



Research Article

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**The GPV-TL1, a Snake Venom Thrombin-like enzyme (TLE) from a Green Pit Viper (*Trimeresurus albolabris*), shows a strong fibrinogenolytic activity**

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**ABSTRACT**

Snakebites are the important global public health problem. Viper bites cause a low fibrinogen level and a systemic hemorrhage in human victims. Thrombin-like enzymes (TLEs) that degrade fibrinogen are the key molecules contributing to this viper-induced coagulopathy. Furthermore, these venom proteins have potentials to be novel anti-thrombotic agents. In this study, we expressed a TLE from green pit viper (*Trimeresurus albolabris*), GPV-TL1, in *Pichia pastoris* system and characterized its activities on human fibrinogen and platelets. The recombinant protein migrates as an approximately 31.6 kDa on SDS-PAGE under reducing condition. It released both fibrinopeptide A and fibrinopeptide B from human fibrinogen and showed plasma clotting activity of 233.3 thrombin units/mg. However, there was no platelet-aggregating activity. The recombinant GPV-TL1 could directly digest  $\alpha\alpha$ - and  $B\beta$ -chains of plasminogen-free human fibrinogen within 30 minutes and later degrade the fibrinogen  $\gamma$ -chain. Therefore, the GPV-TL1 displayed a strong  $\alpha\alpha$  and  $B\beta$  fibrinogenolytic activities while presenting a relatively weaker coagulant effect. This information gives us a deeper insight into the pathogenesis of GPV bites. The GPV-TL1 should be further investigated for the role in the treatment of thrombosis.

**Key words:** Fibrinogenolytic activity, *Trimeresurus albolabris*, Thrombin-like enzyme, Viper Venom, Clotting activity

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**INTRODUCTION**

Green pit vipers (GPV, *Trimeresurus spp.*) are the most frequent culprits of snakebites in Thailand [1]. Clinical manifestations range from mild local pain and inflammation to severe manifestations, such as local tissue necrosis, systemic hemorrhage, shock and death. A systemic bleeding is the combined result of fibrinogen consumptions, low platelet counts and direct vascular wall damages by snake venom metalloproteinases [2, 3].

Ninety percent of viper venoms are peptides that belong to a few families of proteins. They include serine proteinases, phospholipases A<sub>2</sub>, C-type lectin-like proteins (Snaclecs) and snake venom metalloproteinases that are the precursors of disintegrins [4, 5, 6]. To adapt for various preys in different areas, these venom genes underwent duplications and numerous nucleic acid substitutions resulting in a wide variety of proteins with very diverse sequences and effects on the hemostatic system [7].

Thrombin-like snake venom serine proteases (TL-SVSPs) or thrombin-like enzymes (TLEs) are serine proteases commonly found in pit viper venoms. TLE effects on blood clotting system partially mimic those of a thrombin [6, 8]. Whereas a TLE usually frees either fibrinopeptide A or fibrinopeptide B, a thrombin releases both fibrinopeptides from fibrinogen and activates coagulation factor XIII. As a result, the thrombin-produced fibrin becomes firmly polymerized and stabilized by the activated factor XIII (factor XIIIa), while the TLE-produced fibrin merely turns into soft clots, which will be degraded rapidly by the patient fibrinolytic system, resulting in

hypofibrinogenemia [9]. Furthermore, some TLEs can directly degrade fibrinogen [10, 11]. However, the fibrinogenolytic activities of TLEs have been rarely reported. In addition, the relative importance of the endogenous fibrinolytic response to coagulation effects and the exogenous fibrinolysis directly by the venoms for fibrinogen consumption *in vivo* remains unclear.

With their fibrinogen-lowering ability, the recombinant TLEs have potentials as anti-thrombotic agents [6]. In contrast to using thrombin that releases both fibrinopeptides, a study of an appropriate TLE will help revealing mechanisms and consequences of the releasing one of the fibrinopeptides from fibrinogen. In addition, deeper insights in the structure-function relationship of these serine proteases can be gained by comparing the amino acid sequences and functions among TLEs from various species. This may be useful to engineer proteins with desired properties in the future.

