CHAPTER V
DISCUSSION

In this study, we found that the recombinant *C. albolabris* snake venom metalloprotese (SVMP) class P-II with N-terminal 6-histidine-tag could be expressed in *Pichia pastoris* system. The other systems that had been used before were Baculovirus and *E.coli* systems yielding different amounts of proteins and functions. The expressed proteins, such as the P-II class SVMP Jerdonitin, were compareed with native proteins that comprise metalloproteinase domain and disintegrin. Our P-II SVMP is another novel protein that will be discussed in this part.

Snake venoms have been investigated for their wide variety of activities and functions. Many studies were perform using crude or lyophilized venoms to determine effects on hemostatic system and hemorrhagic activity. The functions of native proteins have been compared among others. On the other hand, other studies used recombinant venom proteins from various expression systems. In the present study, molecular technique is utilized to insert the cloned cDNA and express the SVMP form *C. albolabris*. The advantages of using a recombinant protein are that there is no potential contamination and, more importantly, the activities can be correlated with their amino acid sequences. The latter information will be very useful for future protein engineering for research, diagnostic or therapeutic uses.

The eukaryotic system of *Pichia pastris* was applied because its correct post-translational modifications could lead to a functional protein that would be secreted in to the media. The histidine insertion was used to aid protein purification using affinity chromatography. The N-terminal polyhistidine was utilized because the C-terminus of the disintegrin was reported to be important for protein function (Fujii *et al.*, 2003). The expected molecular weight of the metalloprotease and disintegrin domain was 30-35 kDa from calculated sequence by protein molecular weight program. After induction by 5% methanol for 96 hour at 30° C, the protein was produced showing approximately 32 kDa in size, which was close to the prediction. The yield of recombinant protein was 8.5

mg/Liter of culture media, which is higher than that of Singjamatr, 2006, which yielded 3.3 mg/Liter of media and close to that of Guo et al,2001 who produced rhodostomin in *Pichia pastoris* yielding 5 – 10 mg/Liter of media. Because of the eukaryotic system was used and the correct size of the protein was produced, it should show the activities. The recombinant protein displayed an inhibitory activity on platelets aggregation in a dose-dependent manner. After incubation with PRP for 5 minutes and instant addition of collagen, an agonist, a inhibitory activity was detected. Figure 9 showed that collagen-induced aggregation was markedly inhibited suggesting that our recombinant protein was active. This might have been mediated by the binding to integrins on the platelets surface.

The roles of proteases in snakebite pathology had been noted since 1881 showing that snake venoms contained proteolytic activities (Porges, 1953). In 1960, Japanese investigators isolated proteins from viperid venom. These proteinase activities could be inhibited by EDTA and, therefore, these were in a metalloproteinase family (Maeno, 1960, Okonogi *et al.*, 1960). SVMPs possess many biological activities, such as hemorrhagic, fibrinolytic, fibrinogenolytic and pro-apoptotic functions, as well as activation of factor X and inhibition of platelets aggregation (Fox and Serrano, 2005.). Activities of SVMPs depend on their domain compositions that categorized their types into P-I to P-IV. (Fox and Serrano, 2009) Our novel recombinant protein belonged to class P-II of SVMPs composing of metalloproteinase together with disintegrin domain . The cDNA sequence of our SVMPs was the most homologous to Jerdonitin from *Trimeresurus jodonii*. Jerdonitin expressed in *E.coli* system using pMD18-T vector dose-dependently inhibited ADP- induced human platelets aggregation (Chen *et al.* 2003). Our *C. albolabris* SVMPs could not inhibit ADP-induced but could inhibit collagen-induced platelet aggregation. This may suggest that the mechanisms of inhibition were dissimilar, although the sequences were homologous. Jerdonitin and Akistin from *Akiston haly* were homologous to each other. In contrast to other P-II SVMPs, metalloproteinase and disintegrin domain of Jerdonitin do not separated from each other, similar to the two domains of Akistin. This is because Jerdonitin and Akistin contain 2 extra cysteine residues in spacer and disintegrin domains. They probably form a disulfide bond preventing the disinterin domains to be processed away from

metalloproteinase. (Fox *et al.*, 1995 ,Siiguret *et al.*, 1996, Jeon *et al.*,1999,). Our SVMP 038 contains all conserved cysteines similar to jerdonitin. Therefore, it was predicted to form a disulfide bond that holded metalloproteinase and disintegrin domains together

