การแสดงออกและลักษณะสมบัติของตัวยับยั้งซีรีนโปรตีเนส SPI*Pm*1 และ SPI*Pm*2 ที่กลายพันธุ์ของกุ้งกุลาดำ *Penaeus monodon*

นาย วีรภัทร ค่ำอำนวยสุข

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EXPRESSION AND CHARACTERIZATION OF SPI*Pm*1 AND MUTATED SPI*Pm*2 SERINE PROTEINASE INHIBITORS OF BLACK TIGER SHRIMP *Penaeus monodon*

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ตัวยับยั้งซีรีนโปรตีเนสถูกพบในสิ่งมีชีวิตหลายชนิด และทำหน้าที่ควบคุมแอคติวิตีของโปรตีเนสที่เกี่ยวข้องกับกระบวนการทางชีวภาพต่างๆ ตัวยับยั้งซีรีนโปรตีเนสชนิดคาซอลแบบสี่และห้าโดเมน ชื่อ SPI*Pm*1 และ SPI*Pm*2 ถูกพบจากห้องสมุด cDNA จากเม็ดเลือดกุ้งกุลาดำ งานวิจัยก่อนหน้านี้พบว่า SPI*Pm*2 มีฤทธิ์การยับยั้งต่อ subtilisin และ elastase และมีฤทธิ์การยับยั้งอย่างอ่อนต่อ trypsin เพื่อให้ทราบถึงฤทธิ์ยับยั้งโปรตีเนสของโดเมน งานวิจัยนี้จึงได้ทำการกลายพันธุ์ยีน SPI*Pm*2 อย่างจำเพาะ โดยเปลี่ยนแปลงกรดอะมิโน P1 ของโดเมนที่ 1, 2 และ 3 จากทรีโอนีนเป็นลิวซีน (T35L) แอลานีนเป็นทริปโทเฟน (A83W) และกลูทาเมตเป็นลิวซีน (E131L) ตามลำดับ ทำการผลิตโปรตีน wild type และโปรตีนกลายพันธุ์สามตัว และศึกษาฤทธิ์การยับยั้งโปรตีเนส พบว่า T35L และ E131L ยับยั้ง elastase และ trypsin ได้น้อยกว่า wild type เล็กน้อย มีเพียง A83W เท่านั้นที่ไม่สามารถยับยั้ง elastase โปรตีนกลายพันธุ์ทั้งสามตัวไม่สามารถยับยั้ง chymotrypsin เช่นเดียวกับ wild type เฉพาะโดเมน 3 ที่กลายพันธุ์ถูกผลิตเพื่อศึกษาผลของการกลายพันธุ์ที่อะมิโน P1 ต่อการยับยั้งโปรตีเนสและพบว่ามีฤทธิ์การยับยั้งต่อ subtilisin และ elastase นอกจากนี้ SPI*Pm*1 ซึ่งประกอบไปด้วย open reading frame ขนาด 669 เบส แปลรหัสให้กรดอะมิโน 222 ตัว ได้ถูกโคลนเข้าสู่ pET-28b(+) เวกเตอร์และแสดงออกในระบบ *E. coli* จากการวิเคราะห์โปรตีนที่ได้จากการแสดงออก พบโปรตีนรีคอมบิแนนท์ rSPI*Pm*1 ในรูปของ inclusion body และได้ยืนยันการแสดงออกด้วยการทำ Western blot analysis ทำการละลาย inclusion body โดยการใช้ denaturing condition และทำให้โปรตีนบริสุทธิ์โดยการใช้คอลัมน์ nickel-NTA จากการทดสอบฤทธิ์การยับยั้งโปรตีเนส พบว่า SPI*Pm*1มีฤทธิ์การยับยั้งอย่างอ่อนต่อ subtilisin, elastase, trypsin และ chymotrypsin ที่อัตราส่วนโมลของตัวยับยั้งต่อโปรตีเนสเท่ากับ 80 ต่อ 1

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VEERAPAT KAMAMNUAYSUK : EXPRESSION AND CHARACTERIZATION OF SPI*Pm*1 AND MUTATED SPI*Pm*2 SERINE PROTEINASE INHIBITORS OF BLACK TIGER SHRIMP *Penaues monodon.* THESIS ADVISOR : ASSOC PROF. : VICHIEN RIMPHANITCHAYAKIT, Ph.D., THESIS CO-ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., 85 pp.

Serine proteinase inhibitors (SPIs) are found widely in multicellular organisms and function as regulators of proteinase activities involving in many biological processes. The four- and five-domain Kazal-type serine proteinase inhibitors, SPI*Pm*1 and SPI*Pm*2, were identified from the hemocyte cDNA library of the black tiger shrimp, *Peneaus monodon*. From the previous study, The SPI*Pm*2 was found to strongly inhibited subtilisin and elastase, and weakly inhibited trypsin. In this study, the SPI*Pm*2 gene was mutated by site-directed mutagenesis in order to delineate the domain inhibitory activities. The P1 amino acid residues of domains 1, 2 and 3 were changed from Thr to Leu (T35L), Ala to Trp (A83W) and Glu to Leu (E131L), respectively. The wild type and three mutated SPI*Pm*2 proteins were produced and their inhibitory specificities against proteinases were determined. We found that T35L and E131L slighty inhibited trypsin less than the wild type. Only A83W had no elastase inhibition activity. All mutant proteins had no inhibitory activity against chymotrypsin like the wild type. The individual mutated domain 3, D3E131L, was produced to study the effect of P1 residue mutation on the inhibitory specificity. The D3E131L had the strong inhibitory activity against subtilisin and elastase.

Moreover, the SPI*Pm*1, which have 669 bp of open reading frame coding for 222 amino acids, was cloned in to pET-28b(+) and expressed by using *E. coli* system. The protein was expressed as the inclusion bodies and the identity of the expressed recombinant protein was confirmed by Western blot analysis. The denaturing condition was used to solubilize the inclusion bodies and the solubilized protein was further purified using nickel-NTA column. From the proteinase inhibitory assay, the SPI*Pm*1 showed the weak inhibition against all proteinases when the mole ratio of inhibitor against proteinases was 80 : 1.

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LIST OF ABBREVIATIONS

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

CHAPTER I

INTRODUCTION

1.1 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 response provides the vertebrate immune system with the ability to recognize and remember specific pathogens (to generate immunity), and to mount stronger attacks each time the pathogen is encountered. It is adaptive immunity because the body's immune system prepares itself for future challenges. The 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 pathogen-associated molecular patterns (PAMPs) that are present on the microorganisms (Fig. 1.1) such as bacterial lipopolysaccharide (LPS), peptidoglycan and β-1, 3-glucan (Janeway Jr, 1998). Innate immune responses include phagocytosis, complement, antimicrobial peptides and proteinase cascades, which lead to melanization and coagulation (Kim, 2006). Whereas adaptive immune system is a more sophisticated and complicated mechanism including immunological memory (Lee et al., 2001).

1.2 Crustacean immune system

Crustacean immune system is innate immune system based on cellular and humoral components of the circulatory system (Lee et al., 2002). The hard cuticle covering all external surfaces of crustaceans is the first line of defense between them and the environment. Moreover, the innate immune system can respond rapidly if microorganisms invade the animals. Major defense systems are carried out in the hemolymph containing cells called hemocytes. Hemocytes and plasma protein

recognize large groups of pathogens by means of common molecular patterns of particular microbes. Hemocytes as effectors of the cellular immune response are also involved in synthesizing the majority of humoral effectors. Cellular response, the actions with direct participation of blood cells, includes phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting (Jiravanichpaisal et al., 2006). On the contrary, humoral factors act in the defense without direct involvement of the cells even though many of the factors are originally synthesized and stored in the blood cells. These factors include enzyme and proteins involved in prophenoloxidase (proPO) system, clotting proteins, agglutinins, hydrolytic enzymes, antimicrobial peptides, and proteinase inhibitors.

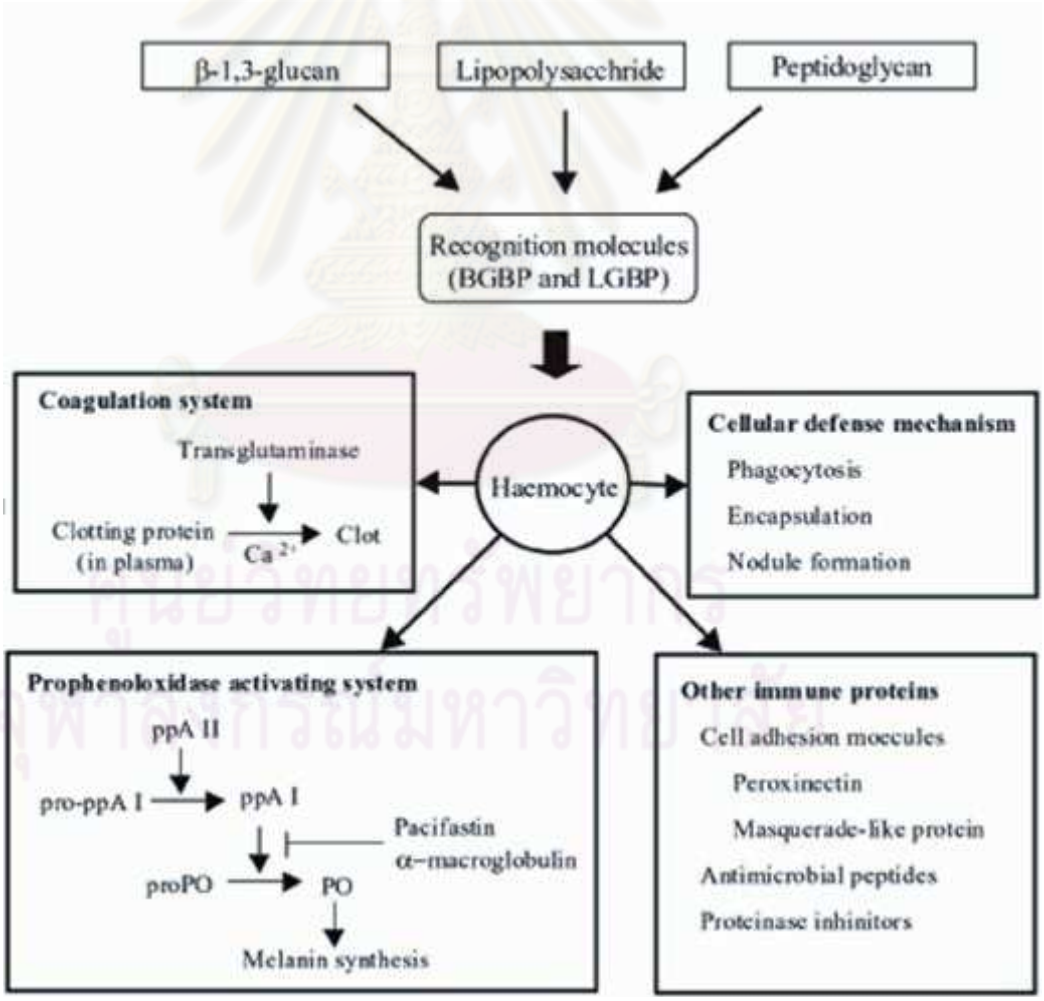

Figure 1.1 Schematic overview of crayfish defense reactions.(Janeway Jr, 1998)

1.2.1 Pattern recognition proteins

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

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

In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin that, besides its bacterial agglutination ability, increases phagocytic rate (Vargas-Albores, 1995). Recent findings indicate that, in decapods, LGBP and BGBP have retained the crucial components for glucanase activity, and share a common ancestor with glucan receptors, as well as with the glucanase proteins of a wide range of invertebrates; however, these LGBP and BGBP lack glucanase activity, implying that these proteins might have lost their glucanase properties during evolution, but retained their glucan-binding activity as an adaptive process. The cloned cDNA of LGBP and BGBP from hemocytes and hepatopancreas

of the white shrimp *Litopenaeus vannamei* indicates the presence of two putative integrin-binding motifs (Arg–Gly–Asp) and a potential recognition motif for β-1,3 linkage of polysaccharides (Padhi et al., 2008). Quantitative real-time RT-PCR analysis revealed that the LGBP transcript in *L. vannamei* hemocytes increases a few hours after *Vibrio alginolyticus* injection (Cheng et al., 2005), confirming that gene expression of this family of proteins is up-regulated in bacterial and fungal infections; similar findings were obtained by injecting the *P. stylirostris* shrimp with the white spot virus (Roux et al., 2002). In some crustaceans, such as the crayfish, the most important mechanism that activates the proPO system involves the participation of PGBP, LGBP, and BGBP; whereas, in the horseshoe crab, these receptors seem to be less important in the proPO activating system (Iwanaga et al., 2005).

1.2.2 Cell-mediated defense reactions

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

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

1.2.3 The prophenoloxidase (proPO) system

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

In vitro studies have shown that phenoloxidase (PO) exists as an inactive precursor prophenoloxidase (proPO) which is activated by a stepwise process involving serine proteases activated by microbial cell wall components such as low quantities of lipopolysaccharides or peptidoglycans from bacteria, and β-1,3-glucans from fungi through pattern-recognition proteins (PRPs) (Kurata et al., 2006). An enzyme capable of activating the proPO in vivo is called prophenoloxidase activating enzyme (factor) (ppA, PPAE, PPAF). In crayfish, ppA is a trypsin-like proteinase present as an inactive form in the hemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA will convert proPO to an active form, phenoloxidase (PO) (Aspan, 1991; Aspan, 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Soderhall et al., 1998; Shiao et al., 2001). It both catalyses o-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can non-enzymatically polymerise to melanin. PO is a sticky protein and can adhere to the surface of parasites leading to melanisation of the pathogens. Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites such as crayfish plague fungus, *Aphanomyces astaci* (Sodërhäll et al., 1982). The production of forming insoluble melanin deposits involving in the process of sclerotisation, wound healing, and encapsulation of foreign materials (Theopold et al., 2004). To prevent excessive activation of the proPO cascade, it is needed to be regulated by proteinase inhibitors.

The prophenoloxidase activating enzyme (PPA) is a zymogenic protein (proppA). The C-terminal half of the proppA comprises a typical serine proteinase domain with a sequence similar to other invertebrate and vertebrate serine proteinases. The N-terminal half consists of a cationic glycine-rich domain, a cationic proline-rich domain, and a clip-domain, in which the disulfide-bonding pattern is likely to be

identical to those of the horseshoe crab big defensin and mammalian β-defensins. The clip-domains in proppAs may function as antibacterial peptides (Wang et al., 2001).

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

1.2.4 The coagulation system/ the clotting system

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

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

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

CPs are synthesized in the hepatopancreas and released to hemolymph. In crustaceans, CPs were found in several species: the freshwater crayfish (Kopacek et

al., 1993), *P. monodon* (Yeh et al., 1998), and the lobster *Panulirus interruptus* (Kollman et al., 2005).

1.2.5 Antimicrobial peptides (AMPs)

The peptide antibiotics are defined as anti-microbial agents made by animals, including humans, with a function that is important for the innate immunity of that animal. Most of the AMPs are small in size, generally less than 150-200 amino acid residues, amphipathic structure and cationic property. However, the anionic peptides also exist. For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupturing the membrane or perturbing the membrane lipid bilayer that allows the leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

AMPs are active against a large spectrum of microorganisms; bacterial and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Murakami et al., 1991; Hancock et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991).

There are a few reports on antimicrobial peptides in shrimp. Penaeidins which act against Gram-positive bacteria and fungi were reported in *L. vannamei* (Destoumieux et al., 1997). The cDNA clones of penaeidin isoform were also isolated from the haemocytes of *L. vannamei*, *P. setferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2004). Crustins, the antimicrobial peptides, were identified from *L. vannamei* and *L. setiferus*. Several isoforms of crustins were observed in both shrimp species. Like the 11.5 kDa antibacterial protein from *Carcinus maenas*, crustins from shrimp show no homology with other known antibacterial peptides, but possess sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Peptide derived from the hemocyanin of *L.vannamei*, *P. stylirostris*

and *P. monodon* possessed antiviral activity has been identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Recently, the histones and histone-derived peptides of *L. vannamei* have been reported as an innate immune effectors because they can inhibit growth of Gram-positive bacteria (Patat et al., 2004).

1.3 Serine proteinases (SPs)

Serine proteinase (SP) is a group of endopeptidase that cleaved peptide bond in protein (Neurath, 1985) in which one of the amino acid at the active site is serine (Phillips et al., 1992). They are found in both single-cell and complex organisms, in both cells with nuclei (eukaryotes) and without nuclei (prokaryotes). In previous study, serine proteinases were originally digestive enzymes. Nowadays, serine proteinases have been studied intensively and their role in a wide array of vital physiological processes, such as food digestion, blood clotting, embryogenesis and immune response (complement activation), has been well documented. Many of these processes are in essence proteolytic cascades, which, once 'turned on', lead very rapidly and irreversibly to a specific cellular response. As a consequence, the normal physiology of an organism is likely to be impaired if these proteolytic cascades are not well regulated. Therefore, most organisms synthesize a set of proteinase inhibitors, whose function is to prevent (regulate) unwanted proteolysis (Simonet et al., 2002a).

Serine proteases are grouped into clans that share structural similarities (homology) and are then further subgrouped into families with similar sequences. The major clans found in humans include the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase clans. The SPs belong to one of the four protease families. Generally SPs can be classified to 6 clans (Othman et al.). The six clans are clan A to clan F. Especially, clan A contains a family that share a common origin with chymotrypsin such as trypsin, elastase and the enzymes of blood clotting system (Barrett et al., 1995). These enzymes typically are synthesized in inactive forms which require activation by cleavage of a peptide bond near the N-terminus (Neurath, 1989). In chymotrypsin, this is between Arg-15 and Ile16; the free, protonated amino group of Ile16 is important for the mechanism. The amino-terminal peptide with residues 1 through 13 stays attached to the rest of the protein through a

disulfide bond. In trypsin, the activation cuts off an amino-terminal hexapeptide, which does not remain attached (Neurath et al., 1976) whereas thrombin does not have its amino terminal domain attached by a disulfide bond and goes free in the plasma to attack fibrinogen and generate clots (Dunn et al., 1982).