## EXPERIMENTAL SECTION

### *The Sequence Alignment and Computational Searching Analysis*

The nucleic sequence of the GPV-TL1 cDNA from the *Trimeresurus albolabris* venom gland cDNA library in the previous study [5] was conceptually translated. Homologous proteins were searched in GENBANK database using the BLAST tool. Their amino acid sequences were aligned using the CLUSTALW multiple sequence alignment program [12] and re-created with BOXSHADE 3.21.

### *Plasmid Constructions of the GPV-TL1 gene*

The mature GPV-TL1 protein coding sequence was amplified using the Advantage 2 polymerase proof-reading PCR (Clontech Laboratories, USA) and ligated to the pGEM-T vector (Promega, USA). The forward primer contained an *EcoRI* site: 5'-cgg aat tcG TCA TTG GAG GTG ATG AAT GCA ACA TAA A-3'. The reverse primer contained an *XbaI* site: 5'-gct cta gag cTG GGG GGC ATG TCA CAG TTG TAT TT-3' (Sigma-Aldrich, USA). The vector was then digested by *EcoRI* and *XbaI* restriction enzymes. Subsequently, the insert was put into the pPICZαA yeast expression plasmid with a polyhistidine tag (Invitrogen, USA) and subjected to sequencing verification using the BigDye® system (Applied Biosystems, UK).

### *The Expression of the GPV-TL1 gene in Pichia pastoris*

The constructed plasmids were linearized by *SacI* restriction enzyme (Promega, USA) and integrated into the X-33 wild-type *Pichia pastoris* using a chemical method of the *Pichia* EasyComp™ transformation kit (Invitrogen, USA). The expression condition was optimized as followed. The integrated yeast cells were cultured in BMGY medium to increase the cell mass at 30°C with shaking at 250 rpm for 12-16 hours. Then, the yeasts were transferred to BMMY medium to reach an A<sub>600</sub> OD of 1.0 and the recombinant protein expression was induced by 0.5% v/v methanol at 28°C with shaking at 250 rpm for 96 hours. The cultured medium was then centrifuged at 9000 x g to separate the medium from cells.

The supernatant from culture was concentrated by 10K Vivaspin (GE Healthcare Life Sciences, UK) and purified using the MagneHis™ protein purification system (Promega, USA) that selectively bound polyhistidine-tagged proteins.

### *The Characterization of the Expressed Protein*

The recombinant protein was quantified by Micro BCA™ protein assay (Thermo Scientific, USA). The purified recombinant protein was run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a reduced condition, blotted into polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) under a semi-dry condition and stained with 0.1% India ink in 3% phosphate buffered saline-tween™ 20 (PBS-T) buffer.

In addition, Western blots were performed using the mouse anti-histidine antibody (1:2000), the anti-mouse antibody tagged with horse radish peroxidase (1:1000, Dako Cytomation, Denmark) and the Amersham ECL Prime Western blotting detection system (GE healthcare Life Sciences, USA) to detect and estimate the recombinant protein size relative to the set of molecular weight standards (Bio-Rad, USA).

### *Thrombin-like Activities*

The coagulation activity was determined by the method of thrombin time (TT), which was performed in triplicate. The procedure was done by combining the 0.2 mL of a pooled normal plasma-saline mixture (1:1) pre-incubated at 37°C in a glass tube with 0.1 ml of GPV-TL1 or various concentrations of the thrombin (Sigma-Aldrich, USA) as a set of positive control. The clotting time was noted when the clot appeared. The equivalent thrombin activity was determined on a semi-log plot of thrombin concentrations against the clotting times.

