcrus5F	ACCAGGGCCAAGGAAACTAT	FJ380049
RTcrus5R	GCAGCATTTGTCGTTTGAGG	FJ380049
RTcrus1F	CTGCTGCGAGTCAAGGTATG	CD766060
RTcrus1R	AGGTACTGGCTGCTCTACTG	CD766060
RTcruslikeF	GCGGCCGCTTAATGCTCTACTGACATGTT	EF654658
RTcruslikeR	TGTCGGAGCCGAAGCAGTCA	EF654658
actinF	GCTTGCTGATCCACATCTGCT	DW042525
actinR	ATCACCATCGGCAACGAGA	DW042525

[a] All primers were designed using the SECentral computer program.

2.5. Recombinant protein expression and purification

The cDNA sequence of crustin*Pm*5 (accession no. FJ380049) was retrieved from the *P. monodon* EST database (http://pmonodon.biotec.or.th). The ORF corresponding to the mature protein was amplified and cloned into the *E. coli* expression vector, pET19b. The PCR amplification was performed using the crusF primer, which introduced an *Nco*I site and a 6xHis-Tag at the amino terminus, and the crusR primer, which introduced an *Xho*I site (Table 1), as described previously (Supungul et al., 2008). The gel-purified PCR product was restriction digested and cloned into the pET19b vector at the *Nco*I and *Xho*I sites. The recombinant plasmid was transformed into the *E. coli* K12 strain BL21(DE3) and the recombinant protein was expressed by induction with 1 mM IPTG. Cells were harvested by centrifugation 8000 ×g for 15 min and resuspended in phosphate-buffered saline, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4). The cell suspension was disrupted by French Press at 9000 psi (Jorgensen et al., 1995). The cell lysate was centrifuged at 12,000 g for 15 min at 4 °C to collect the inclusion bodies which were then solubilized in denaturing solution (20 mM phosphate buffer, pH 7.4, 8 M urea, 0.5 M NaCl and 20 mM imidazol), and the recombinant crustin*Pm*5 (rcrustin*Pm*5) protein was purified using Ni-NTA agarose under denaturing conditions and eluted with denaturing solution containing 100 mM imidazole. The purified protein was dialyzed against 50 mM phosphate buffer pH 12 and analyzed by a denaturing SDS-15% (w/v) PAGE gel and stained by coomassie blue. The concentration of the dialyzed protein was determined by the Bradford method (Bradford, 1976).

2.6. Western blot analysis

Western blot analysis was used to confirm the expression of rcrustin*Pm*5 protein. The proteins isolated from the *E. coli* inclusion bodies as well as the Ni-NTA agarose purified rcrustin*Pm*5 proteins (see above) were separated by SDS-PAGE as described above and then electro-

transferred onto a nitrocellulose membrane in a semidry electrophoretic transfer cell (Trans-blot SD, Bio-Rad) at 10 V for 30 min. The membrane was probed with mouse anti-His antibodies (GE Healthcare) followed by the alkaline-phosphatase conjugated rabbit anti-mouse IgG. The recombinant protein was visualized as a purple band with chromogenic-base detection using NBT/BCIP. The molecular weight of the purified protein was determined by mass spectrometric analysis, MALDI-TOF spectra.

2.7. Antimicrobial activity assay

The spectrum of antimicrobial activity of rcrustin*Pm*5 was assayed by screening against the Gram-positive bacteria: *Aerococcus viridans*, *Bacillus megaterium*, *Micrococcus luteus*, *Staphylococcus haemolyticus* and *Staphylococcus aureus*; and the Gram-negative bacteria: *Enterobacter cloacae*, *Erwinia carotavora*, *E. coli* 363, *V. harveyi* 639, *Klebsiella pneumoniae* and *Salmonella typhimurium*, using the standard liquid growth inhibition assay (Patat et al., 2004). Two fold serial dilutions of peptide were added to the suspension of mid-log phase bacteria. Cultures were grown for 18 h. The growth of bacteria was evaluated by measurement of the optical density at 595 nm. The minimum inhibitory concentration (MIC) value was recorded as a range between the highest concentration of the protein where bacterial growth was observed and the lowest concentration that caused 100% inhibition of bacteria growth (Casteels et al., 1993).

