การก่อกลายพันธุ์ของโปรตีนที่มีโดเมน WAP จากกุ้งกุลาดำ *Penaeus monodon*

นางสาวปราณิศา สุเทียนทอง

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MUTAGENESIS OF WAP DOMAIN-CONTAINING PROTEINS FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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ปราณิศา สุเทียนทอง: การก่อกลายพันธุ์ของโปรตีนที่มีโดเมน WAP จากกุ้งกุลาดำ *Penaeus monodon* (MUTAGENESIS OF WAP DOMAIN-CONTAINING PROTEINS FROM THE BLACK TIGER SHRIMP *Penaeusmonodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.วิเชียรริมพณิชยกิจ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.เปรมฤทัย สุพรรณกูล, 128 หน้า.

โปรตีนหลายชนิดที่ประกอบด้วย whey acidic protein (WAP) domain ซึ่งมีหน้าที่ทางชีวภาพที่หลากหลายรวมถึงสมบัติในการยับยั้งการเจริญของเชื้อจุลชีพ และการยับยั้งการทำงานของเอนไซม์โปรตีเนส crustin*Pm*1 SWD*Pm*2 และ*Pm*DWD ประกอบด้วย WAP domain เช่นกัน แต่มีสมบัติที่แตกต่างกัน ในการยับยั้งการเจริญของเชื้อจุลชีพ และการยับยั้งการทำงานของเอนไซม์โปรตีเนส ในการศึกษานี้ ได้ทำการกลายพันธุ์ crustin*Pm*1 ที่กรดอะมิโนตำแหน่ง P_1และ P_1' และตัดบริเวณไกลซีนจำนวนมาก และซีสเทอีนจำนวนมากด้านปลายอะมิโนของ crustin*Pm*1 ออก โดยโพรลีนที่ตำแหน่ง P_1 ถูกแทนที่ด้วยลิวซีน และโพรลีนที่ตำแหน่ง P_1' ถูกแทนที่ด้วย ลิวซีน ฮิสทีดีน และเมไทโอนีน โดย crustin*Pm*1 ที่ถูกกลายพันธุ์ที่กรดอะมิโนตำแหน่ง P_1และ P_1' ไม่มีแอคทิวิตีในการยับยั้งเอนไซม์ subtilisin trypsin chymotrypsin และelastaseขณะที่โปรตีนรีคอมบิแนนท์ crustin*Pm*1_WAP มีแอคติวิตีดีที่สุดในการยับยั้งการทำงานของเอนไซม์ subtilisin รองลงมาคือ โปรตีนรีคอมบิแนนท์ crustin*Pm*1_delC และ crustin*Pm*1_WAP_LM ตามลำดับ โปรตีนรีคอมบิแนนท์ SWD*Pm*2_E30R และ SWD*Pm*2_E30P มีสมบัติเป็นตัวยับยั้งการทำงานของเอนไซม์ subtilisinเช่นเดียวกับโปรตีนรีคอมบิแนนท์ SWD*Pm*2 wild type โปรตีนรีคอมบิแนนท์ crustin*Pm*1_WAP ไม่สามารถยับยั้งการเจริญของเชื้อ *Staphylococcus aureus* ได้ขณะที่โปรตีนรีคอมบิแนนท์ crustin*Pm*1_delG และ crustin*Pm*1_delC สามารถยับยั้งการเจริญของเชื้อจุลชีพได้ แต่มีฤทธิ์ต่ำกว่าเมื่อเทียบกับ crustin*Pm*1 wild type จากผลการทดลองชี้ว่า บริเวณไกลซีนจำนวนมากและซีสเทอีนจำนวนมากด้านปลายอะมิโนของ crustin*Pm*1 มีความสำคัญต่อสมบัติในการยับยั้งการเจริญของเชื้อจุลชีพ และน่าจะเป็นสาเหตุหนึ่งที่ทำให้ crustin*Pm*1 ไม่มีสมบัติในการยับยั้งการทำงานของเอนไซม์โปรตีเนส โปรตีนรีคอมบิแนนท์ *Pm*DWD ไม่สามารถยับยั้งการทำงานของเอนไซม์ซีรีนโปรตีเนสทั้ง 4 ชนิดที่นำมาทดสอบได้ ขณะที่โปรตีนรีคอมบิแนนท์ *Pm*DWD_F70R มีฤทธิ์ในการยับยั้งเอนไซม์ subtilisin ยีน *Pm*DWD มีการแสดงออกเพิ่มมากขึ้น ในอวัยวะ lymphoidของกุ้งที่ถูกตัดขา และกุ้งที่ติดเชื้อตัวแดงดวงขาว โปรตีนรีคอมบิแนนท์ *Pm*DWD สามารถที่จะยับยั้งการทำงานของเอนไซม์โปรตีเนสจากอวัยวะ lymphoid ของกุ้งที่ถูกตัดขา และกุ้งที่ติดเชื้อตัวแดงดวงขาวเช่นเดียวกัน นอกจากนี้ โปรตีนรีคอมบิแนนท์ *Pm*DWD ยังสามารถยับยั้งการทำงานของเอนไซม์โปรตีเนสจากเชื้อ *Bacillus subtilis*

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....ปราณิศา สุเทียนทอง.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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PRANISA SUTHIANTHONG: MUTAGENESIS OF WAP DOMAIN-CONTAINING PROTEINS FROM THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: ASSOC. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., CO-ADVISOR: PREMRUETHAI SUPUNGUL, Ph.D., 128 p.

The whey acidic protein (WAP) domain in various proteins has diverse biological functions including antimicrobial and antiproteinase activities. The crustin*Pm*1, SWD*Pm*2 and *Pm*DWDall contain WAP domain but have differentactivities both antimicrobial and antiproteinase activities. In this study, the P_1or P_1' or both and the N-terminal glycine-rich and cysteine-rich regions of crustin*Pm*1 were mutated and deleted. Substitution of P_1 from Pro to Leu and $P_1'$from Pro to either Leu, His or Metdid not make the protein inhibitory against subtilisin, trypsin, chymotrypsin and elastase while the rcrustin*Pm*1_WAP exhibited the strongest subtilisin inhibition followed by rcrustin*Pm*1_delC and rcrustin*Pm*1_WAP_LM. The rSWD*Pm*2_E30R and rSWD*Pm*2_E30P were as active the subtilisin inhibitor as the wildtype. The rcrustin*Pm*1_WAP did not exhibit antimicrobial activity against *Staphylococcus aureus* while the rcrustin*Pm*1_delG and rcrustin*Pm*1_delC exhibited lower antimicrobial activity than the rcrustin*Pm*1indicating that the glycine-rich and cysteine-rich regions were important for antimicrobial activity and probablyone of the reasons that crustin*Pm*1 is proteinase inhibitory inactive. The r*Pm*DWD was not active against all four serine proteinases while its mutant r*Pm*DWD_F70R was active against the subtilisin.The *Pm*DWD was up-regulated in lymphoid tissue upon leg amputation and WSSV infection. The r*Pm*DWD could inhibit the proteinase activities in lymphoid lysate from leg amputated and WSSV infected shrimp as well. In addition, it inhibited the crude proteinases from *Bacillus subtilis*.

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LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EtBr	ethidium bromide
h	hour
kb	kilobase
M	molar
mg	milligram
ml	millilitre
mM	millimolar
MT	metric ton
ng	nanogram
nm	nanometre
O.D.	optical density
^{o}C	degree Celcius
ORF	open reading frame
PCR	polymerase chain reaction

RNA	ribonucleic acid
RT	reverse transcription
sec	second
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

Shrimp are one of the most economically important aquatic species due to their high price and world-wide demand. By the development of aquaculture and production technologies, they become an important export product for many countries along the Indo-Pacific coast. In Thailand, the shrimp species mainly produced is the Pacific white shrimp, *Litopenaeus vannamei*, though it was once the black tiger shrimp, *Penaeus monodon*.

The shrimp farming in Thailand started in early 1980s and really began to expand in the mid 1980s. Since then, Thailand has been the world leader for exporting shrimp produces in the forms of frozen and value-added products to several countries, e.g. Japan, USA and the European Union. The industry has been worth approximately 300,000-400,000 metric tons annually providing an income of nearly 85,000 million baht yearly for the country (Source: Office of Agricultural Economics in cooperation with the Customs Department).

Till these days, the once successful farming of the black tiger shrimp *Penaeus monodon* has been seriously affected by many factors, for example, the outbreaks of bacterial and viral diseases, the water quality problem and the very rare high-quality broodstocks. Consequently, the shrimp species for farming has been switched to another species, namely the Pacific white shrimp, *Litopenaeus vannamei*. This is because the white shrimp species is a genetically improved strain that possesses several great advantages over the *P. monodon*. The white shrimp has rapid growth

rate, high stocking density tolerance, low salinity and temperature tolerance, lower protein requirements (and, therefore, production costs), certain disease resistance (if specific pathogen resistance stocks are used), and high survival rate during larval rearing (50-60% comparing to 20–30% for *P. monodon*). However, the *L. vannamei* may have certain disadvantages for it is an alien species for Thailand. It possibly acts as a carrier of various pathogens new to the culture areas in Thailand. Its broodstocks have to be imported mainly from the strain stocking institute at the Hawaii Marine Institute. From these reasons, the shrimp farming of native rather than the alien shrimp species should be considered as essential for Thailand.

Although the overall biological systems of *P. monodon* are progressively studied at the molecular level, the immune system should be intensively studied considering its importance in fighting the shrimp diseases. Most of the studies are to characterize the factors involved in shrimp response to the pathogenic infection. The knowledge is not only applied for the protection of shrimp from the diseases but also for the selective breeding for healthy shrimp for the aquaculture industry in Thailand.

1.1 Shrimp diseases

The loss of productivity of shrimp cultured industry is due mainly to the major disease outbreaks from both viruses and bacteria. Because of the artificial conditions in the shrimp hatcheries and farms where water quality, microbiological flora and nutrition are vastly different from those in the natural habitat, the shrimp face physiological disturbances and immunodeficiency that increase their sensitivity to the pathogens. The main causes of infectious disease are white spot syndrome virus (WSSV), yellow head virus (YHV) and luminescent bacteria, *Vibrio* species (Jiravanichpaisal et al., 1994; Chou et al., 1995; Flegel et al., 1997).

In Thailand, there are five viral pathogens considered to be the main hazards to the cultivated shrimp *Penaeus monodon*. These are white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV).

1.1.1 Viral diseases

The most severe problem of marine penaeid shrimp farming industry is mostly caused by virus infection. The first shrimp virus, *Baculovirus penaei* (BP), was isolated from wild penaeid shrimp in the early 1970s. In *P. monodon*, the major virus species that have been reported are WSSV and YHV. They cause white spot syndrome disease (WSS) and yellow head disease (YH), respectively (Boonyatatpalin et al., 1993; Wongteerasupaya et al., 1995).

1.1.1.1 White spot syndrome virus (WSSV)

White spot syndrome (WSS) is a viral disease affecting most of the commercially cultured marine shrimp species, not just in Asia but globally (Chou et al., 1995; Wongteerasupaya et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Spán et al., 1997). It can infect both freshwater and marine species, for instance shrimp, crab and crayfish (Chou et al., 1995; Wang et al., 1998). Several commercial penaeid shrimps that the virus is able to infect have been observed, including *P. monodon*, *Marsupenaeus japonicus*, *P. chinensis*, *P. indicus*, *P. merguiensis*, *P. vannemei*, *P. stylirostris*, *P. penicillatus* and *P. setiferus*. The disease can be spread via contaminated water, decomposing fecal matters or tissues, cannibalism and fluid from infected females. Shrimp may be indirectly infected by exposing to previous

hatchery or pond growing cycles, contaminated water supplies, contaminated food, equipment surfaces and clothing, or ingesting the diseased shrimp.

WSSV can cause up to 100% mortality of shrimp within 3 to 10 days (Karunasasagas, 1997). The clinical signs of WSSV-infected shrimp were indicated by the red or pink body surface and appendages, loose shell and white calcium deposits embedded in the shell, white spots 0.5-2.0 mm in diameter on the exoskeleton and epidermis for which the disease is named, reduction in food consumption and slow movement. Major targets of the virions are ectodermal and mesodermal origins such as the gills, lymphoid organ, cuticular epithelium. WSSV can be detected in early larvae stages of *P. monodon* but significant mortality was observed in post-larvae and juveniles shrimp (Yoganandhan et al., 2003).

Several diagnostic methods that can be used to detect the WSSV infection are PCR (Lu et al., 1996), in situ hybridization (Wang et al., 1998), observation of tissues subjected to fixation or negative staining (Inouye, 1994), and immunological methods using monoclonal and polyclonal antibodies to WSSV or their protein components (Okumura et al., 2005).

1.1.1.2 Yellow head virus (YHV)

YH disease was known to cause mass mortality in shrimp farming operations throughout South East Asian countries. In Thailand, the disease was first reported in 1990 and called Hua leung (Chantanachookin et al., 1993; Lightner, 1996). This syndrome occurs in the juvenile to sub-adult stages of shrimp, 5 to 15 grams in size, especially at 50-70 days of grow-out (Lightner, 1996). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley et al., 1999). It has been

classified into a new genus *Okavirus*, family *Roniviridae* and order *Nidovirales* (Mayo, 2002).

The typical signs of YH disease include light yellow coloration of the dorsal cephalothorax area and a pale or bleached appearance (Limsuwan, 1991) Infected shrimp frequently have whitish or pale yellowish to brown gills and often a pale yellow hepatopancrease (Chantanachookin et al., 1993; Lightner, 1996). Moribund shrimp with YHV generally appear pallid in color, with a yellowish and often swollen cephalothorax, and die within a few hours. YHV may occur as latent, asymptomatic infections in broodstock shrimp and may possibly transfer from these shrimp to their offspring in larval rearing facilities (Chantanachookin et al., 1993). The mortality rate may reach as high as 100% of affected populations within 3-5 days from the onset of disease.

1.1.2 Bacterial diseases

Species of *Vibrio* are commonly found in aquatic environments as the bacterial flora and formerly considered to be mostly opportunistic pathogens (Lightner, 1998). However, the *Vibrio* that causes bacterial diseases in the larval and postlarval stages of *P. monodon* behaves more like true pathogen than opportunist (Lightner et al., 1992). The virulence of this species has been recognized in Asia and Australia (Vandenberghe et al., 1998). The disease is called vibriosis which is a major disease problem in shrimp aquaculture. It causes high mortality and severe economic loss in all shrimp producing countries (Crosa et al., 1980; Brock et al., 1992; Mohney et al., 1994.) The luminous *V. harveyi*, has been implicated as the main bacterial pathogen of shrimp (Baticados, 1990). This bacterial outbreak causes up to 100% mortality of the affected shrimps whether they are larvae, post-larvae, juveniles, sub-

adults or adults (Lightner, 1983). In Thailand, vibriosis is the main cause of production loss in penaeid shrimp farms (Nash et al., 1992).

The *V. harveyi* is a Gram-negative bacterium. It has a rod shape, 0.5-0.8 μm in width and 1.4-2.6 μm in length. It is able to emit light of a blue-green color. The diseased shrimp have milking white body and appendages, weakness, disoriented swimming and lethargy. These symptoms eventually lead to death. Presumptive diagnosis is made on the basis of clinical signs and culture of the suspensions of hepatopancreas or blood on trytic plate with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* show strong luminescence in dim light.

Control of luminous *Vibrio* by supplementation of antibiotics has become less and less effective and an occurrence of bacterial resistance to a number of antibiotics is increased. In addition, use of excessive antibiotics has also been implicated in shrimp growth retardation, abnormal morphogenesis and rejection of the exported shrimp due to the antibiotics residuals. The development and use of a probiotics, a marine bacterial strain *Pseudomonas* I-2 and *Bacillus subtilis* BT23, can alleviate the infection for it can produced a compound with inhibitory property against shrimp pathogen (Chythanya et al., 2002; Vaseeharan and Ramasamy, 2003).

1.2 The crustacean immune system

The major defense system of crustaceans is the innate immune response which is based on humoral and cellular components of the circulatory system. The recognition of pathogens depends on a limited number of germ-line encoded receptors, which recognize conserved pathogen-associated molecular patterns (PAMPs) found in microorganisms such as bacterial lipopolysaccharide (LPS), peptidoglycan and β-1,3-glucan (Fig. 1.1.) (Janeway et al., 1998). During pathogen

invasion, the recognition molecules may interact with and activate the hemocytes. Hemocytes are the effectors of cellular immune response and involved in the synthesis of the majority of humoral effectors (Fig. 1.1.). The direct participation of blood cells in cellular immune response is demonstrated in phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting.

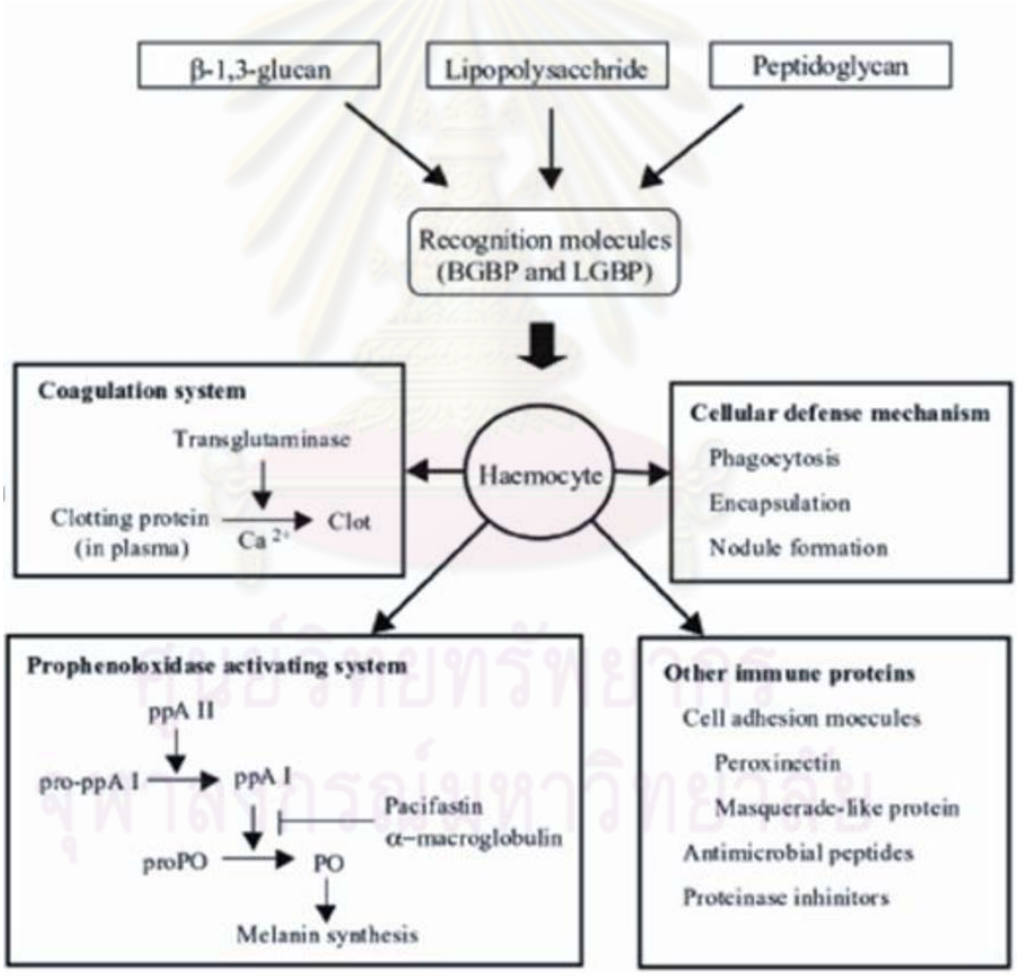


Fig. 1.1. Schematic overview of crayfish defense reactions.

Hemocytes have been classified into three types by their differences in morphology. First, hyaline cells lack the cytoplasmic granules and are involved in phagocytosis (Söderhäll et al., 1986). Second, semigranular cells are the most abundant type of hemocyte and contain a variable number (1-40) of small granules of 0.4 μm diameter. These hemocytes function in phagocytosis and encapsulation (Persson et al., 1987). Third, granular cells contain a large number of secretory large granules of 0.8 μm in diameter.

On the other hand, the humoral factors comprise molecules that act in the defense without direct involvement of cells, although 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.

1.2.1 Cell-mediated defense reactions

Cellular defense actions include phagocytosis, encapsulation and nodule formation (Millar, 1994). Phagocytosis is a common cellular phenomenon in all organisms. It includes the attachment to foreign body, 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 freshwater crab, *Parachaeraps bicarinatus*, and the shore crab, *Carcinus maenas*, phagocytes are the main type of cells that participate in the elimination of foreign circulating particles in the hemocoel.

Encapsulation is a process where layers of hemocytes surround the foreign material. It occurs when a foreign object, such as parasite, is too large to be ingested by phagocytosis. Destruction of encapsulated organisms takes effect by the decrease

in oxygen concentration and the action of hydrolases, or by the toxic action of quinones (Söderhäll et al., 1984). Nodule formation, which appears similar to capsule formation, occurs when the number of invading bacteria is high. However, these capsule and nodule structures are always melanized in arthropods.

1.2.2 The prophenoloxidase (proPO) system

The proPO activating system consists of several proteins involved in melanin production, cell adhesion, encapsulation and phagocytosis (Söderhäll and Cerenius, 1998; Sritunyalucksana and Söderhäll, 2000). It is an efficient immune system for non-self recognition and is initiated by the recognition of lipopolysaccharides or peptideoglycans from bacteria and β–1,3-glucans from fungi. This system contains a proteinase cascade consisting of pattern-recognition proteins (PRPs), several zymogenic proteinases and proPO (Söderhäll and Cerenius, 1998) (Fig. 1.2.).

In crayfish, a trypsin-like proteinase, ppA, is present as an inactive form in the hemocyte granules. After degranulation, the pro-enzyme is released together with the proPO and becomes activated into an active form in the presence of microbial elicitors. The active ppA converts proPO into an active form, phenoloxidase (PO) (Aspan et al., 1991; 1995). The PO is a bifunctional copper-containing tyrosinase that catalyses two successive reactions: the hydroxylation of a monophenol to *o*-diphenol (monophenoloxidase activity) and the oxidation of the *o*-diphenol to *o*-quinone (diphenoloxidase activity) (Söderhäll and Cerenius, 1998; Decker and Tuczek, 2000). Production of *o*-quinones by PO is an initial step in the biochemical cascade of melanin biosynthesis.

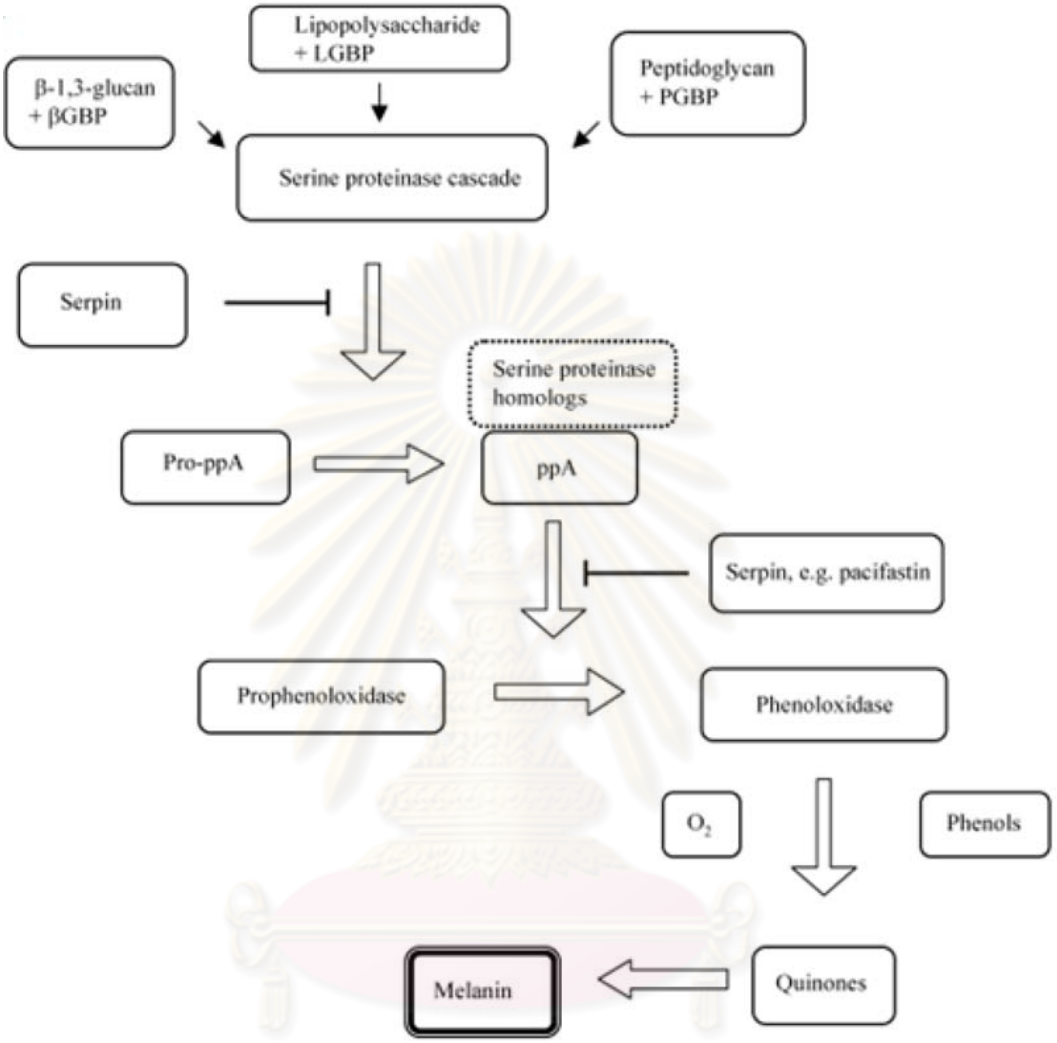


Fig. 1.2. Overview of the arthropod prophenoloxidase (proPO)-activating system.

