Characterization of a Lectin-Like protein isolated from *Lachesis muta* Snake venon

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Abstract: A lectin-like protein was isolated from *L. muta* venom by gel filtration on BIO Gel P-100 followed by column Chromatography on DEAE-Sephadex A-50. The protein eluted at 0.4 M NaCl in 0.01 Tris pH 7.3 and exhibited agglutinin activity toward 0^+ human erythrocytes. The protein is a dimer with Mr 28 kDa. Amino acid analysis revealed high content of tryptophan and acid recidues and low content of cysteine and methionine residues. No neutral carbohydrates and sialic acid were detected. Circular dichroic spectrum shows 78% of B structure and 1% of α structure. *In vitro* experiments with erythrocytes from rat, rabbit and dog revealed strong agglutination while red blood cells from mice, sheep and goat were not agglutinated. *In vivo* experiments using non-anesthetized rats, a sharp and prolonged fall in the blood pressure was observed at protein dose of 1.5 mg/kg. Double dose of protein caused the death of the animal.

It has been reported (Gómez-Leiva and Aragón-Ortiz 1986) that *Lachesis muta stenophyrs* venon induced aggregation of bromelain sensitized human erythrocytes at a concentration of 1 mg/ml. The purified protein exhibited a non-specific lectin-like activity toward human RBC. This action was inhibited by galactose, lactose and inositol. Most likely the protein binds to galactosyl receptors of the red cell membrane. It was also shown to be non-mitogenic and not to aggregate human platelets.

In this work, we further characterize the lectin-like protein from *L. m. stenophyrs* venom and discuss its possible role during human envenomenation.

MATERIAL AND METHODS

The venom was obtained from Instituto Clodomiro Picado, University of Costa Rica. It consisted of pooled lyophylized samples obtained from *L.m. stenophyrs* snakes captured at the Atlantic zone of Costa Rica. BIO Gel P-100 was from BIO Rad laboratories, Richmond California (U.S.A.). Protein molecular weight standards and DEAE-Sephadex A-50 was from Pharmacia, Uppsala (Sweden). Tris-Ncl was from Sigma Chemical Company, St. Louis, MO (USA). Sodium Chloride and sodium acetate were from Zorka, Sabac (Yugoslavia). The gel electrophonesis apparatus, Ge-2/4LS, was from Pharmacia Fine Chemicals. Amino acid analysis was performed on a Beckman 118 CL amino acid analyzer. Samples were hydrolyzed with 4 molar methansulfonic acid containing 0.2% 3-(2-aminoethylindole) at 115° C in a nitrogen atmosphere for 22 hrs. Polyacrylamide gel electrophoresis in Tris-glycine, pH 4.4 was perfomed according to the method of Davis. Polyacrylamide gel electrophoresis in the presence of SDS was perfomed according to the method of Laemmli (1970) in a 4-30% (W/V) gradient gel.

Sialic acid content was quantified by the method of Warren (1959). Protein bound hexose was determined according to the procedure of Winzler (1961). Coagulant activity was measured according to the method of Baughman (1979). Hemorrhagic activity was meassured according to the method of Kondo (1960). Oedema was assayed according to the method of Yamakawa *et al.* (1961). Phospholipase A_2 activity was messured according to the method of Gómez-Leiva (1975). Proteo-

lytic activity was meassured according to the nethod of Anson (1939). Mionecrotic test was done as described by Ownby (1976).

The absorbance coefficient of the protein was determined after exhaustive dialysis against pyridine-acetic (pH 5.0) and subsequent lyophilization. Weighed amounts of the enzyme were then dissolved in 0.1 M Tris (pH 7.0) and the optical density recorded at 280 nm. For the meassurment of circular dichroism spectra a Jobin-Yvon dichrographe III was used. The apparatus was calibrated with a solution of epiandrosterone in dioxane. Protein was dissolved in 0,1 M Tris HCL (pH 7.0) at a concentration of 1 mg/ml. Cells of 1.0, 0.2 and 0.05 cm path length were used for the meassurements. The mean residual ellipticity was calculed using an average residue molecular weight of 110. The secundary structure was estimated according to the procedure of Chen *et al.* (1974). The spectrum was run between 200 and 250 nm.

