Fibrinogenolytic toxin from Indian monocled cobra (*Naja kaouthia*) venom

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A fibrinogenolytic toxin of molecular weight 6.5 kDa has been purified from the venom of Indian monocled cobra (*Naja kaouthia*) by repeated cation exchange chromatography on CM-sephadex C-50. The purified toxin did not show any phospholipase activity but was mildly hemolytic on human erythrocytes. This toxin, called Lahirin, cleaved fibrinogen in a dose- and time-dependent manner. The digestion process apparently started with the Aα chain, and gradually other lower-molecular-weight chains were also cleaved to low-molecular-weight peptides. The fibrinolytic activity was completely lost after treatment with ethylene di-amine tetra acetic acid (EDTA). However, exposure to 100°C for 1 min or pre-treatment with phenyl methyl sulfonyl fluoride (PMSF) did not affect the fibrinolytic activity. Cleavage of di-sulphide bonds by β-mercaptoethanol or unfolding the protein with 4 M urea caused complete loss of activity of pure Lahirin.

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1. Introduction

Monocled and spectacled cobras are the most frequently encountered venomous snakes in India. These snakes are also responsible for most of the mortalities and morbidities caused by envenomation. The most prominent symptoms of cobra bite are those involving the nervous system. Within a short time, the victims start to hypersalivate. Slurring of speech and ptosis follow, which confirm central nervous system breakdown (Warrell 2010). The victim gradually develops paralysis of the limbs. If untreated with an appropriate dose of antiserum, the patient may die of asphyxia due to respiratory muscle paralysis. In contrast, viper envenomation primarily causes hemotoxicity and hemorrhage (Warrell 1989). The hemorrhagic effect is frequently fortified by noncoagulability of blood. Although cobra envenomation primarily causes neurotoxicity, coagulopathy has also been noted in bite victims (Khandelwal *et al.* 2007). The strong neurotoxic symptoms frequently overshadow the hemotoxic activities of cobra venom. However, in the course of the present study, these authors came across several anticoagulant/fibrinogenolytic factors of wide-ranging molecular weights (MWs) in monocled cobra venom. The specific toxins adversely affecting blood coagulation process may inhibit platelet aggregation or may digest either fibrin(ogen) or some other clotting factors in the plasma. The fibrin(ogen)-digesting toxins have gained importance because of their probable therapeutic potential against intravascular coagulation. This is probably the first low-MW fibrin(ogen)olytic toxin reported from Indian monocled cobra *Naja kaouthia*.

2. Materials and methods

Dry-pooled venom (Chakrabarty *et al.* 2000; Doley and Mukherjee 2003; Pook and McEwing 2005) of Indian monocled cobra venom (ICV) was purchased from Calcutta Snake Park, Kolkata, India. All fine chemicals used were purchased from Sigma Chemicals, USA. All other reagents

Keywords. Aα chain; cobra; fibrin(ogen)olytic; fibrinogenolysis; venom

Abbreviations used: BSA, bovine serum albumin; EDTA, ethylene di-amine tetra acetic acid; ICV, Indian monocled cobra venom; MW, molecular weight; PLA, phospholipase A; PMSF, phenyl methyl sulfonyl fluoride; RBCs, red blood cells

were of analytical grade. Polyvalent antiserum (against Russell's viper, monocled cobra, spectacled cobra and saw-scaled viper) were obtained as generous gift from Serum Institute of India, Pune, India.

2.1 *Fractionation of Indian cobra venom*

Dry cobra venom (50 mg) was reconstituted in 20 mM phosphate buffer, pH 7.4 (running buffer) overnight at 4°C. The solution was then centrifuged at 5000 rpm for 5 min at 4°C to discard the cell debris and other particulate matters. The clear solution was then subjected to cation exchange chromatography on a column of CM-sephadex C-50 (1.5 cm× 10.5 cm). The column was first washed with two column volumes of running buffer only and the unadsorbed fractions were collected in microfuge tubes (1 ml each) at a flow rate of 0.5 ml/min. The adsorbed fractions were then eluted with a linear gradient of NaCl (0–0.5 M) in three column volumes of running buffer. The fraction size was restricted to 1 ml.

Fractions with fibrin(ogen)olytic activity were pooled and subjected to further fractionation on a column of CM-sephadex C-50 (1.5 cm×10.5 cm). The sample solution was allowed to be adsorbed slowly on the column resin. Adsorbed proteins were then eluted with a linear shallow gradient of NaCl (0.2–0.5 M) in three column volumes of running buffer. One milliliter fractions were collected in each tube at a flow rate of 0.5 ml/ min.

