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Expression and characterization of a novel plaminogen activator

from green pit viper (*Cryptelytrops albrolabris*)

Miss Wannaporn Phankham

A Thesis Submitted in Partial Fulfillment of Requirements

for the Degree of Master of Science Program in Medical Science

Faculty of Medicine

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พิษงูเขียวหางไหม้ท้องเหลือง (คลิปเทไลทรอปส์ อัลโบลาบรีส) ประกอบด้วยโปรตีนหลายชนิด ที่มีผลต่อระบบการแข็งตัวของเลือด ซีรีนโปรตีเอสเป็นกลุ่มของโปรตีนที่สำคัญชนิดหนึ่ง ที่พบได้ในพิษงูชนิดนี้ การศึกษานี้เราได้โคลนยีนซีรีนโปรตีเอสตัวใหม่จากห้องสมุดยีนของต่อมพิษงู จากการวิเคราะห์ลำดับกรดอมิโน จากการวิเคราะห์ลำดับกรดอมิโน คาดว่าโปรตีนน่าจะมีคุณสมบัติเป็นตัวกระตุ้นพลาสมิโนเจน จากนั้นยีนได้ถูกโคลนโดยใช้ pPICZαA เป็นพาหะ และ แสดงออกโปรตีนที่ติดฉลากด้วยฮิสทิดีนในยีสต์ Pichia pastoris พบว่าการเหนี่ยวนำให้สร้างโปรตีนโดยใช้เมทานอลความเข้มข้นร้อยละ 1.0 โดยปริมาตรทำให้ยีสต์สามารถสร้างโปรตีนนี้ได้ปริมาณมากกว่าใช้เมธานอลความเข้มข้นร้อยละ 0.5 และเมื่อใช้แอนติบอดีต่อฮิสทิดีนบน Western blots พบว่าโปรตีนที่แสดงออกมีขนาดประมาณ 37 กิโลดาลตัน และการตรวจคุณสมบัติพบว่าโปรตีนมีฤทธิ์ในการกระตุ้นพลาสมิโนเจนเป็นพลาสมินจริง แต่ไม่มีผลต่อการทำงานของเกล็ดเลือด โปรตีนนี้อาจสามารถพัฒนานำมาใช้เป็นยาละลายลิ่มเลือดเพื่อการรักษาโรคในอนาคต

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Snake venome is a mixture of several proteins affecting hematostatic system. Serine protease is one of the important protein families found in Green pit viper (GPV, *Cryptelytrops allbolabris*) venom. In this study, a novel serine protease gene was cloned from the GPV venom gland cDNA library. Amino acid sequence analysis predicted that it was a plasminogen activator. The serine protease cDNA was cloned in the pPICZαA vector and, then, expressed in the *Pichia pastoris* system. The histidine-tagged recombinant protein production was higher after the induction using 1.0% (v/v), compared with 0.5%, of methanol. This system was able to produce the 37 kDa protein on a Western blot probed by anti polyhistidine antibody. *In vitro* activities of the protein was tested and found that it contained a plasminogen activator activity. However, there was no effect on platelet aggregarion. This protein has a potential to be a therapeutic thrombolytic agent in the future.

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LIST OF ABBREVIATIONS

ABUS-PA	*Agkistrodon blomhoffii Ussurensis* – plasminogen activator
ADAMs	a disintegrin and metalloproteinase
ATP	adenosine triphosphate
APSAC	acylated plasminogen-streptokinase activator complex
Arg	arginine
bp	base pair
BCA	bicinchoninic acid
BGM	bio geno med
BJ-48	*Bothrops jararacussu*-48
$^{\circ}$C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CRD	calcium regulation domain
Cys	cysteine
DNA	deoxyribonucleic acid
DSPA	*Desmodus rotundus* plasminogen activator
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra acetic acid
FDP	fibrin-fibrinogen degradation product
g	gram
GPV-PA	green pit viper plasminogen activator
GSPs	gene specific primers
HPLC	high performance liquid chromatography
HD	histidine-aspartate
HK	histidine-lysine

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IC_{50}	concentration of inhibitor required to inhibit 50 % aggregation
IPTG	isopropy1-β-D-thiogalactopyranoside
kDa	kiloDalton
L	Liter
LAAO	L-amino acid oxidase
LB	Luria-Bertani media
LV-Ka	*Lachesis muta muta* venom kallikrein-like
mg	milligram
ml	milliliter
mM	millimolar
M	molar
nM	nanomolar
N	normal
OD	optical density
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PLA_2	phospholipase A_2
PPP	platelet poor plasma
pro-UK	prourokinase
PVDF	polyvinylidine difluoride
RACE	rapid amplification of cDNA end
RGD	Arg-Gly-Asp
rt-PA	recombinant tissue type plasminogen activator
SAK	Staphylokinase
scu-PA	single chain urokinase-type plasminogen activator
SDS	sodiumdodecylsulphate

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SDS-PAGE	sodiumdodesylsulphate polyacrylamide gel electrophoresis
SK	streptokinase
SVTLEs	snake venom thrombin-like enzyme
SVMPs	snake venom metalloproteinases
SVSPs	snake venom serine proteinases
tcu-PA	two-chain derivative urokinase-type plasminogen activator
t-PA	tissue-type plasminogen activator
Tris-HCl	tris-(hydroxymethyl)-aminoethane
TSV-PA	*Trimeresurus stejnegeri* snake venom plasminogen activator
u-PA	urokinase-type plasminogen activator
Val	valine
YPDS	yeast extract peptone dextrose medium
v/v	volume/volume
w/v	weight/volume
α2-AP	α2 antiplasmin
μg	microgram
μl	microliter
μm	micrometer

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CHAPTER I

INTRODUCTION

1.1 Background and Rationale

1.1.1 Green pit viper venom

Venomous snakes have been classified to 5 majors families. Venom toxins from the Hydrophiladae family mainly affect skeletal muscle, while venom toxins from the Colubridae family have not been well characterized. Venom from the Elapidae family affects neurological system and venom toxins from the Crotalidae family and Viperidae families interfere with the hemostatic system (1).

The snake venom toxins affecting hemostasis have been sub-classified according to their overall effects: coagulants (thrombin-like enzymes and prothrombin activating toxins), anticoagulants (toxins activating protein C etc.), platelet-activating proteins (C-type lectin-like proteins) and anti-platelet agents (disintegrins, a group of RGD-containing proteins), fibrinolytic activators and hemorhagins (snake venom metalloproteases that directly degrade blood vessel walls).

Green pit viper venoms contain major effects on the hematological system. In Thailand, green pit viper species that are most commonly found are *Cryptelytrops albolabris* and *Cryptelytrops macrops*. They account for 40% of all venomous snakebites in Thailand and are responsible for almost all bites in the Bangkok and nearby areas (2). There was a report on a group of patients who had been bitten by green pit viper (*C. allbolabris and C. macrops*). The study found that fribinolytic system activation was very common, as indicated by low plasminogen, low antiplasmin, and elevated fibrin-fibrinogen degradation product (FDP) levels. In addition, the significant decreases in total platelet counts and in mean platelet volume (MPV) were demonstrated in envenomated patients (3).

1.1.2 Components of green pit viper

Proteins comprise approximately 90% of pit viper venoms. They are categorized according to the protein families into serine proteinases, phospholipase A_2 (4), C-type lectin-like proteins (5), snake venom metalloproteinses, and disintregrins (6,7,8).

Serine protease is one type of snake venom proteases and also be found in dung beetles (*Catharsius molossus*) (9) and centipeds (*Scolopendra subspinipes mutilans*) (10), as well as blue green algae (*Spirulina fusiformis*)(11).

Serine proteases from snake venoms affect mainly hematostatic system interfering with prey hemostasis by specific cleavages of the factors involved in blood coagulation, fibrinolysis and the kallikrein–kinin systems. For example, thrombin-like serine proteases (SVTLEs) that are able to cleave fibrinogen (factor I) to fibrin clots by releasing fibrinopeptide A from the Aα chain or fibrinopeptide B from Bβ chain or both fibrinopeptides from fibrinogen(12,13). However, the activation of factor XIII to cross-link fibrin clot is weak. These proteins are termed 'thrombin-like' because the thrombin possesses many more functions than those of these SVTLEs on fibrinogen. In addition to the action on fibrinogen, thrombin is also involved in the stimulation of blood coagulation by activating coagulation factor V, VIII and XIII. Its complex with thrombomodulin on endothelial cells can, in turn, activate protein C to inhibit blood coagulation by inactivating the activated forms of factor V and VIII as a negative feedback mechanism. Furthermore, thrombin also inhibits fibrinolysis and activates platelet aggregation(14).

There are also other serine proteases that are isolated, structurally characterized and found to contain unique activities. Kallikrein-like serine proteases are able to cleave of the Bβ chain at Arg42 and slowly degrade the Aα chain of fibrinogen resulting in the inhibition of normal fibrinogen clotting(15,16). Furthermore, some serine proteases may directly digest fibrinogen by limited proteolysis and they are

called fibrinogenase. TSV-PA (plasminogen activator) from the *Trimeresurus stejnegeri* has been demonstrated to have a fibrinolytic activity(17). The venom from this species is closely homologous to green pit viper venom. Therefore, fibrinolytic agents from green pit viper venom are still waiting for characterization and this protease may be developed to be a novel thrombolytic agent in the future.

Phospholipase A_2 (PLA_2) is a non-glycosylated protein that can be found in snake venoms. The snake venom PLA_2 exhibits a wide variety of pharmacological effects including neurotoxicity, myotoxicity, and anticoagulant activities. Snake venom PLA_2 are classified into 2 groups based on their amino acid sequences and their disulfide bond patterns. The first group is the HD (histidine-aspartate) PLA_2 that contains enzymatic activity and the other is HK (histidine-lysine) PLA_2 that has no phospholipase activity and exerts their functions through protein-protein interaction mechanisms.

Snake venom C-type lectin-like compounds show amino acid sequence homology to the calcium regulation domain (CRD) of mammalian lectins. Proteins in this family contain disulfide-linked $\alpha\beta$ heterodimers. C-type lectin-like proteins promote platelet aggregation by targeting von Willebrand factor, platelet glycoprotein Ib-IX-V, platelet glycoprotein VI and probably other platelet receptors.

Snake venom metalloproteinases (SVMPs) are multi-domain proteins that compose of a catalytic domain and one or several non-catalytic domains. These proteins have a molecular mass of 20 to 100 kDa comprising a signal peptide, a pro-sequence, a metalloproteinase domain, a disintegrin-like or disintegrin domain with or without a cysteine-rich carboxyl terminus. SVMPs are homologous to mammalian proteins in a disintegrin and metalloproteinase (ADAMs) family. However, ADAMs proteins have other domains besides those of SVMPs that are an epidermal disintegrin-like domain, a transmembrane domain and a cytoplasmic

domain. The metalloprotenase domain of SVMPs contains a zinc-binding consensus sequence, HEXXHXXGXXH, which makes it belongs to the metzincins family of zinc-dependent metalloproteinase. Chelation of the Zn^{2+} ion with EDTA or 1, 10-phenanthroline abolishes its proteolytic and hemorrhagic activities(18).

1.1.3 Plasminogen activator effects

Plasminogen activators are fibrinolytic enzymes that cleave plasminogen into plasmin(19,20). We can divide plasminogen activators into 2 groups.

The first group is composed of endogenous activators: tissue–type plasminogen activator (t-PA), the principle endogenous activator of plasminogen in blood that can convert plasminogen to plasmin(21) and urokinase – type plasminogen activator (u-PA) that is mainly produced in the kidney. It can be in the form of a single-chain molecule (single chain u-PA, scu-PA) or as an active two-chain derivative (tcu-PA, urokinase) generated by specific cleavage of the Lys'58-Ile'59 peptide bond by plasmin(22).

The other group of plaminogen activator is exogenous activators, such as Streptokinase (SK) that is derived from Streptococcal bacteria, Staphylokinase (SAK) that is derived from *Staphylococcus aureus*, etc(23).

The endogenous plasminogen activator (PA) – plasmin system is responsible for maintenance of hemostasis and vascular patency through the degradation of fibrin(24). Plasmin is regulated by tissue-type plasminogen activator (t-PA), urokinase–type plasminogen activator (u-PA) and plasminogen activator inhibitor–1 (PAI-1). In the presence of fibrin, tPA converts the proenzyme plasminogen within the thrombus into its active form, plasmin. PAI-1 regulates plasminogen activation by inhibiting free tPA and forming an enzymatically inactive tPA/ PAI-1 complex, which result in a loss of plasminogen activation potential and thereby a decrease level

of proteolytic and fibrinolytic activity. Fig 1 displays an overview of the endogenous fibrinolytic system.

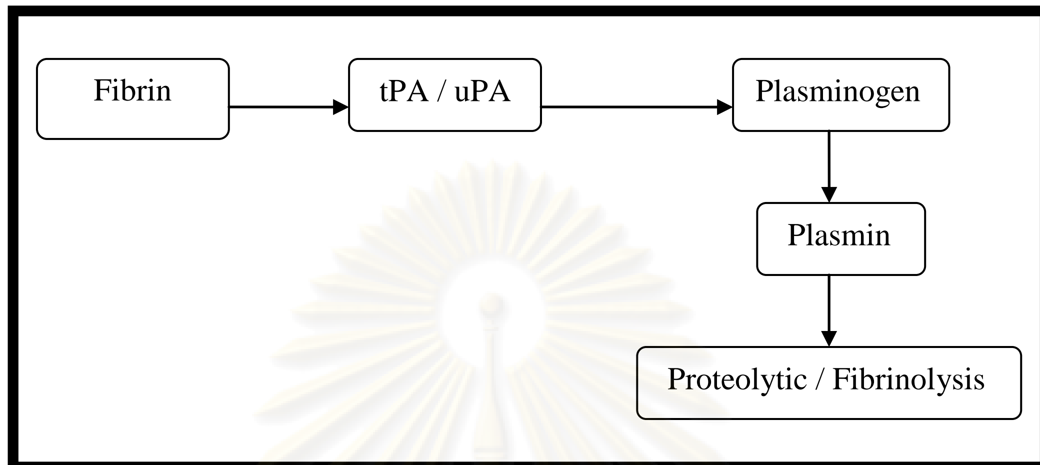

Fig 1. An overview of the fibrinolytic system. *tPA,* Tissue plasminogen activator; *uPA*, urokinase-type plasminogen activator.

