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Isolation and partial characterization of *Lachesis muta melanocephala* coagulant proteinase: biochemical parameters of the venom

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Abstract: The coagulant proteinase of *L. m. melanocephala* was purified by DEAE Sephadex A-50 followed by agmatine CH- Sepharose and gel filtration on Sephadex G-100. The enzyme exhibited many of the properties adscribed to "mudasa", the coagulant proteinase from *L. m. stenophirs* venom. Its molecular weight by gel filtration was 35 kDa and its specific clotting activity was 702 NIH/mg of protein. A 32 fold increase in the clotting activity was obtained by purification. The coagulant proteinase exhibited esterolytic activities toward lysine and arginine esters as well as amidolytic activity. Significant differences are observed when compared with the activities of "mudasa", the former is less esterolytic and amidolytic, although its activity toward TLEME is higher. Significant differences in the activities are also observed when the venom from the Pacific and Atlantic *L. muta* populations (corresponding to the subspecies *L. m. melanocefala* and *L. m. stenophyrs*) are compared toward the same substrates. The Pacific type is less amidolytic and more esterolytic toward BAME and BAEE, although toward the lysine and tyrosine esters no significant differences are observed. The vemon from the Pacific population is more coagulant and less proteolytic than the venom from the Atlantic population. Analytical isoelectric focusing of both populations of venom revealed important differences in the number and intensity of the protein bands. The results here given further substantiate the taxonomical differentiation already given to the Pacific and Atlantic Costa Rican population of *L. muta.*

In Costa Rica, *L. muta* is distributed along the Atlantic and Southern Pacific zones of the country (Vial and Jiménez Porras 1967; Taylor *et al.* 1974). Morphological and inmunoelectrophoretical differences as well as different behavioural patterns have been described for both types of snakes (Bolaños *et al.* 1978, 1982). Recently a new subspecies of the bushmaster, *Lachesis muta melanocephala* has been described from southeastern Costa Rica (Solórzano and Cerdas 1986).

Lachesis muta venom exhibites many of the biochemical and pharmacological properties that characterize bothropic venom, mainly: edema, hemorrhagic, mionecrosis, phosphalipase, esterolytic and thrombin like activities (Jiménez-Porras *et al.* 1973; Bolaños *et al.* 1982). The coagulant enzyme from *L. m. stenophyrs* venom was recently characterized (Aragón 1985; Aragón and Gubensek 1986).

In this paper some properties of the Pacific type coagulation proteinase are described as well as some biochemical parameters of the venom of the Atlantic and Pacific population of *Lachesis muta* venom from Costa Rica.

MATERIAL AND METHODS

The venom was provided by Instituto Clodomiro Picado, Universidad de Costa Rica. It consisted of pooled lyophilized samples obtained from *Lachesis muta* snakes captured at the Pacific and Atlantic zones of Costa Rica. Benzoyl- arginine ethyl ester HCl (BAEE), tosyl arginine methyl ester HCl (TAME), tosyl- lysine methyl ester HCl (TLEME), benzoyl-arginine-dl-p- nitroamilide HCl (BAP-NA), benzoyl-tyrosine ethyl ester (BTEE), benzoyl arginine methyl ester HCl, guanidine HCl, Hemoglobin, casein and heparin were

purchased from Sigma Chemical Co. (St, Louis, Mo., USA). DEAE- Sephadex A-50, Sephadex G-100 and Sepharose 4 B were from Pharmacia (Uppsala, Sweden).

The procedure for the isolation of the coagulant proteinase was as follows: 500 mg of venom were dissolved in 5 ml of 0.01 M Tris (pH 7.3). The solution adjusted to pH 7.3 was applied to a column (2.7 x 40 cm) packed with DEAE-Sephadez A-50, equilibrated with the same buffer. The unbound material was eluted at a flow of 15 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-25. 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 (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 and Gubensek (1981). The fractions with the highest coagulant activity toward citrated human plasma were pooled and concentrated by Sephadez 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 and fractions of 2 ml/tube were obtained. The eluted protein was followed at 280 mm and the coagulant activity measured. The Atlantic type coagulant proteinase was prepared as described by Aragón (1985). 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 standars. Proteolytic activity was measured using 2% casein, 2% hemoglobin and 1% fibrinogen. After precipitation with 3% TCA, and centrifugation, the absorbance of protein free filtrate was recorded at 280 nm. The determination of the specific esterolytic activity was made according to the method of Hummel (1979), using BAEE, TAME, TLEME, BAPNA, and BTEE as substrates. Hemmorrhagic activity was measured according to the method of Kondo *et al.* (1960) slightly modified. Samples of the purified ensyme up to 75 μg were inoculated subcutaneously in depilated areas of albino rats of about 0.2 kg weight. The area of the hemorrhage was the measure of the activity. Edema was assayed according to the method of Yamakawa *et al.* (1976), 70 μg of the enzyme were inoculated in the left hind foot, the other foot was inoculated with distilled water. The increase in the weight of the affected foot was a measure of the activity.