2.8. Tissue expression analysis of crustin*Pm*5

The expression level of crustin*Pm*5 transcripts in various shrimp tissues was determined by semi-quantitative RT-PCR. Total RNA was extracted from hemocyte, hepatopancreas, lymphoid organ, gills, intestine, heart, epipodite, eyestalk and antennal gland using the TRI REAGENT® (MRC) according to the manufacturer's instructions. Semi-quantitative PCR was performed to determine the level of crustin*Pm*5 mRNA using the gene specific primers, RTcrus5F and RTcrus5R, which were designed using the SECentral computer program (Scientific & Educational Software, Durham, NC, USA) (Table 1). Beta-actin was used as the internal reference control for cDNA levels and was detected with the gene specific primers, actinF and actinR (Table 1). One microlitre of the first strand cDNA was subjected to PCR in a 25 μl reaction volume containing 10 mM Tris–HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each specific primers and 1.5 unit of DyNazymeTM II DNA polymerase (DyNazyme, Finnland). The reaction was predenatured at 94 °C for 1 min following by 30 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Ten microlitres of the amplification product was analyzed by resolution through a TAE-2% (w/v) agarose gel and visualized by UV transillumination after ethidium bromide visualization.

2.9. Determination of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA expression levels after heat-shock treatment

The experimental *P. monodon* shrimp (~20 g wet body mass) were acclimatized at 28 ± 1 °C for one week before heat treatment. Nine shrimp were transferred to the tank at 33 ± 1 °C for 2 h and allowed to recover at 28 ± 1 °C for 6 h. For the control group, nine shrimp were maintained in the tank at 28 ± 1 °C. After heat treatment, shrimp tissues were collected. Total RNA was extracted, cDNA was synthesized

Fig. 1. (A) Nucleotide and deduced amino acid sequences of crustin*Pm*5. An asterisk indicates the stop codon. The putative signal peptides are underlined. The shaded amino acids are the predicted WAP domain. The conserved 12 Cys residues are in boxes. (B) Multiple sequence alignments of deduced amino acid sequences of crustin*Pm*1, crustin*Pm*5 and crustin-like*Pm* were performed using the ClustalX program. The putative signal peptides are underlined. The Gly-rich region, Cys-rich region and the WAP domain are indicated by shaded amino acids, an open box and a black box, respectively. (*), (.) and (:) indicate amino acid similarity in terms of all, strong or weaker amino acid groups are fully conserved, respectively.

and the expression level of crustin*Pm*5 transcripts was determined by RT-PCR analysis as described above. Transcript levels of the two other *P. monodon* crustin homologs, crustin*Pm*1 and crustin-like*Pm*, were assayed by RT-PCR as described before (Amparyup et al., 2008b; Supungul et al., 2008) using specific primers of crustin*Pm*1 (RTcrus1) and crustin-like*Pm* (RTcruslike) (Table 1). The crustin gene transcripts from control and heat-induced shrimp were normalized against the expression of β-actin. The relative expression levels of heat-induced shrimp were also normalized against the expression of the control shrimp and tested using one-way analysis of variance (ANOVA) (Steel and Torrie, 1980) followed by Duncan's new multiple range test (Duncan, 1955) using SPSS software.

2.10. Determination of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA expression levels following salinity changes

P. monodon (about 20 g of body mass) were maintained in a 500-liter tank with aeration at 25 ppt salinity for two weeks. They were then separated into 3 groups; the control group (25 ppt), the groups reared in low salinity (3 ppt) and in high salinity (40 ppt). Epipodites were excised and collected from three individual shrimps per treatment group. The samples from the control group were collected at zero time point whereas the samples from the low salinity and the high salinity groups were collected at 6 h, 24 h and 2 weeks. RNA was extracted from epipodite using TRI Reagent® (MRC) for the cDNA synthesis. The expression of the crustin*Pm*1, crustin*Pm*5 and crustin-like*Pm* was examined using the PCR condition and the primers, RTcrus1, RTcrus5 and RTcruslike, as mentioned above. The target gene transcripts were normalized against the expression of β-actin and the relative expression levels of both low and high salinity groups were also normalized against the expression at 25 ppt taken at the zero time point. The differences in expression were analyzed using one-way analysis of variance (ANOVA) (Steel and Torrie, 1980) followed by Duncan's new multiple range test (Duncan, 1955) using SPSS software.