1.3.1 Chymotrypsin-like

The three serine proteases of the chymotrypsin-like clan that have been studied in greatest detail are chymotrypsin, trypsin, and elastase. All three enzymes are synthesized by the pancreatic acinar cells, secreted into the small intestine, and are responsible for catalyzing the hydrolysis of peptide bonds. All three enzymes are similar in structure, as shown through their X-ray structures. They share closely similar structures (tertiary as well as primary). In fact, their active serine residue is at the same position (Ser-195) in all three. The differing aspect lies in the peptide bond that is being cleaved; this is called the scissile bond. The different enzymes, like most enzymes, are highly specific in the reactions they catalyze. Each of these digestive serine proteases targets different regions of a polypeptide chain, based upon the side chains of the amino acid residues surrounding the site of cleavage (Kurth et al., 1997; Hung et al., 1998).

Chymotrypsin is responsible for cleaving peptide bonds following a bulky hydrophobic amino acid residue. Preferred residues include phenylalanine, tryptophan, and tyrosine, which fit into a snug hydrophobic pocket. Trypsin is responsible for cleaving peptide bonds following a positively-charged amino acid residue. Instead of having the hydrophobic pocket of the chymotrypsin, there exists an aspartic acid residue at the base of the pocket. This can then interact with positively-charged residues such as arginine and lysine on the substrate peptide to be cleaved. Elastase is responsible for cleaving peptide bonds following a small neutral amino acid residue, such as alanine, glycine, and valine. (These amino acid residues form much of the connective tissues in meat). The pockets in "trypsin" and "chymotrypsin" can partially accommodate these smaller amino acid residues rendering it a mere depression.

1.3.2 Subtilisin-like

Subtilisin is a serine protease secreted by the bacterium *Bacillus subtilis*. Subtilisins found in higher eukaryotes fall into two families: the pyrolisins and kexins. Subtilisin-like serine proteases or subtilases constitute a protease superfamily that is prevalent in various organisms such as archaea, protozoa, bacteria, yeast, vertebrates and plants (Hamilton et al., 2003) having diverse roles. Subtilases occur in distinct parts of plant ranging from seeds to fruits in various plant species such as melon (Yamagata et al., 1994), lily (Kobayashi et al., 1994), *Alnus glutinosa* (Ribeiro et al., 1995), *Arabidopsis thaliana* (Neuteboom et al., 1999), tomato (Tornero et al., 1996) and soybean (Nelsen et al., 2004). Subtilisin-like serine proteases have been associated with many physiological processes such as microsporogenesis (Taylor et al., 1997), hypersensitive response (Taylor et al., 1997), signal transduction (Yano et al., 1999), cell differentiation (Batchelor et al., 2000) and lateral root development (Neuteboom et al., 1999). In performing their respective roles, LIM9 in lily (Taylor et al., 1997), *Arabidopsis* AIR3 (Neuteboom et al., 1999) and tomato P69A (Tornero et al., 1996) use structural proteins in the cell wall as their substrates. Although it has the same mechanism of action as the serine proteases of mammals, its primary structure and tertiary structure are entirely different. Subtilisin is evolutionary unrelated to the chymotrypsin-clan, but shares the same catalytic mechanism utilizing a catalytic triad, to create a nucleophilic serine. This is the classic example used to illustrate convergent evolution, since the same mechanism evolved twice independently during evolution. The structure of subtilisin has been determined by X-ray crystallography. It is a 275 residue globular protein with several alpha-helices and a large beta-sheet.

1.4 Mechanism of action of serine proteinases

The peptide bond is cleaved by nucleophilic attack of the serine hydroxyl group on the scissile carbonyl bond, forming an acyl enzyme intermediate (Figure 1.2). The carbonyl carbon of this bond is position near the nucleophilic serine. The serine-OH attacks the carbonyl carbon, and the nitrogen of the histidine accepts the hydrogen from the -OH of the serine and a pair of electrons from the double bond of the carbonyl oxygen moves to the oxygen. As a result, a tetrahedral intermediate is generated. The bond joining the nitrogen and the carbon in the peptide bond is now

broken. The covalent electrons creating this bond move to attack the hydrogen of the histidine, breaking the connection. The electrons that previously moved from the carbonyl oxygen double bond move back from the negative oxygen to recreate the bond, generating an acyl-enzyme intermediate. Now, water comes into the reaction. Water replaces the N-terminus of the cleaved peptide, and attacks the carbonyl carbon. Once again, the electrons from the double bond move to the oxygen making it negative, as the bond between the oxygen of the water and the carbon is formed. This is coordinated by the nitrogen of the histidine. This accepts a proton from the water. Overall, this generates another tetrahedral intermediate. In a final reaction, the bond formed in the first step between the serine and the carbonyl carbon moves to attack the hydrogen that the histidine just acquired. The now electron-deficient carbonyl carbon re-forms the double bond with the oxygen. As a result, the C-terminus of the peptide is now ejected. In trypsin, the catalytic triad is composed of Ser195, His57 and Asp102 (Phillips et al., 1992).

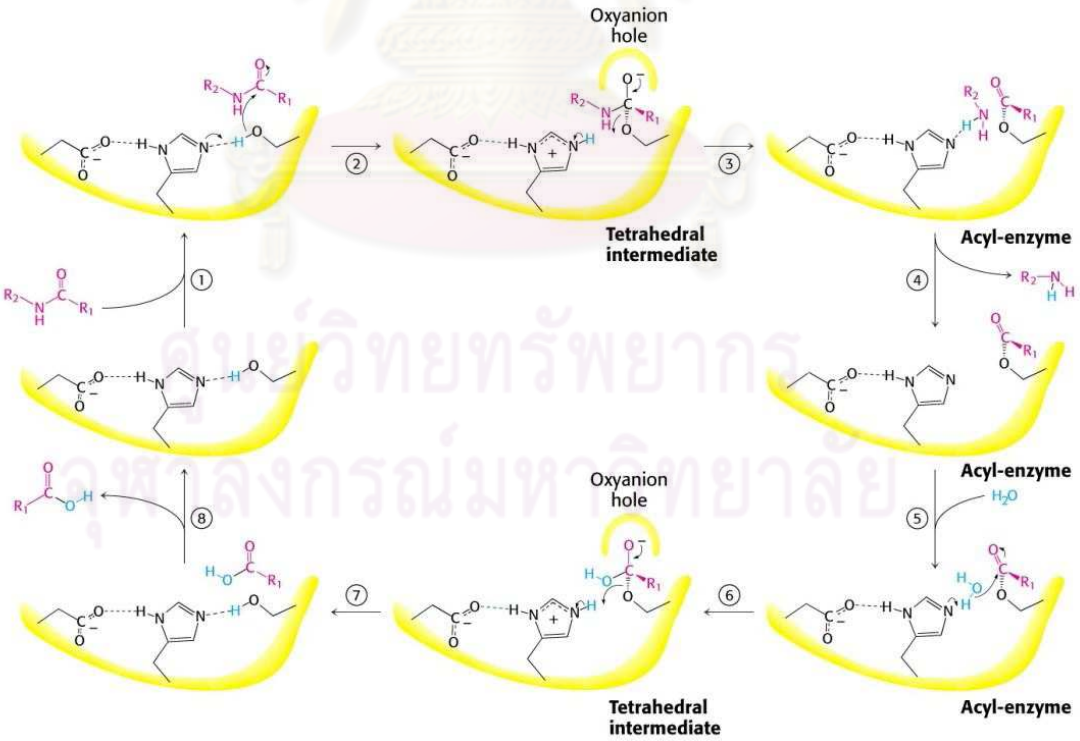

Figure 1.2 A detailed mechanism for the chymotrypsin-like SP reaction.

1.5 Proteinase inhibitor

In multicellular organisms, serine proteinase inhibitors (SPIs) are essential factors involving in controlling the various proteinase mediated biological processes, such as the complement system, blood coagulation, melanization, apoptosis, etc. (Iwanaga et al., 2005; Jiravanichpaisal et al., 2006). Not only do they control the extent of deleterious protease digestion in such processes, they potentially fight as part of the humoral defence of the innate immune system against the invading pathogens (Christeller, 2005). Injury and microbial infection in vertebrates 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.

The SPIs are also involved in direct defense against proteinases from invading pathogens. For example, a subtilisin inhibitor, BmSPI, from *Bombyx mori* might function as an inhibitor to the microbial proteases and protected the silkworm pupae from infection by pathogens (Zheng et al., 2007). Some microbial pathogens and parasites use the SPIs to counterdefense the host protective proteinases. For example, the oomycete *Phytophthora infestans*, a cause of disease in potato and tomato, produces an extracellular protease inhibitor to counter-defense the plant defensive proteinases (Tian et al., 2004; Tian et al., 2005). The obligate intracellular parasite of human *Toxoplasma gondii* produces a serine protease inhibitor to protect itself from the digestive enzymes during its residency in small intestine (Morris et al., 2002).

Some other SPIs are involved in reproductive processes. A male reproduction-related SPI is isolated from *Macrobrachium rosenbergii* with inhibitory activity on sperm gelatinolytic activity (Li et al., 2009). Another reproductive SPI was from the turkey male reproductive tract (Slowinska et al., 2008). For haematophagous insects such as *Dipetalogaster maximus* and *Triatoma infestans*, they secrete potent thrombin inhibitors dipetalogastin and infestin, respectively, to prevent blood clotting during blood meal (Campos et al., 2002; Mende et al., 2004).

Based on the primary and three-dimensional structures, topological functional similarities (Laskowski et al., 1980; Bode, 1992) and inhibition mechanisms, the proteinase inhibitors are classified into at least 18 families according to Laskowski and Qasim (Laskowski Jr et al., 2000). Among them, the following six families: Kazal, BPTI-Kunitz, α-macroglobulin, serpin, pacifastin and bombyx (Pham et al., 1996; Kanost, 1999; Simonet et al., 2002b) have been described in invertebrate haemolymph or also in saliva. Although the primary structure, with the number of amino acids ranging from 29 to approximately 400, and the structural properties of these inhibitors differ significantly, only two fundamentally different inhibiting mechanisms exist. Most inhibitors bind to their cognate enzyme(s) according to a common, substrate-like standard mechanism. They are all relatively small (from 29 to 190 amino acids) and share an exposed, rigid binding loop with a very characteristic 'canonical' conformation (Laskowski et al., 1980; Bode, 1992).

One of the well-known SPIs is the Kazal-type SPIs (KPIs) which are grouped into family I1 (Rawlings et al., 2004; Rawlings et al., 2008). The Kazal inhibitors are usually multi-domain proteins containing more than one Kazal domain. Each domain binds tightly and competitively via its reactive site loop to the active site of cognate proteinase rendering the proteinase inactive. Structural studies reveal that there are several contact positions responsible for the interactions between Kazal domains and the proteinases (Lu et al., 1997; Bode et al., 2000). However, the inhibitory specificity is determined mainly by the P1 amino acid residue resided at the second amino acid residue after the second cysteine residue of the domain.

1.6 Kazal-type SPIs (KPIs)

The invertebrate KPIs can be single or multiple domain proteins with one or more Kazal inhibitory domains linked together by peptide spacers of variable length. A typical or canonical Kazal domain is composed of 40–60 amino acid residues including some spacer amino acids. Generally, the vertebrate Kazal domains are slightly larger than the invertebrate ones but their domain architecture are quite similar. The Kazal motif has a general amino acid sequence of C-X_a-C-X_b-PVCG-X_c-Y-X_d-C-X_e-C-X_f-C where the subscripts *a*, *b*, *c*, *d*, *e* and *f* are integral numbers of

amino acid residues. Though a few amino acid residues in the Kazal motif are relatively conserved, most of them are quite variable both within and among the invertebrate species (Cerenius et al., 2010). Within the Kazal domain reside six well-conserved cysteine residues capable of forming three intra-domain disulfide bridges between cysteine numbers 1–5, 2–4, 3–6 resulting in a characteristic three-dimensional structure (Figure 1.3) (Krowarsch et al., 2003). There are one α-helix surrounded by an adjacent three-stranded beta-sheet and loops of peptide segments. The structure can be viewed as consisting of three loops A, B and C. The B loop harbours the specificity determining P1 amino acid and the scissile peptide bond, and is, thus, called the reactive site loop. The convex reactive site loop is extended out providing easy access for the active site of the cognate proteinase to fit in and, then, get stuck. To emphasize on the scissile bond, a special numeral description of the primary structure of a Kazal domain is generally used. The numerals start from P1, P2, P3, … to the N-terminus and P1′, P2′, P3′, … to the C-terminus (Schechter and Berger, 1967; Jering and Tschesche, 1976).

The Kazal proteinase inhibitory domain inhibits the proteinase by a standard mechanism (Laskowski and Kato, 1980). Each Kazal domain acts as a substrate analogue that stoichiometrically binds competitively through its reactive site loop to the active site of cognate proteinase forming a relatively stable proteinase–proteinase inhibitor complex, much more stable than the Michaelis enzyme–substrate complex. Though the binding is non-covalent, it is very tight as the association constant is extremely high (10^{7}–10^{13} M^{-1}) and, therefore, the inhibition is very strong. In invertebrate, the typical inhibition constants (K_is) are in the range of nanomolar compared to the millimolar range of K_m values of the synthetic proteinase substrates (Somprasong et al., 2006; Wang et al., 2009; Visetnan et al., 2009; Li et al., 2009a; Li et al., 2009b; González et al., 2007a; González et al., 2007b; Campos et al., 2002).

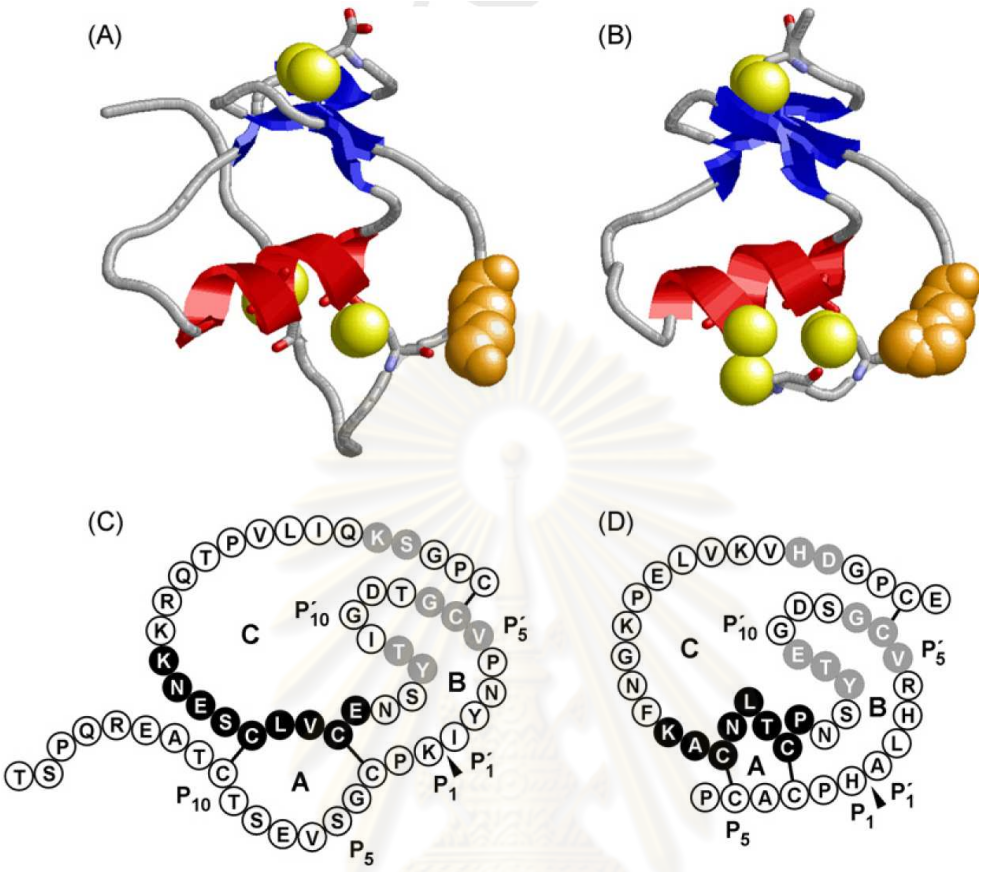

Figure 1.3 Structural comparison of Kazal proteinase inhibitors from vertebrate and invertebrate. The structure of porcine pancreatic secretory inhibitor derived from the PDB file 1TGS (Bolognesi et al., 1982) (A) with its covalent primary structure (C) is a representative of vertebrate KPIs. The structure of rhodniin domain 1 derived from the PDB file 1TBQ (van de Locht et al., 1995) (B) and its covalent primary structure (D) is used as representative for invertebrate KPIs. The structures are derived from their complexes with cognate proteinases. Helices and sheets are shown in red and blue but in black and gray circles in the covalent primary structures, respectively. The sulphur atoms are presented as yellow balls. The space-filled side chains of the P1 amino acids are in orange colour. The scissile peptide bonds are indicated by arrows.

1.7 Biological and physiological functions of KPIs

The KPIs are believed to play regulatory roles in the processes that use serine proteinases. However, it is not easy to define their biological functions in a complex biological system like in the higher eukaryotes. Also very little work has been done to

pursue the roles of these KPIs. Nevertheless, the origin of KPIs, the target proteinases and the inhibitory specificities can be used as clues to the actual functions of the KPIs.

1.7.1 Blood feeding

Hematophagous animals such as leeches, mosquitoes, ticks and bugs, feed on blood and are in need of anticoagulants to prevent the blood from clotting during their blood meal drawn from their prey or digestion of blood in their gut. They actively synthesize the anticoagulants and secreted into the saliva or stomach juice.