The melanin pigment can often be seen as dark spots in the cuticle of arthropods. It is involved in the processes of sclerotisation, wound healing and encapsulation of foreign materials (Sugumaran, 1991; Lai-Fook, 1996). In addition, the melanin and intermediates in the melanin formation can inhibit growth of

microbial parasites, such as the crayfish plague fungus, *Aphanomyces astaci* (Söderhäll et al., 1982). Several components and associated factors of the proPO system have been found to play important roles in the defense reaction of the freshwater crayfish (Söderhäll and Cerenius, 1998). In penaeid shrimp, enzymes of the proPO system are localized in the semigranular and granular cells (Perazzolo and Barracco, 1997). This is in agreement with a recent study showing that the *P. monodon* proPO mRNA is expressed only in the hemocytes (Sritunyalucksana et al., 2000). To prevent excessive activation of the proPO cascade, proteinase inhibitors are needed for its regulation.

1.2.3 The coagulation system/the clotting system

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

Two different coagulation mechanisms have been characterized in molecular details in invertebrates, those are the hemocyte-derived clotting cascade in horseshoe crab, *Tachypleus tridentatus* (Kawabata et al., 1996) and the transglutaminase (TGase)-dependent clotting reaction in crayfish, *Pacifastacus leniusculus* (Hall et al., 1999). In crayfish, clotting occurs through polymerization of clotting proteins in plasma. The crayfish CP is a dimeric protein in which the subunit has both free lysine and glutamine side chains. They are recognized and become covalently linked to each other by a calcium ion dependent TGases (Fig. 1.3.) (Yeh et

al., 1998; Hall et al., 1999; Wang et al., 2001). The CP is synthesized in the hepatopancreas and released into the hemolymph.

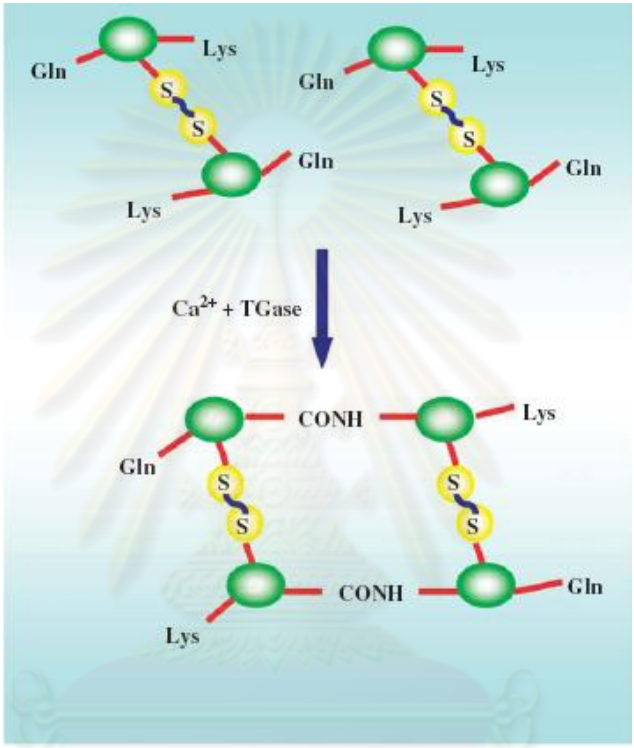


Fig. 1.3. The clotting systems of crayfish and shrimp (Jiravanichpaisal et al., 2006).

1.2.4 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) have been considered to play an important role in innate immune system. They are widely found in multi-cellular organisms. Most of the AMPs are small in size, generally less than 150-200 amino acid residues, with amphipathic structure and cationic property. However, the anionic peptides also exist. Despite their diversity in amino acid sequences, most AMPs adopt an

amphipathic secondary structure that is believed to be essential for their antimicrobial action (Bulet et al., 2004). AMPs display a broad spectrum of activity against bacteria, fungi, viruses and eukaryotic parasites (Hancock et al., 2000; Boman et al., 2003). Moreover, depending on their distribution, the expression of antimicrobial peptide appears to be regulated by different tissue-specific pathways, and these effectors may consequently participate in either local or systemic reactions. Some of them are constitutively expressed in the secretory cells, others are induced upon microbial stimulation (Hancock et al., 2000).

Because of the net positive charge together with the amphipathic structure of AMPs, they preferentially bind to the negatively charged membrane interface of microorganisms. These features give the AMPs the selective toxicity on microbial cells, short bacterial killing time, broad antimicrobial spectra, and no bacterial resistance developed (Matsuzaki, 2001). For many of these peptides, there is evidence that the target for the peptides is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of internal aqueous content of liposomes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis by rupturing the membrane or perturbing the membrane lipid bilayer, and allows the leakage of cellular components as well as dissipating the electrical potential across the membrane (Brogden, 2005).

There are many reports on antimicrobial peptides in crustaceans. In 1997, a small peptide, named calliectin, observed in the hemolymph of blue crab, *Callinectes sapidus* was reported to be responsible for the majority of antimicrobial activity (Khoo et al., 1999). The first shrimp AMP family, penaeidin, which displayed

antifungal and Gram-positive antibacterial properties, was discovered in the Pacific white shrimp *L. vannamei* (Destoumieux et al., 1997). These antifungal and Gram-positive antibacterial peptides were later found in several penaeid shrimp including the Atlantic white shrimp *L. setiferus* (Cuthbertson et al., 2004), the black tiger shrimp *P. monodon* (Supungul et al., 2004) and the Chinese shrimp *F. chinensis* (Kang et al., 2004). In fact, among the AMP family, the penaeidins are considered the most well characterized in terms of the level of gene expression and biological activities. There are four classes of penaeidins (penaeidins 2, 3, 4, and 5) that have been characterized so far (Bachère et al., 2004; Kang et al., 2007).

Antilipopolysaccharide factors (ALFs), first described in the horseshoe crab, *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka et al., 1982), specifically inhibit the lipopolysaccharide (LPS)-mediated activation of *Limulus* coagulation system. ALFs are also identified in shrimp: *L. vannamei* (Gross et al., 2001), *F. chinensis* (Liu et al., 2005) and *P. monodon* (Somboonwiwat et al., 2005). The ALF has a broad spectrum of activity against Gram-positive, Gram-negative bacteria and fungi.

Crustin is an antimicrobial peptide that contains a WAP domain. The first crustin isolated was an 11.5 kDa cationic and hydrophobic protein isolated from the hemocytes of shore crab, *Carcinus maenas* (Relf et al., 1999). This crab protein was first named as carcinin and active against Gram-positive bacteria. Up till now, there may be more than 50 crustins or crustin-like peptides reported from a variety of decapods, including crabs, lobsters, shrimp, and crayfish (Smith et al., 2008).

In penaeid shrimp, crustin homologs have mainly been identified by EST analysis of hemocyte cDNA libraries from *L. vannamei* and *L. setiferus* (Bartlett et al.

2002), *P. monodon* (Supungul et al. 2004; Supungul et al. 2008; Tassanakajon et al. 2008), and the kuruma shrimp, *M. japonicus* (Rattanachai et al. 2004). Moreover, some crustin cDNAs from various shrimp have additionally been obtained from cDNA cloning from *F. chinensis* (Zhang et al., 2007; Liu et al., 2008), *L. schmitti*, the Caribbean brown shrimp, *Farfantepenaeus brasiliensis*, the pink shrimp, *F. paulensis*, and the southern brown shrimp, *F. subtilis* (Rosa et al., 2007). Most crustins exhibit strong antimicrobial activity against Gram-positive but not Gram-negative bacteria.

Other AMPs, for example lyzozymes, C-type lectins, histones, anionic hemocyanins, peritrophins, etc., have been isolated from the penaeid shrimp and characterized.

1.2.5 Proteinase inhibitors

Proteinase inhibitors can fight as part of humoral defense of the innate immune system against the invading pathogens (Christeller, 2005). The serine proteinase inhibitors in the hemolymph may inhibit the proteinases secreted from the pathogens. The proteinase inhibitors are also required for regulation of the proteinase cascades, for example, in coagulation, prophenol oxidase activation, or cytokine activation. Serine proteinase inhibitors from the Kazal, Kunitz, α-macroglobulin, and serpin families have been identifed in arthropod hemolymph and have been characterized biochemically.

Proteinases in many pathogenic fungi function to help the fungi penetrate the cuticle of their arthropod hosts. Proteinases also contribute to the virulence of bacterial pathogens. Proteinase inhibitors in the host hemolymph defend the host against these microbial proteinases. For instance, the silk worm (*Bombyx mori*) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi et al.,

1993). Several *Manduca sexta* serpins inhibit bacterial and fungal serine proteinases (Jiang et al., 1998). Proteinase inhibitors in the cuticle or at the surface of the integument also function in protection against fungal infection. An external secretion of grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski et al., 1996).

The recently reported Kazal-type proteinase inhibitor (KPI) is involved in the reproductive process in the fresh water prawn *Macrobrachium rosenbergii* for it is required to inhibit the sperm gelatinolytic activity (Li et al., 2009). A KPI from *P. monodon* possesses bacteriostatic activity (Donpudsa et al., 2009). The KPI usually contains more than one Kazal domains. Each Kazal domain is able to bind tightly and competitively via its reactive site loop to the active site of cognate proteinase rendering the proteinase inactive. Structural studies reveal that there are several contact positions responsible for the interactions between Kazal domains and the proteinases (Lu et al., 1997; Bode et al., 2000). However, the inhibitory specificity is determined mainly by the P_1 amino acid residue resided at the second amino acid residue after the second cysteine residue of the domain.

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

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

1.3 Crustins

Crustins are cysteine-rich antimicrobial peptide of 7-14 kDa, with an isoelectric point usually in the range of 7.0-8.7. They are present in crustaceans and, hence, the name crustin. Crustins contain one whey acidic protein (WAP) domain at the carboxyl terminus (Smith et al., 2008). The WAP domain is about 50 amino acid residues in length with eight cysteine residues in a conserved arrangement that form a tightly packed structure (4-DSC). The term ‘WAP’ is derived from the name given to a family of proteins, originally discovered in the whey fraction of mammalian milk. Analysis of numerous WAPs from vertebrates reveals a high degree of similarity

between the WAP domain structures (Ranganathan et al., 1999). The PROSITE definition of domain structure has been proposed to be as follows:

$$C_1-(Xn)-C_2-(Xn)-\underline{C_3}-(X5)-\underline{C_4}-(X5)-\underline{C_5}-\underline{C_6}-(X3,X5)-C_7-(X3,X4)-C_8.$$

X indicates any amino acid residue and Xn is a stretch of n residues. The signature motif of the central four cysteines that form the basis of 4-DSC is underlined.

The regions between the signal sequence and WAP domain are variable and can be grouped into at least three main subgroups designated as Types I–III (Fig. 1.4.) (Smith et al., 2008). In Type I crustins, the region that lies between the signal sequence and the WAP domain is of variable length and cysteine-rich but has rarely more than six residues which cannot form a full 4DSC configuration. The region might be thought of as an incomplete 4-DSC. These types of crustins are present mainly in crabs, lobsters and crayfish (the Pleocyemata).

Type II crustins, on the other hand, possess not only a cysteine-rich region but also a long glycine-rich domain of approximately 40–80 amino acid adjacent to the signal region (Fig. 1.4.). Type II crustins are found mainly in shrimp (Dendrobrachiata). The number of glycine residue varies between species but is usually between 20 and 50. In shrimp, it is often arranged as repeat VGGGLG motifs that vary in number from 5 to 8. However, some Type II crustin from *P. monodon* (Amparyup et al., 2008b), though contains some 22 glycine residues, does not show the same VGGGLG repeat.

A third group of WAP domain-containing proteins from decapods resemble crustins but contain the Pro-Arg region instead (Fig. 1.4.). They are called single

WAP domain (SWD) proteins (Jimenez-Vega et al., 2004). Type II and III crustins are mainly present in shrimp.

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

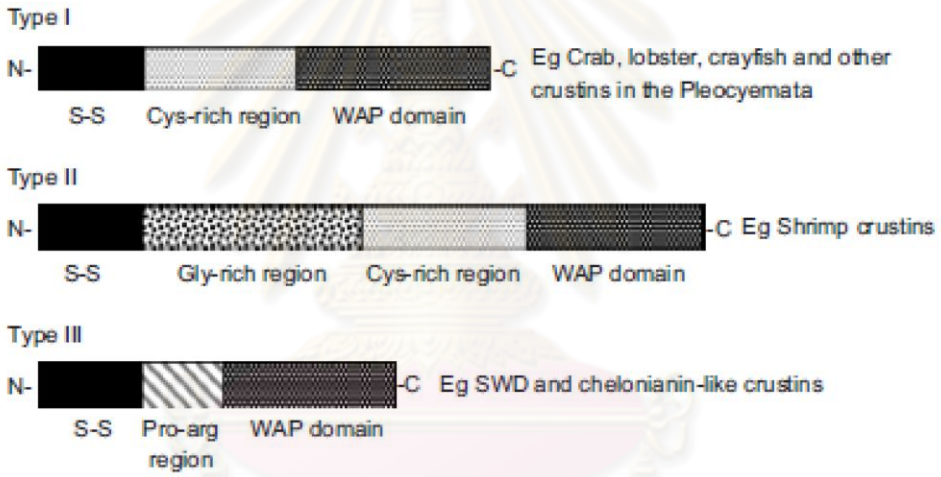


Fig. 1.4. Schematic representation (not to scale) of the domain organization of the three main crustin types from decapods. S-S is signal sequence (Smith et al., 2008).

In the black tiger shrimp, different isoforms of Type II crustins have been identified by homology screening of the EST libraries (Supungul et al., 2004; Tassanakajon et al., 2006). The two major isoforms are identified, crustin*Pm*1 and crustin-like*Pm*. Crustin*Pm*1 and crustin*Pm*5 protein displays antimicrobial activity

against only Gram-positive bacteria whilst crustin-like*Pm* protein inhibits the growth of both Gram-positive and Gram-negative bacteria, including the *V. harveyi*, a shrimp pathogen (Amparyup et al., 2008b; Supungul et al., 2008; Vatanavicharn et al., 2009). In addition to the type II crustins, three isoforms of SWD proteins (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) from the hemocytes cDNA libraries are identified (Amparyup et al., 2008a). The SWD*Pm*2 protein exhibits antibacterial activity against several Gram-positive, but not Gram-negative, bacteria and is a competitive inhibitor of subtilisin A.

1.4 WAP domain-containing proteins

In other multi-cellular animals, the proteins with WAP motif are found widespread. It is well documented that the WAP motif is capable of inhibit certain proteinases and exhibits antimicrobial activity (Vargas-Albores et al., 2004).

The WAP domain is found in 18 human proteins, many of which are poorly characterized. Of the well-characterized WAP domain-containing proteins, secretory leukocyte protease inhibitor (SLPI), protease inhibitor 3 (PI3 or elafin) and Kallmann syndrome 1 (KAL-1) have been extensively studied (Fitch et al., 2006; Moreau et al., 2008; Rugarli et al., 2002). SLPI and elafin are found in multiple cell types, including epithelium and mucosal secretions from a wide range of tissues, including the male reproductive tract (Schalkwijk et al., 1999), as well as in inflammatory cells (Sallenave et al., 1994; Bingle et al., 2006). Their functions are the control of inflammation and antimicrobial activities, both through their antiprotease activity (Sallenave et al., 1994; McNeely et al., 1997; Tomee et al., 1997; Hagiwara et al., 2003; Bingle et al., 2006; Williams et al., 2006; Nishiyama et al., 2008; Iwamori et al., 2009). In the control of inflammation, the primary target is the neutrophil elastase.

SLPI and elafin can be elevated by inflammatory mediators and serve to limit damage of excessive inflammation or can be elevated in response to infection through pattern-recognition receptors and serve as anti-infective agents (Sallenave et al., 1994; Bingle et al., 2001; 2006). Recently, the expression of elafin and SLPI was found to be modulated in response to the onset of cancer (Devoogdt et al., 2004; Bouchard et al., 2006; Nukumi et al., 2007; Liu et al., 2009).

In crustaceans, a number of WAP proteins are isolated and characterized. These are crustins and double WAP domain proteins (DWDs). It was only recently that the DWDs had been identified from penaeid shrimp *Litopenaeus vannamei* (Jiménez-Vega and Vargas-Albores, 2007), *Marsupenaeus japonicas* (Chen et al., 2008) and *Fenneropenaeus chinensis* (Du et al., 2009). They are called double WAP domain proteins or DWD for the whole molecule contains two WAP domains without any other extra motifs unlike those of crustins. The protein primary structure is analogous to the mammalian double WAP-containing protein, the SLPI. The penaeid DWDs are believed to be involved in innate immunity for their expression is altered upon bacterial and viral infections. The up-regulation of DWD synthesis was observed in the hemocytes during an early phase after heat-killed *Vibrio alginolyticus* injection in *L. vannamei* and WSSV infection in *M. japonicas*, and the up-regulation was gradually reduced to more or less normal level afterwards (Jiménez-Vega and Vargas-Albores, 2007; Chen et al., 2008). Similar results were observed with *F. chinensis* infected with *V. alginolyticus* and WSSV (Du et al., 2009). Although the antimicrobial activities have not been experimentally shown, DWDs from *M. japonicas* and *F. chinensis* were shown to inhibit secretory proteinases from bacterial cultures.

1.5 Serine proteinases

Serine proteinase (SP) is a group of endopeptidase that cleaved peptide bond in protein (Neurath, 1985) in which one of the amino acid at the active site is serine (Phillips et al., 1992). Serine proteinases have been studied intensively and their role in a wide array of vital physiological processes, such as food digestion, blood clotting, embryogenesis and immune response (complement activation), has been well documented. Many of these processes are, in essence, proteolytic cascades, which, once 'turned on', lead very rapidly and irreversibly to a specific cellular response. As a consequence, the normal physiology of an organism is likely to be impaired if these proteolytic cascades are not well regulated. Therefore, most organisms synthesize a set of proteinase inhibitors, whose function is to prevent unwanted proteolysis (Simonet et al., 2002).

Serine proteinases are grouped into clans that share structural similarities (homology) and are then further subgrouped into families with similar sequences. The major clans found in humans include the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase clans. The SPs belong to one of the four protease clans.

1.5.1 Chymotrypsin-like clan

The three serine proteinases of the chymotrypsin-like clan that have been studied in great detail are chymotrypsin, trypsin, and elastase. The three enzymes are synthesized by the pancreatic acinar cells, secreted into the small intestine, and are responsible for catalyzing the hydrolysis of peptide bonds. They are similar in structure, as shown through their X-ray structures. In fact, their active serine residue is at the same position (Ser-195). The difference lies in the peptide bond that is being

cleaved or the scissile bond. Each of these digestive serine proteases targets different regions of a polypeptide chain, based upon the side chains of the amino acid residues surrounding the site of cleavage (Kurth et al., 1997; Hung et al., 1998).

Chymotrypsin is responsible for cleaving peptide bonds following a bulky hydrophobic amino acid residue. Preferred amino acid residues include phenylalanine, tryptophan and tyrosine, which fit into a snug hydrophobic pocket. Trypsin is responsible for cleaving peptide bonds following a positively-charged amino acid residue. Instead of having the hydrophobic pocket like the chymotrypsin, there exists an aspartic acid residue at the base of the pocket which can then interact with the positively-charged residues such as arginine and lysine on the substrate. Elastase is responsible for cleaving peptide bonds following a small neutral amino acid residue, such as alanine, glycine, and valine.

1.5.2 Subtilisin-like clan

Subtilisin is a serine protease secreted by the bacterium *Bacillus subtilis*. Subtilisins found in higher eukaryotes fall into two families: the pyrolisins and kexins. Subtilisin-like serine proteases or subtilases constitute a protease superfamily that is prevalent in various organisms such as archaea, protozoa, bacteria, yeast, vertebrates and plants (Hamilton et al., 2003) having diverse roles. Subtilisin-like serine proteases have been associated with many physiological processes such as microsporogenesis (Taylor et al., 1997), hypersensitive response (Taylor et al., 1997), signal transduction (Yano et al., 1999), cell differentiation (Batchelor et al., 2000) and lateral root development (Neuteboom et al., 1999).

Although it has the same mechanism of action as the serine proteases of mammals, its primary structure and tertiary structure are entirely different. Subtilisin

is evolutionary unrelated to the chymotrypsin-like clan, but shares the same catalytic mechanism utilizing a catalytic triad, to create a nucleophilic serine. This is the classic example used to illustrate convergent evolution, since the same mechanism evolved twice independently during evolution.

The structure of subtilisin has been determined by X-ray crystallography. It is a 275 residue globular protein with several alpha-helices and a large beta-sheet. It is a serine endoproteinase with a broad specificity towards native and denatured proteins, and is active under alkaline conditions.

1.6 Objectives of the thesis

In this study, the WAP domain-containing proteins from *P. monodon*: crustin*Pm*1, SWD*Pm*2 and *Pm*DWD, were characterized for their antibacterial and antiproteinase activities. The three proteins contain similar WAP domains but have different activities. By using mutagenesis approach, the amino acids involved in such activities were examined. The reactive P_1 and P_1' residues and adjacent amino acid residues in the WAP domain will be altered to see if any changes in activities are observed.

The involvement of extra motifs, the glycine-rich and the cysteine-rich regions at the N-terminal half, in antibacterial and antiproteinase activities in crustin*Pm*1 will be studied by deletion mutagenesis.

The function of *Pm*DWD will be elucidated through its expression in various tissues of normal, leg-amputated and WSSV-infected shrimp, and it antiproteinase activity

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

Amicon Ultra-4 concentrator (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 safety cabinets (Nuaire)

Biophotometer (Eppendorf)

Centrifuge 5804R (Eppendorf)

Centrifuge Avanti™ J-301 (Beckman Coulter)

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Thermo Electron Corporation)

Gel document (Syngene)

GelMate2000 (Toyobo)

Gene pulser (Bio-RAD)

Hoefer™ miniVE (Amersham Biosciences)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Microcentrifuge tube 0.6 ml and 1.5 ml (Bio-RAD Laboratories)

Microplate reader: FLUOstar OPTIMA (BMG Labtech)

PD-10 column (GE Healthcare)

pH meter model #SA720 (Orion)

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

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

Refrigerated incubator shaker (New Brunswick Scientific)

Trans-Blot® SD (Bio-Rad)

Thermal cycler mastercycler gradient (Eppendorf)

Touch mixer model # 232 (Fisher Scientific)

White/UV transilluminator: UVP ImageStore 7500 (Mitsubishi Electric Corporation)

2.1.2 Chemicals and Reagents

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

1 kb DNA ladder plus (Fermentas)

2-Mercaptoethanol, C_2H_6OS (Fluka)

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Fermentas)

5-Bromo-4-chloro-indolyl phosphate (BCIP) (Fermentas)

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Adenosine-5'-triphosphate potassium salt (ATP) (Sigma)

Agarose (Sekem)

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.)

Ammonium persulfate, $(NH_4)_2S_2O_8$ (USB)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

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 G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

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

Ethidium bromide (Sigma)

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

Formaldehyde (BDH)

GeneRuler™ 100 bp DNA ladder (Fermentas)

Glycerol, $C_3H_8O_3$ (BDH)

Hydrochloric acid, HCl (Merck)

Isopropanol (Merck)

Isopropyl-β-D-thiogalactoside (IPTG) (Sigma)

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

0.22 µm millipore membrane filter (Millipore)

Nitrocellulose membrane (Bio Rad)

Phenol crystal, C_6H_5OH (Carlo Erba)

Potassium chloride, KCl (Ajax)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajax)

Prestained protein molecular weight marker (Fermentus)

RNase A (Sigma)

Sodium acetate, CH_3COONa (Merck)

Sodium bicarbonate, $NaHCO_3$ (Ajax)

di-Sodium carbonate, Na_2CO_3 (Ajax)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma)

Sodium dihydrogen orthophosphate, $NaH_2PO_4 \cdot H_2O$ (Carlo Erba)

di-Sodium hydrogen orthophosphate, Na_2HPO_4 (Carlo Erba)

Sodium hydroxide, NaOH (Eka Nobel)

TRI Reagent (Molecular Research Center)

Tris-(hydroxymethyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

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

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

2.1.3 Kits

Geneaid High-speed Plasmid Mini Kit

NucleoSpin® Extract II Kit (Macherey-Nagel)

QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene)

RevertAid™ First Strand cDNA Synthesis Kit (Fermentas)

2.1.4 Enzymes

*Age*I (Biolabs)

*Ava*I (Biolabs)

*Bgl*II (Biolabs)

*Bsi*WI (Biolabs)

α-chymotrypsin, bovine pancreas (Sigma)

*Eco*RI (Biolabs)

*Eco*RV (Biolabs)

Elastase, porcine pancreas (Pacific Science)

Enterokinase, light chain (Biolabs)

*Mfe*I (Biolabs)

*Nco*I (Biolabs)

Phusion® Hot Start High-Fidelity DNA polymerase (Finnzymes)

*Pml*I (Biolabs)

*Pst*I (Biolabs)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

Subtilisin Carlsberg, *Bacillus licheniformis* (Sigma)

Taq DNA polymerase (Fermentas)

T4 DNA ligase (Fermentas)

T7 DNA polymerase

Trypsin, bovine pancreas (Sigma)

*Xho*I (Biolabs)

2.1.5 Substrates

N-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Sigma)

N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma)

N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma)

2.1.6 Antibiotics

Ampicillin

Chloramphenicol

Kanamycin

Tetracycline

2.1.7 Bacterial and virus strains

Bacillus megaterium

Bacillus subtilis

Enterobacter cloacae

Erwinia carotovora

Escherichia coli 363

Escherichia coli BMH71-18*mut*S

Escherichia coli Rosetta(DE3)pLysS

Escherichia coli XL-1 Blue

Klebsiella pneumonia

Micrococcus luteus

Salmonella thyphimurium

Staphylococcus aureus

Staphylococcus haemolyticus

Streptococcus iniae

Vibrio alginolyticus

Vibrio cholera

Vibrio fluvialis

Vibrio harveyi 639

Vibrio mimicus

Vibrio parahaemolyticus

White spot syndrome virus (WSSV)

2.1.9 Vectors

pET-28a(+)

pET-28b(+)

pUC119

pVR500, a pET-32a(+) derivative

2.2 Softwares

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

ClustalX (Thompson et al., 1997)

GENETYX (Software Development Inc.)

