CHAPTER I
INTRODUCTION

1.1 Background and Rationale

1.1.1 Snake bites and green pit viper venom

Green pit viper bites are common in Thailand especially in Bangkok (Mahasadana and Jintakune, 1990). The two most common species are *Crytelytrops albolabris* and *Cryptelytrops marcrops*, which are the same genus in the Crotalinae subfamily. Therefore, venoms of these two species are closely related. The victims' severity ranges from minimal local pain and swelling to severe pain, marked edema, tissue necrosis, systemic hemorrhage, shock and death.

Two important families of the venomous snakes in Thailand are elapidae and viperidae that are neurotoxic and hematotoxic, respectively. Elapidae consists of Cobra, Spitting cobra, King cobra, Banded krait and Malayan krait. They are widely distributed throughout Thailand. Viperidae family can be classified into two subgroups: the true viper (Viperinae) and the pit viper (Crotalinae). Green pit vipers are the most common venomous snakes, which are found most frequently in the central part of Thailand. Green pit vipers are members of the genus *Cryptelytrops* that are most prevalent in Bangkok and the nearby areas (Mahasandana and Jintakune, 1990). *Cryptelytrops albolabris* is a tree-dwelling snake with a red tail and a yellow belly. It is almost always responsible snake bites in Bangkok.

Snake venom can be classified by their effects into three categories: neurotoxin, myotoxin and hematotoxin. Green pit viper venom affects the hemostatic system. The clinical characteristic of green pit viper bites, similar to other venomous snakes, is the presence of one or more fang marks that look like puncture wounds with or without scratches. The toxic symptoms can be divided into two categories: local and systemic effects. The local effects start to appear within 30 to 60 minutes after pit viper envenomation. These effects include pain, edema, blister, hemorrhage and ecchymosis at the bite sites (Trishnanaada, 1979). The systemic effects include coagulopathy, hypofibrinogenemia and thrombocytopenia (Rojnuckarin *et al.*, 1999).

Green pit viper bites may also result in hypotension, respiratory distress, or severe tachycardia.

1.1.2 Components of green pit viper venom

Ninety percent of pit viper venom is proteins that can be categorized into serine proteases, phospholipases A_2, C-type lectins, metalloproteinases, and disintegrins. Snake venom metalloproteinases (SVMPs) are multi-domain proteins that are composed of a catalytic and one or several non-catalytic domains. SVMPs are homologous to mammalian proteins in a disintegrin and metalloproteinase (ADAMs) family which found in eukaryotic cells displaying different expression patterns (Sagane *et al.*, 1998). They are involved in cell-cell and cell-matrix interactions by catalytic degradation of extracellular matrix protein and integral membrane proteins to reduce cell adhesion (Duffy *et al.* 2003).

Both SVMPs and ADAMs are parts of the "metzincin" family of zinc-dependent metalloproteinases, together with matrix metalloproteinases (MMP), astacins, and serralysins. Snake venom metalloproteinase (SVMPs) are members of the Reprolysin subfamily of these zinc-dependent enzymes. They are responsible for hemorrhagic effects and local myonecrosis after snakebites, as well as involved in provoking inflammatory reactions. All of them exhibit an identical zinc-dependent motif, the consensus sequence HEXXHXXGXXH with the presence of a methionine turn. SVMPs are readily classified according to their peptide domain structures into four basic groups, P-I to P-IV. All four groups share homologous signal, pro-domain and protienase domains. While P-I comprises only metalloproteinase domain; P-II have a metalloproteinase domain followed by a disintegrin domain; P-III comprises metalloproteinase, disintegrin-like and cystien-rich domain, and P-IV is a P-III enzyme that contains an additional lectin-like domain linked by disulfide bonds. These protienase are synthesized in the venom gland as zymogens, which are subsequently processed to the active forms.

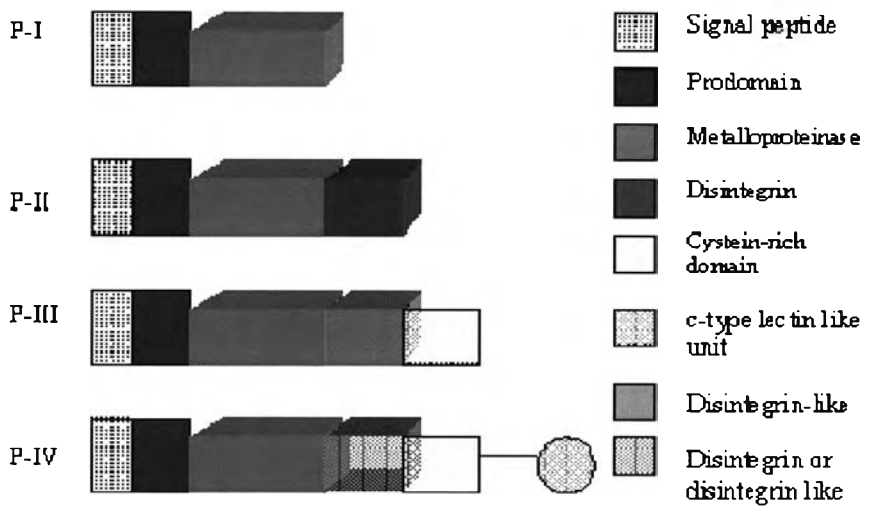


Figure 1 Classification of venom metaloproteinase

Signal peptide is composed of eighteen amino acids that mostly are hydrophobic residues and functions as a protein secretion marker.