The European medical leech *Hirudo medicinalis* and some other leech species have been used for clinical bloodletting for thousands of years because they can prevent blood clotting, thereby stimulate the blood circulation. Nowadays, they are used in medicine to stimulate blood flow in the area of injuries or surgeries where the blood veins are damaged. The ability to prevent blood clotting of the leeches has been investigated extensively in search for the anticoagulants to be used for medical therapy. Besides the hirudin, a non-Kazal specific thrombin inhibitor, which is a potent anticoagulant and now used in medicine to prevent thrombosis in patients (Nowak et al., 2007), two one-domain "non-classical" Kazal-type proteinase inhibitors, namely the bdellin B-3 and leech-derived tryptase inhibitor (LDTI) are also isolated from the medical leech *Hirudo medicinalis* (Sommerhoff et al., 1994; Fink et al., 1986). The bdellin B-3 with it P_1 Lys is able to inhibit trypsin, plasmin and sperm acrosin while the LDTI with the same P_1 Lys inhibits trypsin and chymotrypsin. The LDTI is not involved in the blood coagulation since it does not inhibit any of the proteases in the cascade (Fritz and Sommerhoff, 1999). The bdellin B-3 with its inhibitory activity against plasmin prevents the plasmin-mediated platelet activation that leads to platelet clot (Quinton et al., 2004). The bdellin B-3, thus, helps the hirudin to completely block the coagulation.

The blood-sucking triatomine bug *Rhodnius prolixus* synthesizes a double-headed Kazal-type proteinase inhibitor, rhodniin, to prevent the host blood coagulation. It is a high affinity inhibitor highly specific for thrombin (Friedrich et al., 1993). Another blood-sucking insect *Dipetalogaster maximus* produces a cDNA coding for a six-domain Kazal-type thrombin inhibitor, dipetalogastin. The natural dipetalogastin is double-headed of domains 3 and 4 with the P_1 Arg and Asp,

respectively. The natural inhibitor strongly inhibits thrombin (Mende et al., 1999; 2004). The domain 3 is responsible for the inhibitory activity against thrombin (Schlott et al., 2002).

Still another blood-sucking bug *Triatoma infestans* which is the most important vector of Chagas disease expresses a Kazal-type thrombin inhibitor infestin in the midgut. The full-length cDNA encodes seven Kazal domains but the natural infestins identified so far are the one-domain KPI, named the infestin 1R, corresponding to domain 1 and the double-headed KPI, named the infestin 1-2, corresponding to domains 4 and 5 (Campos et al., 2002; Lovato et al., 2006). The native infestin 1R with its P_1 Leu does not have anticoagulation activity. It inhibits neutrophil elastase, subtilisin A, proteinase K and chymotrypsin but not thrombin (Lovato et al., 2006). The native infestin 1-2 with its P_1 Arg and Asn inhibits thrombin and trypsin (Campos et al., 2002). The recombinant infestin 3-4 and 4 with the P_1 Lys and Arg corresponding to domains 6 and 7 inhibit trypsin, plasmin, factor XIIa and factor Xa. Particularly, the recombinant infestin 4 exhibits very strong anticoagulant activity (Campos et al., 2004b). Thus, the infestin in the midgut plays an important role as anticoagulant during the ingestion of blood meal in *T. infestans*.

Brasiliensin, an eight-domain KPI from a related blood-sucking bug *Triatoma brasiliensis*, is very similar to infestin (Araujo et al., 2007). The reduction of brasiliensin in the midgut by RNA interference reduces the ingestion of blood stressing the important role of anticoagulant in blood feeding in these *Triatoma* bugs.

1.7.2 Reproduction

In the male reproductive tract especially the vas deferens of the prawn *Macrobrachium rosenbergii*, a Kazal-type peptidase inhibitor (MRPINK) is expressed (Cao et al., 2007). By using suppression subtractive hybridization technique, a cDNA of the MRPINK was cloned. The cDNA sequence indicated that the MPPINK was a double-headed KPI with the P_1 Leu and Pro. As expected from the P_1 amino acids, the MRPINK inhibits chymotrypsin but not trypsin or thrombin. Domain 2 (MRTKPIa-1) is solely responsible for this inhibition and domain 1 (MRTKPIa-1) seems to be inactive (Li et al., 2009a). The MRPINK also has an inhibitory effect on the gelatinolytic activity of sperms supposedly catalyzed by a *M. rosenbergii* sperm

gelatinase (MSG) (Li et al., 2008). Therefore, the MRPINK may be involved in the male reproductive process and further investigation is needed.

1.7.3 Prevention of excessive autophagy

A tightly regulated process that cell degrades and recycles its own components using lysosome is called autophagy. Excessive autophagy was observed in gland cells and endodermal digestive cells of *Hydra magnipapillata* when the expression of an evolutionarily conserved gene *Kazal1* was silenced through double-stranded RNA feeding (Chera et al., 2006). The *Kazal1* is a three-domain KPI (P_1 Met, Arg and Arg) expressed from the gland cells. The *Kazal1* silencing affects homeostatic conditions, for instances disorganization and death of gland cells and highly vacuolation of digestive cells. The final result of prolonged *Kazal1* silencing is death. Therefore, the *Kazal1* gene is required for the modulation of autophagy like the pancreatic secretory trypsin inhibitor *SPINK1* and *SPINK3* genes in human and mice, respectively (Masaki et al., 2009).

1.7.4 Protection from microbial destruction

To protect their cocoons from predators and microbial degradation, the wax moth *Galleria mellonella* produces two types of proteinase inhibitors from the silk glands, which are found as components in the cocoon silk besides the major silk proteins, fibroins and sericins, and a few other proteins. The two serine proteinase inhibitors belong to the Kunitz and Kazal families named silk proteinase inhibitors 1 (GmSPI 1) and 2 (GmSPI 2), respectively (Nirmala et al., 2001a). Both serine proteinase inhibitors are single-domain proteins, which inhibit subtilisin and proteinase K. Two similar serine proteinase inhibitors, a Kunitz-type BmSPI 1 and Kazal-type BmSPI 2, are also present in the cocoon silk of *Bombyx mori* (Nirmala et al., 2001b). The Kazal domain of GmSPI 2 is atypical lacking the 1–5 disulfide bridge. The presence of serine proteinase inhibitors and their activities against the bacterial subtilisin and fungal proteinase K in the cocoon silk suggests the protection of silk cocoon from the microbial proteinases.

1.7.5 Protection against pathogen proteinases and antimicrobial activity

It is well known that many invasive pathogens produce extracellular proteinases as virulent factors during the development of the infectious diseases for they help to penetrate through physical barrier of their hosts (Travis eet al., 1995; Christeller, 2005). To defend against such pathogens, the host organisms produce the proteinase inhibitors to combat the microbial proteinases. Some of these inhibitors also exhibit antimicrobial activity.

One of the four KPIs from the hepatopancreas of Chinese white shrimp, *Fenneropenaeus chinensis*, namely FcSPI-1, is able to inhibit the microbial proteinases, subtilisin and proteinase K, indicating that the KPI may participate in the immune defense response (Wang et al., 2009). Besides, the FcSPI-1 can also inhibit the chymotrypsin from the Chinese white shrimp suggesting that it may function as a regulator of the shrimp proteinase activity.

In *Hydra magnipapillata,* the endodermal gland cells are involved in innate immunity by producing a three-domain KPI, kazal2 (Augustin et al., 2009). The KPI strongly inhibits trypsin and subtilisin, and possesses bactericidal activity against *Staphylococcus aureus*. The observations that the KPIs can inhibit or even kill the bacteria may indicate another mechanism of host defense led by the KPIs as well as other serine protease inhibitors.

The potential role of several KPIs in invertebrate immunity can also be implied from their responses against microbial challenge. Semi-quantitative RT-PCR or quantitative real time RT-PCR analysis were performed and showed that the mRNA expression of invertebrate KPIs was up-regulated upon bacteria or viral challenges. Nevertheless, the actual functions of the up-regulated KPIs are to be confirmed by further investigation. A few examples are as follows. A mollusk KPI (AISPI) mRNA from the bay scallop *Argopecten irradians* which encodes a six-domain KPI is up-regulated after *Vibrio anguillarum* injection (Zhu et al., 2006). A twelve-domain KPI gene from the Zhikong scallop *Chlamys farreri* (CfKZSPI) is highly expressed after *V. anguillarum* challenge (Wang et al. 2008). A double-headed KPI from the hemocyte cDNA library of the oriental white shrimp *Fenneropenaeus chinensis* is up-regulated in shrimp infected with the white spot syndrome virus (WSSV) (Kong et al., 2009).

1.7.6 Protection from host proteinases

Toxoplasma gondii is an obligate intracellular parasitic protozoon which infects almost all warm blooded animals as intermediate hosts. The definitive host, however, is the feline. The infection occurs via oral route when a person ingests meat contaminated with the oocysts or the parasites. The parasites pass through the digestive system where the epithelial cells of intestine are infected. The parasites in the infected intestinal epithelium produce more oocysts for new infection and spreading through faeces. Being transit through and multiply in the digestive tract, the parasite produces protease inhibitors to protect itself from the proteolytic enzymes found within the lower intestine. The parasite produces a four-domain KPI, namely the *Toxoplasma gondii* protease inhibitor 1 (TgPI-1) which strongly inhibits trypsin, chymotrypsin, pancreatic elastase and neutrophil elastase (Pszenny et al., 2000; Morris et al., 2002). The natural TgPI-1 probably functions as two double-headed KPIs. The protection against digestive enzymes gives the parasite an opportunity to proliferate in the intestine before disseminating to other host tissues. The parasite also produces another four-domain KPI, namely TgPI-2, which inhibits only trypsin. The actual function of TgPI-2 is not known (Morris and Carruthers, 2003).

In *Neospora caninum*, a closely related species of *T. gondii*, the parasite produces a single-domain KPI, NcPI-S, that inhibits potently the subtilisin but has no or low inhibitory activities against trypsin, chymotrypsin and elastase (Morris et al., 2004; Bruno et al., 2004). The function of NcPI-S is not known either.

1.8 Objectives of the thesis

In previous studies, the expressed sequence tag (EST) libraries from the hemocytes of normal and *Vibrio harveyi*-infected black tiger shrimp *Penaeus monodon* were generated in order to identify genes associated with shrimp immunity (Supungul et al., 2002). Two types of Kazal-type SPIs, SPI*Pm*1 and SPI*Pm*2 containing four and five Kazal-domains, were identified. The SPI*Pm*2 was over-expressed and its activity has been studied (Somprasong et al., 2006). The recombinant SPI*Pm*2 exhibits strong inhibitory activity against subtilisin and elastase, weak inhibitory activity against trypsin, and no activity against chymotrypsin. To gain more insight into the domain inhibitory specificity, in this study, the site-directed

mutagenesis of domain 1, 2 and 3 of the P1 residues was carried out. The inhibitory activities of the mutated SPI*Pm*2 proteins were tested comparing with the wild type protein. Furthermore, the SPI*Pm*1 was over-expressed and characterized its inhibitory activity.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Amicon Ultra-4 concentrators (Millipore)

Autoclave model # LS-2D (Rexall industries Co. Ltd., Taiwan)

Automatic micropipettes P10, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)

AVANTI® J-30 I Centrifuge (Beckman Coulter)

Balance Sartorius 1702 (Scientific Promotion Co.)

Bransonic 32 (BANDELINSONOPULS, Germany)

Centrifuge 5804 R (Eppendorf)

-20 ºC Freezer (Whirlpool)

-80 ºC Freezer (Thermo Electron Corporation)

Gel documentation (SYNGENE)

Gene pulser (Bio-Rad)

Incubator 37 ºC (Memmert)

LABO Autoclave (SANYO)

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

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

Microplate reader : FLUOstar OPTIMA (BMG Labtech)

Minicentrifuge (Costar, USA)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

PD-10 column (GE Healthcare)

pH meter model # SA720 (Orion)

Pipette tips 10, 20, 200, and 1000 µl (Bio-Rad Laboratories, USA)

Power supply Power PAC 3000 (Bio-Rad Laboratories, USA)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Sterring hot plate (Fisher Scientific)

Transilluminator 2011 Macrovue (LKB)

Trans-Blot® SD (Bio-Rad)

Touch mixer model # 232 (Fisher Scientific)

Vertical electrophoresis system (Hoefer™ miniVE)

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

96-well plate (Bio-Rad)

2.1.2 Chemicals and reagents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

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

Agar

Agarose (Sekem)

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

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

Ampicillin (Sigma)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bovine serum albumin (Fluka)

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

Bromophenol blue (Merck, Germany)

Calcium chloride, ($CaCl_2$) (Merck)

Chloramphenicol (Sigma)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue G-250 (Sigma)

Coomassie brilliant blue R-250 (Sigma)

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

di-Sodium hydrogen orthophosphate anhydrous, Na_2HPO_4 (Carlo Erba)

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

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA) (Fluka)

GeneRuler™ 100bp DNA ladder (Fermentas)

GeneRuler™ 1 kb DNA ladder (Fermentas)

Glucose, $C_6H_{12}O_6$ (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine, NH_2CH_2COOH (scharlau)

Hydrochloric acid, HCl (Merck)

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

Kanamycin (Sigma)

2-Mercaptoethanol, C_2H_6OS (Fluka)

Methanol, CH_3OH (Merck)

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

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Phenol crystals, C_6H_5OH (Carlo Erba)

Prestained protein molecular weight marker (Fermentas)

Sodium acetate, CH_3COONa (Merck)

Sodium carbonate anhydrous, Na_2CO_3 (Carlo Erba)

Sodium chloride, NaCl (BDH)

Sodium dodecyl sulfate, $C_{12}H_{25}O_4SNa$ (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

TEMED, $(CH3)_2NCH_2CH_2N(CH_3)_2$ (Amresco)

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

TritonX-100 (Merck)

Tween-20 (LABCHEM)

2.1.3 Kits

NucleoSpin® Extract II kit (Macherey-Nagel)

QIAprep® Miniprep kit (Qiagen)

2.1.4 Enzymes

*Bam*HI (Biolabs)

*Bgl*II (Biolabs)

α-chymotrypsin, bovine pancreas (Sigma)

Elastase, porcine pancreas (Pacific Science)

*Eco*RI (Biolabs)

*Eco*RV (Biolabs)

*Hin*dIII (Biolabs)

pfu DNA polymerase (Fermentas)

*Nco*I (Biolabs)

RNase A (Sigma)

*Sac*II (Biolabs)

*Sal*I (Biolabs)

*Sca*I (Fermentas)

Subtilisin Carlsberg, *Bacillus licheniformis* (Sigma)

T4 DNA ligase (Fermentas)

T4 polynucleotide kinase (Biolabs)

T7 DNA polymerase (Biolabs)

Trypsin, bovine pancreas (Sigma)

*Xho*I (Biolabs)

2.1.5 Substrates

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

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

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

2.1.6 Bacterial strains

Escherichia coli BMH 71-18 *mut*S

Escherichia coli JM109

Escherichia coli Rosetta (DE3) pLysS

M13KO7 bacteriophage

2.1.7 Plasmid vectors

pET-22b(+) (Novagen®, Germany)

pET-28b(+) (Novagen®, Germany)

pSPI*Pm*2-D3 (Donpudsa et al., 2009), a pVR500 containing SPI*Pm*2 domain 3

pSPI*Pm*2-NS2 (Somprasong et al., 2006), a pET-22b(+) containing the SPI*Pm*2 gene

pSPI*Pm*2-T35L (Wongwarangkana., unpublished), a pET-22b(+) containing the SPI*Pm*2 gene mutated at P1 residue of domain 1

p118*Pm*2 and p119*Pm*2, a pUC118 and pUC119 carrying the DNA fragment of SPI*Pm*2 gene between *BamH*I and *Sal*I site in the different orientation

pVR500, (Donpudsa et al., 2009), a pET-32a(+) derivative

2.1.8 Software

GENETYX version 7.0 program (Software Development Inc.)

SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/)

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

2.2 General techniques

2.2.1 Competent cells preparation

The starter of *E. coli* strain JM109, BMH71-18*mut*S was prepared from a single colony inoculated in 20 ml of LB broth (1% (w/v) bacto tryptone, 1% (w/v) NaCl , and 0.5% (w/v) bacto yeast extract) and incubated at 37 °C with shaking at 250 rpm overnight. The starter was 1 : 25 diluted in 500 ml of LB broth and incubated at 37 °C with vigorous shaking until the OD_{600} of the cells reaches 0.3-0.5. Cells were then chilled on ice for 30 min and harvested by centrifuged at 5,000×g for 10 min at 4 °C. The cell pellet was washed twice with 1/2 volume of the ice-cold 10 mM $CaCl_2$

with gently mixing and centrifugation. The pellet was suspended with 1/10 volume of the ice-cold 0.1 mM $CaCl_2$ and left on ice at least 30 min. This cells were ready for transformation or added glycerol with the final concentration of 15% (v/v), the cells were divided into 100 µl aliquots and stored at -80 °C until used. For the procedure of *E. coli* strain Rosetta (DE3) pLysS, the LB broth containing 34 µg/ml of chloramphenicol was used instead.

2.2.2 Calcium chloride transformation

The competent cells were trawed on ice and mixed with 1-2 µl of mutagenesis or ligation reaction, mixed well and placed on ice for 30 min. The mixture was incubated at 42 °C for 1 min and 0.9 ml LB broth was added immediately. The cell suspension was incubated at 37 °C with shaking at 250 rpm for an hour. After that, 100 µl of transformant was spread on a LB agar plate containing appropriate antibiotic.

2.2.3 Extraction of plasmid DNA using QIAprep® Miniprep kits

The plasmid was isolated from the cell culture by a QIAprep® Miniprep kits. A single colony was inoculated into 2 ml of BL broth containing appropriate antibiotic and cultured overnight at 37 °C. The cells were harvested by centrifugation and resuspended in 250 µl P1 buffer containing RNase A. Next, the 250 µl P2 buffer was added and the reaction was mixed thoroughly by inverting the tube 4-6 times to lyse the cells. The N3 buffer was added to neutralized the cell lysate. After maximum speed centrifugation for 10 min, the supernatant containing the plasmid was applied to a QIAprep spin column. The column was centrifuged for 30-60 s and the flow-through was discarded. The column was washed twice with 500 µl Buffer PB and 750 µl Buffer PE, respectively, and centrifuged to remove residual ethanol from Buffer PE. After that, the column was placed in a clean 1.5 ml microcentrifuge tube. The 50 µl EB buffer was added to the center of the column and incubated at room temperature for 1 min. Finally, the plasmid DNA was eluted by centrifugation for 1 min and stored at -20 ºC until used.

