สมบัติการจับและการตกผลึกของ crustin*Pm*1 และ crustin*Pm*7 ของกุ้งกุลาดำ *Penaeus monodon*

นาย ภาสพงศ์ พูลพิพัฒน์

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BINDING PROPERTIES AND CRYSTALLIZATION OF CRUSTIN*Pm*1 AND CRUSTIN*Pm*7 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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ภาสพงศ์ พูลพิพัฒน์: สมบัติการจับและการตกผลึกของ crustin*Pm*1 และ crustin*Pm*7 ของกุ้งกุลาดำ *Penaeus monodon* (BINDING PROPERTIES AND CRYSTALLIZATION OF CRUSTIN*Pm*1 AND CRUSTIN*Pm*7 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. เกื้อการุณย์ ครูส่ง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. อัญชลี ทัศนาขจร. 91 หน้า.

ครัสทิน (crustin) จัดเป็นเปปไทด์ต้านจุลชีพ (Antimicrobial peptides, AMPs) ประเภทหนึ่งที่พบในระบบภูมิคุ้มกันแบบไม่จำเพาะของสัตว์จำพวกกุ้ง (crustacean) ก่อนหน้านี้มีการศึกษาความสามารถในการต่อต้านเชื้อแบคทีเรียของครัสทินพบว่า crustin*Pm*1 มีฤทธิ์ในการต้านเชื้อแบคทีเรียชนิดแกรมบวกได้ดี ในขณะที่ crustin*Pm*7 สามารถต้านเชื้อแบคทีเรียได้ทั้งแบคทีเรียชนิดแกรมบวกและแกรมลบ งานวิจัยนี้มีวัตถุประสงค์คือ (1) ศึกษาสมบัติการจับของรีคอมบิแนนท์ crustin*Pm*1 และ crustin*Pm*7 กับเซลล์แบคทีเรียและองค์ประกอบของผนังเซลล์แบคทีเรีย lipoteichoic acid (LTA) และ lipopolysaccharide (LPS) (2) ศึกษาฤทธิ์ในการเหนี่ยวนำให้เกิด bacterial agglutination ของครัสทินทั้งสองชนิด (3) ศึกษาผลของครัสทินต่อการเปลี่ยนแปลง Inner membrane (IM) permeability (4) ศึกษาผลของครัสทินต่อการเปลี่ยนแปลงทางกายภาพของเซลล์แบคทีเรีย และ (5) ศึกษาสภาวะที่เหมาะสมในการตกผลึก crustin*Pm*1 และ crustin*Pm*7 ในงานวิจัยนี้ได้ทำการผลิตรีคอมบิแนนท์โปรตีนครัสทินทั้งสองไอโซฟอร์มใน *Escherichia coli* ในสภาวะที่โปรตีนเสียสภาพ (denaturing condition) จากนั้นทำให้โปรตีนคืนสภาพ (renature) เพื่อใช้ทดสอบฤทธิ์ในการต้านเชื้อแบคทีเรีย จากการทดสอบคุณสมบัติการจับของครัสทินกับเซลล์แบคทีเรีย และองค์ประกอบของผนังเซลล์แบคทีเรีย พบว่าครัสทินทั้งสองไอโซฟอร์มสามารถจับกับแบคทีเรียแกรมบวกและแกรมลบและจับกับองค์ประกอบของผนังเซลล์ lipoteichoic acid และ lipopolysaccharide ได้ นอกจากนี้ครัสทินยังสามารถเหนี่ยวนำให้เกิด bacterial agglutination ในแบคทีเรียบางสายพันธุ์ และทำให้เกิดการรั่วซึมของสารภายในไซโตพลาสซึมของ *E. coli* สายพันธุ์ MG1655 ออกสู่ภายนอกเซลล์ได้ เมื่อผสม crustin*Pm*7 กับ *Staphylococcus aureus*, *Escherichia coli* และ *Vibrio harveyi* แล้วนำไปตรวจสอบด้วยเทคนิค Scanning Electron Microscopy (SEM) พบว่า เกิดการเปลี่ยนแปลงผนังเซลล์แบคทีเรียทุกสายพันธุ์ ในขณะที่ crustin*Pm*1 สามารถทำให้เกิดการเปลี่ยนแปลงผนังเซลล์ของ *S. aureus* และ *E. coli* เท่านั้น จากการทดลองหาสภาวะที่เหมาะสมในการตกผลึกครัสทิน พบว่า crustin*Pm*1 ตกผลึกในสารละลายที่มีองค์ประกอบของ 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350 pH 8.4 อย่างไรก็ตาม ยังไม่พบสภาวะที่เหมาะสมในการตกผลึก crutin*Pm*7

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PASAPONG POOLPIPAT : BINDING PROPERTIES AND CRYSTALLIZATION OF CRUSTIN*Pm*1 AND CRUSTIN*Pm*7 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR : KUAKARUN KRUSONG, Ph.D., CO-ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., 91 pp.

Crustin is one of several antimicrobial peptides (AMPs) in innate immune system of crustacean. In previous study, crustin*Pm*1 strongly inhibited Gram-positive bacteria, whilst crustin*Pm*7 acted against both Gram-positive and Gram-negative bacteria. The aim of this study is to investigate (1) Binding properties of rcrustin*Pm*1 and rcrustin*Pm*7 to bacterial cells and cell wall components, lipoteichoic acid (LTA) and lipopolysaccharide (LPS) (2) Induction of bacterial agglutination by rcrustin*Pm*s (3) Inner membrane permeabilization (4) Effects of rcrustin*Pm*s on bacteria cells and (5) Crystallization of rcrustin*Pm*s. In this study, the recombinant crustin*Pm*1 and crustin*Pm*7 were produced in *Escherichia coli* and purified under denaturing condition. The purified rcrustin*Pm*s were then renatured and used in antimicrobial activity test. Binding study suggested that both crustin isoforms can bind to both Gram-positive and Gram-negative bacteria, as well as cell wall components LTA and LPS. Crustin*Pm*s can induce bacterial agglutination in some strains of bacteria. They can also change inner membrane permeability of *E. coli* strain MG1655, resulting in cytoplasmic β-galactosidase release. Scanning Electron Microscopy (SEM) revealed physical change on cell surface of *Staphylococcus aureus, Vibrio harveyi* and *E. coli* treated with rcrustin*Pm*7. Recombinant crustin*Pm*1; however, can cause physical changes on cell surface of *S. aureus* and *E. coli* only. Crystallization experiments were carried out. Recombinant crustin*Pm*1 was crystallized in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4. However, no crytallization condition for rcrustin*Pm*7 was found.

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LIST OF ABBREVIATIONS

bp	base pair
DNA	deoxyribonucleic acid
EtBr	ethidium bromide
h	hour
kb	kilobase
LPS	lipopolyscaccharide
LTA	lipoteichoic acid
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
nm	nanometre
OD	optical density
^{o}C	degree Celcius
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
rcrustin*Pm*s	Recombinant crustin*Pm*s
sec	second
μg	microgram
μl	microliter
μM	micromolar

CHAPTER I

INTRODUCTION

1.1 General introduction

The black tiger shrimp *Penaeus monodon* farming is one of the most important aquaculture in Thailand. In Asia, Thailand is the one of top ten countries in shrimp production. The black tiger shrimp hatcheries and farms are dispersed along the coastal area of the country. Nakorn Sri Thammarat and Surat Thani are the provinces that has highest yield of shrimps. Thailand location has several advantages for shrimp cultivation, including no typhoon or cyclone seasons, small variable of sea water during season and ideal soy and terrain for pond construction. Previously, the main cultured species shrimp in Thailand was the black tiger shrimp *P. monodon*. This made *P. monodon* shrimp an economically important species in Thailand (Source : FAO Fishstat 2006). Since the year 2002, the production of the black tiger shrimp in Thailand was decreased continuously due to infectious disease. Until now, the export value of the black tiger shrimp in Thailand fell down over 10 times from 28,299.90 million baht in 2002 to 2,749.10 million baht in 2010 (Table 1.1).

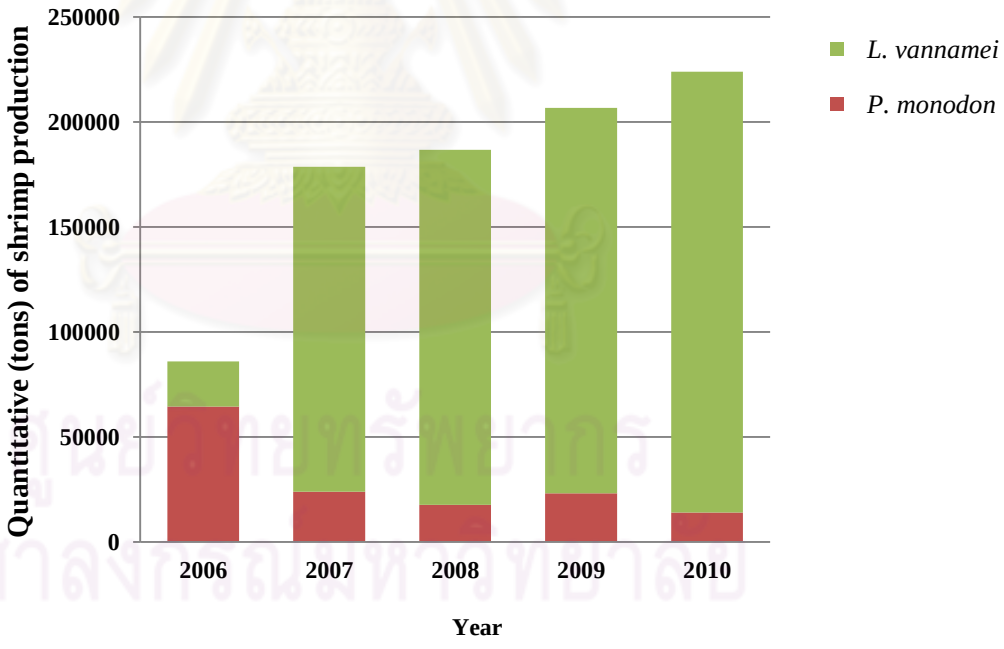
The black tiger shrimp farming has been replaced with white shrimp *Litopenaeus vannamei* farming (Figure 1.1) as the white shrimp has higher growth rate, higher survival rate lower production cost than that of the black tiger shrimp. However, replacing the black tiger shrimp farming with the white shrimp is not an idea solution as the white shrimp is not native species and it carries various viral pathogens such as *Baculovirus penaei* (BP) and *Taura Syndrome virus* (TSV). These viruses can be transmit to the *P. monodon*, and cause a catastrophic lost in *P. monodon* production. In addition, the white shrimp *L. vannamei* has lower export price than the black tiger shrimp, and this affects the country's shrimp export in term of value. The demand of the black tiger shrimp in overseas markets remains high and it is likely to grow annually.

By these reasons, it is important to study the shrimp immune-related genes and their products as it would lead to better understanding of the immune system in the black tiger shrimp. This could effectively solve the outbreaks to revive the black tiger

shrimp industry. New technology to help increase the *P. monodon*'s survival rate and lower its production cost is unavoidably required.

Table 1.1 Aquaculture production of black tiger shrimp in Thailand (Source: office of Agricultural Economics, www.oae.go.th/oae_report/export_import/export.php)

Year	Total	
	Quantity (tons)	Value (million baht)
2002	83,036	28,299.90
2003	77,922	24,201.40
2004	53,012	15,013.00
2005	33,036	8,568.00
2006	28,987	6,965.80
2007	19,314	4,450.30
2008	14,283	2,680.90
2009	11,238	2,355.00
2010	14,099	2,749.10



Source: office of Agricultural Economics
(www.oae.go.th/oae_report/export_import/export.php)

Figure 1.1 The black tiger shrimp and white shrimp export of Thailand during 2006 to 2010.

1.2 Shrimp diseases

In many countries, the serious problem in shrimp industry is infection of shrimp diseases due out lack of proper knowledge on shrimp biology, farm management, and shrimp diseases. In Thailand, the outbreaks of infectious disease have become the most serious problem since 1993. *P. monodon* are mainly infected by virus and bacteria. The major infectious viruses are yellow-head virus (YHV), spreading in central and southern Thailand during 1993 to 1994 (Hasson et al., 1995) and white spot syndrome virus (WSSV), outbreaking during 1994 to 1996 (Flegel, 1997). Taura syndrome virus (TSV) and infectious hypodermal and hematopoeitic virus (IHHNV) have now become important (Flegel, 2006). The major virulent strains of *Vibrio* in shrimp e.g. *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram* cause the Vibrisis disease. This most prevalent bacterial disease could cause mass mortalities both in hatcheries and in shrimp growout ponds (Saulnier et al., 2000).

1.3 Viral diseases

Shrimp farming industry in Thailand encountered a severe problem of viral infectious diseases for over a decade. The harmful virus species that have been reported in *P. monodon* are white spot syndrome virus (WSSV) and yellow-head virus (YHV) which cause white spot syndrome (WSS) and yellow-head disease (YH), respectively (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995). The outbreak of these virus diseases causes great losses in the shrimp industry.

1.3.1 White spot syndrome (WSS) disease

Lightner (1996) has called this virus white spot syndrome baculovirus (WSSV) because its morphological characteristics are similar to insect baculovirus. However, by phylogenetic analysis of ribonucleotide reductase and protein kinase genes, WSSV does not share a common ancestor with baculovirus (Van Hulten et al., 2000; Van Hulten et al., 2001). The WSSV genome contains double-stranded DNA of about 292 to 305 kb in length (Van Hulten et al., 2001; Yang et al., 2001). Original outbreaks were reported from China in 1993 and spread rapidly thereafter to Japan, Thailand, Korea, India, USA, Central and South America. The disease is spread by contaminated water, decomposing fecal matter or tissue, cannibalism and fluid from infected females. Shrimp may be indirectly infected by exposure to previous hatchery

or pond growing cycles, contaminated water supplies, contaminated food, equipment surfaces and clothing , or animals which have ingested diseased shrimp.

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

1.3.2 Yellow head (YH) disease

In Thailand, the disease was first reported in 1990. Yellow head disease occurs in the juvenile to sub-adult stages of shrimp, especially at 50-70 days of grow-out (Lightner, 1996). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley et al., 1999). Infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and have a generally pale or bleached appearance (Limsuwan, 1991). The swim slowly near the surface at the pond edges in moribund shrimp. Mortality may reach as high as 100% of affected populations within 3-5 days from the onset of disease after infected.

1.4 Bacterial diseases

Vibrio species are a normal part of the bacterial flora in aquatic environments and formerly considered to be major pathogens (Lightner, 1988). The bacteria cause the most serious diseases of the larval and postlarval stages of *P. monodon* (Lightner, 1983; Lightner, 1988; Lightner, 1996).

The luminescent bacterium, *Vibrio harveyi*, frequently related to the outbreaks of luminous vibriosis in cultured *P. monodon* in hatcheries in many countries such as Australia, China, India, Indonesia, Thailand, Philippines, and Taiwan (Vandenberghe

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

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

The disease caused by *V. harveyi* is widely known as luminous disease. The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease. These superficial infections can develop into systemic infections under some circumstances. These systemic infections can cause mortality. Other gross features of the infected shrimp are milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite, eventually leading to death.

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

1.5 The immune responses in invertebrates

All living organisms have developed immune system for defending themselves against microbial invasion or other foreign substances. Immune system can be evolutionarily classified into two types: adaptive (acquired) and innate

(natural) immunity. Vertebrates possess both adaptive and innate immune systems, whereas invertebrates have only innate immune system. The adaptive immune system functions by producing highly specific recognition molecules, known as antibodies, which can memorize foreign molecules after the first exposure. The innate immune system involves a large number of generalized effector molecules.

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

1.6 Crustacean immune system

Crustacean immune system is innate immune system based on cellular and humoral components of the circulatory system. The hard cuticle covering all external surfaces of crustaceans is the first line of defense against the environment. The innate immune system can respond rapidly if microorganisms invade the animals. Despite their relatively short life and assumed lesser complexity, crustaceans have mechanisms to detect foreign matter. Crustacean haemocytes play important roles in the host immune response including recognition, phagocytosis, melanization, cytotoxicity and cell-cell communication. Classification of the haemocyte types in decapod crustaceans is based mainly on the presence of cytoplasmic granules into hyaline cells, semigranular cells, and granular cells. Each cell type is active in defence reactions, for example, the hyaline cells are chiefly involved in phagocytosis, the semigranular cells are the cells active in encapsulation, while the granular cells participate in storage and release of the prophenoloxidase (proPO) system and cytotoxicity. The haematopoietic tissue has been described in several crustacean decapod species and shown to be the haemocyte-producing organ. Tentative stem cells have been shown to be present in this tissue.Iin situ hybridization demonstrated that proPO is not present in the haematopoietic tissue of crustaceans which suggests that protein expression is different between circulating haemocytes and the cells in the haematopoietic tissue.

1.6.1 Pattern recognition proteins

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

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

In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin that, besides its bacterial agglutination ability, increases phagocytic rate (Vargas-Albores, 1995).

1.6.2 Cell-mediated defense reactions

Cellular defense actions include phagocytosis, encapsulation and nodule formation (Millar, 1994). Phagocytosis is a phenomenon that in all organisms. It includes attachment to the foreign body, ingestion and destruction. Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation, which appears similar

to capsule formation, occurs when the number of invading bacteria is high. These structures, capsules and nodules, are always melanized in arthropods.

1.6.3 Hemocyte adhesion molecules

In invertebrate immunity, cell adhesion is essential for the cellular immune responses of encapsulation and nodule formation. Blood cells of the crayfish, *Pacifastacus leniusculus*, can release a cell-adhesive and opsonic peroxidase called peroxinectin (Lin et al., 2007). A site containing the motif, KGD, appears to be adhesive by binding to a transmembrane receptor of the integrin family on the blood cells (Johansson et al., 1995). Peroxinectin also binds to a peripheral blood cell surface CuZn-superoxide dismutase. The peroxidase-integrin interaction appears to have evolved early and seems conserved. Human myeloperoxidase supports cell adhesion via the αMβ2 integrin. There is evidence for peroxinectin-like proteins in other arthropods. Effects by RGD peptides indicate that integrins mediate blood cell adhesion and cellular immunity in diverse invertebrate species (Johansson et al., 1989). Other blood cell molecules proposed to be involved in cell adhesion in invertebrates including the insect plasmatocyte-spreading peptide, as well as soluble and transmembrane proteins which show some similarity to vertebrate adhesive or extracellular matrix molecules. Proteins such as the Ig family member hemolin or proteins found in insect hosts for parasitic wasps, inhibit cell adhesion and may regulate or block cellular immunity (Johansson, 1999).

1.6.4 The prophenoloxidase (proPO) system

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

In vitro studies have shown that phenoloxidase (PO) exists as an inactive precursor, prophenoloxidase (proPO), which is activated by a stepwise process involving serine proteases activated by microbial cell wall components, such as low quantities of lipopolysaccharides or peptidoglycans from bacteria and β-1,3-glucans from fungi, through pattern-recognition proteins (PRPs) (Ariki et al., 2004). An enzyme that is able to activate the proPO *in vivo* is termed prophenoloxidase activating enzyme (factor) (ppA, PPAE, PPAF). In crayfish, ppA is a trypsin-like proteinase present as an inactive form in the haemocyte granules. After degranulation,

the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA will convert proPO to an active form, phenoloxidase (PO) (Aspan et al., 1991; 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Söderhäll et al., 1998; Shiao et al., 2001). It both catalyses O-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can polymerise non-enzymatically to melanin. PO is a sticky protein and can adhere to the surface of parasites, which will lead to melanisation of the pathogen.

Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites, such as the crayfish plague fungus, *Aphanomyces astaci* (Söderhäll et al., 1982). The production of forming insoluble melanin deposits involving in the process of sclerotisation, wound healing and encapsulation of foreign materials (Theopold et al., 2004). To prevent excessive activation of the proPO cascade, proteinase inhibitors are needed for its regulation.

1.6.5 The coagulation/clotting system

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

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

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

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

1.6.6 Proteinase inhibitors

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

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

Like blood clotting, phenoloxidase activation is normally regulated in vivo as a local reaction with brief duration. Also comparable to blood clotting, the regulation may be partly due to serine proteinase inhibitors in plasma (Kanost et al., 1996). Pacifastin and, to the lesser degree, α-macroglobulin inhibit crayfish PPO activation (Aspan et al., 1990). Among the low molecular weight inhibitors from insect hemolymph, Kunitz family inhibitors from *M. sexta*, *Sarcophaga bullata*, and *B. mori* (Sugumaran et al., 1985; Saul et al., 1986; Aso et al., 1994) and the 4 kDa

locust inhibitors (Boigegrain et al., 1992) can interfere with PPO activation. Serpin-1J from hemolymph of *M. sexta* inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang et al., 1997). Recently, the *M. sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang et al., 2004). In addition, its structure and function were further characterized by cloning and expression in *E. coli* expression system (Zou et al., 2005) The results indicated that serpin-6 plays important roles in the regulation of immune proteinases in the hemolymph. It is likely that each proteinase in the PPO cascade is regulated by one or more specific inhibitors present in plasma or in hemocyte granules.

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

1.7 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are effector molecules that play an evolutionarily conserved component of the innate immune system. They are found throughout all kingdoms from bacteria to human. Most of the AMPs are small in size, generally less than 150-200 amino acid residues, with amphipathic structure and cationic property. Anionic peptides also exist. AMPs have a wide variety and diversity in amino acid sequences, structure, and range of activity. AMPs are active against a large spectrum of microorganisms, including bacterial and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities.

The diversity of AMPs is divided into sub-groups on the basis of their amino acid composition and structure. Peptides in the first subgroup are small anionic antimicrobial peptide (721.6–823.8 Da), which present in surfactant extracts, bronchoalveolar lavage fluid and airway epithelial cells (Brogden et al.,1996; Brogden et al.,1998; Brogden et al.,1999). They are requires zinc as a cofactor for antimicrobial activity and can act against Gram-positive and Gram-negative bacteria. The second subgroup AMPs contain ~290 short cationic peptides that lack in cysteine residues and sometimes have a hinge or ‘kink’ in the middle (Gennaro et al., 2000;

Tossi et al., 2000). All or part of their molecules is converted to an α-helix when presence of trifluoroethanol, sodium dodecyl sulphate (SDS) micelles, phospholipid vesicles and liposomes, or Lipid A (Gennaro et al., 2000), e. g. LL-37 (Johansson et al., 1998). The extent of α-helicity correlates with the antibacterial activity against both Gram-positive and Gram-negative bacteria (Park et al., 2000). The third subgroub is cationic peptides which are rich in certain amino acid. (~44 group members) (Otvos et al., 2002) This group includes the bactenecins and PR-39, which are rich in proline (34-49%) and arginine (13-33%) residues. These peptides lack cystein residues and are linear, but some can form extened coils. The fourth subgroup is anionic and cationic peptides (~380 members) which contain cysteine residues and form disulphide bonds and stable β-sheet. This subgroup includes a diverse family of defensins. These are ~55α-defensins, which include human neutrophil peptides (HNPs) and cryptdins, and 90β-defensins from both humans (HBDs) and animals that contain six cysteines which are linked by three intramolecular disulpide bonds (Ganz et al., 1990; Lehreret al., 1993; Lehrer et al.,2002; Ganz et al., 2002).