SECentral (Scientific & Education software)

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

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

2.3 Samples

Black tiger shrimp *Penaeus monodon* 16 to 20 g body weight, purchased from shrimp farms in Surat Thani province.

2.4 cDNA sequence analysis

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

2.5 General procedures for genetic engineering purpose

2.5.1 Quantitative method for DNA determination

The concentration of DNA fragment was determined by measuring the A_{260} and estimated in μg/ml using an equation: [DNA] (μg/ml) = A_{260} × dilution factor × 50 for one A_{260} corresponds to 50 μg/ml of DNA (Sambrook et al., 1989).

2.5.2 Primer design

PCR primer pairs were designed based on nucleotide sequences of the template DNA using the SECentral program (Scientific & Educational Software). Each primer in the pair should have about the same T_m values. They were checked for minimal self-priming and primer dimer formation. The elongation factor–1α (EF–1α) gene was used as an internal control.

2.5.3 Preparation of competent cells

The competent cells were prepared using calcium chloride treatment in the early log phase of growth cells. A single colony of *Escherichia coli* strain XL-1-Blue, BMH71-18*mut*S or Rosetta(DE3)plysS were inoculated into 2 ml of LB broth (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) containing 12.5 μg/ml of tetracycline for *E. coli* XL-1-Blue and BMH71-18*mut*S or 34 μg/ml of

chloramphenicol for *E. coli* Rosetta(DE3)pLysS. The culture was grown overnight at 37 ºC with 250 rpm shaking as starter. The starter was diluted 1:100 in 100 ml of LB broth and incubated until the OD_{600} was about 0.2-0.6. Cells were chilled on ice for 10 min and harvested by centrifugation at 5,000 rpm for 5 min at 4 ºC. The cell pellet was washed with 0.5 volumes ice-cold 10 mM $CaCl_2$ and centrifuged at 5,000 rpm for 5 min at 4 ºC. The pellet was resuspended with 0.1 volume ice-cold 0.1 M $CaCl_2$ and placed on ice at least 30 min. The cells were ready for transformation. For storage, the glycerol was added to the cell suspension to the final concentration of 15% glycerol and stored at -80 ºC.

2.5.4 Calcium chloride transformation

Ten microlitres of the mutagenesis reaction, ligation mixture, or one µl plasmid were mixed with 100 µl competent cells and placed on ice for 30 min. The mixture was heat-shocked for 1 min at 42 ºC and then immediately added 0.9 ml LB broth. The cell suspension was incubated for 1 h at 37 ºC. After that, for the transformation of mutagenesis reaction or ligation mixture, the cell suspension was centrifuged at 12,000 rpm for 1 min to collect the bacterial cell pellet that was resuspended in 100 µl LB broth. The cell suspension was spread on the LB agar plates containing antibiotic that was able to select the desired plasmids and incubated at 37 ºC overnight.

2.5.5 Plasmid preparation

The isolation of plasmid from the cultured bacterial cells was done using the Geneaid High-speed Plasmid Mini Kit. The procedure is modified from the alkaline lysis method and the DNA in the chaotropic salt solution bound to the glass fiber matrix of the spin column. Briefly, cells harboring plasmid were cultured in 1.5

ml of LB broth containing 100 μg/ml of ampicillin for a pUC119 plasmid and pVR500 vector, a derivative of pET–32a(+) or 30 μg/ml of kanamycin for a pET-28a(+). Cells were harvested by centrifugation at 12,000 rpm, 4 ºC for 2 min and resuspended in 200 μl PD1 buffer containing RNase A. then, the 200 μl PD2 buffer was added and mix gently by inverting the tube 10 times until the lysate is homologous. To neutralize the cell lysate, PD3 buffer was added and collected the supernatant after centrifugation at 12,000 rpm for 10 min. The supernatant carrying the plasmid was loaded into the PD column and centrifuged for 30-60 s. The PD column was washed twice with 400 μl of W1 buffer and 600 μl of wash buffer, respectively. After that, the PD column was centrifuged to remove residual ethanol from wash buffer. Finally, the dried PD column was transferred to a new 1.5 ml microcentrifuge tube and added 50 μl of elution buffer to the center of the column matrix to elute the plasmid. After incubation for 1 min at room temp, the PD column was centrifuged and collected the flow-through that containing the plasmid.

2.5.6 Agarose gel electrophoresis

DNA was analyzed using 1-1.5% agarose gel that was prepared by melting the agarose gel in 1× TBE (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). After the gel solution was cooled down to 60 ºC, it was poured into a tray with a well-forming comb. The solidified gel was set into the running chamber. DNA samples with 1/10 volumes of the 10× loading dye (0.25% bromophenol blue and 25% Ficoll in water) were loaded into the wells. The agarose gel electrophoresis was performed in 1× TBE buffer at 100 volts for 30-45 min. The gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 1 min and de-stained with distilled water for 15 min. The DNA bands were visualized under the UV transilluminator and

photographed. The size of DNA fragment was determined by comparing the relative mobility with those of standard DNA.

2.5.7 Extraction of DNA fragment from agarose gel

The DNA fragment was extracted from the agarose gel using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL) according to the kit protocol. Briefly, the DNA fragment was carefully excised from the gel to minimize the gel volume, and weighed. The gel slice was completely dissolved in 200 µl of NT buffer for each 100 mg of agarose gel at 50 °C. The sample was loaded into a NucleoSpin extract column placed into a 2 ml collecting tube and centrifuged at 11,000g for 1 min. The flow-through was discarded. NT3 buffer of 600 µl were added into the column and centrifuged to wash the membrane. The membrane was dried by centrifugation at 11,000g for 2 min. Then, the column was transferred to a clean 1.5-ml tube, added 40 µl NE elution buffer (5 mM Tris-Cl, pH 8.5) and incubated at room temperature for 1 min before centrifugation. The eluted DNA was stored at -20 °C until used.

2.5.8 Alkaline-denaturation of double-stranded DNA plasmid for mutagenesis

Two micrograms of plasmid template was treated with 2 M NaOH, 2 mM EDTA in a final volume of 20 µl at room temperature for 5 min. Then, 2 µl of 3 M sodium acetate pH 5.5 and 70 µl of absolute ethanol were added and incubated at -80 °C for 30 min. The DNA precipitate was collected by centrifugation at 13,000 rpm for 15 min. The pellet was washed with 70% ethanol and centrifuged again as describe above. The pellet was dried and dissolved in TE buffer, pH 8.0.

2.5.9 Phosphorylation of oligonucleotide primers

For mutagenesis procedure, the 200 pmol of oligonucleotide primers were kinased in the final volume of 10 µl reaction mixture containing 1× T4 polynucleotide kinase buffer, 1 mM ATP, and 10 U T*4* polynucleotide kinase, and incubated at 37 °C for 1 h. The reaction was stopped by heating at 70 °C for 20 min.

2.6 Pathogen challenges and sample preparation

Three month-old subadult black tiger shrimp, *P. monodon*, of about 20 g were acclimatized in aquaria at an ambient temperature of 30 °C and a salinity of 15 ppt for a few days before use in the experiments. Shrimps were divided into four groups, three shrimp in each group: one group was the unchallenged shrimp (normal). Two groups were injected either with 100 µl normal saline (0.85% NaCl) as a control group or 100 µl diluted solution of white spot syndrome virus (WSSV) in lobster hemolymph medium (LHM) having the LD_{50} of 3 days. The fourth group was the amputated shrimp which were prepared by removing a couple of legs and reared normally for 24 h. Hemolymph and shrimp tissues were collected from an individual shrimp at 24 h after injection or amputation.

2.7 Tissue expression analysis of *Pm*DWD by semi-quantitative RT-PCR

2.7.1 Tissue collection

For tissue-specific expression experiment, the various tissues: antennal gland, epipodite, gill, heart, hepatopancreas, intestine, lymphoid and eyestalk, of normal, 24-h WSSV-infected and leg-amputated shrimp were dissected and snap-frozen in liquid nitrogen. Hemolymph was collected from the ventral sinus of shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 µl of

anticoagulant (10% (w/v) sodium citrate). Hemocytes were collected by centrifugation of the hemolymph at 800g for 10 min at 4 °C. The dissected tissues and hemocyte pellet from individual shrimp were briefly homogenized by a pestle in 1 ml of ice-cold TRI Reagent® (Molecular Research Center) and kept in -70 °C for total RNA preparation.

2.7.2 Total RNA extraction

Total RNA from tissues and hemocytes were extracted using TRI Reagent® according to the manufacturer's instruction. Briefly, tissues or hemocyte samples were added with 1 ml of TRI Reagent®, homogenized and incubated at room temperature for 5 min to completely dissociate the nucleoprotein complexes. Each homogenate was extracted twice with 200 µl chloroform by mixing gently with chloroform for 15 sec each, incubated at room temperature for 3 min, centrifuged at 12,000g for 15 min at 4 °C and transferred the upper aqueous phase containing total RNA to a new tube. The equal volume of isopropanol was added (approximately 500 µl) to precipitate the total RNA. The sample was incubated at room temperature for 10 min and centrifuged at 12,000g for 15 min at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12000g for 15 min at 4 °C. The ethanol solution was discarded and the RNA pellet was air-dried for 5-10 min. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water.

2.7.3 Determination of the quantity and quality of RNA samples

The total RNA concentration was determined by UV spectrophotometer at 260 nm and estimated in µg/µl using the following equation:

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

An absorbance unit at 260 nm corresponds to approximately 40 ng/µl of RNA (Sambrook et al., 1989). The relative purity of the sample could be assessed by determining the ratio of A_{260}/A_{280}. The good quality RNA sample should not have an A_{260}/A_{280} ratio below 1.6.

The quality of extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis. A 1.0% (w/v) formaldehyde agarose gel was prepared in 1× MOPS buffer (0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0). The gel slurry was boiled until completely solubilized and allowed to cool to 60 °C. Formaldehyde (0.66 M final concentration) and ethidium bromide (0.2 µg) were added to the gel and poured into a gel caster. About 10-20 µg of total RNA in 3.5 µl of DECP-treated water, 5 µl of formamide, 1.5 µl of 10× MOPS and 2 µl of formaldehyde were mixed well and incubated at 70 °C for 10 min. The mixture was immediately placed on ice. One-forth volume of the gel-loading buffer [50% (v/v) glycerol, 1 mM EDTA, pH 8.0, 0.5% (w/v) bromophenol blue] was added to each sample. The sample was loaded to the formaldehyde agarose gel. The RNA marker was used. Electrophoresis was carried out in 1× MOPS buffer at 50 V until the bromophenol blue front migrated to approximately ¾ of the gel length. The gel was stained with ethidium bromide and visualized the total RNA as fluorescent bands under a UV transilluminator.

2.7.4 DNase treatment of total RNA samples

The total RNA was further treated with RQ1 RNase-free DNase (Promega) (1 unit/5 µg of total RNA) at 37 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA was purified by phenol/chloroform extraction and ethanol precipitation. Briefly, the reaction was adjusted to 50 µl with DEPC-treated water and added 250 µl of TRI Reagent®. The reaction was vortexed for 10

sec, added 200 µl chloroform and vigorously shaken for 15 sec. The mixture was kept at room temperature for 2-5 min and centrifuged at 12,000g for 15 min at 4 °C. The total RNA in the upper phase was precipitated with 1 volume of isopropanol and washed with 70% ethanol. The RNA pellet was briefly air-dried and dissolved with an appropriate amount of DEPC-treated water. The concentration of DNA-free total RNA was determined.

2.7.5 First-strand cDNA synthesis

The first strand cDNA was synthesized from 1 µg of total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The reaction was performed in a final volume of 12 µl containing 1 µg of total RNA, 0.5 µg of the oligo$(dT)_{18}$ primer and adjusted the volume by DECP-treated water. The reaction was incubated at 65 °C for 5 min and chilled on ice for 5 min to anneal the primer. After that, 4 µl of 5× reaction buffer, 1 µl of RiboLock™ RNase inhibitor (20 U/µl), 2 µl of dNTP mix (10 mM each) and 1 µl (200 U/µl) of RevertAid™ M-MuLV reverse transcriptase were added and gently mixed. The reaction mixture was incubated at 42 °C for 60 min. The reaction was terminated at 70 °C for 15 min.

2.7.6 Semi-quantification of mRNA expression by RT-PCR

Semi-quantitative RT-PCR was used to examine the expression of *Pm*DWD in different tissues. Total RNA from shrimp tissues was extracted and then subjected to cDNA synthesis as described above. The *Pm*DWD gene was amplified using a pair of primers, DWD_F2 and DWD_R2 (Table 2.1). The elongation factor 1-alpha (EF–1α) gene expression was used as an internal control. The PCR conditions were were carried out in a 25µl reaction volume containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP,

0.2 μM of each specific primer, 1.25 units of *Taq* DNA polymerase (Fermentas) and 4 μl of 10-fold diluted template cDNA. The reactions were predenatured at 94 °C for 1 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec. The final extension was at 72 °C for 5 min. The amplification reactions were analyzed through 1.2% agarose gel electrophoresis.

Table 2.1 Primer pairs used for RT-PCR and cloning of *Pm*DWD.

Primer	Sequence 5′–3′	Gene	Product size
DWD-F2 DWD-R2	GCCATGGGCACGAGAGGCCCGCTAAA CCCTCGAGGACTTCCTGACAACTCC	*Pm*DWD	320 bp
EF-F EF-R	GGTGCTGGACAAGCTGAAGGC CGTTCCGGTGATCATGTTCTTGATG	EF-1α	145 bp

2.8 Construction of recombinant pVR500_*Pm*DWD

The *Pm*DWD gene without the upstream sequence coding for the signal peptide was amplified from a *P. monodon* EST clone HC-V-S01-0446-LF (GenBank accession BI784457) by PCR using *Pfu* DNA polymerase (Promega). The primers used were designed to contain restriction sites for the cloning of *Pm*DWD into an expression vector. The DWD-F2 primer and DWD-R2 primer possessed the *Nco*I and *Xho*I sites at their 5′ ends, respectively (Table 2.1).

The 25 μl reaction contained 25 ng of *Pm*DWD plasmid clone, 0.25 mM each dNTP, 0.4 μM of each primer and 5 U of *Pfu* DNA polymerase. The PCR was as follows: 94 °C for 2 min; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min (30 cycles) and 72 °C for 5 min. The PCR product was gel-purified, digested with *Nco*I and *Xho*I and cloned into an expression vector pVR500, a derivative of pET–32a(+) (Donpudsa

et al, 2009). A recombinant plasmid pVR500_*Pm*DWD was capable of over-expression of *Pm*DWD and used as a template in mutagenesis.

2.9 Mutagenesis

2.9.1 Mutagenesis of crustintin*Pm*1

To make the mutagenesis easier, the wild type *crustinPm1* gene (GenBank accession number CD766060) in a recombinant expression plasmid, pET-28a(+)_crustin*Pm*1 as an *Nco*I-*Xho*I fragment of 1,681 bp (Supungul et al., 2008), was sub-cloned into a smaller plasmid pUC119 giving rise to pUC119_crustin*Pm*1. The coding regions of interest in the wild type crustin*Pm*1 gene were, then, mutated by mutagenesis techniques using various oligonucleotide primers. The gene was mutated by site-directedly changed or deleted at the supposedly P_1 and P_1´ using five mutagenic primers: crustin*Pm*1_LL, crustin*Pm*1_LH, crustin*Pm*1_LM, crustin*Pm*1_M and crustin*Pm*1_del (Table 2.2).

The strategy of site-directed mutagenesis procedure is that a mutagenic primer anneals to one strand of the denatured double-stranded plasmid and acts as primer for the synthesis of the complementary strand with T7 DNA polymerase. The gap in the new strand was sealed by T4 DNA ligase. The mutagenesis reaction was performed in a final volume of 20 µl containing annealing buffer (20 mM of Tris-HCl, 10 mM of $MgCl_2$ and 50 mM NaCl), 0.5 µM kinased mutagenic primer and 100 ng denatured pUC119_crustin*Pm*1 as a plasmid template. The reaction mixture was heated at 95 °C for 5 min, placed at 4 °C for 15 min. Then, it was added 10.5 µl of enzyme mixture containing T4 ligase buffer, 5 U T7 DNA polymerase, 5 U T4 DNA ligase, 1 mM dNTPs, and 1 mM ATP. It was incubated at 37 °C for 3 h. The mutagenesis reaction mixture was transformed into an *E. coli* strain BMH71-18*mut*S

defective in mismatch repair. Transformants were cultured in LB medium containing 100 μg/ml ampicillin at 37 °C overnight with shaking for plasmid DNA preparation. The plasmid mixture was digested with *Age*I to linearize the wild-type plasmid and transformed into an *E. coli* strain XL1-Blue.

For the screening of the mutants, the crustin*Pm*1_LH and crustin*Pm*1_LL primers introduced an adjacent *BsiW*I restriction site. Thus, the mutant acquired the *BsiW*I site. For other primers, an *Age*I site was destroyed upon mutagenesis. The desired mutated plasmid was prepared from a transformant and checked for the absence of *Age*I. The mutation sites were confirmed by DNA sequencing (Macrogen). The mutants were subcloned into an expression vector pET-28a(+) at the *Nco*I and *Xho*I sites.

A glycine-rich repeat region of crustin*Pm*1 was deleted using PCR strategy. The primers crustin*Pm*1_delG_F and crustin-R (Table 2.2) were used to amplify crustin*Pm*1 containing only the cysteine domain and WAP domain (crustin*Pm*1_delG). For the deletion of cysteine-rich domain of crustin*Pm*1, the crustin*Pm*1 gene was amplified as two segments, the glycine-rich and the WAP domain using the primers crustin-F, crustin*Pm*1_delC-R, crustin*Pm*1_delC-F, and crustin-R (Table 2.2). Then, the segments were joined by using the newly created *Ava*I site. The mutant was named crustin*Pm*1_delC. The WAP domain of wild type crustin*Pm*1 and crustin*Pm*1_LM were amplified using the primers crustin*Pm*1_WAP and crustin-R (Table 2.2) and cloned. The mutants were named crustin*Pm*1_WAP and crustin*Pm*1WAP_LM, respectively.

The PCR amplification was performed in a final volume of 25 μl containing 25 ng crustin*Pm*1 or crustin*Pm*1_LM clone as a template, 0.4 μM of each

primer, 0.2 mM dNTPs and 5 unit of *Pfu* DNA polymerase (Promega). The PCR reaction was conducted with the initial denaturation step at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, and the final extension step at 72 °C for 5 min. The amplified products were analyzed using 1.2% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The deletion mutants were cloned into the pET-28a(+) expression vector at the *Nco*I and *Xho*I sites. The mutations were confirmed by DNA sequencing.

2.9.2 Mutagenesis of single WAP domain isoform 2 (SWD*Pm*2)

The Glu30 of SWD*Pm*2, the presumed proteinase-inhibitory reactive amino acid, was mutated to Pro or Arg. The site-directed mutagenesis used the QuikChange® Lightning Site-Directed Mutagenesis Kits (Stratagene). The primers SWD*Pm*2_E30R_F and SWD*Pm*2_E30R_R created a *Pst*I restriction site for selection of the mutant. Using the E30R mutant as template for E30P mutation using primers SWD*Pm*2_E30P_F and SWD*Pm*2_E30P_R (Table 2.2), the loss of *Pst*I restriction site was used for the selection.

The mutagenesis reaction was carried out in a 50 µl final volume containing 100 ng of the pET-28b(+)_SWD*Pm*2 plasmid clone as a template, 125 ng of each primer, 1 µl of dNTP mix, 1.5 µl of QuikSolution reagent and 1 µl of QuikChange Lightning Enzyme. The PCR reaction was conducted with the initial denaturation step at 95 °C for 2 min, followed by 30 cycles at 95 °C for 20 sec, 60 °C for 10 sec and 68 °C for 3 min 30 sec and the final extension step at 68 °C for 5 min. Then, the reaction mixture was added 2 µl *Dpn*I which was specific for methylated and hemimethylated DNA. The enzyme digested the parental DNA template. The nicked plasmid containing the desired mutations was transformed into an *E. coli* XL-1

Blue. The mutant plasmids were then screened by digestion with *Pst*I. The mutations were confirmed by DNA sequencing. The mutant plasmids were named pET-28b(+)_SWD*Pm*2_E30R and pET-28b(+)_SWD*Pm*2_E30P.

Table 2.2 Primer sequence using for mutagenesis

Primer	Sequence (5´-3´)	Selection
Wild type sequence	GCAAGAAAACGTACCGGTGGGCAGGTGTCACGG	AgeI
crustin*Pm*1_LL	GCAAGAAAACGTACGAGTAGGCAGGTGTCACGG	BsiWI
crustin*Pm*1_LH	GCAAGAAAACGTACGTGTAGGCAGGTGTCACGG	BsiWI
crustin*Pm*1_LM	GCAAGAAAACGTACCATTAGGCAGGTGTCACGG	Loss of AgeI
crustin*Pm*1_M	GCAAGAAAACGTACCATTGGGCAGGTGTCACGG	Loss of AgeI
crustin*Pm*1_del	CTGCTCTACTGCAAGGCAGGTGTCACGGAC	Loss of AgeI
crustin*Pm*1_delG_F	GGCCATGGGCCATCATCATCATCATCACAGCGTCACAGCCCCACC	-
crustin*Pm*1_R	GCTCGAGTCAGGCAAAAAATTCATAGAAGG	-
crustin*Pm*1_F	GGCCATGGGCCATCATCATCATCATCACCAGAGTTGGCACGGAGG	-
crustin*Pm*1_delC_F	GGCCCGAGGCACCCGTGGGCACC	Use *Ava*I site for manipulation
crustin*Pm*1_delC_R	GGCTCGGGGGAGGCGGGTGGGGC	
crustin*Pm*1_WAP	GGCCATGGGCCATCATCATCATCATCATAAGATACTTGACTGCCCA	-
SWD*Pm*2_E30R_F	CATCATCACCACCTGCAGAGTGACAGAACGC	*Pst*I
SWD*Pm*2_E30R_R	GCGTTCTGTCACTCTGCAGGTGGTGATGATG	
SWD*Pm*2_E30P_F	CATCATCACCACCTGCCCGGTGACAGAACGC	Loss of *Pst*I
SWD*Pm*2_E30P_R	GCGTTCTGTCACCGGGCAGGTGGTGATGATG	
DWD-F2	GCCATGGGCACGAGAGGCCCGCTAAA	
DWD-R2	CCCTCGAGGACTTCCTGACAACTCC	
ArgDWD-F	TACGGCTTGAACTGCAGAGCTTACATTCACCAG	*Pst*I
ArgDWD-R	CTGGTGAATGTAAGCTCTGCAGTTCAAGCCGTA	

2.9.3 Mutagenesis of double WAP domain (*Pm*DWD)

The supposedly P_1 amino acid, Phe70 in the *Pm*DWD was mutated to Arg using the QuikChange® Lightning Site-Directed Mutagenesis procedure. The two complementary mutagenic primers were designed to contain the desired mutation and a new restriction site *Pst*I for the selection. The primers ArgDWD-F and ArgDWD-R

(Table 2.2) were used to mutate the supposedly P_1 reactive amino acid residue Phe70 to Arg and introduce an adjacent *Pst*I restriction site. The mutant was named *Pm*DWD_F70R.

The PCR amplification was performed in a final volume of 50 µl containing, 0.2 mM each dNTP, 0.5 µM of each primer, 1 U Phusion® Hot Start High-Fidelity DNA polymerase (Finnzymes) and 0.1 µg pVR500_PmDWD. The PCR cycles were as follows: 98 °C for 30 sec; 98 °C for 10 sec, 55 °C for 20 sec, 72 °C for 3 min 30 sec (30 cycles) and 72 °C for 8 min. Two microlitres of the PCR product was transformed into an *E. coli* strain XL-1-Blue. The colonies were screened for a mutant plasmid with added *Pst*I site by restriction enzyme digestion. The mutation site was confirmed by DNA sequencing. The mutant plasmid was transformed into an *E. coli* strain Rosetta(DE3)pLysS for over-production of the mutant recombinant protein.

2.10 Preparation of pET-28 and pVR500 expression clones

The expression vectors for curstin*Pm*1, SWD*Pm*2 and *Pm*DWD were pET-28a(+), pET-28 pET-28b(+) (Fig. 2.1) and pVR500 for (Fig. 2.2), respectively. The expression vectors were digested with *Nco*I and *Xho*I. Approximately 2 µg plasmid DNA was digested in a 10 µl reaction containing 1× NE buffer 2, 5 U of *Nco*I (Biolabs) and 10 U of *Xho*I (Biolabs). The reaction was incubated at 37 °C overnight. The digested plasmid DNA was analyzed with 1.0% agarose gel electrophoresis. The linearized plasmids were purified by NucleoSpin® Extract II Kits (MACHEREY-NAGEL) and used for the cloning of crustin*Pm*1 mutants.

pET-28a-c(+) Vectors

TB074 12/98

	Cat. No.
pET-28a DNA	69864-3
pET-28b DNA	69865-3
pET-28c DNA	69866-3

The pET-28a-c(+) vectors carry an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

pET-28a(+) sequence landmarks

T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.