2.11. Sequence analysis

Based on the consensus identification of a four-disulphide core (4DSC) by the PROSITE and SMART databases (http://smart.embl-heidelberg.de/), the cloned sequence was analyzed for the identity and similarity by BLASTX (Altschul et al., 1990). The signal peptide was predicted by the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments were performed with the predicted crustin*Pm*5 amino acid sequences and those of the two known *P. monodon* crustins, crustin*Pm*1 (GenBank accession no. CD766060) and crustin-like*Pm* (EF654658), using ClustalX (Thompson et al., 1997).

3. Results

3.1. cDNA of crustin*Pm*5 and sequence analysis

Analysis of the expressed sequence tags from cDNA libraries prepared from various tissues of the black tiger prawn, *P. monodon* (Tassanakajon et al., 2006), identified several clones of crustin homologues. These crustin's cDNAs were mainly found in the hemocyte cDNA libraries, as expected, but a unique cDNA, namely crustin*Pm*5, was identified from the gill-epipodite cDNA library of heat-shocked shrimp. The full-length cDNA of crustin*Pm*5 (FJ380049) had a predicted open reading frame (ORF) of 510 bp in length encoding a polypeptide of 169 amino acids (Fig. 1A). Sequence analysis using the signalP program revealed the presence of a signal peptide with 19 amino acids, resulting in a 150 residue mature protein with a calculated molecular mass of 15.8 kDa and a predicted pI of 7.82.

The deduced amino acid sequence of crustin*Pm*5 showed 38% and 37% overall sequence identity with crustin*Pm*1 (Supungul et al., 2008) and crustin-like*Pm* (Amparyup et al., 2008b), respectively. Sequence alignment using the ClustalX program (Thompson et al., 1997) revealed a relatively large variation in the Gly-rich and the Cys-rich regions at the amino terminus and a more conserved carboxyl terminus containing eight cysteine residues which form a four disulfide core (4DSC) or a whey acidic protein (WAP) domain (Fig. 1B). However, the WAP domain of each crustin isoform is quite distinct sharing about 57% to 58% identity.

Fig. 2. The nucleotide sequence of the crustin*Pm*5 gene showing the 5′ upstream genomic sequence and the four exons interrupted by three introns. The 5′-flanking, exon and intron sequences are shown in upper case, shaded upper case and lower case letters, respectively. The nucleotide sequence is numbered starting from the transcription start site (+1) in exon-1 (marked by an arrow) and proceeding as positive numbers in a 3′ direction and negative numbers in the 5′ direction. The putative binding sequence motifs for transcription factors, and the ribosomal promoter site, are all shown as underlined and bold text, with the name of the corresponding factor shown above. The translation initiation codon begins from the first methionine in the exon-2.

3.2. Genomic structure and upstream regulatory region of crustin*Pm*5 gene

The complete genomic sequence of crustin*Pm*5 including the 5′ upstream sequence was obtained by PCR amplification of genomic DNA and genome walking. The crustin*Pm*5 gene (1394 bp) of *P. monodon* contained four exons (31, 75, 242 and 194 bp, respectively) separated by three introns (606, 148 and 116 bp, respectively), conforming with the canonical GT/AG splicing recognition rule at the extreme ends of each intron. The 5′-UTR located was in the first exon and the first nucleotide of the second exon, whereas the protein coding region including the stop codon is contained in the remaining exons (Fig. 2). Interestingly, two long stretches of pure dinucleotide (CT) repeats, $(CT)_{15}$ and $(CT)_{20}$, amongst a CT and $(GT)_2(CT)_n$ rich region were found in the first intron, but the degree of polymorphism of this compound microsatellite region remains to be established. The upstream sequence analysis revealed a putative core promoter region at about 39 base pairs upstream (−39 region) from the putative transcriptional start site. Some potential binding sites of important transcription factors were predicted including two NF-κB, one complete heat-shock regulatory element (HSE), and five GATA factor binding sites (Fig. 2).