The imbalance of hemostatic system causes blood clots in the circulatory system yielding severe outcomes to the patients e.g. stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction(25). These thromboembolic disorders usually require clinical interventions including an intravenous administration of thrombolytic agents. Several plasminogen activators, recombinant tissue type-PA (rt-PA), streptokinase (SK) and urokinase type-PA (u-PA), activate free plasminogen and fibrin-bound plasminogen within the thrombus to be the active plasmin The wide spread systemic activation of the fibrinolytic systems leads to the depletion of α2- antiplasmin (α2-AP)(26). In addition, generation of free plasmin results in degradation of several plasma proteins, for example, fibrinogen, factor V and factor VIII.

We have previously cloned a serine protease that contained high homology to plasminogen activators. In this study, we are interested in sequence analysis, recombinant expression and characterization of the activities of a novel serine protease

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from *C. albrolabris* using methylotropic yeast *Pichia pastoris*. This study will give us deeper insights in pathogenesis of viper bite and may yield a potentially useful thrombolytic agent in the future.

1.2 Research Questions

Does the recombinant snake venom serine protease from Green pit viper (*Cryptelytrops albolabris*) GPV-PA contain the plasminogen activator activity ?

1.3 Hypothesis

The recombinant snake venom serine protease from Green pit viper (*Cryptelytrops albolabris*) is a fibrinolytic enzyme.

1.4 Objectives

1. To analyze the amino acid sequence of GPV-PA
2. To express and purify the snake venom serine protease, GPV–PA, in *Pichia pastoris* system.
3. To study the effects of snake venom serine protease on plasminogen, and platelets.

1.5 Key Words

Serine Protease

Plasminogen activator

Green Pit Viper

Pichia pastroris

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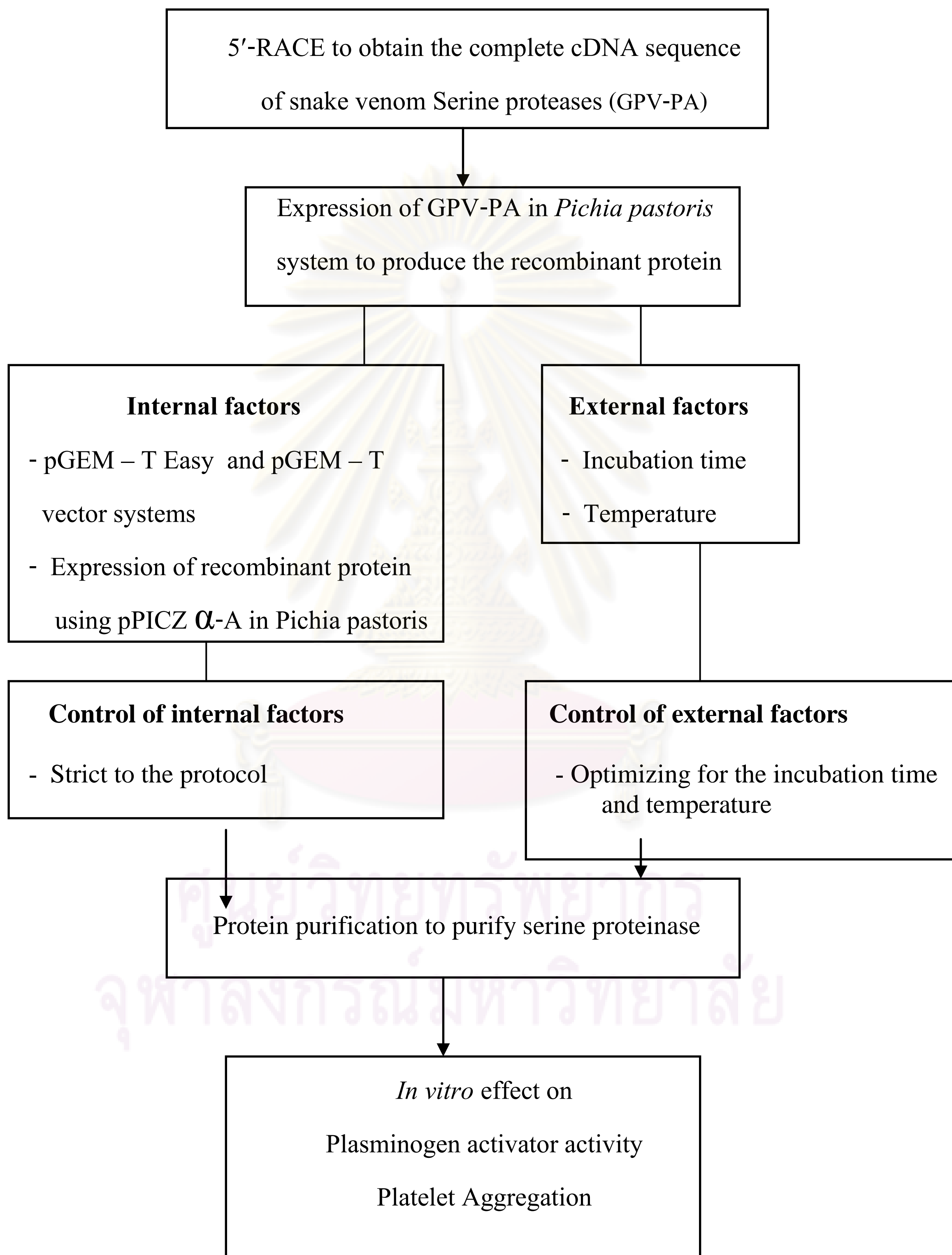
1.6. Conceptual Framework



1.7 Benefits and Applications

1. The study will give us deeper insights in the structure-function relationship of the snake venom serine protease protein, GPV-PA, and the molecular pathogenesis of green pit viper envenomation.
2. This protein is potentially useful as a novel thrombolytic agent.

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CHAPTER II

LITERATURE REVIEW

This thesis studied the expressions and effects of the recombinant snake venom serine protease with fibrinolytic activity from green pit viper (*Cryptelytrops albolabris)*. To the author's knowledge, such study has never been reported in the literature.

Snake Venom Serine Proteases (SVSPs)

Snake venom is a complex mixture of toxins and enzymes, each of which may be responsible for one or more distinct toxic action. They are responsible for hemorrhage, shock and disorders of blood coagulation. They act by activating, inactivating or modifying hemostatic and fibrinolytic system. Because there is a major medical interest in thrombosis and hemostasis, venom proteases that affect the blood coagulation system in human have been extensively investigated and considerable number of venom components acting on hemostasis has been isolated and characterized(1).

Snake venom serine proteinases (SVSPs) are among the best-characterized venom enzymes that affect the hemostatic system. They perform actions on a variety of components of the coagulation cascade, the fibrinolytic and kallikrein – kinin systems as well as cellular components causing an imbalance of the haemostatic system of the prey. Serine proteinases are categorized in the trypsin family S1 of clan SA, the largest family of peptidases. They are present in venoms of various snake families including Viperidae, Crotalidae, Elapidae and Colubridae.

Snake venom enzymes of the serine protease family are characterized by a common catalytic mechanism that includes a highly reactive serine residue that plays a pivotal role in the formation of a transient acylenzyme complex. The complex is stabilized by the presence of histidine and aspartic acid residues within the active site. These histidine, aspartic and serine residues are, therefore, so-called the catalytic triad.

In spite of the high degree of mutual sequence identity, SVSPs are quite specific toward a given macromolecular substrate. Although they show primary substrate specificity similar to that of trypsin, their stringent macromolecular substrate specificity differs from the less specific activity of trypsin. The substrate specificity of clotting factor serine proteases, such as thrombin or factor Xa, is determined by the sequence outside the active sites, so-called exosites. Whether these exosites play important roles in SVSPs remains to be investigated.

Most SVSPs tend to be glycoproteins showing a variable number of N-or O-glycosylation sites in sequence positions that differ from one SVSP to the other. They contain twelve cysteine residues, ten of which construct five disulfide bonds. Based on the homology with trypsin, the remaining two cysteine residues form a conserved disulfide bridge that is unique among SVSPs. It involves Cys245e that found in the C-terminal extension(27).

Snake venom serine proteases (SVSPs) are among the best characterized venom enzymes affecting the hemostatic system. They act on a variety of components of the coagulation cascade including fibrinogen-clotting, fibrinolysis and kallikrein-kinin systems and on cells to cause a disturbance of the hematostatic system of the prey.(12,13)

The venom serine proteases are classified as families of proteins that specifically interact with different targets, on which they exert their physiological actions. The genes have been sequenced and the proteins have been characterized(28).

The venom serine proteinases are categorized in families of proteins which specifically interact with various targets, on which they exert their physiological action. Examples are as following:

Some of venom proteases are frequently referred to as thrombin-like enzymes (SVTLEs) due to their ability to cleave fibrinogen, releasing fibrinopeptide A, fibrinopeptide B or both. Recently, BJ-48, a SVTLE from *Bothrops jararacussu* is

characterized biochemically. It is capable of cleaving either α or β chain of fibrinogen, but shows preference for the β chain(13).

Cryptyletrop jerdonii snake is a pit viper, venom of which contains both fibrinogen-degradation and fibrinogen-clotting serine proteases. Jerdonobin-II, a novel serine protease, was cloned, purified and characterized from the venom. It shares 89 % sequence identity with TSV-PA, displays a distinct biological activity and a weak fibrinogen-clotting activity(30).

Kallikrein-like proteinase of *Lachesis muta muta* (bushmaster) venom designates LV-Ka. Approximately 77% of the protein sequence was determined by sequencing various fragments derived from endoprotease digestion. The obtained partial sequence suggests that LV-Ka is of a similar size to other serine proteinases. Sequence studies on the NH_2- terminal region of the protein indicate that LV-Ka share a high degree of sequence homology with the kallikrein-like enzyme EI and EII from *Crolalus atrox*, with crotalase from *Crotalus adamatues* and significant homology with other serine proteinases from snake venoms and other vertebrates. LV-Ka showed kallikrien-like activity, releasing bradikinin from kininogen as determined by guinea pig bioassay(31).

A recombinant serine protease named albofibrase from Green Pit Viper (*Cryptelytrops albolabris*) is predicted and shown to be a fibrinogenolytic enzyme. Albofibrase degraded fibrinogen by cleavage of peptide bonds in the Aα-chain.

The enzyme called thrombocytin, a serine protease from *Brothrops atrox* venom, is a platelet aggregation inducer, which also activates factor V. In addition, this enzyme activates factor VIII and factor XIII but very weakly.

A serine protease named scolonase was purified and characterized from tissue of a Korean centipede, *Scolopendra subspinipes mutilans.* The study demonstrated that scolonase was able to activate plasminogen to plasmin by specifically cleaving the molecular at Arg^{561} – Val^{562} peptide bond(32).

ABUS-PA from *Agkistrodon blomhoffii Ussurensis venom* has been identified and purified to homogeneity and activates as a plasminogen activator with arginine ester hydrolysis activity(33).

The Expression System

Pichia pastoris is the methylotrophic yeast that has been developed to be a highly successful system for production of a variety of heterologous proteins for both basic researches and industrial uses, e.g. L-amino acid oxidase (LAAO) from Malayan pit viper (*Calloselasma rhodostoma*)(34), vampire bat salivary plasminogen activator α2 (DSPAα2) from *Desmodus rotundus*(35). In addition, prourokinase (proUK) – annexin V chimeras have also been successfully expressed(36).

Pichia is a suitable host for the production of protein for several reasons. *Pichia pastoris* has a strong inducible promoter, *AOX1*, to induce high levels of transcription(37). Moreover, *Pichia pastoris* has the potential to perform many post-translational modifications typically associated with higher eukaryotes, such as processes of folding, disulfide bridge formation and certain types of lipid addition as well as *O*- and *N*-linked glycosylation. In addition, *Pichia pastoris* does not secrete a lot of its own proteins into culture medium. The isolation of the interest protein is, thus, facilitated.

As in the previous study on expression and characterization of recombinant fibrinolytic serine protease from green pit viper (*Cryptelytrops albrlabri*) venom, the recombinant albofibrase produced from *Pichia pastoris* was 0.66 mg/l of culture medium was active as an α fibrinogenase(28).

Albolatin, a novel snake venom mettaloprotease from greem pit viper (*Cryptelytrops albrlabris*), was cloned and its disintegrin domain was expressed in the Pichia system. The recombinant protein was secreted in the culture medium and the

yield of recombinant protein protien was 3.3 mg/l and could inhibit collagen-induced platelet aggregation(38).

In the study of molecular cloning, expression and purification of L-amino acid oxidase from the Malayan pit viper (*Calloselasma rhodostoma*), the recombinant LAAO was purified yielding apparently homogenous protein in quantity of approximate 0.25 mg/l. The recombinant enzyme contained a similar activity as the native one(34).