2.2.4 Agarose gel electrophoresis

The DNA samples were analyzed by using 1% agarose gel prepared by melting the slurry 1% (w/v) agarose gel in 1×TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) using the microwave oven. The gel solution was cooled down at room temperature to 60 ºC before pouring into a tray with a well-forming comb. DNA samples were mixed with one-sixth volume of 6×loading dye (0.25% bromophenol blue and 25% Ficoll in water) before loading into the well. The GeneRuler^{TM} 1 kb DNA ladder (Fermentas) was used as standard DNA marker. The electrophoresis was performed in 1×TBE buffer at 100 volts for an hour. After that, the gel was stained in a 2.5 μg/ml ethidium bromide solution for 30 sec and de-stained by placed in distilled water for 10 min. The DNA bands were visualized under a UV transilluminator.

2.2.5 Extraction of DNA fragment from the agarose gel

The NucleoSpin® Extract II kits (Macherey-Nagel) was used for extracting DNA fragment from the agarose gel and carried out according to the kit protocol. Briefly, The gel slice containing DNA fragment was excised from an agarose gel with a clean sharp scalpel, determined the weight and transferred to a clean 1.5 ml microcentrifuge tube. Two hundred millilitres of NT buffer was added into the tube for each 100 mg of agarose gel. The sample was incubated at 50 ºC for 5-10 min and vortexed briefly until the gel piece was dissolved. The sample was loaded into the A NucleoSpin extract column and centrifuged at 10,000x g for 1 min. The membrane was washed with 600 μl of NT3 buffer and centrifuged at 10,000×g for 1 min. The extra centrifugation at 10,000×g for 2 min was used for completely removal of NT3 buffer and the column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was then eluted by adding 50 μl of elution buffer NE into the column, incubated at room temperature for 1 min, and collected by centrifugation.

2.2.6 Phosphorylation of oligonucleotide primers

Before used in mutagenesis procedure, two mutagenic oligonucleotides, name Pm2-D2 and Pm2-D3 (purchased from Sigma-Proligo, USA), were

phosphorylated. Approximately 100-200 pmol of each oligonucleotide was kinased in 10 µl reaction containing 1× kinase buffer, 1 mM ATP, 10 U T4 polynucleotide kinase, and incubated at 37 ºC for an hour. The reaction was heated at 75 ºC for 20 min to stop the kinase activity and stored at -20 ºC until used.

2.3 Mutagenesis of SPI*Pm*2 gene using the USE (Unique Site Elimination) procedure

The two mutagenic primers, Pm2-D2 and Pm2-D3, were designed in order to alter the codon encoding amino acid at P1 residue of domain 2 and 3 of SPI*Pm*2 gene, respectively. The nucleotide sequences of the mutagenic primers are shown in Fig. 2.1. For the screening of the mutants, Pm2-D2 primer was also designed to contain the *Nco*I site while Pm2-D3 primer was designed to eliminate a *Bgl*II site in the gene.

The procedure of USE mutagenesis is the annealing of two oligonucleotide primers to a plasmid. The mutagenic primer introduces the desired mutation and another primer mutates a unique restriction site of the plasmid for the purpose of selection. A selection primer, named *Sca*I primer, eliminates the *Sca*I restriction site at the ampicillin resistant gene in the plasmid. In this work, each of the two kinased mutagenic primer, the kinased *Sca*I primer and 40 ng of the p119*Pm*2 or p118*Pm*2 for mutagenesis in domain 2 or 3, respectively were mixed in 20 µl reaction containing annealing buffer (200 mM Tris-HCl, 100 $MgCl_2$ and 500 mM NaCl). The reaction mixture was heated at 95 ºC for 5 min, and then placed at room temperature for 15 min. Next, it was added 10 µl an enzyme mixture containing 5 U T4 DNA ligase, 1 U T7 DNA polymerase, 3x ligase buffer, 3 mM ATP, and 3 mM dNTPs. The mixture was incubated at room temperature for 12-16 hours and then transformed into *E. coli* strain BMH71-18*mut*S. The transformants were cultured in 2 ml LB broth containing 100 µl/ml ampicillin overnight. The plasmid mixture was prepared and digested with *Sca*I to linearlize most of the wild-type plasmid. The digestion reaction was transformed into *E. coli* strain JM109 to separate individual clones. Random clones were grown for plasmid preparation using QIAprep® Miniprep kits and screening for the mutant plasmids by using appropriate restriction enzyme digestion.

Furthermore, the mutant clones were sequenced to confirm the correct alteration of the mutated regions. The correct clones containing SPI*Pm*2 gene mutated in domain 2 and 3 were named pUCA83W and pUCE131L, respectively.

Pm2-D2 PRIMER (49mer)

*Nco*I
5′ G AGA TGT CCA GGA ATC TGT CCA TGG GTG TAC GCA CCT GTG TGC GGG ACC 3′

Pm2-D3 PRIMER (33mer)

*Bgl*II
5' CAC GGG AGC ATA GAT GAG AGG GCA CGC GAC AAT 3'

Figure 2.1 The mutagenic primers used in USE mutagenesis procedure. The underlined regions were the added *Nco*I site and eliminated *Bgl*II site in domain 2 and 3, respectively.

2.4 Construction of the protein expression clones

2.4.1 The mutant SPI*Pm*2 expression clones

To express the recombinant mutant SPI*Pm*2 proteins, two mutant plamids, pUC*Pm*2-A83W and pUC*Pm*2-E131L, were digested with *Bam*HI and *Sal*I to yield DNA fragments of mutated SPI*Pm*2 gene. They were gel purified using 1% agarose gel electrophoresis and eluted with NucleoSpin® Extract II kits (Macherey-Nagel). Each DNA fragment was ligated into *Bam*HI-*Sal*I digested pET-22b expression vector (Novagen) (Fig. 2.2). The ligation reactions were composed of 1 µl of 10× T4 DNA ligase buffer, 1 U of T4 DNA ligase, 20 ng of *Bam*HI-*Sal*I digested pET-22b, proper amount of DNA fragment containing mutated SPI*Pm*2 gene, and sterile deionized water was added to a final volume of 10 µl. The reactions were mixed, briefly spun and incubated at 22 °C overnight. The appropriate amount of insert in the ligation reaction was calculated following equation:

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

Next, the ligation mixtures were transformed into *E. coli* strain JM109 using $CaCl_2$ method. The transformants were spread onto the LB agar plates contatining 100 μl/ml of ampicillin and incubated at 37 °C for overnight. Each transformant was grown in LB broth in the presence of 100 μl/ml ampicillin for plasmid preparation using QIAprep® Miniprep kits. The recombinant plasmids were screened using *Bam*HI-*Sal*I digestion. The expression clones of SPI*Pm*2 mutated at domain 2 and 3 were named pSPI*Pm*2-A83W and pSPI*Pm*2-E131L, respectively.

2.4.2 The SPI*Pm*1 expression clone

Two PCR primers (Sigma-Proligo) containing restriction site, Pm1-F; 5' AAGCCATGGATCATGATTGTATCGGCTAC 3' with *Nco*I site (underlined) and Pm1-R; 5' CCACTCGAGTTAGTGGTGGTGGTGGTGGTGAATCTCCAGAAGGT ATTTCC 3' with *Xho*I site (underlined), was designed for the amplification of SPI*Pm*1 gene fragment encoding the mature protein without signal peptides. A plasmid containing SPI*Pm*1 gene was used as template. The *Nco*I and *Xho*I site were incorporated at the 5' and 3' end of the gene fragment were for cloning into the expression vector. The polymerase chain reaction was performed in a final volume of 50 μl containing 0.02 ng of plasmid template, 0.4 M of each primer, 0.2 mM of each dNTP and 3 units of *Pfu* polymerase (Promega). The PCR amplification was carried out at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 45 sec, 50 ºC for 45 sec and 72 ºC for 90 sec, and final extension at 72ºC for 10 min. The PCR product was analyzed using 1.2% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The purified fragment was digested with *Nco*I and *Xho*I, run in 1.2% agarose gel electrophoresis and purified further using NucleoSpin® Extract II Kits. The *Nco*I–*Xho*I fragments containing the SPI*Pm*1 was ligated into the *Nco*I–*Xho*I digested pET-28b(+) expression vector (Novagen) (Fig 2.3). The recombinant clones were screened using *Nco*I–*Xho*I digestion and subjected to nucleotide sequencing to verify the insert sequences of SPI*Pm*1. The SPI*Pm*1 expression clone was named pSPI*Pm*1.

2.4.3 The D3E131L expression clone

A primer pair (Donpudsa et al., 2009), D3F; 5' AAGCCCATGGGACGT

TGTGGTTGC 3', and D3R; 5' AGCCCTCGAGAGGAGTGCAGTCGC 3', was used for the amplification of mutated SPI*Pm*2 domain 3 from pSPI*Pm*2-E131L. The forward and reverse primer contains *Nco*I and *Xho*I sites (underlined) at their 5' terminal ends, respectively. The restriction sites were for the cloning of the mutated domain 3, D3E131L, into the pVR500 expression vector (Fig 2.4). The PCR reaction, cloning and screening of recombinant clones were performed as described in 2.4.2. The expression clone of D3E131L was named pSPI*Pm*2-D3E131L.

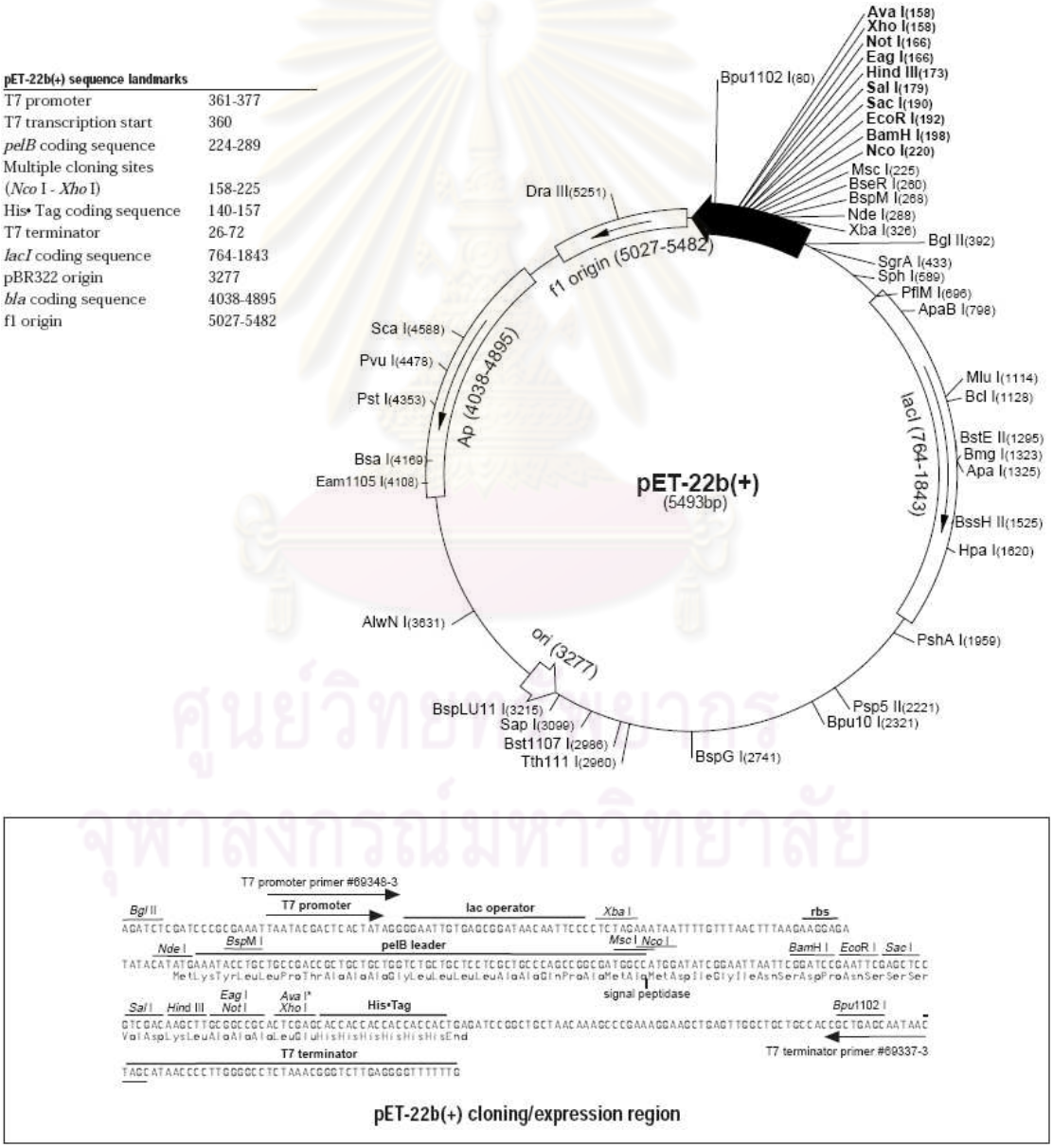

Figure 2.2 The pET-22b(+) vector map (Novagen)

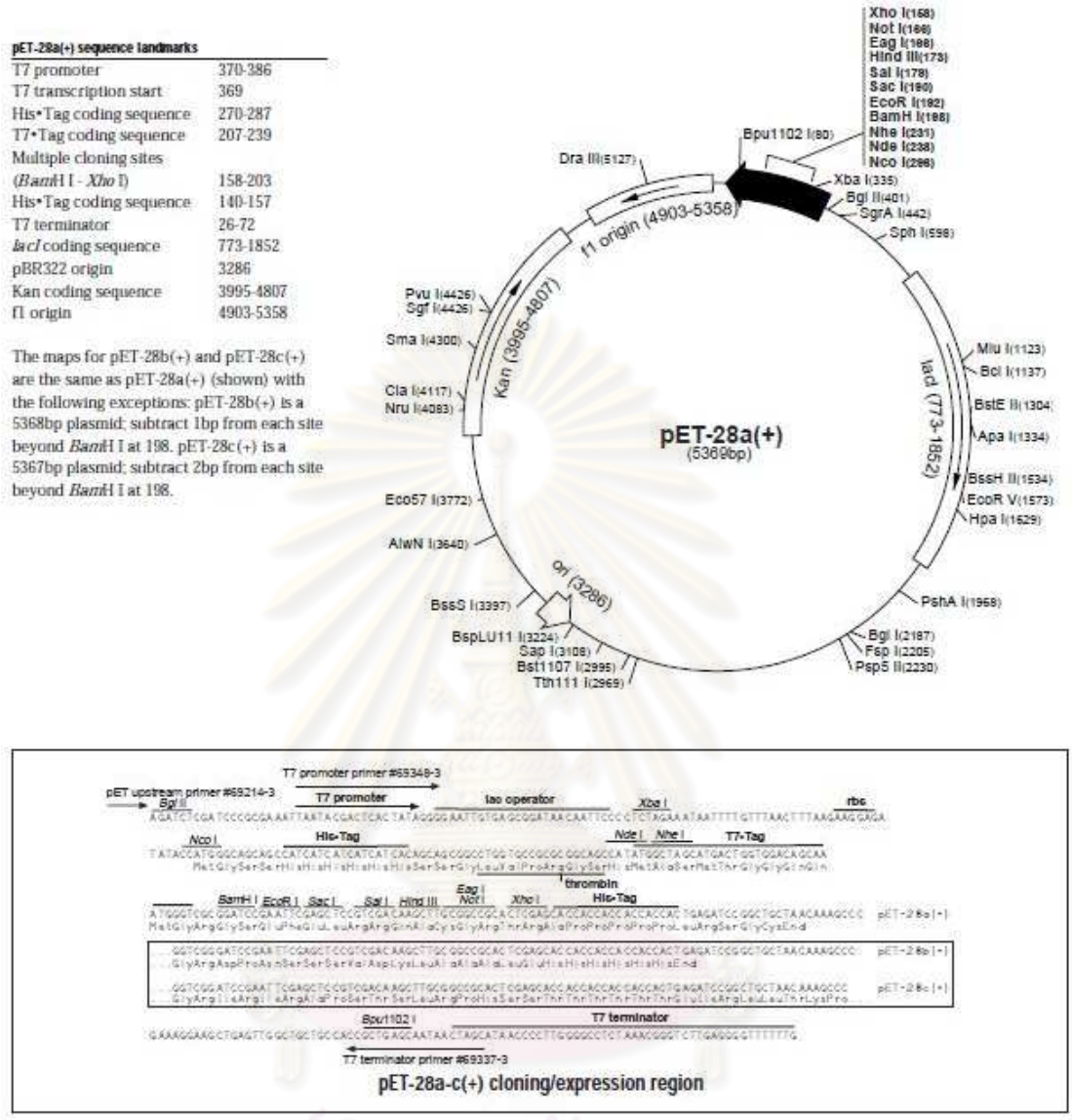

Figure 2.3 The pET-28b(+) vector map (Novagen)

