CHAPTER V

DISCUSSION AND CONCLUSION

This thesıs demonstrates a set of *T.albolabris* venom genes and their predicted toxicities. *T.albolabris* is a rich source of proteins affecting various human physiologic processes they mostly belong to a few protein families, e.g. snake venom serine proteases (SVSPs), snake venom metalloptoteases (SVM), disintegrins, C-type lectins, and PLA_2 **(Table 2).**

Sequences of SVSPs were conceptrally translated and analyzed. The clones of novel serine proteases cDNA can be classified into three groups. The first group was closely homologous to the thrombin-like enzyme, stejnobin (21), comprising GPV-TL1 and GPV-TL2. These two showed 97.7% amino acid identity. The second group, termed albofibrase, was similar to a beta fibrinogenolytic protease, stejnofibrase (50). The third group, GPV-PA, was closely related to plasminogen activator from *T. stejnegeri*, TSV-PA (44). Albofibrase and GPV-PA were more closely related with 76% amino acid identity. GPV-TL2 were approximately 62% identical to either albofibrase or GPV-PA. Amino acid sequence alignment of the four novel serine proteases was shown in **Figure 16**. They were closely homologous among others. The catalytic triad, as well as the key residues of substrate binding subsites 1 (S1) Asp 189, S2 Gly 216 and S3 Gly/Ala 226, was conserved (chymotrypsin numbering).

Like other SVSP, the sodium allosteric site of thrombin, Tyr 225, was substituted by Pro. All five GPV serine proteases used TCT codon for the Ser 195, similar to other Pro 255 proteases that usually use TCN codon (45). Pro 219, similar to glandular kallikrein and all 12 cysteines were conserved.

Most thrombin like SVSPs were FpA-cleaving enzymes (venombin A), except for contortrixobin and bilinebin, which release fpB (venombin B) and both FpA and FpB (venombin AB), respectively. Letters A-H represented nine variable regions (46). According to the homologous alignment, the hydrophobic, basic and extension residues, belonging to the putative anion binding exosite one (ABE 1), were labeled.The putative ABE2 could not be clearly defined. Among residues reported to be unique and constant in all coagulation SVSPs (46), four, Trp 51, Val 75, Gln 104 and 233 (chymotrypsin numbering), were not conserved in GPV thrombin-like enzymes (**Figure 17**).

A comparison of GPV-PA with other snake venom plasminogen activators was shown in Figure 16. Interestingly, Asp 20 and Glu 21 that were conserved in all thrombin-like SVSPs and GPV-PA (46) were replaced by Arg and Pro, respectively. The conserved residues in snake venom plasminogen activators that are Asp 82 (51) and Arg 174 (42) were found in GPV-PA. Asp 96, Asp 97 and Glu 98 (DDE) motif that was reported to be critical for PA activity in TSV-PA (18), as well as Phe 193 that was shown to be important for substrate specificity (42) and the escape from plasminogen activator inhibitor-1 (47) was also noted. Lys 240 unique to snake venom PA was replaced by Gln, which is similar to all thrombin-like SVSPs and LV-PA (48).

In this post-genomic era, molecular cloning of venom proteins may be an alternative to conventional protein purification for investigating the pathogenesis of snakebites. Green pit viper (GPV, *T. albolabris*) venom serine proteases were closely homologous to those from *T. stejnegeri* suggesting that they are closely related on the phylogenetic tree and their functions can be deduced. Our data have shown that the predicted fibrinogen consumption by GPV-TLs, fibrinogen degradation by albofibrase and fibrinolysis by GPV-PA are probably all contributed to defibrination syndrome in patients, supporting our previous finding that plasminogen activator activity was elevated in human (49).

In this research, *Pichia pastoris* expression system was used to produce recombinant albofibrase with methanol induction. There are many reports stated that recombinant proteins produced in *Pichia pastoris* result in high yields corrected folding and post translational modification. In our results, the yield of albofibrase produced in *Pichia pastoris* was 0.658 mg/Liter culture medium. However, there was also reported in the literature that the expression level is strongly affected by induction temperature (53). Optimization of temperature may be helpful.

Albofibrase was predicted to be a fibrinogenolytic enzyme. In general, venom fibrinogenolytic enzymes are more homologous to plasminogen activators than to thrombin-like serine proteases (36). Multiple sequence alignment of the albofibrase with other known viper venom fibrinogenolytic serine proteases was shown in **Figure 18**. They appear to be closely related. However, we cannot find any unique residues common to all fibrinogenases, but distinct from either thrombin-like or plasminogen activating enzymes (46, 36). This study demonstrated that albofibrase is an alpha fibrinogenase. However, upon sequence comparison, we cannot find any distinctive residues that differentiate between alpha fibrinogenases that are stejnefibrase1 (50), *Vipera lebetina* fibrinogenases (52) and albofibrase *versus* Beta fibrinogenases that are stejnefibrase2 (50), mucrofibrase (41) and *Vipera lebetina* fibrinogenase (52). Notably, albofibrase is closely related to stejnefibrase2, mucrofibrase to stejnefibrase1 and *Vipera lebetina* fibrogenases to each other **(Figure 19)**. We found that albofibrase contained a plasminogen-activating activity weaker than that of u-PA. In addition, at 2.3 nM of concentration, it showed fibrinogen clotting activity equivalent to 0.015 unit of thrombin. Because our recombinant expression system was used instead of protein purification, contamination from other snake venom enzymes is not possible. These data demonstrated that the substrate specificity is only relative. A serine protease may possess multiple activities with one predominant action on fibrinogen and/or

fibrinolysis. This may partially explain our inability to recognize any characteristic residues or motifs responsible for any specific activity.

Fibrinogenases have potential as novel anti-thrombotic defibrinating agents. An additional advantage is that they contained no or weak clotting activity, which may aggravate thrombotic problem. Furthermore, albofibrase effects can be rapidly reversed using antivenom that is already commercially available. This is useful for clinical bleeding or over-dosage. Mutagenesis of albofibrase should be performed in future studies in order to study structurefunction relationship identifying critical residues for each effect of the enzyme.

These data give us deeper insight in the mechanism of fibrinogenolysis, thrombin like effect, plasminogen activator, anti-coagulation and bleeding caused by green pit viper venom. In addition, this novel albofibrase should be studied for its potentials to be a new anti-thrombotic agent in the future.