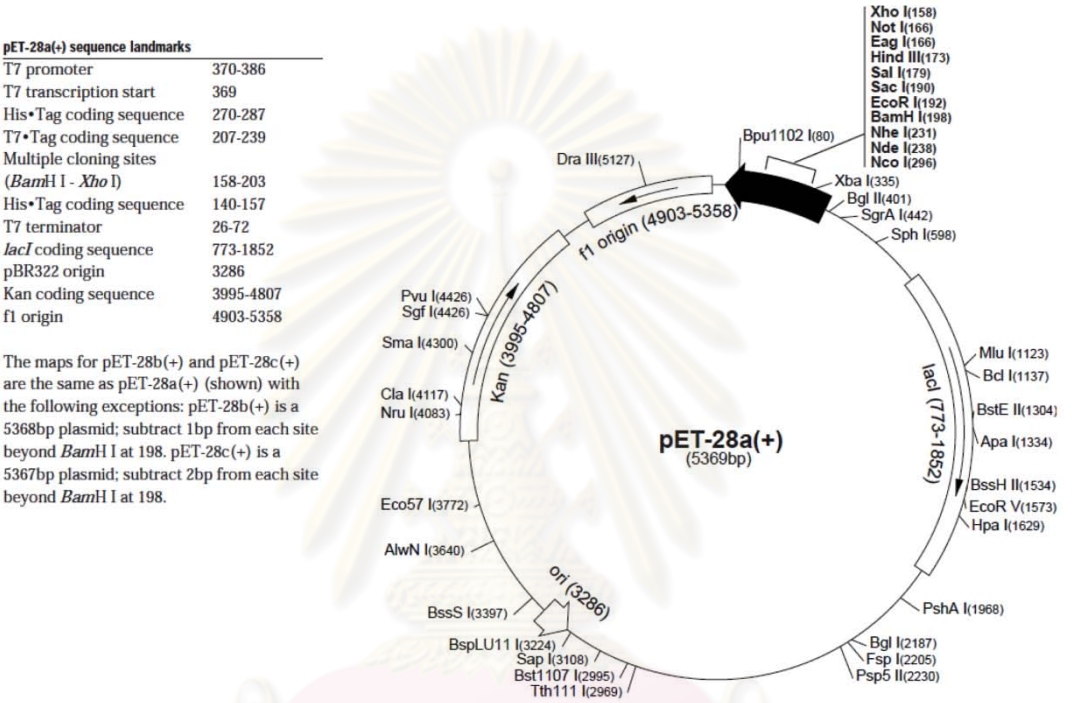


Fig. 2.1. The pET-28a(+) and pET-28b(+) vector map (Novagen®, Germany).

pET-32a-c(+) Vectors

TB122 12/98

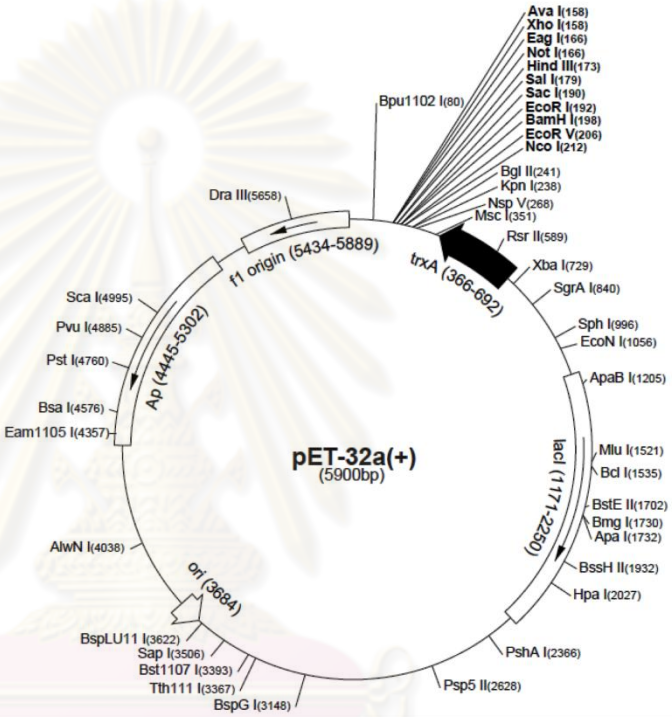
	Cat. No.
pET-32a DNA	69015-3
pET-32b DNA	69016-3
pET-32c DNA	69017-3

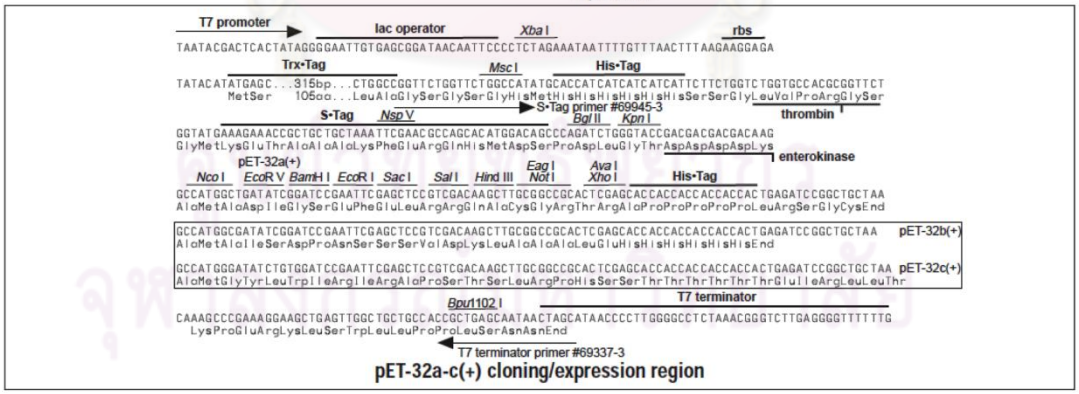
The pET-32 series is designed for cloning and high-level expression of peptide sequences fused with the 109aa Trx•Tag™ thioredoxin protein (1). Cloning sites are available for producing fusion proteins also containing cleavable His•Tag® and S•Tag™ sequences for detection and purification. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

1. LaValle, E.R., DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F. and McCoy, J.M. (1993) *Bio/Technology* **11**, 187–193.

pET-32a(+) sequence landmarks	
T7 promoter	764-780
T7 transcription start	763
Trx•Tag coding sequence	366-692
His•Tag coding sequence	327-344
S•Tag coding sequence	249-293
Multiple cloning sites (*Nco* I - *Xho* I)	158-217
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	1171-2250
pBR322 origin	3684
bla coding sequence	4445-5302
f1 origin	5434-5889

The maps for pET-32b(+) and pET-32c(+) are the same as pET-32a(+) (shown) with the following exceptions: pET-32b(+) is a 5899bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-32c(+) is a 5901bp plasmid; add 1bp to each site beyond *Bam*H I at 198 except for *Eco*R V, which cuts at 209.





pET-32a-c(+) cloning/expression region

Figure 2.2. The pET-32a(+) vector map (Novagen®, Germany). The pVR500 was constructed by deleting the His_Tag and S_Tag between *Msc*I and *Kpn*I sites. The only His_Tag left was at the 3´ side of the reading frame and used for the protein purification. The pET-32a(+) was digested with *Msc*I and *Kpn*I, treated with T4 DNA polymerase to blunt the DNA ends and relegated.

2.11 Construction of recombinant pET-28a_crustin*Pm*1 mutants

To construct the recombinant pET-28a_crustin*Pm*1 mutants for expression, 300 ng of each crustin*Pm*1 mutant: pUC119_crustinPm1_LL, pUC119_ crustin*Pm*1_LH, pUC119_crustin*Pm*1_LM, pUC119_crustin*Pm*1_M or pUC119_ crustin*Pm*1_del, was mixed with 100 ng of pET-28a_crustin*Pm*1 wild type and digested with *Nco*I and *Xho*I in 20-µl reaction containing 1× NE buffer 2, 10 U of *Nco*I (Biolabs) and 20 U of *Xho*I (Biolabs). The reaction was incubated at 37 °C overnight. The enzymes were inactivated at 70 °C for 20 min. Then, 3 µl of the reaction mixture was subjected to ligation in 10-µl reaction containing 1× T4 ligase buffer and 1 U of T4 ligase (Biolabs). The ligation mixture was incubated at 16 °C overnight and the reaction was stopped at 70 °C for 20 min. To eliminate the recombinant pET-28a(+)_crustin*Pm*1 wild type, the ligation reaction was added *Age*I restriction enzyme and incubated at 37 °C for 3 h. The ligation mixture was transformed into an *E. coli* strain XL1-Blue. The cell suspension was spread on the LB agar plate containing 30 µg/ml of kanamycin. The recombinant plasmids were prepared.

The recombinant pET-28a(+)_crustin*Pm*1_LL, pET-28a(+)_crustin*Pm*1_LH and pET-28a(+)_crustin*Pm*1_LM were selected by the present of *Bsi*WI new restriction site; the plasmids were digested with *Bsi*WI and *Eco*RV. The recombinant pET-28a(+)_crustin*Pm*1_M and pET-28a(+)_crustin*Pm*1_del was selected by the absence of *Age*I; the plasmids were digested with *Age*I and *Eco*RV. All five recombinant plasmids were digested with *Nco*I and *Xho*I to confirm the presence of gene insert. The digestions were analyzed by 1.2% agarose gel electrophoresis.

For deletion mutants, the PCR products of crustin*Pm*1_delG, crustin*Pm*1_delC, crustin*Pm*1_WAP and crustin*Pm*1_WAP_LM were digested with *Nco*I and *Xho*I and the DNA fragments were purified by NucleoSpin® Extract II Kit (MACHEREY-NAGEL). Each DNA fragment of 180 ng was subjected to ligation in a reaction volume of 10 µl containing 1× T4 ligase buffer, 1 U of T4 ligase (Biolabs) and 60 ng of *Nco*I-*Xho*I digested pET-28a(+). The reaction was incubated at 16 °C for an overnight. The ligation mixture was transformed into an *E. coli* XL-1 Blue. The recombinant plasmid containing the mutant gene insert was selected and sequenced to ensure the correct DNA insert. The recombinant expression plasmids were named pET-28a(+)_crustin*Pm*1_delG, pET-28a(+)_crustin*Pm*1_delC, pET-28a(+)_ crustin*Pm*1_ WAP and pET-28a(+)_crustin*Pm*1_WAP_LM.

2.12 Recombinant protein expression

The expression plasmid was transformed into an expression host, *Escherichia coli* Rosetta(DE3)pLysS. The starter culture was prepared by inoculating a colony of transformant into a LB medium containing 30 µg/ml of kanamycin for pET-28a(+) and pET-28b(+) derivatives or 100 µg/ml of ampicillin for pVR500 derivatives and 34 µg/ml of chloramphenicol and incubating at 37 ºC overnight with shaking at 250 rpm. The starter was diluted 1:100 into the LB medium with antibiotics and, then, cultured at 37 °C with shaking at 250 rpm to an OD_{600} of about 0.6. The expression of protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was continued for 4 h. At each time point, 1-ml culture was collected. Cells were harvested by centrifugation at 8,000 rpm for 15 min and resuspended in phosphate-buffered saline pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The cell suspensions were frozen at -80

°C and thawed for three times and, then, sonicated for 2 min. Pellet or supernatant were collected by centrifugation at 8000 rpm for 15 min at 4 °C depending on whether the recombinant protein were in the inclusion bodies or soluble.

2.13 Purification of recombinant protein

The frozen cells were thawed and sonicated with a Bransonic 32 (BANDELIN SONOPULS, Germany) three times for 2 min. The cell lysate containing the r*Pm*DWD or the pellet containing the rcrustin*Pm*1 and rSWD*Pm*2 were collected by centrifugation at 8000 rpm for 15 min at 4 °C. The pellet was washed twice with 1% Triton X-100 in 1× PBS pH 7.4 and twice with 1× PBS pH 7.4, respectively. The inclusion bodies were solubilized with alkaline buffer. The recombinant protein was purified using nickel affinity chromatography (GE Healthcare).

For the soluble r*Pm*DWD, the PD-10 column with Ni-NTA agarose was washed with 5 column volumes of distilled water and equilibrated with 5-10 column volumes of binding buffer (20 mM phosphate buffer pH 7.4 containing 20 mM imidazole). The soluble protein fraction was applied to the column and the column was washed with binding buffer to remove the unbound proteins. The column was eluted with an elution buffer (20 mM phosphate buffer pH 7.4 containing 500 mM imidazole). The imidazole was removed by dialysis for at least 10 h at 4 °C against 50 mM phosphate buffer pH 7.4. After purification, the thioredoxin portion was removed from the r*Pm*DWD by enterokinase digestion. The enterokinase reaction was carried out in a final volume of 5 ml containing 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 2 mM $CaCl_2$, 0.01 mg/ml enterokinase (Promega) and approximately 0.6 mg/ml fusion

protein. The reaction was incubated at 23 °C for 16 h. The recombinant protein was analyzed using 15% SDS-PAGE.

For the insoluble rcrustin*Pm*1 and its mutants, the inclusion bodies were solubilized under non-denaturing condition in 50 mM phosphate buffer pH 12. Then, the recombinant proteins were dialysed at 4 °C against 50 mM carbonate buffer pH 10. The proteins were purified using Ni-NTA column. The column was equilibrated and washed with the binding buffer (50 mM carbonate buffer pH 10 containing 20 mM imidazole). The proteins were eluted with 50 mM carbonate buffer pH 10 containing 100 mM imidazole and, then, dialysed against 50 mM carbonate buffer pH 10 at 4 °C.

The purification of rSWD*Pm*2_E30R and rSWD*Pm*2_E30P followed the procedure described by Amparyup et al. (2008a). Briefly, the inclusion bodies were washed twice with 20 mM Tris-HCl pH 8.0 and dissolved in a denaturing buffer (20 mM Tris-HCl pH 8 containing 8 M urea and 20 mM imidazole). The proteins were purified using the Ni-NTA column under denaturing condition and refolded by dialysis against 20 mM sodium phosphate pH 5.8 at 4 °C.

2.14 Protein analysis

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

In a discontinuous system of SDS-PAGE, the gel solutions were prepared as shown in the Appendix B. The glass plates and spacers were assembled. Then, the separation gel solution was pipetted into the gel plate setting and layered on top distilled water to ensure a flat surface of gel. After polymerization, water was poured off. The stacking gel solution was prepared and poured on top of the separating gel. A

comb was placed immediately in position with excess gel solution overflowing the front glass plate. When the stacking gel was polymerized, the comb was removed. The wells were rinsed with distilled water to remove excess un-polymerized acrylamide.

2.14.2 Sample preparation

Protein samples were prepared by resuspending the proteins in 1× sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2.88 mM 0.02% bromophenol blue and 2-mercaptoethanol). The samples were then boiled for 10 min and centrifuged at 12,000 rpm for 1 min. The samples were either held at room temperature or kept at 0 °C until loaded into the gel.

2.14.3 Analysis of the recombinant protein by SDS-PAGE

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

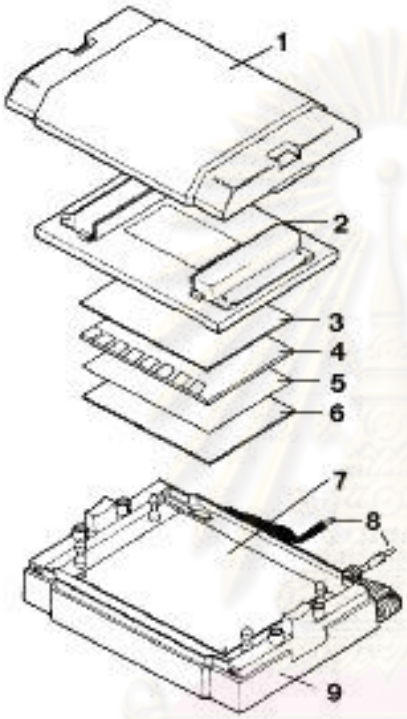
The gels were either stained with 0.1% (w/v) Coomassie brilliant blue R250 or Western blotted. In the Coomassie brilliant blue staining, the gel was immersed in Coomassie blue staining solution (0.1% (w/v) Coomassie brilliant blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol) at room temperature with gentle shaking for 1 h. After that, the gel was dipped into the destaining solution (10% (v/v) acetic acid, 10% (v/v) methanol) and incubated at room temperature with agitation for 1-3 h. Destaining solution was replaced regularly to assist the removal of stain.

2.14.4 Detection of the recombinant protein by Western blot

After the SDS-PAGE, the gel slab was removed from the glass plates. The nitrocellulose membrane, gel and filter paper were soaked in a transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 15 min. The pre-soaked filter paper was placed onto the anode platform. A pipette was rolled over the surface of filter paper to exclude all air bubbles. Then, the nitrocellulose membrane was placed on top of the filter paper and all air bubbles were rolled out. The gel was placed on top and center of the transfer membrane. Finally, another pre-soaked filter paper was placed on top of the gel. Air bubbles were carefully removed. The assembly is shown in Fig. 2.3.

The transfer of protein was performed at constant 90 mA for 1 h from cathode towards anode. After transfer, the nitrocellulose membrane was blocked by dunking in the blocking buffer [1× PBS buffer (10 mM phosphate buffer, 150 mM NaCl) pH 7.4, 0.05% (v/v) Tween™-20 and 5% (w/v) skim milk] at room temperature for an overnight with gentle shaking. The membrane was washed 3 times for 10 min each in washing buffer (1× PBS buffer pH 7.4 containing 0.05% (v/v) Tween™-20) and incubated with anti-His antibody that was diluted 1:3000 in hybridization buffer (1× PBS buffer pH 7.4 containing 0.05% (v/v) Tween™-20 and 1% skim milk) at room temperature with gentle mixing for 3 h. Then, the membrane was washed 3 times for 10 min each in washing buffer and then incubated in a secondary antibody solution, 1:5000 dilutions in hybridization buffer, with agitation for 1 h. The membrane was washed 3 times for 10 min each in washing buffer at room temperature. The protein bands were detected by color development using NBT/BCIP

(Fermentas) as substrate dissolving in 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$.



1. safety lid
2. cathode assembly with latches
3. filter paper
4. gel
5. membrane
6. filter paper
7. spring-loaded anode platform, mounted on four guide posts
8. power cables
9. base

Fig. 2.3. An exploded view of the Trans-Blot SD cell (Bio-Rad).

2.14.5 Protein concentration determination

Protein concentration was determined by a method of Bradford (Bradford, 1976) using bovine serum albumin as standard. The protein solution (maximum 100 µl) was added with the experiment buffer to make a total volume of 100 µl and mixed

with 1 ml Bradford working buffer. The mixture was left for 2 min and then the absorbance at 595 nm was measured. The 500 ml of Bradford working buffer was prepared by mixing 30 ml of Bradford stock solution (350 mg Coomassie blue G250, 100 ml 95% ethanol and 200 ml 85% phosphoric acid), 15 ml 95% ethanol, 30 ml 85% phosphoric acid and 425 ml distilled water.

2.15 Proteinase inhibition assay

2.15.1 Serine proteinase inhibition assay

The proteinase inhibitory activities of recombinant proteins against the proteinases; trypsin (bovine pancreas, Sigma), subtilisin Carlsberg (Bacillus licheniformis, Sigma), elastase (porcine pancreas, Pacific Science), α-chymotrypsin (type II bovine pancreas, Sigma) were assayed in 50 mM Tris-HCl pH 8.0 at 30 °C in a total volume of 100 μl using 147 and 291 mM *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide as a substrate for trypsin and subtilisin, respectively, 886 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide for elastase and 147 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for chymotrypsin. The final concentrations of trypsin, subtilisin and chymotrypsin were 0.04 mM and that of elastase was 0.08 mM. The inhibitor to proteinase ratio of 100:1 was first used to determine the inhibitory potency of the inhibitor against the proteinases.

For the recombinant proteins exhibiting strong proteinase inhibitory activity (r*Pm*DWD_F70R, rSWD*Pm*2_E30R and rSWD*Pm*2_E30P), the ratio of inhibitor to proteinase was varied from 0 to 100 by diluting the inhibitor two-fold. Other recombinant proteins exhibiting lower proteinase inhibitory activity (rcrustin*Pm*1_delG, rcrustin*Pm*1_WAP and rcrustin*Pm*1_WAP_LM), the ratio of inhibitor to proteinase were increased from 0 to 600 and varied by diluting the

inhibitor two-fold. The reaction mixtures were incubated at 30 °C for 15 min and terminated by adding 50 ml of 50% acetic acid. The absorbance of *p*-nitroaniline formed was measured at 405 nm using a microplate reader (BMG Labtech). The percentages of remaining activity were calculated and plotted against the molar ratios of inhibitor to proteinase.

2.15.2 Agar diffusion assay of proteinase inhibition of *Pm*DWD

Secretory proteinases from bacteria were subjected to inhibition test by the *Pm*DWD using the agar diffusion assay similar to that described by Chen et al. (2008). The bacteria, *Bacillus subtilis*, *Enterobacter cloacae*, *Erwinia carotovora*, *Escherichia coli* 363, *Klebsiella pneumoniae*, *Salmonella thyphimurium*, *Vibrio alginolyticus*, *Vibrio harveyi* 639, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio mimicus* and *Vibrio cholerae* were tested for proteinase activities. An overnight culture of bacteria was filtered through a 0.22 mm filter and the filtrate was used as crude preparation. Total protein was determined. The crude preparations were added into the well punched in the skim milk agar plate (0.6% skim milk and 0.75% agar) and incubated at 28 °C overnight. The transparent zone around the well indicated proteinase activity. Those crude preparation exhibited proteinase activities were further tested with the r*Pm*DWD. The appropriate amount of r*Pm*DWD or its mutant *Pm*DWD_F70R were mixed with the crude proteinases and added into the well. Thioredoxin expressed by the same expression system and PBS were used as controls.

Crude proteinases were also prepared from various tissues of normal, 24-h WSSV-infected and amputated shrimp. Various tissues including antennal gland, epipodite, gill, heart, hepatopancreas, intestine, lymphoid, eyestalk and stomach were dissected from the shrimp. Hemocytes were isolated by centrifugation. The hemocyte

pellet and dissected tissues were resuspended in 50 mM Tris–HCl pH 8.0, homogenized and centrifuged at 12,000 rpm for 15 min to collect the supernatant tissue lysate. Hemolymph was used instead of plasma. Total protein was determined. The crude proteinases from various tissue lysates were mixed with the r*Pm*DWD and added into the wells. After incubation at 28 ºC overnight, the transparent zones were observed.

2.16 Antimicrobial activity

2.16.1 Antimicrobial activity of rcrustin*Pm*1 and its mutants by agar diffusion assay

Antimicrobial activities of the rcrustin*Pm*1 mutants were tested against *Staphylococcus aureus* using the agar diffusion assay similar to that described by Takemura et al. (1996). The bacterial density was adjusted to 0.2 at 600 nm with 1× PBS buffer pH 7.4 containing 1% agarose and poured onto the 90-mm plates. Wells with diameter of 0.4 cm were cut into the freshly poured plates after the agar solidified. Then, various two-fold diluted amounts of rcrustin*Pm*1, rcrustin*Pm*1_delG, rcrustin*Pm*1_delC and rcrustin*Pm*1_WAP were added into the well and incubated at 37 °C overnight. As positive and negative controls, 5.0 nmol kanamycin and 50 mM carbonate buffer pH 10 were used, respectively. The diameter of the clear zone surrounding each well was measured. The antibacterial activity was calculated as the difference between the diameter of clear zone from the recombinant proteins and that of the negative control (0.4 cm.) divided by the difference between the diameter of clear zone from the kanamycin and that of negative control.

2.16.2 Antimicrobial activity of r*Pm*DWD and *Pm*DWD_F70R using liquid growth inhibition assay

The antimicrobial activity of *Pm*DWD was tested against the Gram-positive bacteria: *B. subtilis*, *B. megaterium*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Streptococcus iniae*, and Gram-negative bacteria: *E. cloacae*, *E. carotovora*, *E. coli* 363, *V. harveyi* 1526, *K. pneumoniae* and *S. thyphimurium* using liquid growth inhibition assay (Patat et al., 2004). The *Pm*DWD_F70R was tested with *S. aureus*, *B. megaterium* and *S. haemolyticus*. The overnight cultures bacteria were diluted 1:100 with LB and incubated until an OD_{595} was about 0.1 and then diluted further with poor broth (1% bactotryptone, 0.5% NaCl, pH 7.5) to an OD_{595} of 0.001. One-hundred-microliter aliquots of bacteria were mixed with 20 µl of a two-fold serial dilution of *Pm*DWD from 0 to 50 µM in the wells of a 96-well microtiter plate. Aliquots of bacteria and poor broth with PBS were used as positive and negative controls, respectively. The reactions were cultured overnight under vigorous shaking at 30 or 37 °C according to the strains of bacteria and measured the growth of bacteria at 595 nm using a microplate reader (BMG Labtech).

CHAPTER III

RESULTS

3.1 Crustin: crustin*Pm*1 and SWD*Pm*2

3.1.1 Mutagenesis

The amino acid sequence alignment of WAP domains from WAP domain-containing proteins with and without proteinase inhibitory activities (Fig. 3.1.) revealed the differences in the amino acids at P_1 and P_1'. Crustin*Pm*1, crustin type II and SWD*Pm*2, crustin type III also possess different amino acid sequences between the signal peptide and the WAP domain; crustin*Pm*1 contains Gly-rich and Cys-rich regions while SWD*Pm*2 contains Pro-Arg regions (Fig. 3.2.). While the SWD*Pm*2 exhibits proteinase inhibitory activity and antimicrobial activity, the crustin*Pm*1 exhibits only the antimicrobial activity. The differences among WAP domain-containing proteins may cause the differences in their biological activities. To elucidate the importance of these differences, mutagenesis approach was undertaken.

3.1.1.1 Mutagenesis of crustin*Pm*1

The recombinant crustin*Pm*1 gene in pUC119_crustin*Pm*1 was mutated at the codons coding for P_1 and P_1' amino acids using five mutagenic primers. Consequently, the Pro90 was mutated to Leu and Pro91 to Leu, His and Met, respectively. Additionally, the five amino acids from Pro90 to Phe94 were also deleted. The various mutants were generated as shown in (Fig. 3.2.). The mutagenesis was to imitate the P_1 and P_1' amino acids of second domain of human SLPI and the human elafin for they contained Leu-Met and Ala-Met at P_1 and P_1' positions,

respectively (Fig. 3.1). The deletion mutation was to reduce the spacing between the second and third cysteine residues of the WAP domain from 13 to 8 amino acid residues which was the spacing found in the SLPI second WAP domain and elafin. Hopefully, the P_1 and P_1' crustin*Pm*1 mutants would turn out to be proteinase inhibitory activity, especially for chymotrypsin and elastase.