Protein contents of the fractions were monitored in a UV/VIS spectrophotometer (Shimadzu UV-2450) at $A_{280\ nm.}$

2.2 *Purity of fractions and molecular weight determination*

Purity of all fractions was assessed by electrophoresis (SDS-PAGE) on 12% sodium dodecyl sulfate polyacrylamide gel. However, purity of the active fraction after the second chromatography was checked by SDS-PAGE on 15% gel because of its low MW. The protein bands were stained with 1% Coomassie brilliant blue R250 in 7.5% acetic acid and 10% methanol overnight. The gel was then destained by repeated washings with 7.5% acetic acid and 10% methanol.

Molecular weight was determined from a standard curve constructed by plotting mobility in 15% SDS-PAGE versus MW of marker proteins, namely, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, aprotinin and insulin.

2.3 *Protein estimation*

Protein contents of the purified samples were estimated by modified Bradford assay (Bradford 1976). A standard curve was constructed using bovine serum albumin (BSA) as standard protein.

2.4 *Assay of fibrinolytic activity*

The method described by Astrup and Mullertz (1952) (fibrin plate method) was followed with slight modification. 3.3 mg of fibrinogen type I was dissolved in 0.2 ml of 20 mM K-phosphate buffer, pH 7.4. Ammonium sulphate was added to above solution to a final concentration of 70 mM. Five microliter of thrombin was added to the above solution and transferred immediately to a 0.5 ml microfuge tube. The solution was allowed to clot by incubating for 2 h at 25°C. Test samples (20 µl) were applied on the top of the clot and incubated at 37°C for 18 h. Fibrinolytic activity of the samples was observed by the liquefaction of the clot. Whole Russell's viper venom and 0.85% saline were used as positive and negative controls, respectively.

2.5 *Fibrinogenolytic activity*

Fibrinogenolytic activity was confirmed by incubating fibrinogen fraction I (2 mg/ml) with different fractions (20 µg) and 0.85% saline (control) for different time intervals at 37°C and the incubated mixtures were subjected to SDS-PAGE on 12% polyacrylamide gel. The protein bands were viewed by staining with 1% Coomassie brilliant blue R250. Fibrinogenolytic activity was monitored by comparing position and appearance of specific bands with that of fibrinogen incubated with 0.85% saline only (Bos *et al.* 1997).

2.6 *Assay of hemolytic activity*

Blood was collected aseptically from the left radial vein of 'O'+male healthy volunteers in 0.85% saline and centrifuged at 3000 rpm for 3 min. The supernatant was discarded and the pellet containing red blood cells (RBCs) were washed thrice with normal saline by repeated centrifugation at 3000 rpm for 3 min. One percent RBC suspension (0.3 ml) was taken in each tube to which 0.2 ml of fraction was added. Distilled water replaced fractions as positive control and normal saline was added to RBC suspension as negative control. All the tubes were then incubated for 1 h at 37°C and centrifuged at 3000 rpm for 5 min. Absorbance of the supernatants were measured at 540 nm. Values obtained with positive control represented 100% hemolysis (Chakrabarty *et al.* 2000). Volunteers of only 'O' + blood groups were chosen to maintain uniformity of samples.

2.7 *Assay of phospholipase A activity*

Presence of phospholipase A (PLA) activity was tested using egg yolk as substrate (Neumann and Habermann 1954). A suspension was prepared with 9 ml chicken egg yolk, 2.51 ml of 2% NaCl, 1.49 ml of 0.5% EDTA, 4.44 ml

of 1.0% $CaCl_2$, 2.0 ml of Tris-HCl buffer, pH 7.5, and 0.56 ml of 0.85% saline. Two-hundred microliters of fractions were added to 2 ml of egg yolk suspension, mixed well and incubated at 37°C for 1 h. Incubated samples were then placed in a boiling water bath and the time required for coagulation of the samples were recorded. Normal saline (0.85%) and 5 µg Russell's viper venom were used as negative and positive controls, respectively.

