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Characterization of a proteolytic enzyme from *Lachesis muta* venom (Serpentes: Viperidae)

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Abstract: A proteolytic enzyme from *L. muta stenophrys* was isolated by gel filtration on Bio Gel P-100 followed by FPLC on MONO S column. The enzyme exhibited proteolytic activity toward casein,hemoglobin and fibrinogen with a pH optimum around 10. The activity was inhibited by EDTA while trypsin inhibitors were not inhibitory. It is a glycoprotein, Mr 14 kDa with a high content of Asp,Glu,and Leu residues and a low content of Cys and Trp. The protease is devoid of myotoxic, hemorrhagic, esterolytic and amidolytic activities. It lyses the alfa and beta chains of human fibrinogen and releases kinin from L.M.W. kininogen. No release of histamine was observed upon incubation with mast cells.

Key words: Snake venom, protease, fibrinogen, kininogen, kinin.

Lachesis muta is distributed from southern tropical regions of Brazil to the Atlantic and Pacific slopes of Costa Rica (Vial & Jiménez-Porras 1967). Currently *Lachesis muta* includes four subspecies (a) *L. m. rhombeata* present in the rainforest area of the Atlantic regions of Brazil, (b) *L. m. muta* distributed in the equatorial rainforest of Brazil to Panama, (c) *L. m. stenophrys* present in the tropical rainforest of Costa Rica and Panama, and (d) *L. m. melanocephala* recently described as a new subspecies, distributed in the southeastern regions of Costa Rica (Solórzano & Cerdas 1986).

We have been able to isolate and characterize the thrombin-like enzyme (Aragón-Ortiz 1986) and the lectin-like protein from the crude venom of *L. muta stenophrys* (Aragón-Ortiz *et al.* 1989). The venom causes among several clinical alterations a severe hypofibrinogenemia and a prolonged drop in blood pressure followed by human envenoming (Bolaños *et al.* 1981).

In this paper, I characterize the proteolytic enzyme from *L. muta stenophrys* venom and discuss its possible role during human envenoming.

MATERIAL AND METHODS

The venom, kindly supplied by Instituto Clodomiro Picado, Universidad de Costa Rica, consisted of pooled lyophilized samples from *Lachesis muta stenophrys* captured in the Atlantic zone of Costa Rica. Bio Gel P 100 was from Bio-Rad laboratories, Richmond, California (USA). Tosyl-arginine methyl ester HCl (TAME), tosyl-lysine methyl ester HCl (TLME), benzoylarginine methyl ester HCl (BTEE). Soybeen trypsin inhibitors (Kunitz), orcinol, carbohydrate standards meta sodium arsenite, meta sodium periodate, EDTA and Trasylol (BPTI) were from Sigma Chemical Co., St. Louis, Mo (USA). Proteins used as molecular weight standards were from Serva, Heidelberg (Germany). Tris-HCl, 2-thiobarbituric acid, casein, hemoglobin were from Merck, Darmstadt (Germany).

