การวิเคราะห์การแสดงออกของยีนและสมบัติการต้านไวรัสจุดขาวของเพปไทด์ต้านจุลชีพจากกุ้งกุลาดำ *Penaeus monodon*

นางสาวนพวรรณ วรมงคลชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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GENE EXPRESSION ANALYSIS AND ANTI-WSSV PROPERTY OF ANTIMICROBIAL PEPTIDES FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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Thesis Title	GENE EXPRESSION ANALYSIS AND ANTI-WSSV PROPERTY OF ANTIMICROBIAL PEPTIDES FROM THE BLACK TIGER SHRIMP *Penaeus monodon*
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นพวรรณ วรมงคลชัย : การวิเคราะห์การแสดงออกของยีนและสมบัติการต้านไวรัสจุดขาวของเพปไทด์ต้านจุลชีพจากกุ้งกุลาดำ *Penaeus monodon* GENE EXPRESSION ANALYSIS AND ANTI-WSSV PROPERTY OF ANTIMICROBIAL PEPTIDES FROM THE BLACK TIGER SHRIMP *Penaeus monodon* อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร.อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.เปรมฤทัย สุพรรณกูล., 122 หน้า

เพปไทด์ต้านจุลชีพ (Antimicrobial peptide หรือ AMP) ทำหน้าที่สำคัญในการต้านการรุกรานของเชื้อจุลชีพในระบบภูมิคุ้มกันแบบไม่จำเพาะ จากฐานข้อมูล Expressed Sequence Tag database ของกุ้งกุลาดำ (http://pmonodon.biotec.co.th) พบเพปไทด์ต้านจุลชีพ ได้แก่ ครัสติน (crustin) ไลโซไซม์ (lysozyme) แอนติไลโพพอลิแซ็กคาไรด์ แฟกเตอร์ (antilipopolysaccharide factor, ALF) และพีเนียดิน (penaeidin, PEN) เมื่อศึกษาการแสดงออกของยีนเหล่านี้ในกุ้งที่ได้รับเชื้อไวรัสตัวแดงดวงขาว (WSSV) ด้วยเทคนิค semi-quantitative RT-PCR พบยีน ALF*Pm*3, ALF*Pm*6 และ *Penmon*PEN5 มีการแสดงออกเพิ่มขึ้นอย่างมีนัยสำคัญหลังจากกุ้งได้รับเชื้อ WSSV ในการศึกษานี้สนใจศึกษาลักษณะสมบัติและการต้านไวรัสจุดขาวของ *Penmon*PEN5 จากการศึกษาการจัดเรียงตัวของยีน *Penmon*PEN5 ด้วยวิธี PCR และ genome walking พบว่ายีนนี้ประกอบด้วย 1 intron และ 2 exon ส่วนบริเวณปลาย 5′ ของยีนพบโปรโมเตอร์ และส่วนที่ทำหน้าที่ควบคุมการแสดงออกของยีนที่สร้างเพปไทด์ต้านจุลชีพคือ บริเวณ GATA 3 ตำแหน่ง บริเวณ GATA-3 Activator protein 1 (AP-1) และ dorsal transcription binding sites อย่างละ 2 ตำแหน่ง จากการศึกษาการแสดงออกของยีนนี้ในเนื้อเยื่อและอวัยวะต่างๆ ของกุ้ง พบว่ายีน *Penmon*PEN5 มีการแสดงออกมากในเม็ดเลือดกุ้ง และเมื่อวิเคราะห์ด้วยวิธี Quantitative Real-time RT PCR พบยีนนี้มีการแสดงออกมากขึ้นประมาณ 1.8 เท่าที่เวลา 24 ชั่วโมงหลังกุ้งได้รับเชื้อ WSSV เพื่อศึกษาหน้าที่ของ *Penmon*PEN5 ในกุ้งเมื่อได้รับเชื้อ WSSV จึงยับยั้งการแสดงออกของยีนนี้ด้วยอาร์เอ็นเอสายคู่ที่จำเพาะต่อยีน *Penmon*PEN5 ส่งผลให้จำนวนของเชื้อ WSSV ในเม็ดเลือดกุ้งที่ไม่มีการแสดงออกของยีนนี้เพิ่มขึ้นประมาณ 1.9 เท่า เมื่อเทียบกับกลุ่มควบคุม นอกจากนี้ยังได้ทดสอบสมบัติในการยับยั้งการติดเชื้อ WSSV ในเซลล์ปฐมภูมิจากเม็ดเลือดกุ้ง โดยทำการสร้างรีคอมบิแนนท์โปรตีนของ *Penmon*PEN5 (r*Penmon*PEN5) ในระบบของยีสต์ *Pichia pastoris* และทำบริสุทธิ์ด้วยวิธี cation exchange chromatography โปรตีนที่ได้มีฤทธิ์ยับยั้งการเจริญของแบคทีเรียแกรมบวก *Micrococcus luteus* และ *Aerococcus viridans* และนำไปทดสอบสมบัติในการยับยั้งการติดเชื้อ WSSV ในเซลล์ปฐมภูมิจากเม็ดเลือดของกุ้งโดยใช้เทคนิค RT-PCR ติดตามยีนของโปรตีนที่ผิวไวรัสตัวแดงดวงขาว (VP28) พบว่า r*Penmon*PEN5 ความเข้มข้น 6.25 ไมโครโมลาร์ สามารถยับยั้งการเพิ่มจำนวนของ WSSV ได้ แต่ที่ความเข้มข้นสูงไม่เกิดการยับยั้ง จากผลการศึกษาชี้ให้เห็นว่า *Penmon*PEN5 น่าจะมีบทบาทสำคัญในกระบวนการต่อต้านเชื้อ WSSV ในกุ้งกุลาดำแต่จะต้องศึกษากลไกการทำงานต่อไป

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต นพวรรณ วรมงคลชัย

สาขาวิชา ชีวเคมี ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก

ปีการศึกษา 2553 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม เปรมฤทัย สุพรรณกูล

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NOPPAWAN WORAMONGKOLCHAI : GENE EXPRESSION ANALYSIS AND ANTI-WSSV PROPERTY OF ANTIMICROBIAL PEPTIDES FROM THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR : PREMRUETHAI SUPUNGUL, Ph.D., 122 pp.

Antimicrobial peptides or AMPs are small peptides which play an important role in the innate immune system by defending against invading microorganisms. The crustin, lysozyme, antilipopolysaccharide factor (ALF), and penaeidin (PEN) are AMPs identified from the *Penaeus monodon* EST database (http://www.pmonodon.biotec.or.th). Analysis of the *P. monodon* AMP transcripts by semi-quantitative RT-PCR revealed that the expression of ALF*Pm3*, ALF*Pm6* and *Penmon*PEN5 was significantly increased after shrimp were challenged with white spot syndrome virus (WSSV). In this study, we further characterized *Penmon*PEN5 for its role in anti-WSSV. Genomic organization of the *Penmon* PEN5 gene determined by PCR and genome walking revealed two exons interrupted by an intron, while the 5′ upstream sequence contained a putative promoter, TATA box, and regulatory sequences, three GATA, and two each of GATA-3, activator protein 1 (AP-1) and dorsal transcription factor binding. These cis-regulatory elements are reported to be involved in the transcription of several arthropods antimicrobial peptide genes. The *Penmon*PEN5 mRNA was mainly expressed in shrimp hemocytes and was up-regulated about 1.8 fold at 24h after challenge with WSSV as detected by quantitative real-time RT-PCR. The suppression of *Penmon*PEN5 transcript levels by RNA interference mediated gene silencing led to an increase of WSSV copy numbers about 1.9 fold. The recombinant *Penmon*PEN5 protein (r*Penmon*PEN5) over-expressed in the yeast *Pichia pastoris* exhibited antibacterial activity against Gram-positive bacteria, *Micrococcus luteus* and *Aerococcus viridans*. Incubation of the *P. monodon* hemocyte primary cell culture with the mixture of WSSV and r*Penmon*PEN5 inhibited the propagation of WSSV only at 6.25μM but not at higher concentration of the protein. Taken together, the results suggest a possible role of *Penmon*PEN5 in the shrimp's antiviral immunity but its defense mechanism requires further investigation.

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μg	microgram
μl	microliter
ALF	anti-lipopolysaccharide factor
AP-1	activator protein 1
bp	base pair
C-terminal	carboxyl terminal
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EF-1 alpha	Elongation factor-1 alpha
EST	expressed sequence taq
EtBr	ethidium bromide
h	hour
hpi	hour-post injection
hpt	hematopoietic tissue
kb	kilobase
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometre
N-terminal	amino terminal
^{o}C	degree Celcius
OD	optical density
ORF	open reading frame

PCR	polymerase chain reaction
ppt	part per thousand
RNA	ribonucleic acid
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
RT	reverse transcription
RT	reverse transcription
sec	second
UTR	untranslated region
β-actin	Beta actin

CHAPTER I
INTRODUCTION

1.1 General introduction

Between 2000-2008, the shrimp and prawn production were the second highest group reported value in the aquaculture productions (Table 1.1). In 2008, the value reported of shrimps and prawns (8.98% of total aquaculture production) contained 62.87% of Pacific white shrimps (*Litopenaeus vannamei*) followed by 23.46% of black tiger shrimp (*Penaeus monodon*) and the other shrimp (FAO 2008). Thailand is one of the ten leading exporters of shrimps in the world. Cultivated shrimp in Thailand have started from the Department of Fisheries succeeded in the propagation of black tiger shrimp hatcheries since about 1972 (Tookwinas, 1991) and expanded rapidly between 1985-1988, with growth in the province of Central, Eastern and Southern Gulf of Thailand. This rapid growth resulted in the reduction of shrimp cost (1989-1990) and subsequently caused disease outbreaks (1990-1993). The virus that was the major cause of disease outbreaks in the early was yellow head virus and later (1993) white spot syndrome virus (WSSV) which the disease can also widely damage in all area of shrimp culture till today. Previously, the major farmed shrimp species in Thailand was the black tiger shrimp. However, the performance of the black tiger shrimp culture has fallen since 1997 that resulted from the smaller size and the dropped survival rate of shrimp (Limsuwan, 1991; Kongkeo, 1994). Until 2002, the black tiger shrimp production has decreased continuously while that of the pacific white shrimp has increased. Now, the black tiger shrimp export of Thailand declined from 64,565.41 tons in 2006 to 23,158.43 tons in 2009 and the pacific white shrimp expanded from 35,942.76 tons in 2006 to 342,089.05 tons in 2009 (Fig. 1.1).

The Pacific white shrimp, *Litopenaeus vannamei*, are native to the eastern Pacific, Mexico and Peru (Pérez Farfante and Kensley, 1997). It becomes the main cultured species in Thailand because it has much advantage. Examples include rapid growth rate, lower protein requirements, certain disease resistance (pathogen resistant stock), tolerance of high stocking density, tolerance of low salinities, and high survival during larval rearing (Briggs et al., 2004). Although the farms can survive the

crisis by cultivation of the new shrimp species, it does not mean the sustainability of shrimp production in the long run since the actual causes of the infectious diseases are still there in the farm.

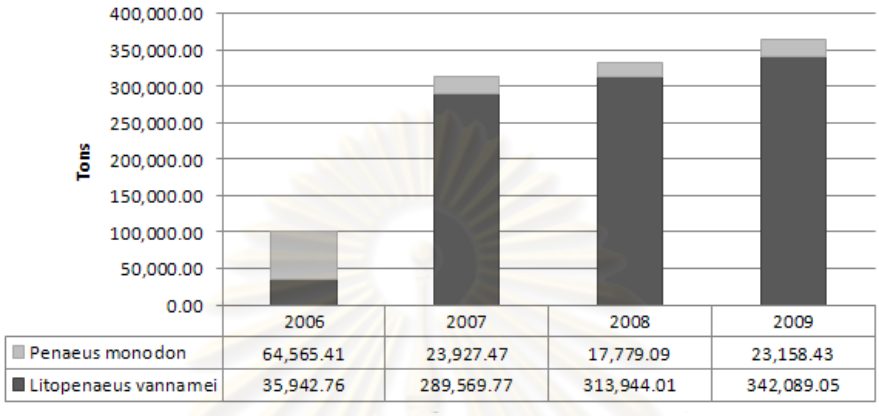


Figure 1.1 The quality export of the black tiger shrimp (*Penaeus monodon*) and white shrimp (*Litopenaeus vannamei*) of Thailand in 2006-2009. (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand)

To solve the above problems and maintain the shrimp production, the overall biological systems of *P. monodon* are continuously studied including shrimp immunity, genetics, pathology, physiology, aquaculture, selective breeding, to name a few. The knowledge can be applied to increase resistance to the pathogens and enhance growth rate.

Table 1.1 World aquaculture productions by species groups in 2000-2008 [Source: FAO Fishstat (2008)] (Q = tons, V = USD 1000)

Species group Groupes d' espèces Grupos de especies		2000	2001	2002	2003	2004	2005	2006	2007	2008
Carps, barbels and other cyprinids	Q	13 859 744	14 575 364	14 939 478	15 635 898	16 890 130	17 766 017	18 532 640	18 950 904	20 593 403
	V	13 336 498	13 252 397	13 164 944	13 988 932	15 967 667	16 389 386	17 717 927	23 177 414	26 694 905
Tilapias and other cichlids	Q	1 190 021	1 303 435	1 418 953	1 587 142	1 795 208	1 996 220	2 238 485	2 551 275	2 797 819
	V	1 614 132	1 715 939	1 696 590	1 853 290	2 052 653	2 266 473	2 651 937	3 638 662	4 021 164
Miscellaneous freshwater fishes	Q	2 562 763	2 742 708	3 459 118	3 134 773	3 556 189	3 963 621	4 568 393	5 146 646	5 359 290
	V	3 478 269	3 728 686	4 239 383	4 677 837	5 167 665	5 854 713	6 989 411	9 207 667	9 799 669
Sturgeons, paddlefishes	Q	3 158	3 091	4 087	13 248	13 856	17 800	19 064	25 706	25 683
	V	27 951	26 770	34 864	51 871	56 811	67 660	67 148	91 692	105 339
River eels	Q	212 284	210 303	209 823	210 179	223 908	217 434	238 981	273 882	265 488
	V	937 751	936 355	891 533	784 653	820 943	979 536	1 054 210	1 295 647	1 232 776
Salmons, trouts, smelts	Q	1 546 995	1 785 098	1 800 097	1 877 156	1 986 810	2 003 755	2 121 941	2 235 580	2 295 523
	V	4 877 241	5 048 707	4 909 119	5 670 287	6 657 302	7 741 964	9 842 895	10 742 197	10 669 958
Shads	Q	47	1	35	206	56	708	2 700	1 292	397
	V	47	1	35	206	56	5 749	14 120	3 721	2 574
Miscellaneous diadromous fishes	Q	488 654	521 685	553 171	580 742	602 623	626 115	617 100	701 818	721 193
	V	797 499	805 782	547 881	578 280	782 213	699 615	734 804	902 284	1 108 401
Flounders, halibuts, soles	Q	26 310	28 459	35 940	83 453	101 742	125 536	118 089	128 752	148 808
	V	327 181	303 187	334 559	493 351	488 559	569 610	702 479	713 138	650 220
Cods, hakes, haddocks	Q	169	1 019	1 450	2 630	3 881	8 193	13 284	13 722	21 387
	V	386	2 259	4 447	8 845	13 283	27 387	50 707	55 089	83 831
Miscellaneous coastal fishes	Q	367 614	362 635	386 315	695 235	709 881	794 078	891 577	973 187	946 499
	V	1 869 939	1 759 053	1 602 231	2 309 207	2 377 167	2 689 948	2 859 412	3 310 597	3 584 327
Miscellaneous demersal fishes	Q	8 701	9 330	16 638	23 938	19 708	21 636	28 013	35 979	33 255
	V	68 711	54 715	73 552	140 313	154 125	197 587	202 970	218 113	195 322
Tunas, bonitos, billfishes	Q	3 513	5 487	6 197	4 727	11 907	9 971	11 812	8 485	8 926
	V	96 618	126 654	130 880	80 986	181 791	111 889	132 986	108 256	123 094
Miscellaneous pelagic fishes	Q	147 029	163 790	172 555	193 422	185 750	197 229	194 721	208 422	209 110
	V	1 288 078	1 355 475	1 430 786	1 421 503	1 350 767	1 437 467	1 400 675	1 461 593	1 439 134
Marine fishes not identified	Q	395 558	451 963	510 887	189 458	202 011	245 935	340 620	321 673	397 522
	V	316 191	357 630	566 638	229 234	212 835	269 937	377 035	552 603	539 797
Freshwater crustaceans	Q	428 614	520 922	577 045	784 807	845 969	913 882	954 846	1 271 864	1 369 626
	V	1 747 949	2 117 013	2 363 357	3 023 962	3 571 100	3 900 496	4 223 033	6 601 763	7 666 533
Crabs, sea-spiders	Q	125 501	145 633	172 101	167 533	178 838	195 995	198 258	231 065	240 781
	V	487 284	573 930	656 777	414 904	454 031	549 773	580 943	648 085	747 840
Lobsters, spiny-rock lobsters	Q	73	47	30	35	39	29	35	70	372
	V	1 128	694	398	502	660	527	710	1 863	5 491
Shrimps, prawns	Q	1 136 168	1 309 710	1 465 538	2 049 011	2 363 575	2 662 411	3 117 978	3 281 558	3 399 105
	V	7 155 901	7 270 406	7 686 571	8 117 559	9 301 246	10 412 153	12 446 851	13 562 178	14 291 651
Miscellaneous marine crustaceans	Q	86	3	91	76	96	89	115	122	109
	V	100	13	143	96	471	536	1 045	1 074	714
Freshwater molluscs	Q	10 220	10 399	13 414	112 985	125 212	127 107	135 124	139 024	153 471
	V	14 014	15 645	20 948	63 595	79 514	73 408	81 677	109 432	138 101
Abalones, winkles, conchs	Q	3 351	3 598	3 079	205 560	251 549	291 985	320 354	374 762	359 432
	V	54 840	52 436	44 836	231 545	296 906	374 327	444 057	545 830	618 584
Oysters	Q	3 610 867	3 786 892	3 883 679	4 016 347	4 142 805	4 156 266	4 263 307	4 402 188	4 164 010
	V	3 165 108	3 157 658	3 251 777	2 481 401	2 625 543	2 870 484	2 943 460	3 016 081	3 174 608
Mussels	Q	1 307 243	1 375 080	1 552 109	1 622 198	1 670 017	1 717 904	1 814 462	1 597 102	1 624 727
	V	592 077	608 814	689 497	966 322	902 530	1 038 622	1 210 865	1 612 734	1 603 171
Scallops, pectens	Q	1 047 884	1 102 345	1 113 078	1 102 063	1 052 561	1 146 909	1 261 693	1 464 157	1 410 830
	V	1 494 942	1 522 453	1 571 536	1 573 024	1 608 214	1 774 700	1 961 565	2 245 720	2 374 631
Clams, cockles, arkshells	Q	2 354 730	2 799 550	3 065 761	3 372 454	3 634 661	3 677 841	3 798 808	4 203 370	4 397 183
	V	2 728 143	3 115 141	3 400 349	3 785 392	2 943 097	3 410 262	3 660 798	3 976 971	4 557 854
Squids, cuttlefishes, octopuses	Q	28	16	14	8	12	16	11	27	30
	V	112	66	56	32	48	64	44	108	254
Miscellaneous marine molluscs	Q	1 423 241	1 209 845	1 234 779	918 025	959 459	995 038	1 124 873	849 754	982 932
	V	617 850	594 983	620 463	486 524	537 148	584 551	662 784	525 524	639 767
Frogs and other amphibians	Q	3 510	3 360	3 121	70 718	67 796	74 539	75 501	80 616	84 944
	V	9 173	7 081	7 039	256 825	243 557	272 852	285 824	380 984	432 930
Turtles	Q	84 969	104 485	106 698	138 698	154 971	174 565	182 611	212 547	228 889
	V	343 278	389 750	399 624	520 459	582 431	661 108	708 896	1 040 544	1 192 310
Sea-squirts and other tunicates	Q	9 966	13 847	18 814	15 602	21 442	17 958	16 931	19 487	18 526
	V	6 884	11 233	15 087	12 339	17 971	21 191	19 337	22 668	18 833
Sea-urchins and other echinoderms	Q	...	5	25	37 482	53 248	62 903	74 867	85 040	95 870
	V	...	22	43	112 530	159 803	207 801	254 153	263 688	332 771
Miscellaneous aquatic invertebrates	Q	57 099	61 267	58 431	69 293	78 673	95 843	73 872	113 560	191 065
	V	142 421	153 748	207 370	136 094	158 377	207 463	166 729	211 725	401 506
Brown seaweeds	Q	4 417 633	4 268 451	4 515 172	5 983 337	6 402 367	6 926 355	6 644 891	6 536 194	6 626 440
	V	2 678 802	2 595 725	2 693 418	3 285 917	3 414 489	3 644 269	3 474 043	3 453 156	3 640 039
Red seaweeds	Q	1 921 737	2 165 849	2 490 122	3 024 222	3 874 913	4 590 595	5 205 149	5 984 393	6 588 144
	V	1 277 831	1 305 934	1 514 932	1 520 727	1 800 128	2 021 881	2 235 477	2 538 285	2 548 867
Green seaweeds	Q	33 891	30 918	20 081	7 952	18 636	12 266	16 785	16 569	26 017
	V	5 846	5 258	6 103	3 608	12 567	6 655	8 363	7 923	16 994
Miscellaneous aquatic plants	Q	2 882 282	3 172 265	3 466 911	2 292 778	2 297 181	1 913 836	2 152 538	2 385 015	2 540 558
	V	1 175 297	1 297 421	1 413 791	947 080	940 729	785 445	904 904	1 031 063	1 220 029

1.2 Taxonomy of *Penaeus monodon*

Penaeus monodon, the giant tiger shrimp, is a penaeid shrimp species that 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 taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 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: Tiger prawn (Africa), Jumbo tiger prawn , Giant tiger prawn , Black 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 Morphology

The exterior of penaeid shrimp contains three major regions, cephalon (head), thorax and abdomen (Fig 1.2). The position of cephalon is first 5 somites and following by 6 somites of thorax that were combined to the cephalothorax bearing all the appendages excepting the pleopods (swimming legs) and uropods. The

cephalothorax is covered by a single, immobile carapace, which shields cuticular structure arising from the posterior margins of the cephalon, extending anteriorly and posteriorly. Many internal organs locate in thorax under the carapace such as gills, digestive system, reproductive system and heart, while the muscles concentrate in the abdomen. Characteristic of carapace is ridges (carinae), grooves (sulci) and prominent sharp teeth of rostrum that locates on dorsal midpoint. Appendages of the cephalothorax differ in feature and function. In the cepharon region, the compound eyes containing cornea and eyestalk responses for vision, antennules and antennae performed sensory functions. Mandibles and two pairs of maxillae form jaw-like structures involved in food uptake (Solis, 1988). In the thorax region including first three pairs of maxillipeds modified for food handling and five pairs of pereopods are the walking legs. The abdomen composes of pleopods in first five pairs (Baily-Brook and Moss, 1992; Bell and Lightner, 1988), the sixth pairs with uropods and final with telson bearing the anus (Dall et al., 1990). A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawn (Anderson, 1993). The mouth is located in ventral and the cephalic appendages surrounding it, plus the first and second maxillipeds and sometimes the third as well, may be referred to collectively as the "mouth parts".

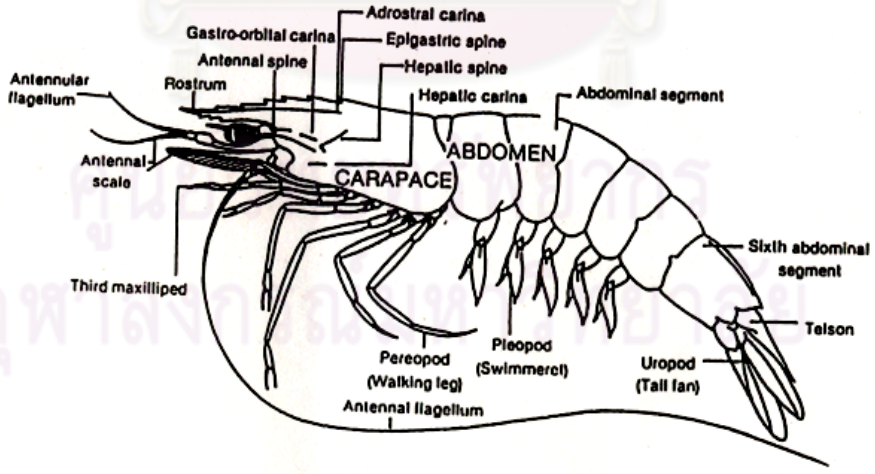


Figure 1.2 Lateral view of the external morphology of *Penaeus monodon* (Anderson, 1993).

The cuticle is secreted by an epidermal cell layer that composes of chitin and protein in which calcium carbonate and calcium phosphate have been deposited.

While the old cuticle is moulted, the epidermis detaches the inner cuticle layer from the inner cuticle layer and begins for secreting a new cuticle. After molting the new cuticle is soft and is extended to accommodate the increased sized of the shrimp.

The black tiger shrimp has the following characteristic coloration: carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackish waters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Moton, 1981: cited in (Solis, 1988).

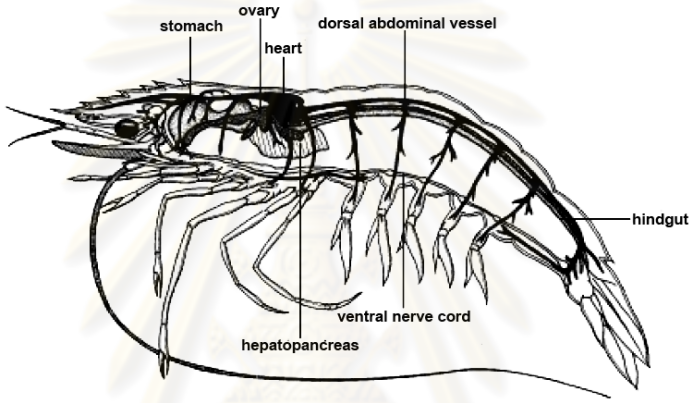


Figure 1.3 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990)

The internal morphology of penaeid shrimp is outlined in Figure 1.3. Muscular, digestive, circulatory, respiratory, nervous, and reproductive systems are all presented. The muscular, many sorts, control movements of the body for example walking, crawling, burrowing, swimming, feeding, and breathing. The complex digestive system, part of the tract is differentiated into a foregut, a midgut, and a hindgut. The circulatory system of penaeids and other arthropods are an open circulatory system consisting of a heart, dorsally located in the cephalothorax, with branching arteries conducting blood to the various organs.

The blood and the blood cells are therefore called hemolymph and hemocytes, respectively. The valve hemolymph vessels leave the heart and branch several times before the hemolymph reaches at the sinus, where exchange of substances take place, scattering throughout the body. When passed the gill, the hemilymp comeback to the heart by means of three wide non-valved openings (Bauchau, 1981). Gills serve on the respiratory process. The nervous system includes two ventral nerve cords, a

dorsal brain, and a pair of ganglia for each somite. A large part of cephalothorax in penaeid shrimps is captured by the hepatopancreas which connect to the gastrointestinal tract via the primary duct. Its main functions are absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the hemolymph vessels that leave the heart ends in the lymphoid organ where the hemolymph is filtered. This organ composes of two dixtinct lobes and locates ventro-anteriorly to the hepatopancreas. The hemocytes are produced in hematopoietic tissue. This organ consists of densely packed lobules located at different parts of the shrimp anterior region, but mainly presented around the stomach and in the onset of the maxillipeds. Lymphoid organ and hematopoietic tissue are shown in Figure 1.4.

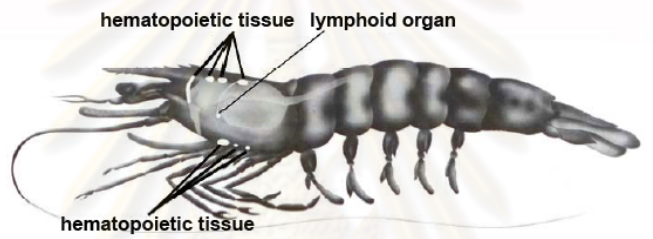


Figure 1.4 Position of hematopoetic tissue and lymphoid organ of penaeid shrimp.