Plasminogen Activator System and Thrombolytic Agents

Plasminogen activators also play an important role in the processes other than fibrinolysis. For example, fibrinolytic factors, tissue-type plasminogen activator (t-PA) and urokinase—type plasminogen activator (u-PA) control plasmin in different processes, such as cell migration, adhesion and aggregation(21). Previous studies suggested the importance of u-PA in development of atherosclerosis in patients. Furthermore, it also plays an essential role in many (patho) physiological processes that require degradation of extracellular matrix(39). Additionally, there is a research demonstrating a significant elevation of t-PA concentration in plasma of patients with abdominal aortic aneurysm(40).

Currently, we can produce recombinant thrombolytic agents that function as plasminogen activators. The fibrinolytic agents available today are serine proteases. They work by converting plasminogen to a natural fibrinolytic agent, plasmin. Plasmin lyses clots by breaking down the fibrinogen and fibrin contained in a clot into fibrin degradation products (FDPs)(41). They are very helpful in treating acute vascular occlusions in human. The examples of these therapeutic agents are Streptokinase(26), Acylated plasminogen – streptokinase activator complex (APSAC), Staphylokinase(42), Urokinase(43) and Prourokinase(44).

The thrombolytic agents, human tPA and uPA, do not stimulate immunological responses. However, their effects are short-lived *in vivo* and consequently of a limited therapeutic effectiveness. On the other hand, Streptokinase is a non-human protein that can illicit severe anaphylactic response, which may be fatal. Uses of streptokinase are related with serious risk of hemorrhage. At present re-administration of streptokinase cannot be recommended beyond 4 days after first dose as a result of a rise in neautralizing anti-streptokinase antibody.

Acylated plasminogen – streptokinase activator complex (APSAC) can also induce fibrinogenolysis. It is antigenic because it incorporates streptokinase and it, therefore, cannot be reused.

Staphylokinase is also a protein of non-human origin and, consequently, triggers an immune response in patients. Antibodies develops in the majority of patients within 2 weeks after initial administration and are persistent for at least 7 months

Urokinase is specified as a fibrinolytic–thrombolytic agent, which is less effective than that in human tissue plasminogen activator (t-PA). In addition, the cost is higher as compared to the streptokinase.

The half-life of Prourokinase is relatively shorter than that of urokinase. However, it is not commercially available(23).

The Green pit viper plasminogen activator (GPV - PA) is a unique protein waiting for expression and characterization. This protease may lead to a novel therapeutic plasminogen activator. Molecular expression of this protein may yield an agent that benefit to thromboembolic patients. Potential advantages include the long half-life as it is not inhibited by the plasminogen activator inhibitor (PAI-1). In addition, it may be neutralized by the commercially available green pit viper

antivenom if there is an overdose of this agent. Nevertheless, immunogenicity will limit the repeated administrations because it is a foreign protein.

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CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cloning of Snake venom serine proteinase

Synthetic oligonucleotide gene specific primers (GSPs) were purchased from Bio Geno Med (BGM).

Table 1 Oligonucleotides and their descriptions.

Name	**Sequence**	**Description**
T7	5′- GTAATACGACTCACTATAGGGC -3′	Sequencing primer from T7 promoter
SP6	5′-ACTCAAGCTATGCATCCAAC –3′	Sequencing primer from SP6 promoter
GPVPAF	5’-TGA AGA ATT CCT GGT CTT TGG AGG TCG TCC ATG TAACAT AAA TGC CCA T-3’	GSP for PCR of the N-terminus of serine protease domain with 6xHis and *Eco*R I recognition site
GPVPAR	5’-TTC ATC TAG ACC CGG GGG GCA GGT TCG ATC TTT ATT TCC-3’	GSP for PCR of the C-terminus of serine protease domain with stop codon and *Xba* I recognition site

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Table 1 Oligonucleotides and their descriptions. (Cont.)

Name	Sequence	Description
5′-*AOX1*	5′- GACTGGTTCCAATTGACAAGC –3′	*Pichia* sequencing primer
3′-*AOX1*	5′- GCAAATGGCATTCTGACATCC –3′	*Pichia* sequencing primer
α-Factor	5′- TACTATTGCCAGCATTGCTGC –3′	*Pichia* sequencing primer

GSP = Gene Specific Primer

3.1.1.2 DNA Extraction and Purification from gel slice

High Pure Plasmid Isolation kit was purchased from Roche Applied Science, Germany

High Pure PCR Product purification kit (Gel Extraction Kit) was purchased from Roche Applied science, Germany

3.1.1.3 Cloning of Snake Venom Serine proteinase Products

pGEM®-T Easy Vector System II was purchased from Promega, U.S.A. The kit contains *Eschericia coli*, JM 109 strain, pGEM®-T Easy Vector, T4 DNA Ligase and 2x Rapid Ligation Buffer.

Isopropyl-β-D-Thiogalactopyranoside (IPTG), Dioxane-Free, Formula weight 238.3 was purchased from Promega, U.S.A.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 100 mg was purchased from Promega, U.S.A.

3.1.1.4 Enzymes

Tag DNA polymerase	(Invitrogen™ Life technologies)
T4 DNA Ligase	(Promega)
*Eco*R I	(Sigma)
Xba I	(Promega)
Sac I	(Pharmacia Biotech)

3.1.1.5 DNA Sequencing

We use ABI PRISM® BigDye® Terminator V.3.1 Cycle Sequencing Kit purchased from AB Applied Biosystems, U.S.A.

3.1.2 Expression of Serine protease in *Pichia pastoris*

3.1.2.1 Polymerase Chain Reaction

Gene specific primers were purchased from Biogenomed.

3.1.2.2 Pichia expression system

EasySelect™ *Pichia* Expression Kit Version G, 122701, was purchased from Invitrogen™ Life technologies.

3.1.2.3 Proteins Detection

Sodium Dodesyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Mini-Protein 3 Electrophoresis apparatus was purchased from Bio-Rad Laboratories, Ltd.

Pre-stained Protein Marker, Broad Range (Premixed Format) was purchased from New England BioLabs Inc.

Coomassie Brilliant Blue R-250 was purchased from USB, U.S.A.

3.1.2.4 Western Blotting Hybridization

Trans-Blot® SD semi-dry electrophoretic transfer cell was purchased from Bio-Rad Laboratories, Ltd.

Polyvinylidene difluoride (PVDF) membrane 0.45 μm was purchased from Bio-active Co., Ltd.

Mouse Anti-His antibody was purchased from Amersham Biosciences, Ltd.

Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP was purchased from Dako Cytomation, Denmark.

ECL Plus Western blotting detection system was purchased from Amersham™, UK.

Hyperfilm™ ECL high performance chemiluminascence film was purchased from Amersham™ UK.

3.1.2.5 Protein Purification

Protein purification using Immobilized Metal Affinity Chromatography (IMAC). Talon Super-flow Metal Affinity Resin was purchased from BD Biosciences.

MagneHis™ Protein Purification System was purchased from Promaga, USA

3.1.2.6 Concentration of Protein

Amicon ®Ultracentrifugal Filter Devices was purchased from Millipore, USA

3.1.2.7 Protein Quantitative Assay

Micro BCA™ Protein Assay Reagent Kit was purchased from PIERCE Biotechnology.

3.1.3 Activity Assay

Chromogenic substrate (Sigma)

Urokinase Plasminogen Activator (Sigma)

Human plasminogen (Sigma)

3.2 Methods

3.2.1 Expression of GPV-PA in *Pichia pastoris*

3.2.1.1Amplification of GPV-PA by Polymerase ChainReaction (PCR)

PCR was used to amplify the cDNA fragment encoding the serine proteinase domain. Two primers, GPVPAF and GPVPAR, were used to amplify the serine proteinase. The *Eco*RI and six histidine residues were incorporated into the forward primer for facilitating purification and detection. The *Xba*I recognition site and UAA stop codon were incorporated into the reverse primer. The PCR reaction was carried out in a 50 μl containing 10X PCR buffer (100mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 1.25 units of Tag DNA polymerase (Clonetech), 10 pM of each primer, 25 mM $MgCl_2$, 25 mM of each dNTPs, and 200 ng DNA template. After incubation at 95 °C for 10 minutes, amplification was carried out for 30 cycles with the following temperature cycling parameters: 95 °C for 30 seconds of denaturation, 66 °C for 30 seconds of annealing, 68 °C for 1.5 minute of extension and a final extension at 68 °C for 10 minutes. The PCR products were electrophoresed in 1.2 % agarose gel. Subsequently, the DNA was extracted and purified from the gel.

3.2.1.2 DNA Extraction and Purification from Gel Slice

After amplification, the products were electrophoresed on 1.2% agarose gel. A band of DNA was excised from agarose gel with a sterile blade. The PCR products were purified by High Pure Plasmid Isolation kit (Roche) according to the manufacture's instruction.

3.2.1.3 Cloning of PCR Products

3.2.1.3.1 Ligation of PCR Products into pGEM® - T Vector.

After the PCR product was purified by an extraction kit, it was cloned into pGEM® - T Vector. The ligation procedure was carried out in a 10 µl ligation reaction mixture containing 5 µl of 2X Rapid Ligation Buffer (60 mM Tris – HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG), 50 ng of pGEM® - T Vector, 3 Weiss units of T4 DNA ligase and an appropriate amount of the PCR product that was optimized from the insert: vector ratio of 3:1.

Subsequently, de-ionized water was added to the final volume of 10 µl. Finally, the ligation reaction was mixed by pipetting and incubated at 4 °C for 16 – 18 hours.

3.2.1.3.2 Transformation into E. Coli, JM 109

The 10-µl ligation reaction was added to sterile falcon tube Cat. # 2059 on ice. JM 109 competent cells that were placed on ice until just thawed were mixed with DNA by gently flicking. Subsequently, 50µl of competent cells were carefully transferred into Falcon tube and gently mixed and placed on ice for 20 minutes. The reaction tube was then subjected to heat-shock for 45 seconds in a water bath at exactly 42 °C and immediately returned to ice for 10 minutes. The transformed cells were mixed with 450 µl of SOC medium and incubated at 37 °C for 1.5 hour with shaking at 150 rpm. Finally, 500 µl of the transformed cells were plated on LB agar plate with 100 µg/ml ampicillin supplemented with 100 mM IPTG and 50 µg/ml of X-gal for blue/white screening. The plate was incubated at 37 °C for 16 – 24 hours.

3.2.1.3.3 Preparation of plasmid DNA by High Pure Plasmid Isolation kit

High Pure Plasmid Isolation kit (Roche) were used for extraction of plasmid pGEM® - T Vector in *E.Coli*, JM 109.

3.2.1.3.4 Restriction Endonuclease and Electrophoresis

Approximately 500 ng of plasmid DNA were digested with 5 units of EcoR I and Xba I according to manufacturer's protocol (Sigma), 1 µl of 10X Buffer (300 mM Tris – HCl pH 7.8, 100 nM $MgCl_2$, 100 mM DTT, and 10 mM ATP) and 0.1 mg/ml BSA. The digestion reaction was incubated overnight at 37 °C. After digestion, the reaction was electrophoresed on 1.5 % gel. Clones containing the inserts were selected for sequencing.

3.2.1.3.4 DNA sequencing

The sequencing was performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The PCR reaction was carried out in a 10 µl reaction containing 4 µl of the terminator ready reaction mix (Amplitag DNA polymerase and FS with thermostable pyrophosphosphatase), 1 pM sequencing primer (T7) and 1µg DNA template. After incubation at 95 °C for 30 seconds, amplification was carried out for 25 cycles of the following thermal cycling parameters: 95 °C for 10 seconds of denaturation, 50 seconds of annealing, and 60 °C for 4 minutes of extension. The DNA was then precipitated by 95% ethanol and 3 M sodium acetate pH 8.0 on 4 °C. Then, the solution was centrifuge at 25,000 x g for 20 minutes and the supernatant was removed by pipetting. The pellet was then washed with 1 ml of 70% ethanol, and centrifuge tube at 25,000 x g for 10 minutes. Then, the supernatant was removed. The pellet was dried in a heated incubator at 95 °C for 2 minutes. Finally, the DNA pellet was re-suspended in 10 µl. Template Suppression Reagent (Pekin-Elmer) and loaded to the ABI PRISM sequencer.

3.2.1.3.5 Alignment and Computational Searching Sequences Analysis

The nucleotide sequences and their conceptual translation obtained from the clones of interest were compared against nucleotide or protein sequences in online databases using BLAST N (Basic Local Alignment Search Tool) program available

in the World Wide Web. An alignment of sequence were made using Genious™ program.

3.2.1.4 Digestion Plasmid DNA and Expression Vector

After the plasmid clone was confirmed by sequencing the insert, plasmid DNA and expression vector, pPICZαA, were digested with *Eco*R I and *Xba* I, respectively. The digestion reaction was electrophoresed in 1.2 % agarose gel. Then, gel was extracted and purified as described in Section 3.2.1.2. After that, the DNA is precipitated by 0.3 M sodium acetate in 90 % ethanol. Then, the solution was centrifuged at 25,000 x g for 20 minutes. The pellet was washed by 1 ml of 70 % ethanol, and centrifuged at 25,000 x g for 10 minutes. The pellet was then dried and dissolved in sterile distilled water.

3.2.1.5 Ligation of GPV-PA into pPICZαA Vector

Appropriate amounts of plasmid DNA and pPICZαA vector were optimized as described before. The ligation reaction was carried out in a 10-μl reaction. The ligation reaction mixture contained 3 μl of 2xRapid Ligation Buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol), 1 μl of pPICZαA vector, 5 μl of digested construct plasmid DNA, and 3 Weiss units of T4 DNA Ligase. The ligation reaction was incubated at 4 °C overnight.

