CHAPTER I

INTRODUCTION

1.1 Background and Rationale

There are more than 160 species of snakes in Thailand, 46 of which are venomous. Venomous snakes can be divided in 3 major groups. The first group is neurotoxic snakes, such as Naja, Ophiophagus and Bungarus. The second group is myotoxic snakes, such as sea snakes. The third group is hematotoxic snakes, such as Russell viper, green pit vipers and Malayan pit viper *(1)*. There are two important families of the venomous snakes in Thailand that are elapidae and viperidae, respectively. Elapidae consists of Cobra, Spitting cobra, King cobra, Banded krait and Malayan krait. Viperidae family can be classified into two subgroups: the true viper (Viperinae) and the pit viper (Crotalinae). The true viper subgroup consists of Russell's viper or *Daboia russelli*. They are most commonly found in the central and eastern parts of Thailand. The pit viper subgroup consists of Malayan pit viper and Green pit viper. Malayan pit vipers are found in the southern, eastern and northern parts of Thailand. The incidence of venomous snakebite in Thailand is about 11,000 per year with the mortality rate around 0.02 per 100,000. Most important species are Malayan pit viper (40%), Green pit vipers *Trimeresurus spp.* (34%), Cobra (12%), and Russell's viper (10%) (2,3) Green pit viper are hematotoxic, The patients are suffured from mild to severe pain, local edema and systemic hemorrhage. Green pit viper (GVP) is one of the most common species responsible for venomous snake bites in Thailand *(2)*. More than 300 new cases came to the snake bite clinic at Chulalongkorn hospital each year. Green pit viper is small to medium sized and usually stays on a tree. Among green pit vipers, *Trimeresurus albolabris* has the most toxic venom *(3)* and the most common biting species. Therefore, it is a major health problem. *T. alb*olabris is in

Phylum Chordata and Family viperidae. They have a pair of pit organs that can detect minimal temperature changes especially in warm-blooded preys.

1.1.1 Green pit viper

Green pit vipers (GPVs) are venomous snakes having long fangs that can fold in normally up against the upper jaw and be used to transfer venom when they catch the preys. GPVs have a pair of heat-sensing pit organs located between nostrils and eyes on each side of the head. The pit organ is a thermo-receptor sensitive to very small changes in temperature when they stay near their preys. It is supplied with nerves and blood vessels and partially enclosed in a cavity on the side of the maxillary bone. GPVs characteristically have a broad, lance-shaped head and vertical pupils. They are generally slender in shape. GPVs body is green in color with yellow markings and tail is brown in color. They are small snakes, usually attaining less than 1.5 meter in length. They usually stay on tree and hang on by their tail. GPVs are members of the genus *Trimeresurus*. The two most common species are *Trimeresurus albolabris* and *Trimeresurus macrops (4)* that are most prevalent in Bangkok and the nearby areas. *Trimeresurus albolabris* is a tree-dwelling snake with a red tail and a yellow belly.

1.1.2 Clinical signs and symptoms of green pit viper bites

Clinical features and laboratory investigations following bites by Russell's viper and Green pit viper were described (4). Bleeding was the most important manifestation of envenomation. Clinically, toxic patterns were classified into local and systemic. The local effects start to appear within 30 to 60 minutes after pit viper envenomation. Local effects include pain, edema, blister, hemorrhage and ecchymosis at the bite sites. The local toxicities were more marked in Green pit viper victims than in Russell's viper. The systemic effects include coagulopathy, hypofibrinogenemia and

thrombocytopenia (*6*, *7*, *8*). GPV bites may also result in hypotension, respiratory distress, or severe tachycardia. Digital bites developed gangrene more frequently than other bitten sites *(4)*. Endogenous fibrinolysis is activated causing bleeding from low fibrinogen. The clinical characteristic of GPV b ites, similar to other venomous snakes, is the presence of one or more fang marks that look like puncture wounds with or without scratches. Identification of these venom components gives us not only deeper insights in the pathogenesis of snakebites, but also potential novel anti-thrombotic agents. Although the ELISA test for diagnosis of *Trimeresurus spp.* is available in Thailand, it is impractical for general use. Characterization of the specific venom components will lead to more rapid immuno-diagnosis and prognostication because active components can be specifically measured. The production of antivenom specifically to the active venom components may lower severe reactions.