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

There are many reports on antimicrobial peptides in crustaceans. In 1997, a small peptide named, calliectin observed in the hemolymph of blue crab *Callinectes sapidus*, was reported to be responsible for the majority of antimicrobial activity (Khoo et al., 1999). Penaeidins, antimicrobial peptides acting against Gram positive bacteria and fungi, have been reported in penaeid shrimp *L. vannamei* (Destoumieux et al., 1997). cDNA clones of penaeidin isoform were also isolated from the hemocytes of *L. vannamei*, *P. setiferus* (Gross et al., 2001), and *P. monodon* (Supungul et al., 2004). Crustins, an antimicrobial peptide, were identified from 2 species of *Penaeid shrimp*: *L. vannamei* and *L. setiferus*. Several isoforms of crustins were observed in both shrimp species. The 11.5 kDa antibacterial protein from

Carcinus maenas, showed no homology with other known antibacterial peptides, but possessed sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Peptides derived from the hemocyanin of *L. vannamei*, *P. stylirostris*, and *P. monodon* possessing antiviral activity has also been identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Moreover, histones and histone derived peptides of *L. vannamei* has also been reported as innate immune effector. They can inhibit growth of Gram-positive bacteria (Patat et al., 2004).

Like most invertebrates, shrimps lack a true adaptive immune system and rely on innate immune responses, including the production of antimibrobial proteins. Various antimicrobial peptides have been identified in shrimps such as penaeidins, anti-lipopolysaccharide factors (ALFs), lysozymes and crustins.

1.7.1 Mechanisms of antimicrobial peptide

The modes of action by which antimicrobial peptides kill bacteria is varied, including disrupting membranes, interfering with metabolism and targeting cytoplasmic components (Brogden, 2005).

Initial contact between the peptide and the target organism relies on electrostatic, since most bacterial surface are anionic. Amino acid composition, amphipathicity, cationic charge and size of AMPs allow them to attach to and insert into membrane bilayers. Transmembrane pore-forming are the mechanisms of antimicrobial peptide insertion and form pores into membrane to kill bateria by ‘barrel-stave’, ‘carpet’ or ‘toroidal-pore’ models. Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to the lysis of microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing (Brogden, 2005, Bolintineanu et al., 2010). Intracellular killing are the mechanism of antimicrobial peptide penetrate into the cell to bind intracellular molecules which are crucial to cell living, includes inhibition of cell wall synthesis, alteration of cytoplasmic membrane, activation of autolysin, inhibition of DNA, RNA, protein synthesis, and inhibition of certain enzymes (Figure 1.2)

In many cases, the exact mechanism of killing is not known. In contrast to many conventional antibiotics, these peptides appear to be bacteriocidal (bacteria killer) instead of bacteriostatic (bacteria growth inhibitor). In general, the

antimicrobial activity of these peptides is determined by measuring the minimal inhibitory concentration (MIC), which is the lowest concentration of antimicrobialpeptide that inhibits bacterial growth

In many instances, cellular damage lags substantially behind the time required for antimicrobial killing, indicating that some of the ultrastructural damage is artefactual. In other instances, cellular damage occurs at the same rate as that of killing. Lehrer et al. (1989) noted that membranous blebs on HNP-treated *E. coli* continued to accumulate as viable counts decreased and concluded that the appearance of blebs followed, rather than caused, the loss of bacterial viability (Lehrer et al., 1989). In similar studies, *E. coli* incubated with DEFB118 were killed in 15 minutes but cellular damage continued 30–120 minutes after exposure to the peptide (Yenugu et al., 2004). In the same fashion, *P. aeruginosa* cells incubated with SMAP29 or CAP18 were killed in 15 minutes, whereas cellular damage continued for up to 8 hours (Kalfa et al., 2001).

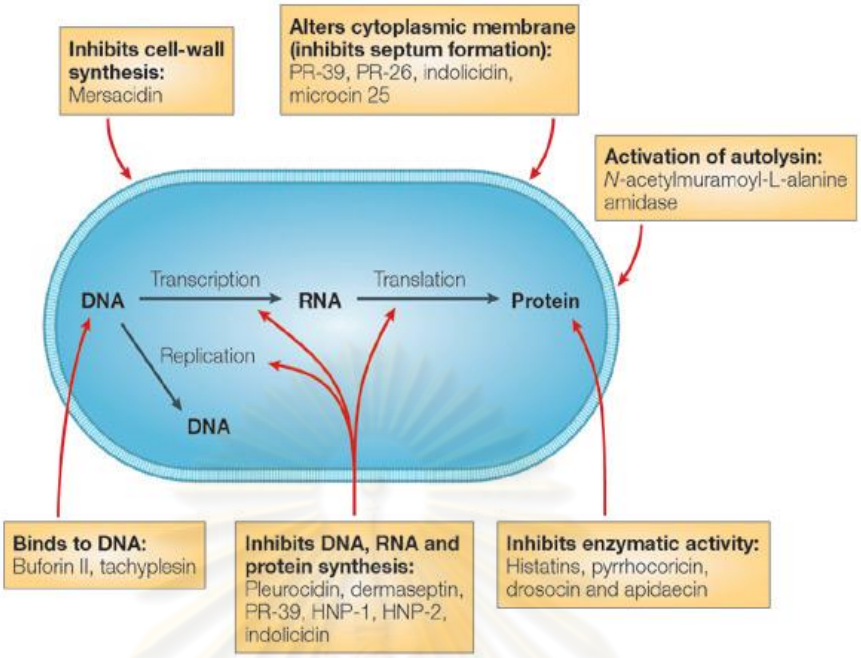


Figure 1.2 Mode of action for intracellular antimicrobial peptide activity.
In this figure *Escherichia coli* is shown as the target microorganism (Brogden, 2005).

1.8 Crustin

Crustin is one type of antimicrobial peptides (AMPs) that have been identified in various crustaceans (Hauton *et al*., 2006, Zhang *et al*., 2007) including several shrimp species e.g. *P. monodon* (Amparyup *et al*., 2008, Supungul *et al*., 2004, Supungul *et al*., 2008), *L. setiferus* (Bartlett *et al*., 2002), *L. vannamei* (Bartlett *et al*., 2002) and *Fenneropenaeus chinensis* (Zhang *et al*., 2007). This peptide is mainly found in the plasma and hemocyte granules of shrimps. The present review considers crustin as a cationic cysteine-rich antimicrobial peptide which contains a single whey acidic protein (WAP) domain at the carboxyl terminus and 50 amino acid residues with eight cysteine residues in a conserved arrangement that forms a four-disulphide core (4DSC) tightly packed structure (Valerie *et al*., 2008). According to Smith et al. (2008), crustins can be classified into three main types based on the structure of the central region. Type I crustins contain cysteine-rich region and the WAP domain. These types of crustins are present mainly in crabs, lobsters and crayfish (Brockton et al, 2007; Christie et al, 2007; Hauton et al, 2006; Jiravanichpaisal et al, 2007; Stoss et al, 2004). Type II crustins have cysteine-rich region and the WAP domain, as well as

a glycine-rich region between signal sequence and cysteine-rich region. Crustins from shrimp is classified in this group. Unlike Type I and II, Type III proteins contain only the WAP domain so the proteins identified in this group are called single-whey domain (SWD) protein, rather than crustins (Figure 1.3)

The term 'WAP' is derived from the name given to a family of proteins, originally discovered in the whey fraction of mammalian milk. All crustins described to date possess a leader/signal sequence at the N-terminus and the WAP domain at the carboxyl end. The WAP domain has also been found in proteins with diverse functions, including protease inhibition (Sallenave, 2000) and antimicrobial activity (Shugars, 1999, Wiedow *et al*., 1998). The WAP domain, in contrast to the signal sequence, is highly conserved between species and in several crustins, especially those from shrimp, aspartic acid and lysine residues are positioned as follows:

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

This arrangement, however, is not true in every case. For example, glycine substitutes for aspartic acid after the first cysteine in a *L. vannamei* EST (AAS57715), and serine replaces lysine before the double cysteine in the *Callinectes sapidus* EST (CV462984). The region between the signal sequence and WAP domain is variable but conforms to one of a small number of distinct structural patterns with regard to the presence or absence of other domains. The arrangements of these are largely, but not entirely, conserved within taxonomic sub-groups of arrangements within the decapoda. At least three main subgroups appear to exist (Figure 1.3) (Smith et al., 2008). Type I crustins have the region that lies between the signal sequence and the WAP domain is of variable length and cysteine-rich but has more than six of these residues. Type II crustins, on the other hand, possess not only a cys-rich region but also a long gly-rich domain of approximately 40–80 aa adjacent to the signal region (Figure 1.3). A third group of WAP domain-containing proteins from decapods resembles crustins but lack not only the gly-rich domain of the Type II molecules but also the cys-rich region present in both Type I and Type II.

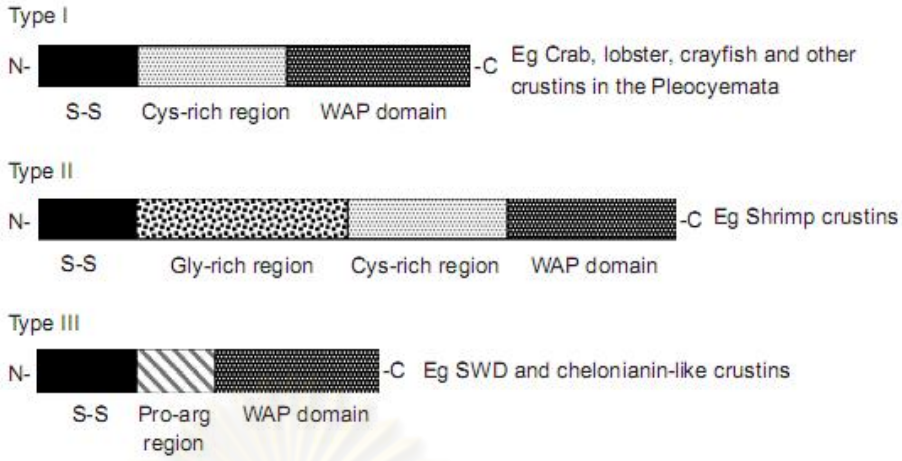


Figure 1.3 Schematic representation (not to scale) of the domain organisation of the three main crustin types from decapods (Smith et al., 2008). S-S is a signal sequence.

The first crustin, an 11.5 kDa antibacterial peptide was first described in the granular hemocytes of the shore crab, *Carcinus maenas* (Schnapp *et al*., 1996). The 11.5 kDa antibacterail protein was characterized as a hydrophobic molecule, which has cysteine-rich and antimicrobial activity against Gram-positive bacteria, named 'carcinin' (Smith *et al*., 2001).

From Expressed Sequence Tag (EST) database, the different isoforms of crustins have been found in *P. monodon* (Supungul *et al*., 2004 and Tassanakajon *et al*., 2006). The two major isoforms are crustin*Pm*1 and crustin-like antimicrobial peptide (crustin*Pm*7), were expressed as recombinant proteins in *E. coli* and characterized for their antimicrobial properties (Amparyup et al., 2008; Supungul et al., 2008).

Crustin*Pm*1, the most abundant isoform, contains an open reading frame of 435 bp encoding a precursor of 145 amino acids that comprises 17 amino acid signal peptides and 128 amino acid mature peptides. The peptides contain a Gly–Pro rich region at the amino-terminus and a single whey acidic protein (WAP) domain at the carboxyl-terminus. The recombinant crustin*Pm*1 has a molecular mass of 14.7 kDa with a predicted pI of 8.3. Antimicrobial assays demonstrated that recombinant crustin*Pm*1 showed strong inhibition against *Staphylococcus aureus* and *Streptococcus iniae*. In addition, the study of inhibition mechanism revealed that the antimicrobial activity of crustin*Pm*1 was a result of bactericidal effect (Supungul et al., 2008).

Crustin*Pm*7 consists of 124 amino acid residues of the mature peptide and a signal peptide of 17 amino acid residues. The mature peptide contains a glycine-rich domain at the N-terminus and 12 conserved cysteine residues containing a single WAP domain at the C-terminus. The recombinant crustin*Pm*7 has a molecular mass of 12.96 kDa with a predicted pI of 7.81. Antimicrobial assays demonstrated that recombinant crustin*Pm*7 showed strong antimicrobial activity against both Gram-positive and Gram-negative bacteria including *V. harveyi*, a major pathogenic bacteria in shrimp aquaculture (Amparyup et al., 2008).

Furthermore, a unique isoform of crustin, crustin*Pm*5, was identified from a gill-epipodite cDNA library of the black tiger shrimp. This peptide was expressed as a recombinant protein in *E. coli* with the expected size (approximately 17 kDa) and exhibited antimicrobial activity against some Gram-positive bacteria, but did not inhibited the growth of any Gram-negtive bacteria (Vatanavicharn et al., 2009).

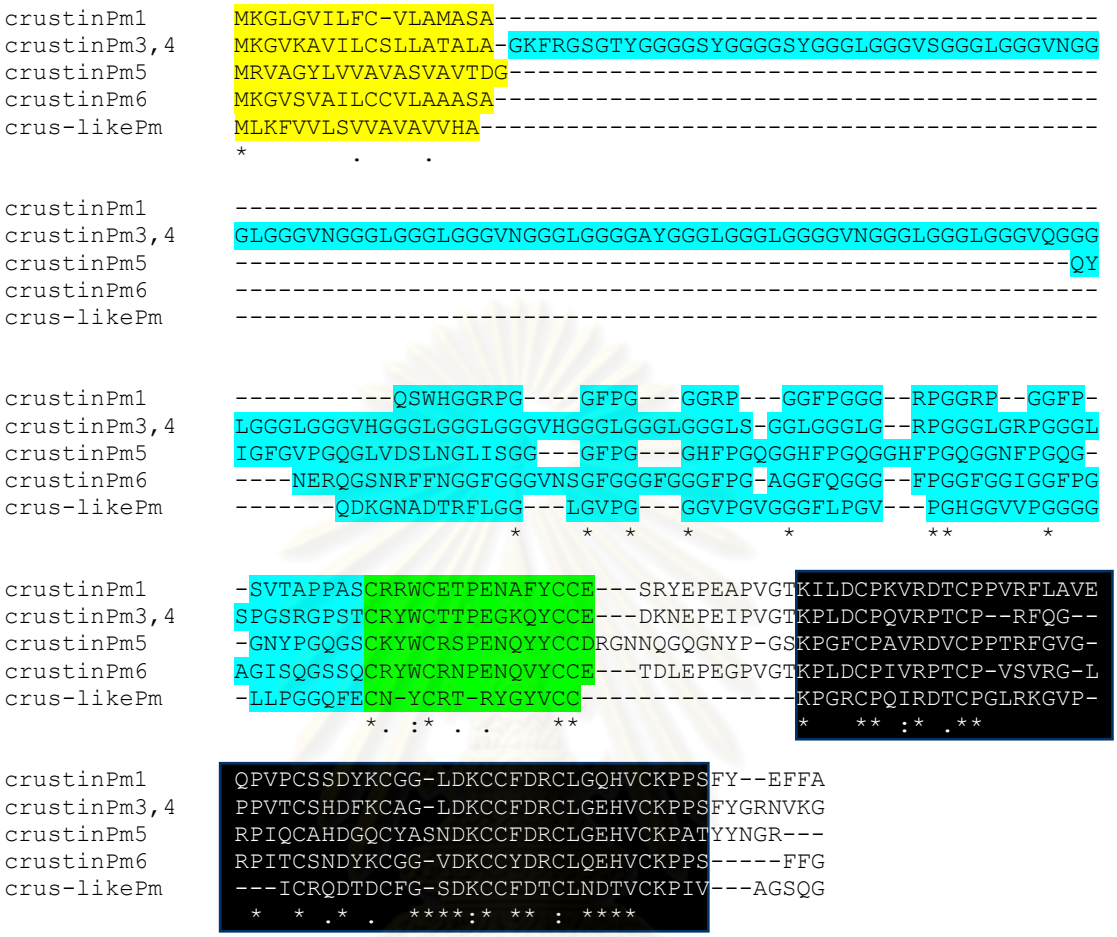


Figure 1.4 Comparison of *Penaeus monodon* crustin amino acid sequences. (*) indicates amino acid identify and (.) and (:) indicate amino acid similarity. The signal peptides, glycine-rich regions, and WAP domains are indicated by yellow, blue, and black boxes, respectively. The bolds letters correspond to the cysteine rich regions. Crus-like*Pm* is known as crustin*Pm7* in this literature.

1.9 Objective of the dissertation

From literature reviews, antibacterial mechanism of AMPs involves in membrane disruption and pore formation (Yeaman and Yount. 2003, Jenssen *et al*., 2006, Fernández-Vidala *et al*., 2007 and Bolintineanu *et al*., 2010). In this research, we proposed to take detail study on binding properties of rcrustin*Pm*1 and rcrustin*Pm*7 using (i) Bacterial and cell component binding assay (ii) Bacterial agglutination assay (iii) Inner membrane (IM) Permeabilization assay (iv) Surface Plasmon Resonance (SPR) and (v) Scanning Electron Microscopy (SEM); In addition, we attempt to crystallize the rcrustin*Pm*1 and rcrustin*Pm*7 for futher atomic structure study.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

-80°C Freezer (Thermo Electron Corporation)

-20°C Freezer (Whirlpool)

4°C Freezer (Whirlpool)

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

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

Amicon Ultra-15 10K concentrators (Millipore)

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

Autolab – Twingle Serface Plasmon Resonance, (Autolab)

Automatic micropipette P10, P20, P200 and P1000 (Gilson Medical Electronic S.A. France)

Balance PB303-S (Metter Toledo)

Centrifuge JA-14 (Beckman)

Centrifuge 5417C (Eppendorf)

Centrifuge 5804R (Eppendorf)

Gel documention System (GeneCam FLEX1, SYNGENE)

Incubator shaker 4000 (New Brunswick Scientific)

Incubator 30°C (Heraeus)

Incubator 37°C (Memmert)

JOEL Scanning electron microscopy, Model JSM-5410LV (JOEL Ltd.)

Laminar Airflow Biological Safety Cabinet Class II Model NU-440-400E (NuAire, Inc., USA)

Microcentrifuge tube 1.5 ml (AxygenMCT-150-C, USA)

Microtiter plate reader (BMG Labtech)

pH meter, Model # SA 720 (Orion)

Pipette tips 10, 20, 100, 1000 µl (AxygenMCT-150-C, USA)

Power supply, Model Power PAC 300 (Bio-RAD Laboratories)

Spectrophotometer DU800 (Beckman coulter)

Trans-Blot® SD (Bio-RAD Laboratories)

Sonicator, Model VCX-500-220 (SONICS Vibracell$^{T.M.}$)

Vertical electrophoresis system (Beckman)

Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

2.1.2 Chemicals and regents

5-Bromo-4chloro-3-indolyl phosphate (BCIP), $C_8H_6BrClNO_4P$ (Fermentus)

Absolute ethanol, C_2H_5OH (BDH)

Absolute methanol, CH_3OH (Scharlau)

Acrylamide, C_3H_3NO (Merck)

Acetic acid glacial, CH_3COOH (BDH)

Agarose (Sekem)

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

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

Ampicillin (Biobasic)

Anti-Histidine tag monoclonal antibodies (GE-healthcare)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Bovine serum albumin (Fluka)

Calcium chloride, $CaCl_2$ (Merck)

Chloramphenicol (Biobasic)

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

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

GeneRuler™ 100bp DNA ladder (Fermentus)

GeneRuler™ 1kb DNA ladder (Fermentus)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine, NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (Merck)

Imidazole (Fluka)

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

Kanamycin sulfate (Biobasic INC.)

Nitro blue tetrazolium chloride (NBT), $C_{40}H_{30}Cl_2N_{10}O_6$ (Fermentus)

MES, Free acid monohydrate (Biobasic INC.)

Methanol, CH_3OH (Merck)

N, N, N', N'-Tetramethylethylenediamine (TEMED) (BDH)

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

Ni Sepharose 6 Fast Flow (GE Healthcare)

millipore membrane filter 0.22 and 0.45 µm (Millipore)

Prestained protein molecular weight marker (Fermentus)

Sodium chloride, NaCl (BDH)

Skim milk powder (Mission)

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

Sodium hydroxide, NaOH (Eka Nobel)

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

Skim milk

Triton® X-100 (MERCK)

Tween™-20 (Flula)

Unstained protein molecular weight marker (Fermentus)

Urea (Fluka, Switzerland)

2.1.3 Enzymes

Nco I, *Not* I and *Xho* I restriction enzyme (New England Biolabs, USA)

T4 DNA ligase (Promega)

2.1.4 Microorganisms

Bacillus megaterium

Escherichia coli Rosetta (DE3)

Escherichia coli strain XL-I blue

Escherichia coli strain MG 1655

Escherichia coli strain 363

Enterobacter cloacae

Erwinia carotovora

Micrococcus luteus

Pichia pastoris

Staphylococcus aureus

Staphylococcus haemolyticus

Vibrio harveyi 1526

2.1.5 Kits

Crytal Screen I&II (Hamton Research)

NucleoSpin® Extract II kit (Macherey-Nagel)

PEG Rx™ 1&2 (Hamton Research)

PEG/Ion 1&2 Screen (Hamton Research)

Salt Rx™ (Hamton Research)

Wizard I, II, III, and IV random sparse matrix crystallization screen (Emerald Biosystems)

Pierce® BCA Protein Assay Kit (Thermo scientific)

Qiaprep® Spin Miniprep Kit (QIAGEN)

2.1.6 Vectors

pET-28b(+) (Novagen)

pVR 500

pPIC9K (Invitrogen)

2.1.7 Software

Autolab SPR Data and Kinetic Software (Metrohm , USA Inc.)

Graphpad Prism® version 5.0 (Graphpad software, USA Inc.)

