CHAPTER II

LITERATURE REVIEW

This thesis studied the amino acid sequences, expressed the recombinant protein and investigated the effects of snake venom metalloproteinase (SVMPs) from *Cryptelytrops alborabris* on hemostasis system. To the author's knowledge, such study has never been reported in the literature. In the following sections, a literature review related to the characteristics and activities of various SVMPs, the studies of the effects of native and recombinant SVMPs from variety of species *in vivo* and *in vitro* and the production of recombinant SVMPs in *Pichia pastoris* expression system are presented

2.1 Characteristics and activities of Snake Venom Metalloproteinase (SVMPs)

Snake venom metalloproteases, belonging to the metzincin family, are classified into four major groups by their protein domain structures or cDNA sequences (See Chapter I). SVMPs are varying in sizes from 20 to 100 kDa and composed of multiple domains that are a pro-peptide, a metalloproteinase, a disintegrin (-like), and a cysteine-rich domain. The signal peptide causes the protein to be secreted. The pro-domain interact with the metalloproteinase catalytic domain preventing its activities. Disintegrins are cysteine-rich low molecular weight polypeptides, which contain tripeptide sequences, such as RGD or KGD motif, recognized by integrin receptors. The metalloproteinase and disintegrin proteins in snake venom can be derived by proteolysis from a common metalloproteinase/disintegrin precursor.

Metalloproteinases are synthesized as zymogens, and are proteolytically processed to yield the active enzymes (Bjarnason and Fox, 1994). Studies of crystal structures of venom P-I class metalloproteinases reveal similar structures, being ellipsoidal molecules with a shallow active site clef that separates a lower subdomain from the main domain. The main domain is composed of five stranded β - sheets and four α-helices (Gomis *et al.*, 1993; Zhang *et al.*, 1994; Kumasaka *et al.*, 1996; Gong *et al.*, 1998). The catalytic Zn2+ ion in the active-site clef is surrounded by three histidine residues and one water molecule is anchored to glutamate143 in a tetrahedral manner.

Metalloproteases from several snake venoms induce hemorrhage by digesting components of the extracellular matrix and by hydrolyzing various blood coagulation factors. In addition HV1, VAP-1, graminelysin I, and agkistin were reported to induce apoptosis of human endothelial cells, leading to vascular injury. Besides hemorrhage, snake venom metalloproteinases induce local myonecrosis, skin damage, and inflammatory reactions in experimental models. Local inflammation is an important characteristic of snake envenomations by viperine and crotaline snake species. P-I or P-III class hemorrhagic metalloproteinases have been shown to induce inflammatory reactions in experimental models both *in vivo* and *in vitro*. Interestingly, large hemorrhagic metalloproteinases containing disintegrin-like and high-cysteine domains of the class P-III are more active at inducing hemorrhage than enzymes comprising only the metalloproteinase domain alone as in P-I class.

Snake venom disintegrins represent a family of RGD (Arg-Gly-Asp) or KGD (Lys-Gly-Asp) containing proteins, while disintegrin –like domains contain ECD motifs (Glu-Cys-Asp). The disintegrin-like domain shows high sequence identity with venom disintegrins (Markland, 1998), which are proteolytically released from P-II SVMP precursors. The disintegrin-like domain does not contain the typical RGD sequence found in disintegrins but shows different sequences in this region. A disintegrin-like domain represents another super-family of proteins. However, many of them have been demonstrated to have similar ability to inhibit platelet aggregation and integrin-mediated cell adhesion similar to disintegrins.

Around a RGD/KGD sequence, an amino acid hairpin loop is maintained by disulfide bridges. The study of several short- and medium-sized disintegrins by NMR technique showed that the active tripeptide is located at the apex of a mobile loop protruding 14 – 17 A^{o} from the protein core. The crystal structure of trimestatin similarly shows that the RGD sequence is located at the tip of a long loop and there is the irregular 15-residue hairpin loop from Thr43 to Cys57 (Markland, 1998). In addition, changing of the amino acids adjacent to the C-terminus of the disintegrins alters its affinity to integrins.

ADAM (a disintegrin-like and metalloproteinase) proteins are members of the metazincins that also include the related matrix metalloproteases (MMPs). Some of ADAM proteins have shown to contain integrins, and the disintegrin-like domain that may be crucial part in their function as proteases. The RGD motif confers the disintegrin property and the ability to selectively bind to integrins in different cell systems (Oliveira *et al.*, 2003). The SVMP-derived disintegrins have also been shown to interact with and activate integrin-signaling pathways in inflammatory cells. Integrin-signaling pathways mediate important functions in leukocytes, including migration, spreading, activating the respiratory burst, binding of complement, cell adhesion, cytokine gene expression and apoptosis (Williams and Solonkin, 1999; Lowell and Berton, 1999; Larsson *et al.*, 2000). Activation of distinct signaling pathways by these domains appears to be dependent on the structure of each domain and on the types of cell surface receptors.