1.1.3 Component of green pit viper venom

Approximately 90 percent of pit viper venom components are proteins that can be categorized into serine proteinases, phospholipases A_2 (*9*), C-type lectins (10), metalloproteinases, and disintegrins (11, 12, 13).

Serine protease is a family of proteins that contain histidine, aspartate and serine residues on the catalytic sites. Most fibrinogen clotting (thrombin-like), fibrinogenolytic and plasminogen activating enzymes from snake venoms of *T. Albolabris* belong to this family. Coagulant effect is the strongest effect of the venom. It makes blood clotted through its 'thrombin like effect'. Similar to thrombin, fibrinogen is cleaved, releasing a small fragment called fibrinopeptide A (FpA) and initiating polymerization. However, fibrinopeptide B (FpB) is not released and fibrin-crosslinking factor XIII is weakly stimulated, as opposed to thrombin effect (7*)*. GPV causes hypofibrinogenemia although other clotting factors levels are normal (8,9,10*)*. The closely related viper, Malayan pit viper also causes FpA cleavage but not FpB, resulting in defibrination syndrome. The thrombin-like proteases from GPV have not

been purified. By protein purification, fibrinolytic activator in GPV venom was shown to be in a different fraction from the coagulant *(11)*. Therefore, the fibrinolytic agents in GPV venom are unique and wait for characterization. Molecular cloning of cDNA of venom proteins will give more complete and accurate protein sequences and provide an opportunity to express and engineer recombinant proteins for future uses. These proteases may lead to novel anticoagulant and thrombolytic agents.

Phospholipase A_2 (PLA_2) is the major component in the snake venom. PLA_2 is a non-glycosylated proteins that can induce a wide variety of pharmacological effects by interfering with several physiological functions, including endothelial injury, myotoxicity, thrombosis and hemostasis. Several isoforms of PLA_2 have been found in a single species and various isoforms are different among different species.

Snake venom C-type lectins contain amino acid sequence homology to the calcium regulatory domain of mammalian lectins. Proteins in this family typically comprise disulfide-linked $\alpha\beta$ heterodimers. C-type lectins have been demonstrated to promote platelet aggregation by targetting von Willebrand factor, glycoprotein Ib-IX-V, glycoprotein VI, and possibly 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 spacer region, 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, e.g., 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 (*14*).

1.1.4 Coagulation effects

Blood coagulation is a system that maintains the combination of the high-pressure and closed circular system. After tissue injury, destruction of the capillary bed and injury of venules and arterioles lead to extravasculation of blood into soft tissues or external bleeding. Blood coagulation is initiated by tissue factor (TF) expressed on extravascular cell surfaces. When plasma comes in contact tissue factor, factor VIIa in plasma binds to this receptor. The factor VIIa/tissue factor complex activates factor IX to IXa and factor X to Xa factor Xa and the cofactor (Va) changes prothrombin (II) to thrombin. The proteolytic activation of factor XI to XIa, factor VIII to VIIIa, and factor V to Va through positive feedback mechanisms by thrombin accelerates the rate blood clotting.

In vitro, the generation of thrombin and the formation of a fibrin clote propagate through two separate pathways, the intrinsic pathway and extrinsic pathways that were showed in **Figure 1**.

The intrinsic pathway of blood coagulation includes protein co-factors and enzymes. The pathway is initiated by the activation of factor XII by kallikrein on negatively charged surfaces, including glass in vitro. These contact-activated factors of the intrinsic pathway activate factors XII, XI, IX, VII, X and V, prothrombin and fibrinogen respectively.

The extrinsic pathway of blood coagulation also includes protein cofactors and enzymes. The pathway is initiated by the formation of a complex between tissue factor on cell surfaces and factor VIIa. Glycoprotein components of the extrinsic

pathway, initiated by the action of tissue factor located on cell surfacesans activate factors VII, X, and V, prothrombin, and fibrinogen, respectively.