1.4 Shrimp diseases

Diseases are one of the main obstacles to the shrimp aquaculture. Shrimp diseases can be divided into two etiologies, noninfectious and infectious (Lightner and Redman, 1998). The examples for noninfectious diseases are environmental extremes, nutritional imbalances, toxicants and genetic factors (Lightner, 1988; Johnson, 1995). The most serious problem is infectious diseases that are caused by viruses, bacteria, fungi and parasites. Especially, Viral and bacterial outbreaks have decimated the shrimp industries in the world.

The most prevalent bacterial in shrimp, that cause mass mortalities, is Vibrionacea family such as *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram*. Vibrio infections often result from the environmental stresses or viral diseases, and are not the primary disease problem (Nash, 1990). Viral diseases are serious outbreaks. The outbreak of the viral disease often occur from various stress factors, such as low dissolved oxygen, abnormal temperatures and overcrowding. Shrimp diseases have affected the economic viability such as losses of production,

reduced earning and decreased confidence of consumers. Hence, the disease prevention and control is of prime important to the shrimp industry. Improvement of farm management and study of the shrimp immunity can be applied in cultivation of shrimp.

1.5 Viral diseases

Viruses are known to be the most important pathogens in shrimp. Viral pathogens can infect shrimp at different life stages that cause mortality, slow growth and deformations. More than 20 viruses have been reported as pathogenic to shrimp and classified into seven families including Parvoviridae, Baculoviridae, Iridoviridae, Picornaviridae, Rhabdoviridae, and Togaviridae (Jittivadhna, 2000). In Thailand, seven viral pathogens are discovered and studied: white spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), Taura syndrome virus (TSV), Laem Singh virus (LSNV) and infectious hypodermal and hematopoeitic virus (IHHNV). For cultivation of *P. monodon* in Asia, the serious viral pathogens are WSSV, YHV and HPV causing the production losses. Losses from the viruses MBV and IHHNV are less clearly evident. While the losses of *P. vannamei* to IHHNV and TSV should be increase consideration (Flegel, 2006). This study is focused on WSSV infection in shrimp thus WSSV is described in details.

1.5.1 White spot syndrome (WSS)

White spot syndrome (WSS) was first detected in shrimp farms of northern Taiwan in 1992 (Chou et al., 1995) and then it rapidly spread to almost all Asian and the Indo-Pacific countries. The pathogen causing this syndrome names white spot syndrome virus (WSSV). The WSSV infection in shrimp reaches to 100% mortality within 7-10 days and causes enormous economic losses of shrimp farming industries (Wongteerasupaya et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Spann et al., 1997). The clinical signs observed in infected shrimp are sudden reduction in feeding, lethargic, reddish discoloration of exoskeleton, white spot of 0.5-2.0 mm diameter in the cuticle (Lightner, 1996; Wang, Y. et al., 1999). However, these clinical signs are uncertainly diagnosis of WSS (Flegel, 2006) because the reduction in feeding is

observed in uninfected shrimp before and after molting (Jory et al., 2001), white spots in the carapace can also be caused by other condition such as bacterial infection and high alkalinity on shrimp pond water (Wang Y. et al., 2000).

White spot syndrome virus has been re-classified as a new virus family, the Nimaviridae, and the genus *Whispovirus* (van Hulten et al., 2001b; Mayo, 2002). It is a bacilliform, rod-shaped and nonoccluded enveloped virus (Chou et al., 1995; Wang C. H. et al., 1995; Wongteerasupaya et al., 1995). The virions range between 210 and 420 nm in length and 70-167 nm in diameter. They contain one nucleocapsid with a typical striated appearance and five majors and at least other 39 structural proteins (Chang et al., 1996; Lu et al., 1997; Park et al., 1998; Rajendran et al., 1999; van Hulten et al., 2000a; van Hulten et al., 2000b; van Hulten et al., 2002) and one non-structural protein VP9 (Liu Y. et al., 2006) (Fig. 1.5). The viral envelope is the external cover of the mature virus that protects the virion from degradation. It is 6-7 nm thick and is a lipidic bilayer membrane structure with two electrontransparent layers divided by an electronopaque layer (Wongteerasupaya et al., 1995; Durand et al., 1997). The nucleocapsid is situated inside the envelope and is stacked ring structure composed of globular protein subunits (Durand et al., 1997; Nadala and Loh 1998). The size of nucleocapside varies according to the viral isolates and ranges between 180- 420 nm in length and 54-85 nm in diameter (Durand et al., 1997; Kasornchandra et al., 1998). Inside the nucleocapsid, there is a highly electrondense core containing the DNA binding protein VP15 and the viral DNA (Durand et al., 1997; Wang Q. et al., 1999; van Hulten et al., 2001a).

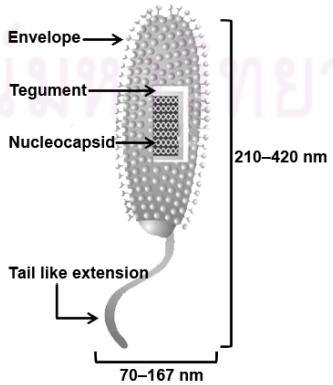


Figure 1.5 Schematic diagram of WSSV (modified from Sánchez-Paz, 2010)

The WSSV genome is a large circular double-stranded DNA and ranges from 292 kb to 307 kp in size, the 292.2 kb isolate (AF369029) from Thailand (WSSV-TH), the 307.2 kb isolate (AF440570) from Taiwan (WSSV-TW) and the 305.1 kb isolate (AF332093) from China (WSSV-CN) (van Hulten et al., 2001a; Yang F. et al., 2001; Chen et al., 2002; Sánchez-Martínez et al., 2007). The major differences among the three genomes are two polymorphic regions of about 14 kb that are not virulence related (Marks et al., 2004; Sánchez-Martínez et al., 2007). Some of the most studied genes and encode proteins of the WSSV genome are classified into four groups according to their assumed function (Table 1.3). The first group is composed of the structural genes encoding the envelope and the nucleocapsid or tegument. The second group contains functional or physiological genes that involves in virus proliferation or life cycle functions, such as replication and phosphorylation of host proteins, and nuclease activity. The third group consists of latency and sequesters genes, whose expression can be detected even though the structural genes might not be active. Moreover these genes involve in the persistence of the virus within a host cell. The temporary regulatory genes belong to the fourth group, which participate at specific times during infection. Indeed, they do not require viral protein for their transcriptional processes and are expressed using the host molecular machinery in the first hours after infection (Flint et al., 2000; Sánchez-Martínez et al., 2007). Transcription of viral genes during infection can be broadly divided into three kinetic phases of gene expression according to an ordered cascade of events. Three phase are immediate-early (IE), early (E) and late (L). The IE genes are expressed relying primarily on host proteins and factors for their expression, which occur in the absence of viral DNA replication. Thus, the proper expression of these genes, during the early stages of infection, controls the accurate cascade of viral replication. The E gene expression mainly encodes enzymes about the synthesis of viral DNA and the proteins which can regulate the expression of L genes. Synthesis of viral DNA is initiated, L gene expressions are encoded enzymes and structural proteins necessary for virion assembly (reviewed in Sánchez-Paz, 2010).

WSSV can infect various tissues of shrimp including hemolymph, gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ, antennal glands, connective tissues, muscle tissues, hepatopancreas, heart, midgut,

hindgut, nervous tissues, compound eyes, eye stalks, pleopods, pereiopods, testes and ovaries (Wongteerasupaya et al., 1995; Chang et al., 1996; Lo et al., 1997; Sahul Hameed et al., 1998; Yoganandhan et al., 2003; Escobedo-Bonilla et al., 2007). However, the major target tissues for replication are gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ and antennal glands (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007). Some tissues, midgut cecum, could not be detected that infected with WSSV (Sahul Hameed et al., 1998; Escobedo-Bonilla et al., 2007). Recently, Escobedo-Bonilla et al. (2007) had shown the primary replication sites and spreading mechanism among the tissues of WSSV. Cells in the gills and epithelial cells of the foregut in *L. vannamei* are portals of entry after oral inoculation of WSSV. After primary replication in these tissues, the WSSV crossed the basal membrane to reach the underlying connective tissues and associated hemal sinuses. The WSSV spreads to the other organs from via hemolymph circulation. Nevertheless, mechanism of WSSV infection has not yet been clearly identified. The transmembrane proteins recognition a large variety of extracellular and cell surface proteins that called integrins, have emerged as receptors or co-receptors for a large number of viruses. Several studies have reported that many RGD (Arg-Gly-Asp) containing viral proteins serve as ligands through which these viruses bind to the integrins on the cell surface and then gain entry into the cells. Many envelope proteins of WSSV contain RGD motifs including VP281, VP31, VP36A, VP110, VP136A, VP664 and VP187 (Huang et al., 2002; Tsai et al., 2004; Li et al., 2006). Lately, Li et al. (2007) found the interaction between WSSV envelope protein, VP187, with the shrimp intergrin. It is believed that β-integrin might function as a cellular receptor for WSSV infection. At the early stage of WSSV infection, the interaction between viral and integrin activated phosphorylation of MjFAK (a focal adhesion kinase of *Penaeus japonicas*) to help viral entry (Zhang et al., 2009). The PmRab7 might be a receptor and bind directly to VP28 envelope protein of WSSV in shrimp. Additionally, the suppression of PmRab7 by using dsRNA injection into the shrimp inhibited viral propagation (WSSV and YHV) suggesting that the PmRab7 is a common cellular factor required for WSSV or YHV propagation in shrimp (Sritunyalucksana et al., 2006; Ongvarrasopone et al., 2008).

Table 1.2 Functions of WSSV genes (Sánchez-Martínez et al., 2007)

Type	Function/protein encoded	gene/ORF/PROTEIN	Report
Structural	Envelope	VP19, VP466, VP281	Rout *et al.* (2004), Huang *et al.* (2002), Van Hulten *et al.* (2002)
	Envelope/actin interaction	VP26/VP22	Xie and Yang (2005)
	Envelope/attachment and cell-penetration	VP28/VP27.5	Yi *et al.* (2004)
	Envelope	VP110/*wssv092*	Li *et al.* (2006)
	Nucleocapsid	VP15, VP24	Witteveldt *et al.* (2005), Zhang *et al.* (2004), Van Hulten *et al.* (2001a)
	Nucleocapsid/assembly	VP664/*wssv419*	Leu *et al.* (2005)
	Nucleocapsid	VP35/*wssv019*/ORF687, ORF5	Tsai *et al.* (2004), Chen *et al.* (2002), Lo *et al.* (1999)
		VP136B/*wssv524*	Tsai *et al.* (2004)
	Envelope, cytokine receptor	VP76/ORF112 or 220	Huang *et al.* (2005)
	Actin interaction	VP51C/*wssv364*	Tsai *et al.* (2004)
Functional	Met Prim/ATPase S/ER	VP95/*wssv502*	Tsai *et al.* (2004)
		VP75/*wssv388*	Tsai *et al.* (2004)
		VP73/*wssv275*	Tsai *et al.* (2004)
	Vitellogenin-like	VP60A/*wssv381*	Tsai *et al.* (2004)
		VP60B/*wssv474*	Tsai *et al.* (2004)
		VP55/*wssv051*	Tsai *et al.* (2004)
	Hemocyanin	VP53A/*wssv067*	Tsai *et al.* (2004)
		VP53B/*wssv171*	Tsai *et al.* (2004)
		VP53C/*wssv324*	Tsai *et al.* (2004)
		VP51A/*wssv294*	Tsai *et al.* (2004)
		VP51B/*wssv311*	Tsai *et al.* (2004)
		VP41A/*wssv293*	Tsai *et al.* (2004)
		VP41B/*wssv298*	Tsai *et al.* (2004)
		VP39A/*wssv362*	Tsai *et al.* (2004)
		VP39B/*wssv395*	Tsai *et al.* (2004)
		VP38A/*wssv314*	Tsai *et al.* (2004)
		VP38B/*wssv449*	Tsai *et al.* (2004)
		VP36A/*wssv134*	Tsai *et al.* (2004)
	Photosystem reaction	VP36B/*wssv309*	Tsai *et al.* (2004)
		VP32/*wssv253*	Tsai *et al.* (2004)
	RING-H2 motif/sequester ligase	*wssv249*	Wang *et al.* (2005)
		VP24/*wssv480*	Tsai *et al.* (2004)
		VP13A/*wssv339*	Tsai *et al.* (2004)
		VP13B/*wssv377*	Tsai *et al.* (2004)
		VP12B/*wssv445*	Tsai *et al.* (2004)
		VP12A/*wssv065*	Tsai *et al.* (2004)
		VP11/*wssv394*	Tsai *et al.* (2004)
	dUTPase, Nucleotide metabolism	*wsv112*/*wdut*	Liu and Yang (2005), Li *et al.* (2005a)
	Non-specific nuclease	*wsv191*	Li *et al.* (2005a)
	Nucleotide metabolism	*wsv067, 172, 188, 395*	Yang *et al.* (2001)
	Anti-apoptotic	ORF390	Wang *et al.* (2004)
Temporal	Putative transcription factor	ORF126/*ie1*	Liu *et al.* (2005)
	–	ORF242/*ie2*	Liu *et al.* (2005)
	–	ORF418/*ie3*	Liu *et al.* (2005)
	GTP-binding activity	*wsv447*	Han *et al.* (2007)
Latency	Shrimp phosphatase interact	ORF427	Lu and Kwang (2004)
	Auto-repressor	ORF89/ORF151	Hossain *et al.* (2004)
	Protein kinase	*Pk wssv*	Liu *et al.* (2001)

Nomenclature: Genes appear call in italics letters. Name of proteins appear in capitals letters and open reading frames (ORFs) appear according to original reports.

Several reports shown an important major envelope protein of WSSV, is VP28. It is encoded by open reading frame (ORF) 421 (wsv421) (van Hulten et al., 2001a; Yang F. et al., 2001) expressing in the late phase (Sánchez-Paz, 2010) as a structural protein and very concern with the initial steps of systemic WSSV infection in shrimp (Van Hulten et al., 2001b). The VP28 bind the surface of shrimp cells and help the viral to enter into the cytoplasm (Yi et al., 2004) that is assumed to contribute to the recognition of receptors at the shrimp cell surface due to some potential glycosylation sites (Tsai et al., 2004); however, this has not yet been demonstrated. Moreover, feeding with recombinant VP28 can inhibit WSSV infection into shrimp and is developed to the vaccination (Witteveldt et al., 2004a; Witteveldt et at., 2004b). Some studies on VP28 gene structure and their functions would facilitate to develop diagnostic techniques and strategies for the control of the virus infection.

The WSSV infection can be detected by several diagnostic methods such as in situ hybridization (Wang C. S. et al., 1998), miniarray (Quéré et al., 2002), a reverse passive latex agglutination assay (RPLA) (Okumura et al., 2004), loop mediated isothermal amplification (LAMP) (Kono et al., 2004), application of serological methods (employing polyclonal and monoclonal antibodies), histopathological and PCR. In this study, the PCR methods are chosen for WSSV detection because it can detect as little as 5 fg of WSSV DNA (20 viral particles) (Kiatpathomchai et al., 2001). These methods include one step PCR (Lightner 1996; Lo et al., 1996), semi nested PCR (Kiatpathomchai et al., 2001), two step PCR (Hsu et al., 1999; Tapay et al., 1999; Hossain et al., 2004), quantitative competitive PCR (Tang and Lightner, 2000) and real time PCR (Durand and Lightner 2002).

Since, the WSS is the serious problem that loss shrimp production around the world. Thus, researchers have studied and developed several preventive and curative measures such as vaccination (by either the purified WSSV envelop proteins (vp) such as VP28 or vp expression plasmid DNA (Witteveldt et al., 2004a; Witteveldt et al., 2004b; Rout et al., 2007; Satoh et al., 2008; Ha et al., 2008)), immunostimulants (by plant extracts, beta-glucan, vitamin C and seaweed extracts (fucoidan) (Chang et al., 2003; Chotigeat et al., 2004; Citarasu et al., 2006; Balasubramanian et al., 2008)), antimicrobial peptide (by a synthetic mytilin (Dupuy et al., 2004; Roch et al., 2008) and rALF*Pm*3 (Tharntada et al., 2009)), The dsRNA mediated RNA interference

(RNAi) (for example, multiple injections of VP28-siRNA (Xu et al., 2007) and PmRab7-siRNA (Ongvarrasopone et al., 2008)) and General husbandry practices such as avoiding stocking in the cold season, use of specific pathogen free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, and use of biosecure water and culture systems (Withyachumnarnkul 1999).

1.6 The invertebrate immune system

Immune defense is obviously crucial for protecting against microbial, vial or parasite invasion or other foreign substances. It is classified into two types, including adaptive or acquired and innate or natural immunity, by increasing specificity defenses and evolution. Both adaptive and innate immunity are found in the vertebrates, whereas only innate immunity is found in invertebrates. The adaptive immune system produces receptors with ability to highly recognize specific pathogens or antigen and remember foreign molecules after the first exposure, known as antibodies. The innate immune system involves a large number of generalized effector molecules (Medzhitov and Janeway, 1998b).

Innate immune system is a more phylogenetically ancient defense mechanism found in all multicellular microorganisms. It is first immunity to defend and interpret the biological context of antigens and instructs the adaptive immune system to make the appropriate antibody or T-cell pesponse (Medzhitov and Janeway, 1998a) limit infection at an early stage and relies on germline-encoded receptors recognizing conserved molecular patterns that are present in the microorganism (Janeway, 1998). The innate immunity can discriminate between self and non-self recognition molecules. They recognize non-self through a series of pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). PRRs recognize conserved pathogen-associated molecular patterns (PAMPs), which are essential and unique components of virtually all microorganisms, but absent in higher organisms. Albeit, the innate immunity is primarily of immune system, but more sophisticated and complicated mechanism including immunological memory (Lee et al., 2001).

1.7 The crustacean immune system

The defense mechanism of crustacean depends completely on innate immune system based on cellular and humoral components of the circulatory system (Lee et al., 2002). First line of defense between crustaceans and the environment is the hard cuticle that covers all external surfaces of crustaceans. Moreover, the innate immune system can respond immediately 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, synthesis of antimicrobial pepteides (AMPs) and activation of proteolytic cascades that lead to melanization, blood coagulation, release of stress-reponsive proteins and molecules believed to function in opsonization and iron sequestration (Jiravanichpaisal et al., 2006a). 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

The target recognition of innate immunity is called “pattern recognition molecules (PRMs) and shared among groups of pathogens. Responding of host organism to PRMs, the pattern recognition proteins or receptors (PRPs or PRRs) have been developed. PRRs identify conserved pathogen-associated molecular patterns (PAMPs), which are absent in the host but present on the surface of pathogens (Medzhitov et al., 2002). Several known PAMPs are LPS of gram-negative bacteria, PGN of gram-positive bacteria, the mannan of yeasts, glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999; Kurata et al., 2006). This process is mediated by the hemocytes and by plasmatic protein (Medzhitov et al., 1997).

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 (LGBP, βGBP), 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). Lectins can bind to specific carbohydrates expressed on different cell surfaces due to an occurrence of agglutination reaction. The βGBP were reported in many crustaceans and suggested that βGBP is the specific receptor for β-1,3-glucan in the proPO activation system (Lee et al., 2000). LGBP 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 βGBP 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 βGBP 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 (Padhi et al., 2008).

1.7.2 Cell-mediated defense reactions

Cellular defense reactions contain 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 enclose the foreign material, occurs when a large parasite is not ingested by phagocytosis. This process kills pathogens or, at least, limits 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 (Söderhäll and Rented, 1984).

1.7.3 Hemocyte adhesion molecules

Cell adhesion, essential for the function of multicellular organisms, is not only involved in many physiological processes such as development, wound healing and hemostasis, but also in pathological conditions, for examples metastasis of cancer cells and inflammatory disease (Hynes, 1992). Moreover, in invertebrate immunity, it is important for 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 KGD motif 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 and Söderhäll, 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 system is an efficient part of the innate immune response, containing several proteins that are concerned with melanization, cytotoxic reaction, cell adhesion, encapsulation and phagocytosis. This system is triggered by the presence of minute amounts of microbial components, such as LPS and

peptidoglycans (PGN) from bacteria and β-1, 3 glucans from fungi, through pattern-recognition proteins (PRPs). In addition, this system is composed of several serine protease and their zymogens, proPO as well as proteinase inhibitors, which are important regulatory factors to avoid activation of the system where it is not appropriate (Cerenius and Söderhäll, 2004).

In crayfish, when βGBP binds to β-1,3 glucan, it becomes activated and binds specifically to a cell-surface associated protein, a superoxide dismutase (SOD) (Johansson, 1999) or binds to a β-integrin on the hemocyte surface through its RGD motif (Arg–Gly–Asp). The recognition of non-self-triggers degranulation of the semigranular cells (SGCs) and the granular cells (GCs). Among the released proteins are components of the proPO activating system, for example the proprophenoloxidase activating enzyme (proppA), which is further activated to ppA by the presence of PAMPs. Active ppA converts the proPO to PO (monophenol dihydroxyphenylalanine: oxidoreductase; EC1.14.18.1). PO is a bifunctional copper containing enzyme, which is known as a tyrosinase and catalyzes the early steps in the pathway to melanin formation. The active enzyme catalyzes the oxygenation of monophenols to *o*-diphenols and further the oxidation of *o*-diphenols to *o*-quinones and eventually the synthesis of melanin. In addition, PO is also important for pigmentation, sclerotisation in many tissues, wound healing, and encapsulation of foreign materials (Jiravanichpaisal et al., 2006a).

Another function of ppA is found in crayfish whose ppA contains one 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 recombinant clip-domain of ppA has antibacterial activity in vitro against Gram-positive bacteria suggesting a dual function of the crayfish ppA which may be true also for other ppAs (Wang R. et al., 2001). Since this part of the ppA is cleaved during the proteolytic processing of pro-ppA into ppA, it is possible that the antimicrobial activity is produced concomitantly with proPO activation (Jiravanichpaisal et al., 2006a).

In penaeid shrimp, enzymes in the proPO system are localized in the semigranular and granular cells (Perazzolo and Barracco, 1997), according with the study of Sritunyalucksana et al. (2000) demonstrated that *P. monodon* proPO mRNA is expressed only in the hemocytes. Amparyup et al., (2009) reported that the two proPOs from *P .monodon*, *Pm*proPO1 and *Pm*proPO2, were involved in the proPO system and gene silencing resulted in a significant reduction and increase

susceptibility to *V. harveyi* infection. Recently, 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 (Charoensapsri et al., 2009). These results indicate that the proPO system is a major immune reaction in the shrimp defense against bacterial infection.

1.8 Antimicrobial peptides

Antimicrobial peptides (AMPs) are important components of the innate immune system and are ubiquitously found in all kingdoms from bacteria to mammals, including fungi and plants. They are small peptides containing 150-200 amino acid residues which are mostly cationic and amphipatic molecules. However, the anionic peptides also exist. AMPs have a wide variety and diversity in amino acid sequences, structure, and range of activity. These peptides exhibit a broad spectrum of activity against microorganisms: bacterial, yeast and filamentous fungi, parasites, enveloped viruses and even tumor cell (Cruciani et al., 1991; Murakami et al., 1991; Hancock and Diamond, 2000; Tharntada et al., 2008). The AMPs have been shown to have antimicrobial activity and directly kill pathogens, but some appear to participate in immunoregulatory mechanisms by modulating signal transduction and cytokine production and/or release (Hancock and Diamond, 2000; Brown and Hancock, 2006; Guaní-Guerra et al., 2010).

AMPs contain two or more positively charged residues and can be classified on the basis of their amino acid compositions and structure: (i) anionic peptides, rich in glutamic and aspartic acids such as maximin H5 from amphibians, dermcidin from humans; (ii) linear cationic α–helical peptides, lack in cysteine such as cecropins, andropin, moricin, ceratotoxin and melittin from and LL37 from humans (iii) cationic peptides enriched for specific amino acids, rich in proline, arginine, phenylalanine, glycine and tryptophan such as bactenecins, hymenoptaecin, coleoptercin and indolicidin; (iv) anionic and cationic peptides that contain cysteine and form disulfide bonds such as defensin, protegrin and brevinin; and (v) anionic and cationic peptide fragments of larger proteins including lactoferricin and casocidinI (Brogden, 2005).

The accurate mechanism by which AMPs exert their antimicrobial properties has yet unknown, but it is generally accepted that cationic AMPs interact by electrostatic forces with the negatively charged phospholipid headgroups on the bacterial membrane and cause disruption. There are several mechanisms proposed, the widely accepted are the transmembrane pore-forming and intracellular killing (Brogden, 2005). The transmembrane pore-forming mechanisms have three proposed models explaining peptide insertion and membrane permeability (Fig 1.6): (i) the barrel-stave model, the attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core regions and the hydrophilic peptide regions form the interior region of the pore; (ii) the carpet model, the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet; (iii) the toroidal model, the attached peptides aggregate and induce the lipid monolayers to bend continuously through the pore so that the water core is in line by both the inserted peptides and the lipid head groups. Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to lysis of the microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing. There is increasing evidence indicating that antimicrobial peptides have other intracellular targets (Fig 1.7), for example, inhibition of cell wall synthesis, alteration of cytoplasmic membrane (inhibition of septum formation), activation of autolysin, binding to DNA, inhibition of DNA, RNA, protein syntheses and inhibition of enzymatic activity.

Presently, AMPs have been widely discovered in the whole living kingdom identified and are accessible on databases (http://aps.unmc.edu/AP/main.php) comprising more than 1,500 AMPs. In arthropods, several antimicrobial peptides were isolated and characterized, especially *Drosophila* and chelicerates (horseshoe crabs) (Iwanaga et al., 2005). These proteins in horseshoe crabs are mainly synthesized in the hemocyte and 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. The antimicrobial peptide synthesis in insect is mainly synthesized by the fat body that differs from those of the crustaceans (Engstrom, 1999; Hoffmann et al., 1999).

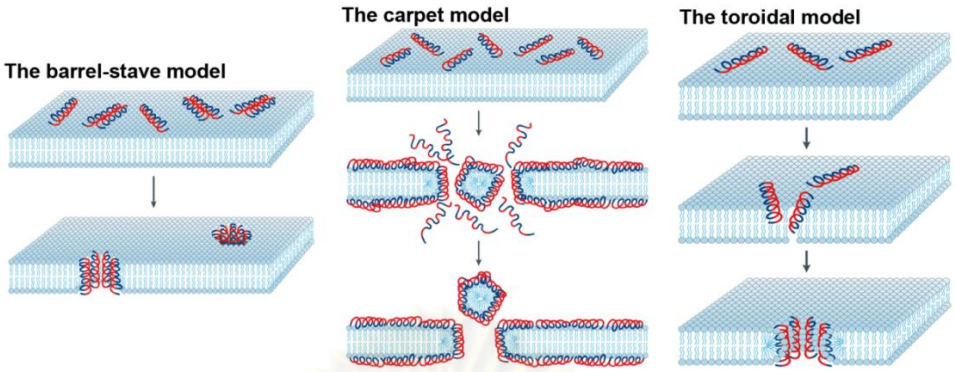


Figure 1.6 Transmembrane pore-forming mechanisms of antimicrobial-peptide-induced killing. Hydrophilic regions of the peptide are shown colored red, hydrophobic regions of the peptides are shown colored blue (Brogden, 2005).