The degree of agglutination was assessed according to the pattern formed by the agglutinated cells on the botton of the test tube. Rat red blood cells (RBC) were washed with 0.1 m phosphate buffered saline (pH7.4). Various amounts of the lectin were added to the wash RBC and the mixture incubated at 37° C for 2 hrs. Human red blood cells as well as RBC from rabbit, dog, mice, sheep and goat were also tested for agglutination.

The physiological action of the lectin-like protein was studied using a Hewlett Packard model 7754A- Thermal tip Recorder, equipped with a pressure amplifier model 8805C. The protein dissolved in physiological saline at a concentration of 3.3 mg/kg was intravenously injected and the carotid arterial pressure recorded. Rats (Sprague-Dowley) of approximately 0.30 kg. were anesthetized with sodium penthobarbital (35 mg/kg).

The lectin-like protein was purified as follows: 0.5 g. of the venom was dissolved in 3 m l of 0.3 M NaCl in 0.1 M NaAc (pH 6.0) centrifuged and applied to a column (2.0 x 90 cm) packed with BIO-Gel P-100, equilibrated with the same buffer. The flow rate was 6 ml/hr and 2.5 ml fractions were collected. Each effluent fraction was read at 280 nm and tested for agglutinating activity. The agglutinin protein was pooled concentrated and equilibrated with 0.01 M Tris (pH 7.3). The solution adjusted to pH 7.3, was applied to a column (2.5 x 40 cm) packed with DEAE-Sephadex A-50 equilibrated with the same buffer. The unbound material was eluted at a flow rate of 25 ml/hr. 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). Fractions were tested for hemagglutinating activity. All column operations were performed in a cold room at 4° C.

RESULTS

The fractionation of 0.5 g. of *L. m. stenophyrs* venom on BIO-GEL P-100 is shown in Fig. 1. The venom was separated into five major fractions. The lectin-like protein eluted in the first peak. Further purification of the agglutinin protein is shown in Fig.2. Hemagglutinating material elutes at 0.4 M sodium chloride. It was devoid of coagulant, proteolytic, hemolytic, oedema, phospholipase, mionecrotic and hemorrhagic activities which were present in the crude venom. No neutral hoxose and sialic acid was detected by the methods described above. Acrylamide gel electrophoresis of the pooled and concentrated material (tubes 55 to 85 Fig. 2) shows a single band, (Figs. 3 and 4) in the presence and in the absence of 2-mercaptoethanol. A single band with Mr 14kDa under reducing conditions and a single band with Mr 28kDa under non-reducing conditions were calculated. Table 1 shows the species specificity of the agglutinin protein. Table 2 shows the amino acid composition of the protein. In Fig. 5 we show the circular dichroic spectrum in the near UV. The lectin-like protein has 78% of β structure and 1% of α structure. Absorbance of a 0.1% solution of the lectin-like protein was 2.00 at 280 nm

In Figs. 6a and 6b we show the hypotensive action of the protein. A fall of 50 mm Hg/mg and 40 mm Hg/mg was recorded under anesthetized and non-anesthethized condition of the animal respectively. If double dose of the lectin-like protein was used the arterial presure feli to zero value causing the death of the animal.