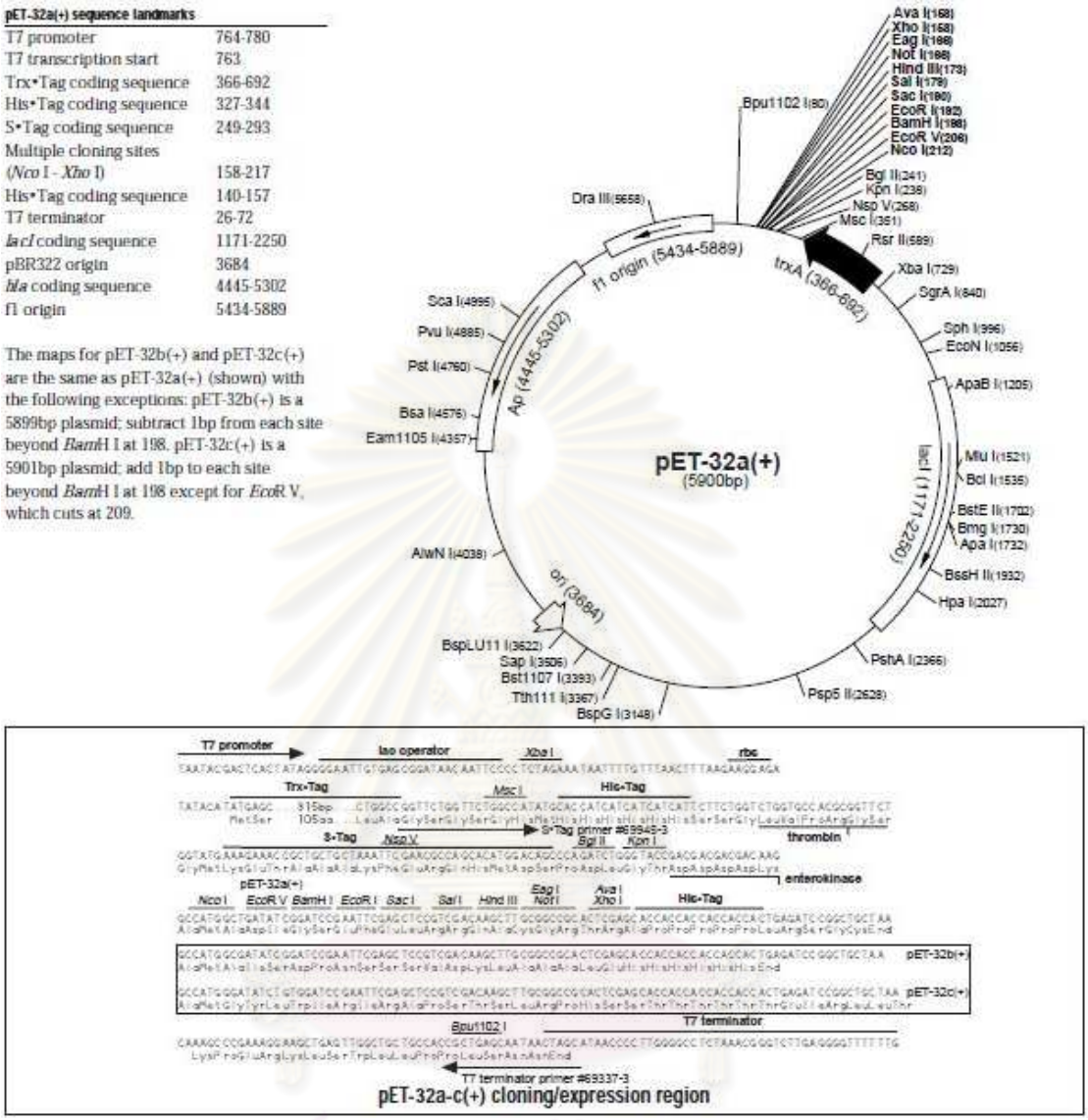

Figure 2.4 The pET-32a(+) vector map (Novagen). The pVR500 was constructed by deleting the His-Tag and S-Tag between *Msc*I and *Kpn*I sites. The pET-32a(+) was digested with *Msc*I and *Kpn*I, treated with T4 DNA polymerase to blunt the DNA ends and religated.

2.5 Recombinant protein expression

The expression plasmids, pSPI*Pm*1, pSPI*Pm*2-NS2, pSPI*Pm*2-T35L, pSPI*Pm*2-A83W, pSPI*Pm*2-E131L, pSPI*Pm*2-D3, and pSPI*Pm*2-D3E131L were transformed into *E. coli* Rosetta (DE3) pLysS. The starter was prepared by inoculating a single colony from a freshly streaked plate into a 2 ml LB broth containing 34 μg/ml of chloramphenicol and 70 μg/ml of kanamycin for SPI*Pm*1 or 100 μg/ml of ampicillin for others and incubating with shaking at 37 ºC for overnight. The starter was diluted 1:100 into LB medium, supplemented with antibiotics and incubated at 37 ºC with shaking at 250 rpm until the OD_{600} reached 0.6. IPTG was then added to the final concentration of 1 mM for induction. The culture was incubated at 37 ºC further with shaking for 0, 1, 2, 3 and 4 h, respectively. The 1 ml cultures were aliquot at each time point and the pellets collected by centrifugation at 6,000×g for 5 min. The cell pellets were stored at -80 ºC until required for further analysis.

2.6 Purification of recombinant proteins

2.6.1 Recombinant SPI*Pm*1

After 4 h of IPTG induction, cells were harvested by centrifugation at 6,000×g for 10 min at 4 ºC. The cell pellet was collected and resuspended by pipetting up and down in phosphate-buffer saline, pH 7.4 (1×PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The cell suspension was disrupted by 3 rounds of freeze-traw and sonicated with a Bransonic 32 (Bandelin Sonopuls, Germany) for 10 min 3 times. The cell lysate was centrifuged at 10,000×g for 10 min to collect the inclusion bodies. The inclusion bodies were washed twice with 1×PBS containing 1% TritonX-100 and twice with 1×PBS without TritonX-100. The washed inclusion bodies were solubilized in 50 mM Tris-HCl, pH 8 containing 4 M urea for overnight at room temperature. The insoluble material was removed by centrifugation. The crude protein solution was purified under denaturing condition using nickel affinity chromatography (GE Healthcare).

The Ni-NTA agarose was packed into the PD-10 column and washed 3 times with distilled water and wash buffer (50 mM This-HCl, pH 8, 300 mM NaCl, 4 M urea and 20 mM imidazole), respectively. The protein solution was applied to the column at room temperature. The flow through was collected by a gravity flow. The column was washed with the wash buffer to remove unbound proteins. After that, the protein was eluted with an elution buffer (50 mM This-HCl, pH 8, 300 mM NaCl, 4 M urea and 500 mM imidazole). The eluted protein was dialysed for at least 10 h at 4 ºC against 50 mM This-HCl, pH 8. The purified protein was analyzed by 15% SDS-PAGE and western blot analysis.

2.6.2 Recombinant wild-type and mutant SPI*Pm*2

The inclusion bodies of intact wild-type and mutant SPI*Pm*2 proteins were prepared as described in 2.6.1., In stead of solubilizing the inclusion bodies with 50 mM Tris-HCl, pH 8 containing 8 M urea, they were solubilized with 50 mM carbonate buffer, pH 10 at room temperature overnight and the protein was purified under non-denaturing condition using nickel affinity chromatography. Briefly, the solubilized protein was loaded into the column before washing by the wash buffer (50 mM carbonate buffer, pH 10 containing 300 mM NaCl and 20 mM imidazole). The protein was eluted with the elution buffer (50 mM carbonate buffer, pH 10 containing 300 mM NaCl and 500 mM imidazole).The eluted fractions were dialysed against 50 mM carbonate buffer, pH 10 at 4 ºC for at least 10 h and determined the purity by 15% SDS-PAGE.

For the recombinant SPI*Pm*2 domain 3 wild-type and D3E131L, the supernatant was collected after the cell lysate was centrifuged. The soluble fraction was purified using the Ni-NTA agarose column. The column was washed with 1×PBS buffer pH 7.4 containing 20 mM imidazole and the protein was eluted with 1×PBS buffer pH 7.4 containing 500 mM imidazole. The purified protein was also dialysed against 50 mM carbonate buffer, pH 10 to remove imidazole and analyzed by 15% SDS-PAGE.

2.7 Protein analysis

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

A discontinuous system of SDS-PAGE was used. The gel solutions were prepared as shown in the Appendices. Firstly, the glass plates and spacers were assembled and the components of the separation gel solution were mixed thoroughly and pipette into the gel plate setting. A small amount of distilled water was then layered over the top of the separation gel solution to obtain a flat surface gel. When the polymerization was complete, the water was poured off. The stacking gel solution was prepared, mixed thoroughly and poured on top of the separating gel. A comb was placed in position with excess gel solution overflowing the front glass plate. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with distilled water to remove excess unpolymerized acrylamide.

Protein samples were prepared by resuspending the proteins in 1× sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2.88 mM 0.02% bromophenol blue, 2-mercaptoethanol). The samples were then boiled for 10 min and either held at room temperature or kept at 4 ºC until loaded into the gel.

The prestained protein marker and the boiled protein samples were loaded into the wells. Electrophoresis was carried out in 1× running buffer (25 mM 52 Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) at a constant current of 20 mA until the tracking dye (bromophenol blue) reached the bottom of the separating gel.

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

2.7.2 Detection of the recombinant protein by Western blot

After running the SDS-PAGE, separated proteins were electrotransferred to a nitrocellulose membrane in the transfer buffer (48 mM Tris-HCl pH 9.2, 39mM glycine, 20% methanol) using Trans-Blot® SD (Bio-Rad) (Fig. 2.5) at 100 mA for 1 h. The membrane, gel and filter paper were soaked in a transfer buffer for 30 min

before they were consequently laid on Trans-Blot® SD. The filter paper was placed on platform, followed by the membrane, the gel and filter paper, respectively. Afer transfer, the membrane was incubated at room temperature for overnight in PBS-Tween buffer (phosphate buffer saline, pH 7.4, 0.05% Tween20) containing 5% skimmed milk. The membrane was washed 3 times for 10 minutes each time in PBS-Tween and incubated in an anti-His antibody solution (1:3000 dilution in washing buffer with 1% skimmed milk) at 37 °C for 3 h. Next, the membrane was washed in PBS-Tween buffer and then incubated in a secondary antibody solution (1:5000 dilutions in washing buffer with 1% skimmed milk) for an hour at room temperature. After washing as above, the bound antibody was detected by color development using NBT/BCIP (Fermentas) as substrate dissolving in 100 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl2, pH 9.5.

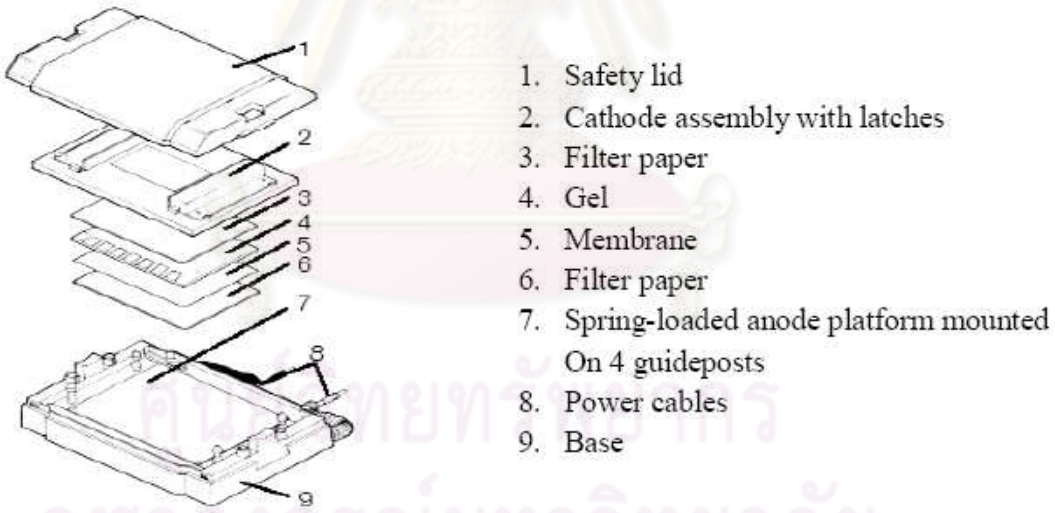

Figure 2.5 Exploded view of the Trans-Blot® SD (Bio-Rad)

2.7.3 Determination of protein concentration

Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard. The 100 µl of diluted sample solution was mixed with Bradford working buffer and left for 2 min before the absorbance at 595 nm was measured. The Bradford working buffer (100 ml) was a mixture of 6 ml Bradford stock solution (350 g Coomassie blue G250, 100 ml 95% ethanol and 200 ml 85% phosphoric acid), 3 ml 95% ethanol, 6 ml 85% phosphoric acid and 85 ml distilled water.

2.8 Serine proteinase inhibition assay

The proteinase inhibitory activities of each recombinant protein towards serine proteinase; subtilisin Carlsberg (Bacillus licheniformis,Sigma), elastase (porcine pancreas, Pacific Science), trypsin (bovine pancreas, Sigma), and α-chymotrypsin (bovine pancreas, Sigma) were assayed using a procedure of Hergenhahn et al. (1987) (Hergenhahn, 1987). The reaction mixture consisted of 50 mM Tris–HCl, pH 8.0; 146.8 µM of N-benzoyl-Phe-Val-Arg-*p*-nitroanilide as substrate for subtilisin and trypsin, 147.3 µM N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for chymotrypsin and 886.2 µM of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide for elastase; 0.04 µM of subtilisin, trypsin and chymotrypsin, and 0.08 µM of elastase, and appropriate concentrations of proteinase inhibitiors in a total volume of 100 ml. The series of 2-fold diluted proteinase inhibitors were used; 3.35–0.01 µM for rSPI*Pm*1, 3.23–0.01 µM for rSPI*Pm*2, 3.05–0.01 µM for rT35L, 3.19–0.01 µM for rA83W, 2.98–0.01 µM for rE131L, 3.14–0.01 µM for rSPI*Pm*2-D3, and 3.30–0.01 µM for rD3E131L. Not all concentrations of proteinase inhibitors were used in the assays. For each substrate, a reaction containing the highest amount of SPI preparation used in the assay but without added proteinase was prepared as a control to make certain that there were no contaminating proteinases from the bacterial host used for the preparation of SPIs. The reaction was incubated at 30 ºC for 15 min and then terminated by adding 50 µl of 50% acetic acid. The proteinase reaction product, *p*-nitroaniline, was measured at 405 nm using microplate reader (BMG Labtech). The percentages of remaining activity were calculated and plotted against the molar ratio of inhibitor to proteinase.

CHAPTER III

RESULTS

3.1 The design of mutagenic primers being specific to SPI*Pm*2 gene

So far we know that the inhibitory specificity of the Kazal-type serine proteinase inhibitors depends on the P1 amino acid residue, which is the second amino acid residue after the second cysteine residue of the domain. From the black tiger shrimp, the P1 residues of SPI*Pm*2 are T, A, E, K, and A for domain 1-5, respectively. This inhibitor exhibits the strong inhibitory activity against subtilisin and elastase. It weakly inhibits against trypsin, but has no the inhibitory activity against chymotypsin. It is suggested that the inhibitory specificities can not be probably assigned to all domains. It is believed that the inhibitory specificities will change if the P1 residue of some domains is altered. In this study, I attempted to create two mutants of SPI*Pm*2 that the P1 residues of domain 2 and 3 were modified. Two mutagenic primers being specific to the second and third domain of SPI*Pm*2; Pm2-D2 and Pm2-D3, respectively, were designed in order to mutagenize the SPI*Pm*2 gene. The primers introduced the base substitutions (Figure 3.1). Upon mutagenesis, the amino acid at position 83 and 131 would be changed from A to W and E to L using Pm2-D2 and Pm2-D3 primers, respectively. For screening of matants, *Nco*I site was designed on Pm2-D2 primer, while *Bgl*II site was eliminated from Pm2-D3 primer. Furthermore, the plasmid carrying the mutated SPI*Pm*2 gene at domain 1, pSPI*Pm*2-T35L, was built by Wongwarangkana et al. (Wongwarangkana, unpublished). The P1 residue of domain 1 was replaced from T to L. It was used in this work for the rT35L production. The three mutants were assayed for the inhibitory activity against the proteinases.

3.2 Mutagenesis of SPI*Pm*2 gene

The plasmid p119*Pm*2 or p118*Pm*2 carrying the SPI*Pm*2 gene was used as the template for mutagenesis procedure. The mutagenic primers, Pm2-D2 and Pm2-D3,

and *Sca*I primer were used to mutagenize the P1 residue of domain 2 and 3 of the SPI*Pm*2 gene. From the mutagenic protocol, the reaction mixture contains wild type and mutated plasmids. So, the wild type plasmid was removed with *Sca*I digestion. The mutated plasmids were separated into individual clones by transformation. The plasmid clones were prepared, and screened for the presence of the mutants. The mutated plasmids at domain 2 and 3 of SPI*Pm*2 were check by *Nco*I and *Bgl*II digestion and confirmed by agarose gel elctrophoresis (Figure 3.2). Two mutated plasmids at domain 2 and 3, were called pUCA83W and pUCE131L, respectively, were obtained as shown in Figure 3.3.

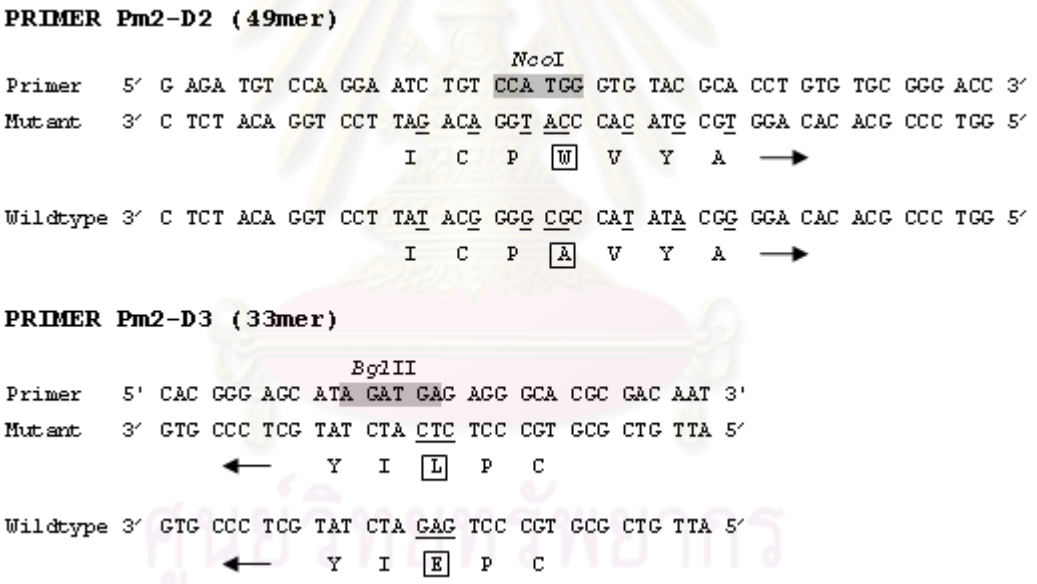

Figure 3.1 The design of oligonucleotides used in the mutagenesis procedure. The nucleotides of wild type to be mutated are underlined. The created and eliminated restriction sites are shaded. The substituted amino acids are boxed.