2.2 Production of rcrustin*Pm*1 and rcrustin*Pm*7 from pET-28b/crustin*Pm*1 and pET-28b/crustin*Pm*7 constructs

2.2.1 Expression of pET-28b/crustin*Pm*1 and pET-28b/crustin*Pm*7

The plasmid containing full-length of crustin*Pm*1 and crustin*Pm*7 were transformed into an *E. coli* strain Rossetta (DE3) for the production of the recombinant crustin*Pm*1 and crustin*Pm*7 (rcrustin*Pm*1 and rcrustin*Pm*7). The rcrustin*Pm*1 and rcrustin*Pm*7 were expressed as described in Supungul et al., 2008 and Amparyup et al., 2008 with some modifications. In brief, a single colony of *E. coli* Rosetta (DE3) containing pET-28b/crustin*Pm*1 and pET-28b/crustin*Pm*7 were cultured with shaking at 37 ºC by shaking speed 250 rpm overnight in fresh LB broth containing 30 μg/mL of kanamycin and 34 μg/mL of chloramphenicol. The overnight

culture was diluted 1:100-fold in LB broth containing 30 µg/mL of kanamycin and 34 µg/mL of chloramphenicol. The culture was grown at 37 ºC with shaking at 250 rpm until the optical density at 600 nm (OD_{600}) of the culture reached 0.5-0.6. IPTG with final concentration of 1 mM was added into the media and further incubated at 37 ºC with shaking. The cells were harvested at 4 h for crustin*Pm*1 and 5 h for crustin*Pm*7, respectively by centrifugation at 8,000xg for 10 minute at 4 ºC. The cell pellet was resuspended with 1× PBS buffer, pH 7.4 by vortexing and then disrupted by freezed-thawed at least 3 rounds followed by sonication using a Bransonic 32 (BANDELIN SONOPULS, Germany) on ice with 60% amplitude pulse 3-5 times for 2.30 minutes each time. The rcrustin*Pm*1 and rcrustin*Pm*7 existed as inclusion bodies, so the pellet was collected and the supernatant liquid was discarded. The pellet was washed with 1×PBS containing 1% TritonX-100 twice followed by 1×PBS twice. The cells lysate were centrifuged at 10,000 rpm for 15 minutes at 4 ºC to remove supernatant. The inclusion bodies were dissolved in 50 mM Tris-Cl buffer pH 8.0, 20 mM imidazole and 8 M urea by gently shaking overnight at room temperature. The remaining precipitant was removed by centrifugation and the supernatant containing crude rcrustin*Pm*1 and rcrustin*Pm*7 were subjected to protein purification.

2.2.2 Purification of rcrustin*Pm*1 and rcrustin*Pm*7 under denaturing condition

The recombinant pET-28b/crustin*Pm*1 or pET-28b/crutin*Pm*7 were expressed with His-tags fussion, so they can be purified under denaturing condition using a Ni-NTA affinity chromatography (GE Healthcare) column.

At First, slurry 2-4 mL of the Ni-NTA agarose in 20% ethanol was packed into the PD-10 column and washed with 10 mL of sterile deionized water 3 times followed by 10 mL of 50 mM Tris-Cl pH 8.0 containing 20 mM imidazole and 8 M urea. The protein solution was then loaded onto the column and fractions were collected immediately by a gravity flow. Subsequently, the column was washed with 50 mM Tris-Cl pH 8.0 containing 20 mM imidazole and 8 M urea to remove unbound proteins. After washing, proteins were eluted with (i) 50 mM Tris-Cl pH 8.0 containing 8 M urea and 150 mM imidazole and (ii) 50 mM Tris-Cl pH 8.0 containing 8 M urea and 500 mM imidazole, respectively. The column was finally washed with 10 mL of 50 mM Tris-Cl pH 8.0 containing 8 M urea and 500 mM imidazole followed by 10 mL of sterile deionized water 3 times. The Ni-NTA agarose was

stored in 20% ethanol at 4 °C. The presence and purity of the purified proteins were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The purified protein fractions with final concentration of 5 mM DTT, were diluted and dialyzed in dialysis buffer (50 mM Tris-Cl pH 8.0 containing 150 mM NaCl, 10% glycerol, 1 mM GSSG (oxidized glutathione) and 0.1 mM GSH (reduced glutathione). Urea was gradually removed from protein solution by reducing urea concentration in 2 M steps (6 M→4 M→2M). Dialysis was kept with 1 mM GSSG and 0.1 mM GSH until the urea concentration was 2 M. At final step, dialysis buffer was changed to MES pH 5.8 buffer and any remaining precipitant was removed by centrifugation.

2.3 Construction of the recombinant plasmids pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7

In order to expressed the recombinant crustin*Pm*1 and crustin*Pm*7 in soluble forms, pVR500, a derivative of pET-32a(+) vector, lacking the nucleotide sequence between *Msc* I and *Kpn* I restriction site. Recombinant crustin*Pm*1 and crustin*Pm*7 were fused to thioredoxin tag (Trx-tag) at the N-terminus with the enterokinase cleavage site between the Trx-tag and the protein (Figure 2.1).

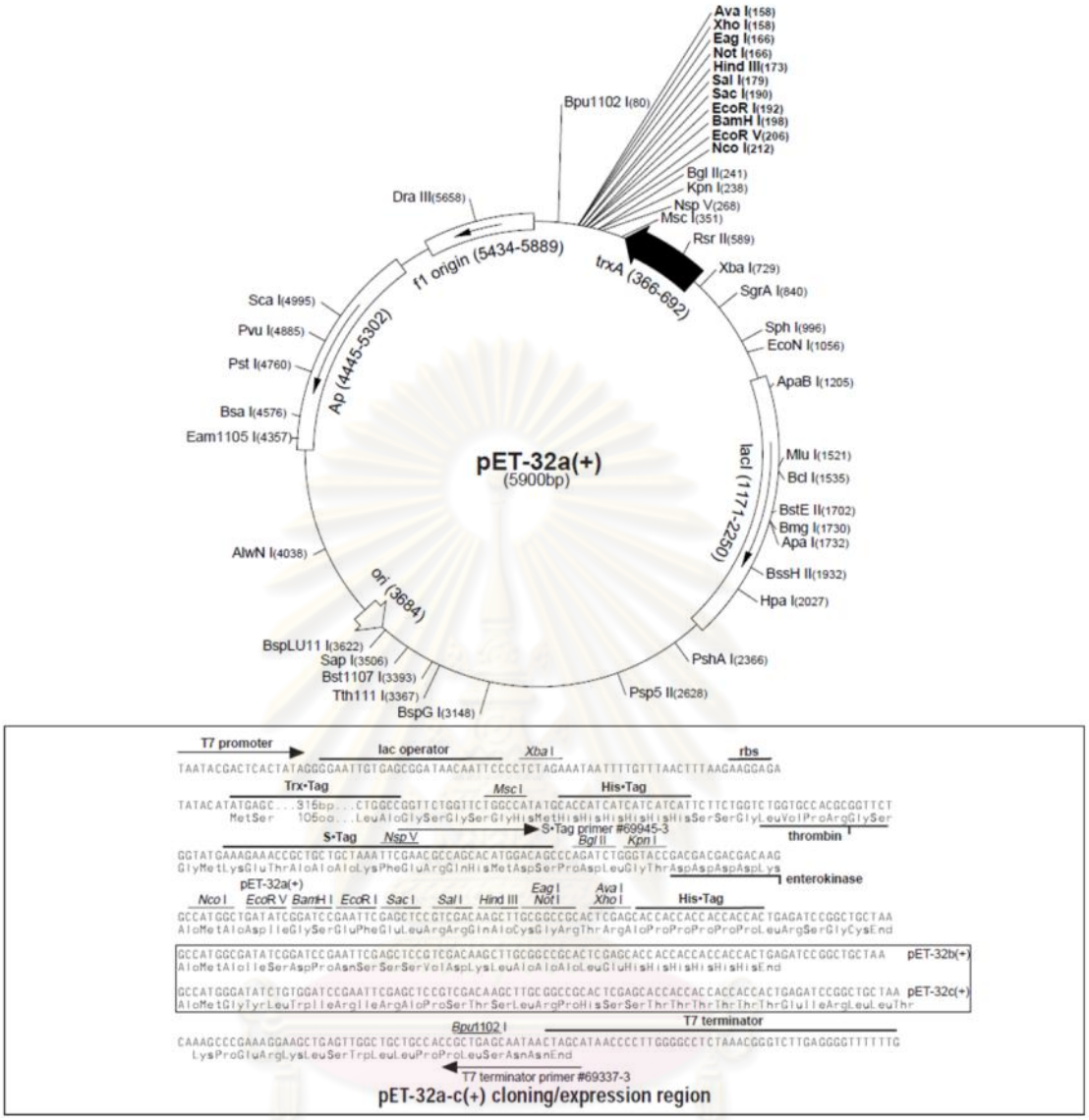


Figure 2.1 The pET-32a(+) vector map (Novagen®, Germany).

2.3.1 pVR500 preparation

pVR500 was transformed into *E. coli* strain XL-1-Blue. The purified vector was double digested with *Nco* I and *Xho* I for *crustinPm1* gene insertion and digested with *Nco* I and *Not* I for *crustinPm7* gene insertion. The double digersted vectors were purified by agarose gel electrophoresis and eluted using NucleoSpin® Extract II kit (Macherey-Nagel). The cut vectors were subjected to DNA concentration determination (OD_{260}) and stored at -20 ^{o}C until used.

2.3.2 Crustin genes preparation

In order to obtain crustin genes, the plasmid pET-28b/crustin*Pm*1 and pET-28b/crustin*Pm*7 were digested with *Nco* I/*Xho* I for crustin*Pm*1 and *Nco* I/*Not* I, respective.

The DNA fragments containing crustin*Pm*1 gene (435 bp) and crustin*Pm*7 gene (426 bp) were analyzed by 1.5 % agarose gel electrophoresis. The positive DNA fragments were eluted from the gel using NucleoSpin® Extract II kit (Macherey-Nagel). In brief, the DNA fragment was excised from an agarose gel and transferred to a clean 1.5 microcentrifuge tube. Buffer NT 200 µL was added per each 100 mg of agarose gel and incubated at 50 °C until the gel was dissolved. A NucleoSpin® Extract II column was placed into a collection tube and loaded with the sample. After centrifuged for 1 minute at 11,000×g, the flow-through was discarded. The silica membrane was washed with 600 µL of Buffer NT3 and centrifuged. The column was then centrifuged for 2 minutes at 11,000×g for completely removal of NT3 buffer and was placed into a new clean 1.5 mL microcentrifuge tube. The DNA was eluted by adding 15-50 µl of Elution Buffer NE (5 mM Tris-Cl, pH 8.5) into the center of each column. The column was left at room temperature for 1 minute and centrifugated for 1 minute at 11,000×g.

2.3.3 Subcloning of crustin genes into pVR500 vector and transformed into *E. coli* XL-1 Blue

The DNA fragment of crustin*Pm*1 and crustin*Pm*7 were ligated into pVR500 vector, which 5,800 bp approximately in length and had unique restriction sites in the multiple cloning regions. The recombinant pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7 plasmids were transformed into an *E. coli* XL1-Blue using the $CaCl_2$ method. The $CaCl_2$ competent cells of 100 µl were mixed with 5 µl of plasmid DNA and incubated on ice for 30 minutes. Then, the mixture was immediately heat-shocked at 42 °C for 1 minute and instantly added with 1 ml of cold LB medium. After shaking at 37 °C for 1 h, appropriate amount of cell suspension was spreaded onto the LB agar plate which contains 100 µg/ml ampicillin. The plates were incubated overnight at 37 °C. The individual colonies grown in 3 ml LB medium containing 100 µg/ml ampicillin at 37 ºC overnight with shaking.

For efficient plasmid DNA extraction, a QIAprep® Miniprep kit (QIAGEN, Germany) was employed. The overnight culture of bacteria was centrifuged at 12,000 rpm for 1 minute. The supernatant was removed. Bacterial cells were resuspended in 250 µl Buffer P1 containing RNaseA. The 250 µl Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times to lyse the cells. The cell lysate was then neutralized by 350 µl Buffer N3. After maximum speed centrifugation for 10 minutes, the supernatant was applied to a QIAprep spin column by pipetting. The column was centrifuged at 12,000 rpm for 1 minute. The flow-through was discarded. The column was the washed twice with 0.5 ml Buffer PB and 0.75 ml Buffer PE, respectively. To remove residual ethanol from Buffer PE, the column was dried by centrifugation at 12,000 rpm for 1 minute. The plasmid DNA was eluted by adding 30-50 µl Buffer EB to the center of the column and left at room temperature for 1 minute prior to centrifugation at 12,000 rpm for 1 minute.

PVR500 containing crustin genes were identified by restriction enzyme digestion, followed by agarose gel electrophoresis. Positive plasmids were heat shock transformed into *E. coli* Rosetta (DE3). The transformants were selected on LB agar with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol.

2.4 Production of rcrustin*Pm*1 and rcrustin*Pm*7 from pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7 constructs

2.4.1 Expression of pVR500/crustin*Pm*1 and pV500/crustin*Pm*7

Prior to large scale protein production, pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7 were transformed into *E. coli* Rosetta (DE3) for small scale expression. The cell suspension was taken at different time points after IPTG induction. The cell was pellet by centrifugation at 8,000×g for 5 min and resuspended with 1×PBS buffer, pH 8.0. The cell suspension was sonicated using microtip probe with 30% amplitude for 3-5 min. The supernatant and pellet were separated and analyzed by 15% (v/v) SDS-PAGE.

For large scale expression, *E. coli* Rosetta (DE3) containing pVR500/crustin*Pm*1 and pV500/crustin*Pm*7 plasmid was culture in 3.2 L of LB-ampicillin-chloramphenicol media. After induced with IPTG at 4 h for rcustin*Pm*1, and 5 h for rcustin*Pm*7, cell pellet was separated by centrifugation and completely

broken by sonication. The supernatant was then added with imidazole to 20 mM final concentration.

2.4.2 Purification of rcrustin*Pm*1 and rcrustin*Pm*7 under non-denaturing condition

The Ni Sepharose affinity column was washed with 10 mL of sterile deionized water 3 times followed by 10 mL of 1×PBS pH 8.0 containing 20 mM imidazole. The protein solution was then loaded onto the column and fractions were collected immediately by a gravity flow. Subsequently, the column was washed with 1×PBS pH 8.0 containing 20 mM imidazole to remove unbound proteins. After washing, proteins were eluted with (i) 1×PBS pH 8.0 containing 150 mM imidazole and (ii) 50 mM Tris-Cl pH 8.0 containing 500 mM imidazole, respectively. The column was finally washed with 10 mL of 50 mM Tris-Cl pH 8.0 containing 500 mM imidazole followed by 10 mL of sterile deionized water 3 times. The Ni-NTA agarose was stored in 20% ethanol at 4 °C.

The eluted protein fraction was dialyzed in dialysis buffer (1×PBS pH 8.0). Dialyzed protein was incubated at 23 °C with enterokinase for 16 hours in order to cleave off the Trx-tag. Any remaining precipitant was removed by centrifugation and the protein was reloaded onto the Ni Sepharose affinity column. At final step, protein was dialyzed with MES buffer pH 5.8 and any remaining precipitant was removed by centrifugation.

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2.5 Expression and purification of rcrustin*Pm*1 in *Pichia patoris*

Pichia partoris clone containing rcrustin*Pm*1 was constructed and provided by Supungul Premruethai. In brief, crustin*Pm*1 gene was inserted into pPIC9K (Invitrogen) and linearized before transforming into *P. partoris.* Yeast colonies with highest rcrustin*Pm*1 expression were then selected by Geneticin (Supungul, unpublished). A single colony was inoculated into BMGY medium and growth at 28-30°C with shaking 250-300 rpm until the culture reaches an OD_{600}=2-6 (approximately 16-18 hours). The culture was inoculated 1:100-fold in BMGY broth and growth at 28-30°C with sharking until OD_{600}=10-20. Harvest the cells by centrifugation and resuspended the cell pellet in 1/5 of the original culture volume of BMMY medium (~100-200 ml). Grow at 28-30°C with shaking. 100% was added to 0.5% every 24 h for 3 days. Protein was harvested by centrifugation and the

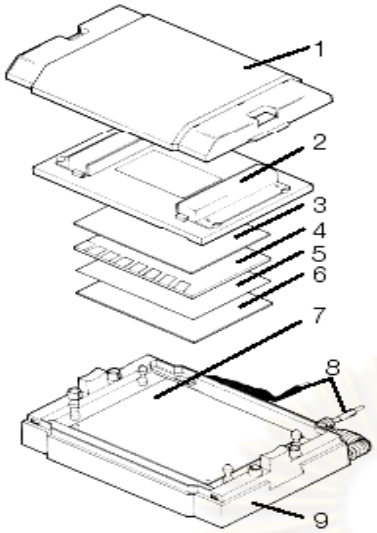
supernatant was kept to purification. The protein solution was purified using the Ni Sepharose affinity column. Protein was eluted with 50 mM Tris-Cl pH 7.4 containing 100 mM imidazole. Finally, protein was dialyzed with 10 mM Tris-Cl pH 7.4.

2.6 Determination of protein concentration

The purified protein was concentrated using centricon columns (Millipore) with a 10 kD cut-off membrane. Protein concentration was determined by Pierce® BCA Protein Assay Kit (Thermo scientific). Bovine serum albumin was used as a protein standard. Sample solution of 25 µl was mixed with 200 µl BCA working buffer and left for 30 minutes before measuring the absorbance at 562 nm. The BCA working reagent was a mixture of solution A: solution B (50:1).

2.7 Western Blot analysis

After the SDS-PAGE, the protein gel can be partly stained with Coomassie brilliant blue R-250 or analyzed by Western blotting. The gel for electroblotting was soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 15-30 minutes together with Whatman® 3MM chromatography paper and nitrocellulose membrane which were cut to the size of the gel. The Whatman® 3MM paper was placed onto the Trans-Blot® SD (Bio-Rad). This is then wet with transfer buffer. The prewetted nitrocellulose membrane was put onto the filter paper and a gel is put on top of the membrane followed by filter paper again (Figure 2.2). Great care should be taken to ensure that there are no air bubbles. Proteins in transfer buffer are negative in charge and move from negative to positive poles. Western blotting was performed for 30-90 minutes at approximately constant 90 mA.



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

Figure 2.2 Exploded view of the Trans-Blot® SD (Bio-Rad).

After finishing the transfer, the membrane was incubated in blocking buffer (1× PBS buffer [10 mM phosphate buffer pH 7.4, 150 mM NaCl] containing 0.05% (v/v) Tween™-20 and 5% (w/v) non-fat dry milk) at room temperature overnight with gently shaking. It was then washed 3 times for 10 minutes each in washing buffer, PBS-Tween buffer [1× PBS buffer containing 0.025% (v/v) Tween™-20]. The nitro cellulose membrane was incubated in an anti-His antibody solution, 1:3000 dilution in washing buffer containing 1% (w/v) non-fat dry milk with volume enough to cover it and allow it to float freely when agitated. After gently mixing at ambient temperature for 3 hours, it was washed 3 times for 10 minutes each in washing buffer and subsequently incubated in a secondary antibody solution, the alkaline phosphatase-conjugated rabbit anti-mouse IgG, 1:5000 dilution in washing buffer with 1% (w/v) non-fat dry milk, with agitation at room temperature for 1 hour. Next, it was washed 3 times as above and detected in darkness by color development using 10 mL of detection buffer (100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) containing 44 µL 4-nitroblue tetrazolium (NBT) and 33 µL 5-bromo-4-chloro-indolyl phosphate (BCIP) as substrate. The BCIP was prepared 50 mg/mL in water or dimethyl formamide and NBT was also 50 mg/mL in water. Reaction product is purple and appears in a few minutes. Incubation time can be up to an hour if the signal is weak. Finally, the development was stopped by washing the membrane with deionized water.

2.8 Antimicrobial assay

Antimicrobial activities and minimum inhibitory concentration (MIC) values of the purified rcrustin*Pm*1 and rcrustin*Pm*7 against Gram-positive bacterium *Staphylococcus aureus* and Gram-negative bacterium *Escherichia coli* 363 were determined by liquid growth inhibition assays as previously described (Destoumieux et al., 1999). Two-fold serially diluted recombinant protein samples, or MES buffer pH 5.8 as a control, were incubated in a sterile micro titration plates with 100 μl of a suspension of mid-logarithmic growth phase culture of bacteria diluted in culture medium to OD_{600} = 0.001. Poor-broth nutrient medium (1% bactotryptone, 0.5% NaCl, pH 7.5) was used for standard bacterial strains cultures. Bacteria were grown overnight under vigorous shaking at 30°C for *E. coli* 363 and 37°C for *S. aureus* according to the strains and measured at 600 nm. The MIC value was recorded as the range between the highest concentration of the peptide where bacterial growth was observed and the lowest concentration that caused 100% of inhibition bacterial growth.

2.9 Bacterial cells and cell wall components binding assays

The antimicrobial peptides rcrustin*Pm*1 and rcrustin*Pm*7 were investigated for their binding properties to Gram-positive bacterial *Staphylococcus aureus, Stapphylococcus haemolyticus, Micrococcus luteus and Bacillus Megterium* and Gram-negative bacterial *Eirwenia carotovara, Enterobacter cloacae, Escherichia coli* 363, *Escherichia coli* MG 1655 and *Vibrio harveyi* 1526. Experiments were set up as described by Lee & Soderhall, 2001 with some modification. Briefly, the 3 ml of logarithmic phase bacteria cultured was used in this experiment. Bacteria cultures were pelleted and the cells were resuspended in MES buffer pH 5.8. Cells were then incubated with 5 μg of purified recombinant crustin*Pm*1 and crustin*Pm*7 at 4 °C for 35 minutes. In addition, cells incubated with MES buffer pH 5.8 were used as a negative control. Binding experiments of *B. megaterium* and *E. coli 363* to purified recombinant ALF*Pm3* were carried out as a positive control (Somboonwiwat et al, 2008), while binding experiments of these bacteria to purified recombinant type-I Lyzosyme were set up as a negative control (Supungul et al, 2010). After incubation, cells are pelleted by centrifugation at 2,000×g for 10 min and the supernatant was removed. The bacteria pellets were resuspended and washed three times with

resuspension buffer (MES buffer pH 5.8). Bound protein was finally eluted with 1×SDS-PAGE sample loading buffer. Supernatant (unbound protein), washed fractions (unbound/weakly bound protein), eluted fractions (bound protein) and cell pellets (strongly bound protein) were analysed by SDS-PAGE and Western blot. Western blot analysis was carried out using anti-Histidine tag monoclonal antibody (GE-healthcare).

