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Purification and properties of a coagulant proteinase isolated from bushmaster (*Lachesis muta*) venom (Serpentes: Viperidae)

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Abstract: The venom of *Lachesis muta* is a rich source of a thrombin-like enzyme. Its coagulant proteinase was purified by DEAE -Sephadex A -50 followed by agmatine CH -Sepharose and gel filtration on Sephadex G-100. On polyacrylamide gel electrophoresis at pH 8.4 a single band was observed. Its molecular weight by gel filtration was 49,000. The coagulant and esterolytic activities toward human fibrinogen and Tame of the mudasa were 662 NIH units/mg of protein and 4.37 Δ OD_{225}/min x 10^{-3}/ μg / ml, respectively. These values represent 23 and 5.7 fold increase over the crude venom. The enzyme mudasa, was evaluated with serum from human patients at Hospital Nacional de Niños Dr. Carlos Sáenz Herrera and found to be a valuable reagent for the quantification of fibrinogen on heparinized plasma.

Lachesis muta is a Central and South American viperid snake. It is localized from Southern Nicaragua to Brazil (Vial and Jimenez-Porras, 1967). So far, three different subspecies are known: *L. muta muta, L. m. noctivaga* and *L. m. stenophyrs.* In Costa Rica, only the latter species is found. It occurs along the Atlantic and Southern Pacific zones (Taylor *et al.*, 1974) but only scanty data have been reported concerning coagulant activity in the venom. The present work describes the purification and some properties of a new coagulant proteinase, mudasa, isolated to homogeneity from the Atlantic type of *L. muta stenophyrs* venom.

MATERIAL AND METHODS

The venom was provided by Instituto Clodomiro Picado, University of Costa Rica. It consisted of pooled lyophilized samples obtained from *Lachesis muta stenophyrs* snakes captured at the Atlantic zone. Benzoyl-arginine ethyl ester HCl (BAEE), agmatine HCl, 1-ethyl-3-3 (3-dimethyl-aminopropyl) carbodiimide HCl (EDC), proteins used as molecular weight standards, human fibrinogen (90% clottable, type 1), bovine thrombin, guanidine HCl and heparin were purchased form Sigma Chemical Co. (St. Louis, Mo., USA). DEAE-Sephadex A-50, Sephadex G-100 and Sepharose 4B were from Pharmacia (Uppsala, Sweden).

The procedure for the isolation of the coagulant proteinase was as follows: one gram of venom was dissolved in 10 ml of 0.01 M Tris -0.05 M Ca-EDTA (pH 7.3). The solution adjusted to pH 7.3 was applied to a column (2.7 x 40 cm) packed with DEAE -Sephadex A-50, equilibrated with the same buffer (Gómez-Aragón, 1986). The unbound material was eluted at a flow rate of 25 ml/h. The eluted protein was followed by spectrophotometric monitoring at 280 nm. After the absorbance decreased below 0.1 units, the elution was continued with a linear gradient toward 0.5 M NaCl in 0.01 M Tris (pH 7.3). The fractions with the highest activity toward citrated human plasma were pooled, and concentrated with Sephadex G-225.

The solution adjusted to pH 8.1 was applied to a column (1.7 x 14 cm) packed with agmatine CH -Sepharose. The unbound material was eluted with 0.01 M Tris -0.05 M Ca -EDTA (pH 8.1), at a flow rate of 5 ml/h. After the absorbance decreased 0.02 units, the elution was continued with 0.2 M NaCl in 0.01 Tris (pH 8.1) and followed by elution with 0.15 M guanidine HCl in 0.01 M Tris (pH 8.1). The affinity chromatography method was essentially as described by Aragón-Ortiz & Gubensek

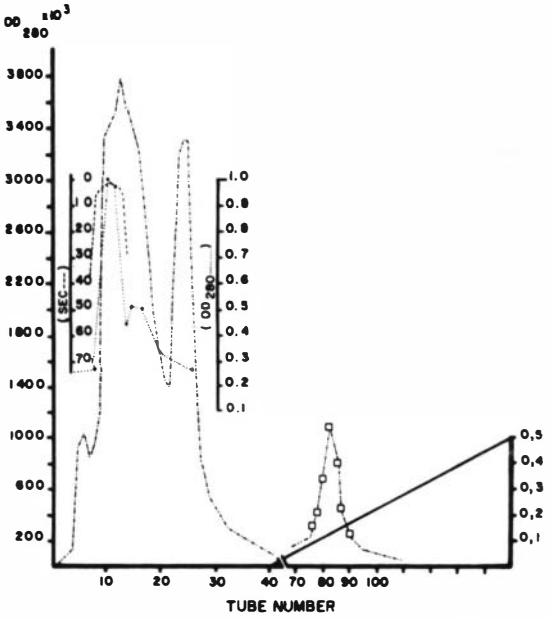

Fig. 1. Fractionation of *Lachesis muta* venom Atlantic type on DEAE-Sephadex a-50 in 0.01 M Ca-EDTA (pH 7.3). Straight line indicates the gradient toward 0.5 M NaCl in 0.01 M Tris HCl pH 7.3.
(--) coagulant activity (....) proteolytic activity.