DISCUSSION

The lectin-like protein isolated from the venom of *L. muta stenophyrs* snake exhibits a dimeric structure around 29kDa. This predominant structure is very likely stabilized by disulfide bridges, as pointed out by the fact that only

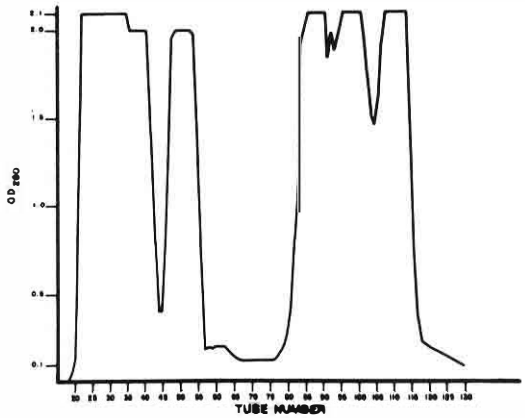

Fig. 1. Gel filtration of 0.5 g *Lachesis muta stenophyrs* venom on BIO-GEL P-100. Agglutination of human RBC was found between tubes No. 20 y 40.

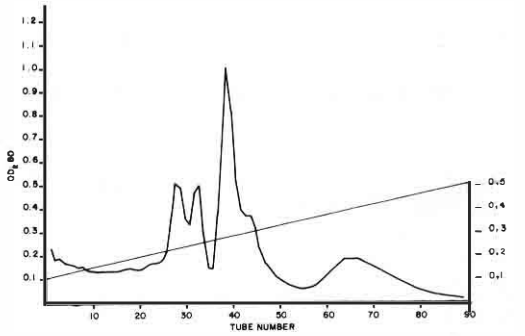

Fig. 2. Purification of the lectin-like protein on DEAE- Sephadex A-50 column. The straight line shows a linear gradiente toward 0.5 M NaCL. Agglutinin activity was found between tubes No. 55 to 85.

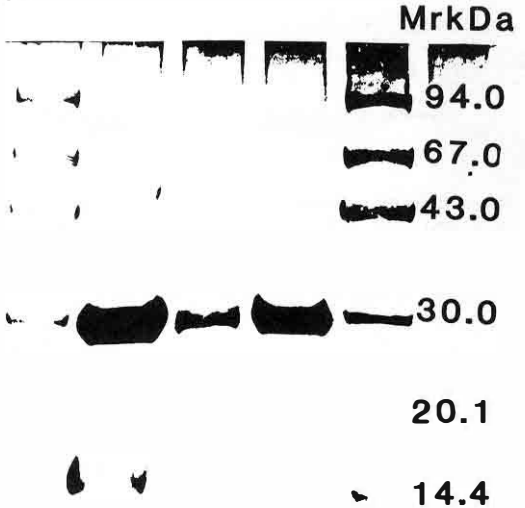

Fig. 3. Photograph shows acrylamide gel electrophoresis at pH 4.4 in the presence of 2-mercaptoethanol.

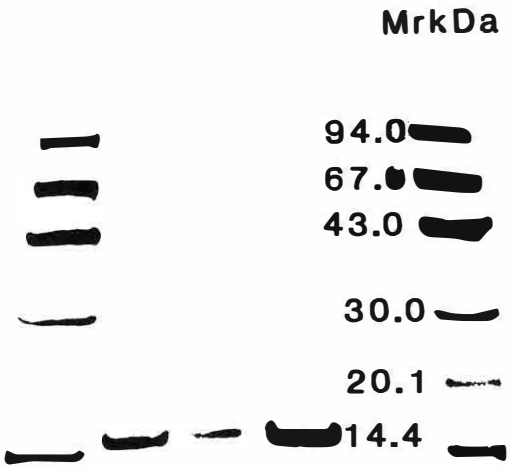

Fig. 4. Photograph shows acrylamide gel electrophoresis at pH 4.4 in the absence of 2-mercaptoethanol.