Quantitative binding assay of purified crustin*Pm*s to cell wall components lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 (Sigma) and lipoteichoic acid (LTA) from *S. aureus* (Sigma) were performed by an enzyme-linked immunosorbent assay (ELISA) using anti-histidine tag monoclonal antibody. Microtiter plates were coated with 100 µl (30 µg/ml) of LPS or LTA. Excess LPS or LTA were washed out with PBS-T (PBS containing 0.1% (v/v) Tween™-20). The plate is blocked with 5% (w/v) BSA in PBS-T prior to incubation with various amounts of purified recombinant crustin*Pm*1 and crustin*Pm*7 overnight at room temperature. The plate was washed with PBS-T twice and 100 µl anti-histidine tag monoclonal antibody in PBS-T 1:5,000 (v/v) was added. A hundred microlitre of alkaline phosphatase-conjugated secondary antibody against IgG in PBS-T 1:10,000 (v/v) was applied to the plate, followed by washing step and addition of 50 µl *p*-nitrophenylphosphate. The reaction was terminated by addition of 0.4 M NaOH. The plate was incubated for 15 min and then analysed in microtiter plate reader (FLUOstar OPTIMA Microreader, SMG Labtechnology) at 405 nm. The apparent dissociation constants (K_d) and the maximum binding (A_{max}) parameters were calculated with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, Calofornia, USA, using nonlinearly fitting as one site – Specific biding with Hill slope (model: Y=Bmax*X^h/(Kd^h + X^h).

2.10 Surface Plasmon Resonance (SPR)

The interaction between rcrustin*Pm*s and cell wall components, *E. coli* LPS and *S. aureus* LTA were studied by Autolab-Twingle SPR instrument. The reation was carried out at 25°C using MES buffer, pH 5.8 as a running buffer. Ten µg/ml of recombinant crutin*Pm*1 or crustin*Pm*7 in 10 mM sodium citrate buffer pH 6.2 was immobilized onto a Mercaptoundecanoic acid (11-MUA) sensor chip using the standard amine coupling method. To determine rcrustin*Pm*s affinity for cell wall

components, 10 to 30 µg/ml of LPS and LTA in MES pH 5.8 were separately flushed onto rcrustin*Pms*-immobilized sensor chip. Bound LPS and LTA were removed from the sensor chip by applying 50 mM HCl solution flow. The kinetic parameters were obtained from sensorgrams using Autolab SPR Data and Kinetic evaluation software.

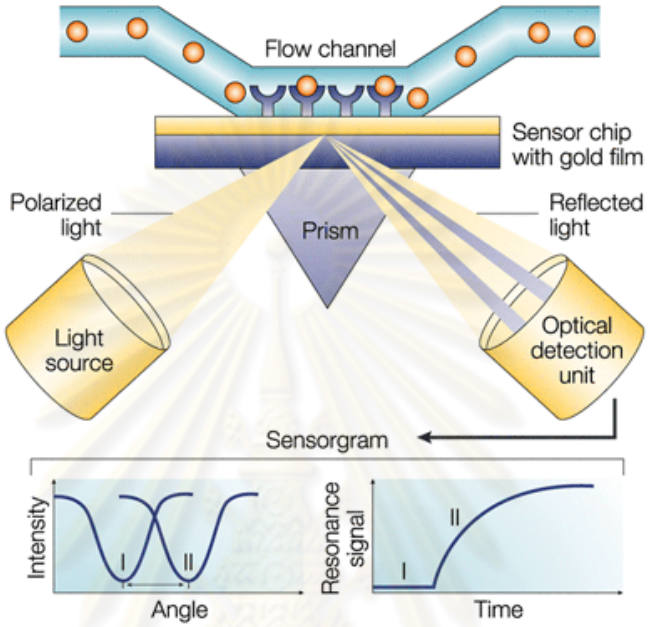


Figure 2.3 Surface Plasmon Resonance Biosensor.

2.11 Bacterial agglutination assay

Bacterial agglutination was studied in 96-wells round bottom microtiterplates and visualized by microscopy. Nine bacterial strains including four Gram-positive bacterial, *S. aureus, S. haemolyticus, M. luteus and B. Megterium* and five Gram-negative bacterial *E. carotovara, E. cloacae, E. coli*363, *E. coli* MG 1655 and *V. harveyi*1526, were used to investigate the agglutination properties of rcrustin*Pm*1 and rcrustin*Pm*7. Bacteria was culture until optimal density (OD) at 600 nm >1. Bacteria culture (1.5 ml) was pelleted by centrifugation at 8000×g for 10 min and the pellets were resuspended with agglutination buffer (50 mM MES buffer pH 5.8 containing 100 mM NaCl and 10 mM $CaCl_2$). The bacterial pellets were resuspended and washed three times with agglutination buffer. Fifty microlitre aliquot of resuspended bacteria were tranfered into 96-wells round bottom plates. The rcrustin*Pm*1 and rcrustin*Pm*7 proteins were diluted into agglutination mixture, in order to obtain the final

concentration of protein 200 µg/ml in each well. Self-or auto agglutination was observed in the well with no proteins. The mixture was incubated for 16 h at 22°C without shaking. After agglutination or sedimentation, 5 µl was taken from the bottom of the well and transferred to a microscope slide. Bacterial samples were then Gram-stained and examined by light microscopy.

2.12 Inner membrane (IM) permeabilization assay

Lactose permease deficient *E. coli* strain MG1655 (lacY: Tn10dKan) was used to test crustin*Pm*s'ability to induce IM permeabilization by measuring β-galactosidase activity. The starter of *E. coli* MG1655 culture was inoculated into LB medium containing 0.1 mM IPTG. When OD_{600} of bacteria culture reached 0.4-0.6, it was splited to two reactions. One was incubated with rcrustin*Pm*1 and another was incubated with rcrustin*Pm*7 Final concentration of rcrustin*Pm*s in the reactions was 100 µM. A 150 µl aliquots of bacteria culture was taken out at 0, 1, 3, 5, 8 and 16 h. The samples at each time point were centrifuged at 8000×g and the supernatants were stored at -20°C.

Supernatant fractions were incubated with 6 µM O-nitrophenyl-β-D-galactopyranoside (ONPG) at 30 °C for 5 min. The reaction was stopped by adding 100 µl of 2 M sodium carbonate into the reation mixture. The absorbance at 405 nm was measured by Microtiter plate reader (BMG Labtech). For control and negative control reations, MES buffer, pH 5.8 and bovine serum albumin (BSA) were added into bacteria culture, respectively.

2.13 Scanning Electron Microscopy (SEM)

In order to observe physical change on baterial cells induced by rcrustin*Pm*s, bacterial samples: *S. aureus*, *E. coli* MG1655 and *V. harveyi* were used. Bacteria were grown overnight in LB media or TSB media in case of *V. harveyi*. The bacterial culture were inoculated into fresh medium and incubated at 37°C for *S. aureus* and *E. coli* MG1655, 30°C for *V. harveyi* with shaking until they reached exponential phase. Five mililitre of bacteria culture were collected by centrifugation, washed twice and re-suspended in MES buffer, pH 5.8. Bacterial samples were incubated with 10-fold over the MIC value of rcrustin*Pm*1 or rcrustin*Pm*7 for 1 h and then pelleted by centrifugation. The specimens were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 1-2 h and washed three times with 0.1 M phosphate buffer prior to dehydration through a graded series of ethanols. After critical point drying, samples were mounted on 1 cm stubs and were gold-coated using an Ion sputter coater (Balzers, model SCD 040). Images were taken from each specimen using JEOL scanning electron microscopy, model JSM-5410LV.

2.14 Crystallization of rcrustin*Pm*1 and rcrustin*Pm*7

Recombinant crustin*Pm*1 and crustin*Pm*7 were screened for crystallization condition using sitting drop vapour diffusion method. Proteins were pipette into the well and mixed with resorvior solutions (Hamton Research and Emerald Biosystems screening kits) in the reservoir of 96-well plate in 1:1 ratio. The 96-well plates were then sealed off and incubated at 20°C. Crystallisation trays were observed at day 1, 3, 5, 7 and every week up to 2 months.

CHAPTER III
RESULTS

3.1 Expression and purification of the recombinant crustin*Pm*1 and crustin*Pm*7 in the *E. coli* expression system

3.1.1 Expression of pET28b/crustin*Pm*1 and pET28b/crustin*Pm*7

The rcrustin*Pm*1 and rcrustin*Pm*7 were over-expressed and purified as described previously (Supungul et al., 2008 and Amparyup et al., 2008). In brief, rcrustin*Pm*1 and rcrustin*Pm*7 were expressed with His-tags fusion at the N-terminus. The rcrustin*Pm*1 and rcrustin*Pm*7 were overexpressed as an in inclusion bodies after induction with IPTG for 4 and 5 h, respectively.

3.1.2 Purification of rcrustin*Pm*1 and rcrustin*Pm*7 under denaturing condition

Recombinant crustin*Pm*1 and crustin*Pm*7 could be purified under denaturing condition using a Ni-NTA affinity chromatography (GE Healthcare). The protein was eluted stepwise with the denaturing solution containing 150 mM imidazole. SDS–PAGE analysis eluted fractions showed a major protein with an approximate size of 14.7 kDa and 12.8 kDa of *r*crustin*Pm*1 and *r*crustin*Pm*7, respectively (Figure 3.1A). Both recombinant crustin proteins were identified by Western immunoblotting using anti-His antibody (Figure 3.1B). Two minor bands were observed on SDS-PAGE even though proteins were treated with β-mercaptomethanol and heated. It is likely that they are dimer and trimer forms of crustin since the protein contain 12 cysteine residues and the reducing condition in SDS–PAGE may not reduce all disulphide linkages.

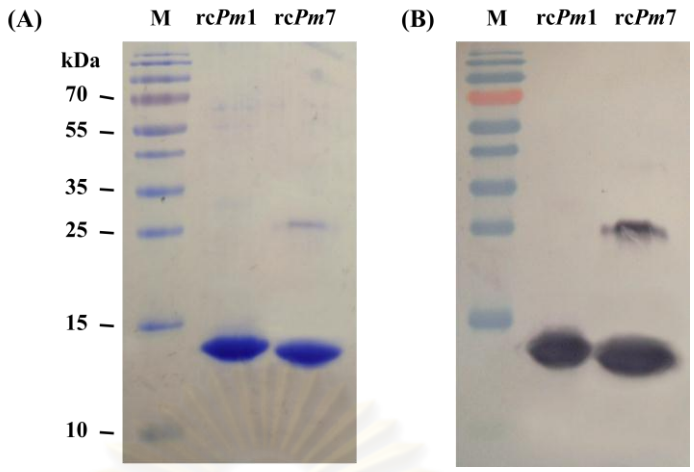


Figure 3.1 15% SDS–PAGE (A) and Western blotting (B) analysis of the purified *r*crustin*Pm*1 (rc*Pm*1) and *r*crustin*Pm*7 (rc*Pm*7).

3.1.3 Construction of pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7

Recombinant pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7 were constructed in attempt to express the crustin genes in soluble forms. The crustin*Pm*1 and crustin*Pm*7 were cloned into pVR500 vector which has the thioredoxin fusion tag (Trx-tag), aid to solubility protein expression. Recombinant plasmids pET28b/crustin*Pm*1 (Supungul et al., 2008) and pET28b/crustin*Pm*7 (Amparyup et al., 2008) from the positive transformants were prepared and cleaved with restriction enzymes *Nco* I and *Xho* I for pET28b/crustin*Pm*1 and *Nco* I and *Not* I for pET28b/crustin*Pm*7. Digested recombinant plasmids were analyzed by agarose gel electrophoresis to identify the DNA fragments insert. The approximately 435 bp of crustin*Pm*1 gene fragment was present in lane 2 and 3 (Figure 3.2A), and 426 bp of crustin*Pm*7 gene fragment in Figure 3.2B.

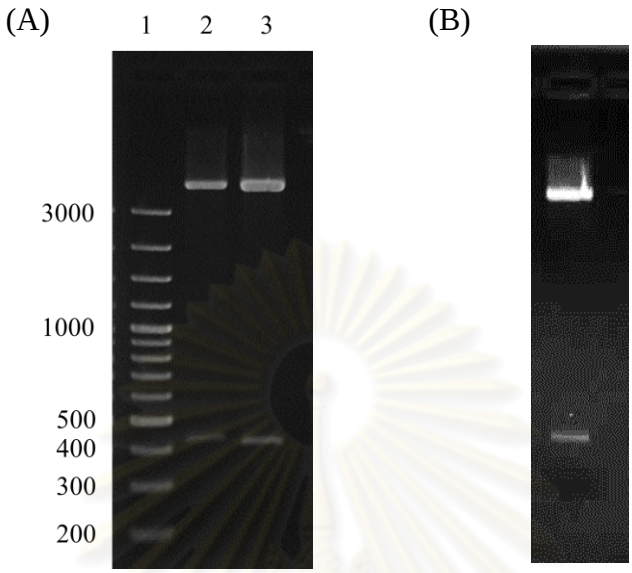


Figure 3.2 Recombinant pET28/crustin*Pm*1 and pET28/crustin*Pm*7 plasmid digested with restriction enzyme.

(A) Lane 1: 100 bp DNA ladder (Fermentus)

Lane 2 and 3: Recombinant pET28b/crustin*Pm*1 after digest with restriction enzyme *Nco* I and *Xho* I

(B) Recombinant pET28b/crustin*Pm*7 after digest with restriction enzyme *Nco* I and *Not* I

The eluted DNA fragment of crustin*Pm*1 and crustin*Pm*7 were ligated into pVR500 vector and transformed into an *E. coli* XL1-Blue. The recombinant plasmids containing crustin genes were extracted and confirmed by digesting with *Nco* I and *Xho* I for rcrustin*Pm*1 (Figure 3.3A) and *Nco* I and *Not* I for rcrustin*Pm*7 (Figure 3.3B)

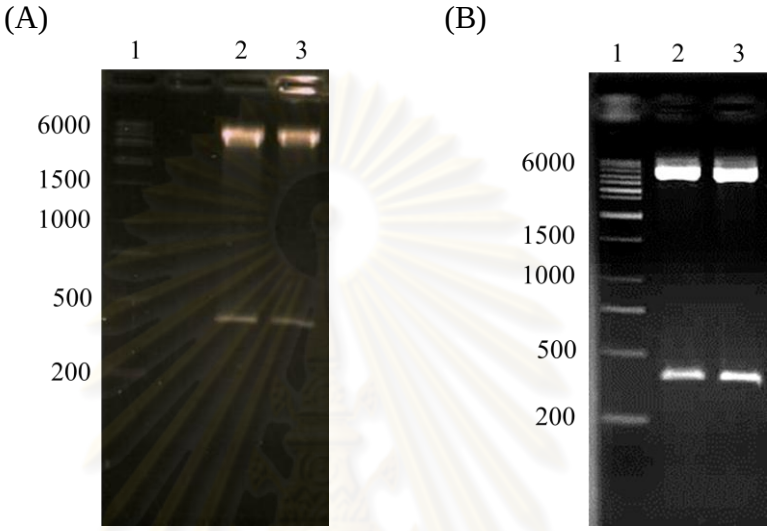


Figure 3.3 The recombinant pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7 plasmid digested with restriction enzyme.

(A) Lane 1: 1 kb DNA ladder (Fermentus)

Lane 2 and 3: Recombinant pVR500/crustin*Pm*1 after digest with restriction enzyme *Nco* I and *Xho* I

(B) Lane 1: 1 kb DNA ladder (Fermentus)

Lane 2 and 3: Recombinant pVR500/crustin*Pm*7 after digest with restriction enzyme *Nco* I and *Not* I

3.1.4 Expression of recombinant pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7

The recombinant pVR500/crustin*Pm*1 and pVR500/crustin*Pm*7 plasmid were transformed into an *E. coli* Rosseta (DE3) using the $CaCl_2$ method. Expression of the transformant was cultured in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chlorampenical IPTG was then added in order to over-expressed the rcrustin*Pm*s. Approximate 27 kDa of rcrustin*Pm*1-Trx tag was analysed by 15% SDS–PAGE at 0, 2 and 4 h (Figure 3.4A1). The rcrustin*Pm*1 mainly expressed in soluble form with Thioredoxin fusion tag (Trx-tag) (Figure 3.4A2). Whilst the recombinant pVR500/crustin*Pm*7 was expressed with approximately as the same size (Figure 3.4B).

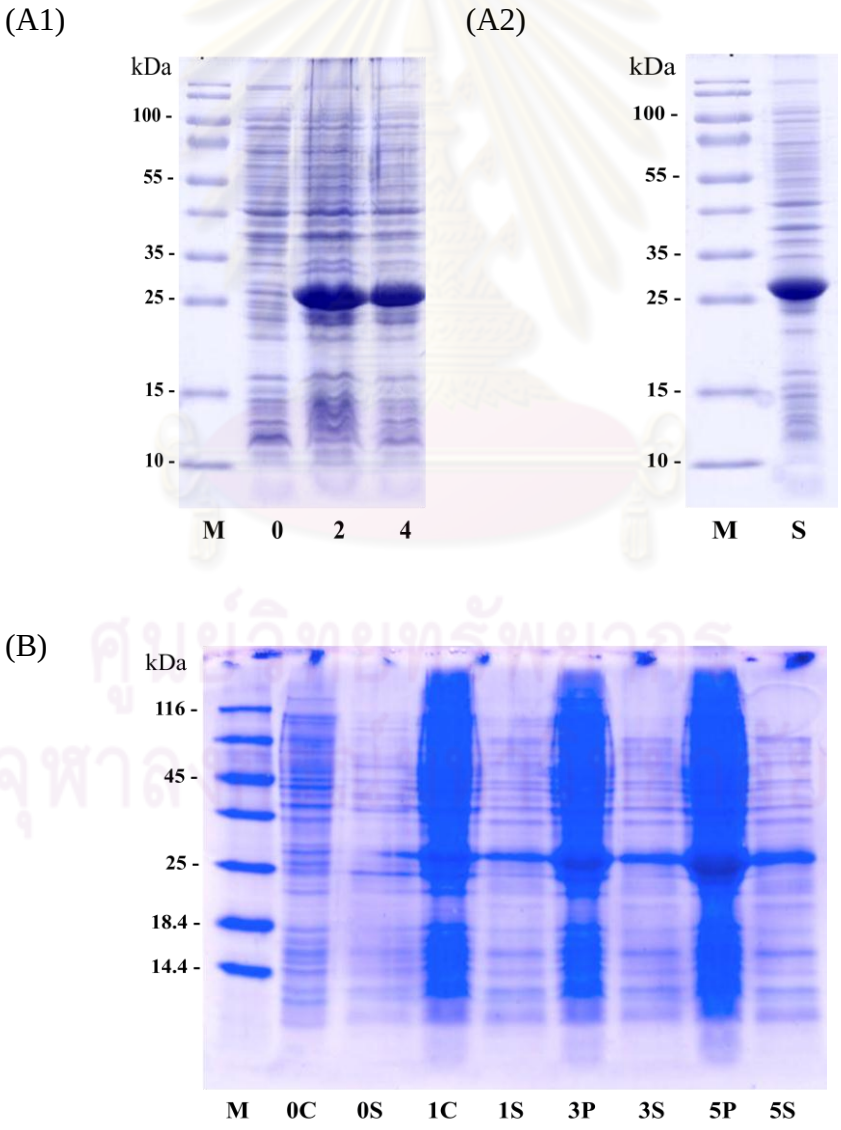


Figure 3.4

Figure 3.4 (cont.) The expression of the rcrustin*Pm*1 and rcrustin*Pm*7 in pVR500 vector. SDS-PAGE 15% (w/v) analysis showing the expressed proteins in pVR500 with *E. coli* Rosetta (DE3) host cells containing rcrustin*Pm*1 expression at difference times, in the soluble fraction.

(A1) Lane M: Prestained protein marker (Fermentus)
Lane 0, 2 and 4: Cell pellet induction fraction of rcrustin*Pm*1

(A2) Lane M: Prestained protein marker (Fermentus)
Lane S: The rcrustin*Pm*1 produced at 4 h in soluble fraction

(B) Lane M: unstained protein marker (Fermentus)
Lane 0C: Cell pellet fraction of rcrustin*Pm*7 at 0 h induction
Lane 0S: The supernatant fraction of rcrustin*Pm*7 at 0 h induction
Lane 1C: Cell pellet fraction of rcrustin*Pm*7 at 1 h induction
Lane 1S: The supernatant fraction of rcrustin*Pm*7 at 1 h induction
Lane 3C: Cell pellet fraction of rcrustin*Pm*7 at 3 h induction
Lane 3S: The supernatant fraction of rcrustin*Pm*7 at 3 h induction
Lane 5C: Cell pellet fraction of rcrustin*Pm*7 at 5 h induction
Lane 5S: The supernatant fraction of rcrustin*Pm*7 at 5 h induction

3.1.5 Purification of rcrustin*Pm*1 and rcrustin*Pm*7 under non-denaturing condition

Since both rcrustin*Pm*1 and rcrustin*Pm*7 contained the Trx-tag and were expressed as the soluble form, they were purified with the nickel-NTA affinity column under non-denaturing condition. The soluble proteins were loaded to the nickel-NTA column, and then washed the column with binding buffer. The purified recombinant proteins with Trx-tag were eluted with 1×PBS buffer containing 500 mM imidazole. The eluted fractions were analyzed using SDS-PAGE (Data not shown) and dialyzed with 1×PBS buffer pH 8.0. After that, proteins were incubated at 23 °C with enterokinase for 16 hours in order to cleave off the Trx-tag. The protein was reloaded onto the by nickel-NTA affinity column. Approximately 14.7 kDa purified rcrustin*Pm*1 and 12.8 kDa rcrustin*Pm*7 were eluted with 1×PBS buffer containing 150 mM imidazole. The eluted protein fractions were checked by 15% (w/v) SDS-PAGE and protein concentration was determined by BCA protein assay kit. Low amount of rcrustin*Pm*s was obtained by this purification method. (Figure 3.5)

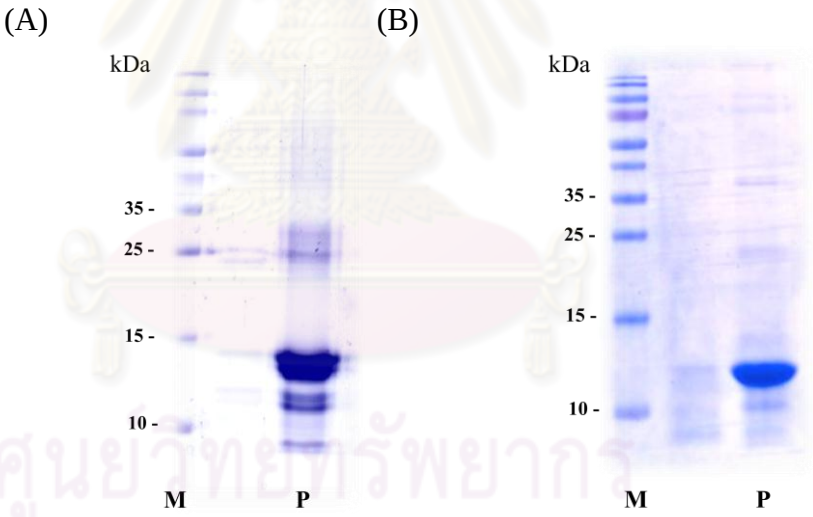


Figure 3.5 15% SDS-PAGE analysis of the purified rcrustin*Pm*1 and rcrustin*Pm*7.