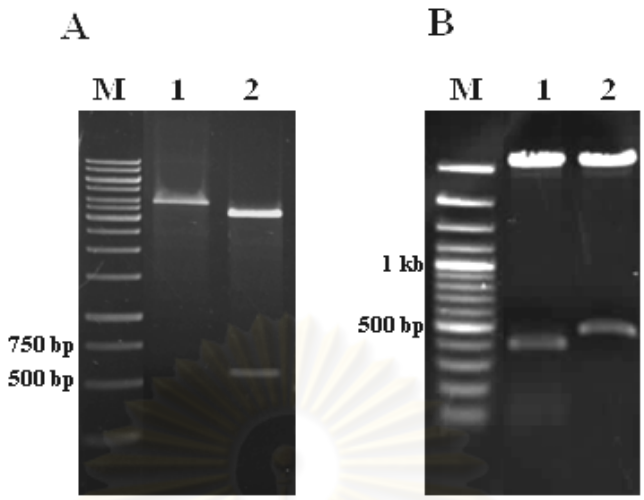

Figure 3.2 The screening of the mutant plasmid by digesting with *Hin*dIII-*Nco*I (A) and *BamH*I-*Bgl*II (B) on 1.2% agarose gel electrophoresis. For domain 2 mutation (A), lane M : GeneRuler™ 1 kb DNA ladder (Fermentas); lane 1 : p119*Pm*2; lane 2: pUCA83W. For domain 3 mutation (B), lane M : GeneRuler™ 100 bp DNA ladder (Fermentas); lane 1 : p118*Pm*2; lane 2 : pUCE131L.

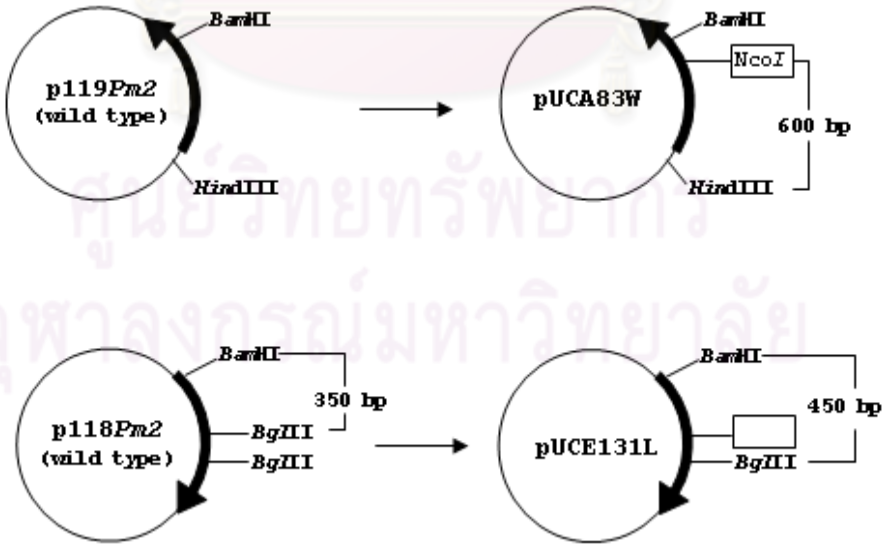

Figure 3.3 The mutated plasmids, pUCA83W and pUCE131L. The correct sites were confirmed by restriction enzyme digestion. The added and eliminated sites are boxed.

3.3 DNA sequencing determination of the mutation regions

The precise DNA sequences around the mutation regions in pUCA83W and pUCE131L were determined using sequencing service of Macrogen. The two mutants had the nucleotide sequences as designed (Figure 3.4). There were no additional mutations in the gene. The mutant genes were subsequently subcloned into expression vector as pET-22b(+) between *Bam*HI-*Sal*I site for protein production.

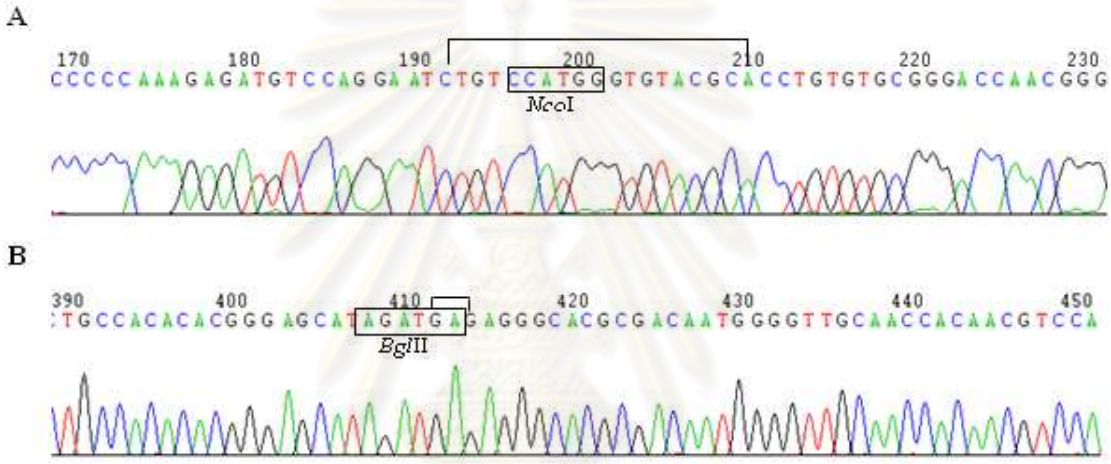

Figure 3.4 Nucleotide sequencing of mutated domain 2 (A) and 3 (B) in pUCA83W and pUCE131L, respectively. The added *Nco*I site and eliminated *Bgl*II site are boxed. Each mutant region is indicated by a line over the regions.

3.4 Construction of the protein expression clones

3.4.1 The mutant SPI*Pm*2 expression clones

To construct the expression clones of the mutant SPI*Pm*2, the 750 bp fragments were excised from pUCA83W and pUCE131L using *Bam*HI and *Sal*I and cloned into pET-22b at the *Bam*HI and *Sal*I sites. After ligation, the reaction mixtures were transformed into the *E. coli* JM 109. The recombinant plasmid containing the mutant gene was checked by restriction enzyme digestion using *Bam*HI and *Sal*I and run on 1.2% agarose gel electrophoresis (Figure 3.5). The correct expression clones of

the mutated SPI*Pm*2 at domain 2 and 3 were named pSPI*Pm*2-A83W and pSPI*Pm*2-E131L, respectively.

3.4.2 The mutated domain 3 expression clones

To determine the inhibitory activity of the mutated domain 3, this domain was separated from SPI*Pm*2. The PCR primers, D3F and D3R containing *Nco*I and *Xho*I restriction sites, respectively, were designed in order to PCR amplify only domain 3 using pSPI*Pm*2-E131L as a template. After digesting with *Nco*I and *Xho*I restriction enzymes, the PCR gene fragment was cloned into an *E. coli* expression vector, pVR500. The recombinant protein was expressed as fusion protein with thioredoxin tag at the 5' terminal and His·Tag at the 3' terminal. The recombinant clone was verified by DNA sequencing. The recombinant expression plasmid was named pSPI*Pm*2-D3E131L.

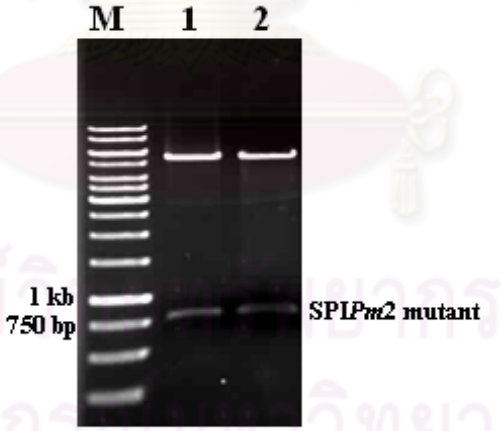

Figure 3.5 The screening of the recombinant plasmid by digesting with *Bam*HI and *Sal*I on 1.2% agarose gel electrophoresis.

Lane M : GeneRuler™ 1 kb DNA ladder (Fermentas)

Lane 1 : pSPI*Pm*2-A83W; lane 2: pSPI*Pm*2-E131L.

3.4.3 The SPI*Pm*1 expression clone

The SPI*Pm*1 (GenBank Accession GO269557), consisting of four Kazal domains, was isolated from the hemocyte cDNA libraries of *P. monodon* (Tassanakajon et al., 2006). The SPI*Pm*1 contains an open reading frame (ORF) of 669 bp encoding a putative protein of 222 amino acid residues (Figure 3.6). Putative signal peptide of the SPI*Pm*1 was identified using the online SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) with the predicted cleavage sites between Ser24 and Asp25, resulting in the 198 residue mature protein with the calculated molecular mass of 22,331 Da and the predicted pI of 4.97. To determine the inhibitory activity of the SPI*Pm*1, the plasmid containing SPI*Pm*1 gene was used as template for the PCR amplification of the SPI*Pm*1 gene. The forward and reverse primers were designed such that the PCR gene products contained the *Nco*I and *Xho*I restriction sites, respectively. The amplified product was analyzed using 1.2% agarose gel electrophoresis (Figure 3.7). After that, the SPI*Pm*1 band was excised from the agarose gel and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). After digesting with *Nco*I and *Xho*I, the purified DNA fragment was ligated with the *Nco*I–*Xho*I digested pET-28b(+) as an expression vector (Novagen). The recombinant plasmid was transformed into an *E. coli* JM 109. The recombinant clone was then selected with LB agar plate containing 70 μg/ml kanamycin and screened by restriction enzyme (*Nco*1-*Xho*I) digestion as shown in Figure 3.8. The resulting expression clone was isolated and subjected to nucleotide sequencing to confirm the insert sequence of SPI*Pm*1.

```
1       ATGTTGTTGTGCAAGATTACTCTTATCCATCTCCTGTTGCAAGGATTTGCTGTCTTTAAT
        M  L  L  C  K  I  T  L  I  H  L  L  L  Q  G  F  A  V  F  N

                                Pm1-F
61      GACGCCAACTCCGATCATGATTGTATCGGCTACTGTCCTGAAGTGTATGATCCTGTGTGT
        D  A  N  S  D  H  D  C  I  G  Y  C  P  E  V  Y  D  P  V  C

121     GCCAGTAACGGCTGGACTTACAACAACGACTGCGAACTACAGGCTATGATAAAGTGCCAG
        A  S  N  G  W  T  Y  N  N  D  C  E  L  Q  A  M  I  K  C  Q

181     GGATGGAATATCACCAAGACACACGACCAAGCATGTGAATGCCTCAAGGCCTGCCCCACG
        G  W  N  I  T  K  T  H  D  Q  A  C  E  C  L  K  A  C  P  T

241     ACCTTTGCCCCTGTGTGTGGGTCAGACAACAAGACCTATCTCAACGAGTGTGTCTTCGAG
        T  F  A  P  V  C  G  S  D  N  K  T  Y  L  N  E  C  V  F  E

301     GTGGCTTCTTGCTGGGATCATTCGCTCGACAAGGCGTCTGAAGGAGCTTGTGGCTGGGGT
        V  A  S  C  W  D  H  S  L  D  K  A  S  E  G  A  C  G  W  G

361     ATCCATTGCCTGCAGTACTGCCCTGAGGTATACGACCCTGTGTGTGGAAGCAACGGCCAG
        I  H  C  L  Q  Y  C  P  E  V  Y  D  P  V  C  G  S  N  G  Q

421     ACTTACACGAACGAATGCGAGTTGCAGGCTGCCATACAGTGTCGTGGGTTGCAGATTGCA
        T  Y  T  N  E  C  E  L  Q  A  A  I  Q  C  R  G  L  Q  I  A

481     AAGAGGCACGACCAGGCTTGTGAGTGCCACGCCACTTGCCCCCTGATCCACGACCCTGTT
        K  R  H  D  Q  A  C  E  C  H  A  T  C  P  L  I  H  D  P  V

541     TGTGGCACTGACGATAGGACTTACTACAACGAGTGCTTCTTCACTAAAGCTTCTTGTTGG
        C  G  T  D  D  R  T  Y  Y  N  E  C  F  F  T  K  A  S  C  W

                                                        Pm1-R
601     GATAGGTCCATTTTGAAGAAGAAAAACGGACCTTGTGACAGGAAATGGAAATACCTTCTG
        D  R  S  I  L  K  K  K  N  G  P  C  D  R  K  W  K  Y  L  L

661     GAGATTTAA
        E  I  *
```

Figure 3.6 The open reading frames of SPI*Pm*1. The putative signal peptide is underlined. The boxed amino acids are P1 amino acids. The shaded nucleotide sequences are the primer annealing sites for the PCR amplification of the SPI*Pm*1 gene fragment for the construction of protein expression clone.

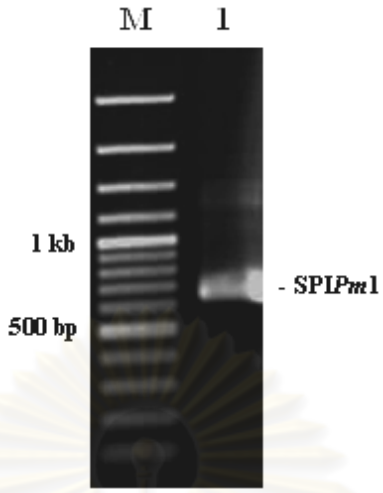

Figure 3.7 Agarose gel electrophoresis of PCR amplification product of SPI*Pm*1.

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

Lane 1 : The PCR product of SPI*Pm*1

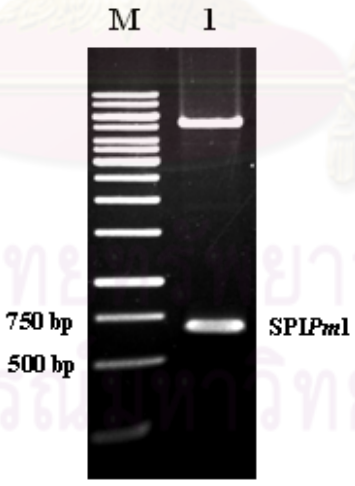

Figure 3.8 The screening of the recombinant plasmid containing SPI*Pm*1gene on 1.2% agarose gel electrophoresis.

Lane M : GeneRuler™ 1 kb DNA ladder (Fermentas)

Lane 1 : pSPI*Pm*1 following digestion with *Nco*I and *Xho*I

3.5 Recombinant protein expression

All expression plasmids were transformed into the expression host, *E. coli* Rosetta (DE3) pLysS. A single colony was cultured in LB medium containing containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol for all SPI*Pm*2 or 34 μg/ml of chloramphenicol and 34 μg/ml of kanamycin for SPI*Pm*1 and incubating at 37 ºC overnight with shaking at 250 rpm. The starter was diluted 1:100 into the culture supplemented with antibiotics and incubated at 37 ºC with shaking at 250 rpm until the OD_{600} reached 0.6. Then, the IPTG was added for the over-expression. The intact wild type and mutated rSPI*Pm*2 were expressed as inclusion bodies. They were solubilized with 50 mM carbonate buffer pH 10. The solubilized recombinant proteins were purified with Nickel-NTA column. The purified wild type and mutant proteins (rT35L, rA83W and rE131L) were analyzed using 15% SDS-PAGE (Figure 3.9). Beside, the rSPI*Pm*2-D3 and rD3E131L were expressed as fusion protein in soluble form. They were purified using Nickle-NTA column and analyzed using 15% SDS-PAGE (Figure 3.10).

For the rSPI*Pm*1, protein expression was induced by the addition of 1 M IPTG to a final concentration 1 mM. The culture was incubated at 37 ºC further with shaking for 0, 1, 2, 3 and 4 h. The cells were harvested at each time point after IPTG induction and then analyzed by 15% SDS-PAGE (Figure 3.11). The results showed that the protein expression was continuously increased after 4 h of IPTG induction. This condition was, therefore, employed for a large-scale expression of the recombinant SPI*Pm*1. The cell pellet at 4 h was resuspended in phosphate-buffered saline, pH 7.4 and sonicated for 2-4 min. The cell lysate was centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet and supernatant were collected and analyzed using 15% SDS-PAGE and Western blot in order to test the inclusion body formation and identify the recombinant protein. The result indicated that rSPI*Pm*1 was expressed as inclusion bodies (Figure 3.12A, B). It was purified with the nickel-NTA column in the denaturing condition. The inclusion bodies were solubilized with 50 mM Tris-HCl pH 8 containing 20 mM imidazole and 4 M urea. The solubilized protein was loaded to the column and washed with washing buffer. The purified recombinant protein, rSPI*Pm*1 protein, was eluted with denaturing solution containing 500 mM imidazole (elution buffer) and analyzed using 15% SDS-PAGE (Figure 3.12C). The result

showed a single band which the approximate size was about 23 kD comparing with the protein marker.

