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Differential action of proteases from *Trimeresurus malabaricus*, *Naja naja* and *Daboia russellii* venoms on hemostasis

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Abstract

The action of venom proteases and their role in hemostasis has been compared in the venoms of *Trimeresurus malabaricus*, *Daboia russellii* and *Naja naja* from the Southern region of Western Ghats, India. These venoms exhibit varying amounts of proteolytic activity and also influence hemostasis differently. Casein hydrolyzing activity of *T. malabaricus* venoms was 16 and 24 fold higher than those of *N. naja* and *D. russellii* venoms, respectively. With the synthetic substrate TAME, the highest activity was observed in *T. malabaricus* venom. *N. naja* venom did not hydrolyze TAME even at higher concentrations. These variations in proteolytic activity also influenced the coagulation process. *T. malabaricus* and *D. russellii* venoms are strongly procoagulant and reduce the re-calcification time from 148 to 14 and 12 s, respectively. Similarly, both *T. malabaricus* and *D. russellii* venoms reduce the prothrombin time from 12.5 to 6.0 s. On the other hand, *N. naja* venom is anticoagulant and prolongs re-calcification time to 600 s and prothrombin time to 42 s. In spite of varied effects on hemostasis, all the venoms hydrolyze fibrinogen. *T. malabaricus* venom hydrolyses both Aα and Bβ subunits. While *D. russellii* and *N. naja* venoms hydrolyse only Aα. None of these venoms hydrolyze the γ subunit of fibrinogen. Inhibition studies with specific protease inhibitors revealed that both *N. naja* and *T. malabaricus* venoms contain only metalloproteases. *D. russellii* venom contained both serine and metalloproteases. Only, *T. malabaricus* venom exhibited thrombin-like activity and induces fibrin clot formation with purified fibrinogen within 58 s. Even though *D. russellii* venom exhibits procoagulant activity, it did not show thrombin-like activity and may act on other coagulation factors.

Keywords: Coagulant activity; Endemic snake; Fibrinogenase; Thrombin like activity; Hemostasis; Metalloproteases; Western Ghats of India; *Trimeresurus malabaricus*

1. Introduction

The Western Ghats is a mountain range that extends along the West coast of India, from Bombay to the Southern tip of the country. The Western Ghat section, due to monsoon rain has mosaic of tropical forest types like wet evergreen forest, mangrove swamp and moist deciduous forests. As the agronomic conditions of the Western Ghats in the state of Karnataka, Kerala and Tamilnadu are suitable for plantation crops like coffee, cardamom, rubber and tea (Ishwar, 2001). Large areas of the region are cleared for the cultivation of these crops. Despite the fact that the forests have been largely cleared they still have an extremely high number of endemic species. *Trimeresurus malabaricus* is one of the endemic venomous snakes found in the Southern tropical rainforests of the Western Ghats. *T. malabaricus* bite cases are common among the plantation workers here and therefore is a major occupation hazard (Sajeeth et al., 2003). *Naja naja* and *Daboia russellii* snakes are widely distributed throughout the entire Indian subcontinent. *T. malabaricus* envenomation, while generally non-lethal, causes extensive local tissue damage compared to bites of *N. naja* and *D. russellii* (Gowda et al., 2006). In contrast to *T. malabaricus*, other *Trimeresurus* species bite is lethal with less or devoid of any local tissue necrosis (Tan et al., 1989). Irrespective of the toxicity, snake venoms affect the human hemostatic system differently. Hemostatic alterations may

Abbreviations: SEM, standard error mean; SDS, sodium dodecyl sulphate.

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exacerbate other venom effects and each venom poses special health challenges (Meier and Stocker, 1991).