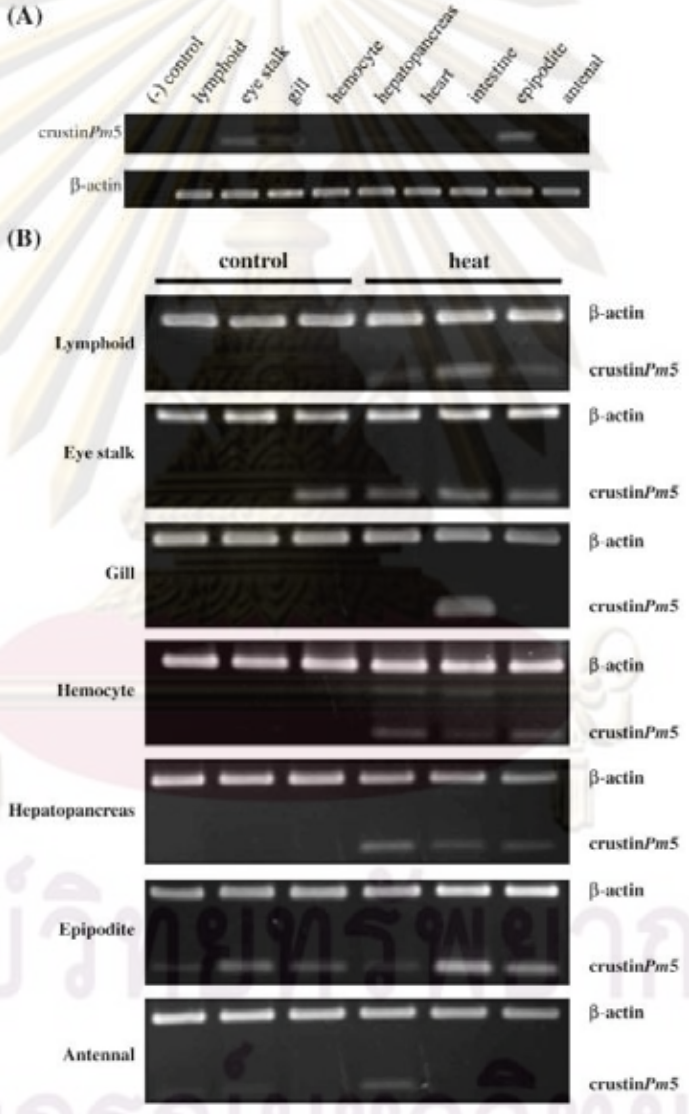

Fig. 3. Tissue distribution analysis of crustin*Pm*5 by RT-PCR analysis. (A) Expression of crustin*Pm*5 in various tissues of healthy shrimps. (B) Comparison of crustin*Pm*5 mRNA in normal (control) and heat-induced shrimp (three individuals each). Beta-actin was used as a house keeping gene.

3.3. Tissue distribution of crustin*Pm*5 transcripts in healthy *P. monodon* shrimp

The tissue distribution of crustin*Pm*5 transcripts in the hemocytes, hepatopancreas, lymphoid organ, gills, epipodite, eyestalk and antennal glands of *P. monodon* was investigated by RT-PCR, using β-actin as the internal reference control for standardization between PCR reactions and templates. From these tissues, crustin*Pm*5 transcripts were found to be expressed at a low level in the epipodite and eyestalk only, and at very low or undetectable expression levels in the remaining tissues including hemocytes (Fig. 3A).

3.4. Relative expression levels of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA after heat treatment

The presence of a putative heat-shock regulatory element (HSE) within the 5′ upstream region of the crustin*Pm*5 gene suggested that this gene is probably heat inducible. Thus, the mRNA transcript level of crustin*Pm*5 was examined in various shrimp tissues by RT-PCR following a transient increase in temperature (heat shock). The heat treatment was performed by transferring the shrimps from a tank at 28 ± 1 °C to 5 °C above (33 ± 1 °C) for 2 h and then back to 28 ± 1 °C for 6 h for recovery. An induction of crustin*Pm*5 gene transcript expression was observed in immune tissues (lymphoid, hemocytes and hepatopancreas), as well as up-regulated expression in the epipodite after heat treatment (Fig. 3B). However, the effect of heat on the expression of crustin*Pm*5 gene in eyestalk, gill and antennal gland was inconclusive (Fig. 3B).

In addition, we further investigated whether heat also affects the expression of the other two isoforms of *P. monodon* crustin, crustin*Pm*1 and crustin-like*Pm*. RT-PCR analysis showed significant up-regulation ($p<0.05$) of crustin*Pm*1, crustin*Pm*5 and crustin-like*Pm* transcripts in the epipodite of the heat-treated shrimps by about 2.7, 2.4 and 1.3 fold, respectively, as illustrated in Fig. 4.

