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Expression and characterization of a recombinant fibrinogenolytic serine protease from green pit viper (*Trimeresurus albolabris*) venom

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Abstract

Viper venom serine proteases (SPs) display several effects on hemostatic system. Molecular cloning showed that *Trimeresurus albolabris* venom comprised a mixture of five SPs with thrombin-like (2), fibrinogenase (2) and plasminogen-activating (1) activities. Because only few fibrinogenolytic SP sequences were reported, we decided to express albofibrase, a novel fibrinogenase from *T. albolabris* using *Pichia pastoris* system. The recombinant active form of enzyme was 30 kDa including 2.2 kDa of glycosylation. Albofibrase showed an α fibrinogenase activity. In addition, a plasminogen activating and clotting effect were detectable. Albofibrase prolonged APTT and PT in a time-dependent manner. The effect was neutralized by pre-incubation with equine antivenom to *T. albolabris*. Therefore, the protein is potentially useful as a new anticoagulant as the antidote is clinically available. Sequence analysis compared with other snake venom fibrinogenases and SPs could not find any unique residues responsible for their various effects. Structure–function relationship should be further studied using mutagenesis in order to explore the mechanisms of venom protease functional diversity.

Keywords: Snake venom; *Trimeresurus albolabris*; Fibrinogenase; Serine protease; *Pichia pastoris*

1. Introduction

Green pit viper (*Trimeresurus albolabris*, GPV) venom is toxic to hemostatic system resulting in hypofibrinogenemia, thrombocytopenia and systemic bleeding (Mitrakul, 1973; Mitrakul and Impun, 1973). The most prominent activity of the crude venom *in vitro* is induction of blood coagulation. In human envenomation, we confirmed that this thrombin-like effect (cleavage of fibrinopeptide A from fibrinogen) was the principle activity. However, accompanying fibrinolytic system over-activation degrades partially clotted fibrinogen causing paradoxical anti-coagulation *in vivo* (Rojnuckarin et al., 1999). To dissect venom components affecting hemostasis, molecular cloning is an attractive mode. The deduced protein sequences are complete and accurate, providing data for a bioinformatic analysis of venom components including the postulation of their possible functions. Our groups have cloned four major families of proteins from *T. albolabris* venom glands: five serine

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proteases (SPs), three snake venom metalloproteases, two C-type lectin proteins and two phospholipases A_2 (Rojnuckarin et al., 2006). *T. albolabris* SPs comprise two thrombin-like enzymes (GPV-TL1 and GPV-TL2), two isoforms of fibrinogenolytic proteases (albofibrases) and a novel plasminogen activator (GPV-PA). Sequence analysis comparing with those of other species suggested that all these SPs were cooperatively responsible for systemic defibrination syndrome seen in patients.

Snake venom SPs contained several activities on blood coagulation, fibrinolysis and platelets. These proteins have potentials to be novel diagnostic or anti-thrombotic agents for clinical uses. Thrombin-like enzymes from snake venom have been used to measure fibrinogen level in the presence of heparin (Reptilase® time, Funk et al., 1971), to characterize fibrinogen functions in dysfibrinogenemia (Cunningham et al., 2002) and to remove fibrinogen from the samples for thrombin-dependent tests (Mullin et al., 2000). Furthermore, hyperfibrinogenemia has been shown to associate with thrombosis and poor outcome in patients with stroke. Recombinant ancrod, a thrombin-like SP from Malayan pit viper (*Agkistrodon rhodostoma*), was found to significantly and cost effectively improve outcomes after cerebral infarction in human (Sherman et al., 2000; Samsa et al., 2002). In addition, Defibrase®, a SP from *Bothrops* spp., is clinically beneficial for ischemic stroke (Guo et al., 2006). Availability of cDNA allows pure recombinant protein production and mutagenesis for detailed structure–function studies. In addition, we can engineer proteins with desirable effects for future clinical or research uses. Relatively few fibrogenolytic SPs have been well characterized. In addition, they are potentially useful as both anticoagulant and fibrinolytic agents. Therefore, a novel enzyme, albofibrase, was chosen for this research.

2. Materials and method

2.1. Expression of albofibrase in pichia pastoris

Green pit viper (GPV) venom SP cDNA have been cloned as previously described (Rojnuckarin et al., 2006). Easy Select Pichia Expression Kit (Invitrogen, Carlsbad, USA) was used according to the instruction. To prepare the construct, the complete coding sequence of albofibrase excluding the signal and pro-peptide was PCR amplified using Taq DNA polymerase (Roche, Basel, Switzerland) with forward, GCG AAT TCC AAA AAT CTT CTG AAC TGG TC, and reverse, TTT CTA GAG CCG AGA GGC AAG TTG CAT C, primers. EcoRI and Xba I recognition sites were incorporated into the forward and reverse primers, respectively. The PCR product was electrophoresed on agarose gel, extracted and purified before subcloned into pGEM-T vector (Promega, Madison, USA) and transformed to *E. coli*, JM109. Plasmid DNA and *Pichia* expression vector, pPICZαA, were subsequently digested with EcoR I and Xba I before ligation. pPICZαA contained *C*-terminal Myc and polyhistidine tag to facilitate protein purification and detection. After transformation into *E. coli*, Zeocin-resistant colonies were extracted for plasmid and verified by sequencing.

Before transformation into *Pichia pastoris*, plasmid DNA was linearized using SacI and precipitated in ethanol. Competent *Pichia pastoris*, X-33 strain, was prepared by chemical method using ethylene glycol and DMSO in the Pichia EasyComp Kit (Invitrogen). Competent cells were kept in −80 °C until use. The linearized plasmid was transformed into the yeast using polyethylene glycol and heat shock at 42 °C for 10 min according to the kit instruction. After an hour of YPD medium incubation, transformed cells were plated on YPDS plates with 100 mg/ml Zeocin and incubated for 3–10 days at 30 °C. The presence of integrants was tested using PCR of *Pichia* genomic DNA with primers GAC TGG TTC CAA TTG ACA AGC and GCA AAT GGC ATT CTG ACA TCC.

For small-scale expression, a single *Pichia* colony was inoculated in 25 ml of BMGY and incubated at 30 °C in a shaking incubator until culture reaches an OD_{600} of 2–6. The culture was adjusted to OD_{600} of 1.0 in BMMY and induced expression by 0.5% (v/v) methanol. An aliquot of culture was collected every 24 h before adding new methanol until reaching 96 h. Recombinant protein was maximally expressed at 96 h as detected by Western blot probed with 1:3000 murine anti-His tag antibody (Amersham Pharmacia, Hong Kong, PRC).

Large-scale expression was then performed in 4 l flasks. Supernatant was concentrated by ultrafiltration using Vivaspin concentrator that had MWCO of 10,000 Da (Vivascience, Sartorius AG, Goettingen, Germany). Recombinant albofibrase was purified according to the protocol of BD TALON Metal Affinity Resins (BD Biosciences, Mountain view, USA). Briefly, the concentrated media is chromatographed on a cobalt-based immobilized