CHAPTER II

LITERATURE REVIEW

This thesis studied the expressions and effects of the recombinant snake venom serine protease with fibrinolytic activity from green pit viper (*Cryptelytrops albolabris)*. To the author's knowledge, such study has never been reported in the literature.

Snake Venom Serine Proteases (SVSPs)

Snake venom is a complex mixture of toxins and enzymes, each of which may be responsible for one or more distinct toxic action. They are responsible for hemorrhage, shock and disorders of blood coagulation. They act by activating, inactivating or modifying hemostatic and fibrinolytic system. Because there is a major medical interest in thrombosis and hemostasis, venom proteases that affect the blood coagulation system in human have been extensively investigated and considerable number of venom components acting on hemostasis has been isolated and characterized(1).

Snake venom serine proteinases (SVSPs) are among the best-characterized venom enzymes that affect the hemostatic system. They perform actions on a variety of components of the coagulation cascade, the fibrinolytic and kallikrein – kinin systems as well as cellular components causing an imbalance of the haemostatic system of the prey. Serine proteinases are categorized in the trypsin family S1 of clan SA, the largest family of peptidases. They are present in venoms of various snake families including Viperidae, Crotalidae, Elapidae and Colubridae.

Snake venom enzymes of the serine protease family are characterized by a common catalytic mechanism that includes a highly reactive serine residue that plays a pivotal role in the formation of a transient acylenzyme complex. The complex is stabilized by the presence of histidine and aspartic acid residues within the active site. These histidine, aspartic and serine residues are, therefore, so-called the catalytic triad.

In spite of the high degree of mutual sequence identity, SVSPs are quite specific toward a given macromolecular substrate. Although they show primary substrate specificity similar to that of trypsin, their stringent macromolecular substrate specificity differs from the less specific activity of trypsin. The substrate specificity of clotting factor serine proteases, such as thrombin or factor Xa, is determined by the sequence outside the active sites, so-called exosites. Whether these exosites play important roles in SVSPs remains to be investigated.

Most SVSPs tend to be glycoproteins showing a variable number of N-or O-glycosylation sites in sequence positions that differ from one SVSP to the other. They contain twelve cysteine residues, ten of which construct five disulfide bonds. Based on the homology with trypsin, the remaining two cysteine residues form a conserved disulfide bridge that is unique among SVSPs. It involves Cys245e that found in the C-terminal extension(27).

Snake venom serine proteases (SVSPs) are among the best characterized venom enzymes affecting the hemostatic system. They act on a variety of components of the coagulation cascade including fibrinogen-clotting, fibrinolysis and kallikrein-kinin systems and on cells to cause a disturbance of the hematostatic system of the prey.(12,13)

The venom serine proteases are classified as families of proteins that specifically interact with different targets, on which they exert their physiological actions. The genes have been sequenced and the proteins have been characterized(28).

The venom serine proteinases are categorized in families of proteins which specifically interact with various targets, on which they exert their physiological action. Examples are as following:

Some of venom proteases are frequently referred to as thrombin-like enzymes (SVTLEs) due to their ability to cleave fibrinogen, releasing fibrinopeptide A, fibrinopeptide B or both. Recently, BJ-48, a SVTLE from *Bothrops jararacussu* is characterized biochemically. It is capable of cleaving either α or β chain of fibrinogen, but shows preference for the β chain(13).

Cryptyletrop jerdonii snake is a pit viper, venom of which contains both fibrinogen-degradation and fibrinogen-clotting serine proteases. Jerdonobin-II, a novel serine protease, was cloned, purified and characterized from the venom. It shares 89 % sequence identity with TSV-PA, displays a distinct biological activity and a weak fibrinogen-clotting activity(30).

Kallikrein-like proteinase of *Lachesis muta muta* (bushmaster) venom designates LV-Ka. Approximately 77% of the protein sequence was determined by sequencing various fragments derived from endoprotease digestion. The obtained partial sequence suggests that LV-Ka is of a similar size to other serine proteinases. Sequence studies on the NH_2- terminal region of the protein indicate that LV-Ka share a high degree of sequence homology with the kallikrein-like enzyme EI and EII from *Crolalus atrox*, with crotalase from *Crotalus adamatues* and significant homology with other serine proteinases from snake venoms and other vertebrates. LV-Ka showed kallikrien-like activity, releasing bradikinin from kininogen as determined by guinea pig bioassay(31).

A recombinant serine protease named albofibrase from Green Pit Viper (*Cryptelytrops albolabris*) is predicted and shown to be a fibrinogenolytic enzyme. Albofibrase degraded fibrinogen by cleavage of peptide bonds in the Aα-chain.

The enzyme called thrombocytin, a serine protease from *Brothrops atrox* venom, is a platelet aggregation inducer, which also activates factor V. In addition, this enzyme activates factor VIII and factor XIII but very weakly.

A serine protease named scolonase was purified and characterized from tissue of a Korean centipede, *Scolopendra subspinipes mutilans.* The study demonstrated that scolonase was able to activate plasminogen to plasmin by specifically cleaving the molecular at $Arg^{561} – Val^{562}$ peptide bond(32).