(A) Lane M: Prestained protein marker (Fermentus)
Lane P: The purified rcrustin*Pm*1 protein after removal of Trx-tag by enterokinase

(B) Lane M: Prestained protein marker (Fermentus)
Lane P: The purified rcrustin*Pm*7 protein after removal of Trx-tag by enterokinase

3.2 Expression and purification of the recombinant crustin*Pm*1 in the *Pichia pastoris* expression system

Pichia pastoris was used in order to produce the rcrustin*Pm*1 in soluble form. Recombinant crustin*Pm*1 was overexpressed with histidine-tag and Ni-NTA column was used to purify the recombinant crustin. The protein was eluted with 50 mM Tris-Cl pH 7.4 containing 100 mM imidazole. The eluted protein fraction was dialyzed in dialysis buffer (10 mM Tris-Cl pH 7.4). The purified protein was concentrated using centricon columns (Millipore) with a 10 kD cut-off membrane. Protein concentration was determined by Pierce® BCA Protein Assay Kit (Thermo scientific). Approximately 15 kDa purified rcrustin*Pm*1 was analyzed by Silver-stained SDS-PAGE (Figure 3.6).

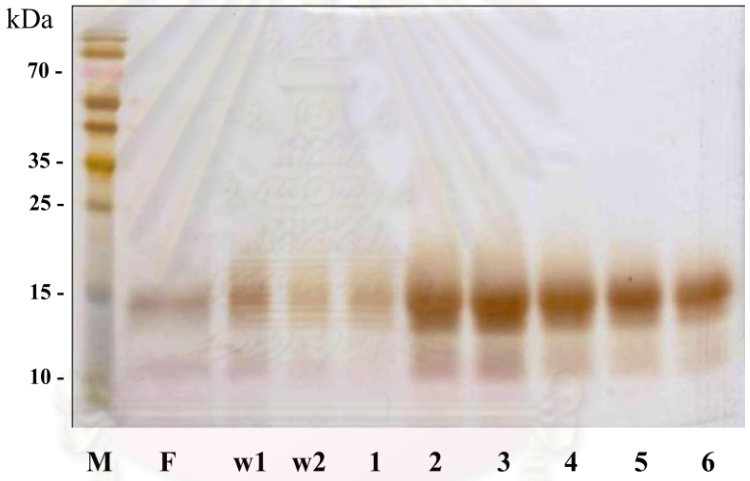


Figure 3.6 Silver-stained SDS PAGE analysis of the purified *r*crustin*Pm*1.
Lane M: Prestained protein marker (Fermentas)
Lane F: Flow through fraction
Lane w1 and w2: Wased fraction 1 and 2
Lane 1-6: Eluted fraction 1-6

3.3 Antimicrobial assay

To confirm antimicrobial activity of purified rcrustin*Pm*s, Gram-positive bacterium *Staphylococcus aureus* was used for minimal inhibitory concentration (MIC). The result showed that both purified rcrustin*Pm*s strongly exhibited antibacterial activity against *S. aureus* with MIC value of 2.5-10 µM for *r*crustin*Pm*1 and 2-8 µM for *r*crustin*Pm*7. These MIC values are in agreement with ones previously reported in Supungul *et al*., 2008 and Amparyup *et al*., 2008.

3.4 Binding of bacterial cells and cell wall components

To determine the binding ability of crustin*Pm*1 and crustin*Pm*7, the purified rcrustin*Pm*1 and rcrustin*Pm*7 were incubated with bacterial cells and cell component, LTA and LPS as described in materials and methods. Silver-stained SDS-PAGE was used to analyse *r*crustin*Pm*s in each fraction. Figure 3.7 shows that rcrustin*Pm*s remains in the supernatant fractions after centrifugation. This indicated that any rcrustin*Pm*s found in the cell pellet fractions was bacterial cell-bound rcrustin*Pm*s. Figure 3.8 shows the supernatant and pellet fractions of *S. aureus* cell incubated with rcrustin*Pm*7 (sample), ALF*Pm*3 (positive control), type-I Lysozyme (negative control) or MES pH 5.8 buffer. The Silver-stained SDS-PAGE showed that the rcrustin*Pm*7 was mostly found in pellet fraction (P) in similar manner to ALF*Pm*3 used as positive control. This indicated that rcrustin*Pm*7 bound to *S. aureus* cells. In contrast to rcrustin*Pm*7 and ALF*Pm*3, type-I Lyzosyme (negative control) was found in supernatant fraction. This confirmed that not all proteins can bind to *S. aureus* cells.

To prove binding of rcrustin*Pm*s to bacterial cells, 5 µg of rcrustin*Pm*1 and rcrustin*Pm*7 were mixed with bacterial cells as described in Materials and Methods. Excess amount of rcrustin*Pm*s was present in the supernatant (S) and the wash (W) fractions (Figure 3.9). 1×loading dye was then added into the cell mixtures in order to elute bound rcrustin*Pm*s. Centrifugation was applied to separate eluted protein and cell pellets. Large amount of rcrustin*Pm*1 and rcrustin*Pm*7 were present in the eluted (E) fractions. Small amount of rcrustin*Pm*s was also found in the cell pellet (P) fractions. This may due to incomplete elution of rcrustin*Pm*s. This result suggested that both crustin*Pm*1 and crustin*Pm*7 bind to both Gram-positive and Gram-negative bacteria (Figure 3.9).

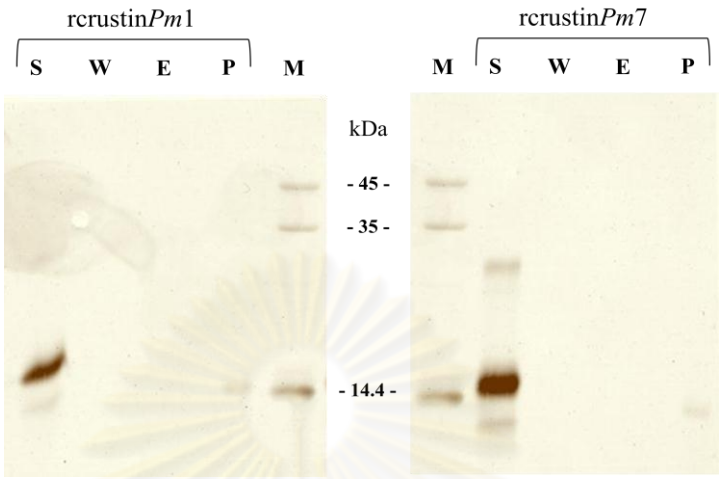


Figure 3.7 Silver-stained SDS-PAGE of rcrustin*Pm*1 and rcrustin*Pm*7 in 50 mM MES buffer pH 5.8. Both of rcrustin*Pm*s were found in supernatant fraction (S).

Lane M: Unstained Protein marker (Fermentus)

Lane S: The supernatant fraction

Lane W: The washed fraction

Lane E: The eluted fraction

Lane P: The cell pellet fraction

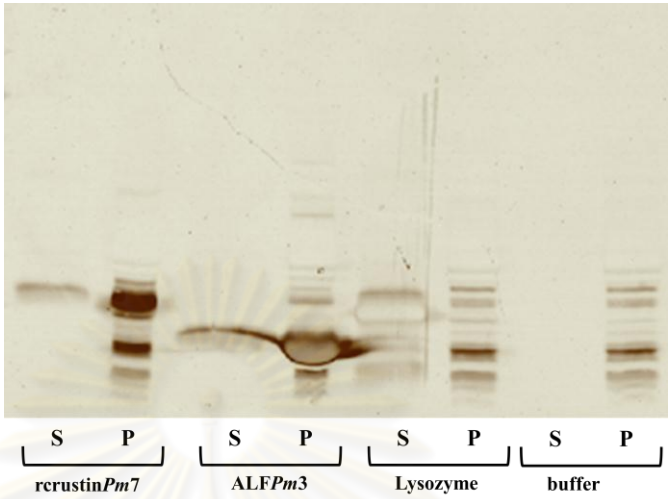


Figure 3.8 The Silver-stained SDS-PAGE of *S. aureus* mixed with rcrustin*Pm*7, ALF*Pm*3, type-I Lysozyme and MES buffer pH 5.8. SDS-PAGE showed that the rcrustin*Pm*7 were mostly founded in pellet fraction (P) in similar manner to ALF*Pm*3 used as positive control. This indicated that rcrustin*Pm*7 bound to *S. aureus* cell. In contrast to rcrustin*Pm*7 and ALF*Pm*3, type-I Lyzosyme (negative control) was found in supernatant fraction. This confirmed that not all proteins can bind to *S. aureus* cell.

Lane S: The supernatant fraction

Lane P: The pellet fraction

Both of rcrustin*Pm*s were incubated with bacterial cells as described in Materials and Methods. The supernatant (S), the washed fractions (W), the eluted fractions (E) and the cell pellet fractions (P) were loaded onto 15% (w/v) SDS-PAGE. The presence of rcrustin*Pm*1 and rcrustin*Pm*7 in each fraction was visualised and determined by Western blot analysis. Bound rcrustin*Pm*s were found in the eluted and cell pellet fractions.

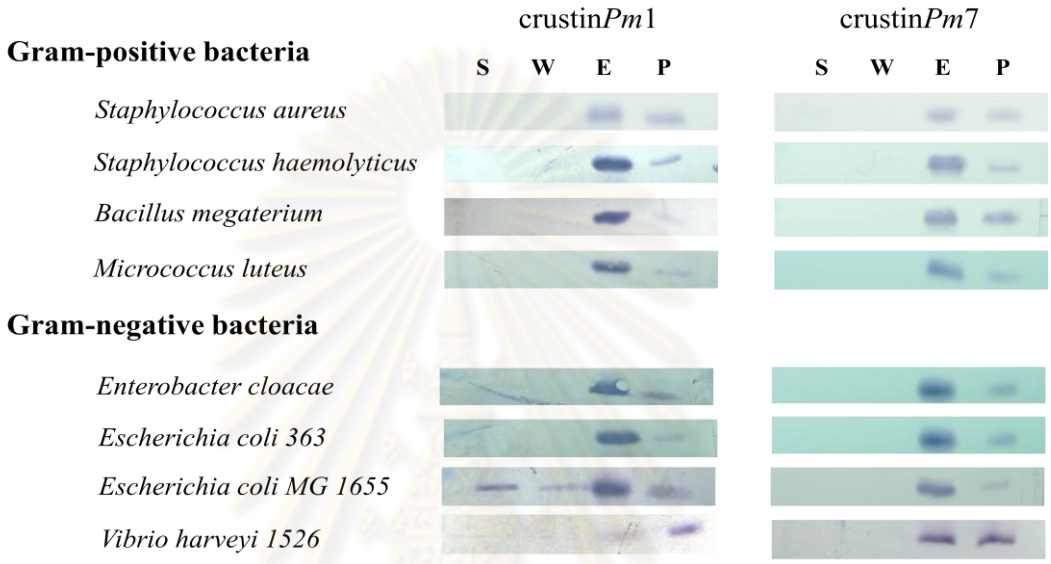


Figure 3.9 The binding of *r*crustin*Pm*1 and *r*crustin*Pm*7 to bacterial cells. *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Bacillus megaterium*, *Micrococcus luteus*, *Enterobacter cloacae*, *Escherichia coli*363, *Escherichia coli* MG1655 and *Vibrio harveyi*1526 were used in this experiment.

Lane S: The supernatant fraction

Lane W: The washed fraction

Lane E: The eluted fraction

Lane P: The cell pellet fraction

The ability of *r*crustin*Pm*1 and *r*crustin*Pm*7 binding to bacterial cells suggested that cell surface components such as lipopolysaccharide (LPS) in Gram-negative and lipoteichoic acid (LTA) in Gram-positive bacteria may play an important role in antimicrobial activity of crustin. Enzyme-linked immunosorbent assay (ELISA) was carried out to quantitatively measure the binding, if any, of *r*crustin*Pm*1 and *r*crustin*Pm*7 to immobilized LPS and LTA. The data revealed that rcrustin*Pm*1 and rcrustin*Pm*7 binds to both LPS and LTA in a concentration-dependent and saturated manner. Nonlinear regression analysis with a One site : Specific binding with Hill slope model showed that the *r*crustin*Pm*s bound to LPS and LTA with an apparent dissociation constant (K_d) of crustin*Pm*1 as 6.204×10^{-6} (LPS) and 5.710×10^{-7} (LTA) M with Hill slope (H) = 2.513 (LPS) and 2.033 (LTA) (Figure 3.10A) and of crustin*Pm*7 as 2.205×10^{-7} (LPS) and 2.072×10^{-7} (LTA) M with Hill slope (H) = 2.147 (LPS) and 2.059 (LTA) (Figure 3.10B). (crutin*Pm*1, R^2 = 0.9816 and 0.9829 for LPS and LTA; crustin*Pm*7 R^2 = 0.9849 and 0.9888 for LPS and LTA)

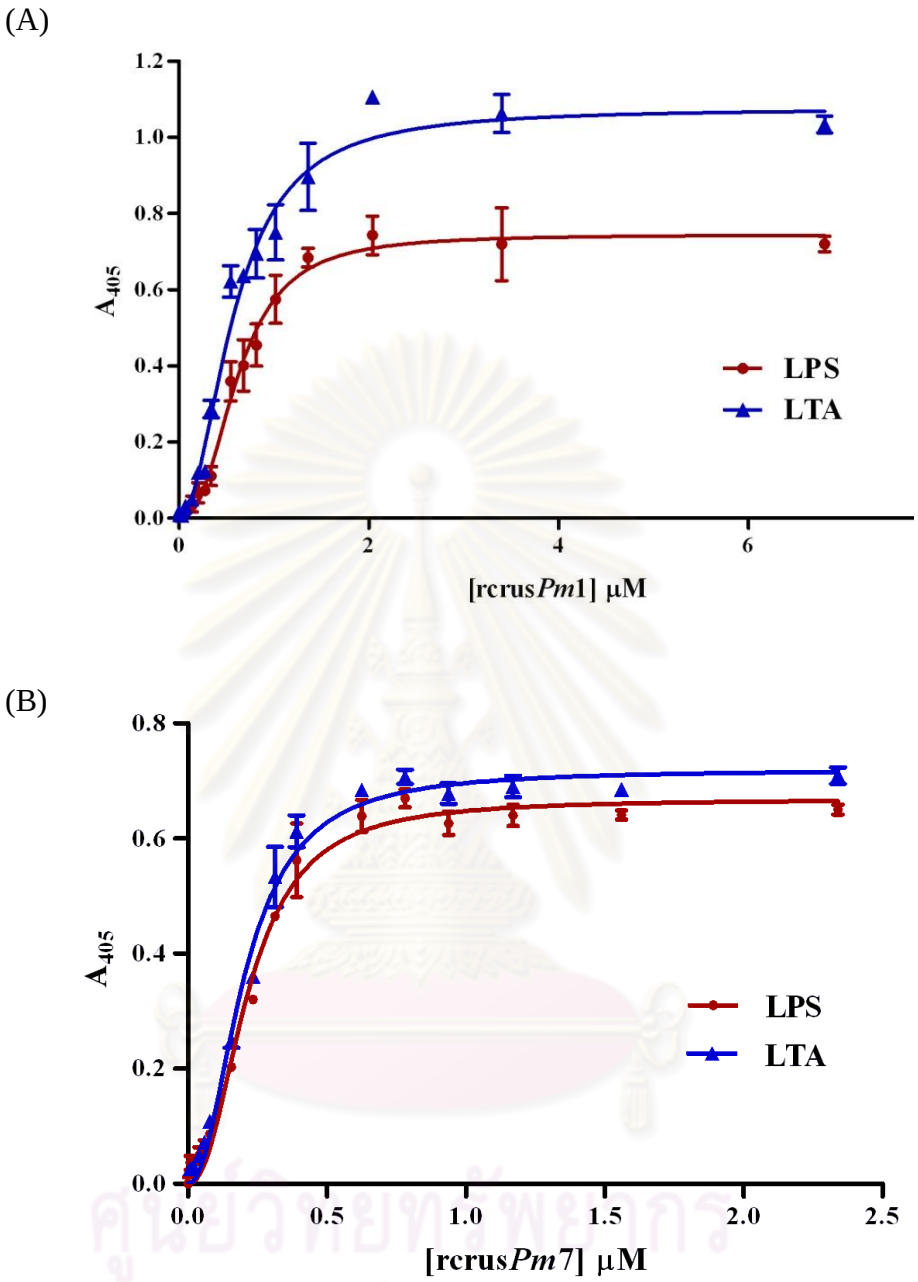


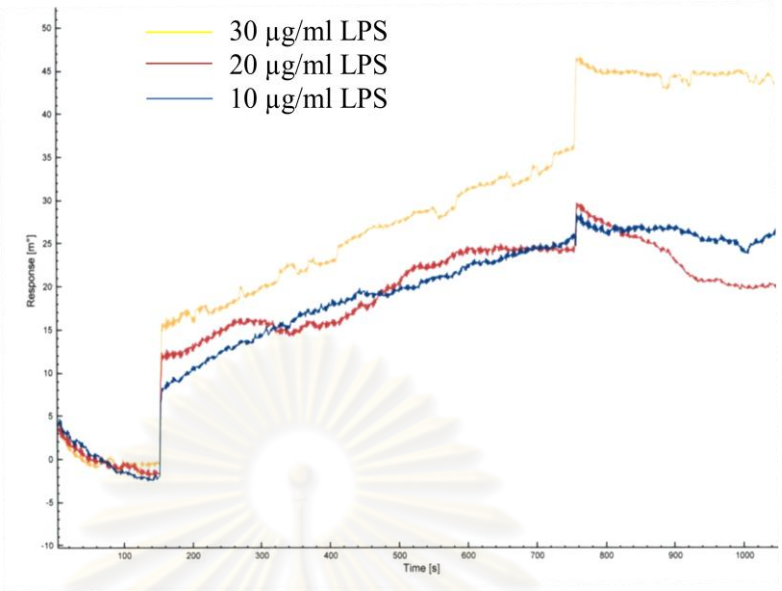
Figure 3.10 Quantitative binding of *r*crustin*Pm*1 (A) and *r*crustin*Pm*7 (B) to immobilized LPS and LTA. The microtiter plates were coated with 3 µg of LPS (red) or LTA (blue) and incubated with increasing amounts of *r*custin*Pm*s.

3.5 Surface Plasmon Resonance

Surface plasmon resonance (SPR) technology enables real-time detection and provides quantitative information on specificity, concentration, kinetics and affinity. In this sudy, Autolab-Twingle SPR was used to determine the interaction between rcrustin*Pm*s and the bacteria cell wall components LPS and LTA. Both LPS and LTA were shown to bind to the rcrustin*Pm*1 (Figure 3.11) and rcrustin*Pm*7 (Figure 3.12) covalently immobilized on 11-MUA sensor chip. The resulting sensorgrams were globally fitted for different amount of ligands using Autolab SPR Data and Kinetic software (Metrohm , USA Inc.) to determine rates and equilibrium affinity constants. Sensorgram of both rcrustin*Pm*1 and rcrustin*Pm*7 suggested interaction between rcrustin*Pm*s and cell wall components LPS and LTA (Figure 3.11 and 3.12).

For rcrustin*Pm*1, affinity constant (K_D) for LTA is a magnitude higher (K_D = $1.3151\times10^{-7}\pm6.7568\times10^{-8}$ M with B_{max} = 52.2240±1.2815) than K_D for LPS (K_D = $1.4771\times10^{-6}\pm3.3716\times10^{-7}$ M with B_{max} = 41.2199±2.6938) (Figure 3.11A and B). In case of rcrustin*Pm*7, K_D for LPS (K_D = $2.2409\times10^{-5}\pm4.6419\times10^{-6}$ M with B_{max} = 52.2017±1.2815) is closed to K_D for LTA (K_D = $1.3161\times10^{-5}\pm1.4070\times10^{-6}$ M with B_{max} = 70.5750±3.2217 (Figure 3.12A and B).

(A)



(B)

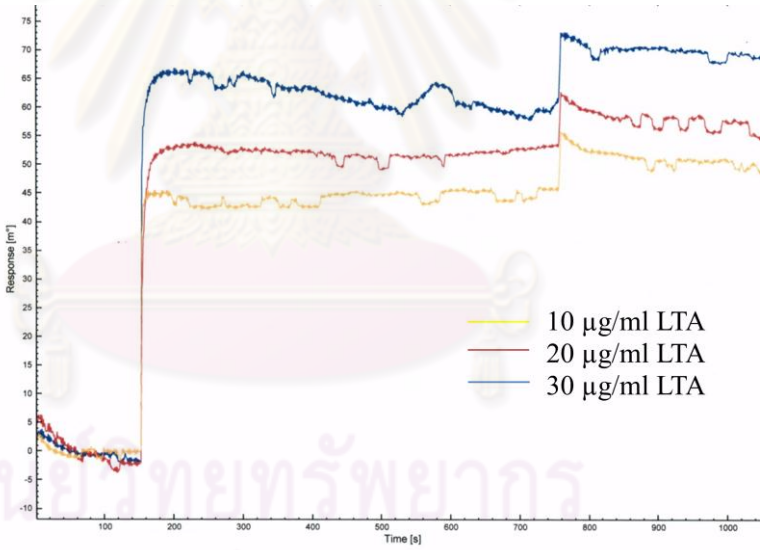
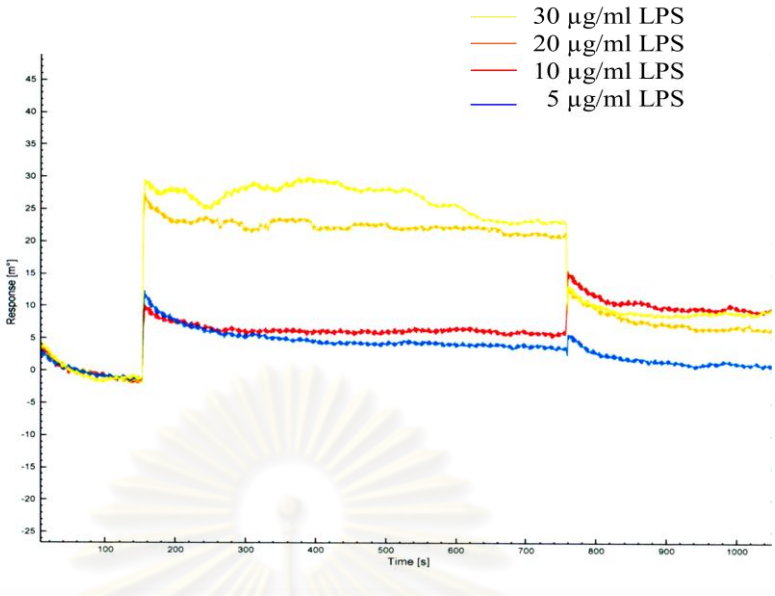


Figure 3.11 Sensorgram of rcrustin*Pm*1 interacting with LPS (A) and LTA (B). Difference concentration of LPS and LTA were used (10, 20 and 30 µg/ml).