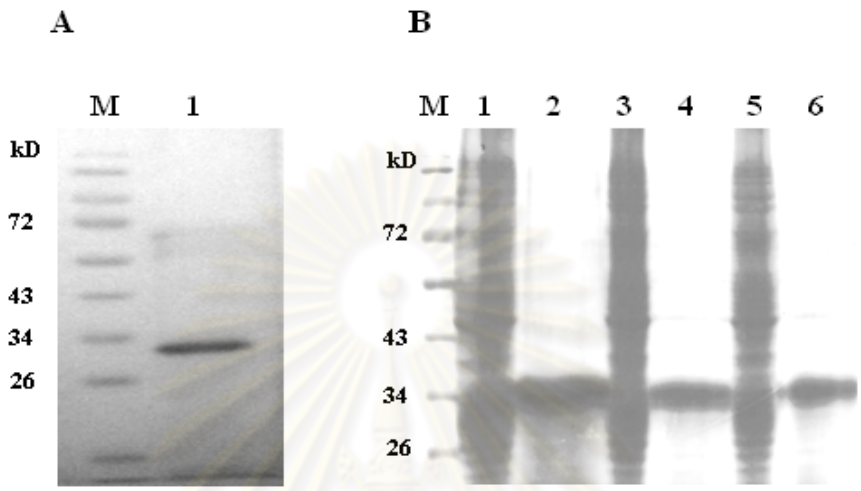

Figure 3.9 SDS-PAGE analysis of the purified recombinant SPI*Pm*2 wild type (A) and mutant proteins (B). For wild type (A), lane 1 is the purified rSPI*Pm*2 protein. For mutant rSPI*Pm*2s (B), lanes 1, 3 and 5 are the lysate of cells carrying pSPI*Pm*2-T35L, pSPI*Pm*2-A83W and pSPI*Pm*2-E131L from induced cells at 4 h., respectively. Lanes 2, 4 and 6 are the purified rT35L, rA83W and rE131L, respectively. Lanes M are prestained protein marker (Fermentas).

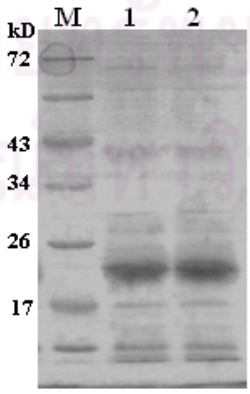

Figure 3.10 SDS-PAGE analysis of the purified recombinant SPI*Pm*2-D3 and D3E131L proteins. Lanes 1 and 2 are the purified rSPI*Pm*2-D3 and rD3E131L, respectively. Lane M is protein size marker.

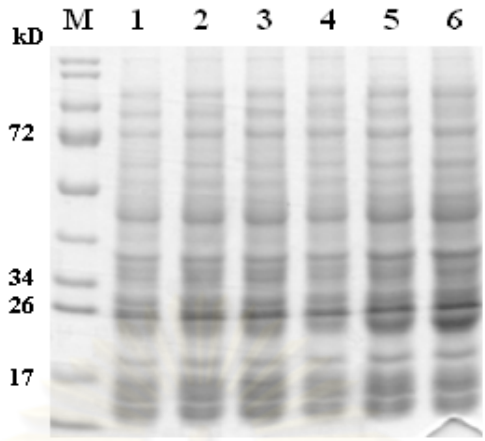

Figure 3.11 SDS-PAGE analysis of the rSPI*Pm*1 expressed in *E.coli* Rosetta (DE3) pLysS at various time of induction. Lane 1 is the lysate of cells containing plasmid pET-28b(+). Lanes 2-6 are the lysate of cells containing pSPI*Pm*1 from induced cells at 0, 1, 2, 3 and 4 hours, respectively.

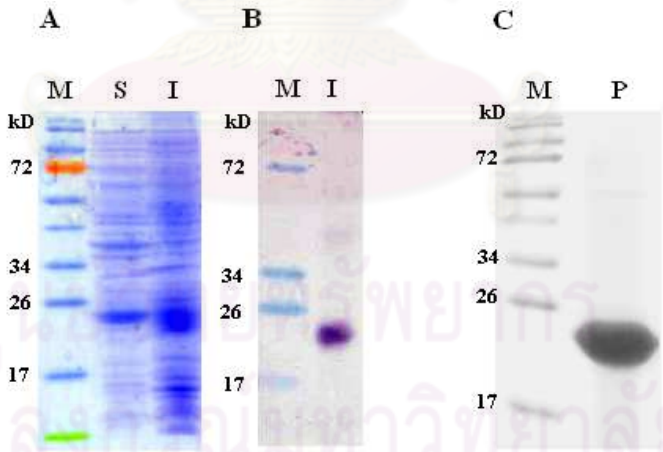

Figure 3.12 The expression and purification of the rSPI*Pm*1 protein. SDS-PAGE analysis of the soluble and inclusion fractions from the cell lysate of *E. coli* containing rSPI*Pm*1 (A). Western blot analysis of the expressed rSPI*Pm*1 protein (B). SDS-PAGE analysis of the purified rSPI*Pm*1 protein (C). Lanes S, I and P are the soluble fraction, inclusion fraction and purified rSPI*Pm*1 protein, respectively. Lanes M are protein marker.

3.6 Serine proteinase inhibitory assay

The inhibitor activity of recombinant serine proteinase inhibitor towards serine proteinase was tested by incubation of recombinant serine proteinase inhibitor and serine proteinase. The residual serine proteinase activity was determined by addition of chromogenic substrate which can be cleaved by the serine proteinase and released *p*-nitroaniline. Absorbance of chromophore p-nitroaniline produced was measured at 405 nm. Therefore, inhibition of serine proteinase hydrolysis of chromogenic substrate can be measured by following the change in absorbance 405 nm. The purified recombinant proteins from nickel-NTA agarose column were tested for serine proteinase inhibitory activity. The recombinant inhibitors were assayed for their inhibitory activities against subtilisin A, elastase, trypsin and chymotrypsin. The mole ratios up to 40 of inhibitor against proteinase were used. For a strong inhibitor, the inhibition was readily seen at the mole ratios less than 10. We found that SPI*Pm*1 showed the weak inhibition against all proteinases when the mole ratio of inhibitor against proteinases was 80 : 1 (Table 3.1).

Table 3.1 Remaining activity of serine proteinases after incubating with rSPI*Pm*1.

Proteinases	**Remaining activity (%)**
Subtilisin	81.3
Elastase	80.6
Trypsin	84.3
Chymotrypsin	88.1

The SPI*Pm*2 with the domain P1 residues T, A, E, K and E was isolated from the hemocyte cDNA libraries and found to strongly inhibit subtilisin and elastase, and weakly inhibit trypsin. To gain further information on the domain inhibitory specificity, the SPI*Pm*2 gene was specifically mutated at the P1 residues of domains 1, 2 and 3. The mutant proteins were over-expressed and their inhibitory activities tested comparing with the wild type protein. The results indicated that T35L and E131L inhibited trypsin less than the wild type (Figure 3.13C). Only A83W has no

inhibitory activity against elastase (Figure 3.13B). All mutant proteins had no inhibitory activity against chymotrypsin like the wild type (Figure 3.13D).

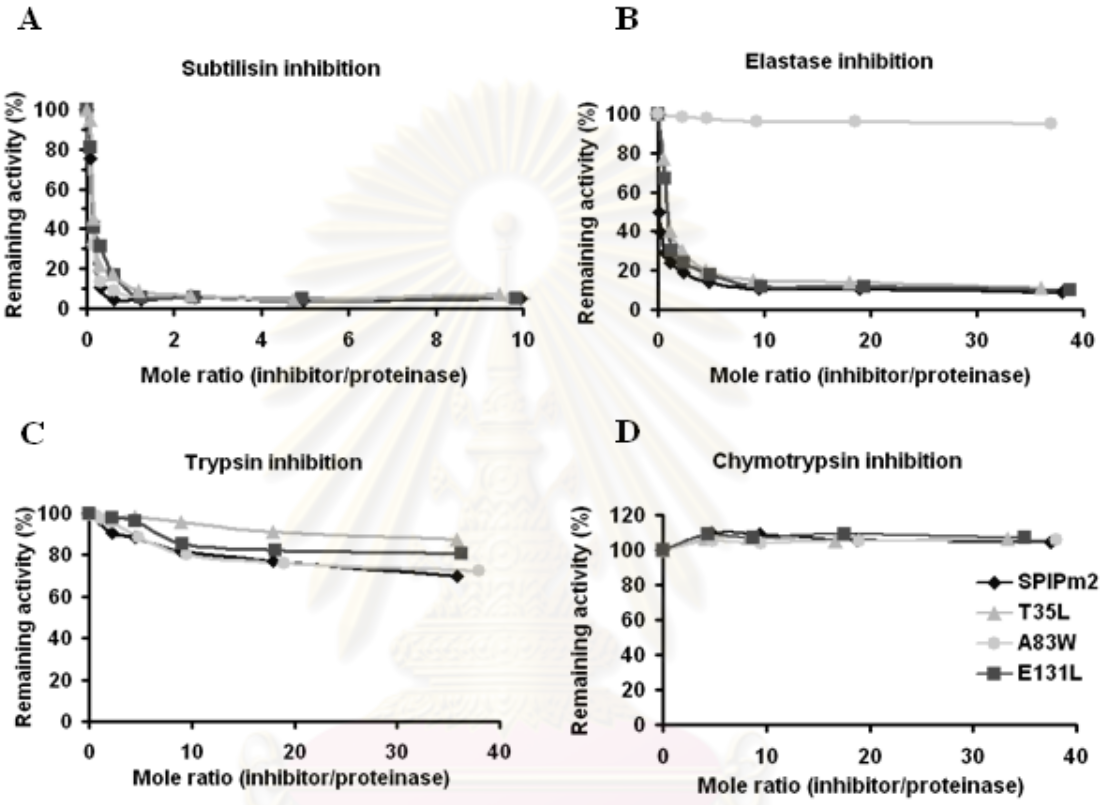

Figure 3.13 Inhibition assays of the SPI*Pm*2 wild type and mutants against subtilisin (A), elastase (B), trypsin (C) and chymotrypsin (D). All SPIs were tested against proteinases at various mole ratio of inhibitor to protease. Symbols ♦, ▲, ● and ■ were for SPI*Pm*2, T35L, A83W and E131L, respectively.

From the proteinase inhibitory assay of the mutants SPI*Pm*2, the inhibitory activity of all mutants against subtilisin didn't change comparing with the wild type (Figure 3.13A). There might be more than one domain that could inhibit subtilisin. In order to confirm the effect of P1 residue of the mutants against proteinases, the mutated SPI*Pm*2 at domain 3 was the choice for study. The mutated domain 3 was separated from E131L by PCR amplification. This mutant was called D3E131L. The

rD3E131L was over-expressed for the proteinase inhibitory assay. The result showed that D3E131L had the stronger inhibitory activity than the wild type, SPI*Pm*2-D3 (Figure 3.15A). D3E131L strongly inhibited elastase comparing with the wild type (Figure 3.15B).

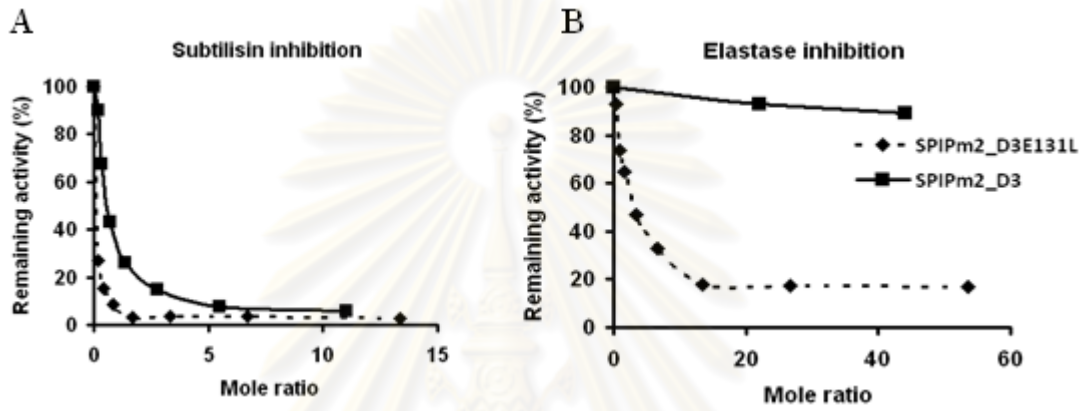

Figure 3.14 Inhibition assays of domain 3 of SPI*Pm*2 and its mutant against subtilisin (A) and elastase (B). All SPIs were tested against proteinases at various mole ratio of inhibitor to protease. Symbols ■ and ♦ were for SPI*Pm*2-D3 and D3E131L, respectively.

CHAPTER IV

DISCUSSION

Proteinase inhibitors are used by organisms to counteract proteinases for balances in several physiological functions, as responses to invading microbes, and for the invasion of hosts (Kanost, 1999; Tian et al., 2005). Among these, serine proteinase inhibitors (SPIs) are known to be widely distributed in living organisms to perform such functions. Several types of SPI reside in the arthropod hemolymph, and their functions are believed to be inhibitors against proteinases from microorganisms and regulators of host-defense reactions involved in blood coagulation, prophenoloxidase activation or cytokine activation. A number of SPIs possess one or more Kazal inhibitory domains and are grouped as the Kazal family of SPIs. The domain of Kazal-type serine proteinase inhibitors is a typical cysteine-rich domain, which is similar to the bovine pancreatic secretory trypsin inhibitor (PSTI) (Kazal et al., 1948). The inhibitory domain is made up of 50 to 60 amino acid residues with six-conserved cysteine residues forming three intra-domain disulfide bridges, which stabilize the domain (Bode et al., 1992). The Kazal domain inhibits a proteinase in a substrate-like manner; it binds tightly to the active site of the proteinase. The inhibitory specificity of a domain is mainly dictated by the reactive P1 amino acid, which varies widely among different Kazal-type SPIs (Laskowski et al., 1980). According to the structural studies, they reveal several contact positions responsible for the interaction between Kazal domains and the proteinases. The side chains of the P1 amino acid residues lie neatly into the S1 cavities of the proteinases (van de Locht et al., 1995; Read et al., 1983; Di Marco et al., 1997). There are twelve contact positions: P6, P5, P4, P3, P2, P1, P1′, P2′, P3′, P14′, P15′ and P18′, responsible for the interactions between Kazal domains and the serine proteases. This means that although the inhibitory specificity is determined mainly by the P1 amino acid residue, amino acid residues in other contact positions influence the potency of the binding as well as specificity of a serine proteinase inhibitor to its cognate proteinase.

Recently, a five-domain Kazal-type serine proteinase inhibitor, SPI*Pm*2, identified from the hemocyte EST libraries of *P. monodon* and its proteinase inhibitory activities was reported (Somprasong et al., 2006; Tassanakajon et al., 2006). The SPI*Pm*2 strongly inhibits subtilisin and elastase probably with the ratio of inhibitor to proteinase 1:2 and 1:1, respectively. It also weakly inhibits trypsin (Somprasong et al., 2006). The reactive P1 inhibitory specificity residues in domains 1-5 are Thr, Ala, Glu, Lys and Glu, respectively. It is known that the inhibitory activities can be predicted from the P1 residue. The SPIs with basic amino acids at P1 position preferentially inhibit trypsin. A heat-tolerant Kazal inhibitor with the P1 Lys residue from the marine snail *Cenchritis muricatus* inhibits strongly both trypsin and human neutrophil elastase (González et al., 2007a). The SPIs with P1 bulky hydrophobic residues tend to inhibit chymotrypsin like the Leu residue(s) in the four-domain Kazal inhibitor from the crayfish *Pacifastacus leniusculus*. The P1 Gln residue in this crayfish inhibitor is supposedly responsible for subtilisin inhibition (Johansson et al., 1994). The three-domain Kazal inhibitor from *Bombyx mori* shows strong inhibition against subtilisin but not thrombin or chymotrypsin (Zheng et al., 2007); its P1 are Thr, Ala and Gln. The 'nonclassical' Kazal-type elastase inhibitor with the P1 Met residue from *Anemonia sulcata* inhibits strongly the porcine pancreatic elastase and moderately inhibits human leukocyte elastase (Hemmi et al., 2005).

From the above information on P1 residue, it is tempting to speculate that the two Glu residues in SPI*Pm*2 are responsible for the inhibition of subtilisin, Thr or Ala residue for elastase and the Lys residues for trypsin. This speculation is also supported well with the work on ovomucoid third domain by Lu et al. (Lu et al., 1997). However, the SPIs with P1 Thr residue from *Bombyx mori* and *Galleria mellonella* turn out to be subtilisin inhibitors (Nirmala et al., 2001a; Nirmala et al., 2001b). It turns out that the inhibitory specificities can not be assigned to all Kazal SPI domain of the SPI*Pm*2. It is then interesting to find out the inhibitory specificity of each Kazal domain.

In this study, SPI*Pm*2 was mutagenized at P1 residue on the domain1, 2 and 3 from Thr 35, Ala 83 and Glu 131 to Leu, Trp and Leu, respectively. Site-directed mutagenesis was carried out to mutate the SPI*Pm*2 gene using the mutagenic primers.

Addition or elimination sites of restriction enzyme were for the purpose of screening for the mutant clones (Figure 3.2 and Figure 3.3). The results indicated that the mutant clones had the bands of expected size after digesting with the restriction enzymes. The mutated SPI*Pm*2 clones were obtained and the DNA sequences of the mutated regions were proved by DNA sequencing (Figure 3.4). This result showed the correct alteration as expected. The mutated genes were subcloned into the expression vector pET-22b(+) and the mutant SPI*Pm*2 proteins were over-expression in *E.coli* system. Three recombinant mutated proteins (rT35L, rA83W and rE131L) along with the wild type were expressed as inclusion bodies that were solubilized before purification by using nickel-NTA column.

The purified wild type and mutated SPI*Pm2* proteins were assayed for their inhibitory activities. We found that only A83W, the mutated SPI*Pm*2 at domain 2, was inactive against elastase. It suggested that this domain contributed to the elastase inhibition. The P1 Ala residue is predicted to be the most specific for porcine pancreatic elastase by the Laskowski algorithm (Lu et al., 2001). The inhibitory activities of T35L and E131L against trypsin slightly decreased comparing with the wild type. According to the recent study of SPI*Pm*2 (Donsudsa et al., 2009), they reported that the SPI*Pm*2 domain 1 was inactive against trypsin, the domain 3 was active against subtilisin and only the domain 4 with P1 Lys residue weakly inhibited trypsin. Thus, we reasoned that mutation at domains 1 and 3 did not affect to the inhibitory activity of T35L and E131L against trypsin and the result was not significant. Beside, all mutants had no inhibitory activity against chymotrypsin like the wild type even through the P1 residues were replaced with the bulky hydrophobic residues that tend to inhibit chymotrypsin like Leu residues in the Kazal inhibitor from the crayfish *P. leniusculus*. It might be that the amino acid residues in other contact positions of domain 1, 2 and 3 were not suitable for the binding between the reactive site loop of these domains and the active site of chymotrypsin.