3.5. Relative expression levels of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNA after salinity changes

Observing the crustin*Pm*5 transcript in the epipodite, it was interesting to study the influence of salinity on its expression. The expression of the crustin*Pm*5 transcript as well as those of the other two crustin isoforms was then examined in the shrimp following salinity induced stress from 25 ppt to low salinity (3 ppt) and to high salinity (40 ppt) at 6 h, 24 h and 2 weeks. Shrimp showed a significant up-regulated expression level of crustin*Pm*5, crustin*Pm*1 and crustin-like*Pm* mRNAs by 3.1, 5.5 and 3.2 fold, respectively, at 6 h after transfer from 25 ppt to 40 ppt (hyperosmotic stress) and returned to normal after 24 h (Fig. 5B) whereas no significantly change was observed when transfer to 3 ppt (hypoosmotic stress) (Fig. 5A).

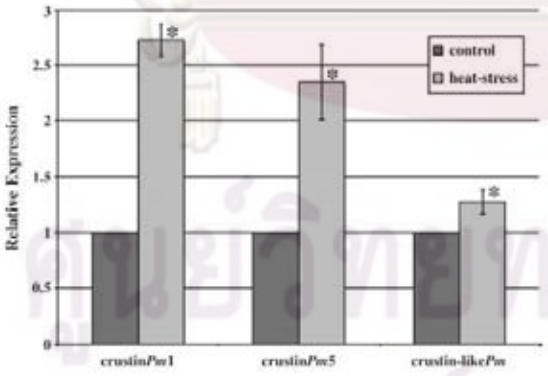

Fig. 4. The expression of crustin genes in epipodite of heat-induced shrimp *P. monodon*. The crustin gene transcripts from control and heat-induced shrimp were normalized against the expression of β-actin ($n=3$). The relative expression levels of heat-induced shrimp were also normalized against the control shrimp. Significant differences at $p<0.05$, are marked with an asterisk.

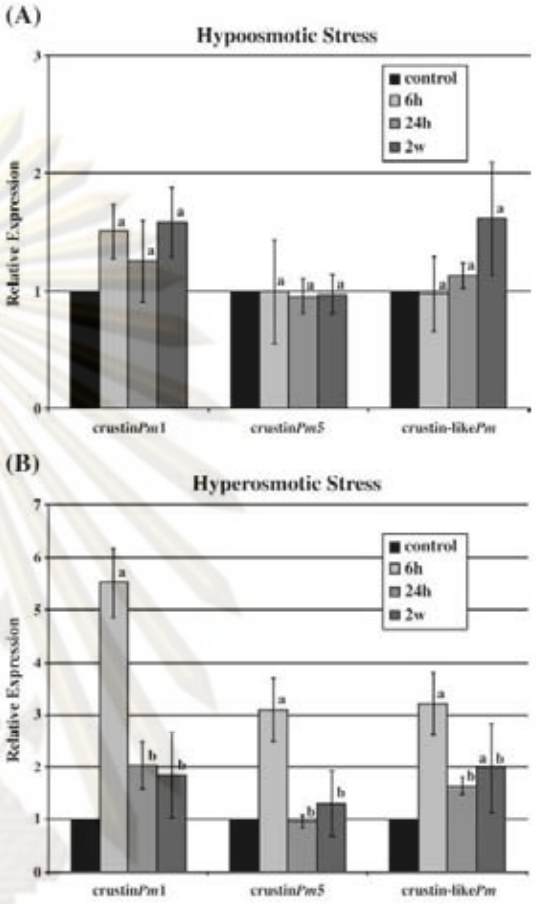

Fig. 5. The relative expression ratio of crustin genes in the epipodite of shrimps that were transferred from 25 ppt to 3 ppt (A, hypoosmotic) and 40 ppt (B, hyperosmotic) for 6 h, 24 h and 2 weeks prior to assay. The crustin transcripts were analyzed by RT-PCR and normalized against the expression of β-actin ($n=3$). The relative expression levels of both low and high salinity group were subsequently normalized against the expression at 25 ppt. Means with the same lower case letters (above each bar) are not significantly different at the $p<0.05$ level.