ABUS-PA from *Agkistrodon blomhoffii Ussurensis venom* has been identified and purified to homogeneity and activates as a plasminogen activator with arginine ester hydrolysis activity(33).

The Expression System

Pichia pastoris is the methylotrophic yeast that has been developed to be a highly successful system for production of a variety of heterologous proteins for both basic researches and industrial uses, e.g. L-amino acid oxidase (LAAO) from Malayan pit viper (*Calloselasma rhodostoma*)(34), vampire bat salivary plasminogen activator α2 (DSPAα2) from *Desmodus rotundus*(35). In addition, prourokinase (proUK) – annexin V chimeras have also been successfully expressed(36).

Pichia is a suitable host for the production of protein for several reasons. *Pichia pastoris* has a strong inducible promoter, *AOX1*, to induce high levels of transcription(37). Moreover, *Pichia pastoris* has the potential to perform many post-translational modifications typically associated with higher eukaryotes, such as processes of folding, disulfide bridge formation and certain types of lipid addition as well as *O*- and *N*-linked glycosylation. In addition, *Pichia pastoris* does not secrete a lot of its own proteins into culture medium. The isolation of the interest protein is, thus, facilitated.

As in the previous study on expression and characterization of recombinant fibrinolytic serine protease from green pit viper (*Cryptelytrops albrlabri*) venom, the recombinant albofibrase produced from *Pichia pastoris* was 0.66 mg/l of culture medium was active as an α fibrinogenase(28).

Albolatin, a novel snake venom mettaloprotease from greem pit viper (*Cryptelytrops albrlabris*), was cloned and its disintegrin domain was expressed in the Pichia system. The recombinant protein was secreted in the culture medium and the yield of recombinant protein protien was 3.3 mg/l and could inhibit collagen-induced platelet aggregation(38).

In the study of molecular cloning, expression and purification of L-amino acid oxidase from the Malayan pit viper (*Calloselasma rhodostoma*), the recombinant LAAO was purified yielding apparently homogenous protein in quantity of approximate 0.25 mg/l. The recombinant enzyme contained a similar activity as the native one(34).

Plasminogen Activator System and Thrombolytic Agents

Plasminogen activators also play an important role in the processes other than fibrinolysis. For example, fibrinolytic factors, tissue-type plasminogen activator (t-PA) and urokinase—type plasminogen activator (u-PA) control plasmin in different processes, such as cell migration, adhesion and aggregation(21). Previous studies suggested the importance of u-PA in development of atherosclerosis in patients. Furthermore, it also plays an essential role in many (patho) physiological processes that require degradation of extracellular matrix(39). Additionally, there is a research demonstrating a significant elevation of t-PA concentration in plasma of patients with abdominal aortic aneurysm(40).

Currently, we can produce recombinant thrombolytic agents that function as plasminogen activators. The fibrinolytic agents available today are serine proteases. They work by converting plasminogen to a natural fibrinolytic agent, plasmin. Plasmin lyses clots by breaking down the fibrinogen and fibrin contained in a clot into fibrin degradation products (FDPs)(41). They are very helpful in treating acute vascular occlusions in human. The examples of these therapeutic agents are Streptokinase(26), Acylated plasminogen – streptokinase activator complex (APSAC), Staphylokinase(42), Urokinase(43) and Prourokinase(44).

The thrombolytic agents, human tPA and uPA, do not stimulate immunological responses. However, their effects are short-lived *in vivo* and consequently of a limited therapeutic effectiveness. On the other hand, Streptokinase is a non-human protein that can illicit severe anaphylactic response, which may be fatal. Uses of streptokinase are related with serious risk of hemorrhage. At present re-administration of streptokinase cannot be recommended beyond 4 days after first dose as a result of a rise in neautralizing anti-streptokinase antibody.

Acylated plasminogen – streptokinase activator complex (APSAC) can also induce fibrinogenolysis. It is antigenic because it incorporates streptokinase and it, therefore, cannot be reused.

Staphylokinase is also a protein of non-human origin and, consequently, triggers an immune response in patients. Antibodies develops in the majority of patients within 2 weeks after initial administration and are persistent for at least 7 months

Urokinase is specified as a fibrinolytic–thrombolytic agent, which is less effective than that in human tissue plasminogen activator (t-PA). In addition, the cost is higher as compared to the streptokinase.

The half-life of Prourokinase is relatively shorter than that of urokinase. However, it is not commercially available(23).

The Green pit viper plasminogen activator (GPV - PA) is a unique protein waiting for expression and characterization. This protease may lead to a novel therapeutic plasminogen activator. Molecular expression of this protein may yield an agent that benefit to thromboembolic patients. Potential advantages include the long half-life as it is not inhibited by the plasminogen activator inhibitor (PAI-1). In addition, it may be neutralized by the commercially available green pit viper antivenom if there is an overdose of this agent. Nevertheless, immunogenicity will limit the repeated administrations because it is a foreign protein.