Snake venoms may be procoagulant, anticoagulant and/or fibrinolytic (Markland, 1991; Siigur and Siigur, 1992). The major proteins affecting hemostasis are proteases (Garcia et al., 2004; Cecchini et al., 2005; Lu and Clemetson, 2005; Sanchez et al., 2005). Several proteases affecting coagulation and fibrinolysis have been characterized from *D. russellii* and *N. naja* venoms. Those from *D. russellii* venom are predominantly procoagulant while that from *N. naja* venom is anticoagulant in nature (Prasad et al., 1999; Shashidharamurthy et al., 2002). The major clinical manifestation of *D. russellii* envenomation is coagulopathy and systemic hemorrhage; however, the precise combination of systemic effects varies markedly within this species. *D. russellii* venom from Northern, Southern, Eastern and Western region of India exhibit procoagulant activity, associated with varying caseinolytic and TAME hydrolyzing activities. Its interesting to observe that *D. russellii* venom from Eastern India exhibit procoagulant activity at lower concentration and anticoagulant activity at higher concentration. In the other three regions, *D. russellii* venom exhibits only procoagulant activity (Prasad et al., 1999). Regional variation in venom composition and toxicity was also described for Indian *N. naja* and *N. Kaouthia* venom (Shashidharamurthy et al., 2002; Mukherjee and Maity, 2002). Eastern *N. naja* venom is neurotoxic and procoagulant whereas Western region venom is myotoxic and anticoagulant (Shashidharamurthy et al., 2002).

Extensive work on composition and toxicity of Indian *N. naja*, and *D. russellii* venoms has been carried out (Jayanthi and Gowda, 1988; Babu and Gowda, 1991; Basavarajappa and Gowda, 1992), but little is known about the composition of *T. malabaricus* venom, even though it is a hazard for plantation workers (Sajeeth et al., 2003). Preliminary studies from our laboratory revealed that *T. malabaricus* venom is several fold rich in proteases than *N. naja* and *D. russellii* venoms, which accounts for the observed local tissue damage (Gowda et al., 2006). However, the effect of these proteases on hemostasis is not known. Present study is intended to help in improving the management of coagulopathy resulting from envenomations in Western Ghats by these snakes.

2. Materials and methods

2.1. Materials

Fat-free casein, bovine serum albumin (BSA), *p*-tosyl-L-arginine methyl ester (TAME), human fibrinogen and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO. USA). Other protease inhibitors-1, 10-phenanthroline, ethylene diaminetetraaceticacid (EDTA) and ethylene glycol-*N*, *N*, *N*′, *N*′-tetraaceticacid (EGTA) were purchased from SRL Chemical Company, Bangalore, India. All other chemicals and reagents purchased were of analytical grade. All solvents were redistilled before use. Fresh human blood samples were collected from healthy volunteers from the Department of Biochemistry, University of Mysore, India.

2.2. Source of venom

Venom collection was performed manually. Venom from each fang was separately collected in a sterile microcentrifuge tube covered with a latex membrane to restrain salivary contamination. Venom samples were freeze dried and stored at room temperature. Venom samples were dissolved in phosphate buffered saline (PBS) and used for all assays.

2.3. Protein estimation

Protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.4. Caseinolytic activity

Caseinolytic activity was assayed according to the method of Murata et al. (1963). 0.4 mL Casein (2% in 0.2 M Tris–HCl buffer, pH 8.5) was incubated with different concentrations of venom (2–100 μg) at 37 °C for 2 h in a 1 mL reaction volume. The reaction was stopped by adding 1.5 mL of 0.44 M trichloroacetic acid and allowed the tubes to stand for 30 min. Tubes were centrifuged at 500 ×*g* for 15 min. An aliquot (1.0 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin reagent (1:2, v/v). The colour developed was measured at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h at 37 °C.

2.5. Arginine esterase activity

Arginine esterase activity was assayed according to the method of Hummel (1959) using TAME as substrate. Different quantities of venom (20–100 μg) were added to 1 mL of 0.5 mM TAME in 0.2 M Tris–HCl buffer, pH 8.5 at 37 °C. Change in absorbance was recorded at 250 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 250 nm/30 min at 37 °C.

2.6. Human fibrinogenolytic activity

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng (1976). The 40 μL reaction mixture contained 50 μg of human fibrinogen in 10 mM Tris–HCl buffer (pH 7.6). It was incubated with 10 μg of venom samples dissolved in PBS for 1 h at 37 °C. In addition dose and time dependencies of fibrinogenolysis were also examined. The reaction was terminated by adding 20 μL of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. Hydrolyzed products were analyzed by 10% SDS-PAGE carried out according to the method of Laemmli (1990). Proteins were visualized by staining with Coomassie brilliant blue R-250 and destained with 25% ethanol and 8% acetic acid in water. Inhibition studies were carried out as described above, after pre-incubation of 10 μg of venom samples dissolved in PBS with