3.6. Expression and purification of recombinant crustin*Pm*5

The nucleotide sequence encoding for the mature peptide (no signal sequence) of crustin*Pm*5 with a 6xHis-tag at the N-terminus was cloned into the pET19b expression vector and the recombinant plasmid was transformed into *E. coli* BL21(DE3). The expression of rcrustin*Pm*5 was induced by 1 mM IPTG and was found to reach a maximum yield at 4 h post induction (data not shown). Analysis of the cell lysate, soluble and insoluble fractions (inclusion body) by SDS-PAGE resolution and coomassie blue staining showed that a major protein with the expected size (approximately 17 kDa) was principally located in the inclusion bodies (Fig. 6A).

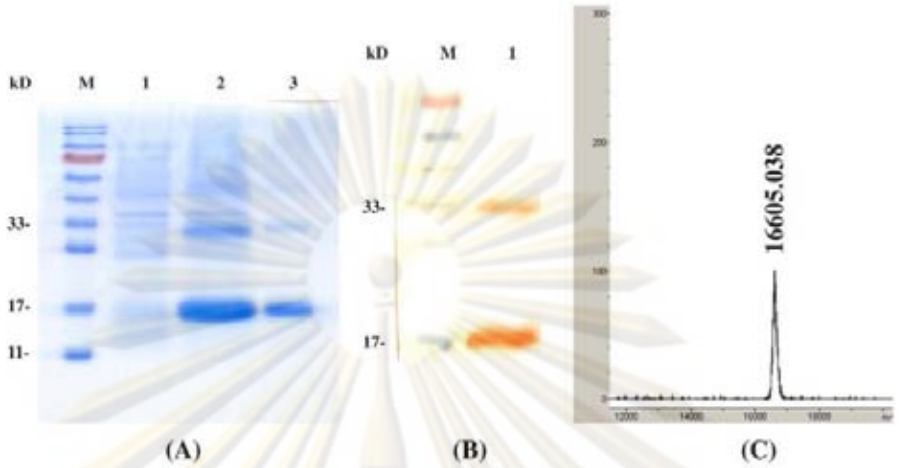

Fig. 6. The expression and purification of the recombinant crustin*Pm*5 protein. (A) SDS-15% (w/v) PAGE analysis showing the expressed proteins in *E. coli* BL21(DE3) host cells containing rcrustin*Pm*5 in the soluble fraction (lane1), inclusion fraction (lane 2), and the purified rcrustin*Pm*5 protein. The molecular masses of some of the protein standards are shown at lane M. (B) Western blot analyses of the expressed rcrustin*Pm*5 protein (lane1). M indicates protein marker. (C) MALDI-TOF spectra.

To obtain the purified rcrustin*Pm*5 protein, the inclusion body was solubilized in a denaturing solution (8 mM urea, 50 mM phosphate buffer pH 7) and purified by Ni-NTA agarose column. The purified rcrustin*Pm*5 protein was refolded by dialyzing against 50 mM phosphate buffer pH 12 and identified by Western immunoblotting using mouse anti-His antibody. A major band was identified at 17 kDa (Fig. 6B) which corresponds to the MALDI-TOF spectra (16,605 Da) (Fig. 6C).

3.7. Antimicrobial activity of crustin*Pm*5

The antimicrobial activity of the purified rcrustin*Pm*5 protein against several strains of Gram-positive and Gram-negative bacteria was determined by a liquid growth inhibition assay (Table 2). Purified rcrustin*Pm*5 was found to be highly active against *S. aureus* (MIC value of 0.78–1.56 μM) and had a lower activity against *S. haemolyticus* (MIC value of 12.5–25 μM) and *M. luteus* (MIC value of 25–50 μM). No significant activity was observed against the other Gram-positive bacteria, or all six species of Gram-negative bacteria tested, including the shrimp pathogen *V. harveyi* 639.

Table 2
Antimicrobial activity of crustin*Pm*5.

Microorganisms	MIC value (μM)[a]
Gram (+) bacteria	
Aerococcus viridans	>100
Staphylococcus aureus	0.78–1.56
Staphylococcus haemolyticus	12.5–25
Micrococcus luteus	25–50
Bacillus megaterium	>100
Gram (−) bacteria	
Enterobacter cloacae	>100
Klebsiella pneumoniae	>100
Salmonella typhimurium	>100
Escherichia coli 363	>100
Erwinia carotovora	>100
Vibrio harveyi 639	>100

[a] MIC are expressed as the interval *a–b*, where *a* is the highest concentration tested at which microorganisms are growing and *b* is the lowest concentration that causes 100% growth inhibition.

