Rev. Biol. Trop. 34(1): 49-53, 1986

Purification and Some Properties of Hemagglutinating Protein Mutina from Bushmaster *Lachesis muta* Snake Venom

M. A. Gómez Leiva & F. Aragón-Ortiz
Depto. de Bioquímica. Universidad de Costa Rica

(Received: March 27, 1985)

Abstract: *Lachesis muta* snake venom induced aggregation of bromelain sensitized human erythrocytes at a concentration of 1 mg/ml. The hemagglutinating protein was purified by DEAE-Sephadex A-50 column chromatography. Polyacrylamide gel electrophoresis revealed at least three bands, whereas SDS electrophoresis in the presence of 2-mercaptoethanol showed a single one. Isoelectric focusing revealed hemagglutinating activity in the range of pH 3-8. The maximun peak (mutina) at pH 5.5. This fraction was active in agglutinating human RBC of types A, B, O Rh (+) and B, O Rh (–).
One mM EDTA and I mM Ca^{++} did not alter the agglutinating time significantly. Lactose and inositol inhibited the agglutination of A, B, O Rh (+) and B, O Rh (–) human RBC.
The present study showed the non specificity of the hemagglutinating activity of mutina. It was also shown that mutina is a non-mitogenic protein.

Lectins are carbohydrate-binding, cell agglutinating proteins, occuring in extracts from certain plants, mainly from seeds. However, they have also been found in bacteria, fish roes, snails, vertebrates and mushrooms (Goldstein and Hayer, 1978; Gold and Baldin, 1975). These agglutinating proteins have been reported to be present in some snake venoms (Flexner and Noguchi, 1902). In this paper the purification and some properties of mutina, the hemagglutinating protein from *Lachesis muta* venom (Atlantic type), is described.

MATERIAL AND METHODS

The venom was obtained from Instituto Clodomiro Picado, University of Costa Rica. It consisted of pooled lyophilized samples obtained from *Lachesis muta* snakes captured at the Atlantic zone. Benzoyl-arginine ethyl ester HCL (BAEE), human fibrinogen (90% clottable, type I), casein, D (+) galactose, α D(+) glucose, D (+) mannose, D (–) fructose, D (+) xylose, lactose saccharose, inositol, β D-mannosamine, N acetyl, β D-galactosamine and concanavalin A were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Carrier ampholytes (ampholine) were from LKB- Productor AB (Broma, Sweden). Acrylamide and ammonium persulfate were from Bio Rad Laboratories (Richmond, Calif. USA).

The procedure for the purification of the hemagglutinating protein was as follows: one gram of venom was dissolved in 10 ml of 0.01 M Tris HCl 0.05 M Ca-EDTA (pH 7.3) and applied to a column (2.7 x 40 cm) packed with DEAE- Sephadex A-50, equilibrated with the same buffer. The unbound material was eluted at a flow rate 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 HCl (pH 7.3). The hemagglutinating protein was eluted with 0.15 M NaCl, in a single peak. The pooled and concentrated material was then subjected to preparative isoelectric-focusing in an LKB 110 column. The ampholyte concentration was 0.5% and the pH range from 3 to 9. Disc electrophoresis was made in 7% acrylamide gel at pH 8.4 as described by Davis (1964).

The agglutination assay was made according to the method of Hartman et al., (1978)

slightly modified. Serial two fold dilution of 25 µl aliquots (mutina 200 µg/ml, fraction that focuses at pH 5.5) were made in 25 µl of Tris buffered saline at pH 7.0 (50 mM Tris HCl 150 mM NaCl-40 mM $CaCl_2$ plus 12.5 µl of 2.5% bromelain sensitized human RBC, using a microtiter V plate. The plates were kept at room temperature and the agglutination titer was read after 30 minutes. Bromelain treatment was made as follows in all tests: 2 ml of washed human RBC incubated at 37°C with 2 ml of 0.1% bromelain, 0.01 ml of 2-mercaptoethanol 98%, during one hour. Cells were then washed six times with buffered saline.

Inhibition studies of the agglutinating activity were carried out using the following carbohydrates at a concentration of 0.1 M in buffered saline: D (+) galactose, D (+) glucose, β D (+) glucose, D (+) mannose, D (−) fructose, D (+) xylose, lactose, saccharose, D-mannosamine, N-acetyl- β D-galactosamine as well as inositol. Serial two fold dilution of aliquots, 25 µl of carbohydrate in buffered saline (pH 7.0) were made in 25 µl of the isotonic hemagglutinating protein. Incubation of 30 min was made at room temperature and the agglutination titer was read after 30 min. A requirement for divalent ions was studied by adding $CaCl_2$ and EDTA (each at a concentration of 1 mM) to the purified lectin preparation and incubated for 15 min prior to addition of bromelain sensitized RBC. The agglutination was recorded as described above. Mitogenic activity of mutina was studied according to the method of Makino (1978).