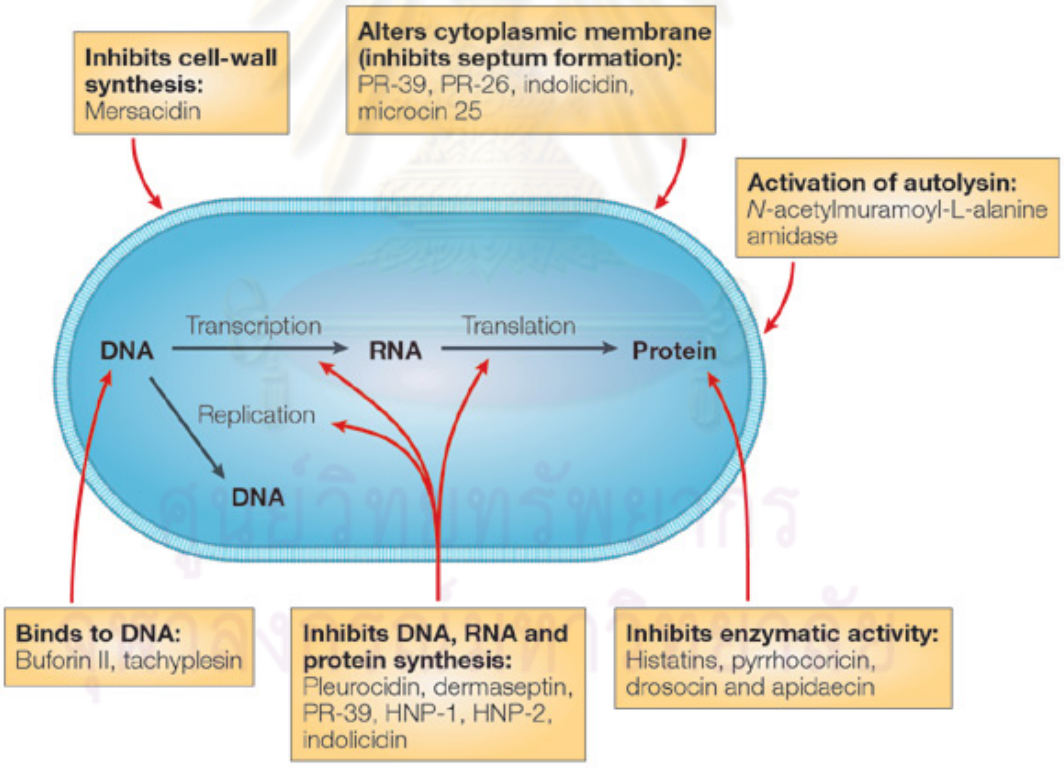


Figure 1.7 Mode of action for intracellular antimicrobial peptide activity. In the figure, *Escherichia coli* is shown as the target microorganism (Brogden, 2005).

In crustaceans which lack an adaptive immune system, these AMP molecules provide an important means to fight such foreign invaders (Hancock and Sahl, 2006).

There are many reports on antimicrobial peptides in crustaceans. In horseshoe crab, tachyplesin family and anti-LPS factors are identified which act against Gram-negative bacteria (Ohashi et al., 1984; Aketagawa et al., 1986; Nakamura et al., 1988; Muta et al., 1990). In 1997, calliectin was found in the hemolymph of blue crab, *Callinectes sapidus* and reported to be responsible for the majority of antimicrobial activity (Khoo et al., 1999). In shrimp, the first AMP family being identified is penaeidin which display antimicrobial activity against Gram-positive bacteria and fungi (Destoumieux et al., 1997). The cDNA clones of penaeidin isoform were also isolated from the haemocytes of *L. vannamei* and *L. setiferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2002). The anti-lipopolysaccharide factor (ALF) was originally isolated from the hemocytes of horseshoe crabs, *Tachypleus tridentatus* (TALF) and *Limulus polyphemus* (LALF) (Tanaka et al., 1982). They are able to specifically inhibit the lipopolysaccharide (LPS)-mediated activation of the *Limulus* coagulation system (Tanaka et al., 1982; Muta et al., 1987) and also showed strong antimicrobial activity against Gram-negative R-type bacteria (Morita et al., 1985) and a hemolytic activity on red blood cells sensitized with LPS (Ohashi et al., 1984). At the N-terminal region, ALF are highly hydrophobic and contain positively charged residues within the conserved disulfide loop, the putative LPS binding domain as predicted in *Limulus* ALF (LALF) (Hoess et al., 1993). Several isoforms of ALF have been identified from the expressed sequence tag (EST) database of *P. monodon* (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006). The expression of ALF*Pm*2 and ALF*Pm*3 was rapidly increased in *V. harveyi* challenged shrimp (Tharntada et al., 2008). Moreover, the recombinant ALF*Pm*3 protein exhibits antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria and fungi (Somboonwiwat et al., 2008) and exhibits antiviral activity (Tharntada et al., 2009). Crustins contains a single whey acidic protein (WAP) domain at the C-terminus (Smith et al., 2008). The WAP domain generally consists of 50 amino acid residues with eight cysteine residues at defined positions that form to four intracellular disulfide bonds creating a tightly packed structure (Ranganathan et al., 1999). Crustin was first identified from shore crab (*Carcinus maenas*), namely carcinin (Smith and Chisholm 2001). They were classified into three types (Type I, Type II and Type III) based mainly on the domain structure between the signal sequence and

the WAP domain (Smith et al., 2008). In *P. monodon*, the recombinant crustin*Pm*1, crustin*Pm*5, crustin-like*Pm* and SWD*Pm*2 (Type III crustin) shown the activity against Gram-positive bacterial (Supungul et al., 2008; Vatanavicharn et al., 2009), whereas crustin-like*Pm* can inhibit the growth of both Gram positive and Gram negative bacteria (Amparyup et al., 2008). The most well-known of AMP is lysozyme that described in numerous phylogenetically diverse organisms. They clave the β-1,4-glycosidic linkage between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan in the bacterial cell walls (Qasba et al., 1997). Lysozymes have been traditionally categorized into three major types: chicken-type lysozyme (c-type), goose-type lysozyme (g-type) and invertebrate-type lysozyme (i-type) (Simpson et al., 1980; Bachali et al., 2002). The i-type lysozyme is found in the invertebrate but is identified from several other species such as earthworm, leech and starfish. Its activity inhibits the bacteria in the digestive system (Jolles and Jolles, 1975). Recently, destabilase-lysozyme, a new menber of i-type lysozyme was identified from salivary gland of the medicinal leech, *Hirudo medicinalis* and the plasma of the eastern oyster (Zavalova et al., 2000). They exhibit the antimicrobial activity against Gram negative and Gram positive bacteria. From the EST libraries of *P.monodon*, the c-type and two forms of i-type lysozymes were identified (Supungul et al., 2010). 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 (Relf et al., 1999; Patat et al., 2004).

1.9 Penaeidin

Penaeidins are shrimp AMPs that first isolated from hemolymph of *L. vannamei* by reverse-phase chromatography (Destoumieux et al., 1997). They contain the unique characteristic compose of two domains, the N-terminal proline-rich domain (PRD) and the C-terminal cysteine-rich domain (CRD) containing six cysteine residues with a molecular mass of 5.5 to 6.6 kDa (Destoumieux et al., 1997; Cuthbertson et al., 2002). Based on their primary amino acid sequence diversity, penaeidins can be classified into four distinct classes: PEN2, PEN3, PEN4 and PEN5. Penaeidin class 1 has since been defined as a class 2 variant (Destoumieux et al., 1999; Cuthbertson et al., 2002).

Moreover, each class has been defined by the conserved key residues of eight specific amino acids located in precise positions that appear to be a signature (Gueguen et al., 2006; Kang et al., 2007). The signatures of penaeidins have been established in PenBase, http://www.penbase. immunaqua.com (Gueguen et al., 2006), whereas the sequence characteristics of PEN-5, initially identified in the Chinese shrimp, *Fenneropenaeus chinensis*, have been reported by Kang et al. (2007). However, the alignment of these known penaeidin sequences (as of December 2008) exposed slightly different penaeidin signatures, as variations in a few key amino acid residues were observed (Fig. 1.8).

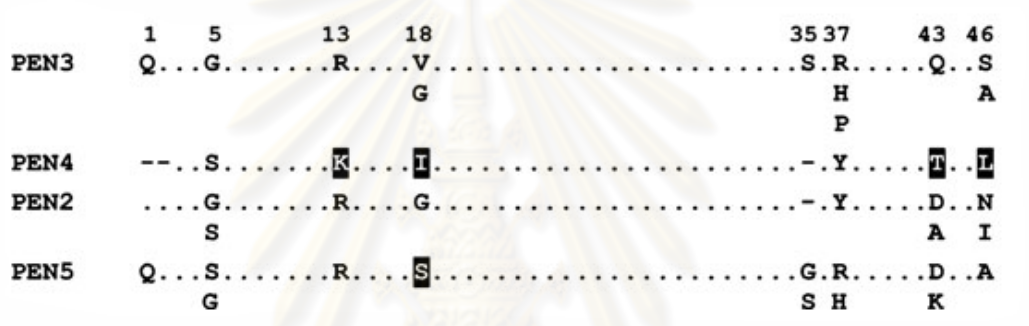


Figure 1.8 Signatures of the penaeidin subgroups. (Tassanakajon et al., 2010)

It has been reported that different shrimp species each express different classes of penaeidins (Gross et al., 2001; Supungul et al., 2002; Rojtinnakorn et al., 2002; Barracco et al., 2005). For example, PEN2, PEN3 and PEN4, were identified in the pacific white shrimp, *L. vannamei*, and the white shrimp, *L. setiferus*, transcripts were found and present to all be expressed in a single individual (Gross et al., 2001). However, quantitative real-time PCR revealed that the expression level of PEN3 transcripts was 10^4-fold and 10^3-fold more abundant than those for PEN2 and PEN4, respectively (O'Leary and Gross 2006). Whilst PEN3 and PEN5 were found only in the Chinese shrimp, *Fenneropenaeus chinensis* (Kang et al., 2007) and the black tiger shrimp, *P. monodon* (Tassanakajon, et al., 2010), with PEN3 being more abundant. Furthermore, PEN3 seemed to be the most abundant class in penaeid shrimps. PEN3 from different shrimp species differ in the coding length and amino acid sequences, whereas PEN4 are highly conserved across species. Most variations were observed in the proline-rich domain, while the cysteine-rich domain was more conserved (Tassanakajon, et al., 2010). In addition to class diversity, each penaeidin class also

displays isoform diversity arising from multiple length and single nucleotide polymorphisms (Cuthbertson et al., 2002).

During larval development, penaeidin transcripts are observed in all larval stages as early as in the nauplii stage but vary in expression levels (Muñoz et al., 2003; Jiravanichpaisal et al., 2007). Penaeidins are synthesized in hemocytes, stored in granular hemocytes and released into the blood circulation in response to microbial stimulation (Destoumieux et al., 2000; Muñoz et al., 2002), which results in a reduction of penaeidin transcripts in the early hours following stimulation and a return to normal levels at 48 h post-challenge (Muñoz et al., 2002; Supungul et al., 2004). The decrease of penaeidin expression in circulating hemocytes is a resulted from migration of penaeidin-expressing hemocytes to the infected sites (Muñoz et al., 2002). In contrast, in *F. chinensis* PEN5 transcript levels were upregulated much later in various tissues, being significantly altered from after 24 h post-microbial challenge (Kang et al., 2007). The non-congruent pattern of penaeidin gene expression within and between shrimp species indicates a likely different regulation of gene expression for each penaeidin class.

In oder to produce large amount of penaeidins, they were over-expressed in a heterologous expression system because they contain disulfide-linked cysteine residues with post-translational modification. Ho et al. (2004) used the insect-baculovirus expression system to produce PEN3 from *P. monodon*, Destoumieux et al (1999) expressed PEN2 and PEN3a from *L.vannamei* in *Saccharomyces cerevisiae*, lastly, Li et al. (2005) and Kang et al. (2007) expressed PEN3 and PEN5 from *F. chinensis* in the yeast *Pichia postoris*. In the initial description of penaeidins and recombinant penaeidins examination, it was evident that penaeidins are active primarily against Gram-positive bacteria, with some effect on fungi at higher concentration (Destoumieux et al., 1999; Cuthbertson et al., 2004), and it appears that different classes exhibit variations in their potency and target specificity against various strains of microorganisms. Although, PEN3 has a broader range of microbial targets and is more effective against certain bacteria species than other classes of penaeidins, it is less effective against fungi (Cuthbertson et al., 2006). A little or no activity against Gram-negative bacteria was observed for PEN2, PEN3, and PEN4 subgroup members, whereas *F. chinensis* PEN5 possesses antibacterial activity

against Gram-negative bacteria as well as Gram-positive bacteria and fungi (Kang et al., 2007). Interestingly, penaeidins exhibit significant activity against human pathogens including multiple species of *Candida* (Cuthbertson et al., 2006) and the herpes simplex virus type (HSV-1) (Carriel-Gomes et al., 2007). Recently, Li et al. (2010) demonstrated that penaeidin from *P. monodon* possess an integrin-β-mediated cytokine feature to promote shrimp granulocyte and semi-granulocyte adhesion.

The solution structures of the recombinant PEN3 from *L. vannamei* and the synthetic PEN4 peptide from *L. setiferus* unveil the N-terminal proline-rich domain that is unconstrained and adopts an extended structure, while the C-terminal domain forms a conserved structure that is constrained by disulfide bonds (Yang Y. et al., 2003; Cuthbertson et al., 2005). While PEN3 and PEN4 share a similar fold at the C terminus, it is likely that the sequence divergence in the N-terminal proline-rich domain and subsequent conformational differences are responsible for the differences in antimicrobial activity and effectiveness (Cuthbertson et al., 2005).

1.10 RNA interference (RNAi)

RNA interference (RNAi) or RNA-based gene silencing is a gene silencing system that found in many eukaryotes. Two types of small RNA molecules, including microRNA (miRNA) and small interfering RNA (siRNA), are important to RNA interference pathway. These small RNAs bind to mRNA and prevent an from producing a protein. In an immune system, the double stranded RNA (dsRNA) can directly prevent eukaryotic cell form viral infection (for example HIV-1, RSV, HPV, poliovirus etc.) and also induced sequence-specific inhibition of gene expression (Bagasra and Prilliman, 2004). First, dsRNA is attracted with a ribonuclease III (RNAaseIII) type protein Dicer. Dicer homologues can be found in *C.elegans*, Drosophila, plants, and mammals, suggesting that small RNA-mediated regulation is evolutionarily ancient and may have critical biological roles (Elbashir et al., 2001; Agrawal et al., 2003; Bernstein et al., 2003). Next, the dicer enzyme will cleave the dsRNA into short interfering RNA (siRNA) of 21-23 nucleotides (Hammond et al., 2000). The siRNA will be unwound into two ssRNA, namely the passenger strand and the guide strand. The passenger strand will be degraded, and the guide strand is incorporated with helicase, RecA, exo-, endo-nucleases and other protein forming

RNAi-induced silencing complex (RISC). The RISC binds to and claves the target mRNA at the center of the region complement to siRNA. As a result, mRNA is suddenly degraded leading to increasing of gene expression. MicroRNA (miRNA) is a special class of siRNA encoded by endogenous gene (Bartel et al., 2004; Ambros et al., 2007). In mammalians, miRNA is function in regulation of specific expression of immune gene (Chowdhury et al., 2005).

RNAi pathway was initially discovered in plant (Matzke et al., 1989) but the effect of dsRNA leading to gene specific silencing was elucidated in *Caenorhabditis elegans* (Fire et al., 1998). Now post-transcriptional gene silencing mechanism by dsRNA has been discovering in various organism (Mello and Conte et al., 2004). The selective and specific of RNAi on gene expression became an important tool for functional genomic studied, cell culture, biotechnology, medicine and other applications. RNAi mediated gene silencing have been used successfully in many organism such as *Drosophila* (Misquitta et al., 1999), Zebrafish (Wargelius et al., 1999), *Planaria* (Sánchez Alvarado et al., 1999) and plants (Jensen et al., 2004). In crustacean, RNAi have become a powerful technique for understanding of functional immune respond in crayfish (Liu H. et al., 2006, 2007) and *P. monodon* (Ongvarrasopone et al., 2008; Charoensapsri et al., 2009; Prapavorarat et al., 2010).

1.11 Aims of the thesis

The objective of this thesis was to investigate the role of shrimp AMPs in antiviral immunity. First, the *P. monodon* AMP genes were primarily analyzed the transcription after WSSV-challenge. Then, the AMP gene responding to WSSV-challenge, *Penmon*PEN5, was further characterized for the anti-viral properties. The viral induction of *Penmon*PEN5 mRNA expression was confirmed by quantitative real-time RT-PCR (qrt-RT-PCR), while RNA interference mediated gene silencing was performed to study the effect of penaeidin knockdown on the susceptibility of shrimp to WSSV infection. In addition, the recombinant *Penmon*PEN5 protein was produced in the yeast *Pichia pastoris* expression system and further characterized for its *in vitro* antimicrobial activity and anti-WSSV activity.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Thermo Electron Corporation)

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

Amicon Ultra-4 concentrators (Millipore)

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)

Gel document (Syngene)

GelMate2000 (Toyobo)

Hitrap SP HP (Amersham Biosciences)

Hoefer^{TM} miniVE (Amersham Biosciences)

Incubator 37 °C (Memmert)

Incubator 30 °C (Memmert)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Microscope eclipse TS100 (Nikon)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

PCR thin wall microcentrifuge tubes 0.2 ml (Perkin Elmer)

PCR workstation model # P-036 (Scientific Co., USA)

pH meter model # SA720 (Orion)

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

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

iCycler iQ™ Real-Time Detection system (Bio-Rad, USA)

CX31 Biological Microscope (Olympus)

Inverted microscope (Nikon)

Refigerated incubator shaker (New Brunswick Scientific)

Sterring hot plate (Fisher Scientific)

Thermal cycler mastercycler gradient (Eppendrof)

Touch mixer model # 232 (Fisher Scientific)

Vacuum pump (Bio-RAD Laboratories, USA)

2.1.2 Chemicals and reagents

0.22 μm millipore membrane filter (Millipore)

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

2-(*N*-morpholino)ethanesulfonic (Sigma)

2-Mercaptoethanol, C_2H_6OS (Fluka)

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (Fermentas)

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Ammonium persulfate, $(NH_4)_2S_20_8$ (USB)

Amplicilin (Biobasic)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast ext ract (Scharlau)

Biotin (Sigma)

Boric acid, BH_3O_3 (Merck)

Bovine serum albumin (Fluka)

Bromophenol blue (Merck, Germany)

Calcium chloride, ($CaCl_2$) (Merck)

Chloroform, $CHCl_3$ (Merck)

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

Diethyl pyrocarbonate (DEPC), $C_6H_{10}O_5$ (Sigma)

Dipotassium hydrogen phosphate, K_2HPO_4 (Ajax)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA)

Formaldehyde, CH_2O (BDH)

G418-sulfate (USB)

GeneRuler™ 100bp DNA ladder (Fermentas)

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Hydrochloric acid, HCl (Merck)

IPTG (Fermentus)

Isoamylalcohol, $C_5H_{12}O$ (Merck)

Isopropanol, C_3H_7OH (Merck)

Magnesium chloride, $MgCl_2$ (Ajax)

Magnesium sulfate, $MgSO_4$ (Carlo Erba)

Methanol, CH_3OH (Merck)

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

Phenol crystals, C_6H_5OH (Carlo Erba)

Phenol:chloroform:isoamyl alcohol (Sigma)

Potassium chloride, KCl (Ajax)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajax)

Prestained protein molecular weight marker (Fermentus)

Sodium acetate, CH_3COONa (Merck)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

TEMED $(CH_3)_2NCH_2CH_2N(CH_3)_2$. (Amresco)

Tricine, $C_6H_{13}NO_5$ (National diagnostics)

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

Trizol reagent (Gibco BRL)

Tryptic soy broth (Difco)

Whatman 3 MM™ filter paper (Whatman)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

Yeast nitrogen base without amino acid (Difco)

2.1.3 Enzymes

Advantage 2 Polymerase Mix (Clontech)

*Bam*HI (Biolabs, UK)

DNaseI (Promega)

*Eco*RI (Biolabs, UK)

Hotstart Phusion *Taq* DNA Polymerase (New England Biolabs)

*Not*I (Biolabs, UK)

Pfu DNA Polymerase (Promega, USA)

Proteinase K (Sigma)

RBC Taq DNA Polymerase (RBC Bioscience)

RNase A (Sigma)

*Sna*BI (Biolabs, UK)

T4 DNA ligase (Promega)

2.1.4 Microorganisms

Aerococcus viridans

E. coli strain XL-1 blue

Micrococcus luteus

Pichia pastoris KM71

2.1.5 Kits

GenomeWalker™ Universal Kit (Clontech)

ImProm-II™ Reverse Transcription system kit (Promega)

NucleoSpin® Extract II kit (Macherey-Nagel)

QIAprep spin miniprep kit (Qiagen)

RevertAid™ First Strand cDNA Synthesis Kits (Fermentas)

SYBR® Green (Bio-Rad, USA)

T & A Cloning vector kit (RBC)

T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega)

2.1.6 Vectors

pPIC9K (Invitrogen)

T&A cloning vector (RBC)

2.2 Software

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

ClustalX (Thompson et al., 1997a)

GENETYX version 7.0.3 program (Software Development Inc.)

SECentral (Scientific & Education software)

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

Genetools (SYNGENE)

Penaeus monodon EST database (http://pmonodon.biotec.or.th/home.jsp)

Neural Network Promoter Prediction

(http://www.fruitfly.org/seq_tools/promoter.html; Reese, 2001,)

MATCH^{TM}/TRANFAC 6.0 program (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi; BIOBASE)

2.3 Samples

Shrimps were obtained from a local shrimp farm in Thailand and acclimatized in laboratory aquaria, at a temperature of 28 ± 4 °C and a salinity of 15 ppt, for at least 1 week before use in experiment. Healthy sub-adult *P. monodon* shrimps of about 15 to 20 g body weight were used for analysis of the transcriptional expression of shrimp antimicrobial genes after WSSV challenge. For the gene silencing experiment, juvenile shrimps of approximate 4 g body weight were used.

2.4 Expression of antimicrobial genes from Penaeus monodon in response to WSSV challenge

2.4.1 Preparation of WSSV infected shrimp

To determine the proper dilution of WSSV for WSSV challenge experiments, three dilutions, 1:6000, 1:8000 and 1:10000 of WSSV stock solution

obtained from the Charoen Pokphand Group of Companies were tested. Shrimp were separated into 4 groups, three shrimp per group. In group 1-3, shrimp were injected with 100 µl of each WSSV dilution in lobster hemolymph medium (LHM: 15 mM $CaCl_2$, 10 mM KCl, 5 mM $MgCl_2$, 8.1 mM $MgSO_4$, 486 mM NaCl, 0.5 mM Na_2HPO_4, 36 mM $NaHCO_3$ and 0.05% (w/v) dextrose in Minimum Essential Medium (Invitrogen)). The last group, the control group, shrimp were injected with 100 µl of LHM. The shrimp mortality was observed daily for 7 days. To confirm the WSSV infection, a small portion of the gill tissue from each shrimp was homogenized in 0.2 ml of lysis buffer (2.5 N NaOH and 10% (w/v) SDS), then boiled for 10 min, incubated on ice for 3 min and centrifuged at 8,000 ×g for 10 min at 4 ºC. The supernatant was diluted 100 fold with distill water and used as the template for PCR amplification with the WSSV specific primers WSSVF/WSSVR (Table 2.1) as described by Kiatpathomchai et al. (2001). Briefly, The PCR reaction (25 µl) included 75 mM Tris–HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of specific primers, 0.2 unit of *Taq* DNA polymerase (Fermentas) and 3 µl of template. The PCR cycle parameters were an initial denaturation at 94 ^{o}C for 5 min, followed by 25 cycles of 94 ^{o}C for 30 sec, 55 ^{o}C for 30 sec and 72 ^{o}C for 30 sec, and then finally 72 °C for 10 min. Five microliters of each PCR reaction was visualized by UV transillumination following electrophoretic resolution through 1.5% (w/v) agarose-TBE gel and staining with ethidium bromide. The WSSV dilution that caused the 100% shrimp mortality within 4 days was chosen for the challenge experiments.

To prepare WSSV infected shrimp, shrimp were divided into four groups, twelve shrimp per group. The three experiment group, shrimp were injected with 1:8000 of WSSV dilutions and a control group, shrimp were injected with LHM. Hemocytes from 3 individual shrimp per group were collected at 0.25, 12, 24 and 48 hour post injection (hpi).

2.4.2 Hemocyte collection and total RNA preparation

Hemolymph was collected from the shrimp ventral sinus at 0.25, 12, 24 and 48 h post challenge (three individual shrimp for each time point) using 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 µl of an anticoagulant

solution of 10% (w/v) trisodium citrate dehydrate, pH 4.6., and immediately centrifuged at 800 ×g for 10 min at 4 ^{o}C to separate the hemocytes from the plasma. The harvested hemocyte pellet was then immediately resuspended in 1 ml of Trizol reagent (Gibco BRL) and briefly homogenized. The homogenate was incubated at room temperature for 5 min to completely separate of nucleoprotein complexes. Then 0.2 ml of chloroform was added into the sample and vigorously shaken for 15 sec. The mixture was incubated at room temperature for 2-5 min and centrifuged at 12,000 ×*g* for 15 min at 4 ℃ resulting two phases, a lower red of phenol chloroform phase and colorless aqueous phase. The RNA remains in the aqueous phase that was transferred into a fresh 1.5 ml centrifuge tube and the same volume of isopropanol was added to precipitate total RNA. The sample was stored at room temperature for 10 min and centrifuged at 12,000 ×*g* for 15 min at 4 ℃ for removing the supernatant. The RNA pellet was washed with 1 ml of cold 75% ethanol and centrifuged at 12000 ×g for 15 minutes at 4 ℃ for discard the supernatant. The RNA pellet was shortly air-dried for 10 min, and then dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water and kept at -80 ℃ until used.

The total RNA was further treated with 1 µl of RNase-free DNase (Promega, 1 unit/µl) at 37 ℃ for 30 min to eliminate the DNA contamination and extracted again with Trizol reagent as described above. The RNA pellet was preserved in 75% ethanol at -80 ℃ until use. Before use, the RNA pellet was air-dried and resuspended in appropriate volume of DEPC-treated water.

The concentration of total RNA was determined by measuring the OD_{260} and estimated in ng/µl using the following equation:

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40$$

One OD_{260} corresponds to approximately 40 ng/µl of RNA (Sambrook et al., 1989). The relative purity of RNA samples was investigated by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at 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 using 1.5% (w/v) agarose gel electrophoresis that prepared in 1× TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH8.0). The agarose slurry was melted by a microwave oven until completely dissolving and placed to cool down at room

temperature to 60 °C. Then, the solution was poured into a casting tray with a well-forming comb. The gel was immersed in a chamber that holds enough amount of 1× TBE buffer for covering the gel.

Approximate 200 ng of the total RNA was mixed with one-sixth volume of 6x loading dye (0.25% bromophenol blue and 30% glycerol in water) before loading into the well. A 100 bp DNA marker was used as the standard markers. Electrophoresis was carried out in 1× TBE buffer at 100 volts about 20 min that the bromophenol blue front was migrated to approximately ½ of the length. 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 total RNA was visualized as fluorescent bands under a UV transilluminator.

2.4.3 First strand cDNA synthesis

One microgram of the DNA-free total RNA sample was used with 0.5 µg of oligo$(dT)_{18}$ as the primer for first-strand cDNA synthesis using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The reaction was adjusted to 12 µl by Nuclease free water and incubated at 70 °C for 5 min. After on ice for 5 min, the reverse transcription reaction mix (4 µl of 5× reaction buffer, 2 µl of 10 mM each of the dNTP, 1 µl of RevertAid™ M-MuLV reverse transcriptase (200 U/µl) and 1 µl of RiboLock™ RNase inhibitor (20 U/µl),) was added and gently mixed. The reaction was incubated at 37 °C for 5 min followed by 42 °C for 60 min and then terminated reverse transcriptase activity at 70 °C for 10 min. All cDNA samples were stored at –20 ºC until use.