3.2.1.6 Transformation of Ligated product into E. coli, JM109

Transformation was performed by the heat shock method. The procedure was described in Section 3.2.1.3.2. 500 μl of the transformation were mixed and plated onto Low Salt LB plate with 25 μg/ml Zeocin™ and incubated at 37 °C, overnight. After that, transformants were isolated and analyzed for the presence and the correct orientation of the insert. Zeocin™-resistant colonies were picked, inoculated into 3 ml of Low Salt LB medium with 25 μg/ml Zeocin™ and

incubated overnight at 37 °C with shaking. The plasmid DNA was isolated by the Miniprep for restriction analysis and sequenced

3.2.1.7 Linearization of the Plasmid DNA

Prior to transformation into *Pichia pastoris*, we prepared 5 – 10 μg of plasmid DNA by minipreparation and linearized with the restriction enzyme, which cut one time in the 5′-*AOX 1* region of pPICZαA. 14 μl of plasmid DNA were mixed with 2 μl of 10X Buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), 0.1 mg/ml BSA and 1 unit of *Sac* I. The reaction was incubated at 37 °C for 16-18 hours. An aliquot of reaction was electrophoresed to verify complete linearization. The reaction was then inactivated using heat at 65 °C for 20 minutes. Then, plasmid DNA was precipitated by 2.5 volumes of 100 % ethanol and 1/10 volume of 3 M sodium acetate. Subsequently, the solution was centrifuged and the pellet was washed with 80 % ethanol, air-dried and re-suspended in 5 μl sterile de-ionized water, and stored at –20 °C until use.

3.2.1.8 Transformation of the Linearized Plasmid DNA into Pichia pastoris, X-33

The transformation was performed using the *Pichia* EasyComp™ Kit from Invitrogen. Solutions II and III were stored at room temperature before use. The 50 μl of competent cells were thawed at room temperature for each reaction. 3 μg of the linearized plasmid DNA were placed with the competent cells. Then, 1 ml of Solution II (PEG solution) was added to the DNA/cell mixture and mixed by vortexing or flicking the tube. After that, the transformation reaction was incubated at 30 °C for 1 hour in a water bath. The tube was vortexed every 15 minutes. Subsequently, the transformation reaction was subjected to heat shock at 42 °C for 10 minutes in water bath. The transformed cells were split into 2 microcentrifuge tubes. Add 1 ml of YPD medium to each tube and incubated the transformed cells at 30 °C for 1 hour to allow expression of

Zeocin™ resistance. After that, the transformed cells were centrifuged at 500 x g for 5 minutes at room temperature, re-suspended in 500 µl of Solution III (Salt solution) and combined into one tube. The transformed cells were then centrifuged at 500 x g for 5 minutes at room temperature, and re-suspended in 100 to 150 µl of Solution III. Finally, the transformed solution was plated on YPDS plate with 100 µg/ml Zeocin™ and incubated for 3 to 10 days at 30 °C.

3.2.1.9 Expression of Recombinant Protein in Pichia pastroris

A single colony was inoculated in 10 ml of BMGY in a 250 ml baffled flask, and incubated at 30 °C in a shaking incubator for 16 – 18 hours or until culture reached an OD_{600} between 2 to 6. Subsequently, 10 ml of culture were inoculated in 100 ml of BMGY in a 500 ml baffled flask and grown at 30 °C with shaking until the culture reached an OD_{600} of 2 to 6. After that, the cells were collected by centrifugation at 500 x g for 5 minutes at room temperature. To induce expression, supernatant was discarded and the cell pellet was resuspended to an OD_{600} of 1.0 in BMMY medium. Then, the culture was aliquoted into several 4 liters baffled flasks covered with two layers of sterile gauze. They continued to grow at 30 °C with shaking. The methanol concentration was maintained at 0.5 % (v/v) every 24 hours for induction expression until the time reach 96 hours. After that, the supernatant and cell pellets were separated by centrifuging at 25,000 x g for 10 minutes at room temperature. The cells were stored at –80 °C and the supernatant was concentrated by a centrifuging concentrator.

3.2.1.10 Concentration of Proteins

The supernatant was separated by centrifugation and concentrated by ultrafiltration using Vivaspin concentrator that have MWCO of 5,000 Da. The supernatant was poured into the concentrator at maximum volume, and then the concentrator was placed in 50 ml centrifuge tube. Subsequently, the assembled

concentrator was centrifuged at 25,000 x g for 40 minutes. The remaining sample from the bottom of the concentrated pocket was recovered using a pipette.

3.2.2 Purification of Recombinant Proteins

Recombinant serine proteinase was purified according to protocol from MagneHis™ Ni-particles from 200 µl of the concentrate (equivalent to 1 ml of culture). The solution was mixed by pipetting up and down approximately 10 times and incubating for 2 minutes at room temperature. The tube was then placed in the appropriate magnetic stand for approximately 30 seconds to allow the MagneHis™ Ni-particles to be captured by the magnet and the supernatant was removed with a pipette. After removal of the tube from the magnet, 150 µl of MagneHis™ binding/ wash buffer were added to the MagneHis™ Ni-particles and mixed by pipetting. Then, it was placed in the magnetic stand again for 30 seconds. After MagneHis™ Ni-particles were captured by the magnet, supernatant was carefully removed with a pipette. This step was repeated twice. Finally, MagneHis™ elution buffer was added and mixed by pipetting and incubated for 1-2 minutes at room temperature. A magnetic stand was placed to allow MagneHis™ Ni-particles to be captured by the magnet and supernatant containing the purified protein was removed using a pipette. The samples were analyzed for expression of the fusion protein by SDS – PAGE or by functional assay or kept at -80 °C until tests.

3.2.3 Protein Detection

3.2.3.1 Sodiumdodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brillient Blue Staining

The 10% resolving and 5% stacking acrylamide gels containing 10% SDS were freshly prepared. After gel setting, the recombinant protein was mixed with ¼

volume of 2X sample buffer (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 200 mM with or without β-mercaptoethanol), denatured at 95 °C for 10 minutes and loaded into gel slots. Electrophoresis was performed at 125 volts for 90 minutes in 1X running buffer, pH 8.3 (0.25 M Tris-HCl, 1.92 M glycine, 1 % w/v SDS). After electrophoresis, the gel was soaked in Coomassie Brillient Blue Solution for 30 minutes with gentle agitation. After the staining solution was removed, the destaining solution (10% glacial acetic acid, 30% methanol) was added and incubated for 2 – 3 hours. The destaining solution was changed 3 to 4 times during incubation

3.2.3.2 Western Blotting Hybridization

After SDS-PAGE, the proteins were transferred to PVDF membrane using electroblotting in the semi-dry system. The polyacrylamide gel and PVDF membrane were soaked in a transfer buffer for 20 minutes. Both of equilibrated gel and wetted membrane were sandwiched between sheets of transfer buffer-soaked thick filter papers and then placed on Trans-Blot® SD cell. The proteins were transferred at 40 volts for 40 minutes. When finished, the blotted membrane was immediately placed into the blocking solution (5 % v/v Non-fat Dry Milk in 1X PBS buffer, pH 7.4) for 1 hour at room temperature with gentle agitation and then washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. The membrane was incubated with 1:3,000 dilution of Anti-His Antibody in blocking buffer for 1 hour at room temperature with gentle agitation. The membrane was, subsequently, washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. After that, the membrane was incubated with 1:1,000 dilution of Horse radish peroxidase-conjugated rabbit Anti-Mouse IgG:HRP in blocking buffer for 2 hours at room temperature with gentle agitation and washed as described previously. For developing the blot, the membrane was soaked in the visualizing solution (1.66 mM

3, 3′-diaminobenzidine (DAB) tetrahydrochloride, 0.04 % $NiCl_2$ and 3 % H_2O_2). The reaction was allowed to occur in the dark for 5 minutes. Finally, the solution was removed and the reaction was stopped with H_2O and let the membrane dry overnight.

3.2.4 Quantitative Assay for Recombinant Proteins

Protein concentration was determined using Micro BCA™ Protein Assay Reagent Kit (Pierce). The method utilized bicinchoninic acid (BCA) as the detection reagent for Cu^+ that was formed when Cu^{2+} was reduced by protein in an alkaline environment. The bovine serum albumin standards (BSA) were diluted into 6 dilutions (0.025 – 0.1 mg/ml). Then fresh working reagent was prepared by mixing 25 parts of Micro BCA™ Reagent MA containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH and 24 parts Reagent MB containing 4% bicinchoninic acid in water with 1 part of Reagent MC containing 4% cupric sulfate pentahydrate in water. 150 µl of each standard or the sample solution replicate were pipetted into microplate wells and 150 µl of the working reagent were added to each well and mixed. The plate was covered and incubated at 37 °C for 2 hours. The reaction was then measured the absorbance at 570 nm on a plate reader.

3.2.5 Plasminogen activator activity assay

The reaction was performed using human plasminogen (0.1 U/ml, Sigma) with serine protease protein to provide the final concentrations ranging from 0.3 to 2.4nM. After 10 min at 37°C, 20 µl aliquots were taken, mixed with 180 µl of chromogenic substrate S-2251 (1 mM) subtrate in buffer, and incubated for 10 min to assay for the amidolytic activity of the active form of plasminogen, plasmin. The same procedure was applied for u-PA for calibrating the plasminogen activating

activity. The increase in absorbance at 405 nm during a 10 min incubation period was defined as ΔO.D.

3.2.6 Platelet Aggregation Assay

Platelet aggregation assay is performed using a Helenna Aggregometer. Venous blood (9 parts) from healthy donor who has not received any medication for at least 2 weeks is collected in 3.2 % sodium citrate (1 part). The whole blood is centrifuged at 1,000 x g for 10 minutes to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP) is prepared from the remaining whole blood by centrifuging at 3,500 x g for 10 minutes. PRP is diluted to 250×10^9 platelets/L with PPP. Different amount of recombinant disintegrins are added to PRP and incubated at 37 °C for 10 minutes. Platelet aggregation is initiated by adding collagen (2 mg/ml). Light transmittance is recorded and the maximum aggregation response is obtained. The maximal aggregation in the absence of recombinant serine protease is given a value of 100 % aggregation

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CHAPTER IV

RESULT

4.1 **Expression of A Novel Snake Venom Serine Proteinase (SVSP) in *Pichia pastoris***

4.1.1 Amplification of GPV-PA using Polymerase Chain Reaction (PCR)

The structural gene of serine proteinse domain was amplified by PCR with a forward primer, GPVPAF, that has an *Eco*R I recognition site and six histidine residues for facilitating purification and a reverse primer, GPVPAR, that has an *Xba* I recognition site and UAA stop codon. After electrophoresis, the PCR product size was approximately 705 bp in length (Figure 2)

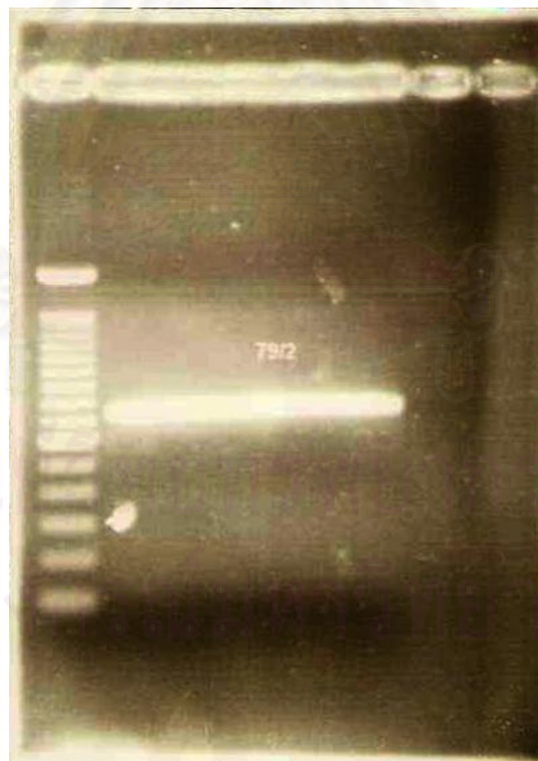

Figure 2 The GPV-PA coding sequence PCR product of approximately 705 bp in length electrophoresed on a 1.5% agarose gel.

4.1.2 **Ligation of GPV-PA into pGEM® T-vector and Transformation of *E. coli*, JM109**

The PCR product was extracted from 1.2% electrophoresis gel using the High Pure Plasmid Isolation kit, cloned into pGEM® T-vector and subsequently transformed to *E. coli*, JM 109. The positive plasmid clones were identified by the blue – white colony screening system. The white plasmid clones were purified and digested with *Eco*R I and *Xba* I to verify the presence of inserts and then sequenced using the T7 sequencing primer to confirm the correct and in-framed sequences of GPV-PA.

The selected clones were grown in LB broth with 100 μg/ml of ampicilin. The plasmid was purified again using High Pure PCR Product purification Kit (Gel Extraction Kit) to obtain a high yield of the purified insert.

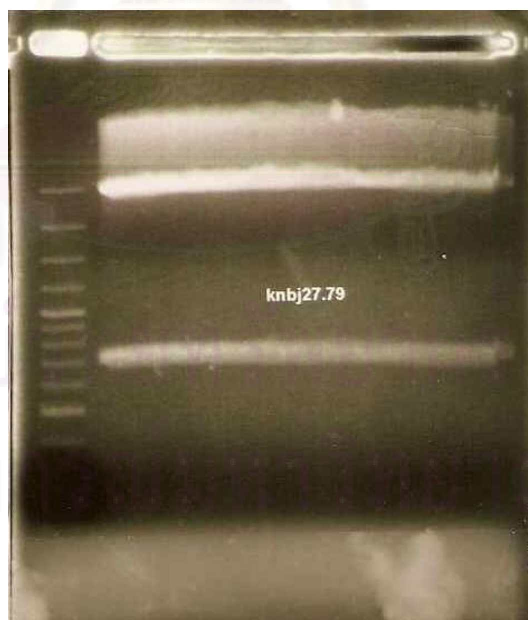

Figure 3 The restriction enzyme digested products of plasmid pGEM® T-vector containing the construct.