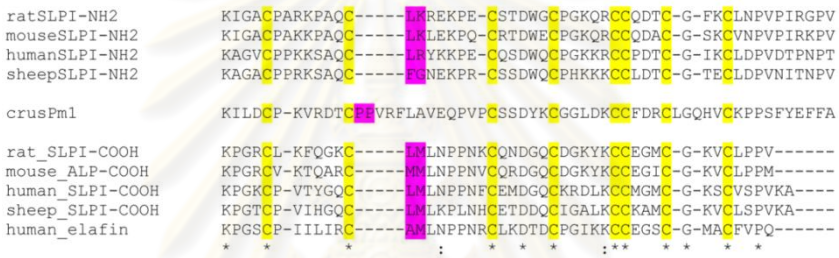


Fig. 3.1. Comparison of WAP domain from crustin*Pm*1 (CD766060) with the N-terminal and C-terminal WAP domains from mouse ALP (GenBank no. NP_035544), rat SLPI (NP_445824), human SLPI (NP_003055) and human elafin (NP_002629). The P_1 and P_1' amino acids of each WAP domains were indicated by shading.

In fact, in site-directed mutagenesis, the procedure generates a mixture of plasmids, mostly mutated and wild type. In this study, the mutation also destroyed the *Age*I site and created the *BsiW*I. Therefore, the proportion of the wild type plasmid was reduced by *Age*I digestion. The enriched mutant plasmids were prepared and screened for the presence of added restriction site, *BsiW*I and the absence of *Age*I restriction site. For example, the crustin*Pm*1_LL and the crustin*Pm*1_LH were digested with *BsiW*I and *EcoR*I and examined by agarose gel

electrophoresis. The digested crustin*Pm*1_LL and the crustin*Pm*1_LH showed two bands at size 3,446 bp and 1,389 bp while the digested wild type plasmid gave linear plasmid (Fig. 3.3.). The mutation sites were confirmed by DNA sequencing (Fig. 3.4.).

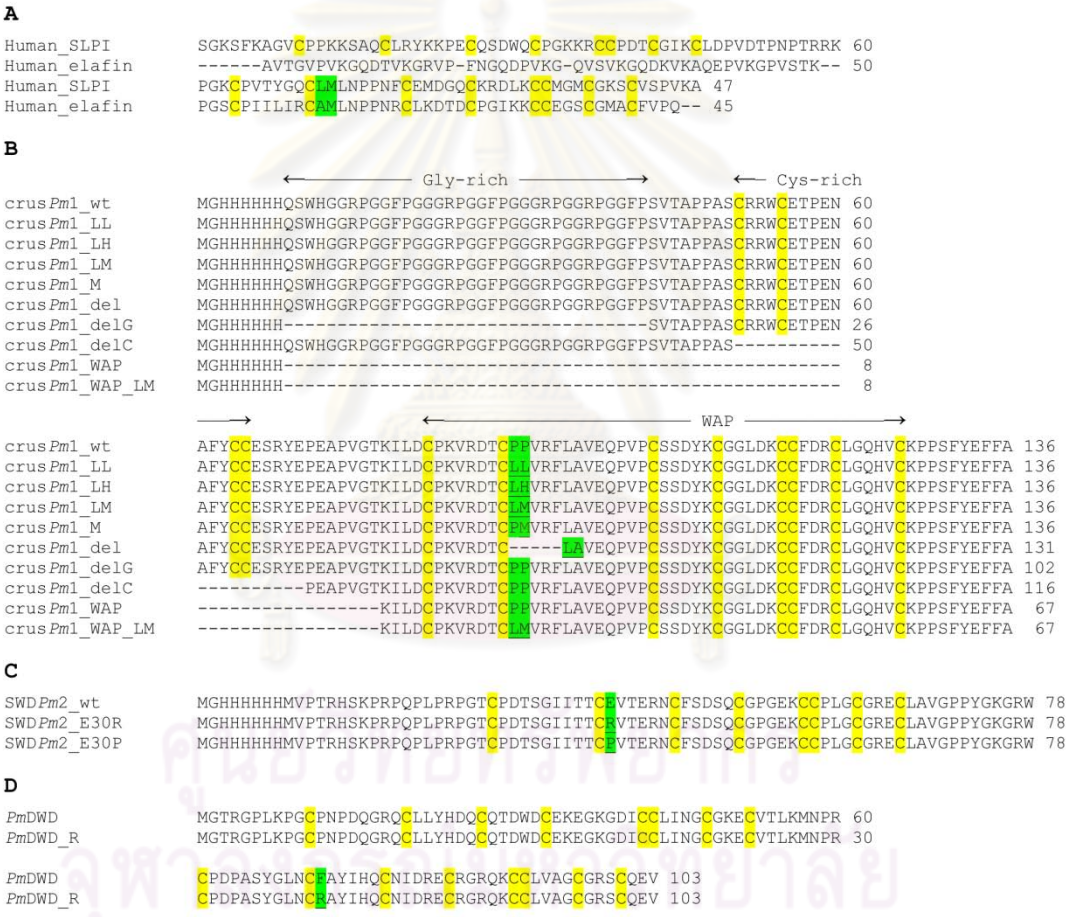


Fig. 3.2. Amino acid sequence comparison of human SLPI (NM_003064) and elafin (NM_002638) (A), crustin*Pm*1 (CD766060) and its mutants (B), SWD*Pm*2 (EU623980) and its mutants (C), and *Pm*DWD (BI784457) and its mutant (D).

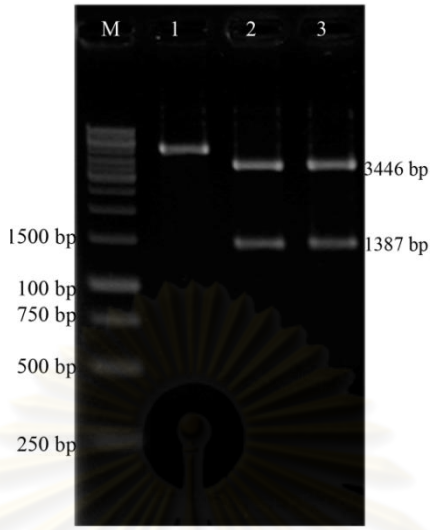


Fig. 3.3. The screening of mutant plasmid by *BsiW*I-*Eco*RI digestion. The digestion are analyzed by 1% agarose gel electrophoresis. Lane M: GeneRuler™ 1 kb DNA ladder (Fermentas); lane 1: pUC119_crustin*Pm*1; lane 2: pUC119_ crustin*Pm*1_LL; lane 3:pUC119_crustin*Pm*1_LH.

For the deletion mutants, the crustin*Pm*1_delG, crustin*Pm*1_delC, crustin*Pm*1_WAP and crustin*Pm*1WAP_LM were amplified from crustin*Pm*1 using specific primers. The crustin*Pm*1_delG contained the cysteine-rich and WAP domains. The crustin*Pm*1_delC was composed of the glycine-rich and WAP domains. The crustin*Pm*1_WAP was the WAP domain only of crustin*Pm*1. The crustin*Pm*1 WAP_LM was derived from the WAP domain only of crustin*Pm*1_LM. The PCR amplified products were analyzed using 1.2% agarose gel electrophoresis (Fig. 3.5.). The deletion mutations were confirmed by DNA sequencing.

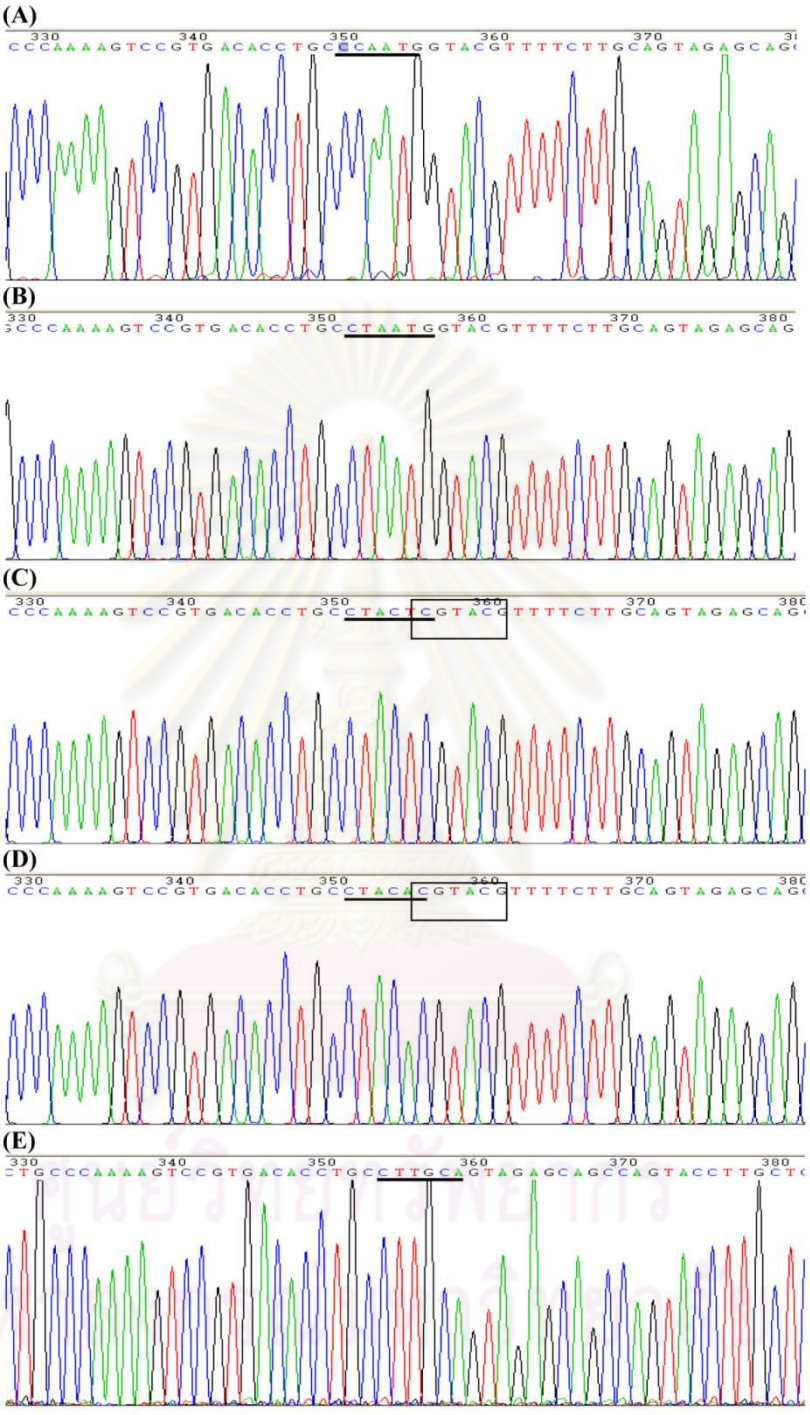


Fig. 3.4. Nucleotide sequencing of crustin*Pm*1 mutants: crustin*Pm*1_M (A); crustin*Pm*1_LM (B); crustin*Pm*1_LL (C): crustin*Pm*1_LH (D) and crustin*Pm*1_del (E). The introduced *BsiW*I site is boxed and the mutant regions are underlined.

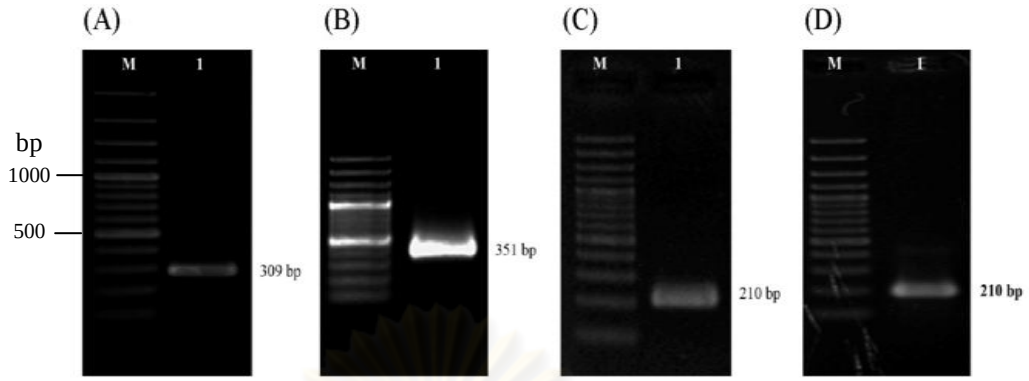


Fig. 3.5. The PCR amplified fragments of crustin*Pm*1_delG (A); crustin*Pm*1_delC (B); crustin*Pm*1_WAP (C) and crustin*Pm*1_WAP_LM (D).

3.1.1.2 Mutagenesis of single WAP domain isoform 2 (SWD*Pm*2)

Since the P_1 of crustin*Pm*1 was Pro and did not has proteinase inhibitory activity, it was suspected that the P_1' Pro might be deleterious to the activity. To test whether the P_1 proline residue had any effect on the proteinase activity, the P_1 residue of SWD*Pm2* gene in pET-28b(+)_SWD*Pm*2 was mutated to Pro and Arg. Changing to Arg was to test the importance of P_1 residue in determining the inhibitory specificity. Hopefully, the SWD*Pm*2_E30R changed its inhibitory specificity from subtilisin to also trypsin and the SWD*Pm*2_E30P turned into inactive inhibitor like crustin*Pm*1.

The P_1 Glu30 was then mutated to Arg using QuikChange® Lightning Site-Directed Mutagenesis Kits. The site-directly change of Glu30 to Arg (E30R) introduced the *Pst*I restriction site for the screening of mutant. To substitute Glu30 with Pro, the SWD*Pm*2_E30P was generated by mutation using the SWD*Pm*2_E30R as a template. The mutation destroyed the *Pst*I site and the absence

of *Pst*I site was used for the selection. The mutants were selected by examining the presence and absence of *Pst*I using 1.2% agarose gel electrophoresis (Fig. 3.6.). The presence of the mutant gene was confirmed by digestion with *Nco*I and *Xho*I (Fig. 3.6.) and DNA sequencing (Fig. 3.7.). The pET-28b(+)_ SWD*Pm*2_E30R and pET-28b(+)_SWD*Pm*2_E30P were able to express the proteins.

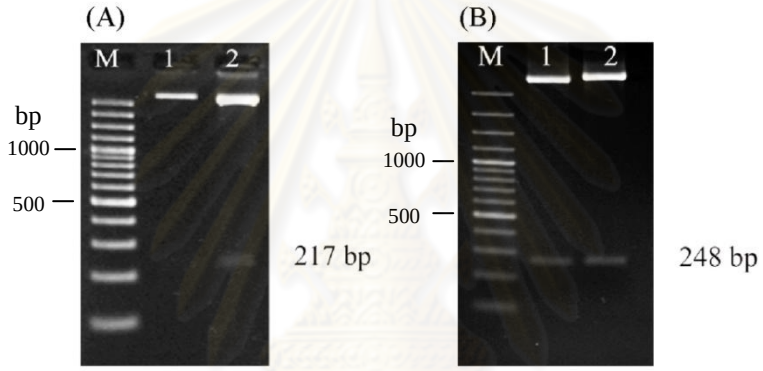


Fig. 3.6. The screening of mutant plasmids by digesting with *Pst*I and *Bgl*II (A) and the confirmation of the mutant gene inserts by digesting with *Nco*I and *Xho*I (B) using 1.2% agarose gel electrophoresis. Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas); lane1: pET-28b(+)_SWD*Pm*2_E30P; and lane2: pET-28b(+)_SWD*Pm*2_E30R.

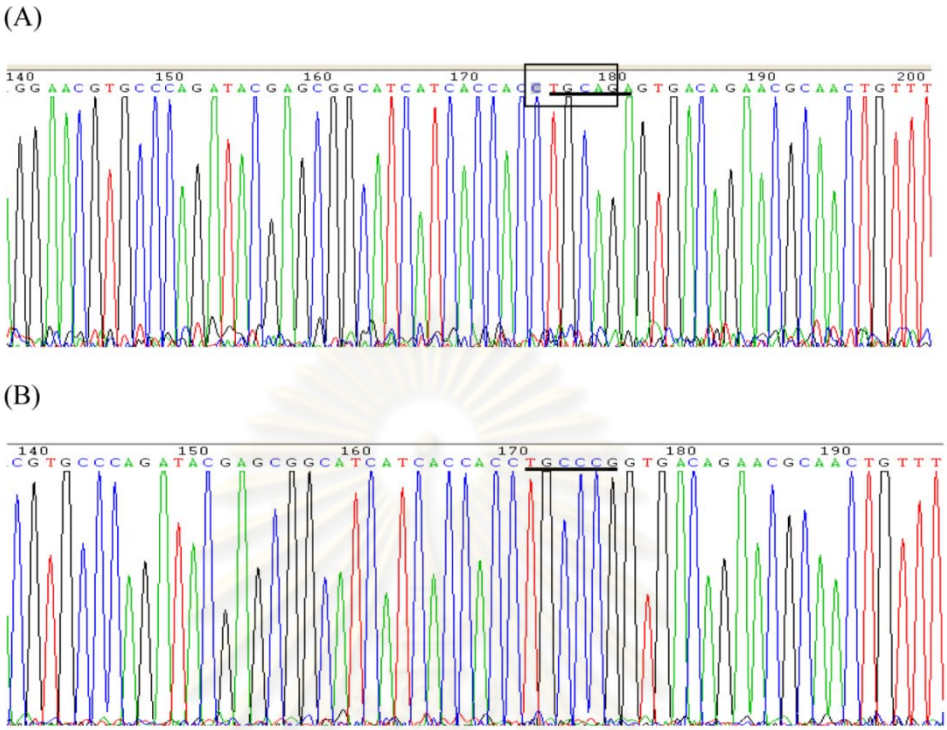


Fig. 3.7. Nucleotide sequencing of SWD*Pm*2_E30R (A) and SWD*Pm*2_E30P (B). The introduced *Pst*I site was boxed and the mutant regions were underlined.

3.1.2 Construction of recombinant pET-28a(+)_crustin*Pm*1 mutants

The crustin*Pm*1 mutant genes in pUC119_crustin*Pm*1_LL, pUC119_crustin*Pm*1_LH, pUC119_crustin*Pm*1_LM, pUC119_crustin*Pm*1_M or pUC119_crustin*Pm*1_del were subcloned into the pET-28a(+) expression vector at *Nco*I and *Xho*I sites. The recombinant plasmids containing the mutant gene were checked with restriction enzyme digestion using *Bsi*WI- *Eco*RV for pET-28a(+)_crustin*Pm*1_LL, pET-28a(+)_crustin*Pm*1_LH, pET-28a(+)_crustin*Pm*1_LM, and *Age*I-*Eco*RV for pET-28a(+)_crustin*Pm*1_M and pET-28a(+)_crustin*Pm*1_del (data not shown). The presence of mutant inserts was confirmed by *Nco*I-*Xho*I digestion (Fig. 3.8.).

The purified PCR products of crustin*Pm*1_delG, crustin*Pm*1_delC, crustin*Pm*1_WAP and crustin*Pm*1WAP_LM were *Nco*I-*Xho*I digested and ligated into pET-28a(+). The recombinant clones were verified by restriction enzyme digestion with *Nco*I-*Xho*I as shown in Fig. 3.9.

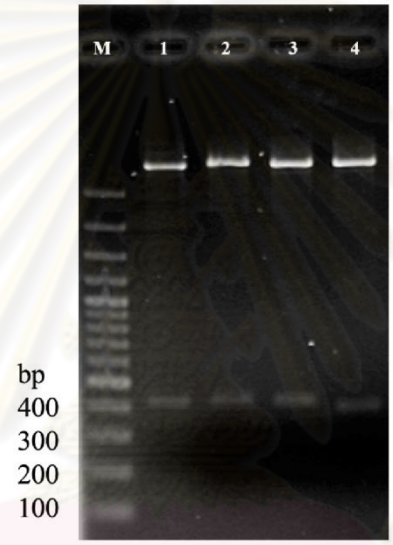


Figure 3.8 The screening of the recombinant plasmid by digesting with *Nco*I and *Xho*I. laneM: GeneRuler™ 100 bp DNA ladder (Fermentas); lane1: pET-28a(+)_crustin*Pm*1_M; lane2: pET-28a(+)_crustin*Pm*1_LM; lane3: pET-28a(+)_crustin*Pm*1_LL; lane4: pET-28a(+)_crustin*Pm*1_del.

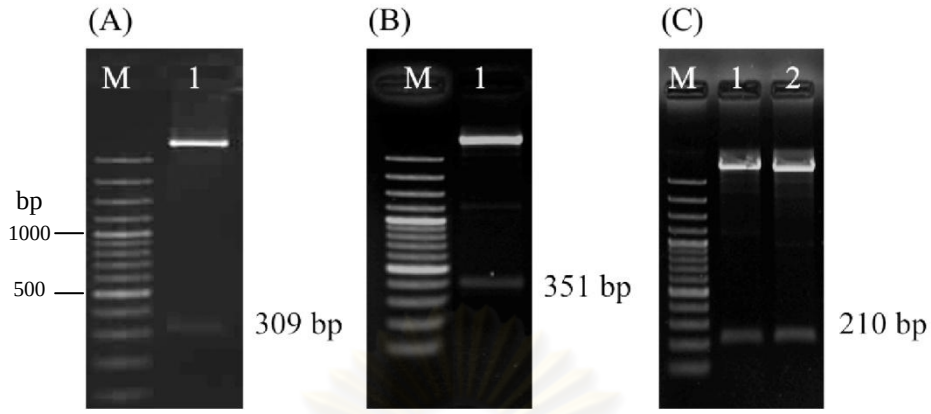


Fig. 3.9. The screening of recombinant plasmid by digesting with *Nco*I and *Xho*I. (A): lane 1, pET-28a(+)_crustin*Pm*1_delG; (B): lane 1, pET-28a(+)_crustin*Pm*1_delC; (C): lane 1, pET-28a(+)_ crustin*Pm*1_WAP and lane 2, pET-28a(+)_crustin*Pm*1WAP_LM.

3.1.3 Recombinant protein expression of crustin*Pm*1, SWD*Pm*2 and their mutants

The recombinant expression plasmids, the pET-28a(+) derivatives of crustin*Pm*1, SWD*Pm*2 and their mutants were over-expressed in an *E. coli* system, Rosetta(DE3)pLysS. The cells were tested for the induction of expression by harvesting the culture at 0, 1, 2, 3 and 4 h after IPTG induction. The cells were solubilized with the SDS-PAGE loading buffer and analyzed by electrophoresis on 15% SDS-PAGE. It was found that the protein gel revealed expression patterns showing the expected protein band which was continuously increased in the induction time dependent manner (Fig. 3.10). For each recombinant protein, the appropriate condition was chosen and used to prepare the recombinant protein for further characterization.

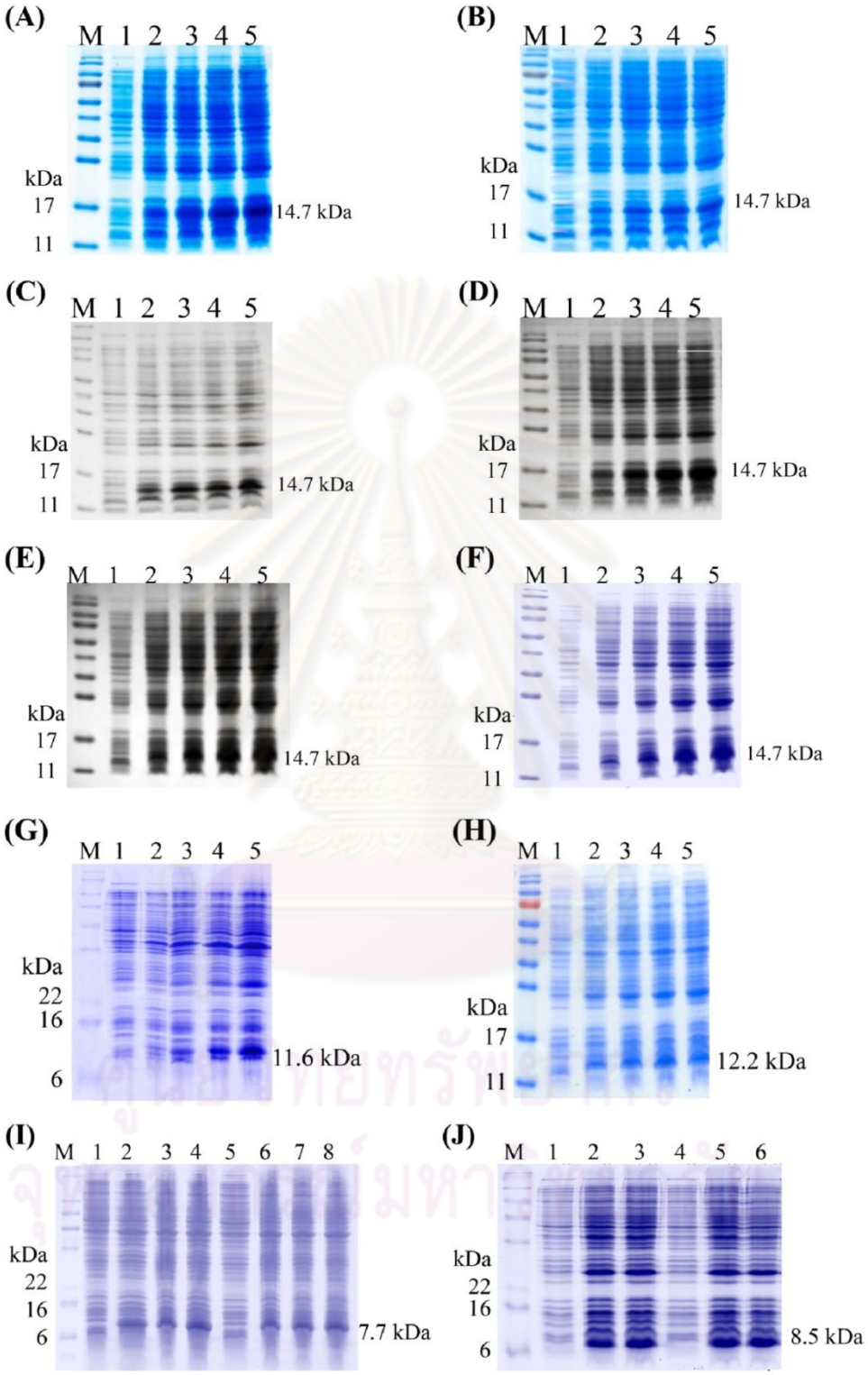


Fig. 3.10. Expression of recombinant crustin*Pm*1 and its mutants at various time points after IPTG induction. (A), rcrustin*Pm*1; (B), rcrustin*Pm*1_M; (C), rcrustin*Pm*1_LM; (D), rcrustin*Pm*1_LL; (E), rcrustin*Pm*1_LH; (F), rcrustin*Pm*1_del; (G), rcrustin*Pm*1_delG; (H), rcrustin*Pm*1 _delC; (I),

rcrustin*Pm*1_WAP and rcrustin*Pm*1_WAP_LM; (J), rSWD*Pm*2_E30R and rSWD*Pm*2_E30P. M is a protein size marker. In (A) to (H), lanes 1, 2, 3, 4 and 5 are crude protein preparations at 0, 1, 2, 3 and 4 h after IPTG induction, respectively. In (I), lanes 1, 2, 3 and 4 are crude protein preparations of rcrustin*Pm*1_WAP and lanes 5, 6, 7 and 8 are crude protein preparations of rcrustin*Pm*1_WAP_LM at 0, 2, 3 and 4 h after IPTG induction, respectively. In (J), lanes 1, 2 and 3 are crude protein preparations of rSWD*Pm*2_E30R and lanes 4, 5 and 6 are crude protein preparations of rSWD*Pm*2_E30P at 0, 3 and 4 h after IPTG induction, respectively.