2.4.4 Semi-quantitative RT-PCR

For analysis of the transcriptional expression of the *P. monodon* antimicrobial genes after WSSV challenge, semi-quantitative RT-PCR was performed. The antimicrobial genes that used in this experiment were identified from *P. monodon* EST database containing ALF*Pm*2, ALF*Pm*3, ALF*Pm*6, crustin*Pm*1, crustin*Pm*4, crustin*Pm*7, penaeidin 3, penaeidin 5 and lysozyme. The gene specific primers of them (table 2.1) were used to amplify the PCR product. An internal control was amplified by the primers of beta-actin gene (table 2.1). Each PCR reactions were

carried out in a total volume of 25 µl including 1× reaction buffer (10 mM KCl, 2 mM $MgSO_4.7H_2O$, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 0.1% (v/v) Triton X-100 and 2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 mM of each primer, 0.7 unit of RBC Taq DNA Polymerase (RBC Bioscience) and an optimal concentration of template cDNA using a Thermal cycler mastercycler gradient (Eppendrof). The reactions were initially denatured at 94 ºC for 3 min followed by optimal cycles of denaturation at 94 ºC for 30 sec, annealing at an optimal temperature for 30 sec and extension at 72 ºC for 30 sec. The final extension was at 72 ºC for 7 min. The primer sequences were displayed in table 2.1 and annealing temperatures, amount of templates and cycle numbers were reported in table 2.2. The PCR products were visualized by UV transillumination following electrophoretic resolution through 2.0% (w/v) agarose-TBE gel and staining with ethidium bromide. The expression level of each transcript at a particular time was normalized with the internal control (actin) using the Genetools analysis software (Syngene). One Way Analysis of Variance (One-way ANOVA) was used for statistical analysis and values were considered to be significant at $p < 0.05$

Table 2.1 Sequences of the PCR primers used in this thesis

Primer name	Sequence (5'-3')	Usage
ALF*Pm*3QF	CCCACAGTGCCAGGCTCAA	RT-PCR
ALF*Pm*3QR	TGCTGGCTTCTCCTCTGATG	RT-PCR
ALF*Pm*2RTF	CAAGCGGTGCAGGACCTCC	RT-PCR
ALF*Pm*2RTR	TTAGTGCTCAAGCCAAATCCTGG	RT-PCR
ALF*Pm*6F	AGTCAGCGTTTAGAGAGGTT	RT-PCR
ALF*Pm*6R	GCTCGAACTCTCCACTCTC	RT-PCR
RT-crus1F	CTGCTGCGAGTCAAGGTATG	RT-PCR
RT-crus1R	AGGTACTGGCTGCTCTACTG	RT-PCR
RT-crus7F	GGCATGGTGGCGTTGTTCCT	RT-PCR
RT-crus7R	TGTCGGAGCCGAAGCAGTCA	RT-PCR
LyF	TCCTCTGGTGCTGCTGGTTG	RT-PCR
LyR	GGTTGCGGTTGCGGTTGATG	RT-PCR
SpPEN3F	GGCTTAGCCCCTTACA	Gene specific, RT-PCR
SpPEN3R	GACCCATACCTACAAATAAC	Gene specific, RT-PCR
CT470F	CAAGGATACAAGGGCGGTTA	Full-length, Recombinant protein expression

Primer name	Sequence (5'-3')	Usage
CT470R	TTATCCTTTCAATGCAGAACAA	RT-PCR, Full-length, Genomic organization, qrt-RT-PCR, Recombinant protein expression
SpPEN5F	ATCCCGACCTATTAGTACTC	RT-PCR, qrt-RT-PCR
Actin-F	GCTTGCTGATCCACATCTGCT	RT-PCR
Actin-R	ATCACCATCGGCAACGAGA	RT-PCR
EF-1αF	GGTGCTGGACAAGCTGAAGGC	qrt-RT-PCR, RT-PCR
EF-1αR	CGTTCCGGTGATCATGTTCTTGATG	qrt-RT-PCR, RT-PCR
VP28-F	TCACTCTTTCGGTCGTGTCG	RT-PCR
VP28-R	CCACACACAAAGGTGCCAAC	RT-PCR
2Gsp1PEN5	GTCAGGTGAGAGCCAAGCACCGACT	Genomic organization
2GSp2PEN5	ACGCCACGCGGTTGTGCAAGCAGTT	Genomic organization
AP1	GTAATACGACTCACTATAGGGC	Genomic organization
AP2	ACTATAGGGCACGCGTGGT	Genomic organization
T7 promoter	TAATACGACTCACTATAGGG	Genomic organization
M13R	GTCATAGCTGTTTCCTGTGTGA	Genomic organization
confirmGW	CCCGGGCTGGTAAAACTGCT	Genomic organization
PEN5RNAiF	TTGGTCTATGCTTTGCAAGG	Gene silencing
PEN5RNAiR	ACAGATAGTTAAAGTGAAAGAC	Gene silencing
WSSVF	AGAGCCCGAATAGTGTTTCCTCAGC	WSSV detection
WSSVR	AACACAGCTAACCTTTATGAG	WSSV detection
WSSV1011F	TGGTCCCGTCCTCATCTCAG	WSSV copy number detection
WSSV1079R	GCTGCCTTGCCGGAAATTA	WSSV copy number detection

Table 2.2 Conditions for the RT-PCR

Gene	Primer name	Amount of template (µl) (1:10 diluted cDNA)	Annealing temperature (°C)	Cycle number
ALF*Pm*2	ALF*Pm*2RTF	1 (undiluted)	58	30
	ALF*Pm*2RTR			
ALF*Pm*3	ALF*Pm*3QF	3	58	28
	ALF*Pm*3QR			
ALF*Pm*6	ALF*Pm*6F	3	55	30
	ALF*Pm*6R			
Crustin*Pm*1	RT-crus1F	3	55	25
	RT-crus1R			
Crustin*Pm*7	RT-crus7F	3	55	25
	RT-crus7R			

Gene	Primer name	Amount of template (µl) (1:10 diluted cDNA)	Annealing temperature (°C)	Cycle number
*Penmon*PEN3	SpPEN3F	3	57	25
	SpPEN3R			
*Penmon*PEN5	SpPEN5F	3	57	25
	CT470R			
Lysozyme	LyF	3	55	25
	LyR			
Beta-actin	Actin-F	2	55	25
	Actin-R			
Elongation factor1 alpha	EF-1αF	2	55	25
	EF-1αR			
VP28	VP28F	3	57	30
	VP28R			

2.4.5 Quantitative real time RT-PCR

The antimicrobial gene that likely to be WSSV responsive genes were investigated using quantitative real time RT-PCR (qrt-RT-PCR). The reference control was elongation factor 1 alpha (EF-1α) gene that was amplified using the EF-1αF/EF-1αR primers (Table 2.1). A standard curve for amplification of the antimicrobial gene fragment was produced and compared to that for the EF-1α fragment. qrt-RT-PCR was carried out in a real-time thermal cycler (Bio-Rad, USA) with SYBR® Green (Bio-Rad, USA). The amplification was done in a 20 µl reaction volume consisted of 3 µl of the 1:10 diluted cDNA template, 0.4 µM of each primer and 1 × iQ™ buffer containing DNA polymerase. PCR amplification was performed by an initial denaturation at 95 °C for 8 min, followed by 40 cycles of 95 °C for 20 sec, 57 °C for 25 sec and 72 °C for 30 sec. For each cDNA sample, the ΔC_t was calculated from the threshold PCR cycle (C_t) of the test gene normalized relative to the C_t of the reference EF-1α gene fragment in the same sample. The $\Delta\Delta C_t$ value was then calculated as ΔC_t (WSSV challenged group) - ΔC_t (control, LHM injected group). Each sample was done in triplicate and presented as $2^{-\Delta\Delta Ct}$, which represents the fold difference relative to the control expression. Data obtained from qrt-RT-PCR analysis were subjected to One-way ANOVA. Data differences were considered significant at $p<0.05$.

According to the differential expression of *P. monodon* antimicrobial gene determined by RT-PCR, penaeidin5 or *Penmon*PEN5 was likely to be one of WSSV responsive genes, therefore this result was confirmed by using qrt-RT-PCR and the expression of elongation factor 1 alpha (EF-1α) was used as an internal control.

2.5 cDNA sequence analysis

Penaeidin 5 cDNA was identified in *P. monodon* EST database (http://pmonodon.biotec.or.th) and translated using the GENETYX 7.0.3 program. The cDNA sequence and deduced amino acid sequence of this gene were analyzed using the NCBI BLAST programs in GenBank. Related multiple nucleotides and deduced amino acid sequences of the penaeidin isoforms were aligned using ClustalX program. The potential cleavage site of the signal peptide was predicted by SignalP software (http://cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004).

2.6 Genomic organization of *Penmon*PEN5

2.6.1 Preparation of *P. monodon* genomic DNA

Genomic DNA was prepared from the pleopods of healthy *P. monodon* using phenol-chloroform extraction. The pleopods were homogenized in 700 µl of extraction buffer (100 mM Tris buffer pH 8.0, 100 mM EDTA pH 8.0, 250 mM NaCl, 1% (w/v) SDS, and 100 µg/ ml Proteinase K) and incubated overnight at 65 ºC. Then, 5 µg of RNaseA were added into the lysate and incubated for 30 min at 37 ºC, 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 fresh 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 pH 8.0 and 1 mM EDTA pH 8.0).

2.6.2 Quality of genomic DNA

The quality of genomic DNA was examined by 0.6% (w/v) 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.

2.6.3 Amplification of genomic *Penmon*PEN5 gene

In order to determine the genomic gene structure of *Penmon*PEN5, the sequence of the *Penmon*PEN5 gene from *P. monodon* was obtained by a PCR approach using genomic DNA as the template and the CT470F/CT470R primers (Table 2.1), so as to amplify from the start to stop codons of the penaeidin gene (183 bp amplicon). Approximately 20 ng of template DNA was used for the PCR amplification in a 50 μl final reaction volume containing one unit Advantage 2 Polymerase Mix (Clonetech, USA), 1× Advantage 2 buffer, 0.2 mM of each dNTP, 0.2 mM each primer and thermocycled with an initial denaturation step at 94 ℃ for 2 min, followed by 30 cycles at 94 ℃ for 30 sec, 55 ℃ for 30 sec and 72 ℃ for 3 min with the final 72 ℃ for 10 min. The PCR product was analyzed on 1% agarose gel electrophoresis in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under UV transillumination to determine the size of PCR product. The expected PCR product was excised and purified using NucleoSpin® Extract II Kits (MACHEREY-NAGEL). The gel slice was weighed and dissolved in three volumes of NT buffer that contain chaotropic salt at 60 ℃. Then, the solution was loaded into the column and centrifuged at 12,000g for 1 min to eliminate 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 new 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 ℃ until used.

2.6.4 Competent cell preparation

A single colony of *E. coli* XL-1 blue was cultured in 10 ml of LB broth (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) to prepare the starter inoculum. The culture was incubated at 37 °C with shaking at 250 rpm overnight. One percent of starter culture was inoculated into 1 L of LB broth and incubated at 37 °C with vigorous shaking for 2-3 h until OD_{600} of the cells reached 0.2-0.6. Cells were then incubated on ice for 10 min and harvested by centrifugation at 5,000 rpm for 5 min at 4 °C. The supernatant was eliminated as much as possible. Cell pellet was washed with cold 10 mM $CaCl_2$ in a total volume of 500 ml, and then centrifuge at 5,000 rpm for 5 min at 4 °C. The pellet was resuspended in a total volume of 50 ml of cold 10 mM $CaCl_2$, chilled on ice for 30 min. This cell suspension was divided into 100 µl aliquots for immediately used or mixed with 60% (v/v) glycerol to a final concentration of 15 % glycerol before divided into 100 µl aliquots and stored at -80 °C until used.

2.6.5 Cloning of the amplified genomic DNA fragments

The DNA fragment was ligated into the T&A cloning vector (Fig. 2.1). The reaction component contained 1 µl of each 10x Rapid A and B ligation buffers, 50 ng of T&A cloning vector, proper amount of PCR product, 1 µl of T4 DNA ligase (3 units/ µl) and distilled water to a final volume of 10 µl. The reaction was mixed, quickly spun and incubated at 4 °C overnight.

2.6.6 Calcium chloride transformation

The ligation reaction was transformed into an *E. coli* XL-1 blue using $CaCl_2$ method. The aliquot competent cells were gently thawed on ice, mixed with 5-10 µl of ligation mixture and then chilled on ice for 30 min. The mixture was incubated at 42 °C for 1 min and added 0.9 ml of LB broth. The reaction was incubated at 37 °C with shaking at 250 rpm for 1 h. The mixture reaction was 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 M13R primers.

2.6.7 Screening of transformant by colony PCR

T7 and M13R primers were used to amplify and analyze the DNA insert cloned size into the multiple cloning regions. Colony PCR was carried out in a 25 µl reaction volume including 1× reaction buffer (10 mM KCl, 2 mM $MgSO_4.7H_2O$, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 0.1% (v/v) Triton X-100 and 2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 mM of each primer, 0.7 unit of RBC Taq DNA Polymerase (RBC Bioscience). For the DNA template, white colonies were picked and resuspended in the reaction mixture. The PCR profile was performed at 94ºC for 3 min, 30 cycles at 94 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 1 min and a final extension at 72 ºC for 7 min. The PCR products were analyzed by 1.0% agarose gel electrophoresis. The clones that contain an expected size of insert were selected to isolate the recombinant plasmid.

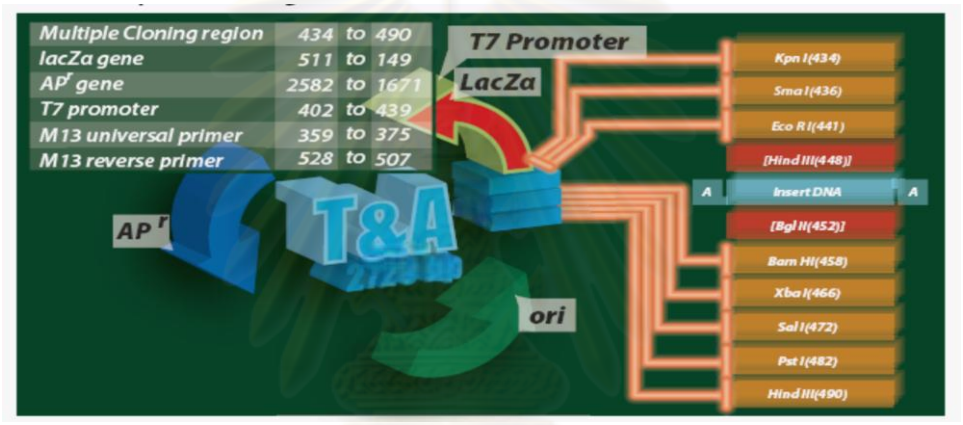
2.6.8 Recombinant plasmid preparation

A positive colony was inoculated into 2 ml of LB broth and incubated at 37 ºC with shaking at 4 ºC overnight. The culture was spun at 8,000 rpm for 3 min and collected the pellet to isolate and purify the plasmid using QIAprep® Spin Miniprep Kit 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, the bacterial cell pellet was resuspended in 250 µl of Buffer P1 containing RNase A. Next, the 250 µl of P2 buffer was added and the reaction was mixed thoroughly by inverting the tube 4-6 times for cell lyses (the solution turned blue). Then, 350 µl of N3 buffer was added and mixed immediately and thoroughly by inverting the tube 4-6 times (the solution turned colorless). After centrifugation at 13,000 rpm 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 of PB buffer and 0.75 ml of PE buffer, respectively, and then centrifuged to remove residual ethanol from PE buffer. Finally, the QIAprep column was placed in a new 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl of EB buffer (10 mM Tris-HCl, pH8.5) to the center of each column.

After incubation at room temperature for 1 min, the DNA eluted fraction was collected by centrifugation for 1 min.

The recombinant plasmid containing interested gene was examined with restriction enzyme digestion using *Eco*RI and *Bam*HI. The digested plasmid was analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp ladder marker). The recombinant plasmid was sequenced by the commercial service (Macrogen Inc., Korea).

(A)



(B)

Figure 2.1 The circular map of the T&A Cloning vector (A) and the linear map that represent the multiple cloning site sequences (B). (Source: T&A Cloning vector kit User Manaul: RBC)

2.6.9 Genomic DNA library construction

Four genomic DNA libraries were constructed by digesting the genomic DNA separately with four blunt-end restriction enzymes (Fig. 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 70% ethanol. The supernatant was decanted and the pellet was air-dried. The pellet was dissolved in 20 µl of TE buffer 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 adaptors, 1.6 µl of 10× ligation buffers and 0.5 µl of T4 DNA ligase (6 units/ µl). The reaction mixtures were incubated overnight at 16ºC. The reactions were stopped by incubating at 70ºC for 5 min. Each reaction was added 72 µl of TE buffer and vortexed at slow speed for 10-15 sec. The four libraries were named *Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries.

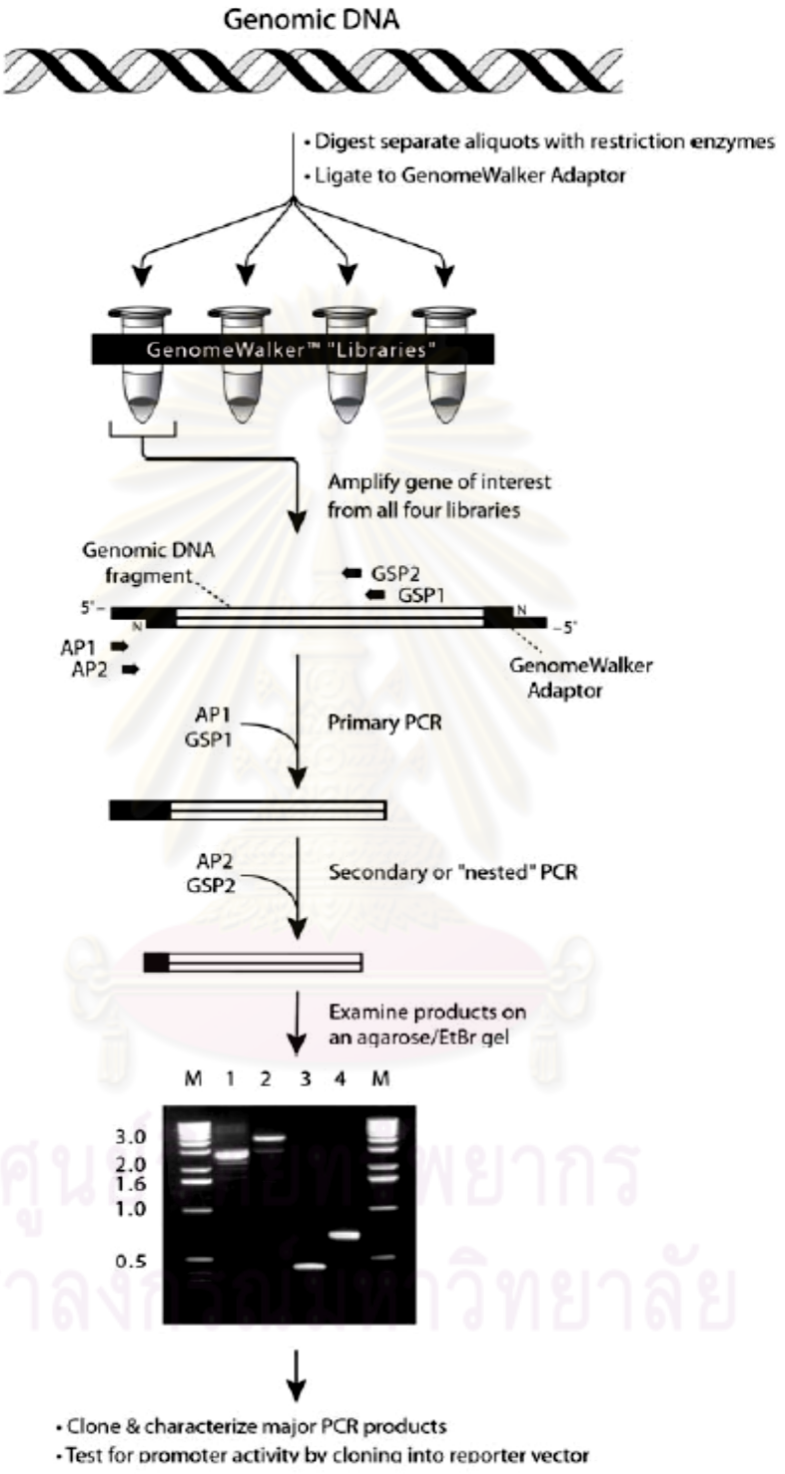


Figure 2.2 Flow chart of the BD GenomeWalker™ protocol. The genomic libraries were constructed for use as templates for nested PCR. (Source: www.bdbiosciences.com)

2.6.10 Determination of the 5′ upstream sequences of *Penmon*PEN5 gene

To obtain the 5´ UTR sequence of the *Penmon*PEN5 gene, the 2Gsp1PEN5 and 2Gsp2PEN5 primers (Table 2.1) were designed from the upstream sequence of the EST clones, CT470. The PCR based genome walking was carried out using universal GenomeWalker^{TM} Universal Kit (Clontech, USA). Four libraries were use as the PCR template. Primary PCR was performed using the AP1 and the gene specific 2Gsp1PEN5 primers (Table 2.1). The PCR reaction contained one unit of Advantage 2 Polymerase Mix, 1× Advantage 2 buffer, 0.2 mM of each dNTP, 200 µM of each primer and was performed with a two stage and two-step PCR cycle comprised of 7 cycles at 94 ºC for 25 sec and 72 ºC for 4 min, followed by 32 cycles at 94 ºC for 25 sec and 67 ºC for 4 min, with a final 67 ºC for 4 min. In the subsequent nested PCR, the primary PCR products that was diluted 50 folds in distill water were used as templates and amplified using the AP2 and gene specific 2Gsp2PEN5 primers (Table 2.1) with PCR cycle parameters of five cycles at 94 ºC for 25 sec and 72 ºC for 4 min, followed by 22 cycles at 94 ºC for 25 sec and 67 ºC for 4 min with a final stage of 67 ºC for 4 min. The PCR products were then separated by 1% (w/v) agarose-TBE gel electrophoresis, the desired band eluted and cloned into the TA vector for sequencing by a commercial service (Macrogen Inc., Korea).

The putative promoter and transcription start site were predicted with Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001) and Match_1.0 Public/TRANSFAC_6.0 program (BIOBASE, http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi), respectively. The Match_1.0 Public program was set to high quality matrices with 0.85 as the matrix and core similarity cut-off value.

2.7 Tissue distribution of *P. monodon* penaeidin5 transcripts

2.7.1 Tissue collection

Hemocytes, eyestalk, gill, epipodite, heart, lymphoid organ, hepatopancreas, intestine and antennal gland were collected from three individual healthy shrimps and immediately frozen in liquid nitrogen (-176ºC). All tissues excepted hemocytes were pulverized until homogenized by 1 ml of Trizol reagent to

extract the total RNA. The RNA extraction and first strand cDNA synthesis method was described above.

2.7.2 Semi-quantification of *Penmon*PEN5 expressions by RT-PCR

The specific primers for *Penmon*PEN5 (SpPEN5F/CT470R) were designed based on the sequence from the CT470 EST clones of *P. monodon* (http://pmonodon.biotec.or.th/) to yield an amplicon of ca 133 bp, and are described in Table 2.1. The PCR reaction (25 µl) included 75 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 mM of specific primers, 1 unit of *Taq* DNA polymerase (Fermentas) and 3 µl of the 1:10 diluted first-strand cDNA. The PCR cycle parameters were an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and then finally 72 °C for 10 min. The EF-1α gene transcript fragment, as the internal control, was amplified as per the *Penmon*PEN5 transcript fragment except with an annealing temperature of 55 ^{o}C. Ten µl of each PCR reaction was visualized by UV transillumination following electrophoretic resolution through 1.5% (w/v) agarose-TBE gel and staining with ethidium bromide. The band intensity of PCR products between the penaeidin gene fragment and the EF-1α gene fragment internal control were analyzed and correlated using the Genetools analysis software (Syngene). The ratios of the PEN5 to EF-1α transcript fragments were presented.

2.8 Antiviral experiment in shrimp by RNA interference (RNAi)

2.8.1 Primer design and double-stranded RNA preparation

Double stranded RNA (dsRNA) that correlated with nucleotide sequence of *Penmon*PEN5 was synthesized by *in vitro* transcription and performed by T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega). Oligonucleotide primers of this gene were incorporated with T7 promoter sequences (5′ GGATCCTAATACGACTCACTATAGG 3′) at the 5′ ends. The template DNA for generating dsRNA was amplified in two separate PCR reactions. The specific primers were designed from the nucleotide sequence of *Penmon*PEN5 gene as shown in appendix B. The sense strand template was synthesized from T7PEN5-RNAiF and PEN5-RNAiR primers (Table 2.1), whereas the anti- sense strand template was

synthesized from the PEN5-RNAiF and T7PEN5-RNAiR primers (Table 2.1). Both PCR reactions were performed with 0.02 units/µl of Hotstart Phusion *Taq* DNA Polymerase (New England Biolabs) with PCR cycle parameters of an initial denaturation at 98 °C for 30 sec, followed by 39 cycles of 98 °C for 10 sec, 55 °C for 30 sec and 72 °C for 20 sec, and with a final extension at 72 °C for 10 min. The PCR product was separated by 1.5% (w/v) agarose-TBE gel electrophoresis and the desired band was eluted and purified using NucleoSpin® Extract II Kits. One microgram of each template was used in an *in vitro* transcription of T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega, USA), according to the manufacturer's protocol. The sense and anti-sense single stranded RNA were then mixed at equimolar amounts and annealed to construct the dsRNA. The DNA template was then eliminated by treatment with RNase-free DNaseI.

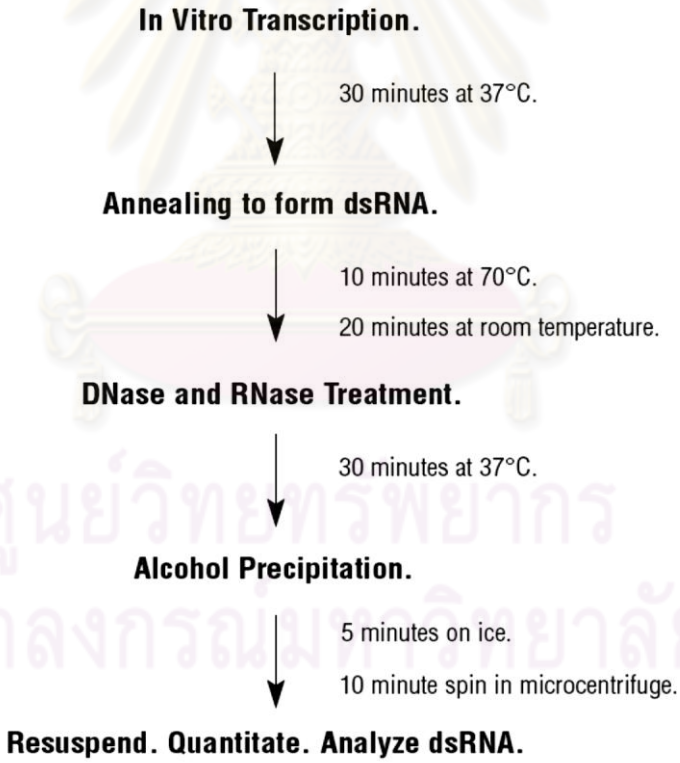


Figure 2.3 Outline of procedure for the production and purification of dsRNA using the T7 RioMAX express RNAi System. (Source: T7 Ribomax(TM) Express RNAi System (Promega)

2.8.2 Determination of quality and quantity of double-stranded RNA

The quality of dsRNA was examined by 1.5 % (w/v) agarose gel electrophoresis. Half of one microliter of dsRNA and each stand template were loaded and run on agarose gel electrophoresis in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under the UV light to estimate the size of product.

The concentration of dsRNA was determined by measuring the OD_{260} and estimated in ng/µl using the following equation:

$$[\text{dsRNA}] = OD_{260} \times \text{dilution factor} \times 50$$

One OD_{260} corresponds to approximately 50 ng/µl of DNA. The relative purity of RNA samples was investigated by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at 280 nm. The good quality of RNA sample should have an $A_{260/280}$ ratio above 1.7.

2.8.3 Specific gene silencing of *Penmon*PEN5 transcript

To assess the sequence-specific knockdown effect of *Penmon*PEN5 dsRNA, the concentration of *Penmon*PEN5 dsRNA and the control, poly(GC) (Invitrogen), were optimised such that they had less effect on other penaeidin transcripts, *Penmon*PEN3. After optimization, double injections of 5 and 2.5 µg of dsRNA per 1 g shrimp were used. Twenty microgram (5 µg per 1 g shrimp) of *Penmon*PEN5 dsRNA or poly(GC) dissolved in 0.85% (w/v) NaCl to a final volume of 30 µl, were injected into each *P. monodon* shrimp (approximately 4 g body weight each) in the lateral area of the fourth abdominal segment using a 0.5 ml insulin syringe with a 29-gauge needle. An additional control group of shrimps were injected with 0.85% (w/v) NaCl only. At 24 h after the first injection, the injection of dsRNA (2.5 µg per 1 g shrimp) or NaCl were repeated. The hemolymph of three individual shrimps from each group were collected and used for total RNA extraction and first strand cDNA synthesis, as described above.