4.1.3 Ligation of GPV-PA into pPICZαA and Transformation into *E.* coli, JM 109

After the plasmid clone was confirmed by sequencing, the inserts in plasmid DNA were digested with *Eco*R I and *X*ba I. The digestion reactions were electrophoresed in 1.2% agarose gel (**Figure 3**). After gel extraction and purification, the digestion product was cloned into *Eco*R I and *X*ba I sites of the expression vector, pPICZαA. The recombinant plasmid was transformed into *E.* coli, JM 109, and the colony was selected on an agar plate of low-salt LB agar with 25 μg/ml Zeocin™. As a result, there were approximately 15 Zeocin™ – resistance tranformants. The recombinant plasmids were digested with *Eco*R I and *X*ba I as shown in **Figure 4**.

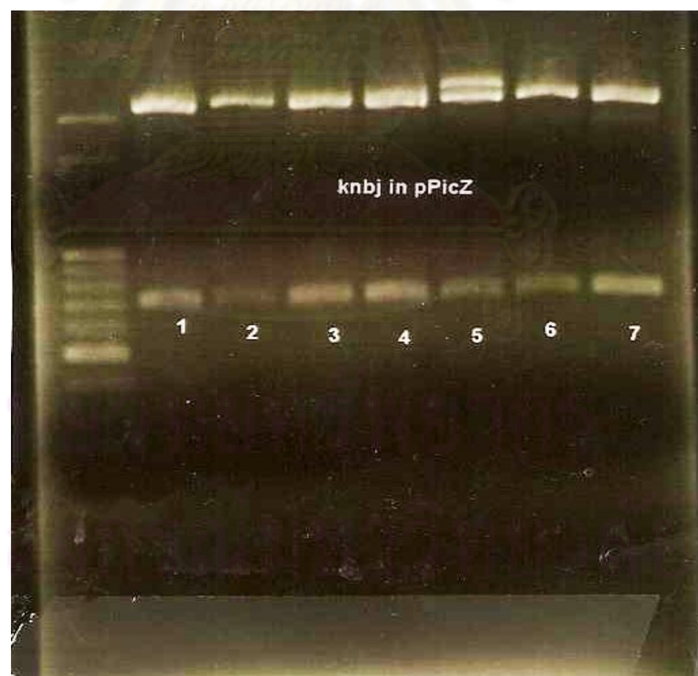

Figure 4 The restriction digested transformed pPICZαA vector. The picture shows inserts in the pPICZαA vector.

4.1.4 Sequence Alignment and Bioinformatic Analysis

The cDNA sequence from recombinant clone of GPV-PA was shown with redundant amino acid sequence after transalation (Figure 5)

```
cgcccagctgcttaatttaatcaaataaagtgctgcttgatcaaaaagtctccgcttgggt
 tatctgattaggttgatacggtacctcaagtttaagtaagggattggaatcttacaggca
 aagagctttctgcgcagagttgaagctatggttctgatcagagtgctagcaaaccttctg
                         M  V  L  I  R  V  L  A  N  L  L
 atactacagctttcttacgcacaaaaatcttctgaactggtctttggaggtcgtccatgt
 I  L  Q  L  S  Y  A  Q  K  S  S  E  L  V  F  G  G  R  P  C
 aacataaatgaacatcgttcccttgttgtcttgtttaactccagcggttttctctgtggt
 N  I  N  E  H  R  S  L  V  V  L  F  N  S  S  G  F  L  C  G
 gggactttgatcaatcaggattgggtggtcaccgctgcacactgtgacagtaataatttc
 G  T  L  I  N  Q  D  W  V  V  T  A  A  H  C  D  S  N  N  F
 cagttgctgtttggtgtgcatagcaaaaagacactaaatgaggatgagcagacaagagac
 Q  L  L  F  G  V  H  S  K  K  T  L  N  E  D  E  Q  T  R  D
 ccaaaggagaagttcttttgtcccaataggaaaaaggatgacgaagtggacaaggacatc
 P  K  E  K  F  F  C  P  N  R  K  K  D  D  E  V  D  K  D  I
 atgttgatcaagctggacagttctgttaacaacagtgaacacatcgcgcctctcagcttg
 M  L  I  K  L  D  S  S  V  N  N  S  E  H  I  A  P  L  S  L
 ccttccagccctcccagtgtgggctcagtttgccgtattatgggatggggcaaaaccata
 P  S  S  P  P  S  V  G  S  V  C  R  I  M  G  W  G  K  T  I
 cctactaaagatatttatcccgatgtccctcattgtgctaacattaacatactcgatcat
 P  T  K  D  I  Y  P  D  V  P  H  C  A  N  I  N  I  L  D  H
 gcggtgtgtcgaacagcttattcatggcggcaggtggcaaacacaacattgtgtgcaggt
 A  V  C  R  T  A  Y  S  W  R  Q  V  A  N  T  T  L  C  A  G
 atcctgcaaggaggcaaagatacatgtcactttgactctgggggacccctcatctgtaat
 I  L  Q  G  G  K  D  T  C  H  F  D  S  G  G  P  L  I  C  N
 gaacaattccatggcattgtatcttgggggtgggcatccttgtggccaaccgcgggagcct
 E  Q  F  H  G  I  V  S  W  G  G  H  P  C  G  Q  P  R  E  P
 ggcgtctacaccaatgtcttcgattatactgactggatccagagcattattgcaggaaat
 G  V  Y  T  N  V  F  D  Y  T  D  W  I  Q  S  I  I  A  G  N
 Aaagatgcaacctgcccccgtgaaaacttttgaaaaagttaagaggagaatatgtaaca
 K  D  A  T  C  P  P  -
 tattagtacatctcttctatatccctaaccatatccgactacattggaatatattcccag
gcagaaaggtt
```

Figure 5 The redundant amino acid sequence of GPV-PA was shown with highlight signal peptide prediction as SYAQK. The predicted N-glycosylation sites was underlined.

The inserted positive clones were sequenced, analyzed and compared with GENEBANK database using the BLAST N. The highest BLAST score of GPV-PA showed highest homology to *Viridovipera stejnegeri*, accession number AAC59686.1. An alignment showed 94 % of amino acids sequence identity (Figure 6).

Sequences producing significant alignments:	Score (bits)	E Value
gb|AAC59686.1| *Viridovipera stejnegeri* venom PA precursor	490	0.0
gb|AAG10789.1| *Trimeresurus jerdonii* serine proteinase2 precursor	485	0.0
gb|AAG10790.1| *Trimeresurus jerdonii* serine proteinase3precursor	450	0.0
gb|ABB76280.1| *Bothrops asper* thrombin-like enzyme	421	0.0
gb|ABD52886.1| *Lachesis muta* serine protease precursor	418	0.0
gb|AAN52350.1| *Viridovipera stejnegeri* venom serine protease 5	395	0.0
gb|AAQ02910.1| *Viridovipera stejnegeri* serine protease PA precursor	392	0.0
gb|AAF76378.1| *Deinagkistrodon acutus* thrombin-like protein DAV-PA..	390	0.0
gb|AAN52349.1| *Viridovipera stejnegeri* stejnefibrase 2 thrombin-like...	384	0.0

Figure 6 The homology search for cDNA sequence of GPV-PA of *C. albolabris*

The selected 4 venom serine proteinase were aligned using Geneous^{TM} commercial software analysis. The consensus sequence indicates as green color with black highlight that indicate the high percentage of identity among these protein sequences. The pairwise statistic analysis of GPV-PA identity with other venom serine proteinase from alignment is 82.6 % (Figure 7).

In this analysis, the TSV-PA and GPV-PA are extensively identical protein with identity score as 94% (Figure 8). Furthermore, the phylogenetic tree analysis of four selected proteins were compared using the same computer program analysis that reveal distinct result of the intensive relation between TSV-PA and GPV-PA (Figure 8). In addition, the conserved cysteine residue alignment and signal peptide cleavage prediction analysisn were reported in GPV-PA (Figure 9 and 10).

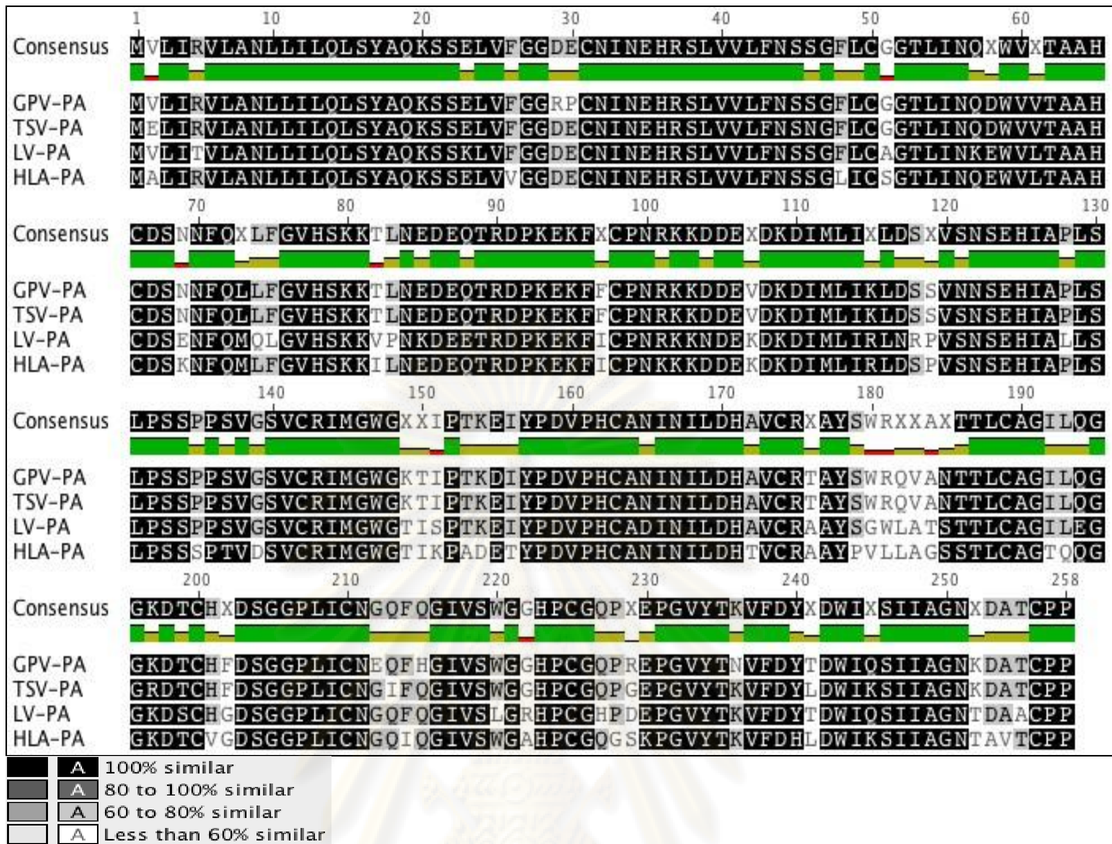

Figure 7 The protein sequence alignment of GPV-PA using Genious™ program.

```
GPV-PA   MVLIRVLANLLILQLSYAQKSSELVFGGRPCNINEHRSLVVLFNSSGFLCGGTLINQDWV
TSV-PA   MELIRVLANLLILQLSYAQKSSELVFGGDECNINEHRSLVVLFNSNGFLCGGTLINQDWV
         *:**************************::***************:**************


GPV-PA   VTAAHCDSNNFQLLFGVHSKKTLNEDEQTRDPKEKFFCPNRKKDDEVDKDIMLIKLDSSV
TSV-PA   VTAAHCDSNNFQLLFGVHSKKTLNEDEQTRDPKEKFFCPNRKKDDEVDKDIMLIKLDSSV
         ************************************************************

GPV-PA   NNSEHIAPLSLPSSPPSVGSVCRIMGWGKTIPTKDIYPDVPHCANINILDHAVCRTAYSW
TSV-PA   SNSEHIAPLSLPSSPPSVGSVCRIMGWGKTIPTKEIYPDVPHCANINILDHAVCRTAYSW
         :*********************************:*************************

GPV-PA   RQVANTTLCAGILQGGKDTCHFDSGGPLICNEQFHGIVSWGGHPCGQPREPGVYTNVFDY
TSV-PA   RQVANTTLCAGILQGGRDTCHFDSGGPLICNGIFQGIVSWGGHPCGQPGEPGVYTKVFDY
         ****************:**************::*:*************:******:****

GPV-PA   TDWIQSIIAGNKDATCPP
TSV-PA   LDWIKSIIAGNKDATCPP
         :***:*************
```

Figure 8 The protein sequence alignment of GPV-PA and *Viridovipera stejnegeri* (TSV-PA) using Genious^{TM} program program. It reveals that they were 94 % identical.

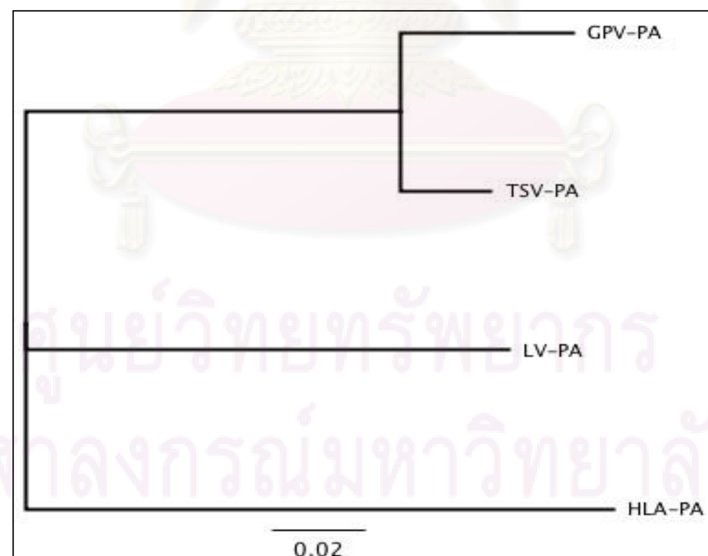

Figure 9 The phylogenetic tree prediction of GPV-PA and other snake venom proteins.