TABLE 1

Species specificity of the Lectin-Like Protein

Erythocytes	Agglutination
HUMAN	+
RAT	+
RABBIT	+
DOG	+
MICE	–
SHEEP	–
GOAT	–

reduction with 2-mercaptoethanol gives a monomeric structure around M r 14kDa. This molecular weight is in accord with the values reported by Gartner, Stocher and Williams (1980) for the thrombolectin isolated from *Bothrops atrox* venom and with the values reported for the three lectins isolated from *Agkistrodon controtix contoriris. Agkistrodon piscivorosis leukostoma* and *Crotalus atrox* by Gartner and Ogilvie (1984). The lectin-like protein shares also the property of not being a glycoprotein (Gartner and Ogilvie) and also to be inhibited preferentially by lactose (Gómez-Leiva and Aragón-Ortíz 1986). Heterogenity in the isoelectric points of the lectin from snake venoms seems to be common as also reported by Gó-

TABLA 2

Amino acid composition of L. muta *stenophyrs lectin-like protein*

Amino Acid	Residues per mole Calculated	Integer
ASP	13	13
THR	3.3	3
SER	5.7	6
GLU	15.9	16
PRO	3.3	3
GLY	6.4	6
ALA	5.0	5
CYS	4.8	5
VAL	3.1	3
MET	1.5	2
ILE	3.3	3
LEU	9.1	9
TYR	7.0	7
PHE	6.7	7
HIS	3.5	4
LYS	10.4	10
TRP	15.6	16
ARG	3.8	4
TOTAL		125
MOL. wt		15,397

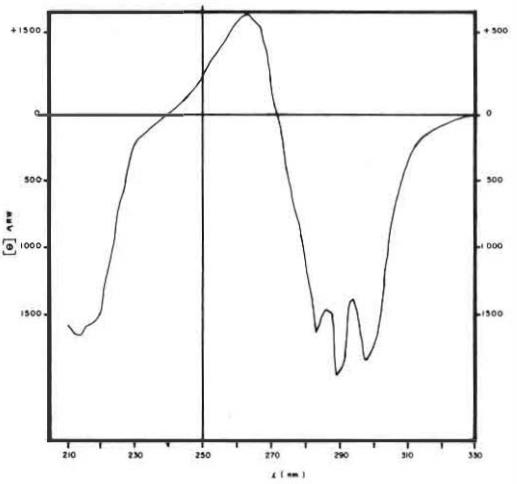

Fig. 5. Circular dichroic spectrum of the lectin-like protein in 0.1 M Tris pH 7.0.

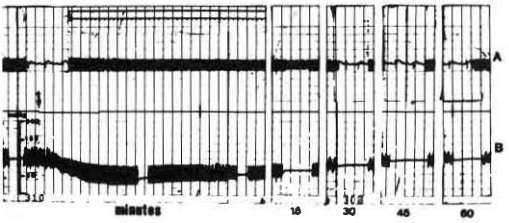

Fig. 6a. Hipotensive action of the lectin-like protein. The protein at a dose of 3.3 mg/kg was intravenously inyected. A fall of 50 nm Hg/mg on the carotid arterial pressure was observed (B). No important change on the ECG was recorded (A).

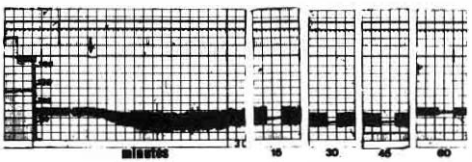

Fig. 6b. In this experimental the same rat, non anethetized was used. A fall of 40 mm Kg/mg was recorded.

mez Leiva and Aragón Ortiz (1986) for mutina and by Gartner and Olgivie (1984) for their three isolated lectins.

It is relevant to point out, that the secondary structure of the lectin-like protein is predominantely a β pleated sheet. This is in accord with the amino acid composition of the lectin, which shows a higher content of residues with B structure forming tendency than B structure brakers with a little content of α formers. The amino acid composition reveals a higher content of acid residues in relation to the basic ones. The presence of cysteine (5 residues) is in accord with the fact that the protein behaves as a dimer linkel by S-S- bridges. The high content of tryptophan may be a characteristic of snake venom lectins and is the reason of the high absorption of the CD spectrum and the high absorption coeffiecient at 280 nm.