2.8 *Inhibition of activity*

Fibrinolytic activity of the purified toxin (Lahirin) was also estimated after treatment with 2 mM EDTA, freshly prepared 1 mM phenyl-methyl sulphonyl fluoride (PMSF) or exposure to 100°C for 1 min. in a boiling water bath. Pure toxin was also exposed to 4 M urea and 1 µl of 98% β-mercaptoethanol to study the importance of secondary and tertiary structure on fibrinolytic activity.

Fibrinolytic activity of the toxin was then estimated by the procedure described above.

3. Results

3.1 *Purification of fibrin(ogen)olytic toxin*

Purification of fibrin(ogen)olytic toxin was achieved by fractionation of the ICV and the semi-purified fraction on a column of CM-sephadex C-50.

The whole ICV fractionated into six protein peaks, two of which were in the unadsorbed region (figure 1a.). All peaks were

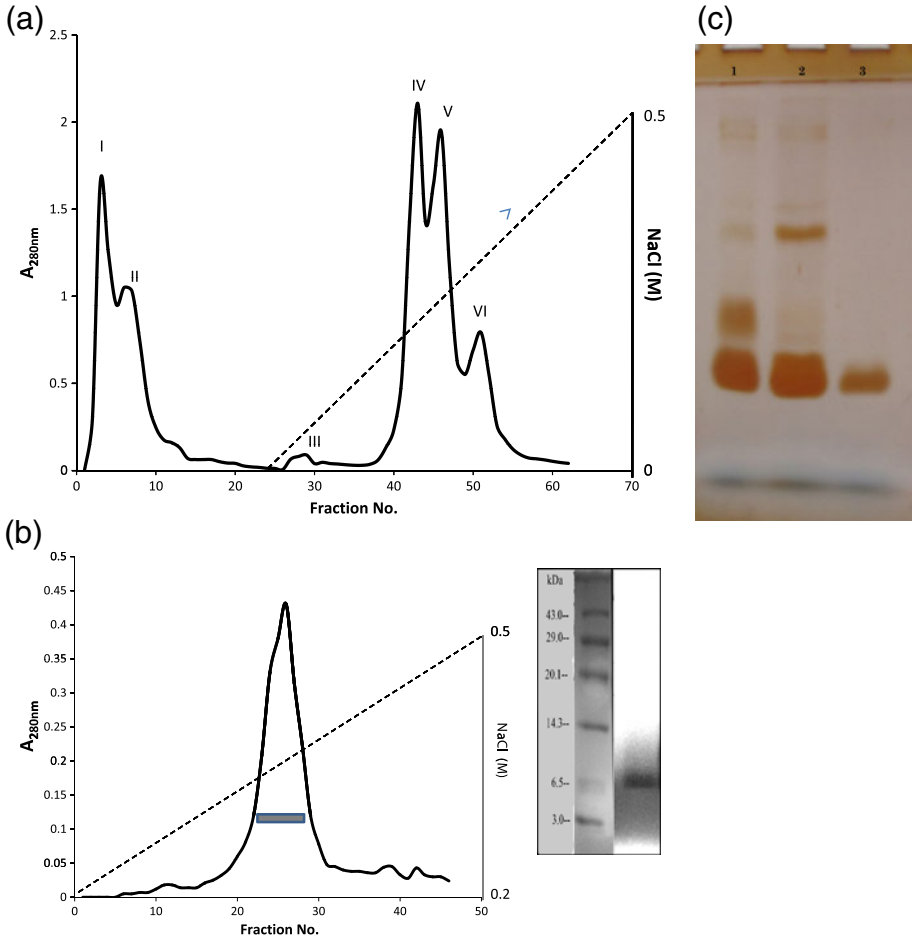

Figure 1. (**a**) Cation exchange chromatography of Indian cobra venom on CM-sephadex C-50. The dotted line represents linear gradient of NaCl. Elution peaks are numbered with roman numerals as mentioned in the results section. (**b**) Rechromatography of Peak V on CM-sephadex C-50. The fractions under Peak V (**a**) were pooled, concentrated and fractionated. Fractions were eluted with a linear gradient of NaCl (0.2 to 0.5 M). The dark bar under the major peak denotes presence of anticoagulant activity. **Inset:** 15 % SDS-PAGE of pure Lahirin with molecular weight markers. (**c**) Purification stages of Lahirin. Lane 1: ICV (25 µg); lane 2: pooled fractions of peak V (30 µg); lane 3: pure Lahirin (10 µg). The samples were run in 15% SDS-PAGE. The gel was silver stained for visualization of bands.

numbered with roman numerals I, II, III, IV, V and VI. Peaks II, IV, V and VI showed fibrinogen degrading activity *in vitro*. Although fibrinogenolytic activity was also noted in some other fractions, studies on only peak V are discussed in detail here.

