CHAPTER II

LITERATURE REVIEW

Snake venoms are probably the most highly concentrated secretory products found in vertebrate. Proteases are important factors in snake venoms, especially in crotalid and viperid. They are responsible for hemorrhage, shock and disorders of blood coagulation. They act by activating, inactivating or other effects on hemostatic and fibrinolytic systems(12) . Because there is a major medical interest in thrombosis and hemostasis, venom proteases that affect the blood coagulation system in humans have been relatively well investigated and a considerable number of venom components acting on hemostasis has been isolated and characterized(13). Since 1950 the complete amino acid sequences of about 40 out of those venom proteases have been determined by protein sequencing or deduced from the nucleotide sequence of the cDNA. Serine proteases have fibrinogenolytic activity and/or fibrinolytic activities. Fibrinogenolytic enzymes are called 'thrombin-like' proteases, only if they show 'fibrinogen clotting' activity. Mammalian α-thrombin (an activated form of prothrombin or blood coagulation factor II) is a multifunctional serine protease. Serine proteases may be kallikrein-like enzymes and release a hypotensive agent, bradykinin, from kininogen in mammalian plasma. Other unique serine proteases are plasminogen activator, such as TSV-PA from the *T. stejnegeri* venom that cleaves the same peptide bond in plasminogen as mammalian plasminogen activators, but it lacks the kringle and finger domains that provide the fibrin binding specificity(14) .

The venom serine proteases are classified as families of proteins that specifically interact with different targets, on which they exert their physiological action. Examples are the followings.

1. Thrombin-like enzymes from crotalid venoms(15) (Pirkle and Theodor, 1991), and Fibrinogen-clotting activity from *T. stejnegeri* venom(16) (Zhang Y et al., 1998)
2. Fibrinogenolytic enzyme from *Vipera lebetina* venom(17) (Swenson S et al., 2005)
3. Plasminogen activator from *Trimeresurus stejnegeri* venom (TSV-PA)(18) (Zhang Y et al., 1995)
4. Kallikrein-like enzymes from *Crotalus atrox* venom(19) (Bjarnason et al., 1983)
5. Protein C activator from *Agkistrodon contortrix controtrix* venom(20,21) (Kisiel et al., 1987), (McMullen et al., 1989)
6. Factor V activator from *Vipera russelli* venom(22) (Kisiel et al., 1979), (Tokunaga et al., 1988)
7. Platelet aggregation from the South American pit viper *Bothrops atrox* venom(24) (Niewiarowski et al., 1977)

The enzyme, called thrombocytin, a serine protease from *Bothrops atrox* venom, is a platelet aggregation inducer, which also activates factor V. Additionally, this enzyme activates factor VIII and factor XIII but very weakly(25). Leucurobin (leuc) is a thrombin-like enzyme from *Bothrops leucurus* venom. The protein sequence exhibits significant similarities with other serine proteases reported from snake venoms, and contains two potential sites of N-linked glycosylation. The enzyme evoked the gyroxin syndrome when injected into the tail veins of mice at levels of 0.143 μg/g mouse(26). The venom of the South American snake *Bothrops jararaca* contains two serine proteinases, bothrombin and the platelet-aggregating enzyme PA-BJ, which share 66% sequence identity. Bothrombin clots fibrinogen but has no direct effect on platelet, unless in the presence of exogenous fibrinogen. PA-BJ induces platelet aggregation by interacting with the protease-activated platelet receptor without clotting

fibrinogen(27). Molecular cloning of serine proteinases from *Bothrops jararaca* venom gland by cDNA library was subsequently screened with a labeled KNBJ2 /cDNA as a probe. Three positive clones, HS 112, HS 114 and HS 120, were selected and sequenced. No apparent match to any of the deposited sequences was found in the current GenBank/EMBL databases, indicating that each of these cDNA clones encodes a serine proteinase distinct from the known enzyme(28).

A thrombin-like serine protease, jararassin–I, was isolated from the venom of *Bothrops jararassin*. The molecular mass of the enzyme was about 30 kD. The jararassin-I is a serine protease similar to coagulating thrombin-like snake venom proteases, degrading the Bβ chain of fibrinogen while the Aα chain and α chain were unchanged(29). In Russell's viper (RV), the active protein components of venom by the cDNA library were made from Thai RV venom glands, and randomly sequenced of cloned cDNA. Phospholipase A_2 (PLA_2), an active enzyme found in several species, is a major component of RV venom, compising up to 70 % of dry weigth(30). This can explain hemolytic anemia and renal failure caused by RV venom in human.