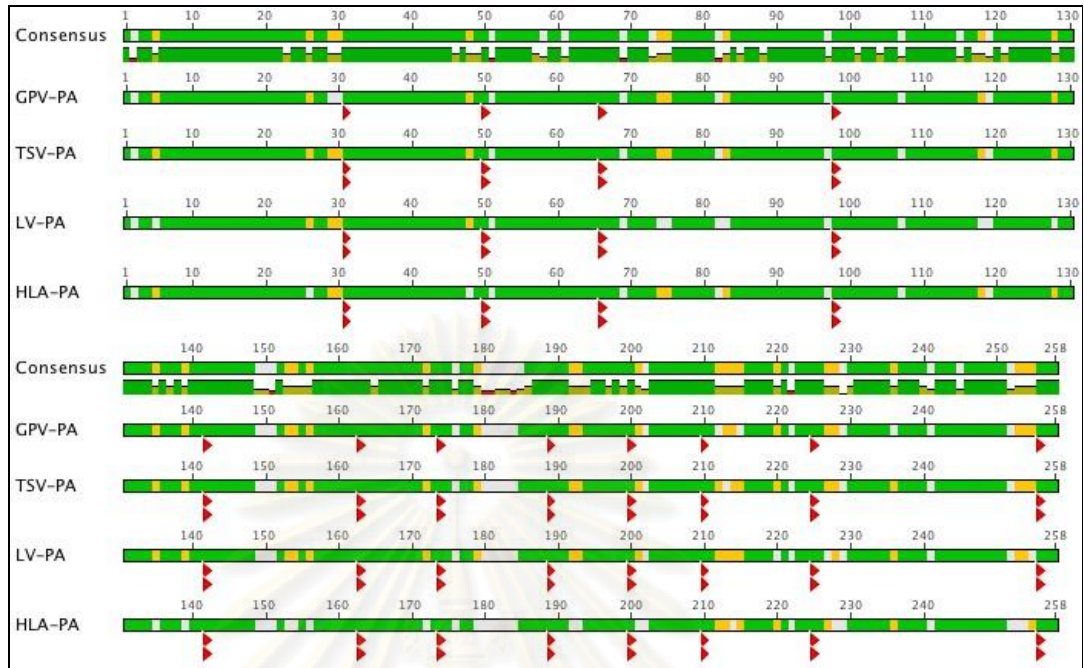

Figure 10 The conserved cysteine residues were represent as red arrow in the identical consensus motif of all protein comparing with GPV-PA.

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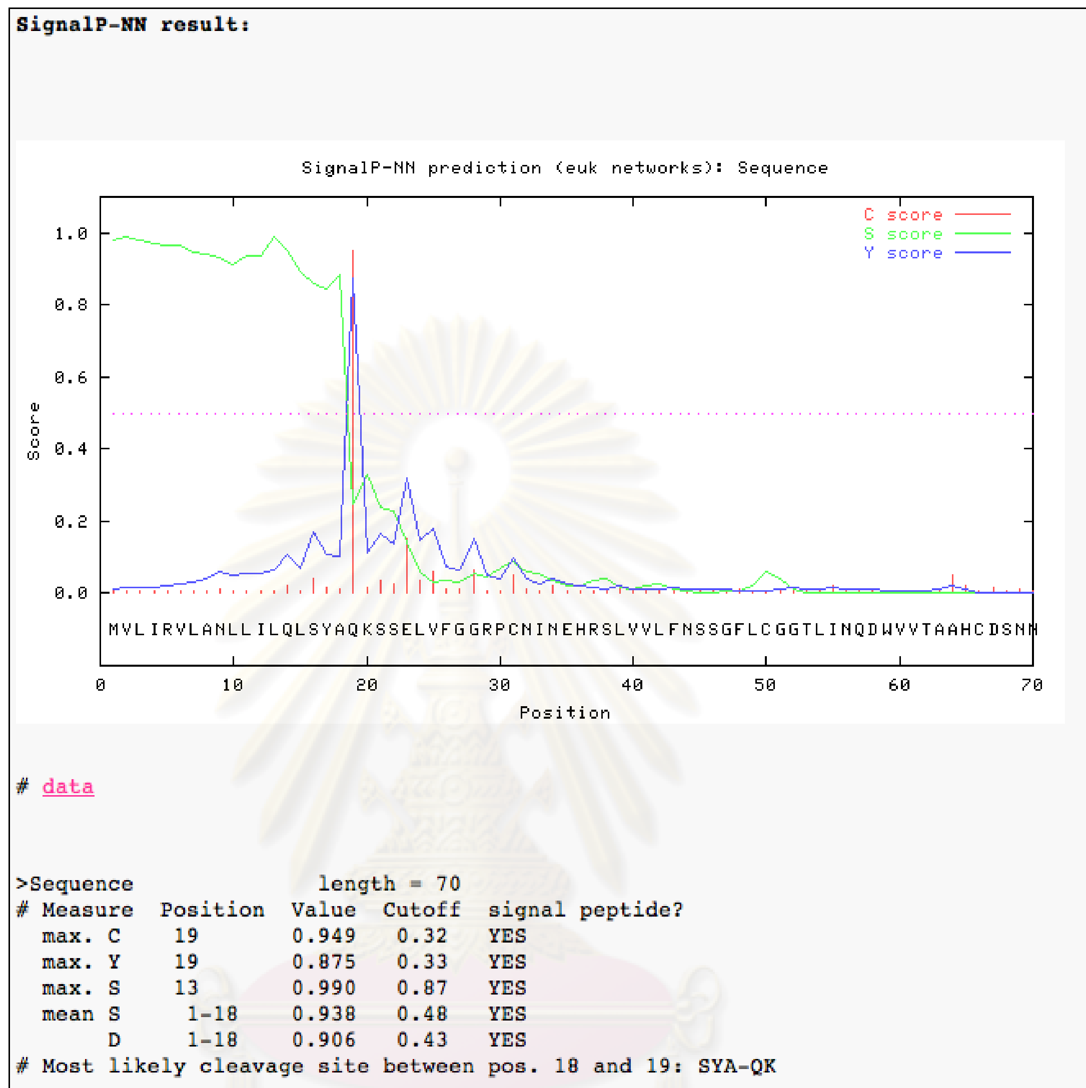

Figure 11 The signal peptide and its cleavage prediction in GPV-PA represent the cleavage site between amino acid residue 18 and 19 (SYA-QK). In this analysis latter sequence of its signal peptide revealed the conserve amino acid residue as KSSEL that determined the post translational protein modification of GPV-PA as secretory protein.

4.1.5 Tranformation of Recombinant pPICZαA into *Pichia pastroris*, X– 33

Prior to transformation into *Pichia pastroris*, recombinant pPICZαA was linearlized with *Sac* I (Figure 8). After that, the linearized recombinant pPICZαA was transformed into competent *Pichia pastroris* cells, X – 33. Approximately, 60 colonies of transformant were found after 4 days as shown in **Figure 13**.

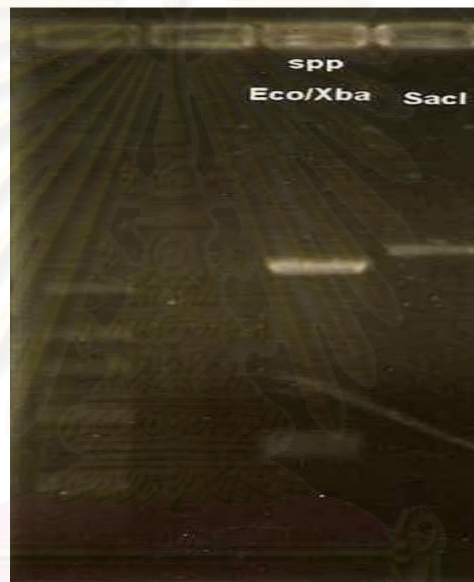

Figure 12 The *Sac* I restriction product with the size of approximately 3000 bp. Lane 2: pPICZαA vector with inserts digested by *Eco*R I and *X*ba I, Lane 3: pPICZαA vector linearized by *Sac* I.

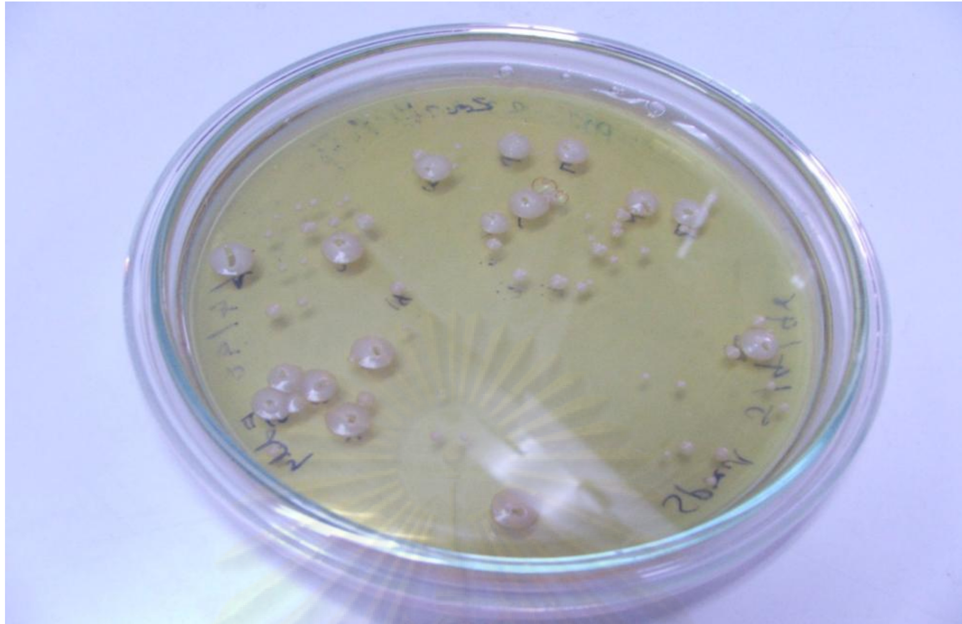

Figure 13 The colonies found on day 4 after transformation of a linearlized vector into *Pichia pastroris,* X-33 on a Zeocin-containing plate

4.1.6 Recombinant GPV-PA from *Pichia pastroris*

After the positive colonies were identified, a clone was selected and cultured. We started to induce the expression of recombinant protein using 0.5% V/V methanol in BMMY media for 3 days. The media were precipitated and concentrated by VIVA spin at 6000 g centrifugation. The concentrated media were purified by using affinity chromatography. Subsequently, the recombinant protein was tested or its characteristics and functions. Moreover, increasing the concentration of methanol for induction as 1% could increase the efficacy of recombinant protein production as

4.2 Characteristics and functions of recombinant protein

4.2.1 Characteristics of GPVPA Recombinant Protein

Purified protein was run on SDS PAGE (10% gel) and subjected to Western blot on PVDF membrane (Figure 10). After incubation with anti-histidine and anti-mouse HRP, the membrane was developed using ECL chemi-luminescence on a photo film. They revealed that recombinant protein size of approximately 37 kDa. Then the concentration of the protein was measured by the micro BCA ™ Protein Assay using absorbance at 562 nm at 1:30 dilution of the protein sample. The calculated protein concentration was 5.45 µg/ml or 163.5 µg/ml for undiluted protein. Therefore, the yield of recombinant serine proteinase product in *Pichia pastroris* was 0.38 mg / Liter of culture medium. Moreover, increasing the concentration of methanol for induction as 1% could effect the efficacy

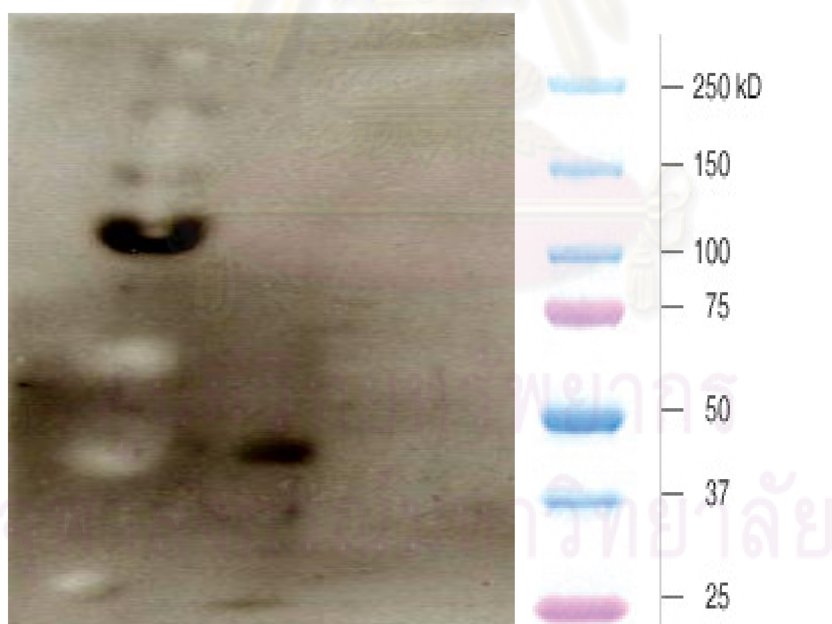

Figure 14 The affinity chromatography purified recombinant GPV-PA on Western blotting analysis of the purify protein visualized by chemi-luminescence.