2.2 Study of the effect of native and recombinant SVMPs *in vivo* and *in vitro.*

SVMPs have been extensively studied on hemostatic activities, such as inhibition of platelet aggregation, coagulation cascade, fibrinolysis and degradation of extracellular matrix around the endothelial cells. Viper venom metalloproteinase and disintegrin, found in various snake venoms especially Viperid and Crotalid families, not only have the platelets inhibiting but also have the matrix degrading activities. Many studies used native and/or recombinant venom protein to investigate their interesting effects.

Metalloproteinases comprise a family of Zn(2+)-endopeptidases that degrade most components of the extracellular matrix. They also digest fibrinogen as well as fibrin, and in most cases, induce hemorrhage in snake bite victims. A few low-molecular weight snake venom metalloproteases (SVMPs) have been described as being devoid of hemorrhagic activity, but they, instead, have a strong direct-acting fibrinolytic activity. This property could be very helpful in development of anti-thrombotic therapy.

In 2003, Ramos OH et al, studied ACLF, a fibrinolytic, non-hemorrhagic metalloprotease from *Agkistrodon contortrix laticinctus*. A recombinant pro-ACLF coding

region including both the pro-enzyme and the mature protein domain was amplified by PCR, sub-cloned into the pET28a vector and transformed into BL21 (DE3) *Escherichia coli* cells. The protein was expressed as insoluble 48kDa-peptide and purified by affinity chromatography under denaturing conditions. Subsequently, the denaturing agent was gradually removed using dialysis. The recombinant protein was active on fibrinogen and on a synthetic substrate. To control the activation step, the denaturing agent was rapidly removed to keep the protein in an unprocessed form, followed later by addition of Ca(2+) and Zn(2+) ions. This allowed controlling the enzyme activation only when it is needed.

Hemorrhagic activity of SVMPs is mostly due to the enzymatic action of metalloproteases on vascular basement membrane components, such as collagen type IV, laminin, and fibronectin. In 2006, Caroline et al studied the biological properties of rACLF. The peptides, which encode the catalytic domain, as well as the pro-ACLF, were expressed. Their result showed that rACLF hydrolyzed laminin, fibronectin, type IV collagen and thrombospondin. Further more rACLF decreased HeLa cell viability, changed cell morphology and induced detachment. For human fibroblasts, there was no cytotoxic effect after a treatment with rACLF. In addition, growth-related oncogene (GRO) and monocyte chemoattractant protein 1 (MCP-1/CCL2), the chemokines, were detectable in the culture supernatant of human fibroblasts incubated with rACLF for 48 h. These chemokines could contribute to the severe local lesions induced by *Agkistrodon contortrix lacticinctus* venom. These findings suggest a relevant role of ACLF in envenomation.

Teraza et al, study insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases. This study compared the action of two SVMPs, BaP1 (the P-I class) and jararhagin (the P-III class), which had a similar proteolytic activity on azocasein and degraded Matrigel with slightly different cleavage pattern. BaP1 exerted a limited proteolysis of both laminin and nidogen, whereas jararhagin predominantly degraded nidogen. Immunohistochemical analysis of laminin, nidogen and type IV collagen, as well as of the endothelial cell marker Vascular endothelial growth factor receptor 2 (VEGFR-2), in the hemorrhagic areas of the muscle

showed reduction in the immunostaining of these three basement membrane components associated with a loss of the endothelial cell marker that was affected to a lesser extent. The drastic loss of these antigens in capillaries *in vivo* is likely to depend on the combination of limited proteolysis of basement membrane in SVMP-induced capillary damage, which may cause a mechanical disruption of basement membrane structure.

Most of the venom disintegrins contain RGD or KGD sequence that is the structural motif recognized by the platelet fibrinogen receptor $\alpha_{IIB}\beta_3$and also act as potent antagonists of several integrins including $\alpha_v\beta_3$ and $\alpha_5\beta_1$ that are expressed on vascular endothelial cells and some tumor cells.

