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A SYSTEMATIC FRACTIONATION OF *CRÓTALUS DURISSUS TERRIFICUS* VENOM +

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ABSTRACT: 1. The venom of *C.d. terrificus* was fractionated to obtain from the same preparation: 5'-nucleotidase, phosphodiesterase, thrombin-like enzyme, phospholipase A2, crotapotin, convulxin and gyroxin. All these proteins were shown to be homogeneous by SDS-PAGE.

2. L-amino acid oxidase, tissue kallikrein-like and NAD-hydrolase activities were detected.

3. One of the homogeneous fractions presents a toxic activity and produces symptoms different of those of known toxins. It has molecular weight of 8,600 Daltons.

4. Phospholipase A2 activity of venom and fraction is completely inhibited by crotapotin, and this inibitions is specific for the venom of Crotalus and does not occur with phospholipase A2 activity of *Bothrops jararaca*, *Bothrops moojeni* and *Tityus bahiensis*.

KEYWORDS: *Crotalus* venom, toxins, enzymes.

INTRODUCTION

Although the venom of *Crotalus durissus terrificus* has already been investigated, with the isolation of toxins and enzymes (Tu, 1977 and 1982),[18]

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a systematic study which would allow the isolation of the several toxins and enzymes from the same batch was not yet been made. In the present investigation a large batch of venom, representative of the venom of this species in Brazil, has been submitted to purification by ammonium sulfate and column chromatographies, with the recovery of known toxins and enzymes as well as of a new toxin.

MATERIALS AND METHODS

Materials

Venom used in this preparation was collected and dried at room temperature by the Section of Venoms of this Institute and was 10 years old. Ion exchange and affinity chromatography resins were obtained from Pharmacia (Sweden) and sepharose-1-4-butenediol-diglycil-p-aminobenzamidine was prepared by Biobraz (Brazil).
Synthetic substrates and purified bovine fibrinogen were obtained from Sigma Chemical Co. (USA).

Enzyme activity determinations

Thrombin-like activity was measured by the clotting time of fibrinogen, using as standard a thrombin preparation, and expressed in NIH units [15].

Phosphodiesterase was measured using bis-p-nitrophenyl-phosphate as substrate[7]. Unspecific phosphatase was measured by the same method, using p-nitrophenyl-phosphate as substrate.

5'-nucleotidase was assayed according to Tatsuki *et al*[17] and phospholipase A2 according to Grassman and Hanning[9]. NAD-nucleotidase was measured by the hydrolysis of NAD^+ and the remaining NAD^+ measured with alcohol dehydrogenase[6].

L-amino acid oxidase was measured according to Wellner[21]. Kallikrein-like activity was measured with D-valyl-L-leucyl-L-arginine-p-nitroanilide and D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide, at pH 8.0 and at 25°C[5] and by the direct method of Henriques *et al*[10] using horse plasma treated according to Werke *et al*[22] as substrate and rat isolated uterus for assaying the kinin formed.

Toxins

Crotamine and crotoxine were assayed by intravenous injections respectively into mice[19] and young chicken.
Crotapotin was assayed by the inhibition of phospholipase A2[3].

Gyroxin was assayed by intravenous injection into mice as described by Barrabin *et al*[1]. Convulxin was assayed by the induction of platelet aggregation[20].

Acrylamide electrophoresis

SDS-PAGE was carried out by the standard method[4]. PAGE at pH 4. O was carried out according to Giglio[8].