Fractions under peak V were pooled, concentrated and subjected to a second chromatography on a separate CM-sephadex, C-50 column, of same dimensions as described earlier. The protein profile revealed one major peak and several small peaks (figure 1b). Only fraction 26 showed fibrinogenolytic activity.

3.2 *Purity and molecular weight*

Peak V from first chromatography separated into several distinct bands of various MWs in 15% SDS-PAGE.

Fraction 26 obtained after second chromatography of peak V showed a single band at approximately 6.5 kDA region (figure 1c). This fraction also showed significant fibrinogenolytic activity and will henceforth be designated as Lahirin, in memory of Prof Sites Chandra Lahiri, a well-known Indian toxinologist.

3.3 *Fibrin(ogen)olytic activity*

Fibrinogen (2 mg/ml) solutions incubated with different fractions (20 µg/ml) obtained from the first chromatography (figure 1a) were run on 12% SDS-PAGE. Clear fibrinogen digestion was seen with peaks II, IV, V and VI (result not shown). The digestion was most prominent on Aα chain of fibrinogen.

Incubation of peak V fraction (20 µg) with 2 mM EDTA for 30 min caused almost total loss of fibrinolytic activity (result not shown).

Lahirin showed preferential digestion of Aα chain of fibrinogen, followed by Bβ and γ chains when incubated with 2 mg/ml fibrinogen solution in a dose-dependent manner (figure 2).

Lahirin also showed digestion of fibrinogen following incubation for different time intervals (figure 3). Fibrinogen solution (2 mg/ml) was incubated with 5 µg/ml of Lahirin for different time periods at 37°C. The figure clearly shows gradual digestion of the Aα chain followed by Bβ and γ chains with longer incubation time. Gradual increase in thickness of the lower-MW bands indicated accumulation of small peptides produced by digestion. The degraded peptides were clearly visualized at the low-MW region of the gel. Complete digestion of the Aα chain was observed after 5 h. No change was noted in the band patterns for fibrinogen incubated with 0.85% saline.

Lahirin (fraction 26) also totally liquefied 200 µl fibrin clots *in vitro* when incubated for 18 h at 37°C. The fibrinolytic activity of Lahirin was totally inhibited by EDTA treatment. However, exposure to 100°C for 1 min or pre-treatment with phenyl methyl sulfonyl fluoride (PMSF) did not affect the fibrinolytic activity (figure 4a). Cleavage of di-sulphide bonds by β-mercaptoethanol or unfolding the protein with 4 M urea caused complete loss of activity of pure Lahirin (figure 4b).

3.4 *Hemolytic activity*

Peak V showed 20% hemolysis on 'O' + RBCs. Treatment of peak V fraction (0.2 ml) with 2 mM EDTA abolished hemolytic activity. Incubation of the same amount of peak V fraction with polyvalent antiserum (v/v) for 1 h also reduced the hemolytic activity to 10% of the control. Lahirin showed only 10% hemolytic activity compared with the control (figure 5).

3.5 *Phospholipase activity*

Phospholipase activity could be detected in all peaks obtained from the first chromatography, except peak VI.