3.1.4 Purification of recombinant protein crustin*Pm*1, SWD*Pm*2 and their mutants

The recombinant crustin*Pm*1 (rcrustin*Pm*1) protein was mainly expressed and aggregated in the insoluble inclusion bodies which were solubilized in a denaturing solution containing SDS and urea (Supungul et al., 2008). In this study, attempt had been made to solubilize the inclusion bodies under the non-denaturing condition. The rcrustin*Pm*1 and its mutants containing the His-tag were solubilized in 50 mM phosphate buffer pH 12. The solubilized protein was dialyzed in 50 mM carbonate buffer pH 10, the pH that still allow the protein to be purified using the nickel-NTA column. The purified recombinant protein was eluted with 50 mM carbonate buffer pH 10 containing 100 mM imidazole (elution buffer) and analyzed the purity on 15% SDS-PAGE (Fig. 3.11). Major bands of about 14.7 kDa of rcrustin*Pm*1, rcrustin*Pm*1_LL, rcrustin*Pm*1_LH, rcrustin*Pm*1_LM and rcrustin*Pm*1_M; 14.2 kDa of rcrustin*Pm*1_del; 11.6 kDa of rcrustin*Pm*1_delG; 12.2 kDa of rcrustin*Pm*1_delC; 7,669.78 Da of rcrustin*Pm*1_WAP; and 7,719.9 Da of rcrustin*Pm*1_WAP_LM were identified. The two minor bands were observed. These

bands were probably dimer and trimer of the protein which could be seen as usual for cysteine-rich proteins.

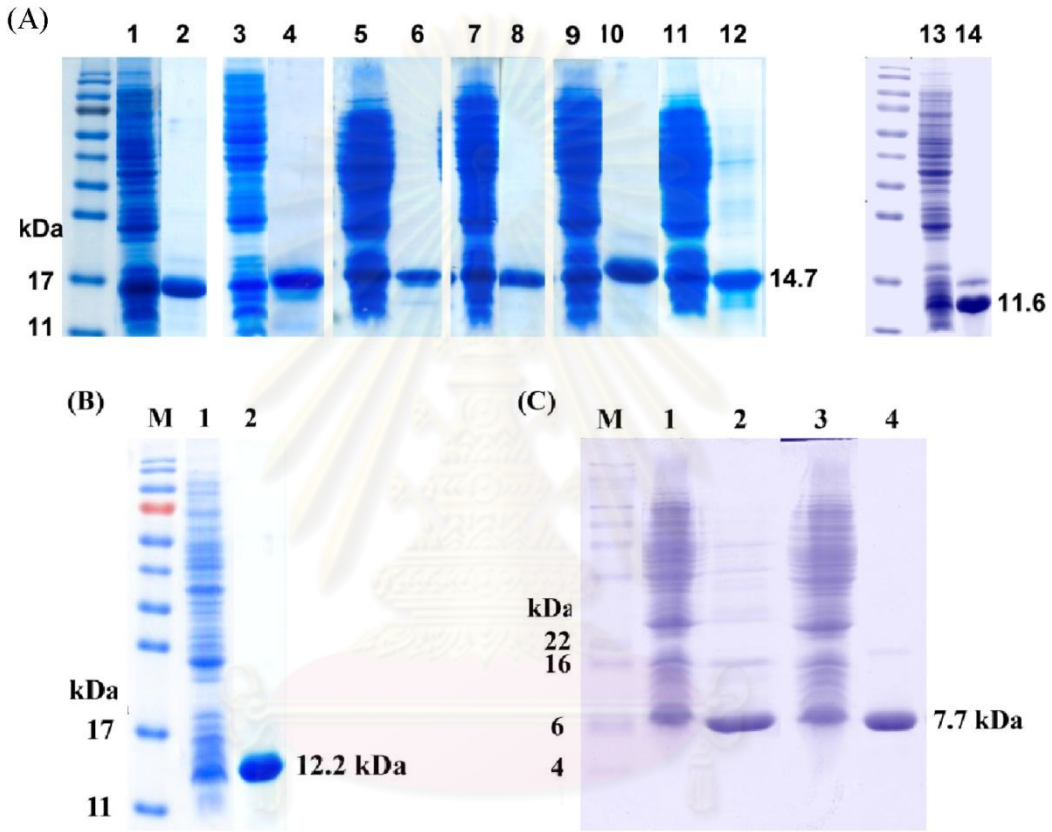


Fig. 3.11. SDS-PAGE analysis of rcrustin*Pm*1and its mutants upon purification. In (A), lanes 1, 3, 5, 7, 9, 11 and 13 are crude protein preparations after IPTG induction of rcrustin*Pm*1, rcrustin*Pm*1_M, rcrustin*Pm*1_LM, rcrustin*Pm*1_LL, rcrustin*Pm*1_LH, rcrustin*Pm*1_del and rcrustin*Pm*1_delG, respectively; lanes 2, 4, 6, 8, 10, 12 and 14 are purified proteins of the same. Lane M is a molecular weight size marker. In (B), lane 2 is the purified protein of rcrustin*Pm*1_delC. In (C), lanes 1 and 3 are crude protein preparations after IPTG induction of rcrustin*Pm*1_WAP and rcrustin*Pm*1_WAP_LM; lanes 2 and 4 are purified proteins of the same.

The rSWD*Pm*2_E30R and rSWD*Pm*2_E30P with their His-tags were purified using Ni-NTA column following the procedure described by Amparyup et al. (2008a). The recombinant proteins were expressed as inclusion bodies which were dissolved in a denaturing buffer and purified under denaturing condition. The eluted fractions were analyzed using 15% SDS-PAGE (Fig. 3.12). The bands of purified rSWD*Pm*2_E30R and rSWD*Pm*2_E30P were shown with approximately 8.5 kDa in size. The purified proteins were refolded by dialysis against 20 mM sodium phosphate pH 5.8 at 4 °C for further characterization of their activities.

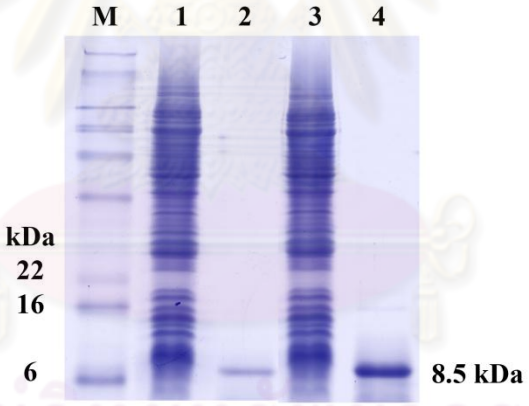


Fig. 3.12. Recombinant expression of rSWD*Pm*2_E30R and rSWD*Pm*2_E30P. The cell cultures were grown for 4 h after IPTG induction and harvested. Lanes 1 and 3 are crude proteins after IPTG induction of rSWD*Pm*2_E30R and rSWD*Pm*2_E30P; lanes 2 and 4 are purified proteins of the same.

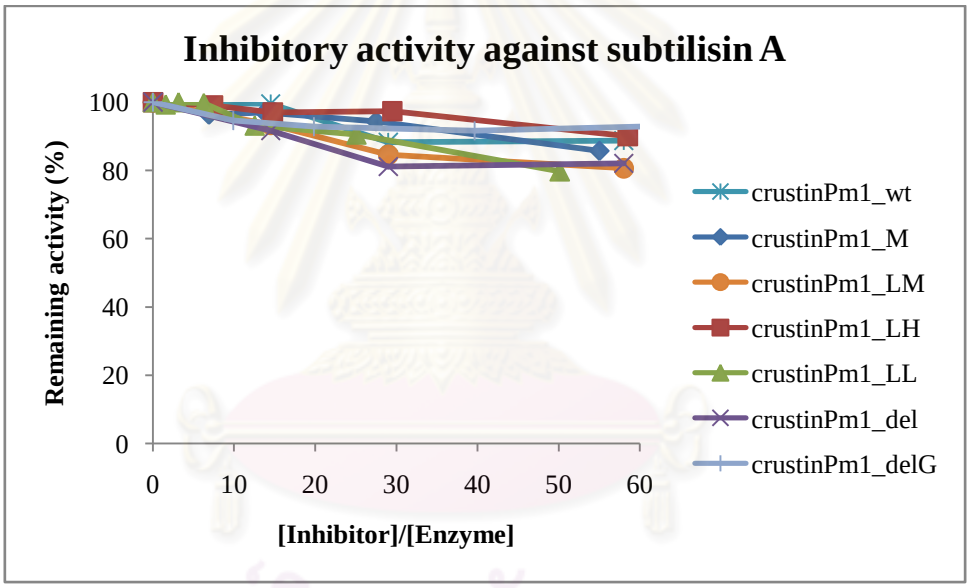
3.1.5 Serine proteinase inhibition assay of crustin*Pm*1, SWD*Pm*2 and their mutants

The serine proteinases: trypsin, subtilisin, chymotrypsin and elastase, can hydrolyse the synthetic chromogenic substrates and release *p*-nitroaniline which can be measured its absorbance at 405 nm. The proteinase inhibitory activity of recombinant proteins was investigated by incubating the recombinant proteins with serine proteinase and chromogenic substrate and measuring the effects on the substrate hydrolysis at 405 nm.

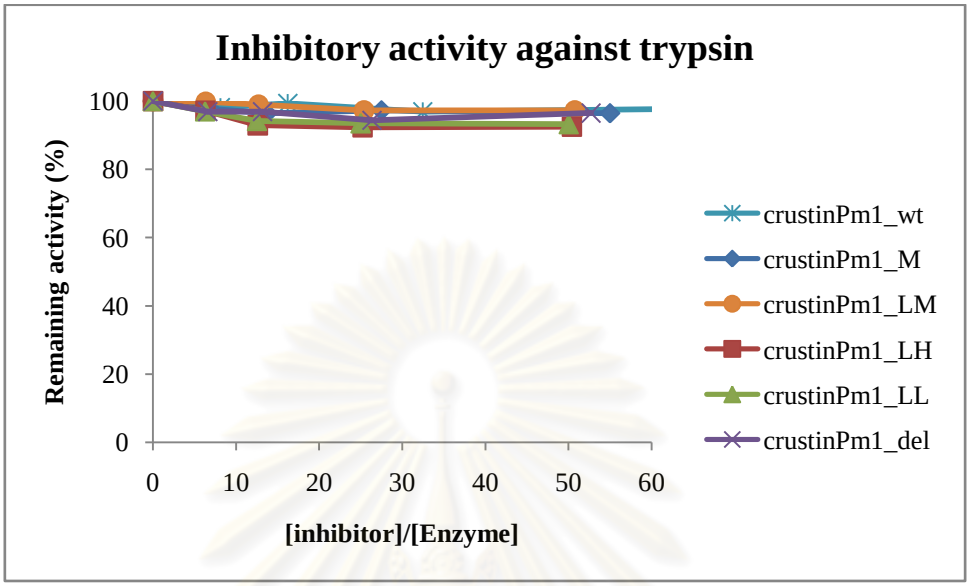
The rcrustin*Pm*1 and its mutants were assayed for their inhibitory activities against trypsin, subtilisin, chymotrypsin and elastase. The inhibitor to proteinase ratio of 100:1 was first used to determine the inhibitory potency of inhibitor against the proteinases. It was found out that neither crustin*Pm*1 nor its mutants inhibited the subtilisin, trypsin, chymotrypsin and elastase (Fig. 3.13). The crustin*Pm*1_LM was slightly inhibitory to the chymotrypsin. Other deletion mutants of crustin*Pm*1 mutants: rcrustin*Pm*1_delC, rcrustin*Pm*1_WAP and rcrustin*Pm*1_WAP_LM exhibited low subtilisin inhibitory activity but not other proteinase (data not shown). Due to the low potency of subtilisin inhibition, the mole ratios up to 600 of inhibitor against proteinase were used. While the crustin*Pm*1 was inactive, the rcrustin*Pm*1_WAP exhibited the strongest subtilisin inhibition followed by rcrustin*Pm*1_delC and rcrustin*Pm*1_WAP_LM (Fig. 3.14). Therefore, the WAP domain itself was capable of proteinase inhibition. Mutation of the P_1 and P_1' amino acids deteriorated the inhibition. It seemed like the glycine-rich and cysteine-rich regions negatively affected the proteinase inhibitory activity.

Both the rSWD*Pm*2_E30R and rSWD*Pm*2_E30P was not changes in their inhibitory specificities but maintained their inhibitory activities against subtilisin as wild type (Fig. 3.15). The inhibition exceeded more than 80% at the ratio about 5. To our disappointment, the rSWD*Pm*2_E30R did not have inhibitory activity against trypsin (data not shown) and the rSWD*Pm*2_E30P was not inactive against subtilisin.

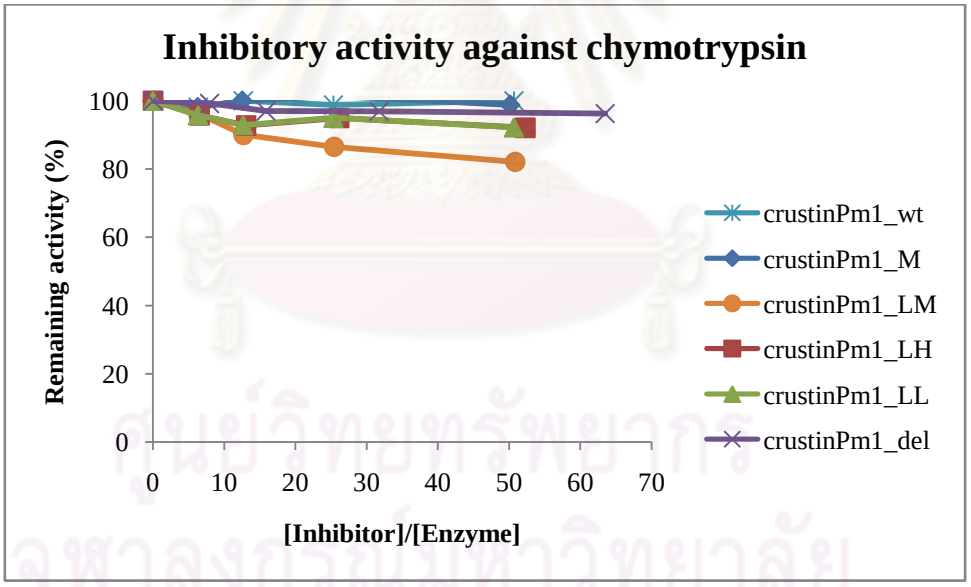
(A)



(B)


Inhibitory activity against trypsin
Remaining activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
[inhibitor]/[Enzyme]
crustinPm1_wt
crustinPm1_M
crustinPm1_LM
crustinPm1_LH
crustinPm1_LL
crustinPm1_del

(C)


Inhibitory activity against chymotrypsin
Remaining activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
[Inhibitor]/[Enzyme]
crustinPm1_wt
crustinPm1_M
crustinPm1_LM
crustinPm1_LH
crustinPm1_LL
crustinPm1_del

(D)

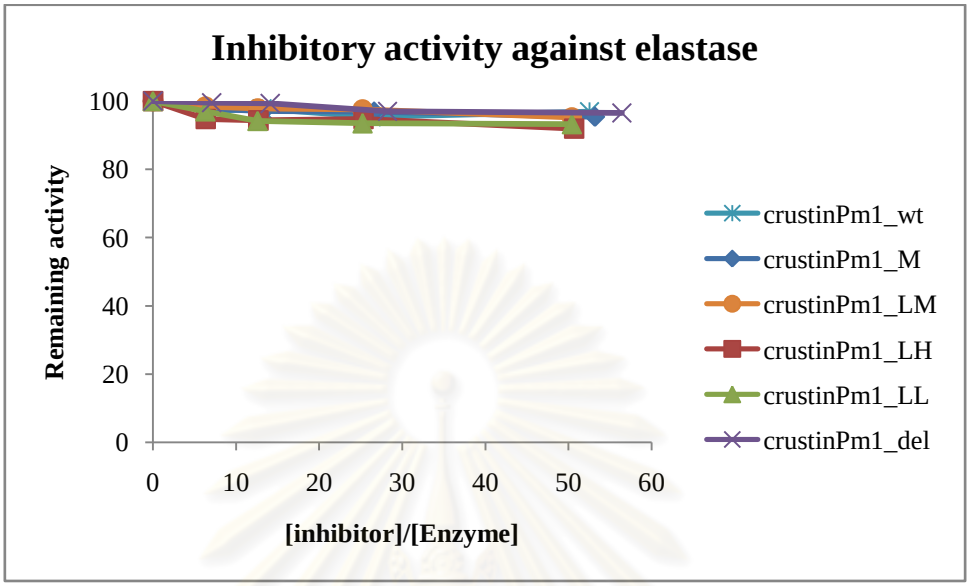


Fig. 3.13. Proteinase inhibition assay of the rcrustin*Pm*1 or its mutants. The recombinant proteins were incubated at various ratios with subtilisin (A), trypsin (B), chymotrypsin (C) and elastase (D) in the reactions containing chromogenic substrates. The reduction in proteinase activity was determined. Results are means ± S.D. for three independent assays each performed in triplicate.

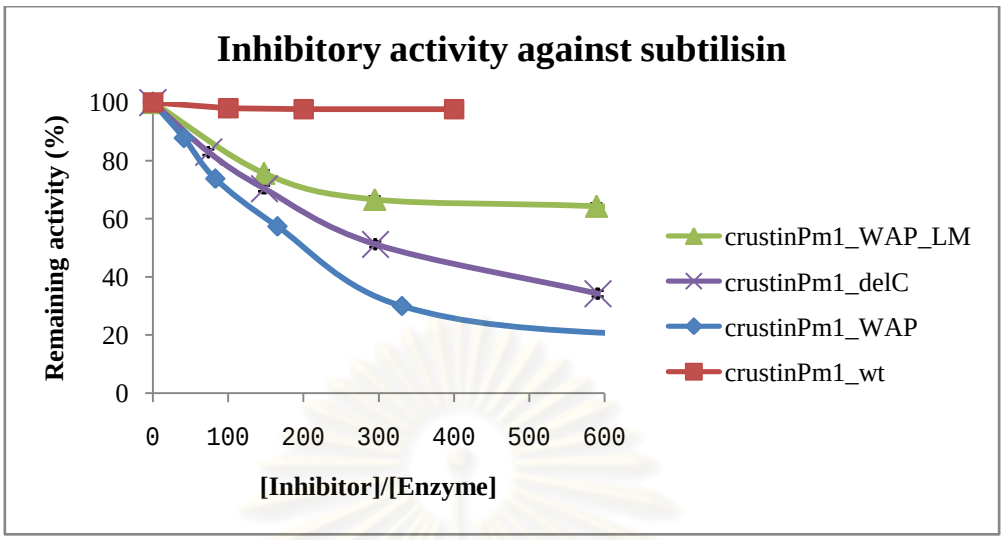


Fig. 3.14. The inhibitory affects of rcrustin*Pm*1_WAP_LM (▲), rcrustin*Pm*1_delC (×), rcrustin*Pm*1_WAP (◆) and rcrustin_wt (■) on subtilisin.

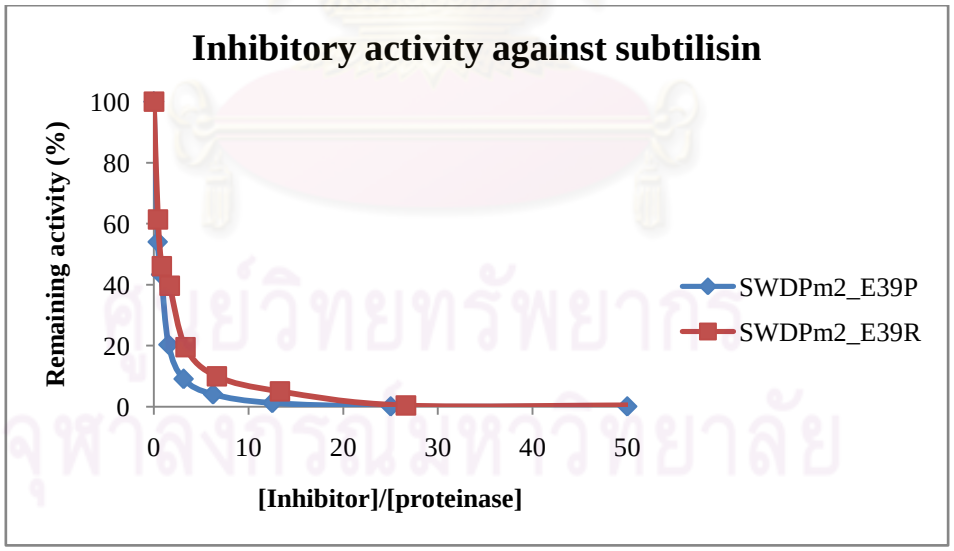


Fig. 3.15. Subtilisin inhibition assay of rSWD*Pm*2_E30P (◆) and rSWD*Pm*2_E30R (■). They were incubated at various ratios with subtilisin in the reaction containing chromogenic substrate. The reduction in proteinase activity was determined.

3.1.6 Assay of antimicrobial activity of rcrustin*Pm*1 and its mutants using agar diffusion assay

Since the crustin*Pm*1 exhibits antimicrobial activity, particularly strong against *Staphylococcus aureus*, the rcrustin*Pm*1 deletion mutants were tested against *Staphylococcus aureus*. Samples containing doses ranging from 0.02-0.17 nmol of rcrustin*Pm*1; 0.02-2.0 nmol of rcrustin*Pm*1_delG; 0.04-3.2 nmol of rcrustin*Pm*1_delC; 0.03-2.5 nmol of crustin*Pm*1_WAP were added into each well. The results showed that the antibacterial activity of the recombinant proteins was observed as clear zones surrounding the wells. The negative control (50 mM carbonate buffer pH 10) gave no clear zone while the positive control (4.9 nmol kanamycin) showed a prominent clear zone around the well.

The antimicrobial activity of crustin*Pm*1 and its mutants: rcrustin*Pm*1_delG and rcrustin*Pm*1_delC were concentration-dependent (Fig. 3.16). The minimum inhibitory dose (MID) which was the minimum dose of crustin*Pm*1 or its mutants forming a detectable zone of clearance larger than the size of the negative control (0.4 cm) was calculated. The MID of rcrustin*Pm*1, rcrustin*Pm*1_delG and rcrustin*Pm*1_delC were 0.04, 0.50 and 0.39 nmol, respectively. The rcrustin*Pm*1_WAP did not show antimicrobial activity while the rcrustin*Pm*1_delG and rcrustin*Pm*1_delC exhibited lower antimicrobial activity than the rcrustin*Pm*1 indicating the importance of glycine-rich and cysteine-rich regions for antimicrobial activity.