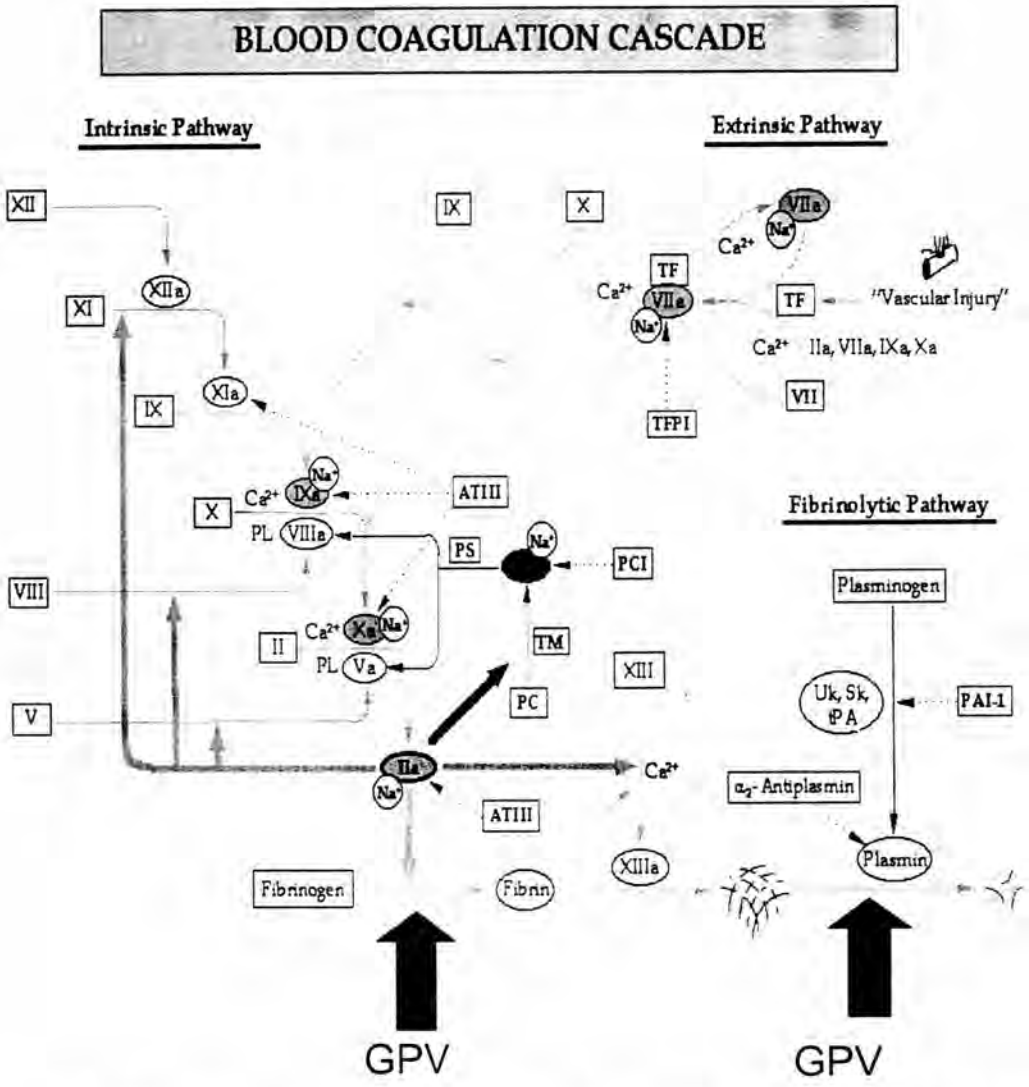


Figure 1. Blood coagulation cascade. Cascade reactions are activated by contact factors or tissue factor (TF) and culminate in the conversion of fibrinogen to fibrin and the formation of a fibrin clot. GPV contains the enzymes that can activate fibrinogen to fibrin and a fibrinolytic enzyme that can activate plasminogen to plasmin. [8].

1.2 Research Questions

1. What is the sequences of *Trimeresurus albolabris* ?

2. What are the complete nucleotide sequences of various serine proteases from *Trimeresurus albolabris* and what are the predicted functions using sequence analysis?

