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# Thermal detoxification of the venom from *Daboia russelli russelli* of Eastern India with restoration of fibrinolytic activity

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ABSTRACT

The fibrinolytic components of venom have been evaluated for long in the enzymatic treatment of thrombosis. Russell's viper venom has fibrinolytic activity that is associated with hemorrhagic activity. Here it has been investigated whether the crude venom could be detoxified by thermal denaturation retaining fibrinolytic activity. The venom at 0.05 mg/ml in 20 mM K-phosphate, pH 7.5 when exposed to 100 °C for 5 min followed by cooling at 25 °C for 1 h led to its detoxification, while 80–85% of the fibrinolytic activity was recovered. Assessment of toxicity of the renatured venom in mice after injection at 5 fold excess of the lethal dose showed no lethality including hemorrhage, myotoxicity, cytotoxicity and liver toxicity. This simple method for preparation of fibrinolytic component for therapeutic use may be developed.



## 1. Introduction

Several fibrinolytic enzymes have been characterized from different snake venoms like *Calloselasma rhodostoma*, *Bothrops atrox*, *Vipera lebetina*, *Crotalus adamanteus*, *Agkistrodon contortrix, Daboia russelli* etc (Chakrabarty et al., 2000; Egen et al., 1987; Gasmi et al., 1991; Willis and Tu, 1988). Efficiency of the fibrinogenase from *V. lebetina* for removal of thrombosis was investigated in rat model of venous thrombosis (Gasmi et al., 1997). Later, fibrinolytic enzymes from *C. rhodostoma* and *B. atrox* were applied in patients with deep vein thrombosis and ischemic stroke under controlled condition (Nuber, 2000; Orgogozo et al., 2000; Sherman et al., 2000). Recently, recombinant fibrinolytic enzymes derived from snake venom are being clinically trialed (Moll et al., 2006; Ouriel et al., 2005; Toombs, 2001,). The *Daboia russelli russelli* venom from Eastern India has one skin and muscle specific hemorrhagin of 73 kDa, named as VRR-73 (Chakrabarty et al., 2000). VRR-73 shows fibrinolytic and esterolytic activities that are independent of hemorrhagic activity. Upon thermal denaturation, its hemorrhagic activity is irreversibly lost while the fibrinolytic activity could be reversibly restored. This fibrinolytic activity of VRR-73 has the potential for development as anticoagulant of therapeutic use upon inactivation of the hemorrhagic activity (Chakrabarty et al., 1997). The aim of this study is to develop a simple method of detoxification and subsequent recovery of the fibrinolytic activity of crude Russell's viper venom (RVV) upon heating. The information might be useful for preparation of a fibrinolytic agent of RVV origin for therapeutic use in future.

## 2. Material and methods

### 2.1. Materials

RVV was procured from Mr Dipak Mitra, a licensed supplier of venom form Calcutta Snake Park, as desiccated

*Abbreviation:* RV, Russell's viper same as *Daboia russelli russelli*; RVV, Russell's viper venom; ht-RVV, heat treated RVV; $PLA_2$, phospholipase $A_2$; DMPC, 1,2-dimyristoyl-sn-glycero-3 phosphocholine.

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yellowish shinning crystals containing approximately 60% of protein (w/w). Fibrinogen fraction I, thrombin (bovine plasma), azocasein, DMPC (1,2-dimyristoyl-sn-glycero-3 phosphocholine), plasminogen (bovine plasma), S-2251 (specific amidolytic substrate for plasmin), NAD and dialysis tubing of cut off range <12 kDa were from Sigma, USA. Pre-stained protein Mw ladder containing 170, 130, 95, 72, 55, 43, 34, 26, 17, and 10 kDa markers for SDS-PAGE (PageRuler) was from Fermentas, USA. Urokinase as lyophilized powder (Korea Green Cross Corporation, Seoul, Korea) was procured from medical stores. Other reagents of analytical grade were purchased locally. Adult male inbred BALB/C mice (20 ± 2 gm) were obtained from animal house facility of this institute. All procedures for animal care, handling and experiments were done according to the guidelines of the ethical committee formed by local legislation.

### 2.2. Heat treatment

To optimize the conditions of heat treatment for maximum recovery of fibrinolytic activity, variable concentrations of RVV (0.025–1 mg/ml) in 5 ml of 20 mM K-phosphate, pH 7.4 were taken in glass tubes (Borosil) of diameter 1.3 cm. The tubes were placed in a boiling water bath at 100 °C for 5 min. Following heating the solutions were left at 25 °C for 1 h for slow cooling. Any precipitate that formed was removed by centrifugation and the supernatant was lyophilized. Hereafter, this supernatant is referred as 'heat-treated RVV' (ht-RVV). Maximum recovery of fibrinolytic activity was observed with 0.05 mg/ml of RVV. Protein concentration was determined using Folin-Phenol reagent where BSA served as reference (Lowry et al., 1951; Oosta et al., 1978). Protein compositions of RVV and ht-RVV were analyzed by 15% SDS-PAGE after staining with Coomassie Brilliant Blue R250. Abundance of proteins was estimated from densitometric scanning of the gels where area of the bands was calculated using 'Image J' software (NIH, USA).