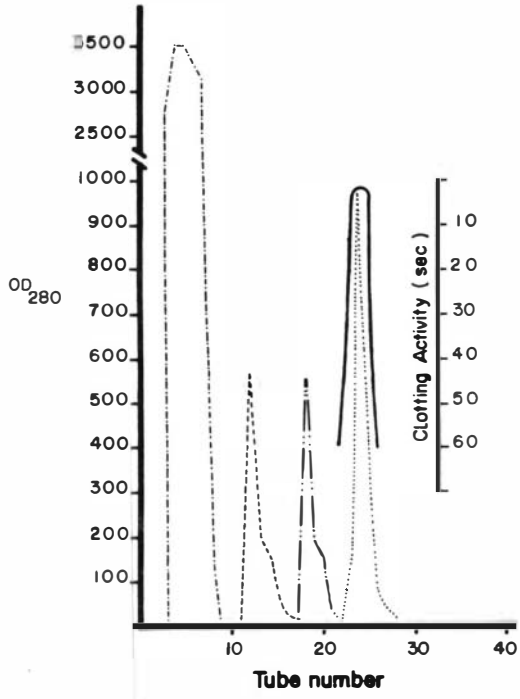

Fig. 2. Affinity chromatography of pooled coagulant material from Fig. 1 on agmatine CH-Sepharose 4 B.
(-.-) Material not bound and eluted in the starting buffer
(—) Material eluted in 0.2 M NaCl in 0.01 M Tris (pH 8.1)
(-..-)Material eluted in 0.15 M quanidine HCl 0.01 M Tris (pH 8.1).
(____) Clotting activity

(1981). The fractions with the highest coagulant activity toward citrated human plasma were pooled and concentrated by Sephadex G-25. The pooled and concentrated coagulant material was applied to a column (2 x 130 cm) packed with Sephadex G-100, equilibrated with 0.1 M NaCl in 0.01 M Tris (pH 7.3). A flow rate of 6 ml/h was applied with the aid of a peristaltic pump, fractions of 2 ml/tube were obtained. The eluted protein was followed at 280 nm and the coagulant activity measured. All fractionations were carried out at 4 °C.

The determination of the specific clotting activity was made according to the method of Baughman (1970), using human fibrinogen and bovine thrombin as standard. Proteolytic activity was measured using 2% casein, 2% hemoglobin and 1% fibrinogen. After precipiting with 3% TCA, readings at 280 nm were made to the protein free filtrate. The determination of specific estereolytic activity was measured according to the method of Hummel (1979), using BAEE (Sigma) as substrate. Hemorrhagic activity was measured according to the method of Kondo, (1969) slightly modified. Samples of the purified enzyme up to 75 µg were inoculated subcutaneously in depilated areas of albino rats of about 0.2 kg weight. Edema was assayed according to the method of Yamakawa *et al* (1976). 70 µg of the enzyme were inocuted in the left hind food, the other foot was inoculated with distilled water. The increase in the weight of the affected foot was a measure of the activity.

Phospholipase activity wss measured by the indirect hemolysis of human erythrocytes (Gómez-Leiva, 1975), caused by the addition of 70 µg of the enzyme to a medium containing egg lecithin. After half hour reaction at 37 °C the tubes were centrifuged and measured at 550 nm. Hemolysis was expressed as percentage related to total hemolysis caused by the addition of distilled water. Agglutination activity was measured according to the method of Hartman *et al* (1978). Seventy µg of the enzyme were mixed with 12.5 µl of 2.5% bromelain-sensitized human red blood cells, using a microtiter V plate. The agglutination titer was read after 30

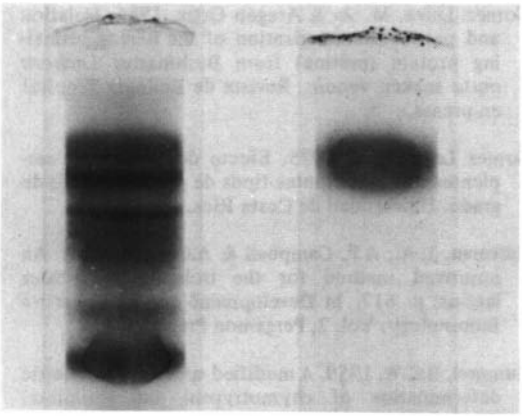

Fig. 3. Photograph shows acrylamide gel of crude venom and of concentrated coagulant material (mudasa).

minutes at room temperature. Heparin inhibition was studied by preparing 10, 20, 30, 40 and 50 heparin international units per ml of citrated plasma. Ten μg of the enzyme were then added per tube and the clotting time was recorded. Disc electrophoresis was made in 7% acrylamide gel at pH 8,4 as described by Davis (1964). The molecular weight of the native enzyme was estimated by the method of Whitaker (1963), using 0.1 M NaCl in 0.01 M Tris pH 7.0 as eluent.