FPLC system and gel electrophoresis apparatus, GE-2/4LS, were from Pharmacia (Sweden). Gel filtration was performed at 3-5°C. Amino acids were analyzed by a Beckman 118 CL amino acid analyser. Polyacrylamide gel electrophoresis in Tris-glycine, pH 4.4 was performed according to the method of Davis (1984). PAGE in the presence of SDS was performed according ton the method of Laemmli (1970) in a 4-30% (w/v) gradient gel. Proteolytic activity was measured by the method of Anson (1939), using 1% casein, 2% hemoglobin and 1% fibrinogen. The substrates were dissolved in the universal buffer of Johnson and Linsday of pH 6-11. Fibrinogenolytic activity was determined as described by Aragón-Ortíz and Gubensek (1987). Esterase and amidase activities were measured using TAME, TLME, BAME, BTEE and BAPNA as substrates (Hummel 1959). Hemorrhagic activity was done according to the method of Kondo (1960). Test for myotoxicity was done as described by Ownby *et al.* (1976). The proteolysis of low molecular weight (LMW) kininogen was performed by incubating 20 µg of the substrate with increasing amounts of the enzyme up to 2 µg. The crude venom was used as positive control incubation, done for 1 h at 37°C prior to electrophoresis. The radioimmunoassay procedure for the detection of the kinin release was done as described by Fink *et al.* (1985). Histamine release was studied by incubating up to 0.3mg/ml of the proteolytic enzyme with mast cells freshly prepared from tonsils, lgE was used as a positive control. The release of tryptase from mast cells by IgE and the proteolytic enzyme was followed by the hydrolysis of the chromogenic substrate S-2238 and used as an indirect estimation of histamine release. Sialic acid content was measured by the method of Warren (1959). Protein bound hexose was determined according to the procedure of Winzler (1961). The effect of protein inhibitors and chelating ions was studied by mixing the protease (35 µg in a final volume of 2 ml) with EDTA (1.0 to 0.01 mM), Trasylol (100 µg/ml), soybean trypsin inhibitor (100 µg/ml). Protein inhibitors were pre-incubated for 10 min. at room temperature prior to the determination of enzymatic activity.

The purification of the proteolytic enzyme from *L. muta stenophrys* venom was performed as follows: 500 mg of the venom were dissolved in 6 ml of the starting buffer (0.2 M sodium acetate, 0.1 M sodium chloride, pH 5.5), centrifuged at 6000 g and applied to a column (2.0 x 90 cm) with Bio Gel P-100 equilibrate and eluted with the same buffer. The flow rate was 6 ml/min and 2.5 ml fractions were collected, read at 280 nm and tested for proteolytic activity toward 1% casein at pH 8.0. The active fractions were pooled, concentrated by ultrafiltration and rechromatographed under the same conditions. A single protein peak was obtained. The active proteolytic material was then concentrated and equilibrated in 0.01 M acetate, pH 5.5 and applied to FPLC using a MONO S column equilibrated with the same buffer. A gradient toward 0.25 M NaCl in the same buffer was applied over a period of 45 min. Proteolytic activity was found in three peaks which were pooled independently for further characterization.

RESULTS

Fig. 1 shows the fractionation of 500 mg of the venom on Bio Gel P-100. The proteolytic activity was eluted in peak B. The FPLC MONO S of the pooled material from peak B appears in Fig.2. The material from peaks I and II show a single polypeptide chain with Mr of 28 and 14 kDa respectively. Peak III shows two strong protein bands which correspond to a mixture of proteins from peaks I and II (Fig 3).

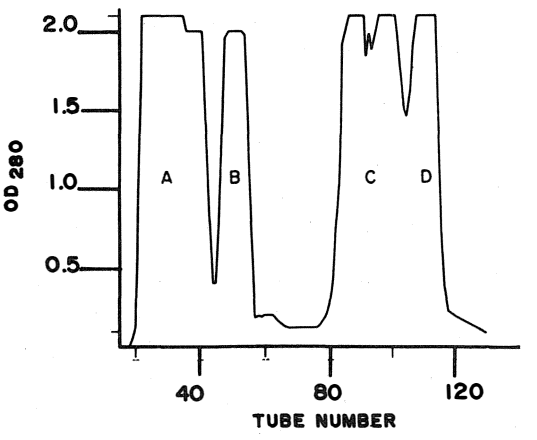

Fig.1. Gel filtration on Bio Gel P-100 at pH 5.5 of 500 mg of *L. m. stenophrys* venom. The proteolytic active material is found in peak B.

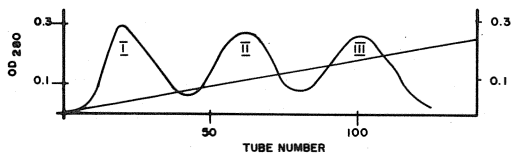

Fig.2. FPLC at pH 5.5 of the proteolytic material. Straight line shows a linear gradient toward 0.25 M NaCl. The proteolytic enzyme is obtained in three different peaks.