The efficiency and specificity of dsRNA-mediated gene knockdown of *Penmon*PEN5 was analyzed by semi-quantitative RT-PCR using the SpPEN3F/ SpPEN3R and SpPEN5/CT470R (Table 2.1) with the same condition for semi-quantification of *Penmon*PEN3, *Penmon*PEN5, respectively except that the amplification cycle was repeated 30 times.

2.8.4 The effect of suppression of the *Penmon*PEN5 transcript on WSSV challenge

To determine the effect of suppression of the *Penmon*PEN5 transcript on WSSV infection, shrimp were double injected with *Penmon*PEN5 dsRNA, poly(GC) or 0.85% (w/v) NaCl as mentioned above and at the second injection, shrimp was injected together with WSSV (10^2 copies). After a further 24 h, the hemolymph of three individual shrimps from each group were collected and used for total RNA extraction and then reverse transcribed to first strand cDNA, as described above.

The effect of suppression of the *Penmon*PEN5 transcript on WSSV infection was investigated by semi-quantitative RT-PCR analysis using the SpPEN3F/SpPEN3R, SpPEN5/CT470R and VP28 primers (Table 2.1). The intensity of the PCR amplicon between the VP28 gene and the internal control (EF-1α gene fragment) were analyzed after electrophoresis through a 1.5% (w/v) agarose-TBE gel. The PCR condition for penaeidin3, -5 and VP28 transcript were described in table 2.2. This experiment was twice examined using two set of shrimps.

2.8.5 Detection of viral copy number

Shrimps were separated into two groups; the control group (poly (GC)-injected shrimps) and the *Penmon*PEN5-downregulated group. Shrimps from both groups were double injected with either poly (GC) or *Penmon*PEN5 dsRNA, respectively, and then with WSSV, as mentioned in section 2.8.4. Twenty-four hour after the WSSV injection, hemocytes from three individual shrimps per group were collected for genomic DNA extraction, as described 2.6.1. The samples were sent to Charoen Pokphand Foods PCL for the detection of the WSSV copy number by real-time PCR with the WSSV1011F/WSSV1079R, as described by Durand and Lightner (2002), using an ABI7000 Sequence Detection System. All assays were carried out in duplicate. A WSSV recombinant plasmid (known copy number) was serially diluted and used as the standard for quantification. Independent Sample T-Test was used to analyze the significance of any difference in the data between groups. This experiment was twice examined using two set of shrimps.

2.9 Expression and characterization of *Penmon*PEN5

The deduced amino acid of *Penmon*PEN5 (accession no. ACQ66008, FJ686018) (Tassanakajon et al., 2008) was regained from *P. monodon* EST database (http://pmonodon.biotec.or.th). The mature *Penmon*PEN5 gene was amplified and expressed in the *Pichia pastoris* methylotrophic yeast system (Invitrogen) for testing the antiviral property in hemocyte cell culture.

2.9.1 Construction of expression plasmid *Penmon*PEN5 (p*Penmon*PEN5)

The multiple copies of the expression vector, pPIC9K (Fig. 2.4), was selected for *Penmon*PEN5 expression. It contains a kanamycin resistance gene which confers resistance to high level of G418 in *P. pastoris.* Spontaneous generation of multiple insertion events, which occur in *P. pastoris* at a frequency of 1-10%, can be identified by the level of resistance to G418. *P. pastoris* transformants are chosen on histidine deficient medium and screened for their level of resistance to G418. The increased level of resistance to G418 indicates multiple copies of the kanamycin resistance gene as well as the cloned of interested gene. The copies number increment of the interested gene in a recombinant *P. pastoris* strain may increase protein expression levels.

2.9.1.1 Primer design

To construct an expression cassette, the forward and reverse primers were designed from the cDNA sequence encoded mature peptide of *Penmon*PEN5 and added the restriction sites. A *Sna*BI site was added to 5'-end of forward PCR primers, thus introducing two additional amino acids (Tyr and Val) to the amino terminus of the recombinant *Penmon*PEN5 protein (r*Penmon*PEN5). Moreover, a *NotI* site was added to 3'-end of reverse primers after the stop codon. Primer sequences were:

CT470F-*Sna*BI: 5´ TACGTACAAGGATACAAGGGCGGTTA 3´

CT470R-*Not*I: 5´ GCGGCCGCTTATCCTTTCAATGCAGAACAA 3´

The primers were used to amplify the mature gene, cloned in-frame into the pPIC9K vector downstream of the sequence for the α-mating factor signal peptide

from *Saccharomyces cerevisiae* and the Glu-Ala-Glu-Ala repeat sequence and then transformed into the *E. coli* XL-1 blue.

2.9.1.2 Amplification of *Penmon*PEN5 gene by PCR

The mature *Penmon*PEN5 gene was amplified by CT470F-*Sna*BI and CT470R-*Not*I primer (Section 2.9.1.1) and using Pfu DNA Polymerase (Promega, USA). PCR conditions were: pre-heat at 94 ℃ for 5 min, follow by 35 cycles of 94 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 30 sec and a final cycle of 72 ℃ for 10 min. The PCR reaction was carried out in the total 50 µl reaction consisting of 200 ng of plasmid containing *Penmon*PEN5 gene, 1× reaction buffer, 0.2 mM of each forward and reverse primers, 0.2 mM dNTPs and 1 unit of *Pfu* DNA polymerase (Fermentas). After PCR amplification, the expected 197 bp PCR product was purified by NucleoSpin® Extract II Kits (MACHEREY-NAGEL).

2.9.1.3 Restriction enzyme digestion of the purified PCR product and expression vector

The pPIC9K vector and purified PCR products from section 2.9.1.2 were digested with *Sna*BI and *Not*I. First, 3 µg of them were double digested at 37 ℃ for 3 h with *Sna*BI and *Not*I in the reacton of 1× FastDigest reaction buffer, 1× BSA and 0.5 unit of *Sna*BI and *Not*I (Fermentas). The reactions were stopped by heat at 70 ℃ for 20 minutes. The completely digested products were separated by agarose gel electrophoresis and purified using NucleoSpin® Extract II Kits (MACHEREY-NAGEL).

2.9.1.4 Ligation

The *Sna*BI/*Not*I digested-PCR and -pPIC9K fragments were ligated prior to transform to *P. pastoris*. The ligation reaction of 20 µl contained 2 µl of 10× ligation buffer, 2 µl of digested-PCR product, 8 µl of *Sna*BI/*Not*I digested-pPIC9K vector, and 2 µl of T4 ligase (New England Biolab), which molar ratio of insert:vector was 7:1. The total volume was adjusted to 20 µl with distilled water. The ligation reaction was then incubated at 16 ℃ overnight.

2.9.1.5 Transformation into *E.coli* and plasmid preparation

The ligation reaction was transformed into *E. coli* strain XL-1 blue cells by calcium chloride transformation as described in section 2.6.6. One hundred microliters of the transformation were spreaded onto LB-amplicillin selected plates and incubated overnight at 37 °C. Transformants grown on LB-amplicillin selected plates were screened by colony PCR as described in section 2.6.7. The α-signal peptide and the 3'-AOX primers were used for colony PCR screening. The positive clone was picked to culture in LB-broth containing 100 mg/ml of ampicillin for overnight and then extracted the recombinant plasmid by QIAprep miniprep.

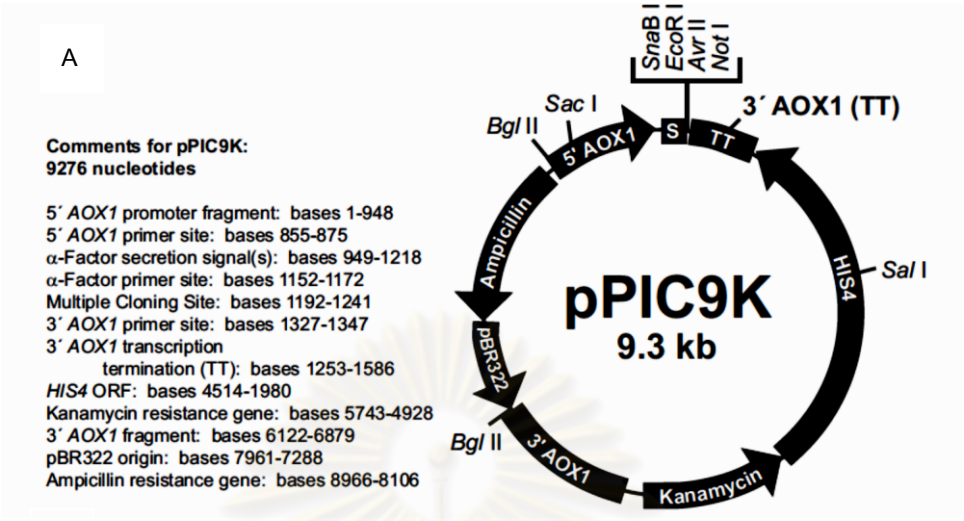
2.9.1.6 Confirmation the p*Penmon*PEN5

A positive transformant was selected to sequence in order to verify the sequence and orient the *Penmon*PEN5 gene in the expression plasmid. The α-signal peptide and the 3'-AOX primers were used in sequencing reactions.

2.9.2 *P. pastoris* transformation

2.9.2.1 Electrocompetent cells preparation

To prepare the *P. pastoris* electrocompetent cells, a single colony of *P. pastoris* KM71 was cultured in 5 ml of YPD (2% (w/v) peptone, 1% (w/v) bacto yeast extract and 2% glucose) at 30 °C with shaking at 300 rpm overnight and used as starter. One hundred microliters of the overnight culture was inoculated in 500 ml of fresh medium in a 2-liter flask and grown overnight again to an OD600 of 1.3-1.5. The cells were precipitated using centrifugation at 3,500 rpm for 5 min at 4 °C. The cell pellet was washed by resuspended in an equal volume of cold sterile water, gently mixing and centrifugation. The pellet was washed further with 250 ml of cold sterile water followed by 20 ml of ice-cold sterile 1 M sorbitol and resuspended to a final volume of 1 ml of ice-cold sterile 1 M sorbitol. The final volume including the cell pellet was approximately 1.5 ml that was divided into 80 µl aliquots and stored at -80 °C until used.



B

AOX1 mRNA 5'end (824)

5' *AOX1* primer site (855-875)

```
TTATCATCAT TATTAGCTTA CTTTCATAAT TGCGACTGGT TCCAATTGAC

AAGCTTTTGA TTTTAACGAC TTTTAACGAC AACTTGAGAA GATCAAAAAA
```

Start (949) α-Factor Signal Sequence

```
CAACTAATTA TTCGAAGGAT CCAAACG ATG AGA TTT CCT TCA ATT
                              Met Arg Phe Pro Ser Ile

TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT
Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala

CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG
Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro

GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT TTC
Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe

GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly
```

α-Factor primer site (1152-1172)

```
TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys
```

Signal cleavage (1203-1204) *Sna* BI

```
GAA GAA GGG GTA TCT CTC GAG AAA AGA GAG GCT GAA GCT TAC
Glu Glu Gly Val Ser Leu Glu Lys Arg Glu Ala Glu Ala Tyr
```

Eco RI *Avr* II *Not* I

```
GTA GAA TTC CCT AGG GCG GCC GCG AAT TAA TTCGCCTTAG
Val Glu Phe Pro Arg Ala Ala Ala Asn ***

ACATGACTGT TCCTCAGTTC AAGTTGGGCA CTTACGAGAA GACCGGTCTT
```

3' *AOX* 1 primer site (1327-1347)

```
GCTAGATTCT AATCAAGAGG ATGTCAGAAT GCCATTTGCC TGAGAGATGC

AGGCTTCATT TTTGATACTT TTTTATTTGT AACCTATATA GTATAGGATT
```

↓ *AOX*1 mRNA 3' end (1418)

```
TTTTTTGTCA
```

Figure 2.4 The circular map (A) and *PAOX1* and multiple cloning site (b) of pPIC9K vector. (Source: www.invitrogen.com)

2.9.2.2 Transforming DNA preparation

The pPIC9K vector with *Penmon*PEN5 gene cloned was linearized with *Sac*I for insertion at AOX1. When *Pichia* strain KM71 was transformed with the *Sac*I-linearized plasmid, Mut^S recombinants would be generated. Both the p*Penmon*PEN5 construct and the pPIC9K vector were digested by mixing 10 μg of plasmid, 10 units of *Sac*I, 1× reaction buffer, and incubating at 37 ℃ for 12-16 h. A small portion of the digest was analyzed by 1.0% agarose gel electrophoresis for confirming the complete digestion of plasmids. The digested DNA was ethanol precipitated and resuspended in 10 μl of water and stored at -20 ℃ until ready to transform.

2.9.2.3 Transformation of *P. pastoris* by electroporation

An aliquot of *P. pastoris* KM71 competent cells was gently thawed on ice and mixed with 8 μg of *Sac*I-linearized plasmid and placed on ice for about 5 minutes. The mixture solution was transformed by electroporation in a cold 0.2 cm cuvette with the apparatus setting as follows; 25 μF of the Gene pulser, 200 Ω of the Pulse controller unit, and 1.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, the cells were immediately resuspended with 1 ml of ice cold steriled 1 M sorbitol and transferred to test tube. Two hundred and fifty microliter of cell suspension was spread on the MD agar plates and incubated at 30 ℃ until colonies appeared.

2.9.2.4 Screening for yeast high expression transformants

To screen transformants of G418 resistance, the transformants that grown on MD plates were pooled by adding sterile water over each plate and running sterile spreader across the top of the agar to resuspend them. The transformants from all plates were pooled and determined the cell density by a spectrophotomerter (1 OD600 = 5 x 10^7 cells/ml). Ten to the fifth power cells transformants were spreaded on the YPD plates which containing G418 at final concentrations of 0, 1 and 2 mg/ml. Plates were incubated at 30 ℃ and checked daily. The G418 resistant colonies appeared after 2 - 5 days. The resistant clones observed on each YPD-G418 plate were chosen. To purify putative G418 resistant clones, they

were streaked on the YPD plates for isolated the single colonies and confirmed G418 resistance on the same concentration of YPD-G418 plates.

2.9.2.5 Determination of the integrated *Penmon*PEN5 gene in *P. pastoris* genome by PCR

To select the transformants that presence the integrated *Penmon*PEN5 gene, a single colony of *Pichia* clones was randomly picked and resuspended in the PCR master mix that contained 1× reaction buffer, 0.2 mM of each dNTP and 0.2 mM of each primer (α-signal peptide and the 3′-AOX) without Taq DNA Polymerase. Next, the reactions were incubated at 94 ºC for 10 min to lyse the cell wall of yeast cells and then added 0.7 unit of RBC Taq DNA Polymerase (RBC Bioscience). The PCR was performed for 30 cycles as above parameters in section 2.6.7. The resulting PCR product was analyzed using 1.2% agarose TBE gel to determine whether the DNA fragment was successfully amplified. The size of the DNA fragment that was amplified including α-factor signal peptide, the *Penmon*PEN5 gene and the 3'sequence of alcohol oxidase (AOX) gene.

2.9.3 Expression of the recombinant clones

A single colony of each positive clone was grown in YPD broth at 30 ºC overnight. The starter was inoculated into 100 ml of BMGY (1% (w/v) Yeast extract, 2% (w/v) Peptone, 100 mM Potassium phosphate buffer pH 6.0, 1.34% (w/v) YPD, $4x10^{-5}$ %(w/v) Biotin and 1% (v/v) glycerol) in a 1 liter flask and grown at 30 ºC with 300 rpm shaking until the OD600 of the culture reached to 4-6. The culture was centrifuged at 3500 rpm for 5 min at room temperature to collect the cell pellets. To induce the expression, the cell pellets were resuspended in 20 ml of BMMY medium (1% (w/v) Yeast extract, 2% (w/v) Peptone, 100 mM Potassium phosphate buffer pH 6.0, 1.34% (w/v) YPD, $4x10^{-5}$ %(w/v) Biotin and 0.5% (v/v) Methanol) and add 100% methanol to a final concentration of 0.5% every 24 h to maintain the induction. The expression culture was collected 1 ml at each time points (0, 1, 2, 3, 4, 5 and 6 days) and separated the supernatant and cell pellet using centrifugation at 9,000 rpm for 2 min at room temperature. The supernatant and cell pellet were kept at -80 ºC

until ready to confirm the r*Penmon*PEN5. Tricine SDS-PAGE and silver stained was used to analyze the r*Penmon*PEN5 expression.

2.9.4 Analysis of recombinant protein by Tricine SDS-PAGE

Fifteen percent of tricine SDS-PAGE gel electrophoresis was used for completed a resolution of small proteins in the range between 5 and 20 kDa (Schagger and von Jagow, 1987). The separating gel in 16.5% (w/v) and 4% (w/v) stacking gel were set into the slab gel system that size about 10 × 10 × 0.75 cm. The gel preparation was described in Appendix A. The anode (0.2 M Tris buffer pH 8.9) and cathode (0.1 M Tris buffer pH 8.25, 0.1 M Tricine, and 0.1% SDS) running buffers were used to run the electrophoresis system. The protein samples were mixed with sample buffer (see in Appendix A) and then boiled for 10 min. After protein boiled, twenty microliters of mixing samples were loaded into the gel and started the electrophoresis at 30 V. When the sample had completely gotten in the stacking gel, the electricity pressure was adjusted to 100 V. The electrophoresis gel was finished when the lower dye had run to the lower edge of gel.

The silver staining gel method, described of Bollag et al. (1996) was used for detection of the protein bands because this method can detect a little protein about 2 ng in a single band. First, the gel was soaked in 50% methanol and 10% acetic acid for at least 1 h with 2-3 changes of methanol/acetic acid solution. After that the gel was washed with water for 30 min with at least 3 changes and then stained in solution C (All solutions for silver staining was described in Appendix A.) for 15 min with gentle constant agitation. After rinsing for 2 times and soaking for 2 min, the gel was developed using Solution D. At less 10 min, the protein bands were appeared and then rinsed with 1% acetic acid to stop the development. Last, the gel was washed in the water for at least 1 hour with at least three changes.

2.9.5 Purification of recombinant protein

To purify the recombinant *Penmon*PEN5 (r*Penmon*PEN5), the cation exchange chromatography was performed and 20 mM of potassium phosphate buffer pH 7.0 was used as the running or start buffer. At this pH r*Penmon*PEN5 with a calculated pI of 9.6 carried a positive net charge and could be purified by cation

exchange. The crude protein was dialyzed in distilled water and concentrate using Calbiochem Aquacides (Merck) to concentrate the protein. Fivefold concentration of the protein can be completed in 3 hours by changing the resin surrounding the dialysis sack every half hour (Schleif and Wensink, 1981). The concentrated protein was dialyzed again in start buffer and clarified by filtration through a 0.45 µm pore size filter (Millipore) before loading into the column.

The Hitrap SP Sepharose (cation exchange) column (GE Healthcare) was washed with 5 column volumes of start buffer at the flow rate of 1 ml/min to eliminate the preservatives. Next, the column was washed with start buffer containing 1 M NaCl, elution buffer, and equilibrated with 10 column volumes of start buffer. The crude r*Penmon*PEN5 in start buffer was loaded into the column and washed with 10 column volumes of start buffer or until the A_{280} read 0. The r*Penmon*PEN5 were eluted using step wise gradient at 0.2, 0.4, 0.6, 0.8 and 1M NaCl. All fractions collected from washing and elution steps were measured the A_{280} to approximate the amount of protein. The fractions were also analyzed by Tricine SDS-PAGE and then the fractions that had the band of protein were pooled.

The protein concentration is then calculated according to the following formula:

$$\text{Protein concentration (mg/mL)} = \varepsilon l / A_{280}$$

where ε = extinction coefficient, and l = optical path length in cm. The extinction coefficient was calculated using ProtParam program from ExPASy Proteomics tools (http://expasy.org/tools/protparam.html).

The pooled purified protein was dialyzed again in start buffer to eliminate NaCl. Then the purified protein was concentrated using ultrafiltration (Amico Ultra 3 k) and kept at -20 °C for antimicrobial and antiviral activity test.

2.9.6 Antimicrobial activity assay

The antibacterial activity of r*Penmon*PEN5 against *Aerococcus viridans* and *Micrococcus luteus* was confirmed before testing the antiviral activity by the solid phase assay described by Minagawa et al. (2001). Bacterial cultures grown overnight for 18 h at 30ºC in 5 ml of 2YT medium (1%w/v NaCl, 1%w/v Yeast extract, 1.6%w/v peptone). The bacterial density was adjusted to 0.2 at 600 nm with

poor broth (0.5% w/v NaCl, 1% w/v peptone) or start buffer (20 mM phosphate buffer pH7.0) for *A.viridans* and *M.luteus*, respectively, containing 1 % agarose and poured onto 90 mm plates. After the solidification of the agar, wells (diameter: 4 mm) were cut into the freshly poured plates. Sixty microgram of purified r*Penmon*PEN5 was added to a punched well and incubated at 30ºC for 24 h. Amplicillin (0.8µg) and lysozyme (10 mg) were used as positive control in *A.viridans* and *M.luteus*, respectively and 100 µl of 20 mM potassium phosphate buffer pH 7.0 was used as the negative control. The diameters of the halo zone of inhibition were measured.

2.9.7 Antiviral activity assay

To investigate the inhibition of r*Penmon*PEN5 against white spot syndrome virus (WSSV) propagation, the primary cell culture of *P. monodon* hemocyte was used. The WSSV infection was detected using RT-PCR of a major envelope protein VP28 expression in the host cells. The presence of the VP28 transcript indicated viral propagation.

2.9.7.1 Preparation of primary hemocyte *P. monodon* cell culture

Hemolymph was collected from the healthy sub-adult shrimp (~20 g body weigth) at ventral sinus using 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 400 µl of an anticoagulant solution (0.82% (w/v) sodium chloride, 0.55% (w/v) citric acid, 1.98% (w/v) glucose, 0.88% (w/v) sodium citrate and adjusted the pH to 5.6 by 10 N sodium hydroxide) and then the hemocytes were harvested by centrifugation at 200 x g for 10 min at 4 ^{o}C. The hemocyte cells were resuspened in 0.7 ml of L-15 culture medium (1.6x Leibovitz L-15 medium (Gibco) supplemented with 20% (v/v) fetal bovine serum (FBS), 1% (w/v) glucose, 0.4% (w/v) sodium chloride, 100 IU/ml penicillin and 100 µg/ml streptomycin; pH 7.6; adjusting the osmotic pressure to 750 ± 15 mOsm/kg with sodium chloride solution). The concentration of a cell suspension was determined using Hemocytometer Slide under a light microscope. Ten microliter of a cell suspension was added to an assembled slide (The coverslip was pressed down onto slide), viewed on microscope and the cell lysates were counted within this 1-mm^2 area that stand on

the top and left hand lines. The cell suspension was calculated the average of the two counts and derived the concentration using the following formula

$$c = n / v$$

where c is the cell concentration (cells/mL), n is the number of cells counted, and v is the volume counted (mL). For this slide, the depth of the chamber is 0.1 mm, and, assuming that only the central 1 mm^2 is used, v is 1 x 10^{-4} mL. The formula then becomes

$$c = n \times 10^4$$

The cell suspension was subsequently seeded at 10^5 cells per 150 µl in a 96-well plate and incubated at 28 ºC for 24 hours. Afterward, the pre-treated hemocytes were ready for used in experiments.

2.9.7.2 Preparation of white spot syndrome virus

The hemolymph was collected from WSSV-infected *P. monodon* and combined with TNE buffer (50mM Tris–HCl, 400mM NaCl, and 5mM EDTA, pH 8.5) in the ratio 1:1. The mixed solution was centrifuged at 3,500*g* for 5 min at 4 ºC to keep the supernatant. After centrifugation of the supernatant at 30,000*g* for 30 min at 4 ºC, the pellet was washed with TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) and then centrifuged at 3,500*g* for 5 min at 4 ºC. The pellet was collected and suspened in TM buffer. Then, the supernatant was harvested using centrifugation at 30,000 *g* for 30 min at 4 ºC and dissolved in TM buffer to split the aliquots and stored at -80 °C until used.

2.9.7.3 Antiviral activity test

In order to better understand the antiviral infection mechanism of *Penmon*PEN5, the r*Penmon*PEN5 was treated with 10^6 copies of WSSV in the primary cell culture. After WSSV mixed with the 25, 12.5 and 6.25 µM (approximately 100, 50 and 25 µg, respectively) of purified r*Penmon*PEN5, they were immediately added to the cell culture and incubated at 28 ºC for 2 h. Then, all medium was wash and replaced with a new culture medium and continuously incubated at 28 ºC for 24 h. Besides, the medium was removed and 50 µl of Trizol reagent was added into a well for RNA extraction. The cell and Trizol reagent was

incubated at room temperature at least 10 min afterwards they were resuspended to lyse the cell and transferred into a new 1.5 ml microcentrifuge tube (10 wells per tube). The RNA was extracted and DNA was eliminated as described in section 2.4.2. The first stand cDNA synthesis was performed as described in section 2.4.3. The VP28 primers were used for detected the WSSV propagation, and the EF-1α primers were used to amplify the housekeeping gene (Table 2.1). The PCR reaction was carried out in a total volume of 25 µl containing 1× reaction buffer (10 mM KCl, 2 mM $MgSO_4.7H_2O$, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 0.1% (v/v) Triton X-100 and 2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 mM of each primer, 0.7 unit of RBC Taq DNA Polymerase (RBC Bioscience) and 3 µl of the 1:10 diluted first-strand cDNA. The parameters of PCR cycle were described in table 2.2. The intensity of the PCR amplicon between the VP28 gene (506 bp) and the internal control (EF-1α gene fragment, 150 bp) were analyzed after electrophoresis through a 1.5% (w/v) agarose-TBE gel.

2.9.7.4 Trypan blue exclusion test for the cell viability

To determine the cytotoxicity of r*Penmon*PEN5, the dye exclusion method was used. The reactivity of this method is based on the dye, such as trypan blue, eosin, propidium, or erythrosine, without interacted with the cell unless the membrane is damaged. As a result, all the cells which exclude the dye are viable. The hemocyte cells were incubated with the mixture of 25, 12.5 or 6.25 µM of purified r*Penmon*PEN5 as described in section 2.9.7.3 without WSSV. After incubation at 28 ºC for 24 hour, 120 µl of medium were eliminated to give an approximate cell concentration of 10^6 cells/ ml and then the cells were resuspended until all cells diffused. The cells suspensions were transferred to the new tube and mixed thoroughly in ratio 1:1 with trypan blue solution (0.4% of trypan blue in 0.81% of sodium chloride and 0.06% of potassium phosphate, dibasic). After standing for 2 min at room temperature, the cells were count in triplicated using the Hemocytometer Slide as described in section 2.9.7.1. The viable cells had clear cytoplasm whereas nonviable cells had blue cytoplasm. Cell viability could be calculated using the following equation:

$$\%\ \textbf{cell viability} = \frac{\textbf{total viable cells (unstained)}}{\textbf{total cells (stained plus unstained)}} \times \mathbf{100}$$

CHAPTER III
RESULTS

3.1 Expression of *P. monodon* antimicrobial peptide genes in response to WSSV-challenge

Previously, genes encoding antimicrobial peptides were identified from hemocyte cDNA libraries of the black tiger shrimp *Penaeus monodon*. These genes include those coding for penaeidins, crustins, antilipopolysaccharide factors (ALF) and lysozymes. The antibacterial activities of these shrimp AMPs have been reported (see a review by Tassanakajon et al., 2010). To investigate the possible antiviral response of *P. monodon* antimicrobial peptide genes, the transcript levels of these genes were determined in hemocytes of WSSV-challenged *P. monodon* compared to those of the control shrimp.