(A)



(B)

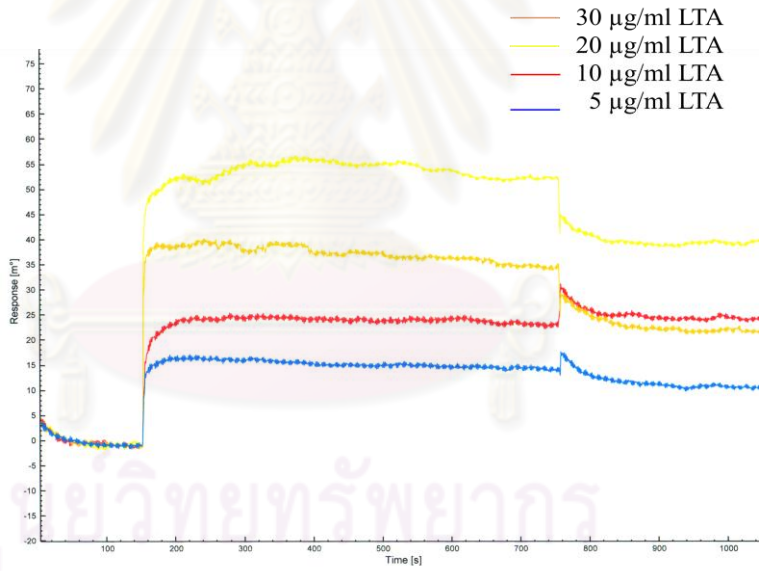


Figure 3.12 Sensorgram of rcrustin*Pm*7 interacting with LPS (A) and LTA (B). Difference concentration of LPS and LTA were used (5, 10, 20 and 30 µg/ml).

3.6 Bacterial agglutination properties

Bacterial binding properties of *r*crustin*Pm*1 and *r*crustin*Pm*7 were tested in soluble phase by monitoring bacterial agglutination properties. Agglutination was analyzed visually in 96-wells round bottom plate. When agglutination occurred, a large solid aggregates were visible microscopically (Figure 3.13).

Regarding Figure 3.14, both rcustin*Pm*s clearly induced bacterial agglutination ag*ainst S. aureus, B. megaterium, M. luteus*, *E. coli 363* and *E. coli* MG 1655. Meanwhile, only rcustin*Pm*7 induced bacterial agglutination against *V. harveyi*. No bacterial agglutination was observed when both rcustin*Pm*s were mixed with *E. carotovara* and *E. cloacae*. Table 3.1 summarises bacterial agglutination properties of rcrustin*Pm*1 and rcrustin*Pm*7.

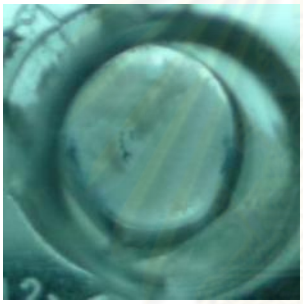
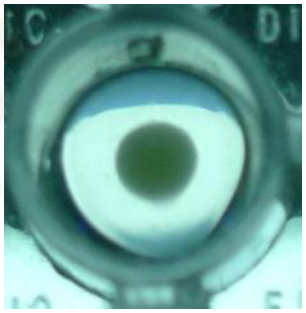
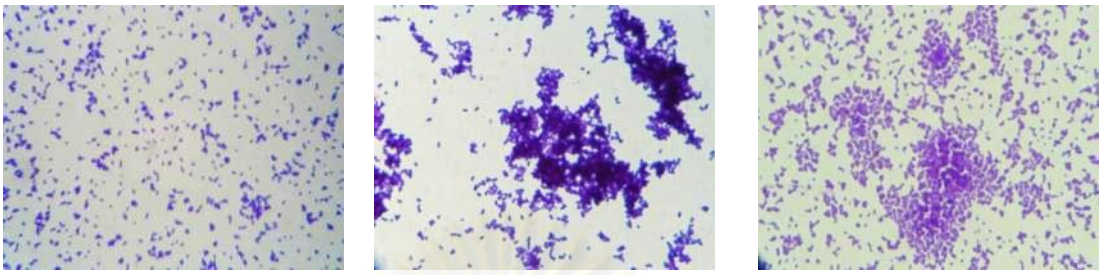
(A)  (B) 

Figure 3.13 Pattern of bacterial agglutination in 96-wells round bottom plate. When agglutination was occurred, bacterial cell was aggregated and glazed around the round bottom plate (A), when there was no bacterial agglutination, bacterial cells were precipitated in the middle of the well (B).

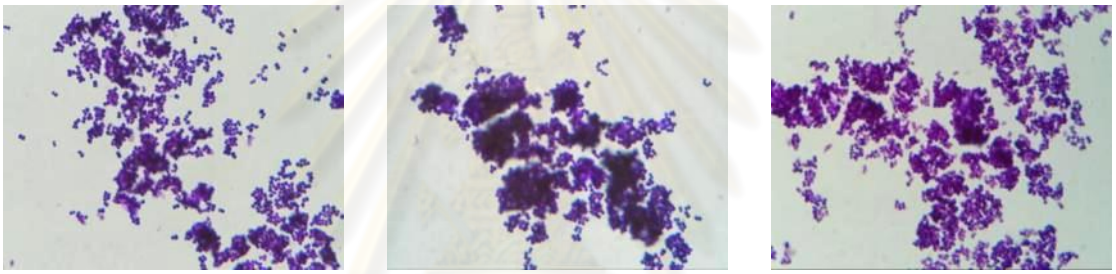
Staphylococcus aureus

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7



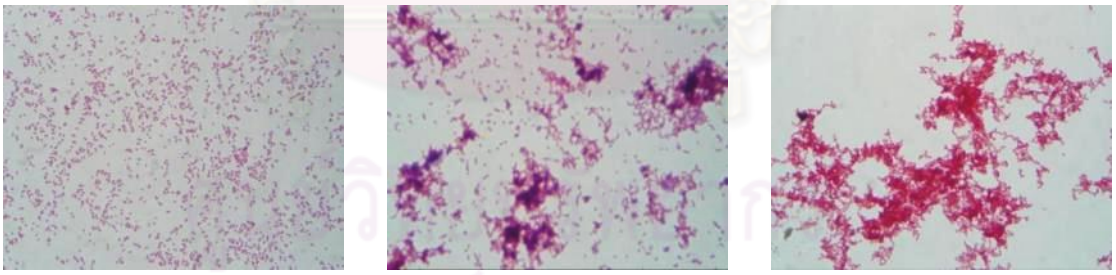
Staphylococcus haemolyticus

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7



Bacillus megaterium

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7



Micrococcus luteus

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7

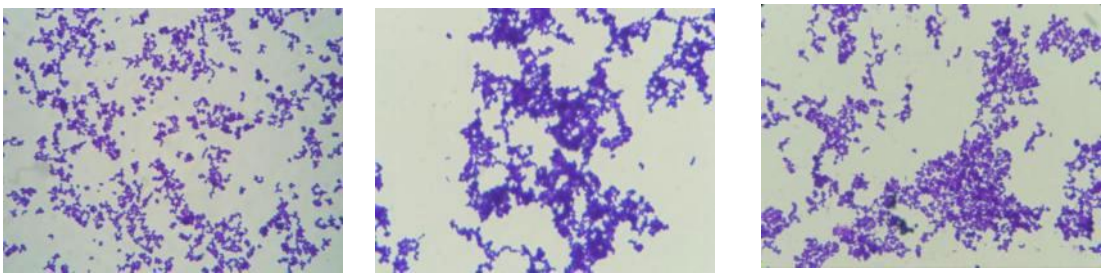
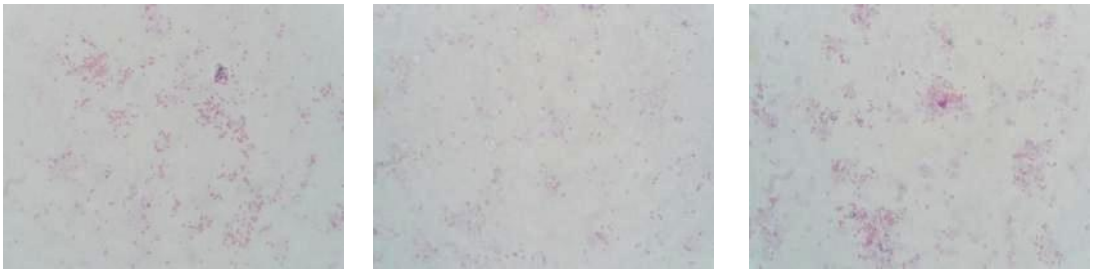


Figure 3.14

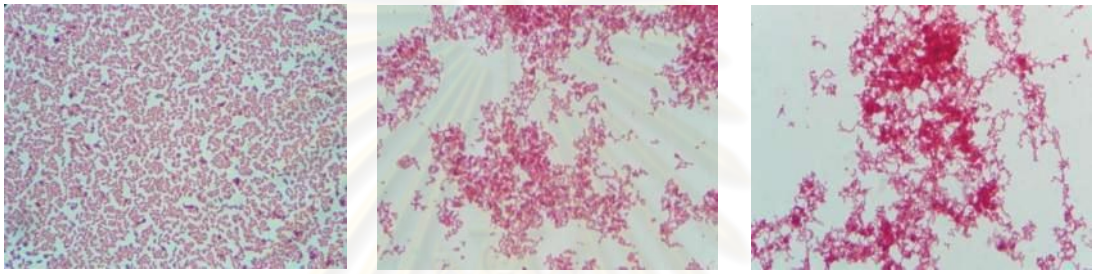
Enterobacter cloacae

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7



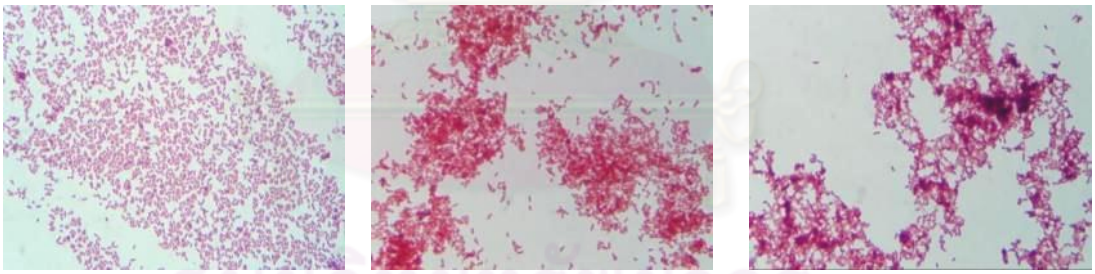
Escherichia coli 363

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7



Escherichia coli MG 1655

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7



Vibrio harveyi 1526

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7

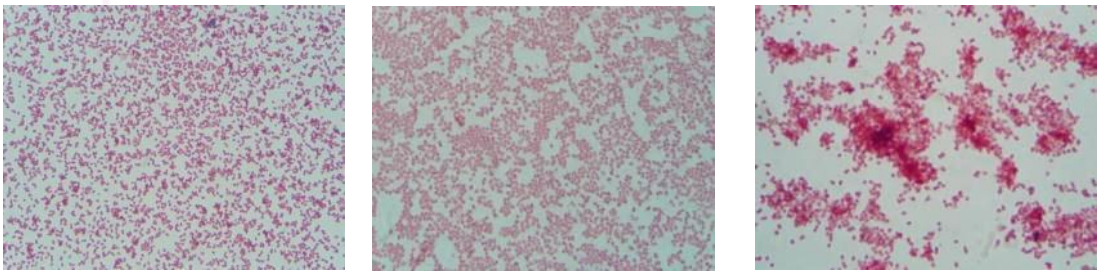


Figure 3.14 (cont.)

Erwinia carotovara

Control (MES buffer pH 5.8)	rcrustin*Pm*1	rcrustin*Pm*7

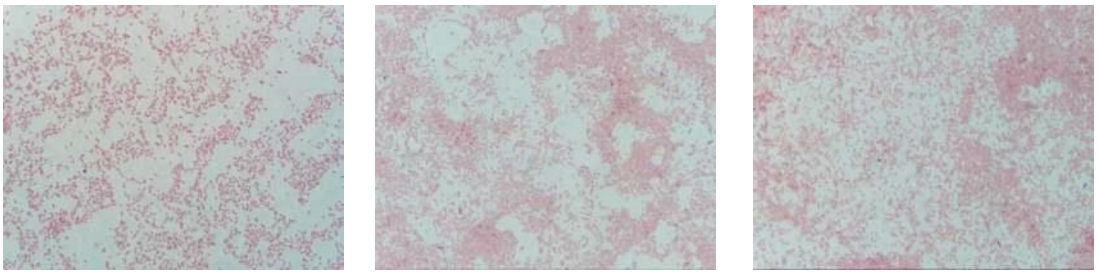


Figure 3.14 (cont.) Bacterial agglutination properties of *r*crustin*Pm*1 and *r*crustin*Pm*7.

Table 3.1 Summary of bacterial agglutination properties of rcrustin*Pm*1 and rcrustin*Pm*7.

Bacteria strain	Bacterial agglutination	
	rcrustin*Pm*1	rcrustin*Pm*7
Gram-positive bacteria		
Staphylococcus aureus	+++	+
Staphylococcus haemolyticus	-	-
Bacillus megaterium	++	+++
Micrococcus luteus	++	++
Gramnegtive		
Enterobacter cloacae	0	0
Escherichia coli 363	+	++
Escherichia coli MG1655	++	++
Vibrio harveyi	0	++
Eirwinia carotovara	0	0

- : auto agglutination/self agglutination
0 : no agglutination
+ : partial agglutination
++ : incompleted agglutination
+++ : completed agglutination

3.7 Inner membrane (IM) permeability

IM permeabilization of *E. coli* strain MG1655 lacY: Tn10dKan was determined by measuring its β-galactosidase activity after incubation with rcrustin*Pm*1 or rcrustin*Pm*7. β-galactosidase is located in the cytosol and hence it will be detected when the bacteria cell membrance is permeabilized. β-galactosidase activity was assayed by mixing supernatant bacteria culture at each incubation time points with O-nitrophenyl-β-D-galactopyranoside (ONPG). When the β-galactosidase cleaves ONPG, O-nitrophenol is released. This compound is yellow color and absorb light at 405 nm.

Figure 3.15 shows the plot of detected β-galactosidase activity against incubation time of rcrustin*Pm*1 and rcrustin*Pm*7 with *E. coli* strain MG1655. It is clear that rcrustin*Pm*7 can induce inner membrane permeabilization of *E. coli* strain MG1655 as β-galactosidase activity increase over time. In contrast to rcrustin*Pm*1, small change in β-galactosidase activity was observed when incubating rcrustin*Pm*1 with *E. coli* strain MG1655 in comparison to the control. This indicated that rcrustin*Pm*7 has higher ability to induce IM permeabilization of *E. coli* MG1655 than rcrustin*Pm*1

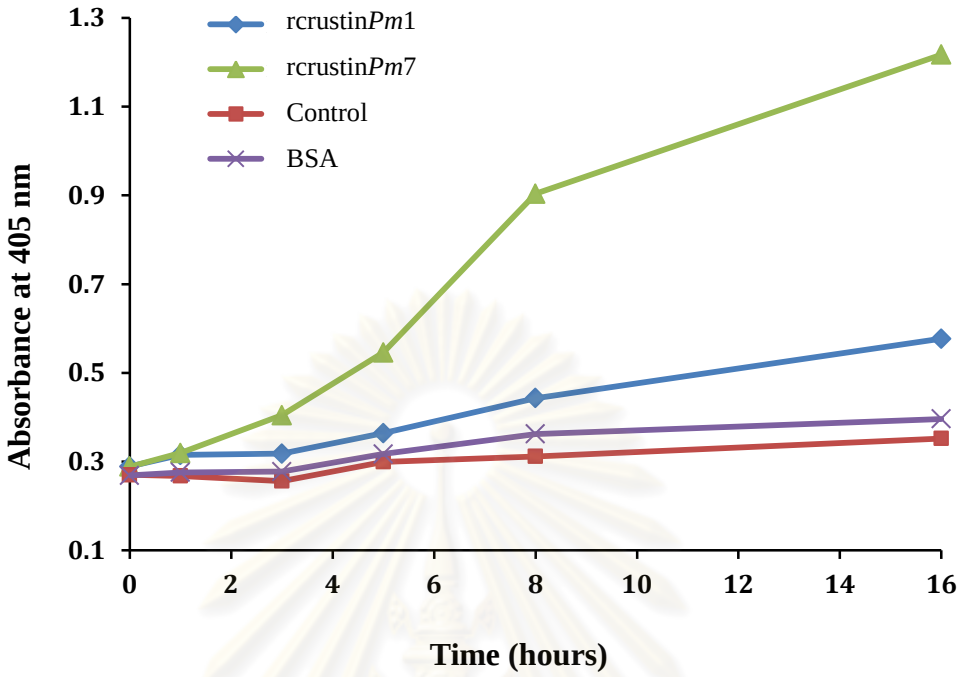


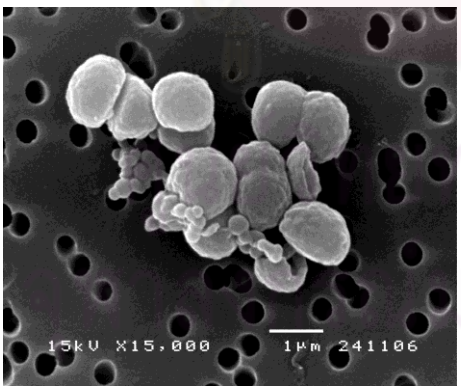
Figure 3.15 A plot of β-galactosidase activity of *E. coli* MG1655 over time. Recombinant crustin*Pm*1 (blue) and crustin*Pm*7 (green) induced inner membrane (IM) permeabilization of *E. coli* strain MG1655. The IM permeabilization was determined by spectroscopy at 405 nm to measuring the β-galactosidase activity of *E. coli* strain MG1655. The absorbance is plotted on Y-axis, which is proportional to the β-galactosidase activity and the incubation time of incubation with rcrutin*Pm*1 and rcrustin*Pm*7.

3.8 Scanning Electron Microscope (SEM)

SEM JSM-5800LV was used to observed the cells surface of Gram-positive bacteria *S. aureus*, Gram-negative bacteria *E. coli* MG1655 and *V. harveyi* after exposure to rcrustin*Pm*1 or rcrustin*Pm*7. Incubation of both rcrustin*Pm*s to *S. aureus* and *E. coli* MG1655 caused remarkable structural changes with rough and distorted cells appearance (Figure 3.16B and C). In addition, rcrustin*Pm*1 and rcrustin*Pm*7 can also induce bleb formation on *E. coli* membrane (Figure 3.17B and C). However, only rcustinPm7 can cause pore formation on *V. harveyi* cells surface (Figure 3.18B and C).

(A)

(B)



(C)

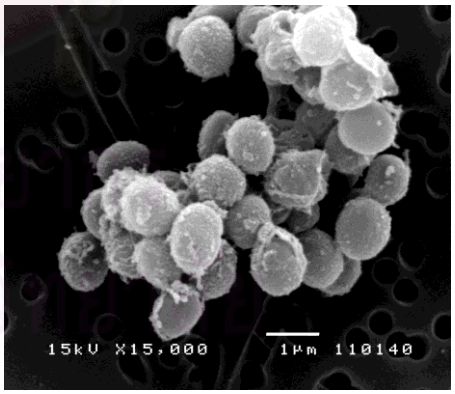
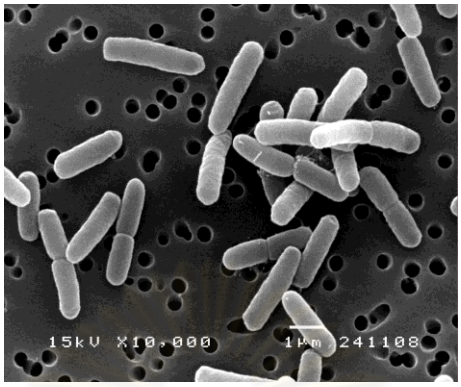
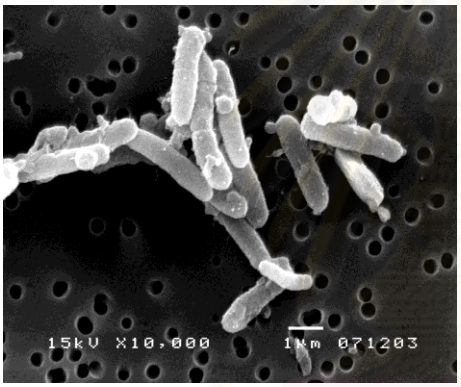


Figure 3.16 Scanning Electron Microscope image of *S. aureus* after the bacteria was incubated with MES buffer pH 5.8 (A), rcrustin*Pm*1 (B) and rcrustin*Pm*7 (C) for 1 h.

(A)



(B)



(C)

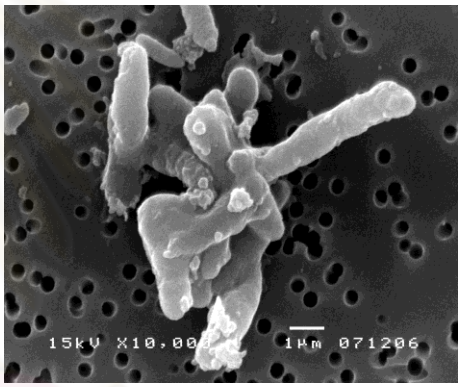
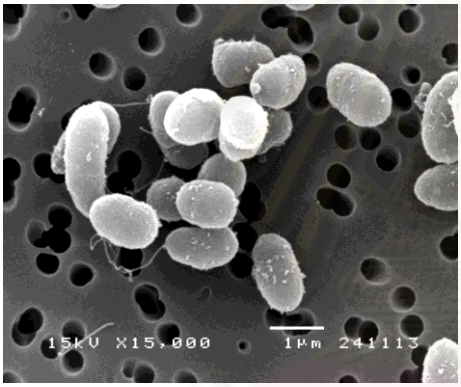


Figure 3.17 Scanning Electron Microscope image of *E. coli* MG1655 after the bacteria was incubated with MES buffer pH 5.8 (A), rcrustin*Pm*1 (B) and rcrustin*Pm*7 (C) for 1 h.