The pro-domain modulates the enzymatic activities through interactions with the catalytic domain. It is highly conserved among the SVMP members.

The metalloprotease or catalytic domain is composed of approximately 215 amino acids. It contains metal-dependent endopeptidase activity and is less conserved than the pro-domain. This suggests that these enzymes have evolved from gene duplication from a common ancestor and that the metalloprotease domain has passed trough an accelerated evolution process.

The spacer peptide can also be found comprising long disintegrins and disintegrin-like domains. In these cases, an extra cysteine residue present in this segment is involved in disulfide bond formation with the downstream domain. In some SVMP members, it can be probably related to the resistance to proteolytic processing (e.g. Jerdonitin).

The disintegrin and disintegrin-like domains vary in lengths from 41 to about 100 residues and contain a high content of cysteines forming disulfide bonds. They have been report to be the strong integrin ligands. Interestingly, this binding can block

integrin ligand binding or trigger integrin-mediated biological effects. This suggests several potential clinical applications. Most disintegrins are released from PII SVMP precursors and have an RGD motif that is relevant for binding to β_3 integrins (e.g. $\alpha_{IIb}\beta_3$, platelet fibrinogen receptor, and $\alpha_v\beta_3$, vitronectin receptor) and β_1 integrins. In P-III class, DCD (Asp-Cys-Asp) or ECD (Glu-Cys-Asp) domains are present at this homologous position, instead of RGD (Arg-Gly-Asp) in P-II class. They do not bind integrins and have no disintegrin folds. Therefore, they are called disintegrin-like domains. (Bjarnason, 1995), (Ramos, 2006)

The cysteine-rich domain, like the disintegrin domain, has a high density of cysteines. It is located C-terminally to the disintegrin domain in PIII SVMPs. The cysteine-rich domains of jararhagin and atrolysin A specifically inhibited the platelet aggregation induced by collagen, ristocetin in the presence of vWF or ADP in the presence of fibrinogen (Ogawa *et al.*, 2005). It was also found that the adhesion of $\alpha_2\beta_1$integrin-transfected K562 cells to collagen was inhibited by these peptides.

The C-type lectin-like subunits were found associated with only two representative members of PIV SVMPs, RVV-X and VLFXA. These proteins are synthesized from three independent mRNAs that codes for a PIII metalloprotease (heavy chain) and two type-C lectins. Therefore, it is appropriate to believe that PIV SVMPs are in fact a modification of PIII SVMPs. (Ramos, 2006)

1.1.3 SVMPs actions on hemostatic system

P-II class SVMPs contain a metalloproteinase domain followed by a disintegrin or disintegrin-like domain. These venom metalloproteinases generally show hemorrhagic activity causing hemorrhage by digestion of components of extracellular matrix (ECM) proteins, e.g. collagen and laminin. In addition, these proteins can also digest some blood coagulation factors, including von Willebrand factor, enhancing its hemorrhagic effect. Inhibition of these proteins may have therapeutic roles in the treatment of snakebites. Disintegrin proteins contain either the Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) conserved sequences. The RGD sequence in these peptides is a potent inhibitor of integrins, such as $\alpha_{IIB}\beta_3$

(fibrinogen receptor on platelets surface). Analogs of these proteins have been used clinically as antiplatelet agents (Hayashi and Camargo, 2005).

Studies of SVMPs have described their activities on inflammation, hemostasis and invasion of cancer cells on extracellular matrices. In general, studies have been focused on the direct affects of crude or lyophilized venoms or proteomic studies on molecular weights, amino acid sequences and their compositions.

In this study, we are interested in cloning and recombinant expression of a snake venom metalloprotease in the yeast *Pichia pastoris* system. The expression products were tested to investigate the function on hemostasis, such as fibrinolytic activity, platelet aggregation, as well as hemorrhagic activities.

1.2 Research Questions

What are the functions of metalloproteinase and disintegrin in P-II class metalloproteinase from *C. albolabris* on the hemostatic system (Platelets aggregation and/or blood coagulation)?

1.3 Hypothesis

There are activities from recombinant protein on hemostatic system. It can inhibit platelets aggregation and/or degrade fibrinogen.