3. Does the recombinant snake venom serine proteinase from *Trimeresurus albolabris* contain the predicted activity?

4. Does albofibrase have the anticoagulant activity in human plasma?

1.3 Objectives of the Study

1. To show cDNA library of *Trimeresurus albolabris*

2. To clone and analyze complete cDNA sequence of snake venom serine proteinase from *Trimeresurus albolabris*.

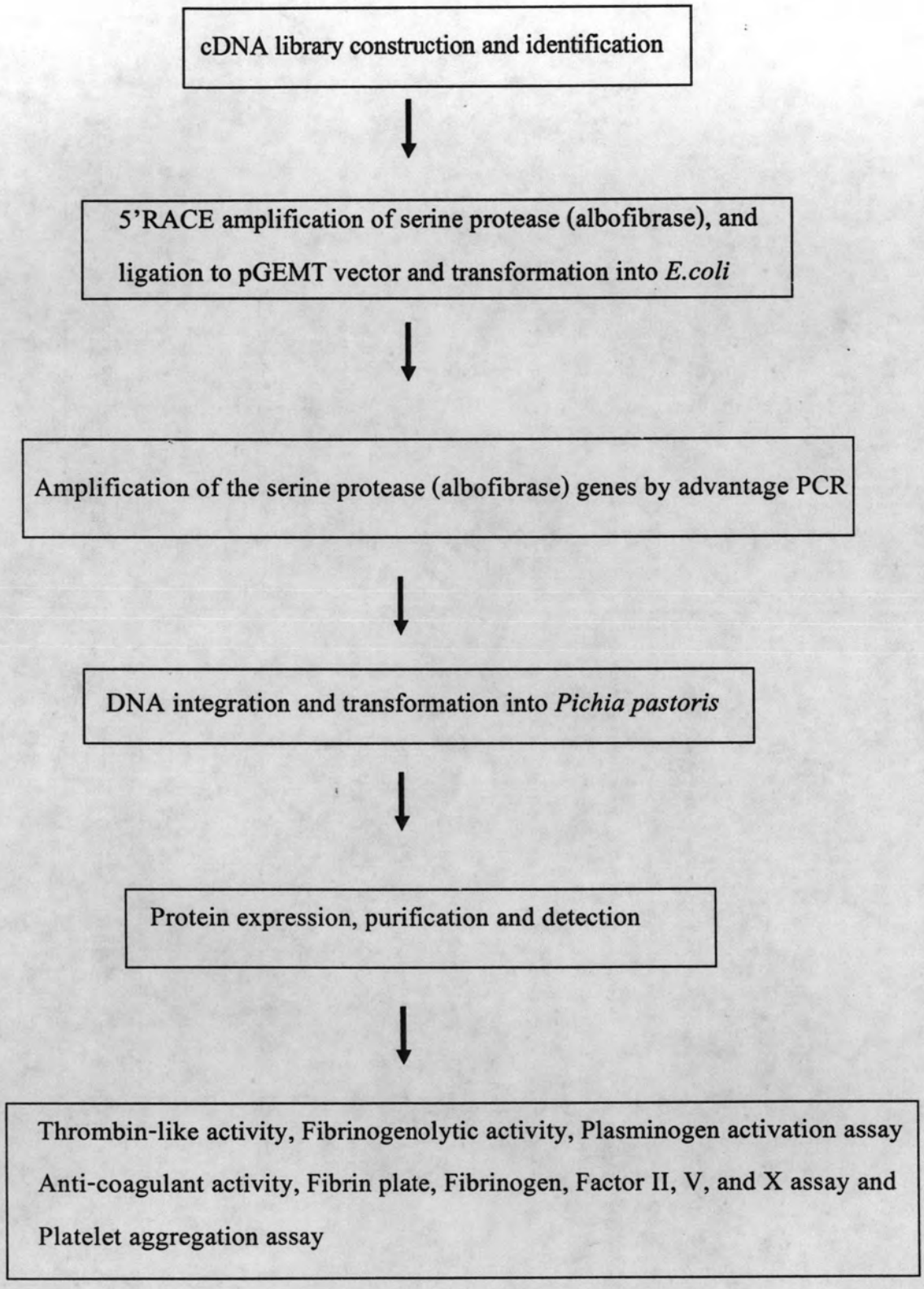
3. To express and purify the snake venom serine proteinase (albofibrase) in *Pichia pastoris* system.

4. T o study the effects of snake venom serine proteinase (albofibrase) on human coagulation.

1.4 Keywords

Snake venom serine proteinase, albofibrase, *Trimeresurus albolabris*.

1.5 Conceptual Framework



1.6 Benefits and Applications

1.The study will give us deeper insights in the structure-function relationship of the snake venom serine protease proteins and the molecular pathogenesis of green pit viper envenomation.

2.These proteins are potentially useful as diagnostic agents and may lead to a novel anticoagulant.