Phospholipase A2 activity was measured by the indirect hemolysis of human erythrocytes (Gómez-Leiva and Aragón-Ortiz 1986) caused by the addition of 70 μg of the enzyme to a medium containing egg lecithin and calcium ions. After half hour reaction at 37C the tubes were centrifuged and the absorbance of supernatant 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 ul of 2.5% bromelain-sensitized human red blood cells, using a microtiter V plate. The agglutination titer was read after 30 minutes at room temperature, Heparin inhibition was studied by adding 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. Analytical isoelectric focusing was made on a 1 mm thick 5% polyacrylamide plate with carrier ampholines of pH 2-11. A mixture of standard proteins with known isoelectric points was run parallel on the same gel. The concentration of the clotting enzyme was calculated from extinction measurement assuming a value of 1.00 at 280 nm for a 0.1% solution at pH 7.0.

Each experiment was made by triplicate. Five different concentrations of the tested material were used for the calculation of the

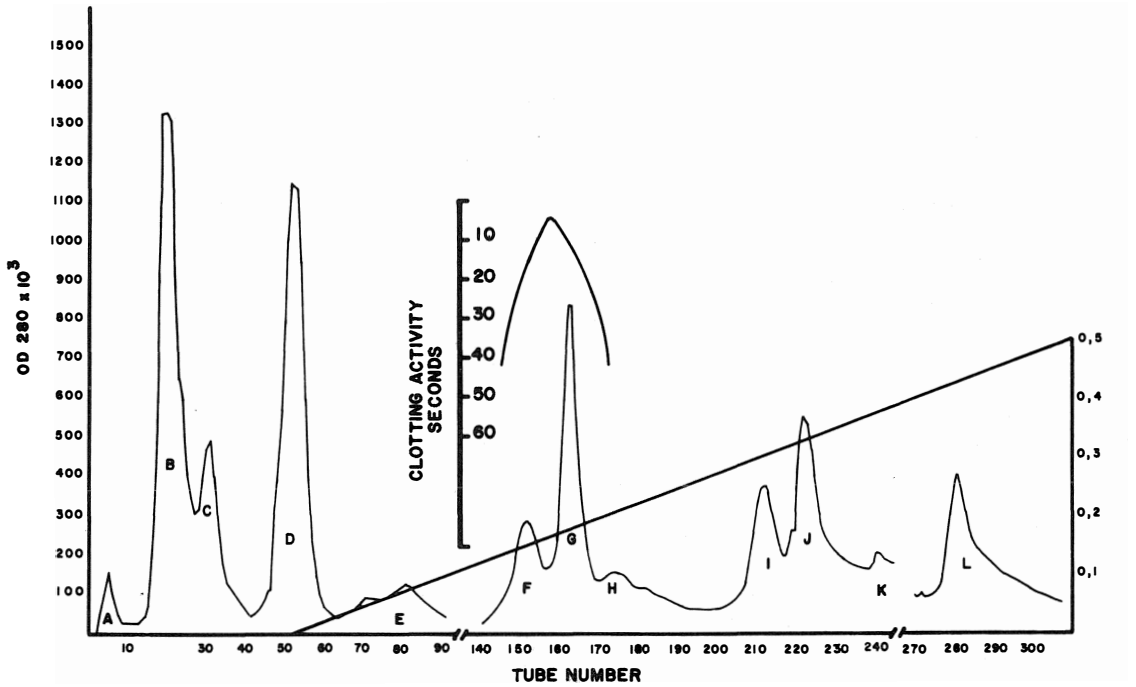

Fig. 1. Fractionation of *L. m. melanocephala* venom on DEAE-Sephadex A-50 in 0.01 M Tris (pH7.3). Straight line indicates the gradient toward 0.5M NaCl in the same buffer.

regression line. The Stundent's t test was done in order to determine the signifance of the statistical differences between the venom of *L. m stenophyrs* and *L.m. melanocephala* populations.

