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.

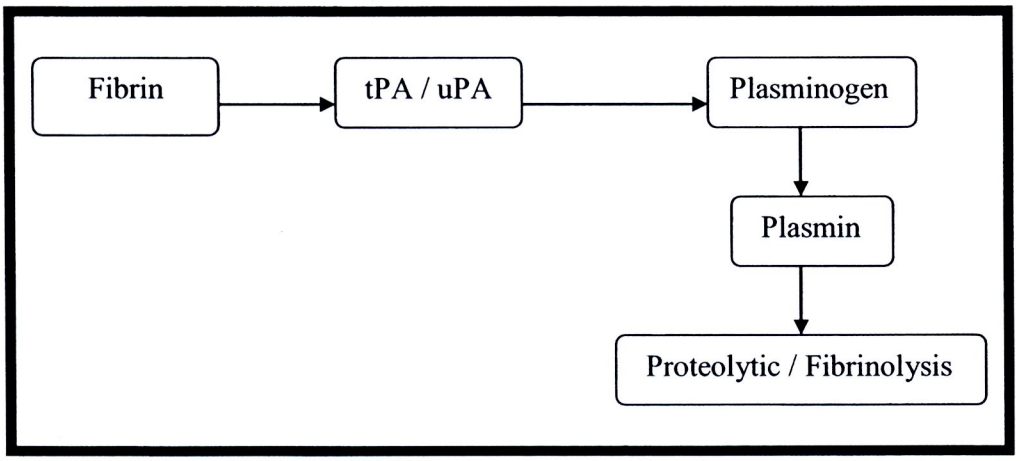


Fig 1. An overview of the fibrinolytic system. *tPA,* Tissue plasminogen activator; *uPA*, urokinase-type plasminogen activator.

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

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

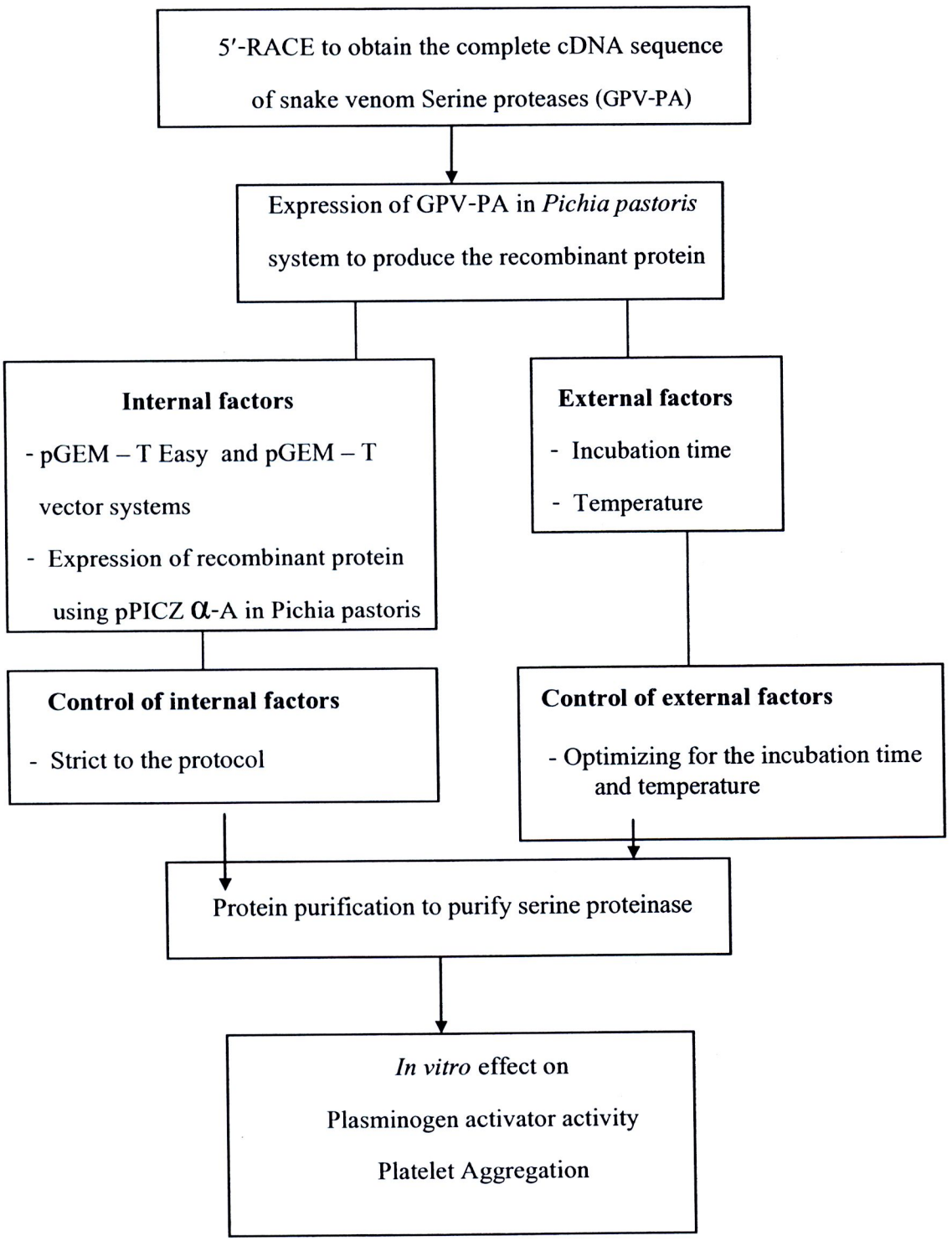
Serine Protease

Plasminogen activator

Green Pit Viper

Pichia pastroris

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.