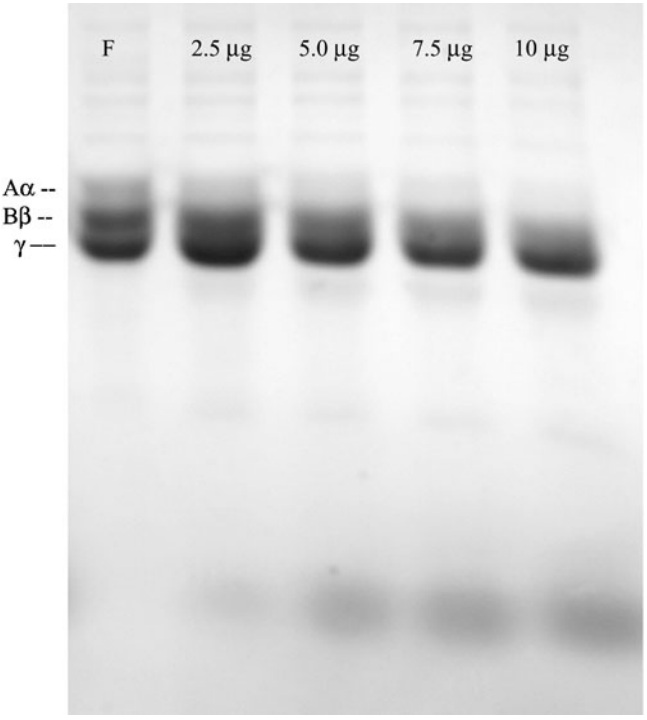

Figure 2. Digestion of fibrinogen by pure Lahirin. Fibrinogen factor I (2 mg/ml) was incubated with different doses of Lahirin (2.5 µg/ml, 5.0 µg/ml, 7.5 µg/ml and 10.0 µg/ml) for 2 h and run on 12% SDS-PAGE. The gel was stained with 1% Coomassie brilliant blue R250.

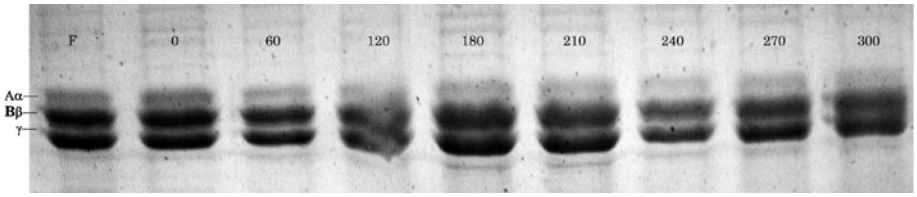

Figure 3. Time-dependent fibrinolytic activity of Lahirin. Fibrinogen (2 mg/ml) was incubated independently with Lahirin (5 µg/ml) for different time intervals in 20 mM potassium phosphate buffer pH 7.4 at 37°C. Samples were kept frozen at −80°C after their incubation period till run on SDS-PAGE. Lane 2: 0 h incubation, lane 3: 1 h, lane 4: 2 h, lane 5: 3 h, lane 6: 3.5 h, lane 7: 4 h , lane 8: 4.5 h , lane 9: 5 h; lane 1: fibrinogen alone after 5 h incubation,

Lahirin (fraction 26) obtained by the second chromatography of peak V was found to be devoid of phospholipase activity.

4. Discussion

Elapid venoms are known for their neurotoxicity. However, many strong fibrin(ogen)olytic toxins of varied specificity have been purified from elapid snakes (Doley and Mukherjee 2003; Kumar *et al.* 2010). Venom of the most common venomous snake in India, the monocled cobra, is least studied in this regard. We describe here a low-MW fibrinogenolytic toxin purified from the monocled cobra venom. Repeated cation exchange chromatography of the whole ICV was used to purify the 6.5 kDa fibrinogenolytic toxin. The pure toxin was named Lahirin as described above. Phospholipase activity was noted in most fractions, including peak V after the first chromatography. However, further fractionation of peak V by second chromatography effectively separated phospholipase activity from the fibrinogenolytic activity.