### 2.3. Fibrinolytic activity

Fibrinolysis was estimated after minor modification of the published protocols (Astrup and Mullertz, 1954; Chakrabarty et al., 2000). White opaque fibrin gels were formed in 0.5 × 11 cm tubes by polymerization of a solution of 160 mg fibrinogen fraction I and 2.4 U of bovine thrombin in 10 ml of 70 mM ammonium sulfate at 25 °C for 2 h. Variable amount of RVV or ht-RVV (0–200 μg) was applied on the surface of the gel keeping the application volume constant at 50 μl. The tubes were incubated at 37 °C up to 120 h and the volume of lysed fibrin was measured. Positive and negative controls were provided by 50 μl of PBS in presence and absence of 4 μg of urokinase. In a separate set, fibrinolytic activity of RVV (200 μg) in presence and absence of 1 mU/ml of plasminogen was compared. The gels were incubated for 24 h after application of venom. Urokinase (4 μg) and PBS served as positive and negative controls respectively.

### 2.4. Fibrin zymography

Fibrinolytic components of RVV were analyzed by fibrin zymography (Kim et al., 1998). Briefly, 15% polyacrylamide gel containing 0.1% SDS was polymerized in presence of 20 mg/ml of fibrinogen and 2.0 U/ml of thrombin. Increasing amount of RVV (0.175–17.5 μg) was applied. After electrophoresis, the gel was soaked in 50 mM Tris–HCl, pH 7.4 containing 2.5% Triton X-100 for 30 min at 25 °C. Then the gel was transferred to reaction buffer, 30 mM Tris–HCl, pH 7.4 containing 200 mM NaCl, 10 mM $CaCl_2$ and 0.02% Brij 35 for 24 h at 37 °C for protease digestion Thereafter, the gel was thoroughly washed with distilled water. Due to fibrin digestion, white bands against dark background were developed after 20 h of staining with Coomassie Brilliant Blue R250.

### 2.5. Plasminogen activation

Activation of plasminogen was estimated after addition of 100 μg of RVV or ht-RVV in 300 μl reaction mixture containing 0.2 mg/ml of plasminogen, 50 mM Tris–HCl, pH 7.8 and 0.01% Tween 80 at 37 °C. Aliquots of 60 μl were withdrawn at stipulated time intervals and added to 940 μl of 50 mM Tris–HCl, pH 7.8 containing 0.01% Tween 80 and 0.09 μM of S-2251 in a spectrophotometer cuvette. Rate of *p*-nitroaniline formation from S-2251 by plasmin was monitored at 405 nm. Formation of plasmin was measured in terms of units where one unit is defined as the amount of enzyme that can raise $A_{405}$ of 0.001/min (Zhang et al., 1995). To evaluate the contamination of plasminogen in fibrinogen, 17.5 mg/ml of fibrinogen was incubated with 200–800 μg/ml of RVV or 40 μg/ml of urokinase in presence of 50 mM Tris–HCl, pH 7.8 containing 0.01% Tween 80 for 1 h at 37 °C. Activation of plasminogen was monitored after addition of 0.09 μM of S-2251 as described above. A Specord 200 spectrophotometer (Analytica Jena, Germany) was used for optical measurements.

### 2.6. Proteolytic activity

Proteolytic activity of RVV or ht-RVV was determined using azocasein as substrate. Venom (50–250 μg) was incubated with 250 μl of 1% azocasein in 0.2 M K-phosphate, pH 7.4 at 37 °C for 2 h. After precipitation of the undigested protein by 10% trichloroacetic acid, the supernatant was diluted with equal volume of 0.5 N NaOH. The absorbance was read at 440 nm.

### 2.7. Phospholipase $A_2$ activity

Phospholipase $A_2$ ($PLA_2$) activity was measured quantitatively as described in Maity and Bhattacharyya (2006). Briefly, 0.295 μmole of 1,2-dimyristoyl-sn-glycero-3 phosphocholine (DMPC) in the form of phospholipid micelle in 20 mM K-phosphate, pH 7.4 containing 8 mM $CaCl_2$ was placed in a spectrofluorimeter cuvette. Hydrolysis was initiated by the addition of 5–25 μg of RVV or ht-RVV to the assay mixture. The decrease in Rayleigh scattering intensity was continuously monitored using a Hitachi F4500 spectrofluorimeter (ex: 650 nm; em: 650 nm).