The fibrinopeptides released from fibrinogen were detected by reverse-phase high performance liquid chromatography (RP-HPLC) (Varian Star, Varian, Inc.). The experiment was modified from Lu *et al.* (2000) [13]. In brief, the 0.5 ml of 0.2% human fibrinogen (Sigma, USA) was incubated with 10  $\mu$ l of recombinant GPV-TL1 protein (10  $\mu$ g/mL) at 37°C for 6 hours. Then, the reaction was stopped by boiling for 5 minutes and centrifuged at maximal speed for 10 minutes. The supernatant was gently collected and analyzed by a RP-HPLC ultraviolet (UV) detector at 214 nm. The 100  $\mu$ L of samples containing soluble fibrinopeptides were analyzed using the C18 column with the linear gradient between 2 eluting solutions: the solvent A (0.025 M ammonium acetate, pH 6.0) and the solvent B (50% acetonitrile in 0.05 M ammonium acetate, pH 6.0), at the flow rate of 1 mL/minute. Purified fibrinopeptides A and B (Sigma, USA) were used as markers. Thrombin-treated fibrinogen and fibrinogen alone were used as positive and negative controls, respectively.

#### *The Platelet Aggregation Assay*

A platelet aggregation assay was performed using the Chrono-Log model 700 CA aggregometer (Chrono-Log Corp., USA). The citrated venous blood from a medication-free healthy donor was collected at the ratio of 9:1 blood to 3.2% sodium citrate. Then, the whole blood was centrifuged at 150 x g for 15 minutes to collect the platelet-rich plasma (PRP). After careful separation of PRP, the platelet-poor plasma (PPP) was prepared from the remaining blood by centrifuging at 2000 x g for 20 minutes. PRP was counted and diluted by PPP to 250  $\times 10^9$  platelets/L. Then, 10  $\mu$ L of GPV-TL1 protein was added to 0.5 mL of PRP and incubated at 37°C for 10 minutes. The platelet aggregation was initiated by adding 10  $\mu$ L of 2 mg/mL collagen as a positive control. Light transmittance was recorded and the aggregation response was obtained.

#### *The Fibrinolytic Activity*

Human plasminogen-free fibrinogen (Sigma-Aldrich, USA) was dissolved in 50 mM tris-HCl buffer, pH 7.8 (containing 0.1 M NaCl). Thrombin was dissolved in normal saline to the final concentration of 50 units/mL. The activity assay was done in triplicate by combining an equal volume of the GPV-TL1 protein (10  $\mu$ g/mL) to 0.2% human fibrinogen and incubated at 37°C for 0, 30, 60 minutes, 2, 3, 4, 6, 12 and 24 hours. Thrombin was also used for comparison. The proteolytic products were analyzed by a reducing 10% SDS-PAGE with colloidal G-250 Coomassie staining.

## RESULTS

#### *The Computational Searching Analysis and Sequence Alignment*

The GPV-TL1 coding cDNA sequence from *Trimeresurus albolabris* was conceptually translated. The GPV-TL1 protein contained a 24-residue signal peptide, a catalytic triad (His-Asp-Ser) and 12 conserved cysteines to form intra-molecular disulfide bonds.

The BLAST search of the GPV-TL1 cDNA (accession number EF690365.1) showed the highest homology score (99% identity) with GPV-TL2 from *Trimeresurus albolabris* (accession number EF690366.1) and the TLE from *Trimeresurus stejnegeri*, stejnobin (accession number AF545576.1) with 96% identity. Additionally, the GPV-TL1 also shared more than 80% homology with the *Crotalus adamanteus* serine proteinase 5 (88% identity, accession number HQ414121.1), the *Trimeresurus mucrosquamatus* serpentokallikrein-1 (88% identity, accession number AF098263.1), the *Gloydus halys* salmobin (88% identity, accession number AF056033.1) and the *Crotalus dirissus terrificus* Gyroxin-like b1.4 serine protease precursor (87% identity, accession number EU360952.1). The GPV-TL1 protein sequence alignment with other snake venom TLEs and their homologies are shown in **Figure 1**.