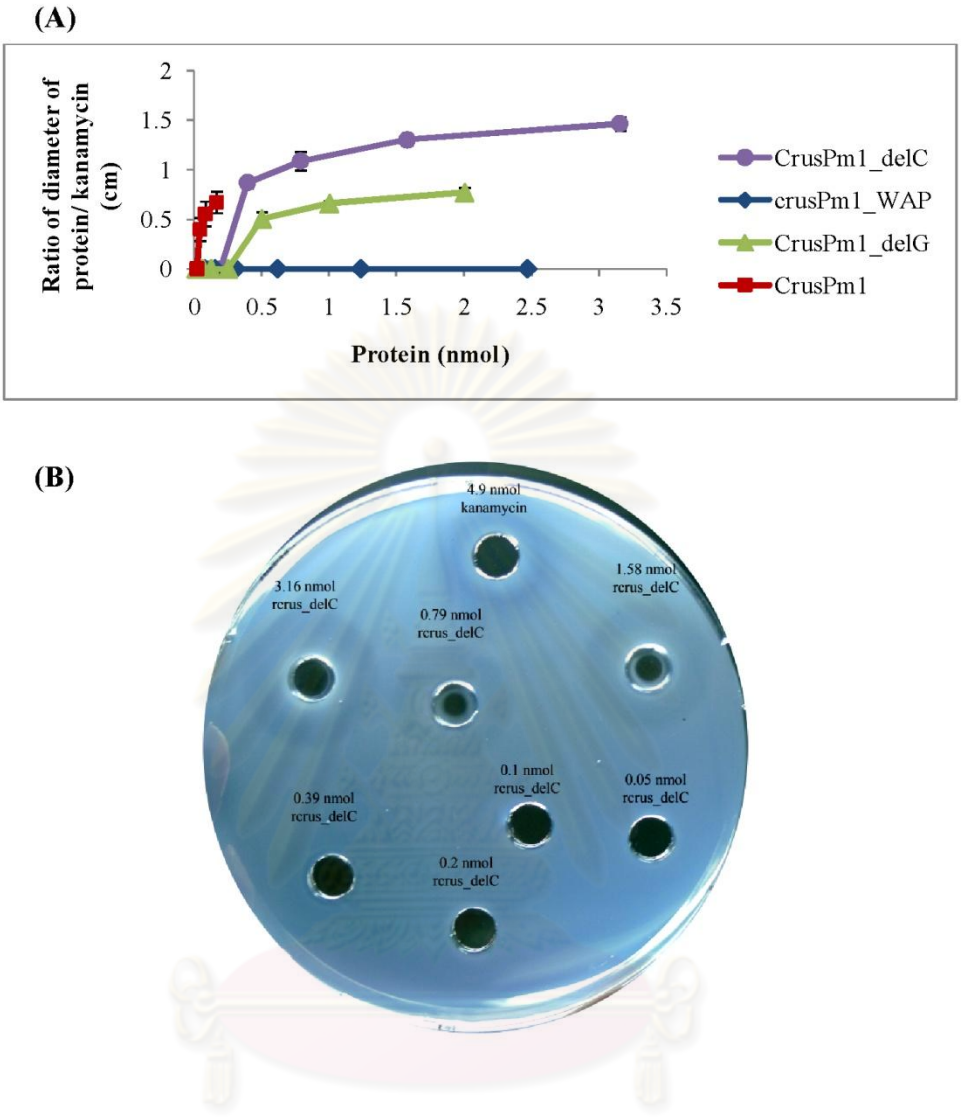


Fig. 3.16. Antibacterial activity of rcrustin*Pm*1, rcrustin*Pm*1_delG, rcrustin*Pm*1_delC, crustin*Pm*1_WAP against *S. aureus* in a radial diffusion assay. (A) The ratios of diameters of clear zones of recombinant proteins to that of kanamycin (as positive control) after subtracting the diameter of well (0.4 cm) were plotted against the amounts of proteins. (B) The radial diffusion assay of *S. aureus*. The rcrustin*Pm*1_delC from 0.04 to 3.2 nmol was added into each well (0.4 cm diameter). Antibacterial activity of recombinant protein is seen as clear zone surrounding the well.

Table 3.1. Summary of the antimicrobial and antiproteinase activities of crustin*Pm*1, SWD*Pm*2, *Pm*DWD and their mutants. The potency of activities is indicated as (+).

Recombinant proteins	Antimicrobial activity against *S. aureus*	Antiproteinase activity
crustin*Pm*1_wt	++++	No
crustin*Pm*1_M	ND	No
crustin*Pm*1_LM	ND	No
crustin*Pm*1_LL	ND	No
crustin*Pm*1_LH	ND	No
crustin*Pm*1_del	ND	No
crustin*Pm*1_delG	++	No
crustin*Pm*1_delC	+++	subtilisin (++)
crustin*Pm*1_WAP	No	subtilisin (+++)
crustin*Pm*1_WAP_LM	ND	subtilisin (+)
SWD*Pm*2_wt	++++	subtilisin (++++)
SWD*Pm*2_E30R	ND	subtilisin (++++)
SWD*Pm*2_E30P	ND	subtilisin (++++)
*Pm*DWD	No	crude proteinase from *B. subtilis*
*Pm*DWD_F70R	No	subtilisin (++++)

ND = not detected

3.2 *Pm*DWD

3.2.1 Data mining of the *Pm*DWD

The *Pm*DWD were identified from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th). The eighty EST clones representing this only one *Pm*DWD. All EST clones were from the hemocyte libraries of normal, heat–treated and WSSV–infected shrimp. The open-reading frame of 354 bp and its deduced amino acid sequence of 117 amino acids are shown in Fig. 3.17. Sequence analysis using the signalP program revealed the presence of a signal peptide with 16 amino acids, resulting in a 101 residue mature protein with a calculated molecular mass of 12.89 kDa and a predicted pI of 7.86. The deduced amino acid sequence of *Pm*DWD contained the two WAP domains. Each domain composed of eight cysteine residues which formed a four disulfide core (4DSC).

```
  1 atgaagattgtgttgcttcttgctcttgctttaccgatggtagtt
    M  K  I  V  L  L  L  A  L  A  L  P  M  V  V
 46 gcaacgagaggcccgctaaagcccggatgcccgaacccggatcaa
    A  T  R  G  P  L  K  P  G  C  P  N  P  D  Q
 91 ggtaggcagtgcctgctctaccatgaccagtgccagacggattgg
    G  R  Q  C  L  L  Y  H  D  Q  C  Q  T  D  W
136 gattgcgaaaaagaaggaaaaggcgacatatgctgcctgataaat
    D  C  E  K  E  G  K  G  D  I  C  C  L  I  N
181 ggctgcggaaaggagtgcgtgacactgaaaatgaacccaagatgc
    G  C  G  K  E  C  V  T  L  K  M  N  P  R  C
226 cccgaccctgcaagttacggcttgaactgcttcgcttacattcac
    P  D  P  A  S  Y  G  L  N  C  F  A  Y  I  H
271 cagtgcaacatcgaccgtgagtgtaggggcagacagaagtgctgt
    Q  C  N  I  D  R  E  C  R  G  R  Q  K  C  C
316 ctcgtggccggatgtggcaggagttgtcaggaagtctaa 354
    L  V  A  G  C  G  R  S  C  Q  E  V  *
```

Fig. 3.17. The open-reading frame and the deduced amino acid sequence of *Pm*DWD. The signal peptide is underlined. The two WAP domains are shaded.

The mature *Pm*DWD sequence was compared with those of other penaeid shrimp: LvDWD from *L. vannamei* (Jiménez-Vega and Vargas-Albores, 2007), Mj-DWD from *M. japonicas* (Chen et al., 2008), and Fc-DWD from *F. chinensis* (Du et al., 2009); as well as those SLPIs from mammals, i.e. mouse (Zitnik et al., 1997), rat (Song et al., 1999), sheep (Brown et al., 2005), human (Eisenberg et al., 1990), rhesus monkey (Magness et al., 2005) and cattle (Mann et al., 2007). The alignment using the ClustalX program is shown in Fig. 3.18. The *Pm*DWD is very close-related to its ortholog Fc-DWD. Nevertheless, it also shares homology, albeit less, with Mj-DWD and LvDWD. Sequence alignment reveals the difference of amino acid among the sequences of DWDs from the penaeid shrimp and those of mammalian SLPIs. The amino acid spacings between the conserved cysteine residues of the WAP domains are almost identical among the penaeid shrimp distinct from those of mammals.

In addition, the amino acid sequences and spacings comparison showed a relatively large variation of the amino acid sequences and spacings among the cysteine residues in the N- and C-terminal WAP domains in either mammalian SLPIs or penaeid DWDs. Some similarities in amino acid sequences are evident but the spacings are different, particularly the spaces between the first and second, and the second and third conserved cysteines. Whether the differences in spacing result in the differences in activities is not known.

The C-terminal WAP domain of mammalian SLPI is proteinase inhibitory active (Kramps et al., 1990) but not in the N-terminal. The reactive amino acids at positions P_1-P_1' of SLPIs are usually Leu-Met while those of DWDs are usually Phe-Ala. This discrepancy raises the possibility of different specificities in proteinase inhibitory activities.

Fig. 3.18. Amino acid sequence comparison among the penaeid DWDs and mammalian SLPIs. The conserved cysteine residues were shaded. The P_1 and P_1' amino acids of the second WAP domain were indicated by dark shading.

3.2.2 Tissue distribution of *Pm*DWD transcripts in normal and pathogen-infected *P. monodon* shrimp

3.2.2.1 Tissue and total RNA preparation

Antennal gland, epipodite, gill, heart, hemocyte, hepatopancreas, intestine, lymphoid and eyestalk of shrimp were collected for total RNA isolation. Then, total RNA was treated with RQ1 RNase-free DNase to remove the genomic DNA. The A_{260}/A_{280} ratio of total RNA samples were 1.5 to 1.8 indicating the acceptable quality of total RNA. The quality of total RNA was analyzed using 1.2% agarose gel electrophoresis as shown in Fig. 3.19.

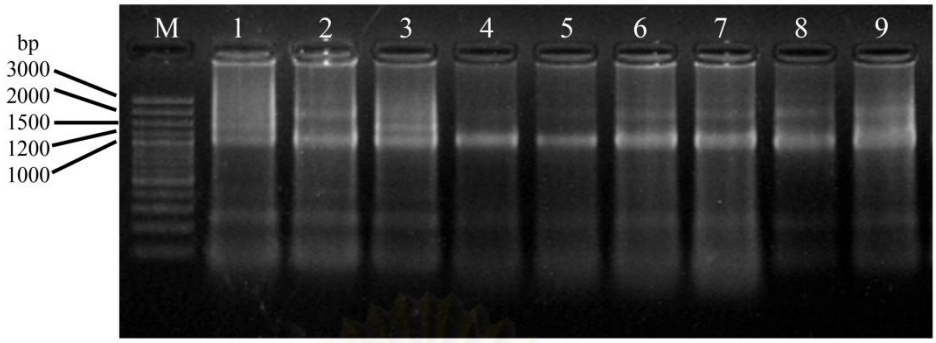


Fig. 3.19. Total RNAs isolated from various tissues of *P. monodon* running on a 1.2% agarose gel. Lane M is RNA marker. Lanes 1-10 are total RNAs from hemocyte, antennal gland, hepatopancreas, epipodite, intestine, gill, lymphoid organ, heart and eyestalk, respectively.

3.2.2.2 Tissue distribution

Tissue specific expression of the *Pm*DWD was examined in various shrimp tissues by RT-PCR analysis. The sizes of PCR products are 320 bp and 145 bp for *Pm*DWD and EF1-α as an internal control, respectively. From normal shrimp tissues, *Pm*DWD transcript was expressed in all tissues examined but predominantly in hemocytes followed by intestine and epipodite (Fig. 3.20.). Much less expression was in hepatopancreas, eyestalk and lymphoid.

With the notion that the mammalian SLPIs were involved in anti-inflammation, tissue repair and cell growth (Hiemstra, 2002) led us to investigate the possible functions of *Pm*DWD. The tissue distributions in response to WSSV infection and leg amputation were determined for clues to the functions. To create a situation like tissue injury to the shrimp, the shrimp legs were amputated and reared normally for 12 h. The tissues of leg amputated and 24-h WSSV-infected shrimp were, then, removed for RT-PCR analysis. The *Pm*DWD was found to be expressed

all over the shrimp body similar to that of normal control and saline-injected control (Fig. 3.20). Nevertheless, the up-regulation was observed clearly in lymphoid of leg amputated and 24-h WSSV-infected shrimp (Fig. 3.20). In the leg amputated shrimp, it was slightly up-regulated in eyestalk for which the significant was not known. Also, slightly up-regulation was in hemocyte of 24-h WSSV-infected shrimp. It should be noted here that the injection of saline solution did somewhat effect the expression of *Pm*DWD in eyestalk.

3.2.3 Construction of the recombinant pVR500_*Pm*DWD

The *Pm*DWD clone was amplified using the primers designed from the cDNA sequence that amplified the open reading frame encoding mature peptide of *Pm*DWD. The PCR primers were designed such that the PCR gene product contained the *Nco*I and *Xho*I restriction sites, respectively, at their 5' and 3' ends. The purified PCR product and expression vector pET-28a(+) digested with *Nco*I and *Xho*I were run on 1.2% agarose gel electrophoresis and the DNA fragments were eluted.

The purified *Nco*I-*Xho*I *Pm*DWD gene fragment was ligated into the *Nco*I-*Xho*I sites of pET-28a(+). For some reasons, the *Pm*DWD was not expressed well. The gene fragment was, then, subcloned into the *Nco*I–*Xho*I sites of a pET-32a(+) derivative, pVR500. The recombinant plasmid containing the gene of interest was screened, selected and checked with restriction enzyme digestion using *Nco*I and *Xho*I for the presence of the gene fragment using 1.2% agarose gel electrophoresis. The correct DNA fragment of 320 bp was observed as showed in Fig. 3.21. The recombinant plasmid pVR500_*Pm*DWD was used further for over-expression and as a template for mutagenesis.

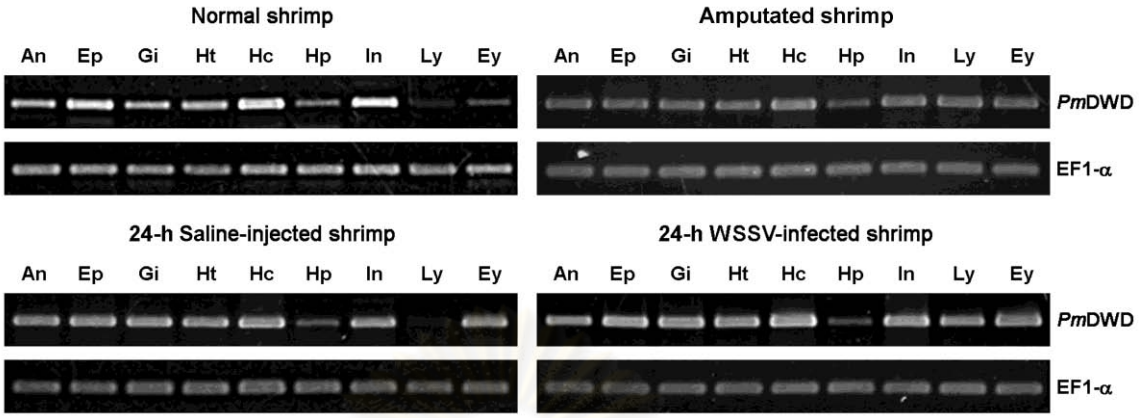


Fig. 3.20. Tissue expression of *Pm*DWD in normal, leg-amputated, 24-h saline-injected and 24-h WSSV-injected shrimp. Tissues tested are antennal gland (An), epipodite (Ep), gill (Gi), heart (Ht), hemocyte (Hc), hepatopancreas (Hp), intestine (In), lymphoid (Ly) and eyestalk (Ey). The EF1-α was used as an internal control.

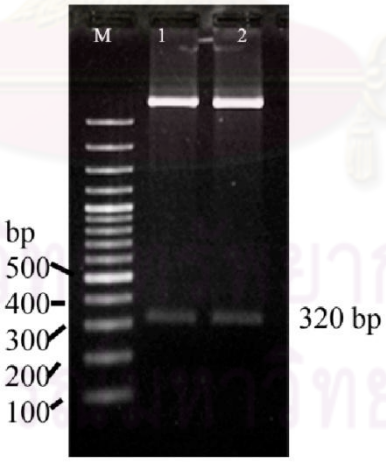


Fig. 3.21. The screening of recombinant plasmid by digesting with *Nco*I and *Xho*I**.** Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas); lane 1: pVR500_*Pm*DWD; lane 2: pVR500_*Pm*DWD_F70R.

3.2.4 Mutagenesis of *Pm*DWD

For the mutagenesis, the presumed P_1 amino acid, Phe70, was mutated to Arg hopefully to change its inhibitory specificity for possibly trypsin or subtilisin. The pVR500_*Pm*DWD was used as a template for mutagenesis using Phusion® Hot Start High-Fidelity DNA Polymerase. The mutant was named *Pm*DWD_F70R selected by the presence of adjacent introduced *Pst*I restriction site. The mutation was confirmed by DNA sequencing (Fig. 3.22).

3.2.5 Recombinant expression of r*Pm*DWD and its mutant

3.2.5.1 Over-expression of the *Pm*DWD and its mutant

Since the *Pm*DWD expression clone in pET–28a(+) in an *E. coli* strain Rosetta(DE3)pLysS failed to produce any recombinant protein, another expression vector pVR500, a derivative of pET–32a(+) was tried. The recombinant plasmid pVR500_*Pm*DWD and pVR_*Pm*DWD_F70R were over-expressed in an *E. coli* strain Rosetta(DE3)pLysS.

A single colony was cultured in LB medium containing 100 mg/ml ampicillin and 34 mg/ml chloramphenicol at 37 °C until the OD_{600} reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to the final concentration of 1 mM to induce the expression. The cells were harvested at 0, 1, 2, 3 and 4 h after IPTG induction and then analyzed by 15% SDS-PAGE. The results showed that the protein expression was continuously increased under the IPTG induction (Fig. 3.23). This appropriate condition was, then, employed for a large-scale expression of r*Pm*DWD and its mutant.

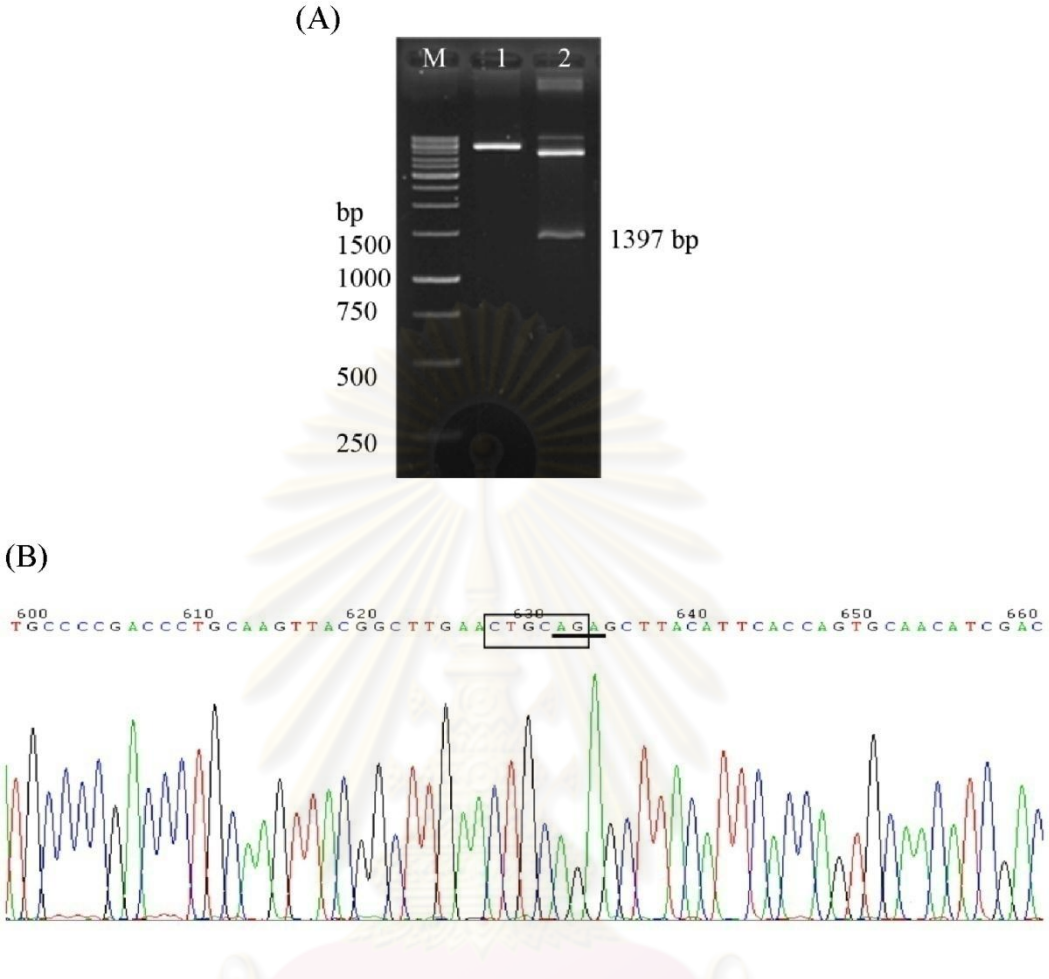


Fig. 3.22. (A) The screening of mutant plasmid by digesting with *Pst*I on 1.2% agarose gel electrophoresis. Lane M, GeneRuler™ 1 kb DNA ladder (Fermentas); lane 1, pVR500_*Pm*DWD; lane 2, pVR500_*Pm*DWD_F70R. (B) Nucleotide sequencing of *Pm*DWD_F70R. The introduced *Pst*I site was boxed and the mutant region was underlined.

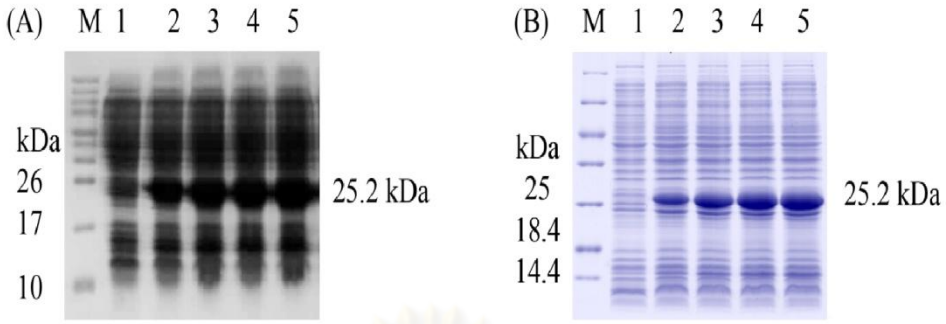


Fig. 3.23. SDS-PAGE analysis of r*Pm*DWD (A) and r*Pm*DWD_F70R (B) expressed in an *E. coli* Rosetta(DE3)pLysS at various times after IPTG induction. Lanes 1-5 are the lysates of cells at 0, 1, 2, 3 and 4 h, respectively. Lanes M are protein marker (Fermentas).

3.2.5.2 Purification of r*Pm*DWD and its mutant

The r*Pm*DWD and r*Pm*DWD_F70R were expressed as a soluble fusion protein of about 25.26 kDa. They were purified with the nickel-NTA column under non-denaturing condition. The purified proteins were eluted and analyzed for their purity on 15% SDS-PAGE. The proteins of expected size were observed (Fig. 3.24). A faint band of dimer is usually seen with high cysteine-containing protein like r*Pm*DWD. From our calculation, the size of thioredoxin and r*Pm*DWD were approximately 12.8 and 12.5 kDa, respectively. Attempt had been made to remove the thioredoxin moiety by enterokinase digestion. It was very unfortunate that once the thioredoxin was removed, the *Pm*DWD readily precipitated and could not be dissolved under mild condition. The fusion proteins were, therefore, used as is in various tests.

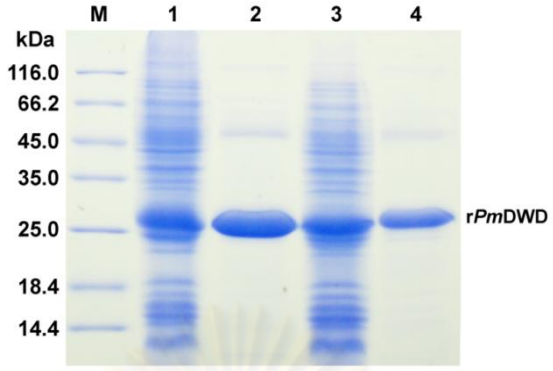


Fig. 3.24. SDS-PAGE analysis of r*Pm*DWD purification. Lanes 1 and 3 are crude proteins after IPTG induction of r*Pm*DWD and its F70R mutant, respectively. Lanes 2 and 4 are purified proteins of the same. Lane M is a molecular weight marker.

3.2.6 Proteinase inhibition assay of *Pm*DWD and its mutant

3.2.6.1 Agar diffusion assay of proteinase inhibition of *Pm*DWD

From other studies, the recombinant DWD proteins were tested against the crude proteinases from bacterial cultures of *B. subtilis*. In this study, the overnight culture media of *B. subtilis*, *E. cloacae*, *E. carotovora*, *E. coli* 363, *K. pneumoniae*, *S. thyphimurium*, *V. alginolyticus*, *V. harveiy* 639, *V. parahaemolyticus*, *V. fluvialis*, *V. mimicus* and *V. cholera* were used as crude preparations of proteinases. The clear zone in the skim milk plate was only observed with crude proteinases from *B. subtilis* and *V. alginolyticus* but not other bacteria. Therefore, an excess amount of r*Pm*DWD was tested against the crude proteinase of two strains. The result showed that the clear zone of crude proteinase of *B. subtilis* was not observed in the presence of r*Pm*DWD. This was due to the proteinase inhibitory activity of r*Pm*DWD against the crude proteinases from *B. subtilis* (Fig. 3.25). However, the *Pm*DWD was not able

to abolish the clear zone of *V. alginolyticus* (data not shown). One-fourth dilution of r*Pm*DWD gave the same result because the recombinant protein was still in excess over the proteinases. Moreover, the rDWD_F70R was found to inhibit the crude enzymes of *B. subtilis* proteinases (data not shown). Since the r*Pm*DWD was a fusion protein with thioredoxin, the thioredoxin was prepared and tested whether it possessed any proteinase inhibitory activity. No inhibitory activity was observed with thioredoxin (Fig. 3.25).