From the result of subtilisin inhibition assay, it demonstrated that the inhibitory activity against subtilisin of all mutants were similar to the wild type. It might be that more than one domain could inhibit subtilisin. In order to prove the effect of mutation at P1 residue, the mutated domain 3 was separately amplified and cloned into expression vector pVR500. The mutated domain 3, called D3E131L, was over-

expressed and purified for the proteinase inhibition assay. We found that the mutated domain 3 had the strong inhibitory activity against subtilisin. This finding agrees with some previous reports, such as the domain 2 with P1 Leu residue of the 3-domain Kazal inhibitor from *Procambarus clarkii* (Li et al., 2009b). It was possible that the P1 Leu residue of this mutated domain 3 contributed to subtilisin inhibition of the intact E131L, that caused its inhibitory activity towards subtilisin did not differ from the wild type. Surprisingly, this mutant showed the new inhibitory activity against elastase. The result was corresponding to the study of a four-domain Kazal SPI TgPI-1 from an obligate intracellular parasite of human *T. gondii* (Morris et al., 2002). The inhibitor with its P1 Arg, Arg, Leu and Leu was able to inhibit trypsin, chymotrypsin, porcine pancreatic elastase, and neutrophil elastase. Therefore, domain1 with the P1 Leu residue of infestin from *Triatoma infestans* was active against chymotrypsin, porcine pancreatic elastase and subtilisin (Campos et al., 2002; Lovato et al., 2006; Campos et al., 2004; Rimphanitchayakit and Tassanakajon, 2010).

From the Expressed Sequence Tags (ESTs) analysis of *P. monodon* haemocytes, six cDNA clones were demonstrated as putative rerine proteinase inhibitors by using BLASTX program (Supongul et al., 2002). They showed 50-58% homology to the 4-Kazal-domain serine proteinase inhibitor (PAPI-1) of crayfish *P. leniusculus* (Johansson et al., 1994). It therefore seems probable that at least in *P. leniusculus* the PAPI-1 group is aimed at preventing microbial proteinases facilitating pathogen establishment (Häll and Söderhäll, 1982, Dieguez-Uribeondo and Cerenius, 1998, Christeller, 2005). The open reading frames (ORF) of the clones were identified using a Genetyx program. Four full-length cDNA clones were identified. In this study, the SPI*Pm*1 clone, HC-N-S01-0010-LF, is of interesting for it contains four complete Kazal domains with diverse reactive P1 residues. This clone consists of an open reading frame of 699 base pairs that encoded polypeptides of 222 amino acid residues with putative signal peptides of 24 amino acid residues and mature proteins of 198 amino acid residues as shown in Figure 4.1.

MLLCKITLIHLLLQGFAVFNDANS♦DHD
CIGYCP*E*VYDPVCASNGWTYNNDCELQAMIKCQGWNITKTHDQACE
CLKACP*T*TFAPVCGSDNKTYLNEC-VFEVASCWDHSLDKASEGACGWGIH
CLQYCP*E*VYDPVCGSNGQTYTNECELQAAIQCRGLQIAKRHDQACE
CHATCP*L*IHDPVCGTDDRTYYNEC-FFTKASCWDRSILKKKNGPCDRKWKYLLEI

Figure 4.1 Amino acid sequences of the SPI*Pm*1 from the *Penaeus monodon* EST database. The conserved cysteine residues are shaded. Italicized and shaded residues are the reactive P1 residues. Diamond indicates the potential signal peptide cleavage site. (Visetnan et al., 2009)

The reactive P1 inhibitory specificity residues in domains 1-4 of SPI*Pm*1 are Glu, Thr, Glu and Leu, respectively. It is known that the inhibitory activities can be predicted from the P1 residue. From the above information on P1 residue, it is tempting to speculate that the two Glu residues in SPI*Pm*1 are responsible for the inhibition of subtilisin, Thr residue for elastase and/or subtilisin and the Leu residues for chymotrypsin (Lu et al., 1997; Nirmala et al., 2001a; Nirmala et al., 2001b. In this study, by using a different *E. coli* expression system, pET-28b(+) and the *E. coli* strain Rosetta(DE3)pLysS, the SPI*Pm*1 was successfully expressed. Upon IPTG induction, an approximate 23 kDa rSPI*Pm*1 was expressed as a major entity in the inclusion bodies. We had used a buffered 4 M urea solution, a denaturing condition, to solubilize the inclusion bodies. The rSPI*Pm*1 was purified by using a Ni-NTA column. The inhibitor was then renatured by dialysis against the Tris-HCl buffer, pH 8. The purified recombinant SPI*Pm*1 proteins from nickel-NTA agarose column were tested for serine proteinase inhibitory activity. The recombinant inhibitors were assayed for their inhibitory activities against subtilisin A, elastase, trypsin and chymotrypsin. We found that SPI*Pm*1 showed the weak inhibition against all proteinases when the mole ratio of inhibitor against proteinases was 80 : 1. The poor inhibitory activity of the five-domain Kazal-type SPI (SPI*Pm*2) was found when SPI*Pm*2 was purified under denaturing condition (Somprasong et al., 2006). Recently, hcPcSPI2 was over-expressed and characterized. It was found that hcPcSPI2 purified under denaturing condition weakly inhibited subtilisin A and trypsin (Li et al., 2010).The results were suggested that the recombinant SPI folding under this condition was not fully active or only certain amount of the SPI protein was correctly folded. It might be due to the presence of several cysteine residues in this protein

rendering the polypeptide more difficult to refold correctly. The expression of SPI*Pm*1 under other conditions such as expression using other vectors and expression in eukaryotic system probably offers a better alternative to obtain the active inhibitor for future characterization.

CHAPTER V
CONCLUSIONS

1. The SPI*Pm*2 gene was successfully mutagenized at the P1 residues of domain 1 2 and 3.
2. Only A83W, the mutated SPI*Pm*2 at P1 residue of domain 2, was inactive against elastase. Thus, the domain 2 contributed to the elastase inhibition.
3. Mutagenesis of the P1 residue of the domain 3 of SPI*Pm*2 significantly altered the inhibitory activity against elastase.
4. The SPI*Pm*1 from the black tiger shrimp was successfully expressed in the *E. coli* strain Rosetta (DE3) pLysS expression system.
5. The rSPI*Pm*1 has a molecular mass of approximately 23 kDa as estimated by SDS-PAGE.
6. The rSPI*Pm*1 was purified by nickel-NTA column under the denaturing condition. The protein had low inhibitory activity against proteinases.

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APPENDICES

Appendix A

1. Preparation for SDS-PAGE electrophoresis

Stock reagents

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

Acrylamide 29.2 g

N,N′-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

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

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8 75 ml

10% SDS 4 ml

Distilled water 21 ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8 50 ml

10% SDS 4 ml

Distilled water 46 ml 118

SDS-PAGE

15 % Seperating gel

30 % Acrylamideml solution 5.0 ml

Solution B 2.5 ml

Distilled water 2.5 ml

10% (NH4)2S2O8 50 µl

TEMED 10 µl

5.0 % Stacking gel

30 % Acrylamideml solution 0.67 ml

Solution C 1.0 ml

Distilled water 2.3 ml

10 % (NH4)2S2O8 30 µl

TEMED 5.0 µl

5X Sample buffer

1 M Tris-HCl pH 6.8 0.6 ml

50% Glycerol 5.0 ml

10% SDS 2.0 ml

2-Mercaptoethanol 0.5 ml

1 % Bromophenol blue 1.0 ml

Distilled water 0.9 ml

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane 3.03 g

Glycine 14.40 g

SDS 1.0 g

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

Appendix B

การแสดงออกและลักษณะสมบัติของตัวยับยั้งซีรีนโปรตีเนส SPI*Pm*2 ที่กลายพันธุ์ของกุ้งกุลาดำ

EXPRESSION AND CHARACTERIZATION OF MUTATED SERINE PROTEINASE INHIBITOR SPI*Pm*2 OF THE BLACK TIGER SHRIMP *Penaeus monodon*

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บทคัดย่อ: ได้ค้นพบตัวยับยั้งซีรีนโปรตีเนสชนิดคาซอลแบบ 5 โดเมน ชื่อ SPI*Pm*2 ในห้องสมุด cDNA ของเม็ดเลือดกุ้งกุลาดำ *Peneaus monodon* ในงานวิจัยที่ผ่านมา ได้ศึกษาลักษณะสมบัติ และพบว่า มีฤทธิ์ยับยั้งซับทิลิซินและอีลาสเทสได้ดี และยับยั้งทริปซินได้เล็กน้อย เพื่อให้ทราบถึงฤทธิ์ยับยั้งโปรตีเนสของโดเมน งานวิจัยนี้จึงได้ทำการกลายพันธุ์ยีนอย่างจำเพาะ โดยเปลี่ยนแปลงกรดอะมิโน P1 ของโดเมนที่ 1, 2 และ 3 จากทรีโอนีนเป็นลิวซีน (T75L) แอลานีนเป็นทริปโทเฟน (A243W) และกลูทาเมตเป็นลิวซีน (E371L) ตามลำดับ แล้วผลิตโปรตีนของ wild type, T75L และ E371L และศึกษาฤทธิ์ยับยั้งโปรตีเนส พบว่า T75L และ E371L ยับยั้งอีลาสเทสและทริปซินได้น้อยกว่า wild type มีเพียง T75L เท่านั้นที่ยับยั้งซับทิลิซินได้น้อยกว่า wild type เช่นเดียวกับ wild type โปรตีนกลายพันธุ์ทั้งสองตัวไม่สามารถยับยั้งไคโมทริปซิน

Abstract: The five-domain Kazal-type serine proteinase inhibitor SPI*Pm*2 was identified from the hemocyte cDNA library of the black tiger shrimp *Peneaus monodon*. In previous study, it was characterized and shown that it strongly inhibited subtilisin and elastase, and weakly inhibited trypsin. In this study, the gene was mutated by site-directed mutagenesis in order to delineate the domain inhibitory activities. The P1 amino acid residues of domains 1, 2 and 3 were changed from Thr to Leu (T75L), Ala to Trp (A243W) and Glu to Leu (E371L), respectively. The wild-type, T75L and E371L proteins were produced and their inhibitory activities were determined. It was found that the inhibitory activities of T75L and E371L against elastase and trypsin were lower than those of wild type. Only T75L showed lower activity against subtilisin than the wild type. Like wild type, both mutants could not inhibit chymotrypsin.

Introduction: Serine proteinase inhibitors (SPIs) are found widely in multicellular organisms and function as regulators of proteinase activities involving in many biological processes, such as protection against microbial proteinases, prophenol oxidase activation, hemolymph coagulation and metamorphosis [1]. The Kazal-type SPIs are well-known among many families of SPIs. Usually, the inhibitors contain multiple domains of Kazal, each with six

conserved cysteine residues forming three intra-domain disulfide linkages and a highly variable P1 amino acid residue in the reactive loop. In black tiger shrimp, 9 Kazal-type SPIs were identified from the hemocyte cDNA library [(Visetnan, Donpudsa et al. 2009)2]. A SPI*Pm*2 is interesting for it contains five Kazal domains. It has been shown that the SPI*Pm*2 strongly inhibits subtilisin and elastase, weakly inhibits trypsin and has no activity on chymotrypsin [3].

To gain further information on the domain inhibitory specificity, the SPI*Pm*2 gene was specifically mutated at the P1 residues of domains 1, 2 and 3. The mutant proteins were over-expressed and their inhibitory activities tested comparing with the wild type protein.

Methodology: Site-directed mutagenesis was carried out to mutate the SPI*Pm*2 gene in a plasmid pUC119 using specific mutagenic primers for domains 1, 2 and 3. The mutagenic primers were 5′ GCCAAACACTGTACGTTGATATCCCCTGTGTGTGGC 3′, 5′ GAGAT GTCCAGGAATCTGTCCATGGGTGTACGCACCTGTGTGCGGGACC-3′ and 5′ ATTG TCGCGTGCCCTCTCATCTATGCTCCCGTG 3′ for domains 1, 2 and 3, respectively. The mutated nucleotides are underlined. The mutant genes were subcloned into an expression vector pET-22b(+). The wild-type, T75L and E371L proteins were over-expressed as inclusion bodies in *E. coli* Rosetta(DE3)pLysS by IPTG induction at 1 mM. The inclusion bodies were solubilized with 50 mM sodium phosphate, pH 12, and dialyzed in 50 mM sodium carbonate, pH 10. The solubulized proteins were further purified using the Ni-NTA agarose column and eluted with an elution buffer containing 300 mM imidazole. The eluted proteins were then dialyzed to remove imidazole in 50 mM sodium carbonate, pH 10. The proteins were analyzed by SDS-PAGE. The inhibitory activities of the wild type and 2 mutant proteins were assayed against subtilisin, elastase, trypsin and chymotrypsin using a procedure of Hergenhahn et al [4]. The percentages of remaining activity were calculated and plotted against the molar ratios of inhibitor to proteinase.

Results, Discussion and Conclusion: The mutant SPI*Pm*2 genes (T75L, A243W and E371L) were constructed using site-directed mutagenesis. The mutant genes were sequenced to confirm the correct alteration. The mutated gene segments were shown along with the wild type gene (Fig. 1).

(A)

```
Wild type       GGATCCGGGCTACGGAAAAGGGGGGAAAATCCGCCTCTGCGCCAAACACTGTACGACCAT 60
T75L            GGATCCGGGCTACGGAAAAGGGGGGAAAATCCGCCTCTGCGCCAAACACTGTACGTTGAT 60
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(B)

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Wild type       ATGTCCAGGAATATGCCCCGCGGTATATGCCCCTGTGTGCGGGACCAACGGGAAAACTTA 240
E243W           ATGTCCAGGAATCTGTCCATGGGTGTACGCACCTGTGTGCGGGACCAACGGGAAAACTTA 240
                ************ ** **   *** ** ** ****************************
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(C)

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Wild type       GCACGATGGACGTTGTGGTTGCAACCCCATTGTCGCGTGCCCTGAGATCTATGCTCCCGT 360
E371L           GCACGATGGACGTTGTGGTTGCAACCCCATTGTCGCGTGCCCTCTCATCTATGCTCCCGT 360
                ****************************************   **************
```

Figure 1 The nucleotide segments of the mutant genes T75L (A), E243W (B) and E371L (C) are shown against the wild type SPI*Pm*2 gene.

The wild type, T75L and E371 proteins were over-produced in *E.* coli Rosetta(DE3)pLysS expression system. The proteins were purified using Ni-NTA column and checked by SDS-PAGE (Fig. 2).

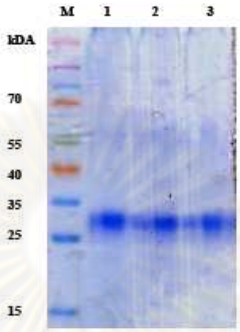

Figure 2 SDS-PAGE analyses of the purified mutant SPI*Pm*2 proteins. A 15% polyacrylamide gel was used. Lane M is the size maker. Lanes 1-3 are wild type, T75L and E371L, respectively.

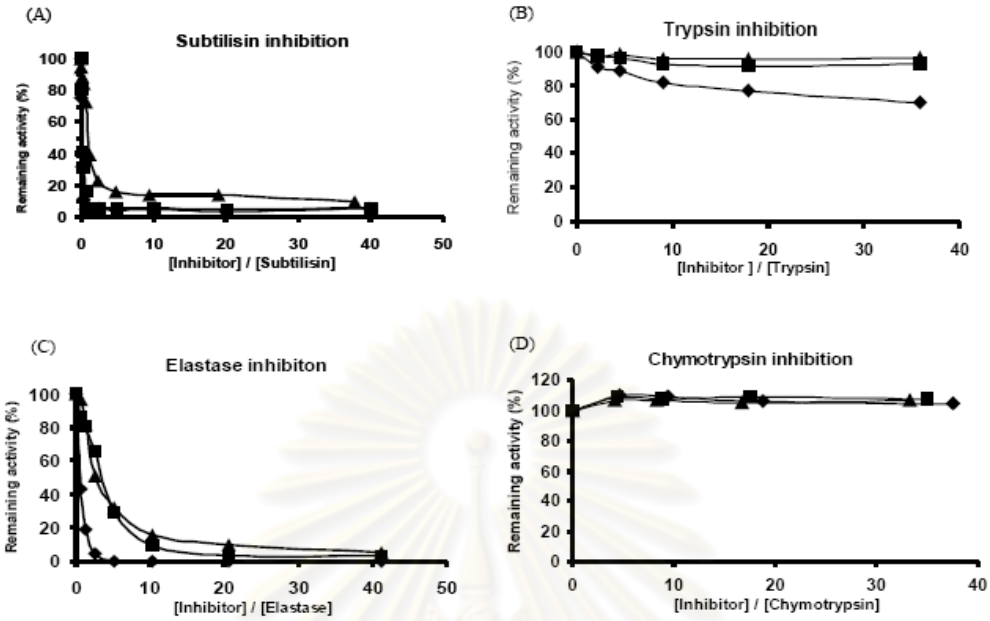

Figure 3 Inhibitory activity assay of wild type (◆), T75L (▲) and E371L (■) against subtilisin (A), trypsin (B), elastase (C) and chymotrypsin (D).

The inhibitory activities of the proteins towards subtilisin, elastase, trypsin and chymotrypsin were assayed (Fig. 3). The results indicated that T75L inhibited subtilisin, elastase and trypsin less than the wild type while the E371L inhibited elastase and trypsin less than the wild type. Both mutant proteins had no inhibitory activity against chymotrypsin like the wild type.

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Keywords: Kazal-type serine proteinase inhibitor, site directed mutagenesis, black tiger shrimp, *Penaeus monodon*

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