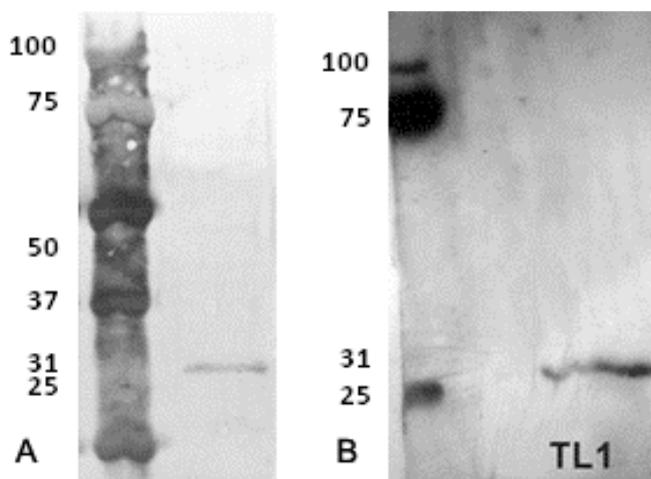


salmobin (80.4% identity, accession number AAC13280.1), *Crotalus dirissus terrificus* Gyroxin-like b1.4 (80.2% identity, accession number ABY65930.1), and human alpha-thrombin (27.3% identity, accession number 1PPB\_H). The sequences contain 24-residue signal peptides. The big letters H, D and S represent the Histidine, Aspartate and Serine of the catalytic triads. The numbers signify the amino acid position according to the chymotrypsin system. Above the sequences, the small letter b, h and e denote the basic, hydrophobic and extension residues of the putative exosite, respectively. The alignment was performed using CLUSTALW program [12] and re-created with BOXSHADE 3.21. The protein homologies were achieved by LALIGN program in SIB ExpASy Bioinformatics Resources Portal [14].

#### *The Characterization of protein expression products*

The optimal condition for recombinant GPV-TL1 expression in *Pichia pastoris* was the induction with the 0.5% v/v final concentration of methanol at the temperature of 28°C with vigorous shaking for 96 hours. The yield of recombinant protein was 1.95 mg per liter of culture medium.

The predicted molecular mass from the amino acid sequence of GPV-TL1 protein was 26.5 kDa as calculated by the protein molecular weight tool [15]. The molecular mass of the protein was approximately 31.6 kDa on reducing SDS-PAGE as shown in **Figure 2**. There was no other contaminant on the protein gel.



**Figure 2.** The affinity-purified recombinant GPV-TL1 protein

The protein was detected after 10% SDS-PAGE in a reduced condition followed by polyvinylidene difluoride (PVDF) membrane transfer.