Aliquots of 50-100 µl (1 mg/ml) were incubated in 5 ml culture media using human leucocytes. Concanavalin A, a known mitogenic lectin was used as positive control.

Human erythrocytes, type O Rh^+, agglutinated by mutina were fixed with 2.5% glutaraldehyde in phosphate buffer of pH 7.0 during two hours at 4°C. Dehydratation was done with ethanol and left in amylacetate. The fixed erythrocytes were dried in a Hitachi HCP-1 critical point drier and put on specimen studs by means of a double sided adhesive tape. The specimen was coated by gold using an ion coater EIKO IB-3, and studied with a scanning electron microscope Hitachi HHS-2R.

Platelet aggregation was studied using mutina at a concentration of 1 mg/ml. The preparation of platelet-rich plasma was essentially as described by Ouyan and Teng (1978). The aggregation was observed with a phase contrast microscope.

The determination of the clotting activity was made according to the method of Baughman (1979), using human fibrinogen as substrate. Proteolytic activity was measured using 2% casein, after precipiting with 3% TCA, readings at 280 nm were made to the protein free filtrate. The determination of estereolytic activity was measured according to the method of Hummel (1979), using BAEE as substrate. Hermorrhagic activity was measured according to the method of Kondo (1960) slightly modified. Samples of the purified protein up to 75 µg were inoculated intracutaneously 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 protein were inoculated in the right hind foot of a mouse (14-16 g), the other foot was inoculated with 0.9% NaCl. Edema was expressed as a percentage increase in the weight of the affected foot related to the weight of the control. Phospholipase activity was measured by the indirect hemolysis of human erythrocytes caused by addition of 70 µg of protein to a medium containing egg lecithin (Gómez-Leiva, 1975). After half hour reaction at 37°C the tubes were centrifugated and measured at 550 nm. Hemolysis was expressed as percentage related 100% hemolysis caused by the addition of distilled water. An absorption coefficient of 1.0 was assumed for a 0.1% solution of mutina read at 280 nm.

RESULTS

The fractionation of one gram of *Lachesis muta* venon on DEAE- Sephadex A-50 column chromatography is shown in Fig. 1. The venom was separated into four major fractions. The hemagglutinating material was obtained in a linear gradient, eluting at 0.15 M NaCl in 0.01 M Tris HCL (pH 7.3). The use of 0.05 M Ca-EDTA in the starting inhibited the proteolytic activity of venoms under fractionating conditions. The hemagglutinating material obtained was devoid of coagulant, proteolytic, hemolytic, edema, phospholipase and hemorrhagic activities which were present in the crude venom. No platelet aggregation was observed by mutina under the conditions of study. Fig. 2 shows the isoelectric focusing

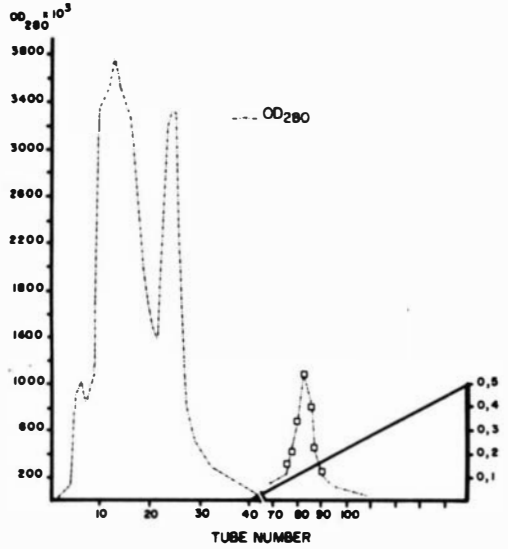

Fig. 1. Fractionation of *Lachesis muta* venom Atlantic type on DEAE-Sephadex A-50 in 0.01 M Ca-EDTA (pH 7.3). Straight line indicates the gradient toward 0.5 NaCl in 0.01 M Tris HCl pH 7.3. ·····□····· hemagglutinating activity.