1.4 Objectives of the Study

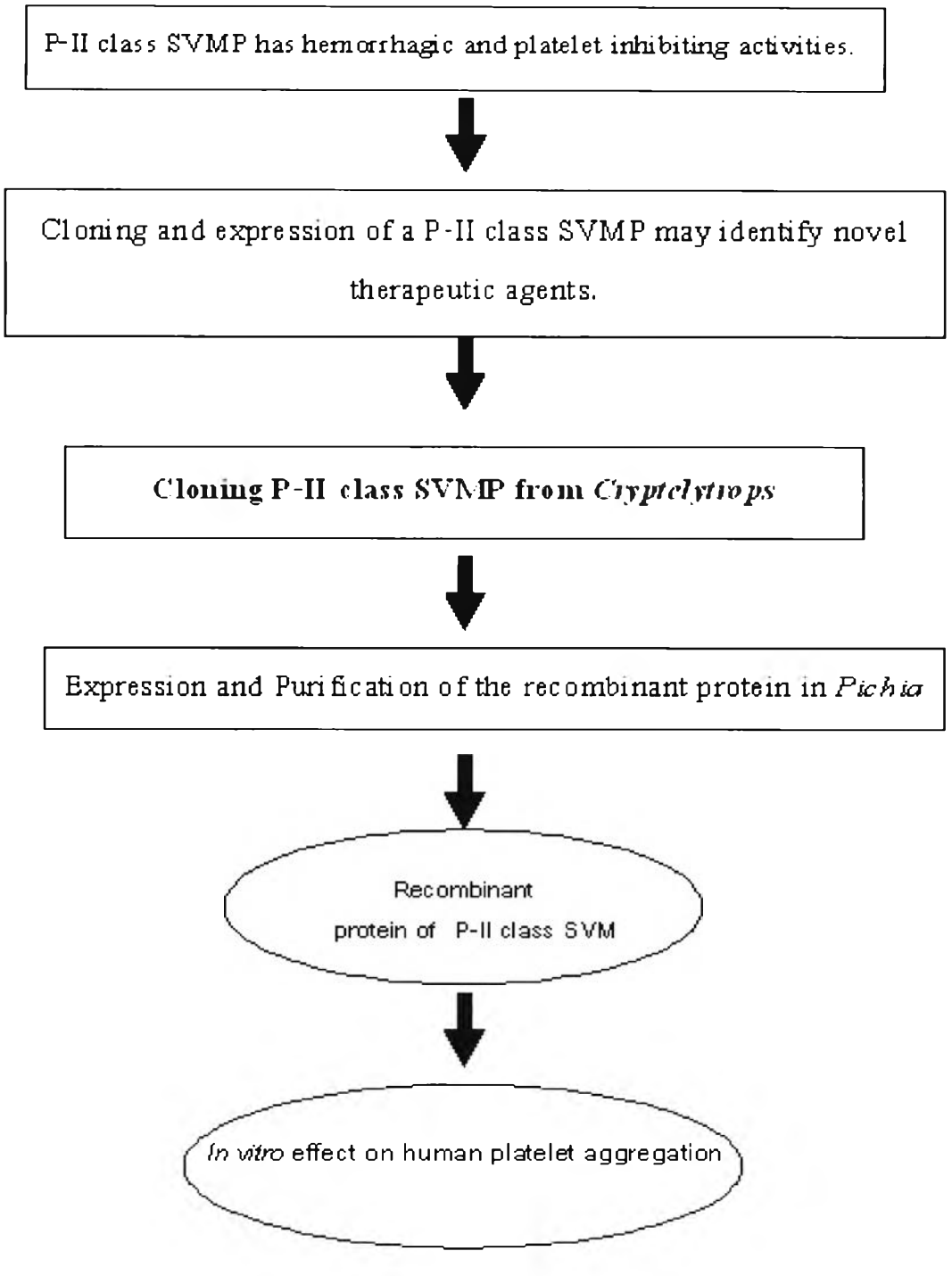
1.4.1 Expression and purification of recombinant metalloproteinase and disintegrin domain of an SVMP *in Pichia pastoris* to investigate the recombinant protein function on hemostatic system.

1.4.2 Identification of protease inhibitors that inhibit their activities in order to investigate for their uses in snakebite patients.

1.5 Keywords

Snake Venom Metalloproteinase, Disintegrin, *Pichia patoris expression*, *Cryptelytrops albolabris*

1.6 Conceptual framework and laboratory process



1.7 Benefits and Applications

1.7.1 Expression of recombinant P-II class SVMPs from *Cryptelytrops albolabris* may allow us to identify the active component of SVMPs that cause hemorrhage and tissue necrosis in snake bite victims. The current antivenoms used clinically are less effective in preventions and treatments of the local tissue damages. Therefore, searching for the inhibitors that can decrease the activities to SVMPs may lead to more efficient ways in managing patients

1.7.2 Expression of recombinant P-II class SVMP may derive potentially novel therapeutic agents affecting hemostatic system. The recombinant protein with inhibitory activity on platelets may be used as an anti-thrombotic agent or developed to be a reagent for testing the mechanisms of platelets aggregation.

1.7.3 Several SVMPs have anti-tumor activities. By inhibiting integrins, they may suppress tumor growth, enhance apoptosis or inhibit tumor angiogenesis. Therefore, our SVMPs should be investigated for the activities on tumor cell lines and experimental models of angiogenesis in the future.