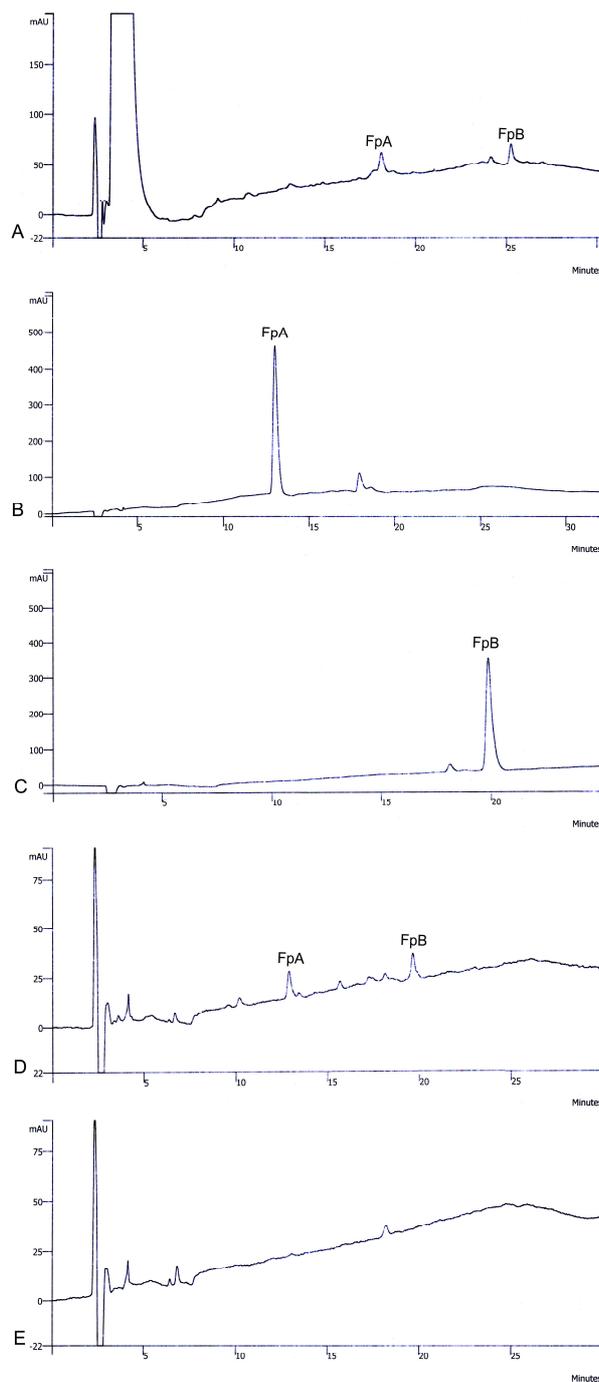
A. GPV-TL1 on membrane stained with 0.1% India ink for total protein detection.

B. Western blot analysis probed with the anti-histidine antibody, Lane 1: The pre-stained molecular weight markers and Lane 2: The GPV-TL1 protein.

#### *Thrombin-like activities*

The clotting activity of the recombinant GPV-TL1 on normal human plasma was determined and compared with thrombin. The average plasma clotting time induced by the GPV-TL1 was 50.7 seconds. In contrast to a firm clot induced by thrombin, a wispy clot appeared after the incubation with GPV-TL1. The clotting activity of the GPV-TL1 was equivalent to 233.3 thrombin units/mg of protein.

The GPV-TL1-induced fibrinopeptide releases were detected by RP-HPLC as shown in **Figure 3**. The GPV-TL1 cleaved both fibrinopeptide A and fibrinopeptide B from human fibrinogen.



**Figure 3.** The fibrinopeptide-releasing activity of GPV-TL1 protein

The fibrinopeptide elution profiles were determined by the reverse-phase HPLC.

A. Human fibrinogen incubated with GPV-TL1

B. Purified fibrinopeptide A as a marker

C. Purified fibrinopeptide B as a marker

D. Fibrinogen incubated with thrombin as a positive control

E. Fibrinogen alone as a negative control.

On the other hand, the recombinant GPV-TL1 protein did not induce any human platelet aggregation as compared with the collagen stimulation (data not shown).

#### *The Fibrinogenolytic Activity*

The direct fibrinogenolytic activity of GPV-TL1 was shown in **Figure 4**. After incubations with plasminogen-free fibrinogen at indicated times, the reactions were run on 10% SDS-PAGE under a reduced condition. Subsequently, the fibrinogen degradation products on the gel were stained by colloidal G-250 Coomassie dye.