RESULTS

The fractionation of 500 mg of *L. m. melanocephala* venom on DEAE-Sephadex A-50 is shown Figure 1. It is observed that the venom was separated into twelve different fractions. The coagulant activity was found only in fractions F and G. The coagulant activity was pooled and passed through an agmatine CH-Sepharose column where the coagulant enzyme was adsorbed. After elution with 0,2 M NaCl in Tris 0,01 M (pH 8,1), the coagulant material was recovered by elution with guandine HCl. Once concentrated with Sephadez G-25 it was repurified on Sephadex G-100 and its molecular weight determined. Under these conditions a value around 69 kDa was obtained. Clotting activity was measured toward human fibrinogen. The crude venom exhibited 22 NIH units/ma and the purified protease 702 NIH units/mg. Acrylamide gel electrophoresis (PAGE) of the enzyme gave a single band. The purified coagulant proteinase was devoid of hemorrhagic, proteolytic, phospholipase A_2, edema and agglutination activities present in the crude venom. Heparin, at a concentration of 50 IU/ml of plasma, did not inhibit the clotting activity of the enzyme.

Table 1 shows the proteolytic and coagulant activities of the crude venom and of the purified proteinases. The esterolytic activities of the clotting enzymes and of the crude venom fron the Pacific and Atlantic populations are shown in tables 2 and 3 respectively. Figure 2 shows the analytical isoelectric focusing of *L. muta* venoms from the Pacific and Atlantic populations.

DISCUSSION

Comparison of the properties of *L.m. melanocephala* coagulant proteinase with those of mudasa, the coagulant proteinase from *L. m. stenophyrs* venom shows many similarities (Aragón 1985). The molecular weight of the Pacific type proteinase is of 69 kDa, calculated by gel filtration. Very likely under

TABLE 1

Specific proteolytic and coagulant activities of Lachesis muta *venom and of their coagulant proteinases*

Substrate	*L.m. melanocephala* (Pacific)	*L.m. stenophyrs* (Atlantic)
Hemoglobin**	5.003 ± 0.476	7.870 ± 0.493
Cassein**	13.690 ± 0.301	23.970 ± 0.597
Fibrinogen Coagulation by crude venom	22	29
Fibrinogen Coagulation by enzyme	702	662

Note: Activities are expressed in OD / min $\mu g \times 10^{-5}$ / 280 (regression coeficient ± S.D.) and in NIH units/mg, ** $p < 0.01$ (Student's t)

TABLE 2

Specific esterolytic activities of Lachesis muta *clotting enzymes*

Substrate	*L.m. melanocephala* (Pacific)	*L.m. stenophyrs* (Atlantic)
TAME	34.905 ± 0.886	35.787 ± 1.002
TLEME*	1.555 ± 0.057	1.388 ± 0.065
BAME**	27.290 ± 0.743	49.934 ± 1.784
BAEE**	23.679 ± 0.866	60.134 ± 1.042
BTEE	NO ACTIVITY	NO ACTIVITY
BAPNA**	0.529 ± 0.029	1.296 ± 0.55

Note: Activities are expressed in OD/ min/ μg/ ml x 10^{-3} (regresision coeficient ± S.D., * $p < 0.05$, ** $p < 0.01$ (Student's t).

TABLE 3

Specific esterolytic activities of Lachesis muta *venom*

Substrate	*L.m.melanocephala* (Pacific)	*L.m. stenophyrs* (Atlantic)	Level of significan
TAME**	16.800 ± 0.720	24.620 ± 1.927	$P < 0.01$
TLEME	5.022 ± 0.213	4.911 ± 0.348	N.S.
BAME**	127.733 ± 1.087	71.113 ± 1.620	$P < 0.01$
BAEE**	128.620 ± 2.220	76.887 ± 1.240	$P < 0.01$
BTEE	2.053 ± 0.096	2.062 ± 0.083	N.S.
BAPNA**	0.789 ± 0.021	1.187 ± 0.026	$P < 0.01$