4.2.2 Plasminogen activation assay

The average of plasminogen activator activity in GPV-PA compare to the activity of control plasminogen activator (U-PA) (Figure 12).

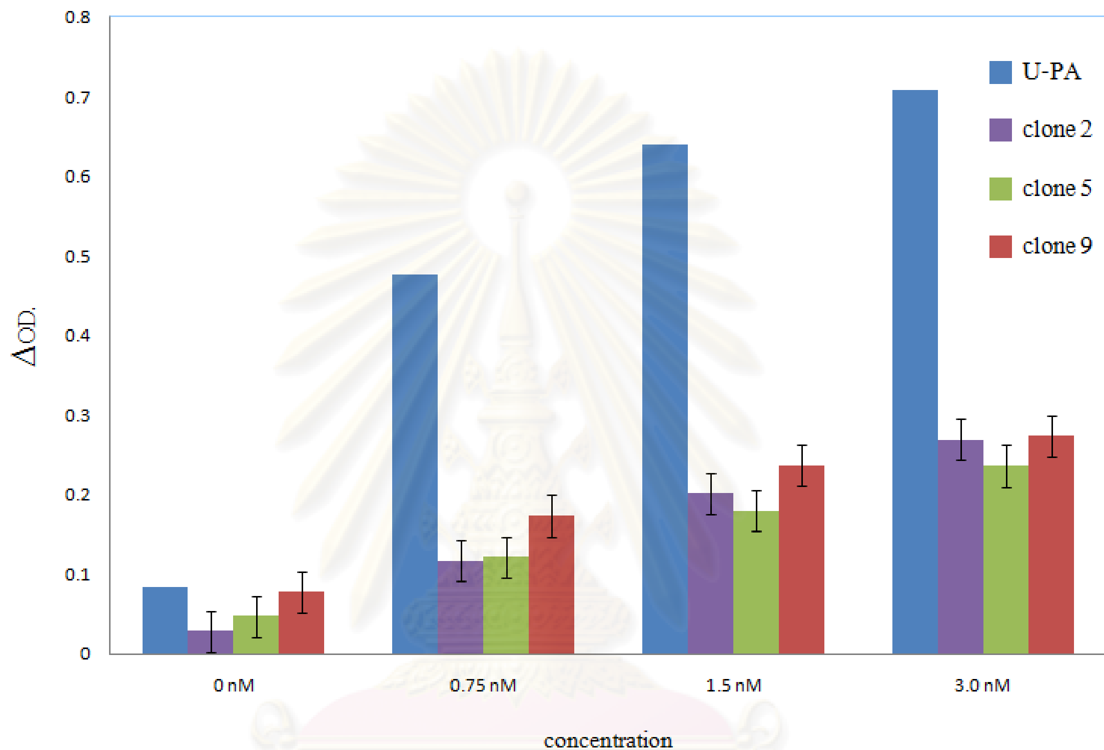

Figure 15. Plasminogen activation by GPV-PA. The delta OD represents absorbance changes GPV-PA added at the indicated concentrations time were measured for the absorbance (405 nm.) at 0 minute and 10 minutes. U-PA was used as standard and S2251 chromogenic substrate was used.

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4.2.3 Platelet Aggregate assay

The purified recombinant protein GPV-PA was tested to investigate the function on platelet aggregation assay. The result showed that recombinant GPV-PA could not inhibit collagen-induced platelets aggregation in a dose dependent manner. At the concentration of 0, 5.0 and 10 μM, there was not aggregation.

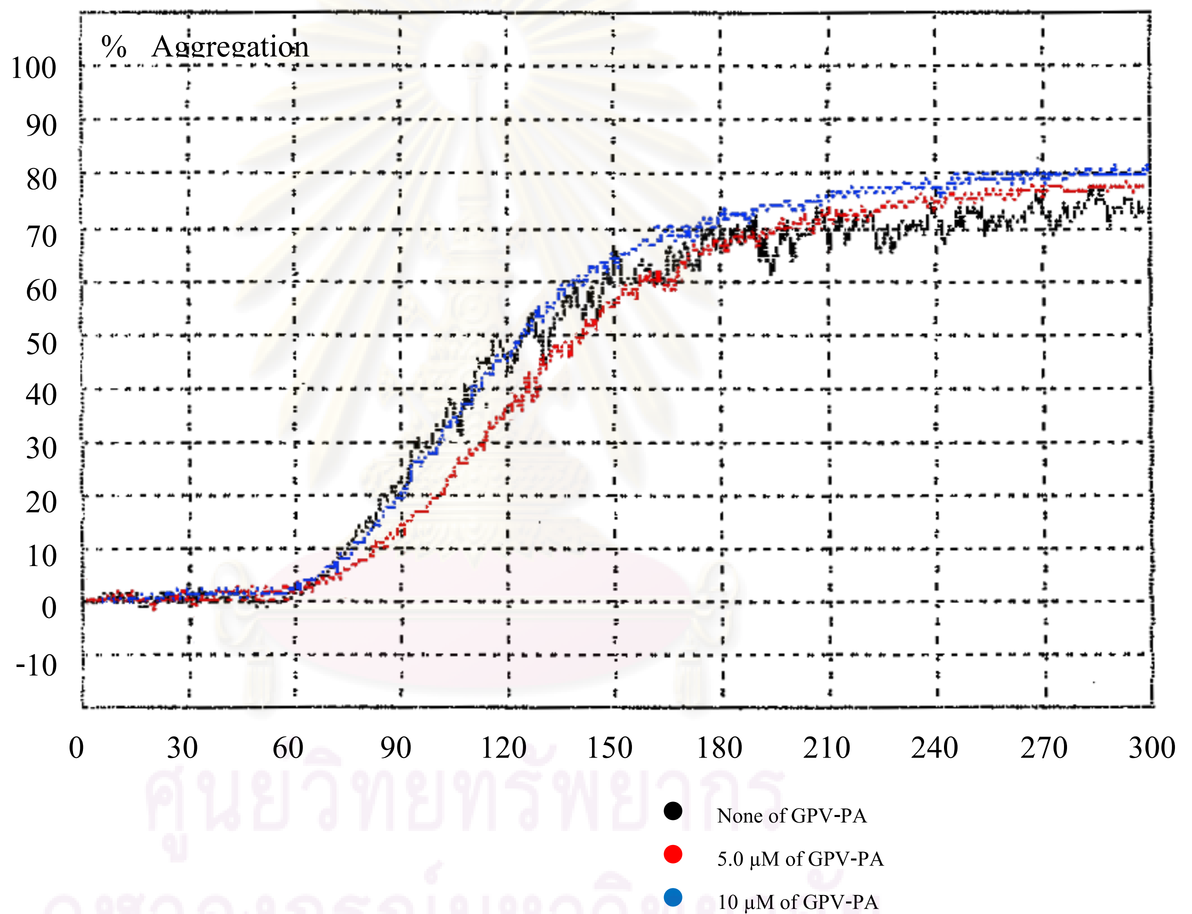

Figure 16. GPV-PA could not induce platelet aggregation by platelet aggregation assay.

CHAPTER V

DISCUSSION AND CONCLUSION

The green pit viper venom is characterized as serine proteinases phospholipase A2, C-type lectin like proteins, metalloproteinase and disintrigrins. To be complementary to the studies of other components of pit venoms, current study was investigated whether GPV-PA could specifically perform a function as plasminogen activator that cleaved plasminogen to plasmin.

The molecular cloning and sequencing revealed that the coding cDNA of GPV-PA consisted of 705 bp. In addition, the bioinformatics tools of the nucleotide sequence alignment and computational searching analysis, such as BlastN, discovered other genes with high BLAST scores with GPV-PA. The 9 proteins with the highest homology were shown in Figure 5. The highest three homologous nucleotide sequences were the venom PA precursor from *Viridovipera stejnegeri*, serine proteinase 2 precursor from *Cryptelytrops jerdonii* and the serine proteinase 3 precursor from *Cryptelytrops jerdonii,* respectively. Our clone from *Cryptelytrops albrolabris* is a novel gene and, therefore, we could not detect this sequence from current version of BlastN. As *Cryptelytrops sp* and *Viridovipera sp.* were newly named separating the, from the same original genus of *Trimeresurus*, this means that our cDNA sequence contained a high similarity with other serine proteases of the close genus classification.

After the cloning process, the GPV-PA protein was conceptually translated to be a 258-residue peptide. The molecular cloning approach predicted the molecular weight of 26.4 kDa of mature GPV-PA, which contain 240 amino acid residues excluding the 18-residue signal peptide. The protein showed the highest identity (94% similarity) with TSV-PA, a plasminogen activator from *Viridovipera stejnegeri*. The TSV-PA is a known plasminogen activator that triggers plasmin production from

plasminogen (46). This suggests that the GPV-PA probably contains a plasminogen activator activity.

The protein sequence was then analyzed using the Genious™ program that determined the protein sequence alignment and phylogenetic tree generation of the selected serine proteinases from venom proteins with plasminogen activator activity including *Trimersurus stejnegeri* (TSV-PA), *Lachesis muta muta* (LV-PA) and *Agkistrodon halys* (HLA-PA) (45,46,47). The result of sequence alignment showed the statistically identity of 82.6 % among all plasminogen activity venom proteins (Figure 6). The cysteine residues of all aligned proteins were examined and found that all the cysteine residue positions were conserved suggesting the identical arrangements of disulfide bond formation, which is important for similar maintaining the molecular structure and the biochemical function of proteins. Figure 8 indicated the identical 12 residues of cysteines in all the analyzed proteins suggesting that these four comparable proteins were similar in term of protein structure and function.

Furthermore, the phylogenetic tree prediction of the 4 snake venom plasminogen activators showed the related evolution of these proteins (Figure 9). This result revealed the close genetic relationship between GPV-PA and TSV-PA that were possibly the same family proteins. On the other hand, LV-PA and HLA-PA were more separated from both of snake venom plasminogen activators.

In this current study, the *Pichia pastoris* expression system was used to produce the recombinant GPV-PA protein using 0.5% and 1% methanol induction. In our experiment, we could produce GPV-PA with the yield of approximately 0.38 mg/Litre and 3.15 mg/Litre respectively. Therefore, the higher concentration of methanol (1%) could increase the production of recombinant protein without the reduction of the *Pichia pastoris* growth rate that was determined during the experimental cell harvests. Methanol is the sole carbon and energy source as well as inducer of heterologous protein production in recombinant *Pichia pastoris*

fermentations. While increasing the concentration of methanol up to 4.5% could not decrease the growth rate of yeast, together with the low level of toxic metabolic compound formation (48). The previous study from our group on the recombinant venom serine protease production using the same expression system revealed that the low level of protein production was correlated with the low concentration of methanol induction (28). In our experiment, we, therefore, adjusted the concentration of methanol from the previous investigation and could increase the production yield of same venom protein for approximately 8.29 times. This finding is very useful for future recombinant expression of other snake venom serine proteases.

After the purification of the recombinant GPV-PA protein using affinity column chromatography, the Western blot analysis was performed using anti-body against the 6-histidine tag. The result showed the 37-kDa band of GPV-PA. The bioinformatics calculation indicated that the molecular weight if GPV-PA with 6 histidines was approximately 27.2 kDa. The signal peptide sequence was present at position 18 to 22 as SYAQK with specific cleavage site between position 18 (S) and position 19 (Y). From computer analysis short amino acid residues after signal peptide sequence determine the possible protein trafficking along the post translational protein modification under secretory pathway through endoplasmic reticulum and golgi body as KSSEL. This discrepancy may be due to the post-tranlational modifications by the yeast, *Pichia pastosis, system.*

GPV-PA was first examined for the plasminogen activator activity. The protein could cleave plasminogen to plasmin resulting in the cleavage of the plasmin chromogenic substrate. Because our recombinant expression system was used to produce the pure recombinant venom plasminogen activator (PA) without contaminations with other PA proteins, the finding demonstrated definitely that this serine protease was specifically involved in fibrinolysis. However, the plasminogen

activity assay found that GPV-PA contained a plasminogen-activating activity that was weaker than the urokinase (u-PA) standard.

The plasmin is an important component inducing the physiologic and pathologic fibrinolysis, as well as proteolytic. Therefore, plasmin activated by GPV-PA can eliminate the coagulation products of the blood coagulation cascade. This probably contributed to bleeding disorders in green pit viper bite patients. Furthermore, the protein should be investigated to be a novel fibrinolytic agent for the treatments of thromboembolic diseases.

GPV-PA was also examined for the platelet aggregation inhibitory activity. The result demonstrated that recombinant GPV-PA did not inhibit the collagen-induced platelet aggregation. In contrast, with snake venom metalloproteinase/disintrigrin from *Cryptelytrops albolabris*, could inhibit platelet aggregation stimulated by collagen (38).

In summary, we characterized the full length cDNA of serine protease from the green pit viper (*Cryptelytrops albolabris*) venom glands and expressed GPV-PA in the *Pichia pastoris* expression system. The bioinformatics analysis of nucleotide and protein sequence demonstrated the high relationship with significant identity score with 3 other snake venom serine proteases with plasminogen activator including *Trimersurus stejnegeri* venom plasminogen activator (TSV-PA), *Lachesis muta muta* venom plasminogen activator (LV-PA) and *Agkistrodon halys* plasminogen activator (HLA-PA). However, GPV-PA from *Cryptelytrops albrolabris* is the highest relationship with TSV-PA by the phylogenetic tree prediction and protein alignment. By increasing the methanol concentration in the culture, the yield of the recombinant protein could be enhanced. Moreover, the recombinant GPV-PA contained plasminogen activator activity. Therefore, this novel recombinant plasminogen activator deserves further investigations to develop the protein into a therapeutic thrombolytic agent in the future.