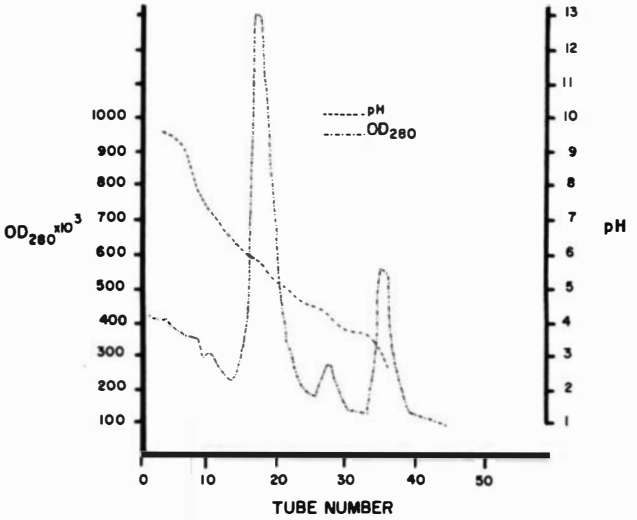

Fig. 2. Isoelectric focusing of pooled and concentrated hemagglutinating material from *Lachesis muta* venom (Fig. 1). in LKB 110 column at 3°C.

of the pooled and concentrated hemagglutinating material from Fig. 1. The hemagglutinating activity was found along the pH gradient, most of the activity focused at pH 5.5 Fig. 3 also shows acrylamide gel electrophoresis of the material that focuses at pH 5.5 and the crude venom. A single protein band is cleary visualized when stained with Coomassie brilliant blue G-250. Fig. 4 shows the agglutinating activity of mutina toward red blood cells as revealed by scanning electron micrograph. The strong agglutinating action of mutina induces a change in the shape of the erythrocytes. Free, non-agglutinated erythrocytes are also clearly visualized. Table 1 shows the activity of mutina toward human RBC. Agglutination of the Rh (+) types occurred at a dilution 1/16 (0.31 μg). The blood types B Rh (+) and 0 Rh (–) were the most sensitive in agglutinating at a dilution 1/64 (0,078 μg). In Table 2 the inhibitory action of the studied carbohydrates in the agglutination activity of mutina toward human RBC, is shown. Inositol exhibited the strongest inhibition, inhibiting A, B, O Rh (+) and B, O Rh (–) human blood types at a dilution of 1/128 (0.781 mM) Lactose inhibited human RBC (+) at a sugar dilution 1/32 (3.12 mM). Toward human RBC Rh (–) the inhibitory effect of lactose was at a dilution 1/16 (6.2 mM). Galactose inhibited human RBC Rh (+) at a dilution 1/64 (1. 56 mM) The 0 and B Rh (–) blood types were inhibited at a galactose dilution 1/32 (3.12 mM). β D (+) glucose, D (+) mannose, α D (+) glucose. D (–) fructose, N acetil β –D mannosamine, N acetil β –D galactosamine, D (+) galactosamine, D (+) xilose, and saccharose, were carbohydrates that did not inhibit the agglutinating activity of mutina.

DISCUSSION

This preliminary work shows that the hemagglutinating protein (mutina) isolated from *Lachesis muta* venom, Atlantic type, exhibited a non-specific lectin like activity toward RBC and that this action can be inhibited by galactose, lactose and inositol. In addition mutina was also shown to be non-mitogenic when incubated with human leucocytes using Concanavalin A as positive control.

Comparison of these results with thrombocytin, the lectin protein isolated from *Bothrops atrox* venom (Gartner and Stocker, 1980), revealed that both of them are inhibited by lactose. In contrast with thrombocytin, mutina is incapable of causing platelet aggregation. The lectin isolated from *Trimeresurus mucrosquamatus* venom is inhibited by α –methyl galactoside (Ouyang and Teng, 1978).

TABLE 1

Hemagglutinating action of Mutina toward human BOR+ and OBR− RED BLOOD CELL Rh+

Dilution in mutina (Initial conc. 5 µg)	A	B	AB	O	B	O
1:1	+	+	+	+	+	+
1:2	+	+	+	+	+	+
1:4	+	+	+	+	+	+
1:8	+	+	+	+	+	+
1:16	+	+	+	+	+	+
1:32	+	+	—	—	+	+
1:64	—	+	—	—	—	+
1:128	—	—	—	—	—	—
1:256	—	—	—	—	—	—

(+) Agglutination after 30 minutes

(—) No detectable agglutination

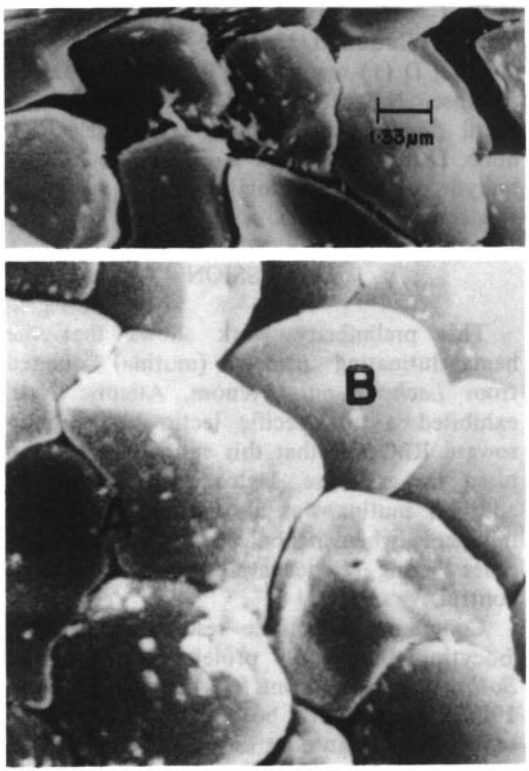

Fig. 4. Electron micrograph of human erythrocytes type o Rh (+), agglutinated by mutina. Bar= 1.33 µm. A. Red blood cells united by the action of mutina. B. Free RBC trapped in the agglutinated pellet.