To determine the appropriate dose of WSSV for experimental-challenge, *P. monodon* were intramuscularly injected with various dilution of WSSV. Subsequently, a gill portion of three individual shrimp was collected for WSSV detection. DNA was extracted and 20 ng was used as the template. The WSSV infection was detected by PCR amplification using primers WSSVF/R (Table 2.1). The expected size of PCR product was about 250 bp (Fig. 3.1). The dilution of WSSV at 1:8000 which caused 100% mortality within 4 days was used for WSSV experimental challenge.

Sub-adult *P. monodon* (approximately 15 g of body weight) were separated into four groups, three shrimps per group. Three experimental groups were injected with 1:8000 WSSV dilutions whereas the control group was injected with Lobster hemolymph medium (LHM). Hemolymph of three individual shrimp from each group was collected at 0.25, 12, 24 and 48 h post injection (hpi). RNA was extracted and used for the first stand cDNA synthesis. The expression of major AMP genes in *P. monodon* was determined by semi-quantitative RT-PCR analysis using β-actin as an internal control. The relative expression was then normalized against the control group and presented in Fig. 3.2

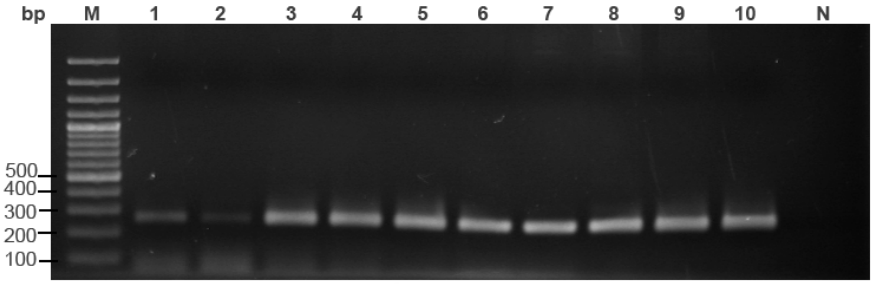


Figure 3.1 Detection of WSSV infection by PCR amplification. Shrimp were injected with various dilution of WSSV. Twenty nanogram of gill DNA was used as a template for PCR amplification using primers WSSVF/R (Table 2.1). The PCR product was analyzed on a 1.5% agarose gel at 100 volts for 30 min.

Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas)
Lanes 1-3: 1:10000WSSV dilutions
Lanes 4-6: 1:8000 WSSV dilutions
Lanes 7-9: 1:6000 WSSV dilutions
Lane 10: positive control (with DNA of WSSV)
Lane N: negative control (without DNA template)

After challenging with WSSV, the expression levels of ALF*Pm*3, ALF*Pm*6 and *Penmon*PEN5 were significantly increased ($p < 0.05$). The transcription of ALF*Pm*3 was significantly up-regulated by approximately 6 folds at 24 and 48 hpi as compared to the control shrimp. Likewise, ALF*Pm*6 mRNA was up-regulated and the highest level of its expression was detected at 48 hpi (increase by 3.3-fold). Significant up regulation of *Penmon*PEN5 transcripts was observed at 24 hpi by approximately 3.6-fold. Nevertheless, the transcription of this gene was undetectable at 48 hpi. Constitutive expression of ALF*Pm*2, crustin*Pm*1, crustin*Pm*7, *Penmon*PEN3 and lysozyme was observed as their expression levels were not significant different during the study period.

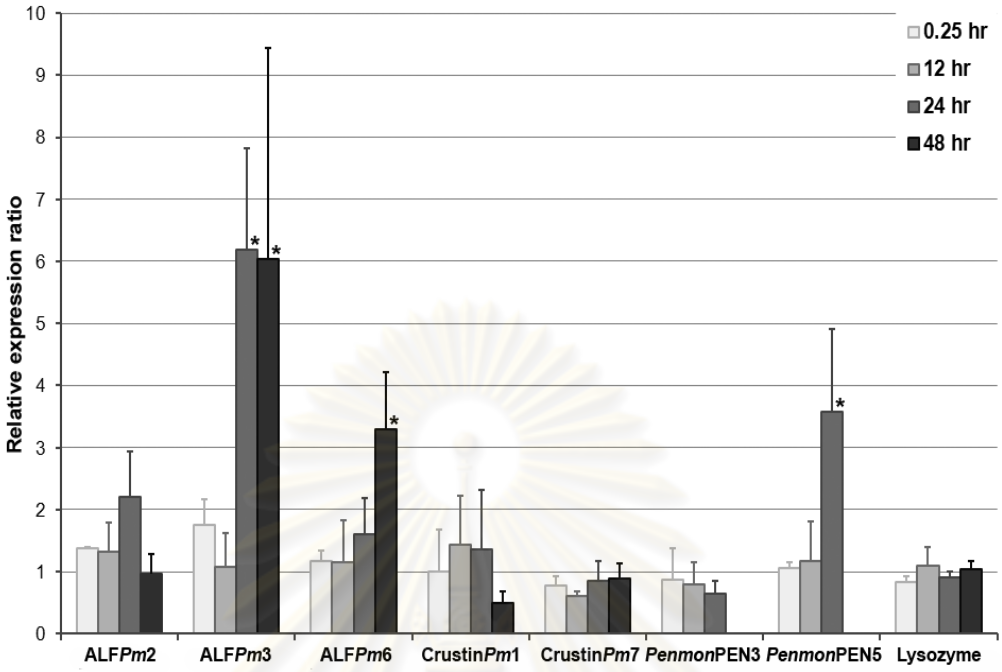


Figure 3.2 The relative expression ratios of *P. monodon* AMP genes, ALF*Pm*2, ALF*Pm*3, ALF*Pm*6, crustin*Pm*1, crustin*Pm*7, penaeidin3, penaeidin5 and lysozyme, in the hemocytes of WSSV-challenged *P.monodon* (15 g) analyzed by semi-quantitative RT-PCR. The relative expression ratios at 0.25, 12, 24 and 48 h post injection were then compared with β-actin and normalized against the control group (LHM-injected shrimp). The data shown as the mean (±1 SD) are derived from three independent experiments. One-Way ANOVA is used as the statistic and significant differences ($p < 0.05$) are marked with an asterisk.

3.2 Sequence analysis of *PenmonPEN5* cDNA from *Penaeus monodon*

In this study, *Penmon*PEN5 was selected for further study its role in antiviral immunity. In *P. monodon* two subgroups of penaeidin genes, *Penmon*PEN3 and *Penmon*PEN5, were identified from 4 contigs of *P.monodon* EST database (http://pmonodon.biotec.or.th) and deposited in the GenBank (accession nos. ACQ66008 and FJ686018). Both of them were mostly identified in the hemocytes cDNA library. The full-length cDNA of *Penmon*PEN5 was predicted the open-reading frame of 237 bp encoding a putative peptide of 79 amino acid residues as shown in Fig 3.3A. The signal peptide of 19 residues was predicted by using the signalP 3.0 program. Thus, a calculated molecular mass and a theoretical pI of the

mature protein of *Penmon*PEN5 (60-amino residues), were estimated to be 6.42 kDa and 9.64, respectively. BLASTX homology searching of the NCBI databases showed that *Penmon*PEN5 significantly matched to PEN5 from the Chinese shrimp, *F. chinensis* (*Fenchi*PEN5) with 72% amino acid sequence identity, whereas it shared only 57% overall amino acid sequence identity to the PEN3 from *P. monodon* (*Penmon*PEN3) (ACQ66006, ACQ66007). Multiple sequence alignment using the ClustalX program (Thompson et al., 1997) revealed a highly conserved signal peptide at the N-terminus follow by a proline-rich domain (PRD) where as a large variation and a cysteine-rich domain (CRD) containing six conserved cysteine residues at the C-terminus (Fig. 3.3B). Amino acid sequence alignment of the two classes, PEN3 and PEN5, from *P. monodon* and *F. chinensis* revealed that the penaeidin sequences from both species contain the conserved eight specific amino acids (Fig. 3.4B and C), which are the signatures of the penaeidin classes (Gueguen et al., 2006; Kang et al., 2007). However, slight variations in certain key residues in the penaeidin class signature were found and seem to be species specific (Fig. 3.3C).

3.3 Genomic organization of penaeidin5 gene from *P. monodon*

3.3.1 Determination of exons and introns of *P. monodon* penaeidin5 gene

The introns and exons within the ORF of *Penmon*PEN5 gene were investigated using PCR amplification with the gene specific primer (CT470F/R, Table 2.1) which designed from the 5′ and 3′ ends of the *Penmon*PEN5 ORF and genomic DNA was used as a template. The PCR product of about 860 bp was detected and cloned into the T&A cloning vector (Fig 3.4). After sequencing, the genomic sequence was analyzed by comparing to the corresponding cDNA sequence. The results presented that the entire coding region contained two exons separated by a single intron of 620 bp (Fig. 3.7). The intron separates the proline-rich domain from the cysteine-rich domain. The classical canonical GT/AG splicing recognition site was found at the boundary of the intron.

(A)

```
1     ATG CGT CTC GTG GTC TGC CTG GTC TTC CTG GTC TCC TTC GCC CTG   45
1      M   R   L   V   V   C   L   V   F   L   V   S   F   A   L   15

46    GTC TGC CAA GGC CAA GGA TAC AAG GGC GGT TAC ACA GGT TCA TAC   90
16     V   C   Q   G   Q   G   Y   K   G   G   Y   T   G   S   Y   30

91    TCC AGA CCA CCC TAT GGA TCC CGA CCT ATT AGT ACT CGA CCA ATC   135
31     S   R   P   P   Y   G   S   R   P   I   S   T   R   P   I   45

136   AGT CGT CCA GCC ACT GGT TGC ACT TCA TGC CAC ACT ATT ACC TTC   180
46     S   R   P   A   T   G   C   T   S   C   H   T   I   T   F   60

181   GAT AAA GCT ATT GCT TGC TGC AGA CAG TTC GGA CGT TGT TGT TCT   225
61     D   K   A   I   A   C   C   R   Q   F   G   R   C   C   S   75

226   GCA TTG AAA GGA TAA   240
76     A   L   K   G   *
```

(B)

```
                                                    ▼  ▼        ▼▼    ▼▼
PenmonPEN3b     MRLVVCLVFLASFALVCQAQGYQGGYTRPFPRPPYGG---GYHPV----PVCTSCHRLSPLQARACCRQLRRCCDAKQTY-G
PenmonPEN3a     MRLVVCLVFLASFALVCQAQGYQGGYTRPFPRPPYGG---GYHPV----PVCTSCHRLSPLQARACCRQLGRCCDAKQTY-G
FenchiPEN3-1    MRLVVCLVFLASFALVCQGQ--KGGYTRPISRPPYGG---GYG------NVCTSCHVLTTSQARSCCSRFGRCCVPRRGYSG
FenchiPEN3-2    MRLVVCLVFLASFALVCQGQ--KSGYTRPISRPPYGG---GYG------NVCTSCHVLTTSQARSCCSRFGRCCVPRRGYSG

PenmonPEN5      MRLVVCLVFLVSFALVCQGQGYKGGYTGSYSRPPYGSRPISTRPISRPATGCTSCHTITFDKAIACCRQFGRCCSALKG---
FenchiPEN5-2    MRLVVCLVFLASFALVCRGQGYKSGHTGPYPRPLYGSRPIGLRPITRPDPSCAGCRILTLDDAIACCRRLGRCCSALKG---
FenchiPEN5-1    MRLVVCLVFVASFALVCRGQGYKSGHTGPYPRPLYGSRPIGLRPITRPDPSCAGCRIITLDDAIACCRRLGRCCSALKG---
FenchiPEN5      MRLVVCLVFLASFALVCRGQGYKSGHTGPYPRPLYGSRPIGIRPITRPDPSCAGCRILTLDDAIACCRRLGRCCSALKG---
                *********:.******:.*  :.*:* . .** **.  .          *:.*: ::  .* :** :: *** . :
                                                    △
```

(C)

```
          1   5       13   18                          35 37     43 46
PEN3      Q...G.......R....V.........................S.R.....Q..S
                          G                            H         A
PEN5      Q...S.......R....S.........................G.R.....D..A
              G                                      S H      K
```

Figure 3.3 (A) Complete nucleotide and amino acid sequences of the penaeidin 5 from *P.* monodon. The signal peptides are underlined and asterisks indicate the stop codons. (B) Amino acid sequence alignment of penaeidin-3 and penaeidin-5 from *P. monodon* and *F. chinensis*. All penaeidin sequences are from the GenBank (Accession numbers ACQ66006, AAQ84721 and AAQ05769 for PenmonPEN3a; ACQ66007 for *Penmon*PEN3b; AAP33450 for *Fenchi*PEN3-1; ABC33920 for *Fenchi*PEN3-2; ACQ66008 and ACH70378 for *Penmon*PEN5; AAZ80041 and ABC33919 for *Fenchi*PEN5-2; AAZ79334 for *Fenchi*PEN5-1; AAV85945 for *Fenchi*PEN5). The signal peptides are underlined. Amino acid conservation across alignments is shown as (*), (:) and (.) for identical, conserved and semi-conserved, respectively. The six conserved Cys residues are identified by dark arrowheads. The amino acids in highlight of grey are identical to the signature of the penaeidin classes according to Gueguen et al. (2006) and Kang et al. (2007). The white arrowhead illustrates the specific signature amino acids of penaeidin class5**.** Black highlights indicate signature amino acids of species. (C) The eight conserved amino acid signatures of the penaeidin classes are illustrated for classes 3 and 5 with their specific amino acid signature in black highlight.

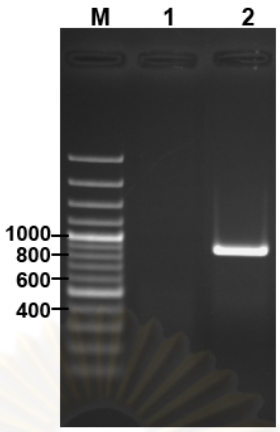


Figure 3.4 Agarose gel electrophoresis of PCR product of *Penmon*PEN5 amplified from the shrimp genomic DNA analyzed on a 1.5% agarose gel at 100 volts for 1 hour.

Lane M: GeneRuler™ 100 bp DNA Ladders (Fermentas)

Lanes 1: Negative control (without DNA template)

Lanes 2: PCR product of *Penmon*PEN5 amplified from shrimp genomic DNA

3.3.2 Determination of promoter and regulatory elements of *P. monodon* penaeidin5 gene

To determine the location of the promoter and regulatory elements at the 5′ upstream sequence, genome walking and nested PCR techniques were performed. The templates of four DNA libraries including *Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries were amplified using nested PCR with specific primers, 2Gsp1PEN5 and 2Gsp2PEN5 primers (Table 2.1), designed from the known gene sequences closed to the 5′ end. The primary and secondary PCR products were analyzed by agarose gel electrophoresis (Fig. 3.5). The secondary PCR products of approximately 400 and 500 bp were found in *Pvu*II and *Stu*I, respectively. The PCR products were then cloned and subsequently sequenced. The genomic sequences of *Penmon*PEN5 were confirmed by amplification of the whole genomic segments using confirmGW and CT470R primers (Table 2.1) and subsequently sequenced.

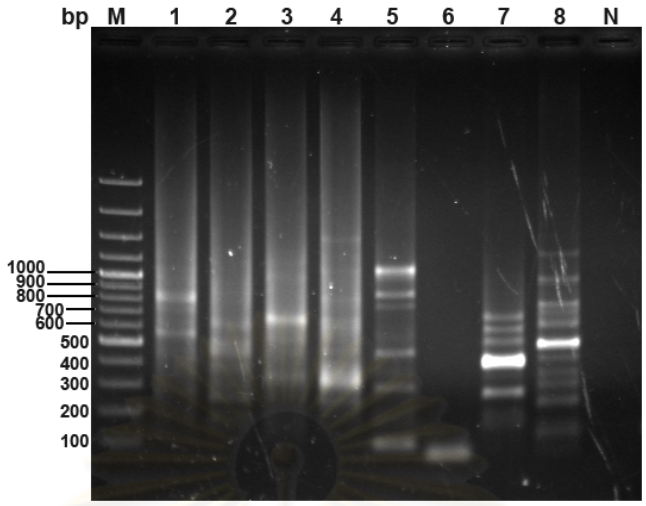


Figure 3.5 Agarose gel electrophoresis of the primary and secondary PCR product of the genome walking of *Penmon*PEN5 gene amplified from the four genomic libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). The PCR products were run on 1.2% agarose gel at 100 volts for 1 hour.

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
Lane N: Negative control (without DNA template)

The putative transcriptional start site and core promoter region were predicted using the Neural Network Promoter Prediction (Reese, 2001). The 5′ upstream sequence of *Penmon*PEN5 contained the putative core promoter region (TATA box) at 32 base pairs upstream (-32 region) of the putative transcriptional start site. The putative *cis*-regulatory elements were indentified by using Match_1.0 Public/TRANSFAC_6.0 program (BIOBASE, http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) with the cut-offs for both core and matrix similarities of 0.85. In this 5′ flanking region, nine putative transcription factor binding sites including three GATA (consensus WGATAR) and two each of GATA-3, activator protein 1 (AP-1) (consensus TGASTCA) and dorsal transcription binding were identified and shown in Fig. 3.6

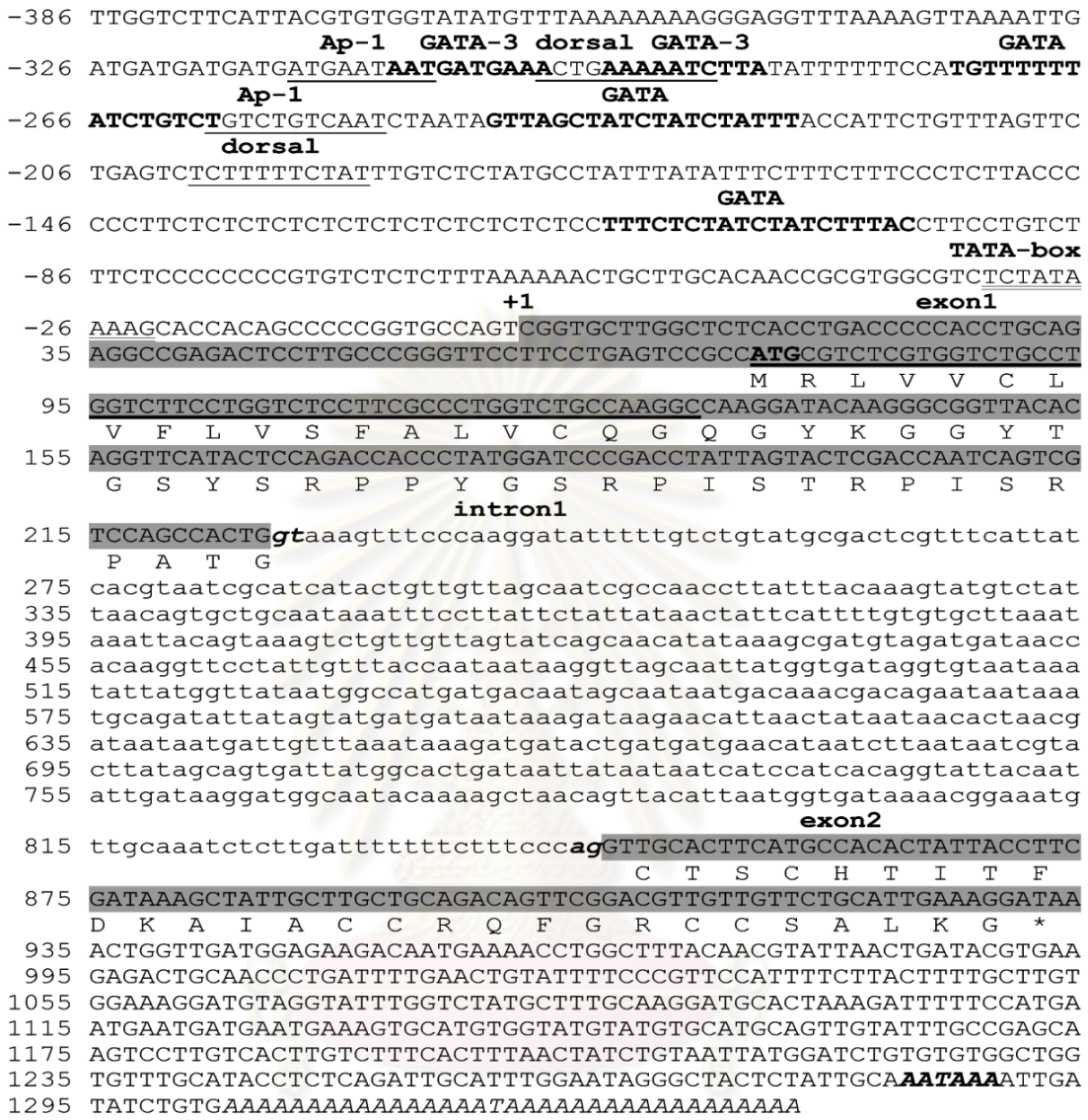


Figure 3.6 The nucleotide sequence of the complete *Penmon*PEN5. The 5′ upstream, exon and intron sequences are presented in upper case, shaded upper case and lower case letters, respectively. The encoding amino acid sequence is bold-faced with the signal peptide underlined. The nucleotide sequence is numbered starting from the putative transcription start site (+1) in exon-1 and proceeding as positive numbers in a 3′ direction and negative numbers in the 5′ direction. The putative binding sequence motifs for transcription factors are shown as underlined and bold text with the name of the corresponding factor shown above. Double underlined and asterisks marked sequences are the TATA box and stop codon, respectively. A polyadenylation site (AATAAA) is shown in bold italic upper case letters. The predicted intron dinucleotide acceptor and donor sites for RNA splicing are in bold italic lower case letters.

3.4 Tissue expression analysis of *Penmon*PEN5

To determine the tissue specificity of *Penmon*PEN5 mRNA expression, semi-quantitative RT-PCR analysis was manipulated with amplification of the EF-1α gene transcript fragment being used as the internal reference control. The total RNAs from hemocytes, hepatopancreas, lymphoid organ, gill, intestine, heart, epipodite, eye stalk and antennal gland were extracted from three healthy shrimp. After cDNA synthesis, the expression of *Penmon*PEN5 gene was determined. The *Penmon*PEN5 transcripts were found to be highly expressed in hemocytes and were also detected to a lesser extent in the intestine and only slightly in the heart (Fig. 3.7). No transcripts were detected in the eyestalk, epipodite, gill, lymphoid organ, hepatopancreas and antennal gland.

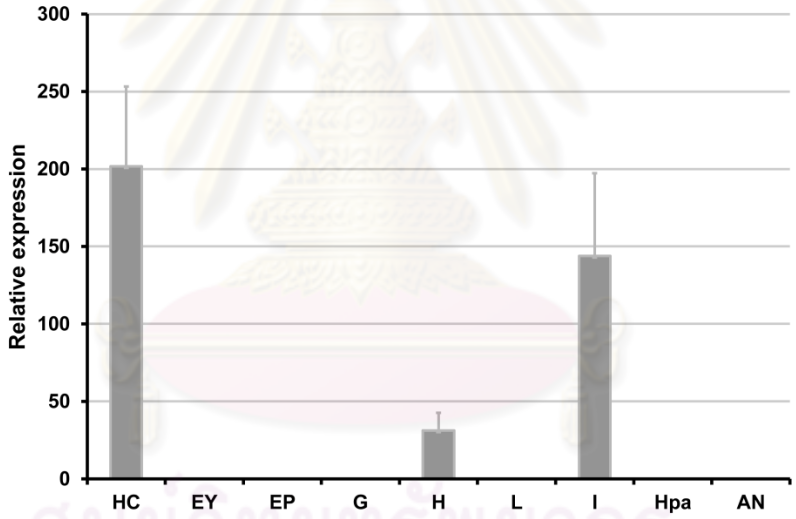


Figure 3.7 The relative expression level of *Penmon*PEN5 mRNA in nine tissues of *P. monodon* detected by semi-quantitative RT-PCR. The total RNA from hemocyte (HC), eye stalk (EY), epipodite (EP), gill (G), heart (H), lymphoid organ (L), intestine (I), hepatopancreas (Hpa) and antennal gland (AN) were extracted form three healthy shrimps (N=3). EF-1α gene was used as an internal control.

3.5 Temporal expression of *Penmon*PEN5 transcripts after systemic infection with a lethal dose of WSSV.

The expression profile of *Penmon*PEN5 transcripts in the hemocytes of WSSV-challenged *P. monodon* was determined by qrt-RT-PCR. The mRNA expression level

of the *Penmon*PEN5 gene found in WSSV (in LHM) injected shrimps was normalized to that of the EF-1α transcript fragment as the reference, and then expressed relative to the control group that was injected with the viral-free LHM media. The results presented that transcripts of *Penmon*PEN5 were rapidly down-regulated after WSSV-challenge (Fig. 3.3) but at 24 hpi the expression level was up-regulated by approximately 1.8-fold. Interestingly, the transcripts of *Penmon*PEN5 were then almost undetectable at 48 h post-challenge indicating a strong down-regulation of the *Penmon*PEN5 transcripts by this time point.

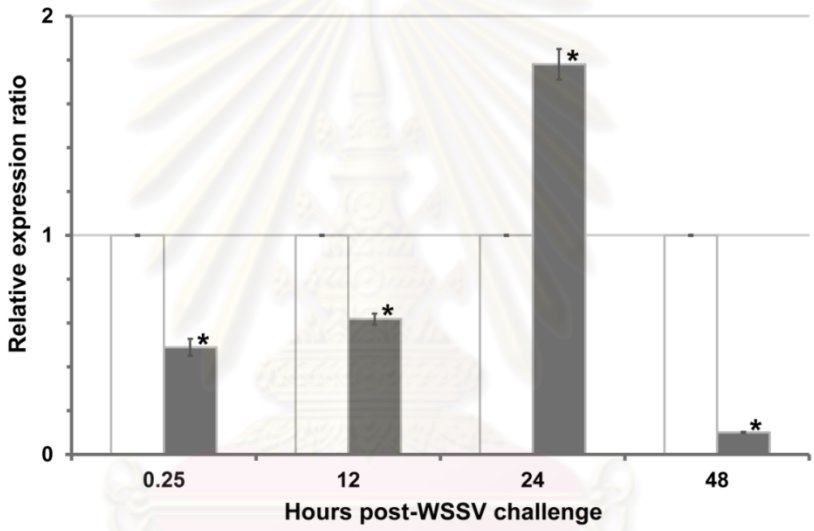


Figure 3.8 Analysis of *Penmon*PEN5 mRNA expression in the hemocytes of *P. monodon* shrimps by qrt-RT-PCR at different times (0.25, 12, 24 and 48 h) after WSSV infection. Data are shown as the mean(±1 SD) of three replicates and represent the fold change of *Penmon*PEN5 after normalization relative to the EF-1α transcript levels and then compared relative to the control group (set as = 1). Significant differences ($p < 0.05$) are marked with an asterisk.

3.6 RNAi-mediated down regulation of the *Penmon*PEN5 transcript levels and the effect on WSSV infection

The shrimp's response to WSSV challenge in terms of *Penmon*PEN5 transcript levels was determined by qrt-RT-PCR. This was initiated in accordance with a previous report from microarray analysis that *Penmon*PEN5 is likely to be a viral responsive gene (Pongsomboon et al., 2010). Therefore, its potential function in the

shrimp's antiviral immunity was investigated in *P. monodon* by RNAi mediated gene silencing

3.6.1 Gene-specific silencing of *Penmon*PEN5 transcript levels in *P. monodon* hemocytes

After optimization, double injections of 5 and 2.5 µg of dsRNA per 1 g shrimp were performed. Shrimps (each of ~4 g) were first injected with 20 µg of *Penmon*PEN5 dsRNA and then 24 h later they were injected with either 10 g of *Penmon*PEN5 dsRNA for the experimental group or with poly(GC) or NaCl for the control group. The *Penmon*PEN5 dsRNA appeared at least partially specific in that suppression of transcription levels at 24 hour post second injection was observed for *Penmon*PEN5 but not *Penmon*PEN3. Moreover, injection of the control poly(GC) or NaCl did not affect the mRNA level of *Penmon*PEN5 (Fig. 3.9).