(A)

(B)



(C)

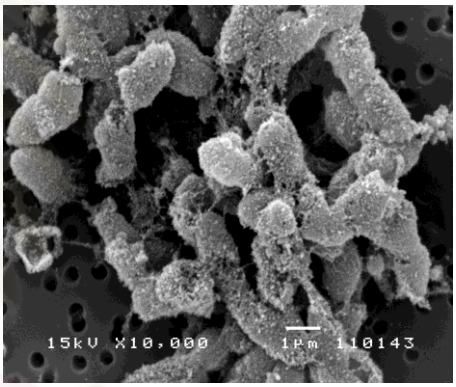


Figure 3.18 Scanning Electron Microscope image of *V. harveyi*1526 after the bacteria was incubated with MES buffer pH 5.8 (A), rcrustin*Pm*1 (B) and rcrustin*Pm*7 (C) for 1 h.

3.9 Crystallization

Recombinant crustin*Pm*1 produced in *E. coli* and *P. pastoris* was used for crystallization screening. Proteins were mixed with resorvior solutions in 96-well plate (1:1). Crystals of rcrustin*Pm*1 appears in 0.2 M Lithium citrate tribasic tetrahydrate, 20% PEG 3350, pH 8.4 (Figure 3.18). Attempts to crystallize rcrustin*Pm*7 were also carried out. So far, no crystallization condition for rcrustin*Pm*7 has been found.

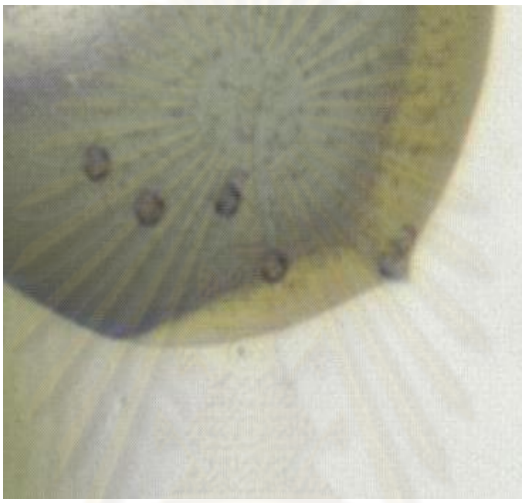


Figure 3.19 Crystals of rcrustin*Pm*1.

CHAPTER IV
DISCUSSION

True mechanisms of AMPs still remain unknown, but it is believed that "…antimicrobial basic proteins and polypeptides combine with cell nucleoproteins or other negatively charged surface constituents of bacteria or viruses, thus disrupting important cell function. The union of the basic substances with negatively charged cell surfaces is believed to occur through electrostatic bonding" (Skarnes et al., 1957).

In a previous study, the pattern recognition receptors MARCO and Spa bound to bacterial LPS and LTA through their SRCR domains (Brannstrom et al., 2002; Sarrias et al., 2005. Anti-lipopolysaccharide factor (ALF*Pm*3) from the black tiger shrimp presented the binding ablilities against Gram-negative and Gram-positive bacterial cells and their major cell wall components as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Somboonwiwat et al., 2008). In 2009, Yang et al. demonstrated that the anti-lipopolysaccharide factor binds to LPS, lipid A and to OM®-174, a soluble analogue of lipid A using Surface Plasmon Resonance. Moreover, they designed an original model of the possible lipid A-binding site by NMR on the basis of striking structural similarities to the FhuA/LPS complex (Yang et al., 2009)

In this study, rcrustin*Pm*1 and rcrustin*Pm*7 were tested for their binding properties to some bacteria (*S. aureus*, *S. haemolyticus*, *B. megaterium*, *M. luteus*, *E. cloacea*, *E. coli* 363, *E. coli* MG 1655 and *V. harveyi*). The result showed that both rcrustins can bind to all bacteria. The binding ability of rcrustin*Pm*7 to *V. harveyi* was higher than that of rcrustin*Pm*1. However, not all binding properties of rcrustins follow the trend of their antimicrobial activity (Table 4.1 and 4.2). It is possible that rcrustins bind to bacterial cells through electrostatic effects. Antimicrobial peptides must be attracted to bacterial surfaces before performing antimicrobial actions. Studies of peptides like magainin 2 and cecropin A showed that, peptides insert into monolayers, large unilamellar vesicles and liposomes that contain acidic phospholipids (Silvestro et al., 1997; Zhao et al., 2001). This is in agreement with the fact that rcrustin*Pm*7 has antimicrobial activity against *V. harveyi*, but rcrustin*Pm*1 does not (Supungul et al., 2008; Amparyup et al., 2008).

Table 4.1 Antimicrobial activity of a rcrustin*Pm*1 (Supungul et al, 2008).

Microorganisms	MIC value (μM)
Gram-positive bacteria	
Staphylococcus aureus	3.13–6.25
Staphylococcus haemolyticus	50–100
Aerococcus viridans	50–100
Bacillus megaterium	6.25–12.50
Micrococcus luteus	25–50
Streptococcus iniae	3.13–6.25
Gram-negative bacteria	
Salmonella thyphimurium	NA
Escherichia coli 363	50–100
Enterobacter cloacea	NA
Vibrio harveyi 1526	NA
Klebsiella pneumonia	NA
Erwinia carotovora	NA

MIC are expressed as the interval a–b, here a is the highest concentration tested at which microorganisms are growing and b the lowest concentration that cause 100% growth inhibition. NA: not active up to 100 mM.

Table 4.2 Antimicrobial activity of a rcrustin*Pm*7 (Amparyup et al, 2008).

Microorganisms	MIC value (μM)
Gram-positive bacteria	
Staphylococcus aureus	5–10
Staphylococcus haemolyticus	2.5–5
Aerococcus viridans	0.312–0.625
Bacillus megaterium	1.25–2.5
Micrococcus luteus	2.5–5
Gram-negative bacteria	
Salmonella thyphimurium	NA[a]
Escherichia coli 363	2.5–5
Enterobacter cloacea	NA[a]
Vibrio harveyi 1526	2.5–5
Klebsiella pneumonia	10–20

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

[a] Not active at 80 μM.

Similar to the bacterial cells binding study, both rcrustin*Pm*s showed binding ability to bacteria cell wall components, LPS from Gram-negative bacteria and LTA

from Gram-positive bacteria. The assays showed that rcrustin*Pm*1 can bind to both immobilized LPS and LTA with dissociation constant (K_d) as $6.204x10^{-6}$ and $5.710x10^{-7}$ M, respectively. This suggested that rcrustin*Pm*1 bind more tightly to LTA than LPS and this may account for the high antimicrobial activity of rcrustin*Pm*1 against Gram-positive bacteria. On the other hand, rcrustin*Pm*7 can bind to both immobilized LPS and LTA with nearby K_d as $2.205x10^{-7}$and $2.072x10^{-7}$ M, respectively. The results can be implied the binding affinity of rcrustin*Pm*7 to LPS and LTA is similar. It is possible that LPS and LTA are one of the target molecules for the rcrustin*Pm*1 and rcrustin*Pm*7 binding. Moreover, the quantitative binding of rcrustin*Pm*1 to LPS and LTA demonstrated the increasing of signal in S-shape with Hill slope (H) as 2.513 and 2.033, respectively. Similarly, rcrustin*Pm*7 binds to LPS and LTA with Hill slope (H) as 2.147 and 2.059, respectively. H value is greater than 1.0, when the receptor or ligand has multiple binding sites with positive co-operativity. This mean the initial binding of LPS or LTA could help.

To confirm the binding ability to the cell wall component, LPS and LTA. Surface Plasmon Resonance (SPR) was used. SPR also showed that both rcrustin*Pm*s can bind to LPS and LTA. Similar to ELISA experiment, rcrustin*Pm*1 exhibits higher affinity for LTA (K_D for LTA = 1.3151×10^{-7} $\pm6.7568\times10^{-8}$ M and K_D for LPS = $1.4771\times10^{-6}\pm$ 3.3716×10^{-7} M). In addition, rcrustin*Pm*7 can bind to LPS and LTA with similar K_D (K_D for LPS = $2.2409\times10^{-5}\pm4.6419\times10^{-6}$ M and K_D for LTA = $1.3161\times10^{-5}\pm1.4070\times10^{-6}$ M)

It is possible that rcrustin*Pm*s bind to bacterial cells by attracting to anionic phospholipids and phosphate groups on lipopolysaccharide (LPS) of Gram-negative bacteria and to the teichoic acids on the surface of Gram-positive bacteria. Artificial chimeric peptides such as CEME bind to LPS (Scott et al., 1999) and lipoteichoic acid (Scott et al., 1999). The ability of CEME-related peptides bind to lipoteichoic acid does not correlate with their ability to kill bacteria, indicating that peptides might use this mechanism to contact other targets, such as the cytoplasmic membrane. High affinity to LTA of the cationic peptides, bee melittin and silk moth cecropin, which have moderate growth-inhibitory activities toward Gram-positive bacteria, has been reported previously. However, the affinity of these peptides to LTA is related to the ability to block LTA-induced inflammation but not to kill bacteria (Scott et al., 1999). Once close to the microbial surface, peptides must traverse capsular polysaccharides

before they can interact with the outer membrane, which contains LPS in Gram-negative bacteria, and traverse capsular polysaccharides, teichoic acids and lipoteichoic acids before they can interact with the cytoplasmic membrane in Gram-positive bacteria. This concept is important but is rarely addressed in mechanistic studies. Once peptides have gained access to the cytoplasmic membrane they can interact with lipid bilayers. *In vitro* studies of antimicrobial peptides incubated with single or mixed lipids in membranes or vesicles show that peptides bind in two physically distinct states (Huang, 2000).

With the ELISA and SPR results, possible candidates for crustin*Pm*1 and crustin*Pm*7 binding are conserved bacteria surface molecules such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). It is assumed that crustin*Pm*s bind to bacterial cells before performing its functions to inhibit or kill the bacterial cells.

Agglutination is the clumping of particles. This occurs in biology in three main examples: 1) The clumping of cells such as bacteria or red blood cells in the presence of antibodies. The antibody or other molecule binds multiple particles and joins them, creating a large complex. 2) The coalescing of small particles that are suspended in a solution; these larger masses are then (usually) precipitated. 3) An allergic reaction type occurrence where cells become more compacted together to prevent foreign materials entering them. Agglutination in microbiology is commonly used as a method of identifying specific bacterial antigens, and in turn, the identity of such bacteria. Because the clumping reaction occurs quickly and is easy to produce, agglutination is an important technique in diagnosis. This is usually the result of an antigen in the vicinity of the cells.

In previous studies, agglutination properties of 76 kDa hemocyanin subunit from mud crab (*Scylla serrata*) against seven bacterial species, was identified as the protein that primarily binds bacterial cells and mediates agglutination through recognition of OmpA and OmpX proteins in bacteria (Yan et al., 2010). However, reported about bacterial agglutination properties by AMPs have never been reported. Our agglutination study suggested that rcrustin*Pm*1 and rcustin*Pm*7 not only inhibit bacteria but also induce or mediate bacterial agglutination. This helps prevent or quarantine the bacterial outbreak in blood system. It is worth noting that agglutination and antimicrobial activity are correlated. Recombinant crustin*Pm*1 causes agglutination in *S. aureus, B. megaterium, M. luteus*, *E. coli 363* and *E. coli* MG1655

and also has antimicrobial activity against these bacteria (Table 4.1), (Supungul et al., 2008). Regarding rcrustin*Pm*7, the correlation between agglutination and antimicrobial activity also occurs in the case of *S. aureus*, *B. megaterium*, *M. luteus*, *E. coli 363*, *E. coli* MG1655 and *V. harveyi* (Table 4.2), (Amparyup et al., 2008). In the case of and *S. haemolyticus*, it is difficult to determine the presence of bacterial agglutination as they undergo self-auto agglutination.

In this study, we also investigate ability of rcrustin*Pm*s to permeabilized inner membrane of *E. coli* MG1655. Lysozyme (BL) has been previously showed ability to permeabilize inner cells membrane of *E. coli* MG1655 by determining of β-galactosidase activity (Archana et al., 2007). In this work, we investigated the ability of rcrustin*Pm*1 and rcrustin*Pm*7 causing IM permeabilization using the same method as described in Archana, 2007. Although 100 μM of rcrustin*Pm*1 was completely inhibited growth of *E. coli*363 (Supungul et al., 2008), small change in β-galactosidase activity was observed when incubating 100 μM rcrustin*Pm*1 with *E. coli* MG1655. Recombinant crustin*Pm*7, meanwhile, exhibited a strong change in β-galactosidase activity over time. This indicates that rcrustin*Pm*7 efficiently induced inner membrane permeabilization in *E. coli* MG1655.

Scanning Electron Microscope (SEM) technique was used to determine the actions of rcrustin*Pm*s against bacterial cell. Previously, effects of melamine, a cationic AMP derived from portions of meliltin and protamine, on bacterial cells were studied by SEM. This work revealed that a major melimine effect was on the integrity of the cytoplasmic membrane to both *P. aeruginosa* and *S. aureus* (Rasul et al., 2010). Microscopic images showed massive change on cytoplasmic membrane of *S. aureus* and *E. coli* after the bacterial cells were incubated with rcrustin*Pm*s. These results supported the IM permeabilization study that rcrustin*Pm*s cause some changes on the membrane of *E. coli* MG1655. Some cells *S. aureus* and *E. coli* were presented in abnormal forms with roughening, bleb formation and distortion. In case of *V. harveyi* strain, the cells were not changing after incubated with rcrustin*Pm*1 in comparison to the control. However, when *V. harveyi* cells were incubated with rcrustin*Pm*7, spot like tiny pores appeared on the cell surface and some cells were distorted.

Rasul et al. (2010) described differences in the relationship between depolarization and the kinetics of viability loss of the two bacteria. For *P. aeruginosa* the rapid loss of cytoplasmic membrane integrity demonstrated by releasing of

membrane potential-sensitive cyanine dye diSC_3-5 correlated directly with loss of cell viability, whilst for *S. aureus* maximal dye release was obtained at concentrations where there was no significant loss of viability. This suggests that either the effects of depolarisation of the cytoplasmic membrane were delayed in *S. aureus* or that a secondary process affecting metabolic activity in the cell may occur.

However, there is increasing many evidence indicating that antimicrobial peptide have many mechanisms to inhibit the bacterial growth. Pore forming on cell membrane is not the only function of AMPs that lead to cell death. AMP may penetrate into the cell to bind intracellular molecules which are crucial for cell living (Brogden, 2005). For example, Bac7 fragment 1-16, 1-23 and 1-35 did not permeabilize *E. coli* but caused a 2-5 log reduction in the number of organisms (Gennaro and Zanetti, 2002). Therefore, the fact that rcrustin*Pm*s can cause inner membrane permeabilization and induce physical change on the cell surface, can not rule out the ability of rcrustin*Pm*s to bind intracellular molecules, resulting in bacterial cell death.

In crystallization experiments, crystallization condition of rcrustin*Pm*1 is 0.2 M Lithium citrate tribaic tetrahydrate, 20% PEG 3350, pH 8.4. The pH of this crystallization condition is closed to pI of crustin*Pm*1 (pI ~ 8.3). However, crystallization condition of rcrustin*Pm*7 has not been found. This may due to homoginity of rcrustin*Pm*7 sample. New methods for expression and purification of rcrustin*Pm*7 should be explored. These may help improve the sample quality and lead to rcrustin*Pm*7 crystals.

CHAPTER V
CONCLUSIONS

In this work, rcrustin*Pm*1 and rcrustin*Pm*7 were expressed in *E. coli* strain Rosetta (DE3) and purified by Ni-NTA column chromatography with denaturing condition. Recombinant crustin*Pm*1 and crustin*Pm*7 were then tested for their antimicrobial activity and used for (i) Bacterial and cell components binding assays (ii) SPR (iii) Bacterial agglutination assay (iv) Inner membrane permeability and (v) Scanning Electron Microscope (SEM).

Bacterial and cell wall components binding assays suggested that both rcrustin*Pm*s can bind to *S. aureus*, *S haemolyticus*, *B. megaterium*, *M. luteus*, *E. cloacae*, *E. coli*363, *E. coli* MG1655 and *V. harveyi*1526 and both cell wall components, LPS and LTA.

It is possible that rcrustin*Pm*1 and rcrustin*Pm*7 initially bind to bacterial cell wall by electrostatic effects as they are cationic antimicrobial peptides. ELISA showed that both rcrustin*Pm*s can bind to LPS and LTA, a cell wall component of Gram-positive and Gram-negative bacteria, respectively. Both rcrustin*Pm*s bind to LPS and LTA in accumulating manners with Hill slope for rcrustin*Pm*1 2.513 (LPS) and 2.033 (LTA) and rcrustin*Pm*7 2.147 (LPS) and 2.059 (LTA). It is possible that binding of LPS and LTA molecule helps stimulate further LPS and LTA binding. SPR also confirms the binding of both rcrustin*Pm*s to LPS and LTA.

Recombinant crustin*Pm*1 and crustin*Pm*7 can induce bacterial agglutination of *S. aureus*, *B. megaterium*, *M. luteus*, *E. coli*363, *E. coli* MG1655 and *V. harveyi*. This agglutination phenomenon is correlated with antimicrobial activity of crustin*Pm*s. For example, rcrustin*Pm*1 neither inhibits growth nor induces agglutination against *V. harveyi* whilst rcrustin*Pm*7 can inhibit growth and induce agglutination against *V. harveyi*. It is likely that bacterial agglutination occurs to prevent bacteria from spreading in blood system.

Recombinant crustin*Pm*1 and crustin*Pm*7 showed ability to permeabilized membrane of *E. coli* strain MG 1655. Permeabilization was justified by an increasing of β-galactosidase activity over time. Furthermore, SEM showed that *S. aureus*, *E. coli* and *V. harveyi* lost cell surface integrity when the bacteria were incubated with

rcrustin*Pm*7, In contrast, rcrustin*Pm*1 can cause a physical change on the cell surface of *S. aureus* and *E. coli*, but not *V. harveyi*.

Recombinant crustin*Pm*1 can be crystallized in 0.2 M Lithium citrate tribaic tetrahydrate, 20% PEG 3350, pH 8.4. No crystallization condition of rcrustin*Pm*7 has been found. This may due to the quality of rcrustin*Pm*7 sample.

References

Amparyup, P., Kondo, H., Hirono, I., Aoki, T. and A. Tassanakajon (2008). Molecular cloning, genomic organization and recombinant expression of a crustin-like antimicrobial peptide from black tiger shrimp *Penaeus monodon*. Molecular Immunology 45: 1085-1093.

Archana, S. G., Gude, J. and Javaregowda, N. (2007) Immune upregulation of novel antibacterial proteins from silkmoths (Lepidoptera) that resemble lysozymes but lack muramidase activity. Insect Biochemistry and Molecular Biology. 37: 655–666.

Ariki, S., Koori, K., Osaki, T., Motoyama, K., Inamori, K. and Kawabata, S. (2004). A serine protease zymogen functions as a pattern-recognition receptor for lipopolysaccharides. Proc. Natl. Acad. Sci. 101: 953-958.

Aso, Y., Yamashita, T., Meno, K. and Murakami, M. (1994). Inhibition of prophenoloxidase-activating enzyme from *Bombyx mori* by endogenous chymotrypsin inhibitors. Biochemistry and Molecular Biology International 33: 751-758.

Aspan, A., Huang T., Cerenius, L. and Soderhall, K. (1995). cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proc. Natl. Acad. Sci. 92: 939-943.

Aspan, A., Sturtevant, J., Smith, V. J. and Soderhall, K. (1990). Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. Insect Biochemistry 20: 709-718.

Aspan, A. and Soderhall, K. (1991). Purification of prophenoloxidase from crayfish blood-cells, and its activation by an endogenous serine proteinase. Insect Biochem 21: 363-373.

Bartlett, T. C., Cuthbertson, B. J., Shepard, E. F., Chapman, R. W., Gross, P. S. and Warr, G. W. (2002). Crustins, homologues of an 11.5-kDa antibacterial

peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*. Marine Biotechnology 4: 278-293.

Boigegrain, R. A., Mattras, H., Brehelin, M., Paroutaud, P. and Coletti-Previero, M. A. (1992). Insect immunity: Two proteinase inhibitors from hemolymph of *Locusta migratoria*. Biochemical and Biophysical Research Communications 189: 790-793.

Bolintineanu, D., Hazrati, E., Davis, H. T., Lehrer, R. I. and Kaznessis, Y. N. (2010). Antimicrobial mechanism of pore-forming protegrin peptides: 100 pores to kill *E. coli*. Peptides. 31: 1–8.

Boonyaratpalin, S., Supamattaya, K., Kasornchandra, J., Dierkkubsaracom, S., Ekpanithanpong, U. and Chantanachooklin, C. (1993). Non-occluded baculo-like virus, the causative agent of yellow head disease in the clack tiger shrimp (*Penaeus monodon*). Fish Pathology 28: 103-109.

Brannstrom, A., Sankala, M., Tryggvason, K. and Pikkarainen, T. (2002). Arginine residues in domain V have a central role for bacteria-binding activity of macrophage scavenger receptor MARCO. Biochem. Biophys. Res. Commun. 290: 1462–1469.

Brockton, V., Hammond, J. A. and Smith, V. J. (2007). Gene characterisation, isoforms and recombinant expression of carcinin, an antibacterial protein from the shore crab, *Carcinus maenas*. Mol Immunol 44: 943-9.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3: 238-50.

Brogden, K. A., Ackermann, M. and Huttner, K. M. (1998). Detection of anionic antimicrobial peptides in ovine bronchoalveolar lavage fluid and respiratory epithelium. Infect. Immun. 66: 5948–5954.

Brogden, K. A., Ackermann, M. R., McCray, P. B. Jr and Huttner, K. M. (1999). Differences in the concentrations of small, anionic, antimicrobial peptides in

bronchoalveolar lavage fluid and in respiratory epithelia of patients with and without cystic fibrosis. Infect. Immun. 67: 4256–4259.

Brogden, K. A., De Lucca, A. J., Bland, J. and Elliott, S. (1996). Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for *Pasteurella haemolytica*. Proc. Natl Acad. Sci. USA. 93: 412–416.

Cheng, W., Liu, C. H., Tsai, C. H. and Chen, J. C. (2005). Molecular cloning and characterisation of a pattern recognition molecule, lipopolysaccharide- and beta-1,3-glucan binding protein (LGBP) from the white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 18: 297-310.

Chythanya, R. and Karunasagar, I. (2002). Inhibition of shrimp pathogenic *vibrios* by a marine *Pseudomonas* I-2 strain. Aquaculture 208: 1-10.