Note: Activities are expressed in OD/min/mg/ml x 10^{-2} (regression coeficient ± S.D.) ** $p < 0.01$ (Student's t)

such conditions the molecules aggregates forming a dimer with a monomeric value around 35 kDa. This value is quite similar to the molecular weight of the Atlantic type proteinase calculated by SDS-PAGE which gives a value of 30 kDa (Aragón and Gubensek 1986). Its specific clotting activity (Table 1) is 6% higher but the specific clotting activity of the crude venom (Pacific type) is 32% less than value of the venom from the Atlantic population. This implies that the clotting enzyme is more abundant in the latter. The esterolytic and amidolytic especific activities of both types of clotting enzymes are significantly higher than similar activities exhibited by the respective crude venoms. This is a consequence of the high degree of purity obtained by purification. Both enzymes are devoid of activities toward BTEE, activity exhibited by the crude venoms.

Statistical differences are observed when the specific esteolytic and amidolytic activities of the two types of clotting enzymes are compared. The clotting enzymes from the Atlantic population is significantly more esterolytic and amidolytic toward BAME, BAEE and BAPNA. Toward TAME both enzymes exhibit the same activity.

Its is revealing that both types of coagulative proteinases exhibit esterolytic activity toward the lysine ester. In this respect they are similar to thrombin and other trypsinlike proteinases (Sherry *et al.* 1965) and differ dramatically from the clotting enzymes of *Bothrops asper* venom, which are not able to hydrolyze the lysine subtrate (Aragón and Gubensek 1981).

It was reported by Aragón and Gubensek (1986), that mudasa releases fibrinopeptide A with higher rate than fibrinopeptide B. In this activity mudasa mimics the action of thrombin and differs from the Bothropic type of clotting enzyme which only releases fibrinopeptide A from fibrinogen (Aragón 1980).

It is very likely that the broader esterolytic activity of the Atlantic and Pacific types proteinases, which is indicative of important differences at the active site of the enzymes, in relation to bothropic clotting enzymes, may be the reason for the thrombin-like activity of "mudasa" toward fibrinogen (Aragón and Gubensek 1986). The Atlantic type of *L. muta* venom is different from the Pacific type of venom in its proteolytic activity toward hemoglobin and casein, being the Atlantic

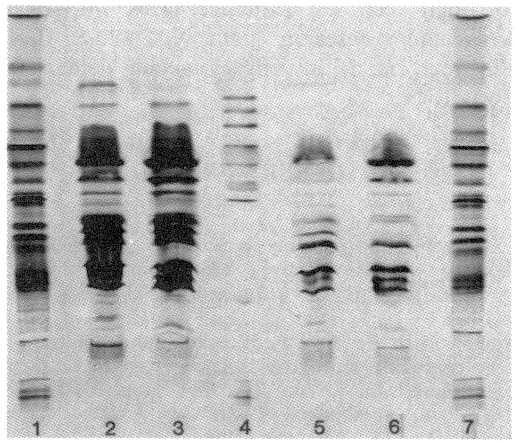

Fig. 2. analytical isoelectric focusing of *Lachesis muta* venom from Atlantic and Pacific populations. Columns 1,4 and 7 are protein standards. Columns 2 and 3- Pacific and Atlantic venoms 1.5 mg each; columns 5 and 6- Pacific and Atlantic venoms 0.5 mg each.

type of venom more proteolytic and coagulant than the Pacific type (Table 1). Statistical differences in their esterolytic activities are also observed (Table 3). Such differences are apparent in the hydrolysis of BAPNA, BAME and BAEE, being the Atlantic type more amidolytic, while the esterolytic activities of the Pacific type are higher. Both venoms hydrolyze TLEME and BTEE to a similar extent.

Analytical isoelectric focusing of both types of venoms (Fig. 2) show striking differences in their protein pattern, both in the number of bands as well as in the intensity when compared at equal protein concentrations. Similar pattern differences were also reported for *B. asper* venom from the Pacific and Atlantic zones of the country (Aragón and Gubensek 1981).

The statistical parameters here shown for the crude venom and for the coagulant proteinases of *L. muta* (Pacific and Atlantic populations) as well as the analytical isoelectric focusing of the crude venoms, show enough differences that further substantiates the taxonomical classification already given to these snakes.

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