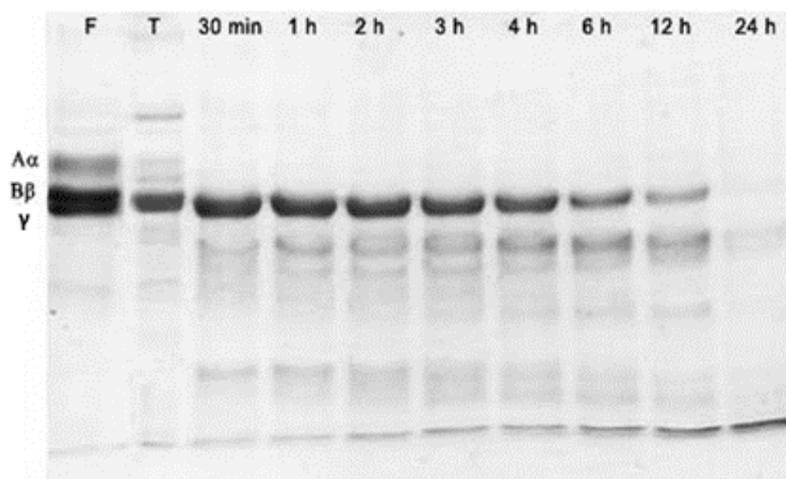


Figure 4. The fibrinolytic activity of GPV-TL1 on plasminogen-free human fibrinogen

The fibrinogen degradation products were analyzed by 10% SDS-PAGE in a reduced condition with colloidal G-250 staining. Lane 1 (F): Fibrinogen alone (negative control), lane 2 (T): 0.2% Fibrinogen incubated with thrombin (50 U/mL) for 24 hours. The rest were 0.2% fibrinogen incubated with GPV-TL1 (10  $\mu$ g/mL) (1:1 ratio) at the indicated durations.

As the intact fibrinogen decreased, the smaller degradation products appeared over time. The GPV-TL1 degraded both A $\alpha$ - and B $\beta$ -chains of fibrinogen within the first 30 minutes while the  $\gamma$ -chain of fibrinogen was cleaved later and more prominent only at 6 hours or after. Most of the 3 chains of fibrinogen were completely cleaved at 24 hours.

## DISCUSSION

In this study, the green pit viper thrombin-like enzyme 1 (GPV-TL1) from *T. albolabris* was successfully expressed using the *Pichia Pastoris* system. In contrast to other reported snake venom TLEs, it displayed a strong fibrinolytic activity. The GPV-TL1 was closely homologous to snake venom TLEs with coagulant activities that were stejnobin [16], salmobin [17] and gyroxin-like b1.4 [18]. However, the direct fibrinolytic effects of these proteins have not been determined.

As predicted from the sequences, we found that GPV-TL1 possessed the coagulant activity of 233.3 NIH thrombin units/mg of protein, which was stronger than that of the native stejnobin (122 units/mg) purified from the snake venom, but much weaker than the 3000 units/mg of thrombin [16]. Furthermore, only fragile plasma clots were formed by GPV-TL1 compared with robust clots induced by thrombin.

This study showed that the GPV-TL1 could release both fibrinopeptides (A and B) from fibrinogen, similar to thrombin. The native stejnobin preferentially released fibrinopeptide B over A [16]. The Gyroxin b1.4 only cleaved fibrinopeptide A and showed esterase and amidase activities [18]. The fibrinopeptide-releasing activities of the serine proteinase 5 [19], salmobin [17] and the serpentokallikrein-1 [20] were still unclear. It has been proposed that the basic and hydrophobic residues out of the active site of TLEs, termed the exosite, may determine substrate specificity of these proteins. The sequence alignment of these homologous proteins (**Figure 1**) showed some disparities in the putative exosite consistent with this hypothesis. A study that directly mutates these residues is required to prove the importance of this site.

Interestingly, the GPV-TL1 showed a prominent fibrinolytic activity by directly cleaving both A $\alpha$ - and B $\beta$ -chains of human fibrinogen within the first 30 minutes of the incubation. In fact, it started to cleave A $\alpha$ - and B $\beta$ -chains as early as 1 minute (data not shown). It also clearly digested  $\gamma$ -chain of fibrinogen at 6 hours. Furthermore, all three chains were almost completely cleaved at 24 hours. Therefore, this protein probably plays a role in causing severe hypofibrinogenemia in green pit viper bite victims. This is more likely mediated by the strong direct fibrinogen degradation in addition to the previously-known coagulant effects that trigger the endogenous fibrinolysis [9]. The direct fibrinolytic activities of other TLEs have been rarely reported. It remains to be determined whether this effect is unique to GPV-TL1 or common in other viper TLEs.