SVMPs have been implicated in systemic bleeding in snakebite patients. The metalloproteinase domain of SVMPs has hemorrhagic activity degrading various substrates in extracellular matrix in vascular basement membrane and can cause endothelial cell apoptosis. In addition, SVMPs have been shown to contain pro-inflammatory effects causing further damages around biting wounds (Teixeira *et a.,l* 2005,Fox, 2008,). The *C. albolabris* P-II class SVMP is likely to contain the hemorrhagic activity as deduced from the sequence data that are homologous to other hemorrhagic SVMPs, It should have degrading activities on extracellular matrix (ECM) components, such as collagen and laminin and probably contribute to local tissue damages of patients after green pit viper bites. However, this activity was not tested in this study.

In addition to metalloprotease domains, disintegrin domain of SVMPs affect blood homeostasis by competitive inhibition of fibrinogen binding to platelets αIIb β3 integrin receptor through their RGD sequence. Albolatin, a disintegrin domain of *Cryptelytrops alborabris* was previously expressed in *Pichia pastoris* system and found to inhibit collagen-induced platelet aggregation. Albolatin contained KGD motif (Singhamatr and Rojnuckarin, 2006). The P-II SVMP in this study possessed the RGD sequence in its disintegrin domain. Notably, it also inhibited collagen-induced platelet aggregation without affecting ADP-induced aggregation, similar to albolatin. The platelet αIIb β3 integrin is important for aggregation by all agonists, except for ristocetin. Therefore, both of the *C. albolabris* were unlikely to inhibit this fibrinogen receptor. The molecular mechanism of platelet inhibition by SVMPs from green pit viper remains to be determined.

Stejnitin, the P-II SVMP from a Chinese snake *Trimeresurus stejnegeri* , was cloned and expressed. It was found to inhibit ADP-inducted platelet aggregation similar to jerdonitin. However, it also contained proteolytic activities on Aα chain of fibrinigen but no effect on γ chain. In addition, stejnitin showed pro-apoptotic activities on treated

ECV304 cells and urinary bladder carcinoma cell line, T-24. Stejnitin contained a KGD sequence instead of an RGD in the disintegrin domain. The characteristic of cysteinyl residue found at position 221 and 240 were similar to Jerdonitin but different from other P-II. This disulfide bond has an importance role in maintaining the disintegrin and metalloproteinase domain together. Therefore, they were classified as P-IIa SVMPs class by Fox and Serrano, 2005.

Agkistin, a P-IIa SVMP, and Akistin-s disintegrin domain from *Akistrodon halys* with HIS-Tag were expressed in baculovirus expression system using the pFastBac-Hbt vector. It inhibited the ADP-induced platelet aggregation and could induce morphological change and apoptosis of human mammary epithelial cells (HMEC cell) after 20 hours incubation. The Akistin-s disintegrin domain with RGD sequence was about two times more efficient than the full-length protein Akistin in term of inhibitory activity on HMEC apoptosis. The result showed that the disintegrin contribute significantly to these activities. (Shoa *et al.*, 2002)

ACLF, the fibrinolytic non hemorrhagic metalloproteinase from *Akistrodon contortrix lacticints*, was insert in the pET28a vector and expressed in *E.coli* BL21(DE3). The expressed protein was successfully refolded, but it rapidly underwent autolysis. Subsequently, pro-ACLF with pro and metalloprotease domains was expressed in same system. After protein purification under a denaturing condition and refolding, it was active on fibrinogen and synthetic substrates. The rapid removal of denaturing agent kept the protein unprocessed and, after addition of Ca^{2+} and Zn^{2+} ion, the enzyme was reactivated. Nevertheless, it was less active than native ACLF. Using this method, they can activate a product immediately and store a pro-enzyme for future experiments (Ramos *et al.*, 2002). This expression system not only has active recombinant protein of SVMPs but also solves the unstable or degraded conditions from other methods. However, it still requires high amounts of recombinant proteins compared with the native form as it contains less activity.