4. Discussion

Crustins are antibacterial proteins containing a four-disulphide core (4DSC) or a whey acidic protein (WAP) domain, and are found in a variety of crustaceans. The cDNA sequences of crustins have been reported in *L. vannamei* (Bartlett et al., 2002; Vargas-Albores et al., 2004), *L. setiferus* (Bartlett et al., 2002), *P. monodon* (Supungul et al., 2004), *Marsupenaeus japonicus* (Rattanachai et al., 2004), *Fenneropenaeus chinensis* (Zhang et al., 2007), *Panulirus argus* (Stoss et al., 2004), *Homarus gammarus* (Hauton et al., 2006), *C. maenas* (Brockton and Smith, 2008) and *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2007). In *P. monodon*, the analysis of crustin cDNA sequences revealed the presence of different isoforms, several of which are abundantly expressed in hemocytes and two isoforms have previously been characterized (Amparyup et al., 2008b; Supungul et al., 2008). Here, the third and unique isoform, crustin*Pm*5, was chosen for further study since unlike the other two isoforms (crustin*Pm*1 and crustin-like*Pm*), crustin*Pm*5 was identified from the gill-epipodite of heat-induced shrimp and not hemocytes. The predicted amino acid sequence of crustin*Pm*5 revealed that it has characteristics of the Type II crustins, as designated by Smith et al. (2008). Specifically, crustin*Pm*5 is predicted to contain a signal peptide, a Gly-rich region, a Cys-rich region and a WAP domain at the C-terminus.

The WAP domain has been described in proteins with diverse functions (Moreau et al., 2008) including antiproteinase and antimicrobial activities (Sallenave, 2000; Hagiwara et al., 2003). So far no proteinase inhibitory activity has been reported for Type I and Type II crustins. On the other hand, it has recently been reported that the shrimp single WAP domain proteins (SWD), categorized as Type III crustins, possess both antibacterial and antiproteinase activities (Amparyup et al., 2008a; Jia et al., 2008). Although the WAP domain of the three crustin isoforms in *P. monodon* shared reasonable overall amino acid sequence similarity (~57% to 58%), they are nevertheless quite distinct suggesting that they might possess different functional properties. Tissue distribution analysis revealed a unique characteristic of crustin*Pm*5 in that, whilst most crustins seem to be constitutively expressed in hemocytes (Smith et al., 2008), no detectable expression of the crustin*Pm*5 transcript was observed in this tissue. Rather constitutive crustin*Pm*5 transcript expression was only found in the epipodite and eyestalks, but it was inducible in most tissues after heat shock.

The finding that the crustin*Pm*5 transcript is heat inducible is in accordance with the presence of a likely heat-shock element (HSE) in the 5′ upstream region of the crustin*Pm*5 gene. HSEs are important components in the promoter region of any heat-shock protein regulated gene. When cells receive heat or other related stress stimulation, activated heat-shock factors will bind to the HSEs leading to the rapid initiation of transcription of those HSE containing genes (Chuang et al., 2007). Besides the HSE, other putative transcription factor binding sites were identified in the 5′ flanking sequence of the crustin*Pm*5 gene, including potential NF-κB and GATA-1 binding sites. NF-κB plays an important role in the Toll signaling pathway and defense system (Anderson, 2000), whilst GATA factor binding sites were reported to be involved in the interactions with NF-κB transcription factor in fat body-specific expression in insects. Both potential GATA factor and NF-κB binding sites, but not a HSE, were also identified in the crustin-like*Pm* gene (Amparyup et al., 2008b). In accordance to the absence of a HSE, a lower level of heat induction of crustin-like*Pm* gene (1.3 fold) was observed as compared to those of crustin*Pm*5 (2.4) and crustin*Pm*1 (2.7). The highest up-regulation of crustin*Pm*1 transcript by heat induction suggests that the crustin*Pm*1 gene is likely to contain heat-shock regulatory element(s) in the 5′ upstream region and this notion required further investigation.