The molecular mass of the recombinant protein was approximately 31.6 kDa, which was larger than the size predicted from the calculated amino acid sequence of 26.5 kDa. This may be due to post-translational glycosylations

that may be vital for enzymatic activities. It was highly possible that the GPV-TL1 glycosylation sites were N-linked at asparagine (Asn or N) residues similar to other snake venom TLEs [21]. Using the NetNGlyc 1.0 server, the GPV-TL1 protein was predicted to have 4 possible N-linked asparagine glycosylation sites at the Asn-X-Ser/Thr motif ( N105, N156, N172, and N253).

In general, a snake venom TLE (SVTLE) has 5-30% carbohydrate molecules in its mass. Not only the glycosylation is significant for the stability of TLEs, but it also affects the enzymatic activities [22]. For example, the high activity of the ancrod is probably from its higher glycosylation than other SVTLEs. Conversely, Paes Leme *et al.* (2008) [23] demonstrated that Bothrops protease A (BPA) had an increased activity when limited deglycosylation was introduced.

The TLE, from *Agkistrodon rhodostoma* venom, ancrod (accession number AAA03254.1), has been used clinically as an anticoagulant for ischemic stroke. Its molecular weight was predicted to be 26.6 kDa from 234 amino acids [15]. However, ancrod possessed a specific oligosaccharide pattern, which brought about a higher molecular weight of over 40 kDa [24]. In contrast to the GPV-TL1, the ancrod cleaves only the A $\alpha$ -chain of fibrinogen while leaves the other chains intact. The plasma clotting activity of the GPV-TL1 was weaker than that of the ancrod (420 units/mg) but the ancrod fibrinogenolytic activity has not been reported [25].

The albobifrase was another protein previously expressed from Thai *Trimeresurus albolabris* cDNA by our group [11]. The recombinant protein had a molecular weight (MW) of 30 kDa including 2.2 kDa of glycosylation. It had an  $\alpha$ -fibrinogenolytic activity, a plasminogen activating ability and a clotting effect of 0.362 thrombin units/mg.

Lin *et al.* (2009) [26] expressed the TLE chitribrisin from Chinese *T. albolabris* using the *Escherichia coli* system. The recombinant chitribrisin was 28 kDa on SDS-PAGE with activities against both fibrinopeptides A and B, but a weak clotting ability of 0.372 thrombin units/mg. The chitribrisin shared 69.6% homology with the GPV-TL1. Both proteins had the AB fibrinogenolytic activity, whereas the chitribrisin had a much lower clotting effect. This activity difference might lie in the amino acids and/or the post-translational modifications.

Notably, the GPV-TL1 activity is promising as a therapeutic anticoagulant because of the relatively weak coagulant effects and the strong fibrinogenolytic activity. Therefore, it does not depend on patient fibrinolytic system for thrombus lysis. Furthermore, the GPV-TL1 did not induce any platelet aggregation and should be beneficial for patients with thrombotic disorders. Although a randomized controlled trial showed that the ancrod failed to improve the outcome in acute ischemic stroke [27], a TLE with a lower coagulation to fibrinolysis ratio may yield a more favorable result.

## CONCLUSION

The recombinant GPV-TL1 showed a strong fibrinogenolytic effect on all 3 chains of human fibrinogen, suggesting its roles in the GPV-induced coagulopathy. Furthermore, the GPV-TL1 should be investigated to be an anticoagulant in the future. The mutagenesis study on the non-conserved region between the H57 and D102 was under way to help clarifying the structure-function relationship of the TLE proteins.

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