Activation of prothrombin to mature thrombin occurs normally by the prothrombinase complex consisting of a serine proteinase factor Xa, and cofactors factor Va, Ca^{2+} ions and phospholipids. Several exogenous prothrombin activators are found in snake venom. The groups were classified by their cofactor requirements into four groups, Group A and B prothrombin activators are metalloproteinases, whereas group C and D prothrombin activators are serine proteinases. Group C prothrombin activators resemble the mammalian factor Xa-factor Va complex, while group D activators are structurally and functionally similar to factor Xa(31). Cerastocytin has been isolated from the venom of the Tunisian viper *Cerastes cerastes*. The recombinant protein was designated rCC-PPP (for *C. cerastes* platelet proaggregant protein). Purified rCC-PPP efficiently activates blood platelets at a nanomolar concentration. In addition, it is able to clot purified fibrinogen and to hydrolyze α-

chains. By comparison with other snake venom serine proteinases, a Gly residue replaces the conserved Cys^{42}. The surface of the rCC-PPP molecule is characterized by a hydrophobic pocket comprising the 90 loop (Phe^{90} – Val^{99}), Tyr^{172}, and Trp^{215} residues, which might be involved in the fibrinogen clotting activity of rCC-PPP(32) .

A novel plasminogen activator from *Agkistrodon halys* venom was found among the 2 serine proteases of 234 and 233 amino acids in length and named as Haly-PA and Haly 2. Haly-PA showed the highest similarity (82% identity) to the previously characterized plasminogen activator, TSV-PA. Haly 2 displayed a 78% similarity to β - fibrinogenase, a fibrinogenolytic enzyme. Haly-PA was successfully expressed using the baculovirus system and secreted into the culture media as a 32 kDa glycoprotein. Haly-PA is a plasminogen activator and displays fibrinogenlytic activity through conversion of plasminogen to plasmin(33). Shedaoenase, a serine protease, was isolated from the venom of *Agkistrodon shedaoenthesis* with an apparent molecular mass of 36 kDa. The N-terminal seqnence of shedaoenase was determined, and its full-length cDNA encoding a protein of 238 amino acid residues was cloned by reverse transcription-polymerase chain reaction from the total mRNA extracted from the snake venom gland. Shedaoenase cleaved the A alpha-chain of human fibrinogen and slowly digested the B beta-chain. This enzyme had arginyl esterase activity using some chromogenic substrate, such as Chromozym PL, S-2266, and S-2160 (34). Lachesis venom plasminogen activator (LV-PA) is a 33-k Da serine proteinase isolated from bushmaster snake venom, which activates the fibrinolytic system in vitro(35).

Green pit viper (*Trimeresurus albolabris* and *T. macrops*) venom was found to have a thrombin-like effect *in vitro* but cause a defibrination syndrome *in vivo*. This is because the fibrinolytic activity in human bodies can rapidly consumes partially clotted fibrinogen. Fibrinolytic system activation is very common as indicated by low plasminogen (50%), low antiplasmin (56.5%) and elevated fibrin-fibrinogen degradation products (FDPs, 97.4%) levels(3). In addition, plasminogen

activator antigen and activity were found to be elevated. Thai *T. albolabris* venom gland cDNA library was closely homologus to *T. stejnegeri*. Two thrombin-like enzymes (GPV-TL1 and GPV-TL2), two isoform of a fibrinogenolytic enzyme and a plasminogen activator may cause in defibrination syndrome in patients by combination of these enzymatic effects(36). The mechanisms of fibrinolytic initiation in GPV bites need further studies. Cloning of profibrinolytic proteins from GPV venom will give us deeper insights in the pathogenesis.

Previous studies on cloning and expression of viper venom serine proteases are followed. Three fibrinogenolytic enzymes from Chinese green tree viper (*T. stejnegeri*) venom, stejnefibrase-1, stejnefibrase-2 and stejnefibrase-3, were purified by gel filtration and molecular weight of 50000, 31000 and 32000. The genes of these 3 proteins have already been cloned. The three purified enzymes degraded fibrinogen to small fragments and rendered it un-clottable by thrombin(37).A fibrinogen-clotting enzyme designed as jerdonobin II was isolated from the venom of *Trimeresurus jerdonii*. Jerdonobin II showed weak fibrinogen clotting activity and its activity unit on fibrinogen was calculated to be less than one unit using human thrombin as standard. The sequence shows high similarity (89% identity) to TSV-PA, a specific plasminogen activator from vemon of *T. stejnegeri*. Despite of the sequence similarity, jerdonobin-II was found devoid of plasminogen-activating effect suggesting that the replacement of Lys^{239} in TSV-PA to Gln^{239} in jerdonobin-II might play an important role on their plasminogen activating activity difference (38). The elegaxobin II was isolated from the venom of *T. elegans*. It consisted of 233 amino acids and showed conservation of the catalytic amino acid residues (His^{57}, Asp^{102}, and Ser^{195}) of chymotrysin family serine protease in its amino acid sequence. Eleganobin II was 91% homologous in sequences with elaganobin and protease I from the same snake venom, and it was 67, 75, 31 and 26% homologous in sequence to Slavoxobin, KN-BJ2, human kallikrein and bovine thrombin respectively. Pre-elegaxobin II contains

glucosamine and an N-linked glycosylation site(39). A novel plasminogen activator from *T. Stejnegeri* venom (TSV-PA) has a single chain glycoprotein with an apparent molecular weight of 33,000 dalton. The activation of human native Glu-plasminogen by TSV-PA is due to a single cleavage of the molecule at the peptide bond Arg^{561} $Val.^{562}$. The deduced complete amino acid sequence of TSV-PA from the cloned cDNA indicates that the mature TSV-PA protein is composed of 234 amino acids and contains a single potential N-glycosylation site at Asn^{161} (40). The venom of Taiwan habu (*T. mucrosquamatus*, denoted as Tm), named Tm-VIG (with Val-Ile-Gly as the first three N-terminal residues) and Tm-IIG (with Ile-Ile-Gly as the first three N-terminal residues) was found to posses relatively specific and strong activities on β-chain of human fibrinogen and kallikrein substrate(41).