The recombinant ACLH zymogen, rProACLH, and the catalytic domain of jararhagin (rDJARA), the hemorrhagic SVMPs from *Brothrops jararaca* and *Akistrodon*

controtrix lacticintus, respectively, were expressed in *E.coli*. This system does not make post-translational modification leading to the misfolded recombinant proteins, which show no hemorrhagic activity on basement membrane. However, the native deglycosylated ACLH and Jararhagin contain proteolytic activities on fibrinogen and fibronectin. (Garcia *et al.*, 2004)

Most SVMPs function via digesting the component of capillary basement membrane or digesting blood coagulation factors (Bjarnason and Fox, 1995). Post translational modifications contribute to hemorrhagic activity and specify substrates of a catalytic domain. Glycan domains of SVMPs were found to affect their active sites. This glycosylation process was performed in endoplasmic reticulum affecting the structure conformation and folding of proteins (Wormald and Dwek,1999; Elbein, 1991; Hart ,1992). The effects of protein glycosylation on hemorrhagic activity were tested by adding an N-deglycosylating enzyme to native Jararrhargin and ACLH. After 27 hours at 37°C incubation, both SVMPs lost their hemorrhagic activity and autolytic degradation. This finding supports the significance of N-glycosylation on enzymatic activity of SVMPs. Therefore, the use of a eukaryotic expression system is required for full protein activities.

Supporting the role of glycosylation, Toshiaki *et al* (2002) isolated bilitoxin-1 from *Akistrodon billineatus*, which was a dimeric SVMP with lethal hemorrhagic and casein hydrolytic activities. It contains several carbohydrates including fucose, galactosamine, glucosamine, galactose, mannose N-acetyl neuraminic acid and N acetyl glycosylneuraminic acid in the molecule. By removal of sialic acid, the hydrolytic activity on casein was unaffected but 75% of hemorrhagic was lost. Furthermore, N-link glycosylation site in bilitoxin-1 play a role in the hemorrhagic activity but not in proteolytic activity.

The RGD-containing disintegrin, Brothrostatin from *Brothrops jararaca*, was expressed in *E. Coli*. Notably, it showed high inhibitory activity on collagen-induced platelets aggregation (IC_{50} =12nM). Its RGD sequence was present very close to C-terminus forming finger –like structure, which was maintained by the Cystein 47-Cystein66 bond. In addition, the C-terminal residues were also involved in the ability to

interact with platelets (Jorge *et al.*, 2005). Therefore, a histidine tag should be put in the N-terminal side of a integrin not to interfere with the function.

Singhamatr and Rojnuckrin (2006) expressed a disintegrin domain of *Crytelytrops alborabris* in *Pichia pastoris* that is a eukaryotic system. The recombinant protein contained Histidine tag at the N-terminus. After purification and activity tests, they found that it contained an inhibitory activity on collagen-induced platelets aggregation (IC_{50} =976nM), but there was no effect on ADP-induced aggregation. It is possible that glycosylation or other post-translational modifications in this eukaryotic expression system contribute to its activity.

Recently, Lili Zhu et al. (2010) expressed a P-II SVMP, Jerdonitin, which was highly homologous to *C. albolabris* protein in *Pichia pastoris* system. This P-II SVMP comprised metalloproteinase and disintegrin that could degrade fibrinogen and inhibit ADP-induced platelets aggregation in a dose dependent manner similar to the native protein. Furthermore, Jerdonoitin affected growth of several cancer cell lines including Bel7402, human leukemia cell (K562), and human gastric carcinoma cell (BG823). Our P-II SVMP protein from *Cryptelytrops alborabris* also contained platelet inhibitory activity but only after collagen induction. This could be explained by the species differences between the two proteins. Previous reports found that sequences around an RGD motif and C-terminus of a disintegrin can determine ligand specificity.

Typical snake venom disintegrin have RGD or KGD sequence that binds to $\alpha_{IIb}\beta_{III}$ integrin, the fibrinogen receptor ligand on platelets membrane. Therefore, this disrupts platelet aggregation. The RGD sequence in structure of our novel disintegrin may cause hemorrhage from inhibitory activity on platelet aggregation.

