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The structure–function relationship of thrombin-like enzymes from the green pit viper (*Trimeresurus albolabris*)

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ABSTRACT

Pit viper venoms can decrease fibrinogen levels in snakebite patients. Studies have shown that the hypofibrinogenemia is a consequence of snake venom thrombin-like enzymes (TLEs), the serine proteases that have the potential to be both diagnostic and therapeutic agents. Exosites of thrombin are the molecular regions that determine the substrate specificities, but its presence and significance in TLEs are unclear. Therefore, the putative exosites of recombinant TLEs derived from Green pit viper (*Trimeresurus albolabris*), GPV-TL1 and GPV-TL2, were mutated in a *Pichia pastoris* system. In a previous report, GPV-TL1 showed a strong fibrinogenolytic activity on the Aα and Bβ chains of fibrinogen, as well as a plasma clotting activity. Compared with GPV-TL1, the GPV-TL1m mutated in the putative exosite (TRN to RRR at residues 60–62) showed a weaker fibrinogenolytic activity with a similar clotting activity of 207.1 thrombin units/mg. GPV-TL2 contained two-residue differences from GPV-TL1 in the putative exosite (N73M and V74Y). GPV-TL2 selectively cleaved only the Aα chain of fibrinogen without detectable clotting activity. The mutated GPV-TL2 (GPV-TL2m) showed a weaker fibrinogenolytic activity compared with that of the wild type. These results support the important roles of the putative exosite in snake venom TLE activities. This information is helpful for future protein engineering.

1. Introduction

Coagulopathy from snakebites ranges from mild laboratory abnormalities to severe systemic bleeding (White, 2005). These are the results of viper venoms that are mixtures of protein families, such as serine proteases, metalloproteinases, C-type lectin like proteins (Snaclec) and phospholipases A_2 (Mackessy, 2009; Lu et al., 2005; Rojnuckarin et al., 2006). Snake venom thrombin-like enzymes (SVTLEs or TLEs) from viper venoms are serine proteases that cause clotting factor consumption and hypofibrinogenemia in human victims (Castro et al., 2004).

TLEs have been studied not only because of their toxic properties, but also their potential as anti-thrombotic medications, topical hemostatic agents, and useful tools to study mechanisms of coagulation *in vitro* (Serrano, 2013). The molecular mechanism of TLEs is the digestion of arginyl bonds, releasing fibrinopeptides (Fp) from fibrinogen. Most TLEs preferentially release the fibrinopeptide A (FpA). However, some proteins selectively cleave out the fibrinopeptide B (FpB) or release both Fps. Most TLEs do not activate factor XIII to cross-link fibrin and hence undergoes rapid fibrinolytic degradation (Serrano, 2013). For this reason, TLEs are clinically used to treat hyperfibrinogenemia, an important risk factor for ischemic stroke and peripheral artery diseases (Sweetnam et al., 1996). A meta-analysis of controlled clinical trials suggested that venom-derived fibrinogen-depleting drugs, ancrod and Defibrase®, might be beneficial in the treatment of ischemic stroke (Hao et al., 2012).

As TLEs can clot fibrinogen *in vitro*, this fibrin clot is used topically to stop bleeding and promote wound healing after surgery or trauma as part of fibrin sealant systems (Barros et al., 2009; Barbizan et al., 2013). Furthermore, TLEs are useful tools to study thrombin-fibrinogen interactions. As thrombin releases both Fps, TLEs allow investigations of the mechanisms and consequences of only one Fp release (Weisel, 2007). In contrast to thrombin, TLE activities are not affected by heparin. Batroxobin is a TLE that is used in clinical laboratories in a test known as Reptilase time.

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Prolonged thrombin time (TT) with normal Reptilase time indicates a heparin effect (Karapetian, 2013).

Thrombin is a multifunctional protein that plays key roles in hemostasis. In addition to Fp releases, thrombin activates coagulation factors V, VIII, XI, XIII, protein C, platelet and thrombin activatable fibrinolysis inhibitor (TAFI). After injury, activated factor X changes prothrombin to the active thrombin. Thrombin recognizes and cleaves the arginyl bonds within the Aα and Bβ chains of fibrinogen releasing Fps (Bode et al., 1992). Interestingly, thrombin activities are controlled by its exosites that are the clusters of hydrophobic and basic residues outside the active site. The exosites bind various substrates contributing to diverse functions of thrombin (Bock et al., 2007).

Because TLEs can perform some functions of thrombin, they may also contain exosites that determine enzymatic activities. Although a region with basic and hydrophobic residues is present in TLEs (Maroun, 2001; Maroun and Serrano, 2004; Pradniwat and Rojnuckarin, 2013), the existence of the functional exosite remains to be proven. We previously cloned and expressed GPV-TL1, a TLE from *Trimeresurus albolabris* (Pradniwat and Rojnuckarin, 2014). It exhibited a strong fibrinogenolytic and a relatively weaker coagulant activities compared with ancrod. Therefore, this study aimed to mutate the putative exosites of GPV-TLs into ancrod-like sequences in order to investigate the role of the exosite in TLE activities. This may give us useful information for future protein engineering.