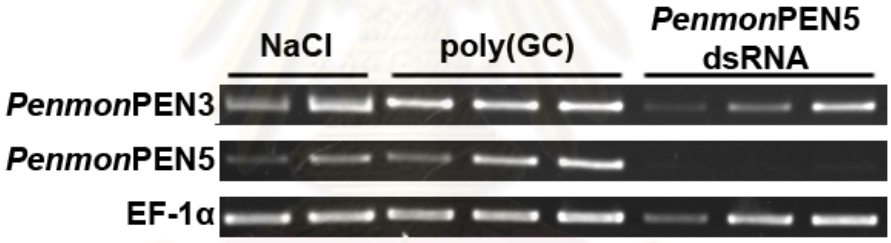


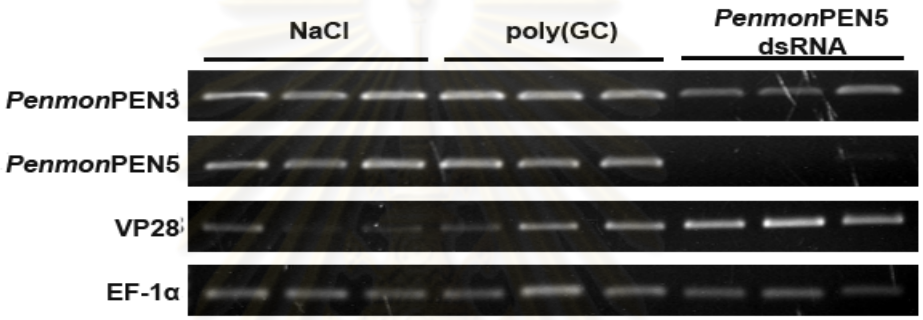
Figure 3.9 Gene-specific silencing of *Penmon*PEN5 transcript levels in hemocytes of *P. monodon*. Shrimp were injected with either the 5 µg of *Penmon*PEN5 dsRNA, poly(GC) per g shrimp or NaCl and then repeated injection with 2.5 µg of *Penmon*PEN5 dsRNA, poly(GC) per g shrimp or NaCl. Total RNA was extracted from *P. monodon* hemocytes 24 h after second injection and assayed for the transcript expression levels of *Penmon*PEN5, *Penmon*PEN3 and VP28 by RT-PCR. EF-1α was used as an internal control to standardize the amount of cDNA template in each reaction.

3.6.2 Relative expression levels of VP28 mRNA after *Penmon*PEN5 gene silencing gene and WSSV challenge

To determine whether the suppression of *Penmon*PEN5 gene transcript levels would affect WSSV-challenge, shrimps were pre-injected with *Penmon*PEN5 dsRNA, poly(GC) (control) or NaCl (control) and subsequently injected with *Penmon*PEN5 dsRNA, poly(GC) or NaCl together with 10^2 copies of WSSV. After 24

hpi, the hemocytes were collected to extract the total RNA.The relative expression of VP28 was normalized against the EF-1α and then the One-Way ANOVA was used to analyze the significance of any difference in the data between groups. The result showed that significant increase in the VP28 transcript level was observed in the *Penmon*PEN5 RNAi knockdown shrimps when compared with the control shrimps injected with either the virus-free saline solution or with the virus and poly(GC) as shown in Fig. 3.10 (A,B).

(A)



(B)

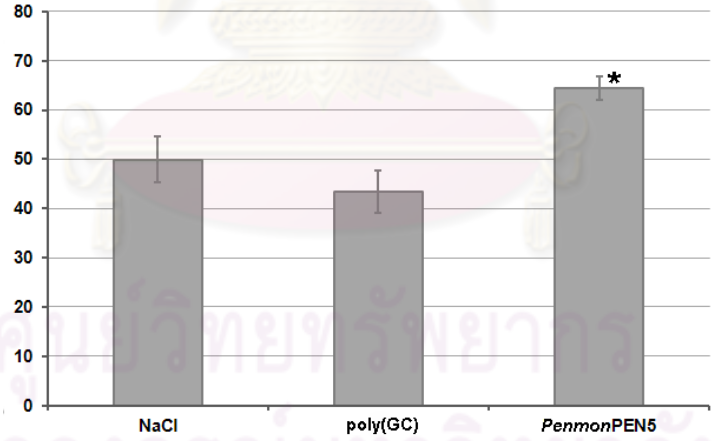


Figure 3.10 *Penmon*PEN5 transcripts down regulation by RNAi results in an increase in the VP28 transcript levels in *P. monodon* shrimps challenged with WSSV (10^2 copies). Shrimps were injected twice with *Penmon*PEN5 dsRNA, poly(GC) or NaCl and then injected with 10^2 WSSV virions. Total RNA was extracted from *P. monodon* hemocytes 24 h after WSSV injection and assayed for the transcript expression levels of *Penmon*PEN5, *Penmon*PEN3 and VP28 by RT-PCR (A). EF-1α was used as an internal control to standardize the amount of cDNA template in each reaction. The One-Way ANOVA was used to analyze the significance of any difference in the data between groups and showed in (B).

3.6.3 Quantification of WSSV copies number by real time RT-PCR

To investigate the viral copy number in infected shrimp, shrimps were double-injection with ds*Penmon*PEN5 or poly(GC) and then injected with 10^2 copies of WSSV. The genomic DNA was extracted from *P. monodon* hemocytes and WSSV infection in shrimp was detected by qrt-RT-PCR. It was found that silencing of *Penmon*PEN5 gene resulted in a 1.9- fold increase in the WSSV copy number in the infected shrimps, from7.8×10^4 to 1.5×10^5 copies, compared to the control shrimps injected with poly(GC) and WSSV, 24 h after the initial WSSV infection (Fig. 3.11).

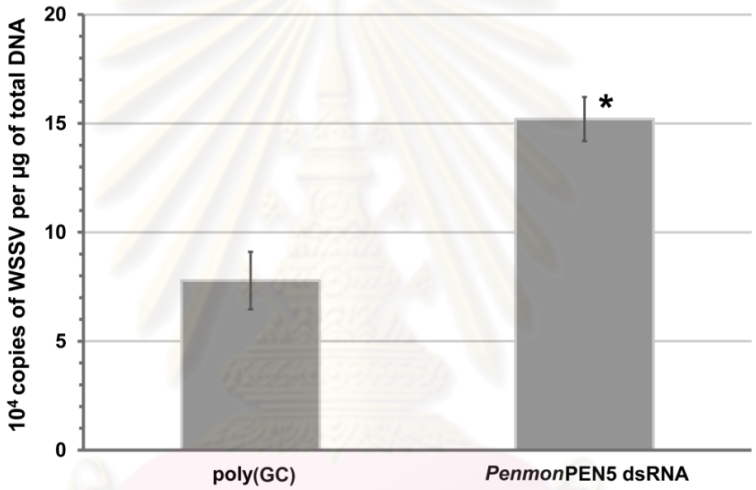


Figure 3.11 The *Penmon*PEN5 transcripts suppression by using RNAi results in an increase in the WSSV copy number in *P. monodon* shrimps challenged with WSSV (10^2 copies). Shrimps were injected twice with *Penmon*PEN5 dsRNA or poly(GC) and then injected with 10^2 WSSV virions. Total DNA was extracted from hemocytes at 24 h post WSSV injection. Data are shown as the mean fold-change of WSSV copy numbers relative to the control group and are derived from three shrimps for each group using qrt-RT-PCR. Significant differences ($p<0.05$) are marked with an asterisk.

3.7 Expression of penaeidin5 in the *Pichia pastoris* expression system

In order to better understand the antiviral mechanism of *Penmon*PEN5, the recombinant protein was produced in *Pichia pastoris* expression system and characterized, before testing the anti-WSSV of r*Penmon*PEN5 in the primary hemocyte cell culture.

3.7.1 Preparation of a DNA fragment encoded mature *Penmon*PEN5

The region encoding mature peptide of *Penmon*PEN5 was amplified from the EST clone homologue of penaeidin5 from the hemocyte EST library. The primers were designed from the cDNA sequence and incorporated with 5' *Sna*BI and 3' *Not*I cleavage sites to the gene. The PCR product was separated on 1.5% agarose gel to determine the size of a specific 183 bp fragment (Fig. 3.12). The PCR product was purified, digested with *Sna*BI and *Not*I, and ligated with an expression vector, pPIC9K. The deduced amino acid sequence at N-terminal of the recombinant protein would be YVQGYKGGYT that Y and V came from the restriction site of *Sna*BI (Fig. 3.13). The calculated molecular mass of the mature protein of *Penmon*PEN5 was about 6.68 kDa.

Figure 3.12 Agarose gel electrophoresis of *Penmon*PEN5 gene amplified by PCR. The PCR product was run on a 1.5 % agarose gel at 100 volts for 30 min.

Lane M: Standard DNA ladder (100 bp marker)

Lane 1: Amplified *Penmon*PEN5 gene products

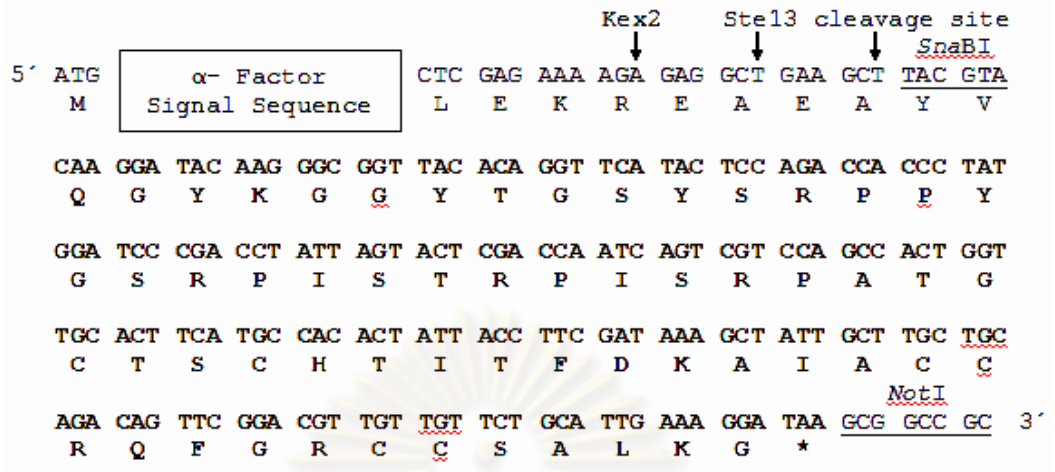


Figure 3.13 The nucleotide and deduced amino acid sequences of *Penmon*PEN5 in the expression vector pPIC9K (p*Penmon*PEN5). The mature peptide of *Penmon*PEN5 gene are shown in bold and was inserted in frame with the α-factor secretion signal sequence between the *Sna*BI and *Not*I sites that are underlined. Arrows above the nucleotide sequence indicate the Kex2 and Ste13 cleavage sites necessary for proteolytic processing of the fusion protein formed by the α-factor secretion signal and *Penmon*PEN5.

3.7.2 Preparation of an expression vector, pPIC9K

To produce the recombinant protein of *Penmon*PEN5, the expression vector, pPIC9K was used. The pPIC9K vector has the generation of multicopy gene insert in *Pichia* and expresses the secretion protein. The vector utilizes the *AOX1* promoter for inducible, high-level expression. The mature *Penmon*PEN5 gene was inserted at the *Sna*BI site after the STE13 protease cleavage site. Thus, the recombinant protein contains extra amino acid residues, Tyr and Val at NH_2-terminus from *Sna*BI, and sometime of double Glu-Ala repeats resulted from the inefficient STE13 protease cleavage (Fig. 3.13).

The pPIC9K vector was double digested with *Sna*BI and *Not*I restriction enzyme. The digested was isolated by 1% agarose gel, product size about 9,283 bp (Fig. 3.14), and excised to purify.

Figure 3.14 The expression vector, pPIC9K, double digested with *Sna*BI and *Not*I. The digested product was analyzed on 1% agarose gel at 100 volts for 1 hour.
Lane M: Standard DNA ladder (1 kb marker)
Lane 1: Double digested pPIC9K

3.7.3 Construction of expression vector

After the PCR product was ligated to the pPIC9K vector, the ligated product was transformed into *E. coli* XL-1-Blue cells. Transformants grown on LB-amplicillin selected plates were randomly screened using colony PCR with the α-signal peptide and the 3'-AOX primers. The positive colonies were selected for plasmid extraction and subjected to sequencing to confirm the correct construction. The plasmid was called the p*Penmon*PEN5.

3.7.4 Transformation of the recombinant plasmid into yeast cells

Before transformed into yeast cells, p*Penmon*PEN5 was linearized with *Sac*I and then transformed into *P. postoris* KM71 by electroporation to generate the recombinant *Penmon*PEN5 (r*Penmon*PEN5). The transformants were spread on the Minimal Dextrose (MD) plates and then screened on YPD that contain various concentration of G418 resistance. Geneticin resistant colonies grew on 1 and 2 mg/ml YPD-G418 plates. Single colony of *Pichia* clones was randomly picked and confirmed to determine the integrated *Penmon*PEN5 expression cassette in *P. pastoris* transformants by PCR amplification. The α-signal peptide and the 3′-AOX primers were used to amplify the *Pichia* genome. The parental plasmid pPIC9K produce a 197 bp PCR product while the expected sizes of p*Penmon*PEN5 of PCR product are 380 bp (197 bp + 183 bp). The results showed that all selected resistant clones have

*Penmon*PEN5 genes integrated into the yeast genome (Fig. 3.15). Since the high-copy number transformants assumed from the G418 hyper-resistance, the positive resistant clones on the YPD containing 2 mg/ml G418 were selected for recombinant expression.

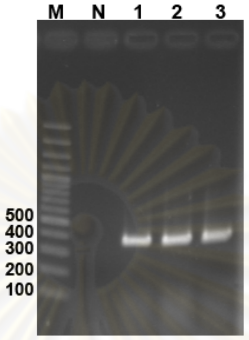


Figure 3.15 Screening of G418 resistant transformant clones containing *Penmon*PEN5 genes by colony PCR. The PCR products were run on 1.2% agarose gel at 100 volts for 45 min.

Lane M: Standard DNA ladder (100 bp marker)
Lane N: Negative control (without DNA template)
Lanes 1-3: PCR product of 2 mg/ml G418 resistant *Pichia* transformant containing the integrated p*Penmon*PEN5.

3.7.5 Expression of recombinant clones

The G418 hyper-resistance clones were selected for expression. Single colonies of each positive clone were grown in YPD broth at 30ºC overnight as the starter. The starter was inoculated in BMGY media to increase biomass. Next, the *Pichia* cells were harvested and resuspended in the BMMY media. Methanol was added into the culture to a final concentration of 0.5% (w/v) every 24 hours for maintenance the induction. Because the pPIC9K consist of the α-factor signal, r*Penmon*PEN5 was secreted into the culture medium. The KEX2 and STE13 proteases proteolytically removed the signal peptide in the *P. pastoris* secretory pathway (Cereghino and Cregg, 2000). Each expression culture was collected at 0, 1, 2, 3, 4, 5 and 6 days and centrifuged for separating the supernatant. The supernatant was analyzed using 16.5% Tricine SDS-PAGE and detected the protein band by silver staining (Fig. 3.16). A major protein band of approximately 6.6 kDa, closed to the

estimated molecular weight (6.685 kDa), was secreted at hightest levels at day 5 after induction. Electrophoresis also revealed the minor band of approximately 7 kDa that may be the recombinant protein containing double Glu-Ala repeats resulted from the inefficient STE13 protease cleavage. Fig 3.16 presented the highest r*Penmon*PEN5 expression clone that was further scaled up for protein production and purification.

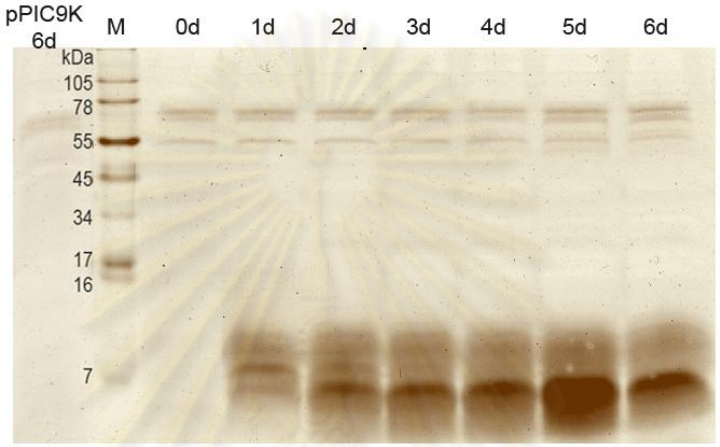


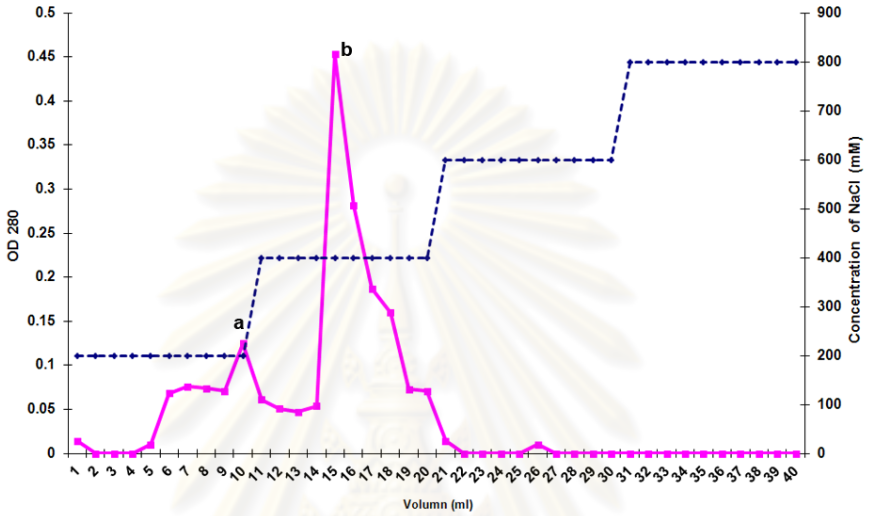
Figure 3.16 The silver stained 16.5% Tricine SDS-PAGE analysis of the r*Penmon*PEN5 expression from *P. pastoris* clone with respective to induction time. The r*Penmon*PEN5 clone was inoculated in BMGY medium and the protein expression was induced with 0.5% methanol. The culture supernatant was sampling every 24 hours within 6 days. Lane M is the protein marker (Invitrogen), lane pPIC9K 6d is the 6 days-expressed protein from *P. pastoris* contains empty the pPIC9K and others are expressed protein from *P. pastoris* contains p*Penmon*PEN5 in each day.

3.7.6 Purification of the recombinant *Penmon*PEN5 (r*Penmon*PEN5)

The culture supernatant of r*Penmon*PEN5 at day 5 post induction was collected to purify the protein by Sp-Sepharose HiTrap cation-exchang chromatography. The crude r*Penmon*PEN5 in 20 mM potassium phosphate buffer pH 7.0 was loaded into the Hitrap SP column and then eluted with step-wise gradient of sodium chloride at flow rate of 1 ml per min. The protein fractions were collected and determined the concentration by A_{280} absorbance (Fig. 3.17A). The purified protein was analyzed using 16.5% Tricine SDS-PAGE and then detected by silver staining (Fig. 3.17B). After analysis of the purified protein with tricine gel, the result illustrated that the purified *rPenmon*PEN5 was eluted in the second peak (b) at 400 mM sodium chloride and the yeast proteins were eluted in the first peak (a) at 200

mM sodium chloride (Fig 3.17A). Silver stained Tricine SDS-PAGE analysis presented two band of purified protein containing a major band of r*Penmon*PEN5 and minor band of r*Penmon*PEN5 joined with the double Glu-Ala repeats. An average of about 2 mg of purified r*Penmon*PEN5 was recovered from 1 L of culture medium.

(A)



(B)

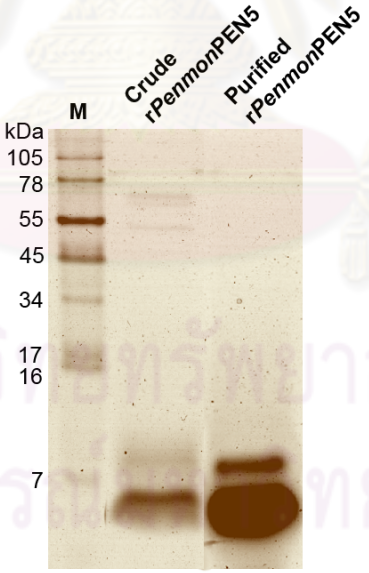


Figure 3.17 The purified r*Penmon*PEN5. (A) The culture supernatant of r*Penmon*PEN5 was purified by cation-exchange chromatography; Sp-Sepharose and eluted by step wise gradient of sodium chloride (dash line). The first peak (a) is the protein of *P. pastoris* and the second peak (b) is the r*Penmon*PEN5 protein. (B) The crude and purified r*Penmon*PEN5 were analyzed using the silver stained 16.5% Tricine-SDS-PAGE.

3.7.7 Antimicrobial activity of r*Penmon*PEN5

To assure the activity of *rPenmon*PEN5, a solid phase assay was employed to evaluate the activity against Gram positive bacteria, *Aerococcus viridans* and *Micrococcus luteus*. Amplicillin and lysozyme were used as the positive control in *A. viridans* and *M. luteus*, respectively. Twenty millimolars potassium phosphate buffer pH 7.0 was used as negative control. After incubation at 30 ºC for 16 h, the result revealed that the purified r*Penmon*PEN5 cleared a halo zone against *M. luteus* and *A. viridans* with diameter of 12 and 18 mm, respectively (Fig 3.18A,B).

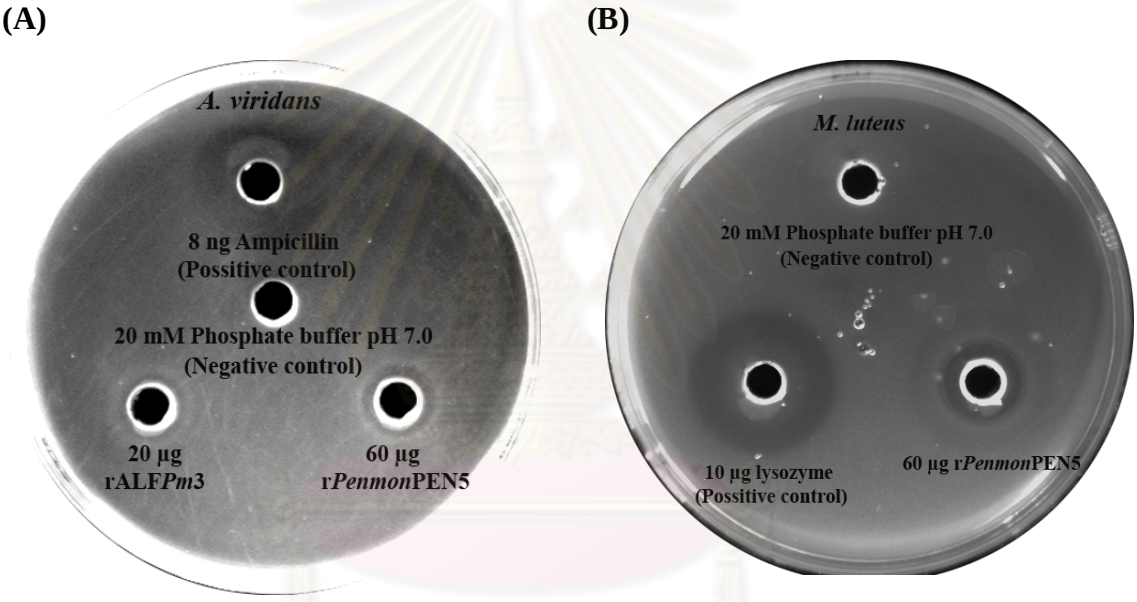


Figure 3.18 Antimicrobial activity of r*Penmon*PEN5 against *Micrococcus luteus* (A) and *Aerococcus viridans* (B) by solid phase assay. Twenty millimolar of potassium phosphate buffer pH 7.0 was used as negative control and 10 µg of lysozyme or 8 ng of ampicillin were used as positive control. After incubation at 30 ºC for 16 h, the diameters of the cleared zones were measured.

3.7.8 Anti-WSSV activity of r*Penmon*PEN5

To assess the anti-WSSV of penaeidin5, the primary cell culture of the *P. monodon* hemocyte was used. The WSSV propagation in hemocyte cells was detected from the VP28 gene, a major envelope protein gene of WSSV using semi-quantitative RT-PCR. The mixtures of WSSV and purified r*Penmon*PEN5 (25, 12.5,

and 6.25 μM) were incubated with cell culture at 28 ºC for 2 hour and then the medium was wash and replaced by fresh medium. The cell culture infected with WSSV and 20 mM potassium phosphate buffer was used as a control. After incubated at 28 ºC for 24 hour, the total RNA was collected to determine the VP28 gene. The EF-1α gene, housekeeping gene, was used as the internal control. At low concentration (6.25 μM) of r*Penmon*PEN5, WSSV propagation was inhibited. However, the transcription level of VP28 was observed at high concentration of r*Penmon*PEN5 (Fig 3.19). The experiment was repeated twice and a representative of one experiment was shown in Fig 3.19.

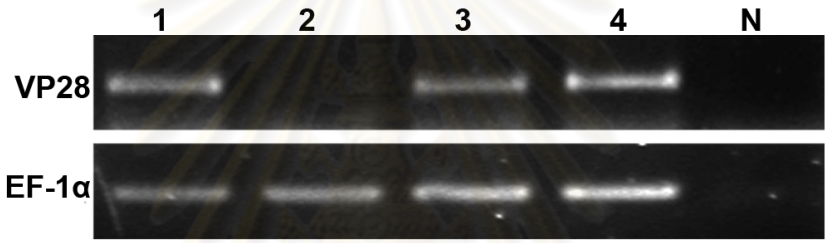


Figure 3.19 The effect of purified r*Penmon*PEN5 in hemocyte cell culture infected with WSSV. Semi-quantitative RT-PCR was used to analyze the transcripts of VP28 gene, a major envelope gene of WSSV. The EF-1α was used as the internal control.

Lane 1: Hemocyte cell culture incubated with WSSV and 20 mM potassium phosphate buffer.

Lanes 2-4: Hemocyte cell culture incubated with WSSV and 6.25, 12.5 and 25 μM of purified *Penmon*PEN5, respectively.

Lane N: Negative control (without cDNA template)

To determine the effect of protein to the cell culture, the cytotoxicity of r*Penmon*PEN5 on hemocyte cells was studied using the trypan blue exclusion test. The viable cells with undamaged cell membranes were not stained with trypan blue, so observed as clear white cells. The dead cells were observed as blue cells that cause the trypan blue interaction with the damaged cell membranes. After incubation the hemocyte cultures with 6.25, 12.5 or 25 μM of r*Penmon*PEN5, the result demonstrated that the viable cells at the ighest concentration of r*Penmon*PEN5 (25

μM) was slightly decreased (65% viability) compared with the control cell incubated with 20 mM potassium phosphate buffer pH 7.0 (69% viability) (Fig. 3.20). Thus, the concentration required to reduce the viable cells to 50% of control, CC50, of *Penmon*PEN5 was more than 25 μM.

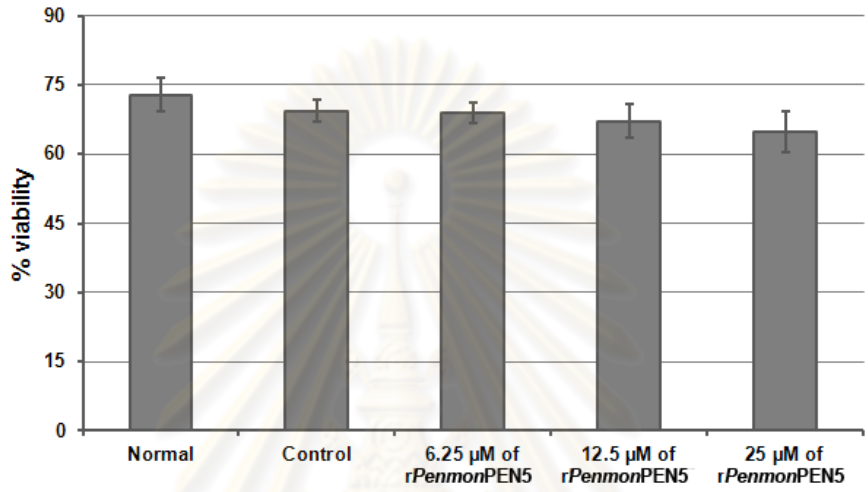


Figure 3.20 The percent viability of hemocyte culture after incubated with 10^6 copies WSSV and 6.25, 12.5 or 25μM of r*Penmon*PEN5. The purified protein was incubated with the cell culture. After incubation with cell culture for 2 hour, the medium was replaced by fresh medium and incubated at 30 ºC for 24 hour. Trypan blue exclusion test was used to exclude the viable and non-viable cells. Cells were counted by Hemocytometer Slide. The experiments were performed in triplicated.