Bilitoxin-1 is a SVMP from *A. billineatus* (Toshiaki et al 2000). Incubation of bilitoxin-1 in EGTA results in the loss of the proteolytic and hemorrhagic activities .This disintegrin protein lacks of RGD sequence but has MGD sequence, which has no platelet inhibitory effect. MGD loop region is quite different in structure from the RGD loop and incapable of engaging the $\alpha_{IIb}\beta_3$ integrin.

Atrolysin has RGD and MVG sequences in its RGD loop region and inhibits both collagen- and ADP-induced platelet aggregation. Additionally, MGD (in the RGD region) sequence can also inhibit the fibronectin receptor, $\alpha_5\beta_1$ integrin, blocking platelet adhesion to this extracellular matrix.

Yvonne et al (2003) found that the disintegrin jararhagin (P-III SVMP) contained RKKH sequence (CTRKKHDC and ARKKHDC), which bound to α_1I domain of the integrin and completely inhibited collagen binding on platelet surface. The binding site of α_1I is partially blocked in close structure, but the open conformation is shifted to fit well to RKKH when the peptide docks. In addition, it also induced hemorrhage by degradation of sub-endothelial matrix proteins and disturbed platelet adhesion function to collagen by binding of the molecule to the alpha(2) subunit I domain of the platelet surface $\alpha 2\beta 1$ integrin (collagen receptor) and also cleaves the $\beta 1$ subunit of the same integrin causing impairment of signal transduction. Our P-II SVMP also inhibited collagen-induced platelet aggregation. However, there was no RKKH motif in the sequence of our protein. Therefore, the mechanism of action of our protein remain to be determined.

Crotarhagin and alborhagin induce platelet aggregation and activation of endogenous metalloproteinase. GP VI, the collagen receptor on platelets, is shed after stimulation by these two SVMPs. Aggregation was inhibited by soluble GP VI ectodomain and GP VI signaling was decreased after shedding. Therefore, these SVMPs activate, followed by inhibit, platelets. However, our protein did not initially stimulate platelets. It remains to be determined if our SVMP causes GP VI ectodomain shedding.

On the other hand, the metlloproteinase domain in SVMPs has proteolytic activity on basement membrane. Collagen type IV is used as an agonist to induce aggregation. It is possible that the agonist is degraded by our SVMPs resulting in impaired platelet-stimulation activity. However, in the experiment we incubated washed platelets at 37 ^{o}C with our protein for only 5 minutes after adding collagen before the light transmission was instantly measured. From other experiments, it would take several hours for most SVMP to digest collagen in vitro. Therefore, the inhibition should be a direct effect on platelet integrins or glycoproteins, rather than collagen digestion.

Uncontrolled platelet activation has been implicated in intravascular thrombosis, the pathology related to peripheral, myocardial and cerebral ischemia. The drugs that target collagen-induced platelet activation are potentially useful for the prevention and/or treatments of these thrombotic problems. Several SVMPs can antagonize collagen-mediated signaling in platelets. For example, Trimucytin from *Trimeresurus mucrosquamatus* act as an antagonist to $\alpha_2\beta_1$ and GPVI leading to the inhibition of phospholipase C, an important signaling molecule in platelet activation (Lin *et al.*, 2002,Hers, 2000). Acurhagin from *A. actus* inhibits collagen- and von Willibrand factor-induced platelet aggregation by inhibiting tyrosine phosphorylation of various signaling proteins (Wang *et al.*, 2005). The effects of our SVMP to platelet signaling proteins remain to be determined.

In conclusion, our SVMP should be developed for potential uses as an antagonist to platelet aggregation and anti-thrombotic agent because it can inhibit platelet activation. However, the exact mechanism of inhibition, i.e. RGD that inhibits platelet $\alpha_{IIb}\beta_3$ integrin vs. another motif that inhibits $\alpha_2\beta_1$ integrin, needs further studies. Moreover, the proteolytic activity of P-II SVMPs should be test for studying the mechanism on hemorrhagic manifestations and necrosis pathology. This will be helpful for research on the treatments of snakebite patients.