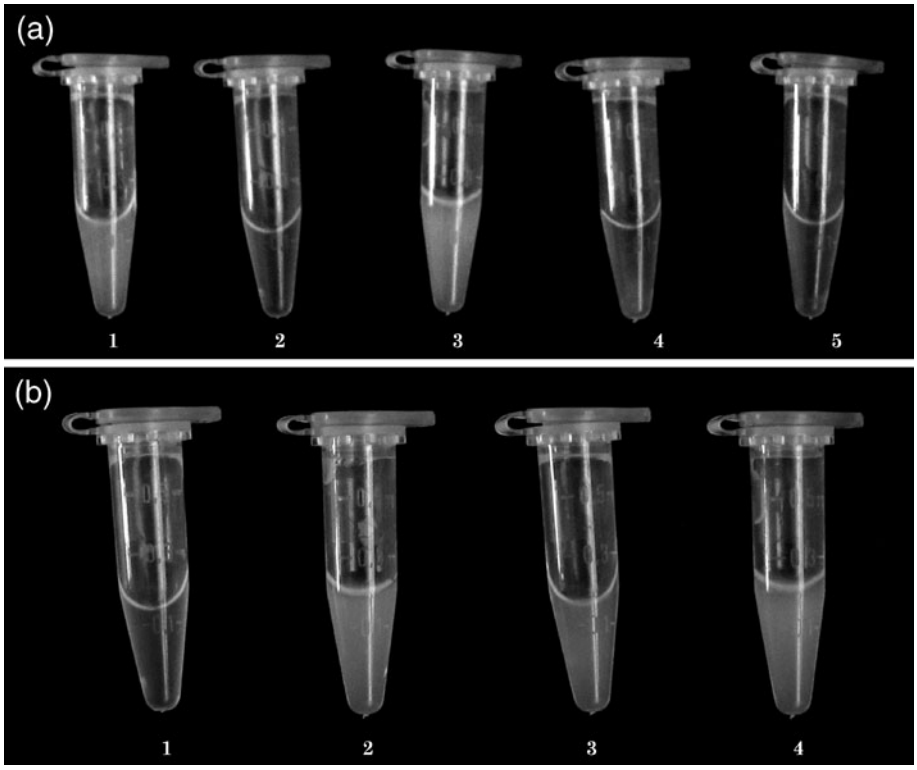

Figure 4. Inhibition of fibrinolytic activity of Lahirin: (**a**) Lahirin (15 µg) was pretreated with inhibitors for 1 h at 37°C or exposed to heat at 100°C for 1 min before incubating with fibrin clot developed in the microfuge tube. The fibrinolytic activity was visualized after 18 h. (**b**) Fibrin clot incubated with (1) saline alone, (2) Lahirin, (3) Lahirin pre-treated with 2 mM EDTA, (4) Lahirin exposed to heat and (5) Lahirin pre-treated with 1 mM PMSF. Fibrin clot incubated with (1) Lahirin, (2) 0.85% saline, (3) Lahirin pre-treated with 1 µl of β-mercaptoethanol and (4) Lahirin pretreated with 4 M urea.

Lahirin was found to be primarily active on Aα chain of fibrinogen. Incubation for longer periods gradually caused digestion of the Bβ and γ chains also (as visualized by thinning of the respective bands) and produced much smaller fragments, which migrated to the lower end of the gel (figure 3). All major bands of fibrinogen were found to be degraded after 24 h incubation with Lahirin (figure 6). This activity of Lahirin is reflected by gradual conversion of a solid fibrin clot first to a very soft gel and then to a complete liquid. Recently, we separated a fibrinogenolytic toxin from jellyfish venom that degraded both Aα and Bβ chains of fibrinogen within 2 h and all major chains of fibrinogen within 3 h of incubation (unpublished observation).The fibrinogenolytic activity of Lahirin is therefore slower compared to some other such toxins. Lahirin also cannot be called a strict α-fibrinogenase. However, it is clear that the digestion starts with Aα chain. Several fibrinogenolytic toxins from elapid snake venoms have been reported to be specific to the Aα chain. However, a few have also been found to degrade the Bβ chain in addition to the Aα chain (Markland 1998). Whereas many viper (*Vipera lebetina, Cerastes cerastes, Echis carinatus* and *Bitis arietans*) venom-derived fibrinogenolytic toxins are known to degrade both Aα and Bβ chains. However, gradual degradation of Aα, Bβ and γ chains is not reported so far for any such toxins (Markland 1998; Kini 2006). Pure Lahirin did not lose its fibrinogenolytic or fibrinolytic activity following exposure to heat. Heat stability of Lahirin is probably dependent on disulphide bonds and the tertiary structure, as disruption of di-sulphide bonds with β-mercaptoethanol or unfolding of the molecule by 4 M urea completely abolished its fibrin(ogen)olytic activity.

The preliminary studies put Lahirin in the category of low-MW metalloprotease anticoagulants (Kini 2006). But unlike other such toxins, it continues to digest other chains of fibrinogen. Many metalloproteinase anticoagulants also show hemorrhagic or hemolytic activities (Kini 2006). Lahirin also showed mild hemolytic activity as an additional effect. The mechanism of hemolytic activity was not pursued in this study.

Previously, Kumar *et al*. (2010) reported NN-PF3 a fibrin(ogen)olytic metalloprotease with similar properties purified from *Naja naja* venom. However, the reported molecular mass of NN-PF3 was 10 times higher than that of Lahirin.

Lahirin is the first low-MW fibrin(ogen)olytic metalloproteinase reported from Indian monocled cobra *Naja kaouthia* venom. It is also unique in digesting all three major chains of fibrinogen molecule, albeit slowly.