In conclusion, snake venom contains a large number of small enzymes and other components that display a broad spectrum of biological activities. In previous studies, cDNAs of snake venom glands were synthesized using SMART cDNA amplification kit and toxin genes were cloned and sequenced(27,31). These characteristic features have led to development of fibrinogenolytic snake venom enzymes as potential clinical agents to treat occlusive thrombi. For example, beta-fibrinogenolytic serine proteinase from *Vipera lebetina* have been tested in clinical trials. A recombinant fibrinolytic enzyme derived from fibrolase was also investigated as a clinical agent(17).

Structural studies on viper venom serine proteases have been determined by x-ray crystallographic analysis and have made it possible to understand their structure-function relationships in more detail. Although the functions of snake venom serine proteases are diverse, the 3-D structures are quite similar. The crystal structure of TSV-PA has been determined to 2.5 A resolution and refined to an R factor of 17.8 (R free, 24.4). The enzyme, showed the overall polypeptide folds of trypsin-like serine proteinases, a phenylalanine at position 193, a C-terminal tail clamped via a disulphide bridge to the 99-loop, and a structurally conserved Asp 97 residue. Asp 97 is crucial

for the plasminogenolytic activity of TSV-PA. The C-terminal cxtension of TSV-PA is conserved among snake venom serine proteinases(42).

The typical folds of a trypsin-like serine proteinase are the active-site cleft is located at the junction of the two six-stranded β - barrels and the pattern of Cys residues and the C-terminal extension unique to snake venom serine protease (SVSP). TSV-PA contains six disulfide bonds that are highly conserved. The bridge and the C-terminal extension of TSV-PA are highly conserved among SVSPs that showed in **Figure 2** (43).

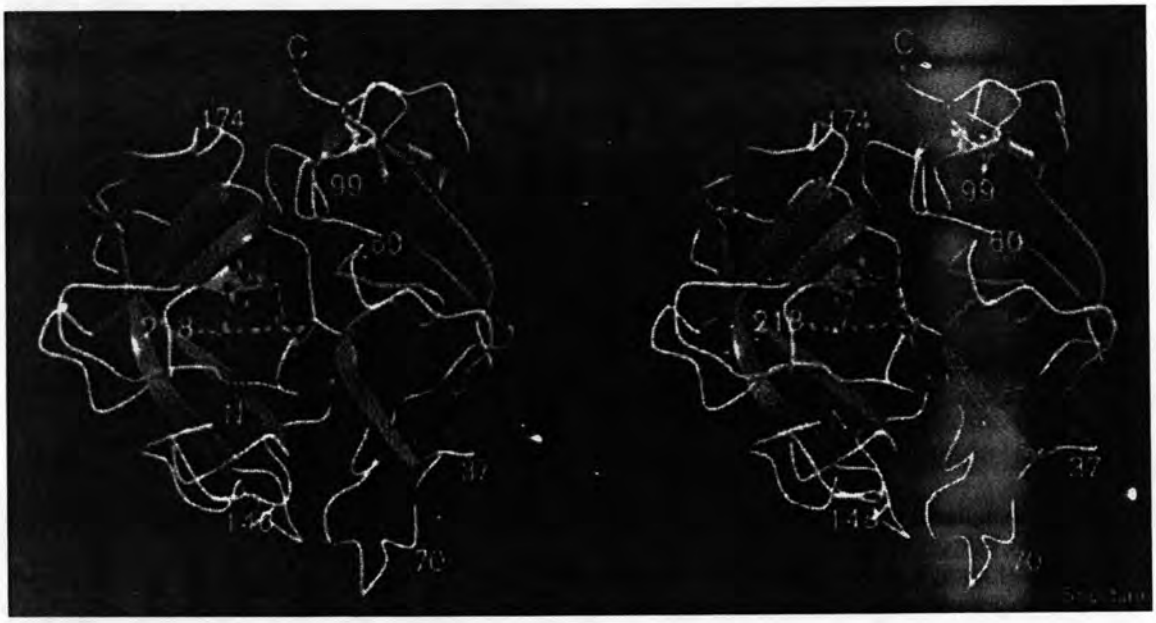

Figure 2. Crossed-eye stereo view ribbon representation of the crystallographic 3D structure of TSV-PA(43) (Solange M.T 2005)

Molecular cloning of cDNA of serine protease venom from *T. albolabris* will be the step of this field, giving more complete and accurate protein sequences. Because structural and functional information of a wide variety of venom protein has been published, functions of cloned gene can be deduced using homology comparison.

Furthermore, this allows production of a large amount of pure recombinant proteins that is potentially useful affecting proteins in coagulation for therapeutic or diagnostic purposes.