Genomic DNA sequence analysis revealed that the crustin*Pm*5 gene is encoded by four exons interrupted by three introns, in contrast to crustin-like*Pm* gene which contains only two exons and one intron (Amparyup et al., 2008b). The data clearly demonstrated that the two isoforms of *P. monodon* crustins are encoded by different genes and that the isoform diversity is not derived from alternative splicing of the same gene. It should be noted that introns 2 and 3 of the crustin*Pm*5 gene are inserted within the coding sequence of exons 3 and 4, whereas the coding sequence of the crustin-like*Pm* gene is contained within one exon and thus is not interrupted by an intron. The splicing of interrupted intron(s) on the protein coding sequence probably resulted in a low level of gene expression. Previously, the carcinin gene has been reported to compose of three exons and four introns (Brockton et al., 2007).

Antimicrobial assays against various strains of bacteria revealed that the recombinant crustin*Pm*5 displayed only significant detectable activity against some Gram-positive bacteria. This finding is in some accordance with the previous reports that most crustins, including carcinin of *C. maenas* (Relf et al., 1999), the crustin-like*Fc*1 proteins of *F. chinensis* (Zhang et al., 2007), crustin*Pm*1 from *P. monodon* (Supungul et al., 2008) and carcinin-like of *Scylla partamamosan* (Imjongjirak et al., 2009), exhibit bactericidal activity against Gram-positive and not Gram-negative bacteria. The current clear exception is that of the crustin-like*Pm* product which also exhibited activity against Gram-negative bacteria (Amparyup et al., 2008b). However, these crustins display variation in both species specificity, and perhaps amongst different strains of the same broad species, and effectiveness for killing or preventing the growth of the bacteria, which may be due to sequence variation in the WAP domain of crustins.

In crustaceans, antimicrobial peptides (AMPs), including penaeidin (Destoumieux et al., 1997) and carcinin (Chisholm and Smith, 1992; Schnapp et al., 1996), are synthesized and stored in the granular hemocytes and released by exocytosis upon microbial invasion. Surprisingly, crustin*Pm*5 transcripts were found to mainly be expressed in the epipodite of *P. monodon* whilst the expression in hemocytes, as well as other tissues, was only found to be induced after heat induction. Generally, antimicrobial molecules are widely distributed in cells of the immune system such as leukocytes/hemocytes and in the tissues that encounter bacterial infections such as the intestine and gills. Most crustins reported so far have been found mainly in the hemocytes but with transcript expression levels that were also observed in the intestine, gills and heart (Supungul et al., 2004; Zhang et al., 2007). Two crustin-like transcripts from the spiny lobster, *P. argus* Latreille (Decapoda: Palinuridae), and the fiddle crab, *Uca pugilator* Bosc. (Decapoda: Ocypodidae), have been found to be expressed in regenerating epithelial tissue in the olfactory organ and regenerating limbs, respectively (Durica et al., 2006; Brockton et al., 2007). Although it appeared that crustins are typically constitutively expressed in multiple tissues, the expression of crustin*Pm*5 in the epipodite is quite distinct. Moreover, it was found that the expression of crustin*Pm*5 as well as the other two crustin isoforms transcripts in shrimp epipodite was up-regulated in the epipodite of *P. monodon* upon hyperosmotic stress (transfer from 25 ppt to 40 ppt). Epipodites of crustaceans are known to play an important role in ionic exchange are involved in osmoregulatory function (Flik and Haond, 2000; Cieluch et al., 2004). The synthesis of crustin*Pm*5 in this organ and its induction upon salinity stress may indicate a diverse function or co-opted functions of AMPs in the crustin family. Previously, it has been shown that crustin expression in the crab, *C. maenas*, was affected by temperature stress suggesting that it might be involved in processes to maintain homeostatic integrity against injury, trauma or environmental stresses, such as heat shock (Brockton et al., 2007; Smith et al., 2008). However, the mechanism remains unclear. A direct antimicrobial action is equivocal, unless the protein works in concert with others, for the reason that this protein only exhibited antimicrobial activity against a few Gram-positive bacteria *in vitro* and not against Gram-negative bacteria including this isolate of *V. harveyi*. Thus, the effect of crustin*Pm*5 may involve its function as a modulator of immune responses or tissue damage and injury related stresses following bacterial pathogen infection. Certainly, it has become evident that several cationic antimicrobial peptides have diverse functions in modulating immunity and having an impact on infections and inflammation (Brown and Hancock, 2006), whilst any role in viral immunity remains unclear.

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