CHAPTER IV
DISCUSSION

Nowadays, the shrimp farming industry has progressed along with the management of cultured system such as biosecurity but it is not absolutely eliminate the outbreaks of infectious diseases in hatcheries and farms. The main outbreak is the virus infection, especially WSSV that causes high mortality in cultured shrimp and has not effectively prevent or heal by chemicals or drugs. Thereupon, the knowledge of shrimp immune system will be very helpful for controlling this disease in shrimp aquaculture.

Antimicrobial peptides (AMPs) are important components of the innate immune system. They are short chain polypeptides that kill or slow the growth of microbes like bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity), or parasites (antiparasites activity) (Boman, 1995; Hancock et al., 2000). The existence of antiviral properties of several peptides has been reported in various marine species including tachyplesin (Murakami et al., 1991; Yasin et al., 2000), polyphemusin (Morimoto et al.,1991; Masuda et al., 1992; Nakashima et al., 1992; Tamamura et al., 1993), PmAV (Luo et al., 2003), LvCTL1 (Zhao et al., 2009), haemocyanin (Zhang et al., 2004; Lie ei al., 2008), mytilin (Dupuy et al., 2004; Roch et al., 2008), PmRab7 (Sritunyalucksana et al., 2006) and ALF (Liu H. et al., 2006; Tharntada et al., 2009). In *Penaeus monodon*, several AMP sequences were identified from the cDNA libraries of the *P. monodon*, consisting of penaeidins (PEN3 and PEN5), crustins (crustin*Pm*1- 2, 4-7), antilipopolysaccharide factor (ALF*Pm*1-6) and c-type lysozyme (Supungul et al., 2002; Tassanakajon et al., 2006; Tassanakajon et al., 2010). These *P. monodon* AMPs have been characterized for their antibacterial and antifungal activities (see a review by Tassanakajon et al, 2010). To investigate the antiviral property of these AMPs, a temporal expression analysis of eight AMP genes in response to WSSV challenge was conducted by semi-quantitative RT-PCR. The results revealed that WSSV infection induced the expression of the three AMP genes: ALF*Pm*3, ALF*Pm*6 and *Penmon*PEN5. Nevertheless, both isoforms of ALFs have been previously investigated their role in the defense against WSSV infection

(Tharntada et al., 2009, Tassanakajon et al., unpublish), hence the *Penmon*PEN5 was chosen for further characterization in this study.

The penaeidin class 5 (PEN5) is the new subgroup of penaeidin and have been reported, until today, from *F. chinensis* and *P. monodon* (Kang et al., 2007; Tassanakajon et al., 2010). Previously, the other penaeidin subgroups (PEN2, 3 and 4), especially PEN3, are well characterized (Destoumioux et al., 1997; Cuthberson et al., 2002; Cuthberson et al., 2004) while PEN5 is less documented (Chen et al., 2004; Kang et al., 2007). Gueguen et al. (2006) classified the penaeidin subgroups according to signature and specific amino acid of each subgroup, excepted penaeidin5 that are classified by Kang et al. (2007). Sequence alignment of the deduced amino acids of the *Penmon*PEN5 with other penaeidin subgroups showed the amino acid signature (Gln1, Ser5, Arg13, Ser18, Gly35, His37, Lys43 and Ala46) similar to the signature of *Fenchi*PEN5 but variations in three of the residues, Gly5, Ser35 and His37, were observed, which were identical to the residues in PEN3 (Fig. 3.3B, C). However, PEN5 contain Ser18, which is a specific class 5 residue (Tassanakajon et al., 2010). It also contains Lys/Asp at position 43 which illustrates the specific subgroup between PEN3 and PEN5 (Fig. 3.3B, C). In addition, the Lys/Asp at position 43 was also species specific in PEN5 (Fig. 3.3B, C). Although a PEN5 from *P. monodon* has been reported before (Chen et al., 2004), this is different from that reported here in this study. From analysis of the conserved amino acid signatures of the penaeidin classes and phylogenetic analysis, this previously reported PEN5 should rather be classified as PEN3 and not PEN5 (Kang et al., 2007; Tassanakajon et al., 2008).

Amplification of the genomic *Penmon*PEN5 gene was also performed to reveal the gene structure. The result demonstrates that the gene structure of *Penmon*PEN5 is very similar to that of the *Fenchi*PEN5 (Kang et al., 2007). The entire encoding sequence was interrupted by only one intron of 620 and 608 bp for *Penmon*PEN5 and *Fenchi*PEN5, respectively, which separated the proline-rich domain from the cysteine-rich domain. This was also similar to the overall gene organization of other penaeidin subgroups, but difference within the variation of intron length (O'Leary and Gross, 2006). Despite the similar gene organization, it has been shown that each penaeidin class is encoded by a unique gene and that class

diversity is not generated by alternative splicing. Indeed, genomic DNA sequence analysis indicates that PEN2, PEN3 and PEN4 of the Pacific white shrimp, *L. vannamei*, are encoded by different genes (O'Leary and Gross, 2006; Cuthbertson et al., 2008).

To gain more information of the *Penmon*PEN5 gene organization, the genome walking technique was performed (Siebert et al., 1995). 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. The regulation of gene expression of *Penmon*PEN5 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 *Penmon*PEN5 contained some nine putative regulatory motifs known to be involved in immune response and /or regulate the expression of antimicrobial peptides in other arthropods. There are three GATA and two each of GATA-3, AP-1 and dorsal.

GATA factors play crucial roles in cell development, containing in cell-fate specification, differentiation and proliferation. They were reported to be involved in the interactions with other transcription factors, transcriptional co-activators and co-repressors such as interactions with NF-κB transcription factor in fat body-specific expression in insects (Patient and McGhee, 2002; Senger et al., 2006). The GATA site is required for the activity of penaeidin 2 promoter of *L. vannamei* (O'Leary and Gross, 2006). Seven and five putative GATA sites were also found in the ALF genes. GATA3 belongs to transcription factor families (GATA1 to GATA6) that share a steroid-hormone-receptor superfamily C4 zinc-finger DNA-binding motif (Merika and Orkin, 1993). The activator protein 1 (AP-1) factor is involved in the cell proliferation, differentiation, apoptosis and also produced in response to a variety of stimuli, including cytokines, growth factors, stress and infection of bacterial and viral (Karin et al., 1997; Douglas et al., 2003). Dorsal is a member of the NF-kB family, involved in the Drosophila host defense (Hoffmann, 2003), and might contribute to the immune responses (Meng et al., 1999).

Some putative regulatory motifs, including dorsal, GATA and AP-1, of *Penmon*PEN5 are similarly found in the upstream region of the *Litvan*PEN4 gene as

reported by O'Leary and Gross (2006). AP-1 and GATA motifs are also found in the upstream region of another antimicrobial peptide, ALF of *P. monodon* (Tharntada et al., 2008). Discovery of these regulatory motifs on 5′ upstream of *Penmon*PEN5 implied the involvement of *Penmon*PEN5 in the shrimp immune system. In 2009, Ho and Song reported two promoter types in *P. monodon*, type536 and type411, and demonstrated that Type411 exhibits a stronger transcriptional activity. According to the 5′ upstream sequence, the *Penmon*PEN5 promoter is nearly identical to the Type536 promoter, bar a few nucleotide variations, and so Type411 could, presumably, be the *Penmon*PEN3 promoter. The analysis of the hemocyte cDNA libraries (>10,000 ESTs) from *P. monodon*, indicated that transcripts of PEN3 are much more abundant than PEN5 (Tassanakajon et al., 2006), which could result from the differences in the promoter activities.

The tissue distribution of *Penmon*PEN5 in healthy shrimp was analyzed by semi-quantitative RT-PCR and the result revealed that *Penmon*PEN5 transcript is mainly expressed in the hemocyte followed by intestine and very low level in heart (Fig 3.7), which implies that hemocyte is the main site of *Penmon*PEN5 synthesis. This was consistent with the previous reports of *Fenchi*PEN5 (Kang et al., 2007), other penaeidins from *L. vannamei* (Destoumieux et al., 2000) and penaeidin 3 from *P. monodon* (Ho et al., 2004). The other tissue of shrimp which were detected the weak expression of *Penmon*PEN5, may resulted from the infiltration of hemocytes.

Penaeidins contain the three disulfide-linked cysteine residues and their chimeric-like overall structure display posttranslational modification such as COOH-terminal amidation and NH_2-terminal cyclization of a glutamine residue in PEN3 (Destoumieux et al., 1997). Besides previously reported, the recombinant of penaeidins were expressed in insect-baculovirus expression system (Ho et al., 2004), in the yeast *Saccharomyces cerevisiae* (Destoumieux et al., 1999), and *Pichia pastoris* (Li et al., 2005) presented a broad spectrum of antibacterial and antifungal properties. The overalls indicating these peptides could perhaps be produced in eukaryotic expression systems. Therefore, in this study, the *Penmon*PEN5 was over-produced using the *P. pastoris* expression system. Also the reasons that described, *P. pastoris* was chosen because it possesses many advantages containing high expression level

that possible to hundreds of mg/L yields, easy scale-up and inexpensive heterologous expression system (Cereghino and Cregg, 2000). The expression of several antimicrobial peptides were also successful in this expression system such as anti-lipopolysaccharide factor (Somboonwiwat et al., 2005; Tharntada, 2007), cecropin (Jin et al., 2006) and penaeidin (Li et al., 2005; Kang et al., 2007). The mature *Penmon*PEN5 gene was ligated into pPIC9K vector at downstream of the alcohol oxidase I (AOX I) promoter that drives the heterologous protein expression in *P. pastoris* and the α-factor which sometimes is removed by the somewhat inefficient STE13 protease cleavage. The yield of recombinant protein depends on the gene copy number that integrated into the host genome as single or multiplie copies or expression cassettes. The kanamycin gene in the expression cassette confers resistance to geneticin or G418 in *P. pastoris* so the level of G418 resistance roughly relies on the number of kanamycin gene to screen the highest expression clone. In this study, the *Pichia* transformant clones which resisted 2 mg/ml G418 was used for the scale-up expression, because it could express higher amount of protein. Proteins secreted into the culture supernatant, were analyzed by Tricine SDS-PAGE resulting the band corresponding to the expected size of the recombinant *Penmon*PEN5 (r*Penmon*PEN5) (6.685 kDa) and then was purified based on charge property using cation exchanger chromatography since at pH 7 which lower than its pI (9.59), the molecule possessed the net positively charge. The r*Penmon*PEN5 contained an additional dipeptide, Tyr and Val while the recombinant of Ch-penaeidin was added tetrapeptide, Tyr-Val-Glu-Phe, at the N-terminal of mature peptide (Li et al., 2005). Tricine SDS-PAGE analysis showed the major band that more than 80% of the elution fractions were the r*Penmon*PEN5 and a minor band probably is the r*Penmon*PEN5 linked with the double Glu-Ala repeats of STE13 cleavage site. These fractions also exhibited antimicrobial activity against *A. viridians* and *M. luteus*.

To confirm the activity of r*Penmon*PEN5 before testing the anti-WSSV, the liquid growth inhibition assay has been used to determine the minimum inhibitory concentration (MIC) of most penaeidins but in this study, an agar diffusion assay was employed because the amount of r*Penmon*PEN5 obtained is too low for the MIC test. The results revealed that r*Penmon*PEN5 exhibited the antimicrobial activity against

M. luteus and *A. viridians* which correspond to the activity of penaeidin 5 from *F. chinensis* that was less effective than other penaeidins (Table 4.1). The antimicrobial activity spectrum of all penaeidin subgroups has been examined and PEN3 has a broader range of microbial targets and is more effective against certain bacteria species than other classes of penaeidins (Cuthbertson et al., 2006; Tassanakajon et al., 2010).

Table 4.1 Range of antimicrobial activity of the recombinant *Litvan* PEN2-1, *Litvan* PEN3-1 (Destoumieux et al., 1999), the chemically synthesized *Litvan* PEN4-1 (Cuthbertson et al., 2004), the recombinant *Fenchi* PEN3-1 (Li et al., 2005), the recombinant *Fenchi* PEN5 (Kang et al., 2007) and the recombinant penaeidin-like (*Penmon* PEN3, Chiou et al., 2005). MIC are expressed as the interval *a-b*, where *a* is the highest concentration tested at which the growth of the microorganism is not inhibited and *b* the lowest concentration that causes the 100% growth inhibition. For *Fenchi* PEN5, *b* the lowest concentration that causes the 50% growth inhibition.

Micro-organism	**MIC (μM)**					
	***Litvan* PEN2-1**	***Litvan* PEN3-1**	***Litvan* PEN4-1**	***Fenchi* PEN3-1**	***Fenchi* PEN5**	**Penaeidin-like (*Penmon* PEN3)**
Bacteria						
Gram positive						
Aerococcus viridans	1.25-2.5	0.3-0.6	1.9-2.92	ND	ND	20
Micrococcus luteus	2.5-5	1.25-2.5	1.9-2.92	3.7	0.78-6.25	ND
Bacillus megaterium	2.5-5	2.5-5	>50	2.0	6.25-25.0	ND
Saphylococcus aureus	> 20	> 40	> 50	3.5	6.25-25.0	ND
Gram negative						
Escherichia coli 363	>40	10-20	22-33	2.1	ND	ND
Vibrio vulnificus	>20	>20	>50	ND	ND	ND
Salmonella thyphimurium	>20	>40	>50	ND	ND	ND
Klebsiella pneumoniae	>20	>40	>50	2.0	3.13-12.5	ND
Filamentous fungi						
Fusarium oxysporum	5-10	5-10	0.84-1.26	12	3.13-6.25	10
Botrytis cinerea	5-10	5-10	4.38-6.57	ND	ND	ND

ND = not determine

To confirm the preliminary study of the expression profile of *Penmon*PEN5 transcript in the hemocytes of WSSV-challenged shrimp, the quantitative real time RT-PCR (qrt-RRT-PCR) was performed. The result illustrated that transcripts of *Penmon*PEN5 were rapidly down-regulated after WSSV-challenge nevertheless at 24 hpi. Its transcript was up-regulated by approximately 1.8-fold. On the contrary, the expression level of *Penmon*PEN5 was then almost undetectable at 48 hpi indicating a strong down-regulation of the *Penmon*PEN5 transcripts by this time point. In 2005, Dong et al. revealed the expression profile of penaeidin as an inverse "U" gradually decreasing to below baseline level after 24 h after WSSV challenged and Pongsomboon et al. (2010) also demonstrated the decrease of this gene in hemocyte at 24 h after WSSV challenge. In *L. vannamei* the transcript of penaeidin3 was decrease four- to fivefold at 3 h after heat-killed microorganism challenge and its expression returned to control levels at 12 hpi. Nevertheless, the concentration of penaeidin3 was increased in the plasma at 3 h post-stimulation (Destoumieux et al., 2000). After microbial infection, the migration of the hemocytes toward injured tissues or to nodule formation leads to decrease the penaeidin transcription. This is in agreement with previous studies on other crustacean species (Martin et al., 1998). On the other hand, microbial stimulation could also trigger hemocyte degranulation and release several peptides into the blood (Bachère et al., 2004). In additon, WSSV might inhibit the expression of genes by yet undiscovered modes (Dong et al., 2005).

To better understand the role of *Penmon*PEN5 against WSSV, *Penmon*PEN5 was knocked-down by RNAi technology to investigate the infected-WSSV of shrimp and was over-produced as recombinant protein to use in the in vitro. RNA interference (RNAi) mediated by long dsRNA has been used to silence the target genes for study the functional genomics. RNAi promotes the study function of immune relate genes in several arthropods (Reynolds et al., 2008) and long dsRNA has been reported that potential activity against viral infection in shrimp (Robalino et al., 2004, 2005; Kim et al., 2006; Yodmuang et al., 2006). In this study shrimps were double-injected with ds*Penmon*PEN5 and then injected with WSSV. The result revealed that the suppression of *Penmon*PEN5 gene transcript resulted in an increase

in the viral infection load in shrimp as compared with the control shrimp indicating the possible role of *Penmon*PEN5 in protecting shrimp from WSSV infection.

Hemocytes of crustaceans are generally known to play a crucial role in the innate immunity (reviewed in Jiravanichpaisal et al., 2006a). Here, the primary hemocyte culture of *P. monodon* was used as an in vitro model for the study of anti-WSSV activity of *Penmon*PEN5. The WSSV propagation in the hemocyte cell culture was detected by semi-quantitative RT-PCR with primers that corresponding with the VP28 genes, an envelope protein gene for WSSV entry into host cells. After incubating the cell culture with the mixture of r*Penmon*PEN5 and WSSV, the effect of r*Penmon*PEN5 on WSSV propagation was examined and the result shown that, the VP28 gene expression was significantly decreased only at 6.25 μM of the r*Penmon*PEN5 protein. Surprisingly, a higher concentration could not inhibit the propagation of WSSV. Likewise the previous report by Li et al. (2010), found that a lower concentration of the recombinant penaeidin (50 pmol) led to a full recovery of hemocyte adhesion after penaeidin knock-down but at higher concentration of the recombinant penaeidin further lowered the number of adhesive hemocytes and no obvious cell lysis and apoptosis phenomena was observed. They presume that the regulation of adhesive hemocytes by penaeidin is concentration-dependent and possesses the feature of a feedback control. This function of penaeidin as a cytokine is an addition function to a bactericide. The inhibition effect of WSSV propagation by the r*Penmon*PEN5 in the shrimp cell culture remains unclear and the understanding of the immune mechanism require extensive investigation.

So far, antilipopolysaccharide factor (ALF) is the only shrimp AMP which has been demonstrated to be involved in protection against shrimp viral pathogens. However, a few studies report the upregulation of penaeidin transcripts in WSSV-challenge shrimps (Rojtinnakorn et al., 2002; Pongsomboon et al., 2010), whilst a significant activity of penaeidins against human herpes simplex virus type 1 (HSV-1) has been reported (Carriel-Gomes et al., 2007). Therefore, it seems likely that AMP in the penaeidin family could play a crucial function in the shrimp antiviral immunity. Due to the high negative impact of viruses play an important role in antiviral

immunity is of great importance and so they should be further characterized to unveil the shrimp's immune mechanisms towards viral infections.

CHAPTER V
CONCLUSIONS

The expression of *Penmon*PEN5 transcript was significantly increased upon WSSV-challenge which implied its possible function in antiviral immunity. Tissue distribution analysis revealed that the transcript of *Penmon*PEN5 was highest in shrimp hemocytes.

The open reading frame of *Penmon*PEN5 contains 237 bp encoding a putative peptides of 79 amino acids with a 19 amino acids signal peptide and a 60 amino acid mature protein. The *Penmon*PEN5 genomic gene contains two exons separated by a single intron located between the proline-rich domain and the cysteine-rich domain. The 5′ upstream sequence of *Penmon*PEN5 contains a putative promoter, TATA box and nine putative regulatory motifs known to be involved in the regulation of immune genes in other arthropods.

Silencing of the *Penmon*PEN5 gene by double-injection of double stranded RNA (dsRNA) corresponding to this gene into shrimp, illustrated a specific *Penmon*PEN5 gene knockdown. Suppression of the *Penmon*PEN5 gene transcript resulted in an increase in viral load in shrimp.

The recombinant protein of *Penmon*PEN5 was successfully expressed in the yeast *Pichia pastoris* system. The purified recombinant protein has the estimated molecular mass of 6.6 kDa and displays antibacterial activity against Gram-positive bacteria, *Aerococcus viridans* and *Micrococcus luteus*. Incubation of shrimp hemocyte cell culture with the mixture of WSSV and r*Penmon*PEN5 could inhibit WSSV propagation only at 6.25 μM but not at higher concentration of the r*Penmon*PEN5 protein. Taken together, this study suggests the possible function of *Penmon*PEN5 in the shrimp's antiviral immunity against the white spot syndrome virus (WSSV).

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APPENDICES

Appendix A

1. Preparation for Tricine-SDS-PAGE electrophoresis

Stock reagents

49.5 % Acrylamide, 3% bis-acrylamide, 100 ml

Acrylamide	48.0 g
N,N′-methylene-bis-acrylamide	1.5 g

Adjust volume to 100 ml with distilled water.

Gel buffer: 3.0 M Tris-HCl, 0.3% SDS pH 8.45

Tris (hydroxymethyl)-aminomethane	36.4 g
SDS	0.3 g

Adjust pH to 8.45 with 1 M HCl and adjust volume to 100 ml with distilled water.

Tricine SDS-PAGE

16.5 % Seperating gel

49.5 % Acrylamide, 3% bis-acrylamide	3.3 ml
Gel buffer	3.3 ml
Glycerol	1.0 ml
Distilled water	2.3 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

4.0 % Stacking gel

49.5 % Acrylamide, 3% bis-acrylamide	0.4 ml
Gel buffer	1.2 ml
Distilled water	3.3 ml
10% $(NH_4)_2S_2O_8$	40 µl
TEMED	5 µl

2X Sample buffer

1 M Tris-HCl pH 6.8	1.0 ml
Glycerol	2.4 ml
SDS	0.8 g
2-Mercaptoethanol	0.4 ml
Commasie blue G	2 ml

Phenol red	2 ml
1 % Bromophenol blue	1.0 ml
Distilled water	0.9 ml

Adjust volume to 10 ml with distilled water

One part of sample buffer was added to one part of sample. The mixture was heated 10 min. in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

Anode buffer: 0.2 M Tris-HCl pH 8.9

Tris (hydroxymethyl)-aminomethane	24.2 g

Adjust pH to 8.9 with 1 M HCl and adjust volume to 100 ml with distilled water.

Cathode buffer: 0.1 M Tris-HCl, 0.1 M Tricine, 0.1%(w/v) SDS

Tris (hydroxymethyl)-aminomethane	12.11 g
Tricine	17.92 g
SDS	1.0 g

adjust volume to 100 ml with distilled water but do not adjust pH

2. Preparation for sliver staining solution

Solution A

Sliver nitrate	0.8 g
Distilled water	4.0 ml

Solution B

0.36% NaOH	21.0 ml
14.8 M Ammonium hydroxide	1.4 ml

Solution C

Add Solution A to Solution B dropwise with constant vigorous stirring, allowing brown precipitate to clear

Solution D

Mix 0.5 ml 1% citric acid with 50 µl 3.8% formaldehyde, add water to 100 ml. Solution must be fresh

Appendix B

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PenmonPEN5    -GCTTGCACAACCGCG--TGGCGTCTCTATAAAAGCACCACAGCCCCC-GGTGCCAGTCG 56
PenmonPEN3    AACCTGC-CGGACGTGGCTGCCCTCCCC-TGGTGGCCTGTCGGGCAGGAGGTGC-AGTCG 57
                * *** *   ** *  ** * ** *  *     **     * * *     ***** *****

PenmonPEN5    GTGCTTGGCTCTCACCTGACCCCCACCTGCAGAGGCCGAGACTCCTTGCCCGGGTTCCTT 116
PenmonPEN3    GTGCTTGGCTCTCACCTGACCCCCACCTGTAGAGGCCGAGACTCCTTGCCCGGGTTCCTT 117
              ************************** ******************************

PenmonPEN5    CCTGAGTCCGCCATGCGTCTCGTGGTCTGCCTGGTCTTCCTGGTCTCCTTCGCCCTGGTC 176
PenmonPEN3    CCTGTGTCCGCCATGCGTCTCGTGGTCTGCCTGGTCTTCCTGGCCTCCTTCGCCCTGGTC 177
              **** ****************************************** ****************

PenmonPEN5    TGCCAAGGCCAAGGATACAAGGGCGGTTACACAGGTTCATACTCCAGACCACCCTATGGA 236
PenmonPEN3    TGCCAAGCCCAAGGGTACCAGGGTGGTTACACACGCCCGTTCCCCAGACCACCCTATGGG 237
              ******* ****** *** **** ********* *  * * * ****************

PenmonPEN5    TCCCGACCTATTAGTACTCGACCAATCAGTCGTCCAGCCACTGGTTGCACTTCATGCCAC 296
PenmonPEN3    -------------------GGAGGAT--ATCATCCAGTTCCTGTTTGCACTTCATGCCAC 276
                               *    **   ** *****   *** ****************

PenmonPEN5    ACTATTACCTTCGATAAAGCTATTGCTTGCTGCAGACAGTTCGGACGTTGTTGTTCTGCA 356
PenmonPEN3    AGGCTTAGCCCCTTACAAGCTCGTGCTTGCTGCAGGCAGTTAAGACGTTGTTGTGATGCA 336
                 *   *** *  *    *****  ************ *****  ***********  ****

PenmonPEN5    TTGAAAGGATAAACTGGTTGATGGAGAAGACAATGAAAACCTGGCTTTACAACGTATTAA 416
PenmonPEN3    AAGCAGACATA---TGGTTGATGGAGAAGACAACGAAAAACTGACTTCACAATGTATTAA 393
                * *   ***   ******************** ***** *** *** **** *******

PenmonPEN5    CTGATACGTGAAGAGACTGCAACCCTGATTTTGAACTGTATTTTCCCGTTCCATTTTCTT 476
PenmonPEN3    TCAGT-TGTGAAGAAAGTGCAACCCTGATTTTGAACTGTATTTTCTAGTTCCATTTTCTT 452
                  *  ******* * ****************************  **************

PenmonPEN5    ACTTTTGCTTGTGGAAAGGATGTAGGTATTTGGTCTATGCTTTGCAAGGATGCACTAAAG 536
PenmonPEN3    ACTTTTGCTTGTGGAAAGGATGTAGGTATTTGGA-------------------------- 486
              ***********************************

PenmonPEN5    ATTTTTCCATGAATGAATGATGAATGAAAGTGTATGTGGTATGTATGTGCATGCAGTTGT 596
PenmonPEN3    --TTTTCCATGAATGTATGATGAATGAAAGTGCATGTGGGATGTATGTGCATACAGTCGT 544
                ************* *************** ****** ************ **** **

PenmonPEN5    ATTTG-CCGAGCAAGTCCT------------------------TGTCACTTGTCTTTC 629
PenmonPEN3    ATTTGTCCCAGCAGGTCCTCGTGTATTCACAGGAGAAAGATATCGTGTTGTTTGACTTTC 604
              ***** ** **** *****                           ***   *** *****

PenmonPEN5    ACTTTAACTATCTGTAATTATGGATCTGTGTGTGGCTGGTGTTTGCATACCTCTCAGATT 689
PenmonPEN3    GTTGTAGTTATTTGTAGGTATGGGTCTGTGTGTGGTTGGTGTTTGCATATTTCCCAAAGG 664
                * **  *** ****  ***** *********** *************  ** ** *

PenmonPEN5    GCATTTGGAATAGGGCTACTCTATTGCAAATAAAATTGATATCTGTGAAAAAAAAAAAAA 749
PenmonPEN3    ACATTCGGAATTGTACTACTCTTTTACAAATAAAATTGATATCTGTGAAAAAAAAAAAAA 724
               **** ***** *  ******* ** ***********************************

PenmonPEN5    AAAAAAAA---------------------------------------------------- 757
PenmonPEN3    AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 784
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A sequence alignment, produced by ClustalW program between *Penmon*PEN5 and *Penmon*PEN3. Nucleotide sequence conservation across alignments is shown as (*). The highlight indicated the specific primers as used to construct the *Penmon*PEN5 dsRNA.

Biography

Miss Noppawan Woramongkolchai was born on January 1, 1985 in Chonburi. She graduated with the degree of Bachelor of Science in 2007. She has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2007.

She had published her works in the research journals, Developmental and Comparative Immunology, on the topics of "The possible role of penaeidin5 from the black tiger shrimp, *Penaeus monodon*, in protection against viral infection".