Several articles have shown that the disintegrins can affect platelet aggregation. Adinbitor, a disintegrin from a Chinese snake (*Agkistrodon halys brevicaudus steineger*) was cloned and characterized by Wang et al. The gene encodes a polypeptide of 73 amino acids with 12 cysteines and an RGD motif. It was expressed in *E. Coli* with polyhistidine tag and purified using the His-Bind affinity chromatography. Wang et al. found that adinbitor could inhibit human platelet aggregation in a dose-dependent manner. Human platelet aggregation is completely inhibited by recombinant disintegrin at the concentration of 8.4 μM. The IC_{50} value of the recombinant adinbitor in inhibiting platelet aggregation was 6 μM. Rhodostomin, isolated from the venom of *Calloselasma rhodostoma*, consists of 68 amino acids including 12 residues of cysteines and an RGD sequence. It was express in *E. coli* and *Pichia pastoris* expression protein and found to inhibit platelet aggregation with a constant of inhibition (Ki) value of 263 nM and 70 nM, respectively. Therefore, the Pichia system seemed to produce more active protein. The recombinant disintegrin domain of jararhagin, a P-III snake venom metalloproteinase isolated from *Bothrops jararaca* venom, was produced in *E. Coli* system. It inhibited collagen-induced platelet aggregation in a dose-dependent manner with IC_{50} value of 8.5 μg/ml .

Disintegrin, bothrostatin, was cloned from a *Bothrops jararaca* cDNA library. It contains a pro, a metalloproteinase, and an RGD disintegrin domain. As expressed

in *Escherichia coli*, it showed potent inhibitory activity on collagen-induced platelet aggregation (IC_{50} of 12 nM). The 3D structure analysis reveals that bothrostatin is a globular, closed structure in solution. The RGD motif is exposed to the solution by the loop formed by residues 45–59 and is very close to the C-terminal domain forming the finger-like structure. The RGD loop and the C-terminal residues is maintained by the Cys47–Cys66 bond suggesting that the C-terminal residues are involved in the ability of bothrostatin to interact with its ligands. These can be used as a helpful tool for studies of integrin–ligand interactions.

The dimeric p-II SVM disintegrin-like domain of bilitoxin-1 isolated from the venom of *Agkistrodon bilineatus* lacks the RGD cell-binding sequence, which is substituted by the MGD motif. The disintegrin domain is auto-proteolytically processed from the native protein. Bilitoxin disintegrin was found to lack a platelet aggregation inhibitory activity, probably reflecting the substitution of RGD by MGD.

Jerdonitin, was purified from *Trimeresurus jerdonii* venom with a molecular weight of 36 kDa. It dose-dependently inhibited ADP-induced human platelet aggregation with the IC_{50} of 120 nM. Metalloproteinase and disintegrin domains of its natural protein were not separated, in contrast to other P-II class SVMPs. Jerdonitin has two additional cysteines (Cys219 and Cys238) located in the spacer domain and disintegrin domain, respectively. They probably form a disulfide bond and therefore the metalloproteinase and disintegrin domains cannot be separated by the posttranslational process.

2.3 Production of recombinant SVMPs in *Pichia* Expression System

Pichia pastoris is methylotrophic yeast that has been developed to be a highly successful system for production of a variety of heterologous proteins. *Pichia pastoris* is a suitable host strain for the production of proteins for several reasons. *Pichia pastoris* has a strong inducible promoter, *AOX1*, to induce high levels of transcription (Xu and Janson, 2002). In addition, it provides potential for producing soluble, correctly folded recombinant proteins that require post translational modifications essential for functions. Because *Pichia pastoris* performs many post-translational modifications, that are

typically associated with higher eukaryotes, such as processing of folding, disulfide bridge formation, certain types of lipid addition as well as *O*- and *N*-linked glycosylation. Moreover, *Pichia pastoris* does not secrete a lot of its own proteins into culture medium. Expression is driven by alcohol oxidase promoter in methanol-added media. The isolation of the protein of interest, thus, can be facilitated.

For example, the metalloproteinase domains of ADAM9, barbourin, rhodostomin, laccase, β-mannanase, and bovine β-casein have been successfully expressed in *Pichia pastoris* system in the correct active forms. This indicate that the recombinant proteins from *Pichia pastoris* are more active than those from *E. coli*.

The disintegrin-like domain from *G. halys*, halydin, was cloned and expressed in *Pichia pastoris*. It contains an Asp-Glu-Cys-Asp (DECD) sequence in place of the RGD motif, and is able to inhibit human platelet aggregation in a dose-dependent manner by suppressing platelet adhesion to collagen rather than by blocking fibrinogen binding to glycoprotein (GP) IIb-IIIa on the platelet surface. This suggests that halydin binds to integrin alpha2beta1 on the platelet surface.

In this study, we have been interested in cloning and expression of prodomain, metalloproteinase and disintegrin of in *Pichia pastoris* system to produce recombinant protein in the correctly-folded active form. We hypothesize that it contains an activity to inhibit platelet aggregation and extracellular matrix degradation.