2. Materials and method

2.1. Sequence alignment and computational searching analysis

The nucleic sequences of GPV-TL1 and GPV-TL2 cDNAs were conceptually translated. Homologous sequences were searched in the GENBANK database using the BLAST tool. Their amino acid sequences were aligned using the CLUSTALW multiple sequence alignment program (Kyoto University Bioinformatics Center, 2013) and re-created using BOXSHADE 3.21.

2.2. Plasmid constructions of native GPV-TL2, mutant GPV-TL1 (GPV-TL1m) and mutant GPV-TL2 (GPVTL2m) cDNAs

The GPV-TL2 cDNA from the previous study (Rojnuckarin et al., 2006) was amplified by Advantage 2 polymerase proofreading PCR (Clontech Laboratories, USA) with a forward primer containing an *Eco*RI recognition site: 5′-cggaattcGTCATTGGAGGTGATGAATGCAACATAAA-3′, and a reverse primer with an *Xba*I recognition site: 5′-gctctagagcTGGGGGGCATGTCACAGTTGTATTT-3′ (Sigma–Aldrich, USA). Afterward, the insert was ligated to the pPICZαA vector that contained a polyhistidine tag and a zeocin resistant gene. The incubation temperatures included the DNA denaturation step at 95 °C for 10 min, followed by 30 amplification cycles: 95 °C denaturation for 30 s, 64 °C annealing for 30 s, 68 °C extension for 1.5 min, and a final extension at 68 °C for 10 min.

The GPV-TL1 and GPV-TL2 genes from our previous work (Rojnuckarin et al., 2006) were mutated using the Quikchange lightning site-directed mutagenesis kit (Stratagene, USA) with forward and reverse primers containing mutation sites: 5′-CACTGCGAAAGAAGACGTATGTACATATACCTTGGTATG-3′ and 5′-GGTATATGTACATACGTCTTCTTTCGCAGTGTGCAGCGGTG-3’ (Sigma–Aldrich, USA) (Zheng et al., 2004). The incubation temperatures included the DNA denaturation step at 95 °C for 1 min, followed by 16 amplification cycles: 95 °C denaturation for 1 min, 53 °C annealing for 1 min, 72 °C extension for 12 min, the second 60 °C annealing for 1 min and a final extension at 70 °C for 30 min.

Subsequently, the constructs were put into the pPICZαA plasmid (Invitrogen, USA) and verified by DNA sequencing using the Applied Biosystems BigDye terminator and ABI Prism 3100 sequence analyzer (Applied Biosystems, UK).

2.3. Expression of the GPV-TL2, GPV-TL1m and GPV-TL2m cDNAs in Pichia pastoris

The constructed plasmids were linearized by the *SacI* enzyme (Promega, USA) and integrated into X-33 wild-type *P. pastoris* using a chemical method (*Pichia* EasyComp transformation kit, Invitrogen, USA).

The integrated yeast cells were cultured in BMGY to increase cell mass at 30 °C with 250-rpm shaking for 12–16 h. The yeasts were then transferred to BMMY to reach A_{600} of 1 OD and were induced by 0.5%, 1% and 1% v/v methanol to express GPV-TL2, GPV-TL1m and GPV-TL2m proteins, respectively, at 28 °C with 250-rpm shaking for 96 h. The cultured media were then centrifuged at 9000 × g to separate the media from cells.

2.4. Purification and characterization of recombinant proteins

The supernatant from the culture media were concentrated by 10K Vivaspin (GE Healthcare Life Sciences, UK) and purified using MagneHis® protein purification system (Promega, USA) that bound the polyhistidine tag.

The purified recombinant proteins were run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a reduced condition, semi-dry blotted into a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad, USA) and stained with 0.1% India ink in 3% phosphate buffered saline-tween 20 (PBS-T) buffer for total protein. In addition, Western blotting was performed using mouse anti-histidine (1:2000), anti-mouse-HRP (1:1000) antibodies (Dako Cytomation, Denmark) and Amersham ECL Prime Western blotting detection (GE healthcare Life Sciences, USA) to detect and estimate protein sizes relative to a set of molecular weight standards (Bio-Rad, USA). The recombinant protein concentrations were measured by Micro BCA protein assay (Thermo Scientific, USA).

The Periodic Acid Schiff (PAS) reagents were used to stained for glycosylation. The purified proteins were run on 10% SDS-PAGE in a reduced condition. The gel was fixed in 12% tricarboxylic acid for 30 min. After washing with water, the gel was placed in 1% periodic acid in 3% acetic acid for 50 min and washed overnight. Subsequently, the gel was put in the filtered Schiff's reagent containing 0.5% basic-fuchsin, 0.05 N HCl, and 1% sodium metabisulphite in the dark for 50 min. The gel was then washed three times (10 min each) with 0.5% sodium metabisulphite and water.

2.5. Platelet aggregation assay

Platelet aggregation assay was performed using the Chrono-Log model 700 CA aggregometer (Chrono-Log Corp., USA). Citrated venous blood from a medication-free healthy donor was collected at the ratio of 9:1 blood to 3.2% sodium citrate. Whole blood was then centrifuged at 150 × g for 15 min 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 × g for 20 min. PRP was counted and diluted by PPP to 250 × 10^9 platelets/L. Then, 10 μL of GPV-TL proteins was added to 0.5 mL PRP and incubated at 37 °C for 10 min. Light transmittance was recorded and the maximum aggregation response was obtained. PPP was given as a value of 100% aggregation. As the positive