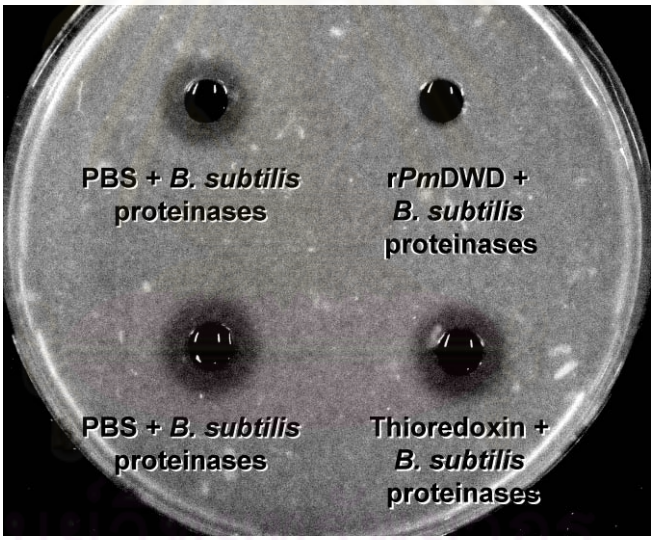


Fig. 3.25. Agar diffusion assay of proteinase inhibition of r*Pm*DWD against *B. subtilis* proteinases. The 0.6 μg *B. subtilis* proteinases were mixed with 8 μg each of r*Pm*DWD and thioredoxin before adding into the wells in a milk agar plate. PBS was used as control. The transparent zones observed after overnight incubation at 28 °C indicate proteinase activity.

To investigate the proteinase inhibitory activity of r*Pm*DWD in the shrimp, the r*Pm*DWD was, then, tested against crude proteinases isolated from various tissues from normal, leg-amputated and 24-h WSSV-infected shrimp using an agar diffusion assay. The crude preparations were added into the wells in the skim milk plate and incubated overnight at room temperature. A clear zone around the well indicated proteinase activities. The proteinase activities were found in the tissue lysates of proteinase-rich tissues like intestine, hepatopancreas and stomach (Fig. 3.26) but hardly found in those of antennal gland, epipodite, gill, heart, eyestalk and hemocyte (data not shown). Interestingly, proteinase activities were found in the tissue lysate of lymphoid organ and hemolymph from leg-amputated and 24-h WSSV-infected but not untreated shrimp (Fig. 3.26). In addition, the r*Pm*DWD was able to inhibit only the proteinases from lymphoid organ. The inhibition was indiscernible when tissue lysates from proteinase-rich tissues were tested with r*Pm*DWD.

3.2.6.2 Serine proteinase inhibition assay

The inhibition of serine proteinase hydrolysis of chromogenic substrate can be measured by following the change in absorbance 405 nm. The purified r*Pm*DWD was tested for serine proteinase inhibitory activity. The recombinant protein was assayed for its inhibitory activities against trypsin, subtilisin, chymotrypsin and elastase. The mole ratios up to 100 of inhibitor against proteinase were used. From its presumed P_1 amino acid, Phe, the *Pm*DWD was expected to inhibit chymotrypsin. To our dissatisfaction, the r*Pm*DWD had no proteinase inhibitory activity against the said commercial proteolytic enzymes. From the result, the *Pm*DWD was mutated at the supposedly P_1 amino acid to elucidate the involvement of this amino acid on proteinase inhibition.

The result showed that the r*Pm*DWD_F70R strongly inhibited subtilisin (Fig. 3.27) but not trypsin, chymotrypsin and elastase (data not shown). The inhibition was so strong that more than 80% inhibition was obtained with the mole ratio of inhibitor to proteinase less than 5.

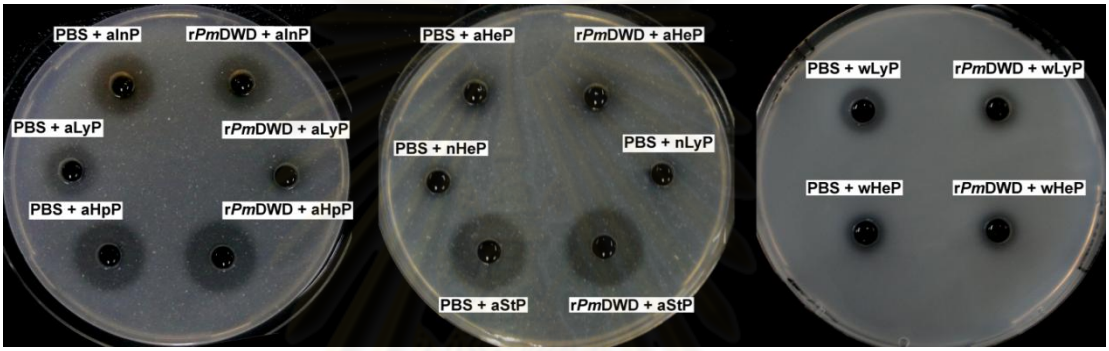


Fig. 3.26. Agar diffusion assay of proteinase inhibition of r*Pm*DWD against shrimp tissue proteinases. The crude proteinases from tissues of amputated shrimp: intestine (aInP, 37.9 μg), lymphoid (aLyP, 17.74 μg), hepatopancreas (aHpP, 5.82 μg), hemolymph (aHeP, 713.4 μg), stomach (aStP, 68.4 μg); normal shrimp: hemolymph (nHeP, 327.92 μg), lymphoid (nLyP, 32.7 μg) and 24-h WSSV-infected shrimp: hemolymph (wHeP, 196.3 μg), lymphoid (wLyP, 33.7 μg) were mixed with 275 μg of r*Pm*DWD before adding into the wells in a milk agar plate. PBS was used as control. The transparent zones observed after overnight incubation at 28 °C indicate proteinase activity.

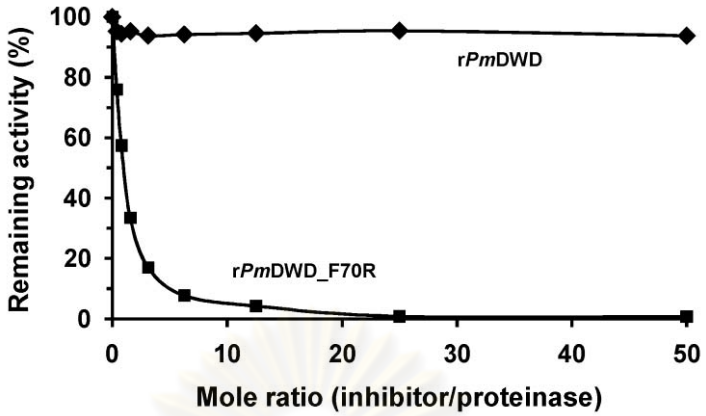


Fig. 3.27. Subtilisin inhibition assays of r*Pm*DWD (◆) and its mutant F70R (■). They were incubated at various ratios with subtilisin in the reactions containing chromogenic substrates. The reduction in proteinase activity was determined.

3.2.7 Antimicrobial activity of r*Pm*DWD and its mutant using liquid growth inhibition assay

The spectrum of antimicrobial activity of r*Pm*DWD was tested against various bacteria both Gram-positive: *B. subtilis*, *B. megaterium*, *M. luteus*, *S. aureus*, *S. haemolyticus*, *S. iniae*, and Gram-negative: *E. cloacae*, *E. carotovora*, *E. coli* 363, *V. harveyi* 1526, *K. pneumoniae*, *S. thyphimurium*, using liquid growth inhibition assay. No significant activity was observed against all bacterial strains tested. The mutant r*Pm*DWD_F70R was also tested negative against *S. aureus*, *B. megaterium* and *S. haemolyticus*.

CHAPTER IV

DISCUSSION

The WAP domains are the part and parcel of numerous proteins in the widespread of multi-cellular organisms which may possess one or two of these domains. These proteins exhibit various biological functions. A large biological diversity exists between the proteins that contain one or two WAP domains, with many being identified as proteinase inhibitors or antimicrobial peptides. In mammal, the SLPI which comprises two WAP domains are proteinase inhibitor and antimicrobial peptides; the N-terminal WAP is seemingly involved in antimicrobial activity while the C-terminal WAP possesses a proteinase inhibitory activity (Fitch et al., 2006; Moreau et al., 2008).

In crustacean, the crustins contains only one WAP domain. They are antimicrobial peptides but some of them also have anti-proteinase activity. For example, the crustin type III, the SWD (Amparyup et al., 2008a; Jia et al., 2008) exhibits antibacterial as well as anti-proteinase activities. In contrast, the crustin*Pm*1 (Supungul et al., 2008) and other crustins type II such as crus-like *Pm* (Amparyup et al., 2008b) and the cruFc (Zhang et al, 2007) lack proteinase inhibitory activity. Although each crustin has the conserved WAP domain, each of them may have different functions including antimicrobial and proteinase controlling activities, among others. The differences may be due to the amino acids in the reactive site like the P_1 amino acid residue, other reactive amino acid residues in the WAP domain and other regions besides the WAP domain.

The primary contact region in proteinase inhibition of SLPI and elafin includes the amino acids around the second conserved cysteine residue of WAP domain from P_5 to P_2'. Vargas-Albores et al. (2004) proposed that the P_1' Met was the key contribution to selected activity. The influence of Met at P_1' in proteinase inhibitory activity was mentioned. The P_1 and P_1' residues of the C-terminal domain of SLPI are Leu72 and Met73, respectively. Modifications of Met73 strongly and negatively affected the proteinase inhibitory activity indicating the importance of Met73 in the interaction between SLPI and its target proteinases (Kramps et al., 1990; Nobar et al., 2005; Doucet et al., 2007). Oxidation of the Met residue in the reactive loops of SLPI (Boudier et al., 1994), α1-PI (Taggart et al., 2000) and elafin resulted in the decrease in proteinase inhibitory activity. Replacing the P1′ Met by Leu in elafin (mutant M25L) slightly decreased the affinity of inhibitor for HNE (Nobar et al., 2005).

To study the importance of the P_1 and P_1' amino acids of crustin*Pm*1, the P_1 amino acid was changed to Leu and the P_1' was changed to either Leu, His or Met which hopefully the mutants would inhibit the chymotrypsin or elastase. To our dissatisfaction, the crustin*Pm*1_M, crustin*Pm*1_LM, crustin*Pm*1_LL and crustin*Pm*1_LH did not inhibit all four serine proteinases: subtilisin, trypsin, chymotrypsin and elastase, tested in contrast to the notion emphasized on the importance of a Met residue at P1 (α1-PI) or P1′ (SLPI or elafin) as a major determinant for proteinase inhibition.

When the amino acid spacing between the conserved Cys2 and Cys3 of crustin*Pm*1 and WAP domain-containing proteins with antiproteinase activities were compared, it revealed that the spacing of crustin*Pm*1 contained thirteen amino acids

which was longer than the spacing of SLPI and elafin that contained eight amino acids. Also, the amino acid spacing between the Cys2 and Cys3 of human whey four disulphide core domain (WFDC) proteins was one amino acid shorter than those of SLPI and elafin (seven amino acids) might be insufficient for the structural fold of these WFDC domains for the generation of an active protease inhibitory site (Bingle et al., 2008). Thus, the five amino acids between the Cys2 and Cys3 of crustin*Pm*1 were deleted. Again, the crustin_del mutant was non-inhibitory for all serine proteinases tested.

The SWD*Pm*2 with six amino acids between the Cys2 and Cys3 and proteinase inhibitory activity against subtilisin (Amparyup et al., 2008a) was used to study the importance of P_1 amino acid. Its P_1 amino acid which was the first amino acid residue C-terminal to Cys2 according to the P_1 position of mammalian SLPIs was mutated. The E30R mutation was expected to change the inhibitory specificity from subtilisin to also trypsin while the E30P mutation inactivated the proteinase inhibitory activity. It turned out that both SWD*Pm*2 mutants were as active the subtilisin inhibitor as the wildtype (Amparyup et al., 2008a). The results were in contrast to those of the *Pm*DWD. The *Pm*DWD, a seemingly SLPI version of the shrimp, contained 8 amino acids between the conserved Cys2 and Cys3 of its second WAP domain and was inactive in proteinase inhibition. Mutation of the P_1 Phe to Arg (F70R) in the second WAP domain rendered it proteinase inhibitory active against subtilisin. Thus, the amino acid spacing between the conserved Cys2 and Cys3 was probably not important as the amino acid at the P_1 site. From the above results, one could conclude that the proteinase inhibitory activity was dependent on several factors not just the P_1' Met residue such as the amino acid spacing between the conserved

Cys2 and Cys3, the architecture of inhibitor and its reactive loop, and most importantly the amino acid at the P_1 site.

The crustin*Pm*1 contains the glycine-rich repeat region and the cysteine-rich region at its N-terminal while the SWDs contain a short proline-arginine-rich region, and SLPIs are two WAP domains without any other extra motifs. The extra region at the N-terminus of crustin*Pm*1 might possibly prohibit its proteinase inhibitory activity. It was, therefore, interesting to see if the deletion of extra motifs might rendered the protein proteinase inhibitory active. It was found out that the deletion mutant, rcrustin*Pm*1_WAP, exhibited subtilisin inhibition. Less inhibitory activities were from the rcrustin*Pm*1_delC and rcrustin*Pm*1_WAP_LM.

Therefore, the WAP domain of crustin*Pm*1 only was capable of proteinase inhibition. However, the potency of inhibition was low comparing with other WAP domain-containing antiproteinases. The mole ratio of rcrustin*Pm*1_WAP to proteinase was as high as 600 for about 80% inhibition which is exceedingly high for the protein to perform its function as proteinase inhibitor. In the penaied shrimps, the crustin*P*m1, thus, did not function as the proteinase inhibitor. Though the potency of inhibition was low, the P_1 and P_1´ amino acids were still proved to be important as the LM mutation in rcrustin*Pm*1_WAP_LM deteriorated the subtilisin inhibition.

For the antimicrobial activity, it is, generally, known that most antimicrobial cationic peptides have the same unique features, that is, they are both polycationic (having a net positive charge of more than +2) and fold into amphipathic structures (having both hydrophobic and hydrophilic regions) (Zhao and Wang, 2008). As such, these characteristics enable them to interact with the negatively charged surface molecules of bacteria and to interact with and penetrate into the negatively charged

cytoplasmic membranes of most bacteria (Hancock, 1997). This is, therefore, a reason for the high anti-microbial activity of rcrustin*Pm*1 compared to that of rcrustin*Pm*1 deletion mutants against *S. aureus*. The WAP domain in rcrustin*Pm*1_WAP exhibited indiscernible antimicrobial activity. The antimicrobial potency of rcrustin*Pm*1_delC deletion mutant with the remaining glycine-rich repeat was inferior to the wild type rcrustin*Pm*1. The rcrustin*Pm*1_delG deletion mutant with the glycine-rich repeat deleted had less antimicrobial activity than rcrustin*Pm*1_delC and wild type rcrustin*Pm*1, respectively. The result showed that the glycine-rich and cysteine-rich regions are essential for the antimicrobial activity of crustin*Pm*1. The results agreed well with those of omwaprin that the WAP domain possessed no antibacterial activity and the extra N-terminal sequence was essential for the antibacterial activity (Nair et al., 2007). The single WAP domain of SWD from *F. chinensis* (Fc-SWD) was shown to have also low antimicrobial activity but had anti-protease activities (Jia et al., 2008).

In the preparation of rcrustin*Pm*1 and its mutants, the rcrustin*Pm*1 was expressed as inclusion bodies. The rcrustin*Pm*1 mutants were purified under denaturing condition. These mutants did not exhibit proteinase inhibitory activity. It was suspected that the denaturing condition might affect its activity. The non-denaturing condition was employed to purify the recombinant proteins. These mutants also failed to exhibit the proteinase inhibitory activity. It was very unfortunate that the recombinant proteins, especially, the rcrustin*Pm*1 and its P_1 and P_1' mutants, were often precipitated. Thus, the concentrations of recombinant proteins were limited for the tests. It should be noted that only the rcrustin*Pm*1_WAP was not precipitated.

In mammals, a double WAP-containing protein, SLPI, is extensively studied. It plays important roles in host defense, wound healing and growth (Ding et al., 1999; Zhu et al., 2002; Bouchard et al., 2006). It is an anti-inflammatory molecule for it specifically inhibits the neutrophil elastase secreted upon infection or tissue injury. The two WAP domains in SLPI are not equal; the N-terminal WAP is seemingly involved in antimicrobial activity while the C-terminal WAP possesses a proteinase inhibitory activity (Fitch et al., 2006; Moreau et al., 2008). Analogous to the mammalian SLPI, a double WAP containing protein has been identified in penaeid shrimp from *M. japonicus*, *L. vannamei*, *F. chinensis* as well as *P. monodon* in this study and named generally as DWD as it contains essentially two domains of WAP like SLPI. It is expected that the shrimp DWD might exert its functions similar to that of SLPI. The antimicrobial and proteinase inhibitory activities were most studied.

In this study, we had compared the amino acid sequence of *Pm*DWD with those DWDs from other shrimp and a few mammalian SLPIs. The comparison reveals that the two 4-DSCs, either in SLPIs or DWDs, were not equal in terms of the spacing among the cysteine residues and the amino acid sequences within. These differences were more extensive between the SLPIs and DWDs. Along with the difference in P_1 reactive residues of proteinase inhibitory activity, mostly leucine and phenylalanine in SLPIs and DWDs, respectively, it is suggested that the DWDs might be different in their activities to a certain extent.

The quantitative RT-PCR revealed that the *Pm*DWD was up-regulated in response to WSSV and *V. harveyi* infection during the first 24 h (Naritsara, unpublished). The response was more pronounced with *V. harveyi* than WSSV. The results were in agreement with those of Mj-DWD (Chen et al., 2008) and Fc-DWD

(Du et al., 2009). Chen et al. (2008) also found that the high level of Mj-DWD was maintained in WSSV-resistant shrimp. It was, therefore, quite conclusive that the DWDs in shrimp were involved in immune response to pathogenic infections.

Tissue distribution analysis revealed that the *Pm*DWD are constitutively expressed in all tissues. The transcription was predominant in hemocytes is in accordance with antimicrobial peptides (AMPs) of the crustintaceans, including penaeidin (Destoumieux et al., 1997) and carcinin (Chisholm et al., 1992; Schnapp et al., 1996), are synthesized and stored in the granular hemocytes and released by exocytosis upon microbial invasion.

Since the response was against both bacteria and virus, it was possible that the DWD might act as anti-inflammatory molecule like the SLPI to control the inflammatory response to infection. If this was to be the case, the DWD would be up-regulated in response to inflammation caused by other means. To create inflammation by injury, we cut off a few legs from the shrimp. The expression of *Pm*DWD in various tissues of these shrimp was compared with that of WSSV-infected shrimp. We found that the *Pm*DWD was expressed in all tissues tested from normal, leg-amputated and WSSV-infected shrimp. Higher expression of *Pm*DWD was clearly observed in lymphoid from leg-amputated and WSSV-infected shrimp. The lesion from leg amputation might make the shrimp susceptible to infection from the surrounding microbes and caused inflammation response. The same response occurred also for WSSV infection. Since the lymphoid is the site of pathogen clearance in shrimp (van de Braak et al., 2002), the up-regulation of *Pm*DWD in lymphoid indicated, therefore, that it is involved in the function of lymphoid.

It was, then, reasoned that the tissues of interest, lymphoid in this case, might contain proteinases that could be inhibited by *Pm*DWD. The r*Pm*DWD was over-expressed using an *E. coli* expression system. The r*Pm*DWD was tested inactive against the commercial proteinases: trypsin, chymotrypsin, elastase and subtilisin. This was rather puzzling for the similar mammalian SLPI was active against certain proteinases. It was possible that the P_1 reactive amino acid was not appropriate. We, therefore, mutated the P_1 residue at position 70 from phenylalanine to arginine to hopefully change the substrate specificity of r*Pm*DWD for trypsin or subtilisin. As expected, the mutant r*Pm*DWD_F70R was shown to inhibit subtilisin quite effectively. Thus, the wildtype r*Pm*DWD might possibly be a rather specific inhibitor not specific to the commercial proteinases tested. The r*Pm*DWD was, then, tested against the proteinases from the shrimp tissues.

Tissues were removed from normal, leg-amputated and WSSV-infected shrimp for the preparation of tissue lysates which were used as crude proteinases. For normal shrimp, the proteinase activities were observed only in proteinase-rich tissues like intestine, hepatopancreas and stomach but not in other tissues tested. These proteinase activities, however, were barely or not at all inhibited by the r*Pm*DWD. Interestingly, additional proteinase activities were observed in hemolymph and lymphoid from leg-amputated and WSSV-infected shrimp indicating that some proteinases were released into the circulation possibly in the process of inflammation. More interestingly, the r*Pm*DWD could inhibit, though partly, the proteinase activities in lymphoid lysate. In agreement with the higher expression of *Pm*DWD in lymphoid upon leg amputation and WSSV infection, the *Pm*DWD might function in controlling the clearance of foreign matters in the lymphoid by certain proteinases activated in

response to infection. We have recently shown that the lysosomal cysteine proteinases; cathepsins B, cathepsins L, peritrophin and thrombospondin are abundantly expressed in the lymphoid organ (Pongsomboon et al., 2008). These proteinases are believed to be involved in the functions of lymphoid and the *Pm*DWD probably helps controlling their activities.

Like its orthologs in *M. japonicus* and *F. chinensis*, the r*Pm*DWD was also able to inhibit the crude proteinases from *B. subtilis* suggesting the host protective function of *Pm*DWD against bacterial invasion.

Most puzzling was the observation that the *Pm*DWD had no antimicrobial activity though its expression was responding to the pathogenic infections. This was in accordance with those observed in DWDs from *M. japonicus* and *F. chinensis*. It might be that the DWDs in penaeid shrimp needed not be antimicrobial since there were several other WAP-containing proteins available for this function. The penaeid DWDs, then, function through their proteinase inhibitory activities only. Along with other results above, we hypothesized that the *Pm*DWD functioned as proteinase inhibitor in the modulation of the degradation of foreign matters including the bacteria, viruses, etc. in the shrimp lymphoid organ.

CHAPTER V

CONCLUSIONS

Crustins: crustin*Pm*1 and SWD*Pm*2

1. The P_1 and P_1' crustin*Pm*1 mutants: rcrustin*Pm*1_M, rcrustin*Pm*1_LM, rcrustin*Pm*1_LL, rcrustin*Pm*1_LH and rcrustin*Pm*1_del, the five-amino acid deletion between the Cys2 and Cys3 did not inhibit against subtilisin, trypsin, chymotrypsin and elastase.
2. The deletion mutants with extra motifs at the N-terminal half of crustin*Pm*1 deleted: rcrustin*Pm*1_delC, rcrustin*Pm*1_WAP and rcrustin*Pm*1_WAP_LM exhibited very low subtilisin inhibitory activity. Therefore, the WAP domain itself was capable of proteinase inhibition but not good enough to be an inhibitor.
3. The rSWD*Pm*2_E30R and rSWD*Pm*2_E30P which were mutated at the presumed P_1 amino acid according to the P_1 position of mammalian SLPIs turned out to be strong subtilisin inhibitor like the wild type.
4. The proteinase inhibitory activity was dependent on several factors not just the P_1' Met residue as speculated in literatures. Other factors could be the amino acid spacing between the conserved Cys2 and Cys3, the architecture of inhibitor and its reactive loop, and most importantly the amino acid at the P_1 site. The P_1 and P_1' amino acids were proved to be important.
5. The rcrustin*Pm*1_WAP did not show antimicrobial activity while the rcrustin*Pm*1_delG and rcrustin*Pm*1_delC exhibited lower antimicrobial

activity than the rcrustin*Pm*1 indicating the importance of glycine-rich and cysteine-rich regions for antimicrobial activity.

***Pm*DWD**

1. Tissue distribution analysis revealed that the *Pm*DWD was constitutively expressed in all tissues but predominantly in hemocytes. Much less expression was in hepatopancreas, eyestalk and lymphoid.
2. The expression of *Pm*DWD from the 24-h WSSV-infected and leg-amputated shrimps was clearly up-regulated in lymphoid.
3. The r*Pm*DWD exhibited inhibition against the crude proteinases from bacterial cultures. *B. subtilis*.
4. The r*Pm*DWD had no proteinase inhibitory activity against all four serine proteinases: subtilisin, trypsin, chymotrypsin and elastase but its mutant r*Pm*DWD_F70R had strongly proteinase inhibitory activity against subtilisin.
5. The proteinase activities were found in the tissue lysate of lymphoid and hemolymph from leg-amputated and 24-h WSSV-infected but not untreated shrimp. The r*Pm*DWD inhibited against only proteinase activities from lymphoid.
6. The r*Pm*DWD did not exhibit the antimicrobial activity against various bacteria both Gram-positive: *B. subtilis*, *B. megaterium*, *M. luteus*, *S. aureus*, *S. haemolyticus*, *S. iniae*, and Gram-negative: *E. cloacae*, *E. carotovora*, *E. coli* 363, *V. harveyi* 1526, *K. pneumoniae*, *S. thyphimurium*.
7. The *Pm*DWD might function in controlling the clearance of foreign matters in the lymphoid by certain proteinases activated in response to infection.

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APPENDIX

1. Preparation for SDS-PAGE electrophoresis

Stock reagents

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

Acrylamide 29.2 g

N,N′-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

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

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8 75 ml

10% SDS 4 ml

Distilled water 21 ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8 50 ml

10% SDS 4 ml

Distilled water 46 ml

SDS-PAGE

15 % Seperating gel

30 % Acrylamideml solution 5.0 ml

Solution B 2.5 ml

Distilled water 2.5 ml

10% (NH4)2S2O8 50 µl

TEMED 10 µl

5.0 % Stacking gel

30 % Acrylamideml solution 0.67 ml

Solution C 1.0 ml

Distilled water 2.3 ml

10 % (NH4)2S2O8 30 µl

TEMED 5.0 µl

5X Sample buffer

1 M Tris-HCl pH 6.8 0.6 ml

50% Glycerol 5.0 ml

10% SDS 2.0 ml

2-Mercaptoethanol 0.5 ml

1 % Bromophenol blue 1.0 ml

Distilled water 0.9 ml

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane 3.03 g

Glycine 14.40 g

SDS 1.0 g

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

BIOGRAPHY

Miss Pranisa Suthianthong was born on August 16, 1984 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Biology, Faculty of Science, Chulalongkorn University in 2006. She has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2007.

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