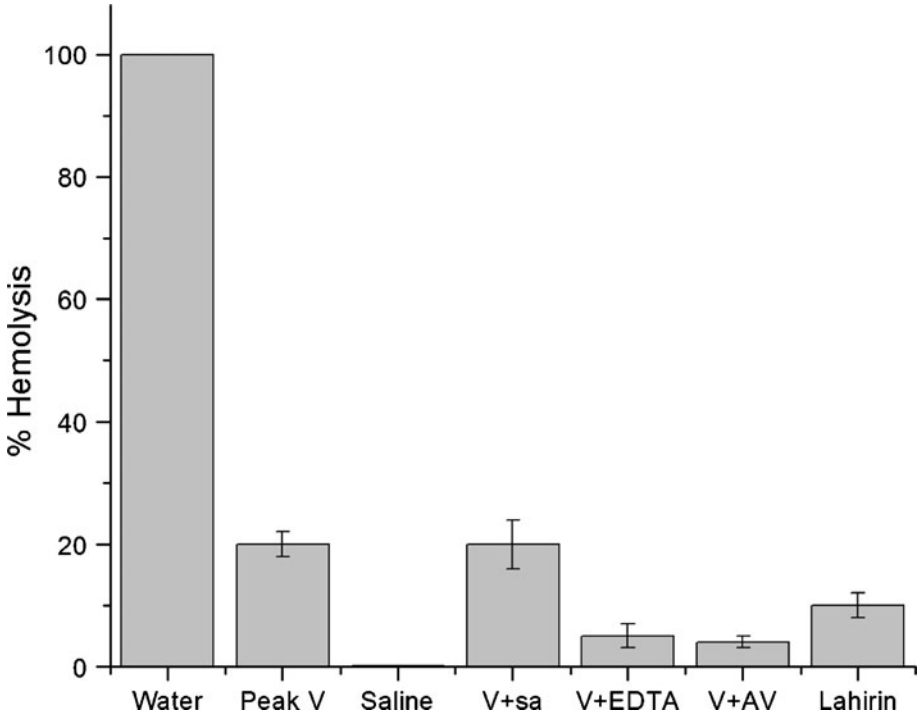

Figure 5. Hemolysis: Hemolytic activity of semi purified (peak V) and purified Lahirin. Peak V and Lahirin were tested for hemolytic activity as described earlier. Distilled water was used as positive control (100% hemolysis) and 0.85% saline was used as negative control. Values represent mean±SD of three independent experiments. V represents peak V, AV represents antivenom, and V+sa represents peak V incubated with 0.85% saline.

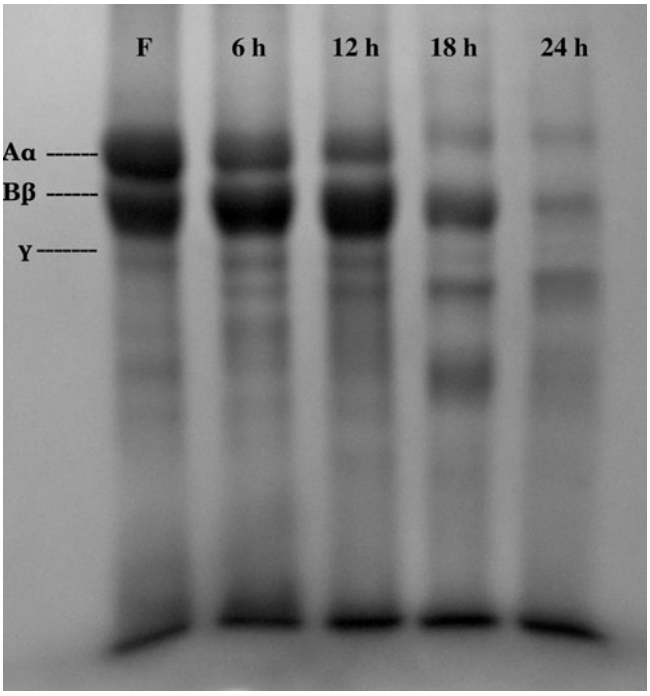

Figure 6. Gradual digestion of major chains of fibrinogen by Lahirin. Fibrinogen (30 μg) was incubated with Lahirin (15 μg) at 37°C, and aliquots were taken out at pre-determined intervals and stored at 4°C until run on 12% SDS-PAGE. Numbers above each lane indicate time of incubation in hours. 'F' indicates fibrinogen only incubated for 24 h at 37°C.

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