Cowley, J. A., Dimmock, C. M., Wongteerasupaya, C., Boonsaeng, V., Panyim, S. and Walker, P. J. (1999). Yellow head virus from Thailand and gill-associated virus from Australia are closely related but distinct prawn viruses. Dis Aquat Organ 36: 153-7.

Bolintineanu, D., Hazrati, E., Davis, H. T., Lehrer, R. I. and Kaznessis, Y. N. (2010). Antimicrobial mechanism of pore-forming protegrin peptides: 100 pores to kill *E. coli*. Peptides. 31: 1–8.

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J. and E. Bachere (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J Biol Chem 272(45): 28398-28406.

Destoumieux, D., Bulet, P., Strub, J. M., Van Dorsselaer A. and Bachere E. (1999). Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. Eur J Biochem 266: 335-46.

Destoumieux-Garzon, D., Saulnier, D., Garnier, J., Jouffrey, C., Bulet, P. and Bachere, E. (2001). Crustacean immunity. Antifungal peptides are generated

from the C terminus of shrimp hemocyanin in response to microbial challenge. J Biol Chem 276: 47070-7.

Donpudsa, S., Tassanakajon, A. and Rimphanitchayakit, V. (2009). Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon*. Developmental and Comparative Immunology 33(4): 481-488.

Eguchi, M., Itoh, M., Chou, L. Y., and Nishino, K., (1993). Purification and characterization of a fungal protease specific protein inhibitor (PIF-F) in the silkworm haemolymph. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology 104: 537-543.

Engstrom, Y. (1999). Induction and regulation of antimicrobial peptides in *Drosophila*. Dev Comp Immunol 23: 345-58.

Fernández-Vidal, M., Jayasinghe, S., Ladokhin, A. S. and White, S.H. (2007). Folding amphipathic helices into membranes: Amphiphiliccity trumps hydrophobicity. J Mol Biol. 370(3): 459–470

Flegel, T. W. (1997). Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J. Microbiol. Biotech 13: 422-433.

Flegel, T. W. (2006). Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture 258(1-4): 1-33.

Ganz, T., Selsted, M. E. and Lehrer, R. I. (1990). Defensins. Eur. J. Haematol. 44: 1–8.

Ganz, T. (2002). Immunology. Versatile defensins Science. 298: 977–979.

Gennaro, R. and Zanetti, M. (2000). Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers 55: 31–49.

Gross, P. S., Bartlett, T. C., Browdy, C. L., Chapman R. W. and Warr, G. W. (2001). Immune gene discovery by expressed sequence tag analysis of hemocytes and

hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. Developmental and Comparative Immunology 25: 565-577.

Hall, M., Wang, R., van Antwerpen, R., Sottrup-Jensen L. and Soderhall K. (1999). The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc Natl Acad Sci U S A 96: 1965-70.

Hasson, K. W., et al. (1995). Taura syndrome in *Penaeus vannamei*, demonstration of a viral etiology. Dis Aquat Organ 23: 115-126.

Hauton, C., Brockton, V. and Smith, V. J. (2006). Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria. Molecular Immunology 43: 1490-1496.

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. Jr. and Ezekowitz, R. A. B. (1999). Phylogenetic perspectives in innate immunity. Science 284: 1313-1318.

Huang, H. W. (2000). Action of antimicrobial peptides: two-state model. Biochemistry. 39: 8347–8352.

Iwanaga, S. and Bok, L. L. (2005). Recent advances in the innate immunity of invertebrate animals. Journal of Biochemistry and Molecular Biology 38: 128-150.

Janeway Jr, C. A. (1998). Approching the asymptole evolution and revolution in immunology Colo. Spring Habour Symp. Quant. Biol. 54: 1-13.

Jenssen, H., Hamill, P. and Hancock, R.E. (2006). Peptide Antimicrobial Agents. Clinical Microbiology Reviews. 19(3): 491–511.

Jiang, H. and Kanost, M. R. (1997). Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. J Biol Chem. 272: 1082-1087.

Jiang, H., Wang, Y. and Kanost, M. R. (1998). Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: A bacteria-inducible protein similar to *Drosophila easter*. Proceedings of the National Academy of Sciences of the United States of America 95: 12220-12225.

Jiravanichpaisal, P., Lee, S. Y., Kim, Y. A., Andren, T. and Söderhäll, I. (2007). Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: Characterization and expression pattern. Dev Comp Immunol. 31(5): 441-445.

Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D. and Agerberth, B. (1998). Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J. Biol. Chem. 273: 3718–3724.

Johansson, M. W. and Soderhall, K. (1989). A peptide containing the cell adhesion sequence RGD can mediate degranulation and cell adhesion of crayfish granular haemocytes in vitro. Insect Biochemistry 19: 573-579.

Johansson, M. W., Lind, M. I., Holmblad, T., Tharnqvist, P. O. and Soderhall, K. (1995). Peroxinectin, a novel cell adhesion protein from crayfish blood. Biochemical and Biophysical Research Communications 216: 1079-1087.

Johansson, M. W. (1999). Cell adhesion molecules in invertebrate immunity. Developmental & Comparative Immunology 23: 303-315.

Kalfa, V. C., et al. (2001). Congeners of SMAP29 kill ovine pathogens and induce ultrastructural damage in bacterial cells. Antimicrob. Agents Chemother. 45: 3256–3261.

Kanost, M. and Jiang, H. (1996). Proteinase inhibitors in invertebrate immunity. New Directions in Invertebrate Immunology S. K, I. S and V. GR. Fair Haven NJ, SOS Publications**:** 155–173.

Kollman, J. M. and Quispe, J. (2005). The 17A structure of the 420 kDa lobster clottable protein by single particle reconstruction from cryoelectron micrographs. J Struct Biol 151: 306-14.

Kopacek, P., Grubhoffer, L. and Soderhall, K. (1993). Isolation and characterization of a hemagglutinin with affinity for lipopolysaccharides from plasma of the crayfish *Pacifastacus leniusculus*. Dev Comp Immunol 17: 407-18.

Khoo, L., Robinette, D. W. and Noga, E. J. (1999). Callinectin, an antibacterial peptide from blue crab, *Callinectes sapidus*, hemocytes. Marine Biotechnology 1: 44-51.

Lee, S. Y. and Söderhäll, K. (2001). Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. J Immunol. 166(12): 7319-7326.

Lee, S. Y. and Söderhäll, K. (2002). Early events in crustacean innate immunity. Fish Shellfish Immunol 12: 421-37.

Lehrer, B. C. and McCray, P. B. Jr. (2002). β-defensins in lung host defense. Annu. Rev. Physiol. 64: 709–748.

Lehrer, R. I., et al. (1989). Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. J. Clin. Invest. 8: 553–561.

Lehrer, R. I., Lichtenstein, A. K. and Ganz, T. Defensins:antimicrobial and cytotoxic peptides of mammalian cells. Annu. Rev. Immunol. **11**, 105–128 (1993).

Li, Y., Qian, Y. Q., Ma, W. M. and Yang, W. J. (2009). Inhibition mechanism and the effects of structure on activity of Male Reproduction-Related Peptidase Inhibitor Kazal-Type (MRPINK) of *Macrobrachium rosenbergii*. Marine Biotechnology 11(2): 252-259.

Lightner, D. V. (1996). A Handbook of pathology and diagnostic procedures for disease of penaeid shrimp. World Aquaculture Society, Baton Rouge.

Limsuwan, C. (1991). Handbook for cultivation of black tiger prawns. Bangkok, Tansetaki Co Ltd.

Lightner, D. V. (1988). Vibrio disease of penaeid shrimp. Amsterdam, Elsevier.

Lightner, D. V. (1983). Diseases of cultured penaeid shrimp. Handbook of Mariculture. I. J. McVey. Boca Raton

Lin, X., Cerenius, L., Lee, B. L. and Soderhall, K. (2007). Purification of properoxinectin, a myeloperoxidase homologue and its activation to a cell adhesion molecule. Biochimica et Biophysica Acta (BBA) - General Subjects 1770: 87-93.

Millar, D. A. and Ratcliffe, N. A. (1994). Invertebrated. Immunology: A Comparative Approach. I. R. J. Turner. Chichester: Wiley**:** 29-68.

Nash, G., Nithimathachobe, C. and Tungmadi (1992). Vibriosis and its control in pond reared *Penaeus monodon* in Thailand. . Disease in Asian Aquaculture I. M. Shariff, R. P. Subasinghe and J. R. Arthur. Manila, Asian Fish Society**:** 143-155.

O'Brien, D. and McVey, J. (1993). Blood coagulation, inflammation, and defence. The natueal immune system, humoral factors. I. E. Sim. New York: IRL Press**:** 257-280.

Otvos, L. Jr. (2002). The short proline-rich antibacterial peptide family. Cell. Mol. Life Sci. 59: 1138–1150.

Park, C. B., Yi, K. S., Matsuzaki, K., Kim, M. S. and Kim, S. C. (2000). Structure–activity analysis of buforin II, a histone H2Aderived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. Proc. Natl Acad. Sci. USA. 97: 8245–8250.

Polanowski, A. and Wilusz, T. (1996). Serine proteinase inhibitors from insect hemolymph. Acta Biochimica Polonica 43: 445-454.

Patat, S. A., Carnegie, R. B., Kingsbury, C., Gross, P. S., Chapman, R. and Schey, K. L. (2004). Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. European Journal of Biochemistry 271: 4825-4833.

Rasul, R., Cole, N., Balasubramaniand, D., Chene, R., Kumare, N. and Willcox, M. D. P. (2010). Interaction of the antimicrobial peptide melimine with bacterial membranes. International Journal of Antimicrobial Agents. 35: 566–572.

Rosenberry, B. (2003). World shrimp farming. Shrimp News International. Sandiago, CA.

Sallenave, J. M. (2000). The role of secretory leukocyte proteinase inhibitor and elafin (elastase-specific inhibitor/skin-derived antileukoprotease) as alarm antiproteinases in inflammatory lung disease. Respir Res 1: 87-92.

Sarrias, M. R., et al. (2005). A role for human SPa as a pattern recognition receptor. J. Biol. Chem. 280: 35391–35398.

Saul, S. J. and Sugumaran, M. (1986). Protease inhibitor controls prophenoloxidase activation in *Manduca sexta*. FEBS Letters 208: 113-116.

Saulnier, D., Haffner, P., Goarant, C., Levy, P. and Ansquer, D. (2000). Experimental infection models for shrimp vibriosis studies: A review. Aquaculture 191: 133-144.

Schnapp, D., Kemp, G. D. and Smith, V. J. (1996). Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur J Biochem 240: 532-9.

Scott, M. G., Gold, M. R, and Hancock, R. E. (1999). Interaction of cationic peptides with lipoteichoic acid and gram-positive bacteria. Infect Immun. 67: 6445–53.

Scott, M. G., Yan, H. and Hancock, R. E. (1999). Biological properties of structurally related α-helical cationic antimicrobial peptides. Infect. Immun. 67: 2005–2009.

Shugars, D. C. (1999). Endogenous mucosal antiviral factors of the oral cavity. J Infect Dis. 179(Suppl 3): S431.

Silvestro, L., Gupta, K., Weiser, J. N. and Axelsen, P. H. (1997). The concentration-

dependent membrane activity of cecropin A. Biochemistry. 36: 11452–11460

Skarnes, R. C. and Watson, D. W. (1957). Antimicrobial factors of normal tissues and fluids. Bacteriol. Rev. 21: 273–294

Shiao, S. H., Higgs, S., Adelman, Z., Christensen, B. M., Liu, S. H. and Chen, C. C. (2001). Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito *Armigeres subalbatus*. Insect Mol Biol 10: 315-21.

Smith, V. J. (2001). Chisholm JR. Antimicrobial proteins in crustaceans. Adv Exp Med Biol. 484: 95–112.

Smith, V. J., Fernandes, J. M. O., Kemp, G. D. and Hauton, C. (2008). Crustins: Enigmatic WAP domain-containing antibacterial proteins from crustaceans. Developmental and Comparative Immunology 32: 758-772.

Söderhäll, K. and Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 10: 23-8.

Söderhäll, K. and Ajaxon, R. (1982). Effect of quinones and melanin on mycelial growth of Aphanomyces spp. and extracellular protease of *Aphanomyces astaci*, a parasite on crayfish. Journal of Invertebrate Pathology 39: 105-109.

Somboonwiwat, K., Bachère, E., Rimphanitchayakit, V. and Tassanakajona, A. (2008). Localization of anti-lipopolysaccharide factor (ALFPm3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties. Developmental and Comparative Immunology. 32: 1170–1176.

Sritunyalucksana, K. and Soderhall, K. (2000). The proPO and clotting system in crustaceans. Aquaculture 191: 53-69.

Stoss, T. D., Nickell, M. D., Hardin, D., Derby, C. D. and McClintock, T. S. (2004). Inducible Transcript Expressed by Reactive Epithelial Cells at Sites of Olfactory Sensory Neuron Proliferation. Journal of Neurobiology 58: 355-368.

Sugumaran, M., Saul, S. J. and Ramesh, N. (1985). Endogenous protease inhibitors prevent undesired activation of prophenolase in insect hemolymph. Biochemical and Biophysical Research Communications 132: 1124-1129.

Supungul, P., Klinbunga, S., Pichyangkura, R., Hirono, I., Aoki, T. and Tassanakajon, A. (2004). Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. Diseases of Aquatic Organisms 61: 123-135.

Supungul, P., Tang, S., Maneeruttanarungroj, C., Rimphanitchayakit, V., Hirono, I., Aoki, T. and Tassanakajon, A. (2008). Cloning, expression and antimicrobial activity of crustin*Pm*1, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. Developmental and Comparative Immunology 32: 61-70.

Supungul, P., Rimphanitchayakit, V., Aoki, T., Hirono, I. and Tassanakajon, A. (2010). Molecular characterization and expression analysis of a c-type and two novel muramidase-deficient i-type lysozymes from *Penaeus monodon*. Fish Shellfish Immunol. 28(3): 490-8.

Tassanakajon, A., Klinbunga, S., Paunglarp, N., Rimphanitchayakit, V., Udomkit, A., Jitrapakdee, S., et al. (2006). *Penaeus monodon* gene discovery project: The generation of an EST collection and establishment of a database. Gene 384: 104-112.

Theopold, U., Schmidt, O., Soderhall, K. and Dushay, M. S. (2004). Coagulation in arthropods: defence, wound closure and healing. Trends in Immunology 25: 289-294.

Tossi, A., Sandri, L. and Giangaspero, A. (2000). Amphipathic, α-helical antimicrobial peptides. Biopolymers. 55: 4–30.

Van Hulten, M. C. W., Westenberg, M., Goodall, S. D. and Vlak, J. M. (2000). Identification of two major virion protein genes of White Spot Syndrome virus of shrimp. Virology 266: 227-236.

Van Hulten, M. C. W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M. et al. (2001). The white spot syndrome virus DNA genome sequence. Virology 286: 7-22.

Vargas-Albores, F. (1995). The defense system of brown shrimp *Penaeus californiensis*: humoral recognition and cellular responses. J Mar Biotechnol 3: 153-156.

Vandenberghe, J., Li, Y., Verdonck, L., Li, J., Sorgeloos, P., Xu, H. S. and Swings, J. (1998). *Vibrios* associated with *Penaeus chinensis* (Crustacea : Decapoda) larvae in Chinese shrimp hatcheries. Aquaculture 169: 121-132.

Vaseeharan, B. and Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36: 83-7.

Vatanavicharn. T., Supungul, P., Puanglarp, N., Yingvilasprasert, W. and Tassanakajorn, A. (2009). Genomic structure, expression pattern and functional characterization of crustin*Pm*5, a unique isoform of crustin from *Penaeus monodon*. Comparative Biochemistry and Physiology. 153: 244-252.

Wiedow, O., Harder, J., Bartels, J., Streit, V. and Christophers, E. (1998). Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes. Biochem Biophys Res Commun. 248(3): 904–9.

Whaley, K. and Lemercier, C. (1993). The complement system. The natural immune system, humoral factors. I. E. Sim. New York: IRL Press**:** 121-150

Wongteerasupaya, C., Vickers, J. E., Sriurairatana, S., Nash, G. L., Akarajamorn, A. and Boonsaeng, V. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and cause high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Organ 21: 69-77.

Yan, F., Zhang, Y., Jiang, R., Zhong M., Hu, Z., Du, H., Lun, J., Chen, J. and Li, Y. (2011). Identification and agglutination properties of hemocyanin from the mud crab (*Scylla serrata*). Fish & Shellfish Immunology. 30: 354-360.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X. and Xu, X. (2001). Complete genome sequence of the shrimp white spot bacilliform virus. Journal of Virology 75: 11811-11820.

Yang, Y., Boze, H., Chemardin, P., Padilla, A., Moulin, G., Tassanakajon, A. et al. (2009). NMR structure of *r*ALF-*Pm3*, an anti-lipopolysaccharide factor from shrimp: Model of the possible lipid A-binding site. Biopolymers. 91(3): 207-220.

Yeaman, M. R. and Yount, N. Y. (2003). Mechanisms of Antimicrobial Peptide Action and Resistance. The American Society for Pharmacology and Experimental Therapeutics Pharmacol Rev. 55: 27–55.

Yeh, M. S., Chen, Y. L. and Tsai, I. H. (1998). The hemolymph clottable proteins of tiger shrimp, *Penaeus monodon*, and related species. Comp Biochem Physiol B Biochem Mol Biol 121: 169-76.

Yenugu, S., Hamil, K. G., Radhakrishnan, Y., French, F. S. and Hall, S. H. (2004). The androgen-regulated epididymal sperm-binding protein, human β-defensin 118 (DEFB118) (formerly ESC42), is an antimicrobial β-defensin. Endocrinology 145: 3165–3173.

Zhang, J., Li, F., Wang, Z. and Xiang, J. (2007). Cloning and recombinant expression of a crustin-like gene from Chinese shrimp, *Fenneropenaeus chinensis*. J Biotechnol 127: 605-14.

Zhang, X., Huang, C. and Qin, Q. (2004). Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*. Antiviral Research 61: 93-99.

Zhao, H., Mattila, J. P., Holopainen, J. M. and Kinnunen, P. K. (2001). Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin. Biophys. J. 81: 2979–2991.

Zou, Z. and H. Jiang (2005). *Manduca sexta* serpin-6 regulates immune serine proteinases PAP-3 and HP8: cDNA cloning, protein expression, inhibition kinetics, and function elucidation. J Biol Chem 280: 14341-14348.

APPENDIX

1. Preparation of Competent cell for $CaCl_2$-transformation

The 10 min pre-chilled culture was centrifuged at 4,000 x g for 5 min. Then, the cells were washed once with 0.5 volume of 10 mM $CaCl_2$ solution. The final cell pellet was resuspended in an appropriate volume of 100 mM $CaCl_2$ solution supplemented with 10% (v/v) glycerol and chilled on ice for about 30 min. One hundred microliters of competent cells were taken into an aliquot and immediately frozen at -80 ^{o}C until used.

2. BMGY **Buffered Glycerol-complex Medium**

and BMMY **Buffered Methanol-complex Medium** (1 liter)

1% yeast extract
2% peptone
100 mM potassium phosphate, pH 6.0
1.34% YNB
4 x 10^{-5}% biotin
1% glycerol or 0.5% methanol

1. Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water.
2. Autoclave 20 minutes on liquid cycle.
3. Cool to room temperature, then add the following and mix well:

   100 ml 1 M potassium phosphate buffer, pH 6.0
   100 ml 10X YNB
   2 ml 500X B
   100 ml 10X GY

4. For BMMY, add 100 ml 10X M instead of glycerol.
5. Store media at 4°C. The shelf life of this solution is approximately two months.

3. Preparation for SDS-PAGE electrophoresis

Stock reagents

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

Acrylamide 29.2 g

N,N′-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

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

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8 75 ml

10% SDS 4 ml

Distilled water 21 ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8 50 ml

10% SDS 4 ml

Distilled water 46 ml 118

SDS-PAGE

15 % Seperating gel

30 % Acrylamideml solution 5.0 ml

Solution B 2.5 ml

Distilled water 2.5 ml

10% $(NH4)_2S_2O_8$ 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

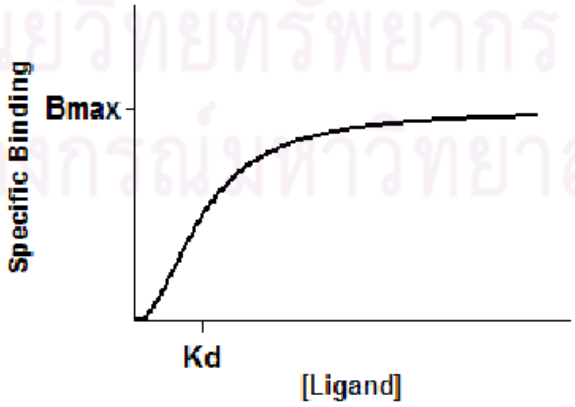
SDS 1.0 g

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

4. Graghpad Prism Equation

Equation: One site -- Specific binding with Hill slope

Model: Y=Bmax*X^h/(Kd^h + X^h)



*Note that the X axis is concentration, not log(concentration).

Interpret the parameters

Bmax is the maximum specific binding in the same units as Y. It is the specific binding extrapolated to very high concentrations of radioligand, and so its value is almost always. higher than any specific binding measured in your experiment.

Kd is the radioligand concentration needed to achieve a half-maximum binding at equilibrium, expressed in the same units as X. If h=1.0, this is the equilibrium binding constant. If h is not equal to 1.0, then the molecular interpretation of the Kd depends on why h is not 1.0.

h is the Hill slope. It equals 1.0 when a monomer binds with no cooperativity to one site. When it is greater than 1.0, you see a sigmoidal look to the graph as shown above. This happens when the receptor or ligand has multiple binding sites with positive cooperativity. The Hill slope is less than zero when there are multiple binding sites with different affinities for ligand or when there is negative cooperativity.

5. Affinity constants from SPR equilibrium analysis

Affinity constant (K_D) equilibrium model; $Req = \left(\frac{[analyte]_{free}}{[analyte]_{free}+K_D}\right) B_{max}$

BIOGRAPHY

Mr. Pasapong Poolpipat was born on January 27, 1984 in Bangkok. He graduated with the degree of Bachelor of Science from the Department of Biology, Faculty of Science, Silpakorn University in 2007. He has studied for the Master's degree of science in Biochemistry Department, Chulalongkorn University. In his forth year of research work, he presented a poster entitle "Binding properties of crustin*Pm*1 and crustinP*m*7 from the black tiger shrimp *Penaeus monodon*" at the 22th Annual Meeting of the Thai society for Biotechnology, Organized by Prince of Songkla University on October 20-22, 2010.