Although the lectin does not show any specific hemagglutinating activy (Gómez-Leiva and Aragón-Ortiz 1986) it is proper to point out that the protein differenciates between RBC belonging to different species. In addition,

the agglutinin protein differenciates between RBC of the mouse and of the rat, agglutinating the latter type of RBC. This difference might be of importance when comparing the LD50 of the crude venom of *L.m.stenophyrs* in relations with other Bothropic and Crotalic venoms (Bolaños 1972). Important differences in the membrane structure of this species are then pointed out, suggesting that during animal maturation galactosidic residues are incorporated in the RBC membrane of the rat.

In vivo experiments showed a dramatic fall in the carotid arterial pressure. Very likely, this fall is caused by the agglutination of the RBC which may produce a blockade at the capillary bed of the lungs, causing a decrease in the venous return to the heart thus decreasing the cardiac output.

It was reported by Bolaños, Rojas and Ulloa (1981) that significant alterations in the arterial blood pressure of humans bitten by *Lachesis muta stenophyrs* snakes occur during envenomenation. The physiological action of the lectin-like protein during human envenomentation must be considered seriously at the light of the present results.

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REFERENCES

Anson, M.L. 1939. Estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. gen. Physiol. 22: 79-89.

Baughman, D.J. 1981. Thombin assay. Methods in Enzymolo. 19: 145-157.

Bolaños, R. 1972. Toxicity of Costa Rican snake venoms for the white mouse. The American Journal of Tropical Medicine and Hygiene 21: 360-363.

Bolaños, R., O. Rojas & C.E. Ulloa Flores. 1982. Aspectos biomédicos de cuatro casos de mordedura de serpiente por *Lachesis muta* en Costa Rica. Rev. Biol. Trop. 30: 53-58.

Chen, Y., Y.T. Yang. & K.H. 1974. Determination of the helix and beta form of proteins in aqueous solution by circular dichrioism. Biochem. 13: 3350-3355.

Davis, B.J. 1964. Disc electrophoresis 2. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404-427.

Gartner, T.K. & M.L. Olgivie. 1984. Isolation and characterization of three Ca^{++} -dependent B-galactoside-specific lectins from snake venom. Biochem. J. 224: 301-307.

Gartner, T.K., K. Stoker & D.C. Williams. 1980. Thrombolectin: A lectin isolated from *Bothrops atrox* venom. FEBS Letters & F.A. 117: 13-16.

Gómez-Leiva, M.A. & F.A. Aragón-Ortiz. 1986. Purification and some properties of hemagglutinating protein mutina from bushmaster *Lachesis muta* snake. Rev. Biol. Trop. 34: 49-53.

Gómez-Leiva, M.A. 1975. Efecto del veneno de serpientes sobre diferentes glóbulos rojos. Tesis de Grado. Universidad de Costa Rica.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277: 680-686.

Kondo, H., S. Kondo, H. Ikezawa, R. Murata & A. Okasaka. 1960. Studies on the quantitative method for determination of hemorrhagic activity of habu snake venom. Jpn. J. med. Sci. Biol. 13: 43-46.

Ownby, C. L., Cameron D. and Tu, A.T. 1976. isolation of myotoxin componen from rattlesnake venon. Am.J. Path. 85: 149-152.

Warren, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234: 1971-1975.

Yamakawa, M., Nozaki & Z. Hokama. 1976. Fractionation of Sakishina-habu (*Trimeresurus elegans*) venom and lethal, hemorrhagic, and edema forming activities of the fractions *In* : Animal, plant and Microbial Toxins, Vol. 1: Biochemistry (A. Ohasada, K. Hayashi & Y. Sawai, eds.). Plenum Press, New York.

Winzler, R.J. 1961. Determination of serum glycoproteins. P.290 *In* D. Glik (ed.). Methods of Biochemical Analysis, Vol.2. Interscience, New York.