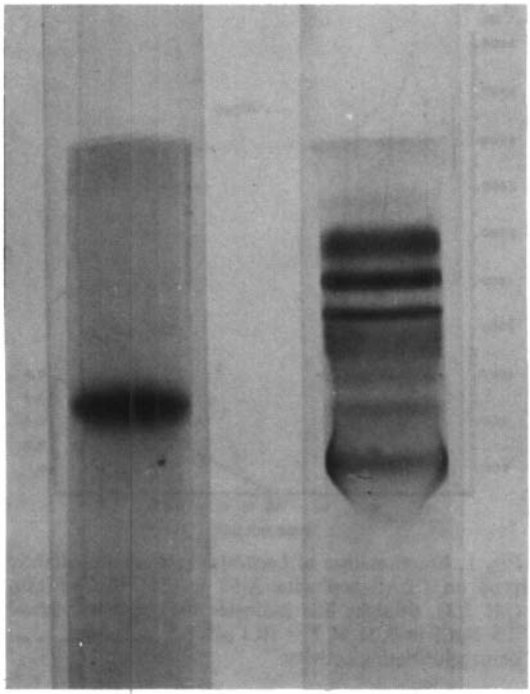

Fig. 3. Photograph shows acrylamide gel of crude venom and of concentrated material (mutina) that focuses at pH 5.5.

TABLE 2

Inhibitory action of carbohydrates on hemagglutinating activity of Mutina, toward human ABO Rh+ and OB Rh− cells

Carbohydrate (0.1 M)	RED BLOOD CELL Rh+ A	B	AB	O	Rh− B	O
Galactose	1:64	1:32	1:64	1:64	1:16	1:32
Lactose	1:32	1:16	1:32	1:32	1:16	1:16
Inositol	1:128	1:128	1:128	1:128	1:128	1:128

Dilution indicated was the final carbohydrate concentration wich provoked inhibition of agglutinating activity.

These results probably point out that these lectins bind to galactosyl receptors of the red cell membrane.

AKNOWLEDGEMENT

The authors are indebted to Dr. Ana Isabel Barrantes for performing the hemagglutinating test. The mitogenic studies were done at the Centro de Biología Celular y Molecular (CIBCM), University of Costa Rica. The electron micrographs were taken at the Electron Microscopy Unit of the University of Costa Rica. This work was done under grand No. 02-07-10-59, University of Costa Rica and

was partially supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT), Costa Rica.

REFERENCES

Baughman, D. J. 1970. Thrombin assay. Methods in Enzymol 19, 145-149.

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

Flexner, S., & Noguchi, H. 1902. In: Venomous animal and their venoms, Vol. 1 p. 129-131. Academic Press, New York.

Gartner, T. K. Stocker, K., & William, C. D. 1980. Thrombolectin: A lectin isolated from *Bothrops atrox* venom FEBS LETTERS. 117, 13-16.

Gold, E. R. & Baldin, P. 1975. Experta Medica, Amsterdam.

Goldstein, I. J. & Hayer, C. E. 1978. Adv. carbohyd. Chem. Biochem. 35, 128.

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

Hartman, L. A., Campbell, A. P., & Abel, A. C. 1978. An improved method for the isolation of lobster lectins. In: Development and Comparative Inmunology Vol. 2, p 617 Pergamon Press.

Humel, B.C.W. 1959. A modified spectrophotometric determination of chimotrypsin, trypsin and trombin. Canad. J. Biochem. Physiol. 37, 1393.

Makino, S. 1978. Human chromosome North-Holland Publishing Co. p. 55.

Ouyang, C. & Teng, C. 1978. Red Blood cell agregation by *T. mucrosquamatus* snake venom. Toxicon, 16, 503-508.

Kondo, H. S., Kondo, H. Ikesawa, R. Murata & A. Osasaka. 1960. studies on the quantitative method for determination of hemorrhagic activity of habu sanke venom Jap. J. Med. Sci. Biol 13, 43.

Yamakawa, M., Nozaki, M. & Hokama, Z. 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). New York: Plenum Press.