CHAPTER V

DISCUSSION AND CONCLUSION

The green pit viper venom is characterized as serine proteinases phospholipase A2, C-type lectin like proteins, metalloproteinase and disintrigrins. To be complementary to the studies of other components of pit venoms, current study was investigated whether GPV-PA could specifically perform a function as plasminogen activator that cleaved plasminogen to plasmin.

The molecular cloning and sequencing revealed that the coding cDNA of GPV-PA consisted of 705 bp. In addition, the bioinformatics tools of the nucleotide sequence alignment and computational searching analysis, such as BlastN, discovered other genes with high BLAST scores with GPV-PA. The 9 proteins with the highest homology were shown in Figure 5. The highest three homologous nucleotide sequences were the venom PA precursor from *Viridovipera stejnegeri*, serine proteinase 2 precursor from *Cryptelytrops jerdonii* and the serine proteinase 3 precursor from *Cryptelytrops jerdonii,* respectively. Our clone from *Cryptelytrops albrolabris* is a novel gene and, therefore, we could not detect this sequence from current version of BlastN. As *Cryptelytrops sp* and *Viridovipera sp.* were newly named separating the, from the same original genus of *Trimeresurus*, this means that our cDNA sequence contained a high similarity with other serine proteases of the close genus classification.

After the cloning process, the GPV-PA protein was conceptually translated to be a 258-residue peptide. The molecular cloning approach predicted the molecular weight of 26.4 kDa of mature GPV-PA, which contain 240 amino acid residues excluding the 18-residue signal peptide. The protein showed the highest identity (94% similarity) with TSV-PA, a plasminogen activator from *Viridovipera stejnegeri*. The TSV-PA is a known plasminogen activator that triggers plasmin production from

plasminogen (46). This suggests that the GPV-PA probably contains a plasminogen activator activity.

The protein sequence was then analyzed using the Genious™ program that determined the protein sequence alignment and phylogenetic tree generation of the selected serine proteinases from venom proteins with plasminogen activator activity including *Trimersurus stejnegeri* (TSV-PA), *Lachesis muta muta* (LV-PA) and *Agkistrodon halys* (HLA-PA) (45,46,47). The result of sequence alignment showed the statistically identity of 82.6 % among all plasminogen activity venom proteins (Figure 6). The cysteine residues of all aligned proteins were examined and found that all the cysteine residue positions were conserved suggesting the identical arrangements of disulfide bond formation, which is important for similar maintaining the molecular structure and the biochemical function of proteins. Figure 8 indicated the identical 12 residues of cysteines in all the analyzed proteins suggesting that these four comparable proteins were similar in term of protein structure and function.

Furthermore, the phylogenetic tree prediction of the 4 snake venom plasminogen activators showed the related evolution of these proteins (Figure 9). This result revealed the close genetic relationship between GPV-PA and TSV-PA that were possibly the same family proteins. On the other hand, LV-PA and HLA-PA were more separated from both of snake venom plasminogen activators.

In this current study, the *Pichia pastoris* expression system was used to produce the recombinant GPV-PA protein using 0.5% and 1% methanol induction. In our experiment, we could produce GPV-PA with the yield of approximately 0.38 mg/Litre and 3.15 mg/Litre respectively. Therefore, the higher concentration of methanol (1%) could increase the production of recombinant protein without the reduction of the *Pichia pastoris* growth rate that was determined during the experimental cell harvests. Methanol is the sole carbon and energy source as well as inducer of heterologous protein production in recombinant *Pichia pastoris*

fermentations. While increasing the concentration of methanol up to 4.5% could not decrease the growth rate of yeast, together with the low level of toxic metabolic compound formation (48). The previous study from our group on the recombinant venom serine protease production using the same expression system revealed that the low level of protein production was correlated with the low concentration of methanol induction (28). In our experiment, we, therefore, adjusted the concentration of methanol from the previous investigation and could increase the production yield of same venom protein for approximately 8.29 times. This finding is very useful for future recombinant expression of other snake venom serine proteases.

After the purification of the recombinant GPV-PA protein using affinity column chromatography, the Western blot analysis was performed using anti-body against the 6-histidine tag. The result showed the 37-kDa band of GPV-PA. The bioinformatics calculation indicated that the molecular weight if GPV-PA with 6 histidines was approximately 27.2 kDa. The signal peptide sequence was present at position 18 to 22 as SYAQK with specific cleavage site between position 18 (S) and position 19 (Y). From computer analysis short amino acid residues after signal peptide sequence determine the possible protein trafficking along the post translational protein modification under secretory pathway through endoplasmic reticulum and golgi body as KSSEL. This discrepancy may be due to the post-tranlational modifications by the yeast, *Pichia pastosis, system.*

GPV-PA was first examined for the plasminogen activator activity. The protein could cleave plasminogen to plasmin resulting in the cleavage of the plasmin chromogenic substrate. Because our recombinant expression system was used to produce the pure recombinant venom plasminogen activator (PA) without contaminations with other PA proteins, the finding demonstrated definitely that this serine protease was specifically involved in fibrinolysis. However, the plasminogen

activity assay found that GPV-PA contained a plasminogen-activating activity that was weaker than the urokinase (u-PA) standard.

The plasmin is an important component inducing the physiologic and pathologic fibrinolysis, as well as proteolytic. Therefore, plasmin activated by GPV-PA can eliminate the coagulation products of the blood coagulation cascade. This probably contributed to bleeding disorders in green pit viper bite patients. Furthermore, the protein should be investigated to be a novel fibrinolytic agent for the treatments of thromboembolic diseases.

GPV-PA was also examined for the platelet aggregation inhibitory activity. The result demonstrated that recombinant GPV-PA did not inhibit the collagen-induced platelet aggregation. In contrast, with snake venom metalloproteinase/disintrigrin from *Cryptelytrops albolabris*, could inhibit platelet aggregation stimulated by collagen (38).

In summary, we characterized the full length cDNA of serine protease from the green pit viper (*Cryptelytrops albolabris*) venom glands and expressed GPV-PA in the *Pichia pastoris* expression system. The bioinformatics analysis of nucleotide and protein sequence demonstrated the high relationship with significant identity score with 3 other snake venom serine proteases with plasminogen activator including *Trimersurus stejnegeri* venom plasminogen activator (TSV-PA), *Lachesis muta muta* venom plasminogen activator (LV-PA) and *Agkistrodon halys* plasminogen activator (HLA-PA). However, GPV-PA from *Cryptelytrops albrolabris* is the highest relationship with TSV-PA by the phylogenetic tree prediction and protein alignment. By increasing the methanol concentration in the culture, the yield of the recombinant protein could be enhanced. Moreover, the recombinant GPV-PA contained plasminogen activator activity. Therefore, this novel recombinant plasminogen activator deserves further investigations to develop the protein into a therapeutic thrombolytic agent in the future.