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APPENDIX

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APPENDIX

1. Bacterial Media

1.1 LB Medium (per liter)

10g	Bacto®-tryptone
5g	Bacto®-yeast extract
5g	NaCl

Adjust pH to 7.0 with NaOH.

1.2 LB Plates with Ampicillin

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50 °C before adding ampicillin to a final concentration of 100 µg/ml. Pour 30-35 ml of medium into 85 mm petri dishes. Let the agar harden. Store at 4 °C for up to one month or at room temperature for up to one week.

1.3 LB Plates with Ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

1.4 SOC Medium (100ml)

2.0 g	Bacto®-tryptone
0.5 g	Bacto®-yeast extract
1 ml	1M NaCl
0.25 ml	1M KCl
1 ml	2M Mg^{2+} stock, filter sterilized

1 ml 2M glucose, filter sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2 M Mg^{2+} stock and 2 M glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile, distilled water. The final pH should be 7.0.

2. *Pichia pastoris* Media

2.1 Low Salt LB (Luria-Bertani) Medium

1 % Tryptone

0.5 % Yeast Extract

0.5 % NaCl

Adjutst to pH 7.0 with NaOH.

For 1 liter, dissolve 10 g tryphone, 5 g yeast extract and 5 g NaCl in 950 ml deionized water. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter. Autoclave for 20 minutes at 15 lb/sq. in. Let cool to ~55 °C and add desired antibiotics at this point. Store at room temperature or at +4 °C.

2.2 Yeast Extract Peptone Dextrose Medium - YPD or YEPD (1 liter)

1 % Yeast Extract

2 % Peptone

2 % Dextrose (glucose)

Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. Note: Add 20 g of agar if making YPD slants or plates. Autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D (20% Dextrose). The liquid medium is stored at room temperature. YPD slants or plates are stored at +4 °C. The shelf life is several months.

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2.3 Yeast Extract Peptone Dextrose Medium – YPDS + Zeocin™ Agar (1 liter)

1 % Yeast Extract

2 % Peptone

2 % Dextrose (glucose)

1 M Sorbitol

2 % Agar

100 μg/ml Zeocin™

Dissolve 10 g yeast extract, 20 g peptone and 182.2 g sorbitol in 900 ml of water. Note: Add 20 g of agar and autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D (20% Dextrose). Cool solution to ~ 60 °C and add 1.0 ml of 100 mg/ml Zeocin™. Store YPDS or plates containing Zeocin™ at +4 °C in the dark. The shelf life is one to two weeks.

2.4 Buffered Glycerol-Complex Medium and Buffered Methanol-Complex Medium – BMGY and BMMY (1 liter)

1 % Yeast Extract

2 % Peptone

100 mM Potassium phosphate, pH 6.0

1.34 % YNB

4×10^{-5} % Biotin

1 % Glycerol or 0.5 % methanol

Dissolve 10 g yeast extract and 20 g peptone in 700 ml of water. Autoclave for 20 minutes on liquid cycle. Cool to room temperature, then add 100 ml 1 M potassium phosphate buffer (pH 6.0), 100 ml 10X YNB, 2 ml 500X B (0.02% Biotin), and 100 ml 10X GY (10% Glycerol) and mix well. For BMMY, add 100 ml

10X M (5% Methanol) instead of glycerol. Store media at +4 °C. The shelf life of this solution is approximately two months.

3. Buffer

3.1 1X Equilibration/Wash Buffer (pH 7.0)

50 mM Sodium Phosphate pH 7.0
300 mM NaCl

3.2 1X Equilibration Buffer (pH 8.0)

50 mM Sodium Phosphate pH 8.0
300 mM NaCl

3.3 1X Elution Buffer (pH 5.0)

50 mM Sodium Phosphate pH 5.0
300 mM NaCl

3.4 Alkaline Lysis Solution I

50 mM Glycose
25 mM Tris-Chloride, pH 8.0
10 mM EDTA, pH 8.0

3.5 Alkaline Lysis Solution II

0.2 N NaOH
1 % (w/v) SDS

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3.6 Alkaline Lysis Solution III

60 ml	5 M Potassium Acetate
11.5 ml	Glacial Acetic Acid
28.5 ml	dH_2O

3.7 STE BUFFER

10 mM	Tris-Cl pH 8.0
0.1 M	NaCl
1 mM	EDTA pH 8.0

3.8 Tris-Glycine Buffer (1X)

25 mM	Tris-Cl
250 mM	Glycine

3.9 10X Tris EDTA (TE) pH 8.0

100 mM	Tris-Cl, pH 8.0
10 mM	EDTA, pH 8.0

3.10 1X Phosphate-Buffered Salive (PBS)

137 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
2 mM	KH_2PO_4

Dissolve 8 g of NaCl, 0.2 of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml of dH_2O. Adjust pH to 7.4 with HCl. Add dH_2O to 1 liter and sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle. Store the buffer at room temperature.

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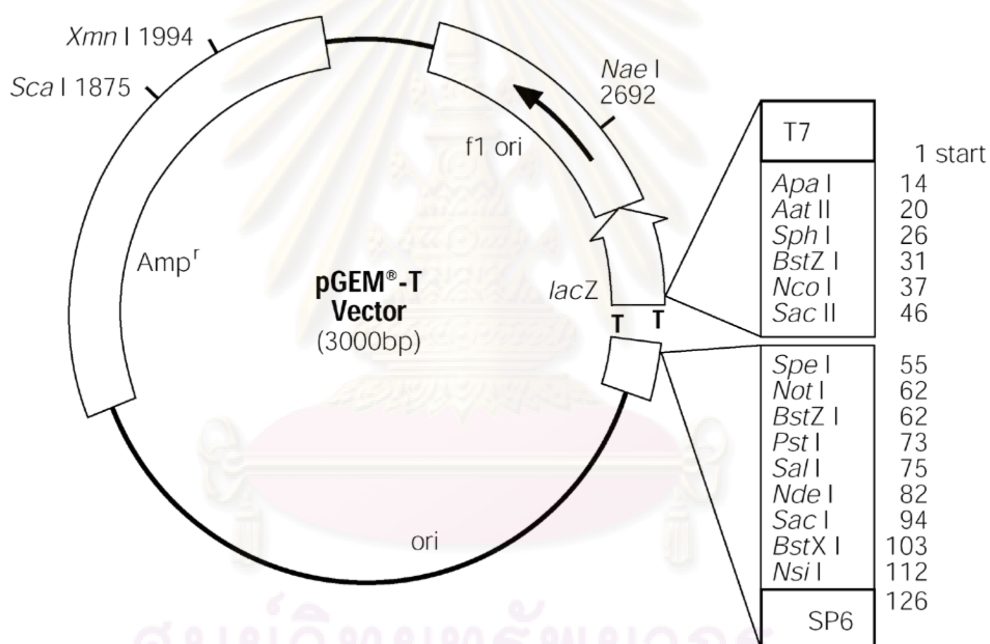
3.11 Blotting Transfer Buffer pH 8.3

20 mM	Tris-Cl
150 mM	Glycine
20 % v/v	Methanol

4. Vector

4.1 pGEM®-T Vector Circle Map and Sequence Reference Points.

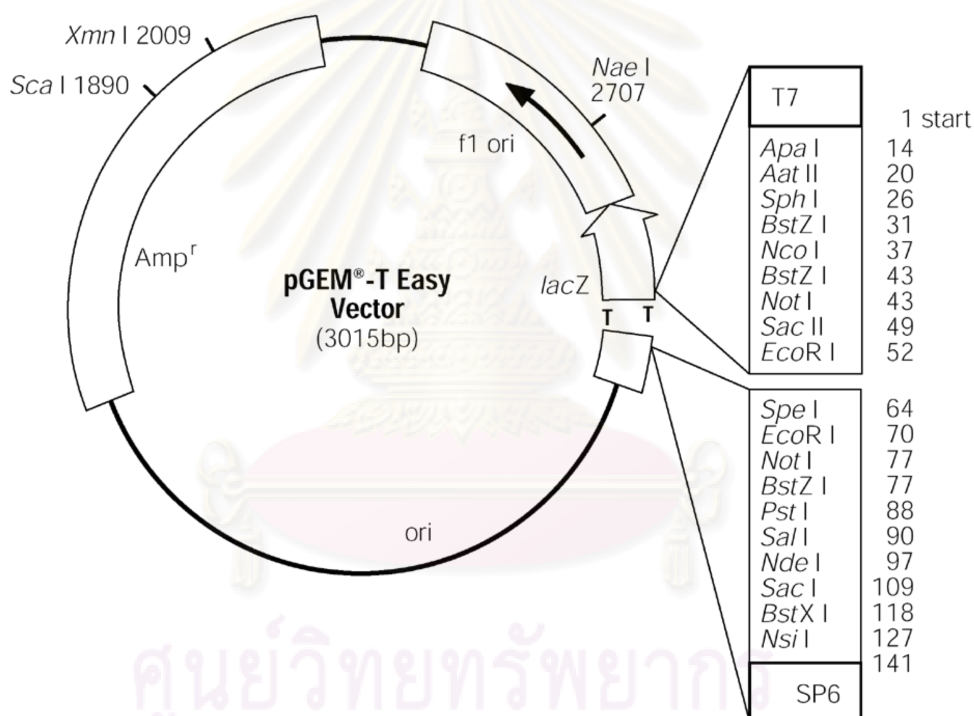


pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165

lacoperator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lacoperon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

4.2 pGEM®-T Easy Vector Circle Map and Sequence Reference Points.



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180

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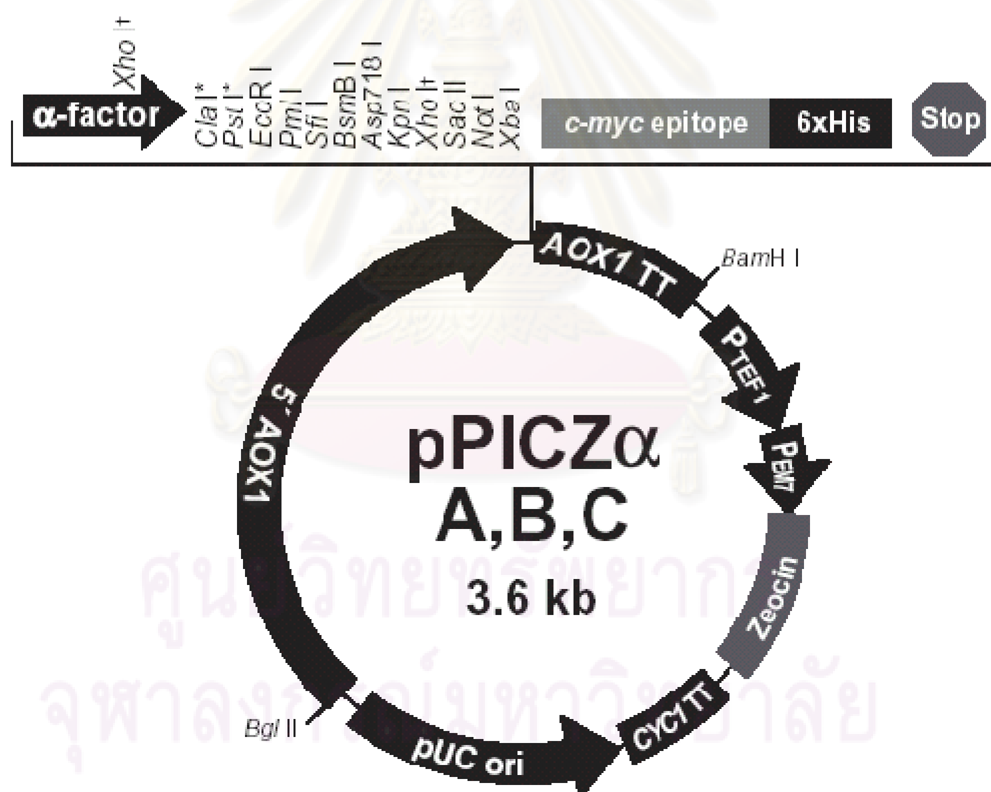
lacoperator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lacoperon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

4.3 Map of pPICZα A, B, C.



5. Others

5.1 DAB / $NiCl_2$ Visualization Solution

5 ml 100 mM Tris-C pH 7.5

120 µl DAB stock (40 mg/ml in H_2O, stored in 100 µl aliquots at –20 °C

25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100 µl aliquots at –20 °C

Mix just before use.

5.2 12 % Gel (5 ml) Resolving Gels for Tris-Glycine SDS-Polyacrylamide Gel Electrophoresis

1.6 ml H_2O

2.0 ml 30 % acrylamide mix

1.3 ml 1.5 M Tris, pH 8.8

0.05 ml 10 % SDS

0.05 ml 10 % ammonium persulfate

0.002 ml TEMED

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5.3 5 % Stacking Gel (1 ml)

0.068 ml H_2O

0.17 ml 30 % acrylamide mix

0.13 ml 1.0 M Tris, pH 6.8

0.01 ml 10 % SDS

0.01 ml 10 % ammonium persulfate

0.001 ml TEMED

0.1 % SDS

5.4 2X SDS Gel-Loading Buffer

100 mM	Tris-Cl, pH 8.8
4 % w/v	SDS
0.2 % w/v	bromphenol blue
20 % v/v	glycerol
200 mM	dithiothreitol or β-mercaptoethanol

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2005	Trainee in Medial Laboratory, Lampang Hospital, Lampang , Thailand.
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