The proteolytic enzyme with Mr 14 kDa purified by chromatography (FPLC, MONO S) was further characterized. The enzyme is a glycoprotein containing 1.0 mole of N-acetylneuraminic acid per mole of protein and 2.0% of neutral carbohydrates. It degrades the alpha and beta chains of fibrinogen in 15 and 6 min, respectively (Fig .4), and degrades L.M.W. kininogen releasing kinin;373 and 695 fmol/ml of the enzyme released 932 and 1737 pmol/ml of kinin, respectively. The enzyme did not release histamine from mast cells. No hemorrhagic nor myotoxic activities were observed.

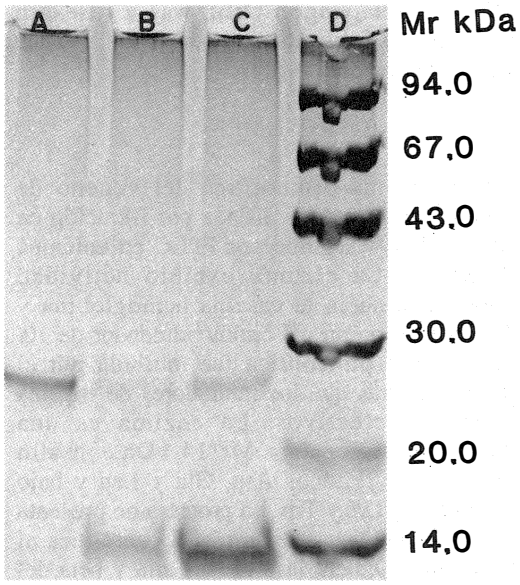

Fig.3. SDS-polyacrylamide gel in the absence of ß-mercaptoethanol after FPLC of the proteolytic material. A) Single protein band at 28 kDa,peak I, B) Strong protein band at 14 kDa,peak II , C) Both proteins bands are observed,peak III.

The optimal pH values for proteolysis of casein, hemoglobin and fibrinogen were found to be around 10. The enzyme was devoid of esterolytic and amidolytic activities toward all

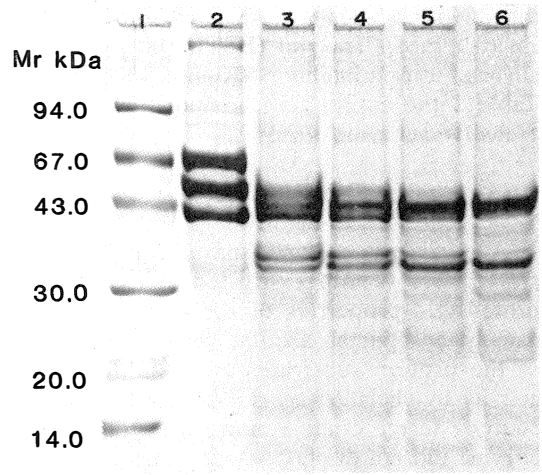

Fig.4. Fibrinogenolytic activity of protease from *L. m. stenophrys* venom. 1) Protein standards, 2) Reduced fibrinogen, 3-6) Fibrinogen incubated with the proteolytic enzyme for 15,30,60 and 120 min, respectively, at 37°C. Anode is at the bottom.

TABLE 1

Amino acid composition of the proteolytic enzyme

Amino acid	Amino acid residue
Asp	12
Thr	5
Ser	8
Glu	11
Pro	4
Gly	6
Ala	6
Cys	3
Val	5
Met	4
Ile	8
Leu	13
Tyr	6
Phe	5
His	6
Lys	7
Trp	2
Arg	5
Total	116

MW=13.5 kDa

of the small molecular weight substrates mentioned in materials and methods. The proteolytic enzyme (30 µg) was inhibited by 0.1 mM EDTA and also by EDTA-Ca (0.1

mM). No inhibition was observed with EDTA-Zn (0.1 mM), Trasylol (BPTI, 100 µg), and soybean trypsin inhibitors (Kunitz, 100 µg). In Table 1 the amino acid composition of the proteolytic enzyme is shown.