The clinical evaluation of this enzyme was made using children's sera at Hospital Nacional de Niños Dr. Carlos Sáenz Herrera by Dr. Cecilia Lizano.

RESULTS

The fractionation of one gram of *L.m. stenophyrs* venom on DEAE-Sephadex A-50 appears in Fig. 1. It is observed that the venom was separated into four major fractions. The coagulant and proteolytic activities were found only in fraction two. The use of Ca -EDTA in the starting buffer inhibits the proteolytic activity of the venom under the fractionating conditions. It was previously observed that the proteolytic activity could be reversed by the addition of 0.1 M Zn^{++}. The coagulant and proteolytic activities were then pooled and passed through an agmatine CH -Sepharose column where the coagulant enzyme was adsorbed. This material was recovered by elution with guanidine HCl (Fig. 2). Once concentrated with Sephadex G- 25 it was repurified on Sephadex G-100. Fig. 1 shows a polyacrilamide gel of the crude venom and that of the purified enzyme; a single band for the purified proteinase is clearly visualized. The molecular weight of the enzyme was determined by gel filtration, under this conditions a value around 49,000 was obtained. Clotting activity was measured toward human fibrinogen. The crude venom exhibited 28.8 NIH unit/mg and the purified enzyme 662 NIH units/mg. The esterolytic activity of the crude venom measured toward BAEE was found to be 7.6 Δ OD 255/min x 10^{-4} / μg /ml. The purified coagulant proteinase exhibited an activity of 4.36 Δ OD/min x 10^{-3} μg/ml. A 5.7 fold increase in the esterolytic activity was obtained. The concentration of mudasa was calculated from extintion measurements assuming a value of 1.00 at 280 nm for a 0.1% solution at pH 7.0. The purified coagulant proteinasa was devoid of hemorrhagic, proteolytic, phospholipase, edema and agglutination activities, present in the crude venom. Heparin, a known inhibitor of thrombin was without effect on coagulation of citrated plasma by mudasa, when present in the amount of 50 IU/ml of plasma.

DISCUSSION

Comparison of the properties of mudasa (Table 1) with thrombin-like enzymes isolated from *B. asper* venom Costa Rica (Aragón-Ortiz & Gubensek, 1981), showed many similarities. The molecular weight calculated by gel filtration revealed that mudasa exhibited 89% of the molecular weight of *B. asper* Atlantic type and 67% of the molecular weight of the Pacific type coagulant enzyme.

TABLE 1

Some properties of Mudasa, the coagulant enzyme from L. m. stenophyrs *snake venom (Atlantic type)*

Acrylamide gel electrophoresis pH 8.4	gives one single band when stained with Coomassie blue
Molecular weight gel filtration	49.000
Specific clotting activity	662 NIH units/mg
Speci ic estereolytic activity (BAEE)	4.35 10^{-3} Δ OD/min/μg/ml
Hemorrhagic activity	Not present
Phospholipase activity	Not present
Proteolytic activity against casein hemoglobin and fibrinogen	Not present
Action toward human ibrinogen	
90% clottable	Direct
Heparin inhibition in citrated plasma	Negative
Edema	Negative
Aglutination of RBC	Negative

Its clotting activity compared with the Atlantic type proteinase was only 44.5%. In relation to the Pacific type proteinase, its activity was very similar, 96%.

Its esterolytic activity expressed as Δ OD/min/mg las 2.1 and 3.9 times that of *B. asper*

Atlantic and Pacific types of clotting enzymes respectively.

By comparison of the results obtained with the thrombin-like enzyme from *L. muta noctivaga*, (Magalhaes *et al.*, 1981), the following observations were possible: *L. muta noctivaga* clotting enzyme was obtained with a 41-fold purification while mudasa exhibited 23-fold purification, however our method of purification was supported on affinity chromatography using agmatine, a competitive inhibitor as a ligand. The specific clotting activity of the Brazilian Atlantic type *L. muta* enzyme was 2.5 times that of mudasa, so far one of the highest clotting activity reported for a thrombin-like enzyme isolated from snake venom (Markland 1977; Stoker, 1978). However, its molecular weight was only 74% of mudasa both calculated by gel filtration.

Due to these properties, to coagulate citrated plasma and 90% human fibrinogen, the new enzyme was tested at Hospital Nacional de Niños, Dr. Carlos Sáenz Herrera. It was found to be an excellent laboratory reagent for the evaluation of heparinized plasma and also an appropriate substitute of bovine thrombin for the clinical quantification of fibrinogen levels.

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