DISCUSSION

A proteolytic enzyme from *Lachesis muta stenophrys* venom exhibited many of the proteolytic properties of the crude venom, namely, an optimal, alkaline pH of hydrolysis of the protein substrates and inhibition of activity by chelating agents such as EDTA. In contrast to the crude venom, the proteolytic enzyme did not display any coagulant activity nor any detectable myotoxic, hemorrhagic, esterase and amidolytic activities. Protein trypsin inhibitors did not inhibit the proteolytic activity of the enzyme. From inhibition studies it can be inferred that the enzyme is a metalloprotease and shows fibrinogenolytic activity, degrading the alpha and beta chains of human fibrinogen. A similar proteolytic enzyme was purified from *Bothrops asper* venom (Aragón-Ortíz and Gubensek 1987).

This enzyme differs from moojeni proteinase A,which exhibited 50% of the hemorrhagic activity of the crude venom of *Bothrops moojeni* (Assakura *et al* . 1985), and exhibited some of the characteristics of a non hemorrhagic zinc metalloprotease from the venom of *Bothrops jararaca* called J protease (Tanizaki *et al.*1989).It also differs markedly from the properties exhibited by the ß-fibrinogenase from the venom of *Vipera lebetina* (SIIgur *et al.*1991),which is an arginine esterase that cleaves the beta chain first and later the alfa chain of fibrinogen. It differs from the kininogenase 1 and 2 from *Agkistrodon caliginosus* (Ohtani *et al.*1988, Yabuki *et al.*1991), both enzymes released directly bradykinin from kininogens and exhibited activity toward arginine and lysine esters.

Recent purifications and characterizations of metalloproteases from snake venom (Assakura *et al.*1985, Kurecki and Kress 1985,Tanizaki *et al.*1989),as well as inhibition studies with soybean trypsin inhibitor and DFP,show that in addition to protease,esterolytic enzymes without proteolytic activity are also present in these venoms (SIIgur *et al.*1991). Comparison of the amino acid composition of *L.m.stenophrys* proteolytic enzyme,with similar snake venom proteases (Aragón-Ortiz & Gubensek 1987, Kurecki and Kress 1985, Assakura *et al.* 1985, Tanizaki *et al.* 1989), shows that this enzyme contains fewer Asp and Glu residues.The low content of Cys seems to be a typical characteristic of some of these proteases.

By degrading fibrinogen, presumably other clotting factors (Barrantes *et al.*1985) and also releasing kinin, this metalloprotease may contribute to the severe hypofibrinogenemia and hypotension reported in snake bitten patients (Bolaños *et al.*1981).

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RESUMEN

Una enzima proteolítica del veneno de *L.muta stenophrys* fue aislada por filtración en Bio Gel P-100 seguido por FPLC en columna MONO S. La enzima exhibió actividad proteolítica hacia la caseína hemoglobina y fibrinógeno con un pH óptimo alrededor de 10. La actividad proteolítica fue inhibida por el EDTA mientras que los inhibidores de tripsina no fueron efectivos. La enzima es una glicoproteína con una Mr 14 kDa,con alto contenido de residuos Asp, Glu y Leu y bajo contenido de Lys y Trp. La proteasa no presenta actividad miotóxica, hemorrágica,esterolítica ni amidolítica. Digiere las cadenas alfa y beta del fibrinógeno humano y libera quinina del quininógeno de bajo peso molecular. Cuando la enzima se incubó con células cebadas no se observó liberación de histamina.

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