RESULTS AND DISCUSSION

A. VENOM FRACTIONATION

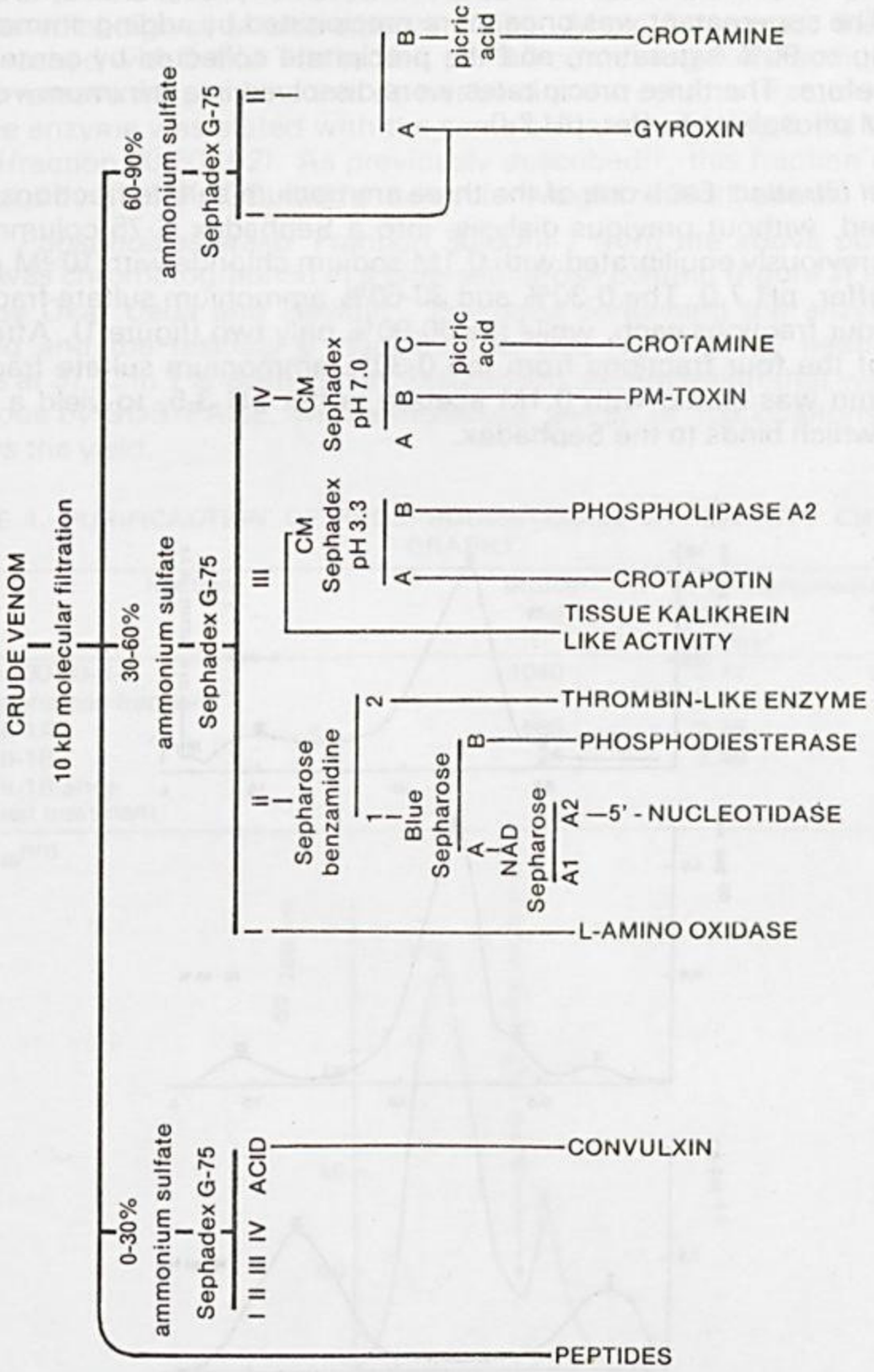

FIGURA 1. Fractionation scheme

1. *Isolation of peptides*: 30g of venom dissolved in 300 ml of 0.9% sodium chloride was filtered through a hollow fiber with a nominal cut of 10 kDaltons. The filtrate, containing the peptides, was lyophylized, yielding 1.2g of dry weight.

2. *Ammonium sulfate fractionation*: After removal of the peptides a protein fraction was precipitated, with stirring and in an ice bath, with solid ammonium sulfate, up to 30% saturation. The precipitate was removed by centrifugation at 10.000g for 15 minutes, at 4°C. More ammonium sulfate was added to reach 60% saturation, and the second precipitate removed as before. The supernatant was once more precipitated by adding ammonium sulfate up to 90% saturation, and the precipitate collected by centrifugation as before. The three precipitates were dissolved in a minimum volume of. 0.01M phosphate buffer, pH 7.0.

3. *Gel filtration*: Each one of the three ammonium sulfate fractions were introduced, without previous dialysis, into a Sephadex G-75 column (5 x 80cm), previously equilibrated with 0.1M sodium chloride with 10^{-3}M phosphate buffer, pH 7.0. The 0-30% and 30-60% ammonium sulfate fractions yielded four fractions each, while the 60-90% only two (figure 1). After the elution of the four fractions from the 0-30% ammonium sulfate fraction, the column was eluted with 0.1M acetate buffer pH 3.5, to yield a small fraction which binds to the Sephadex.

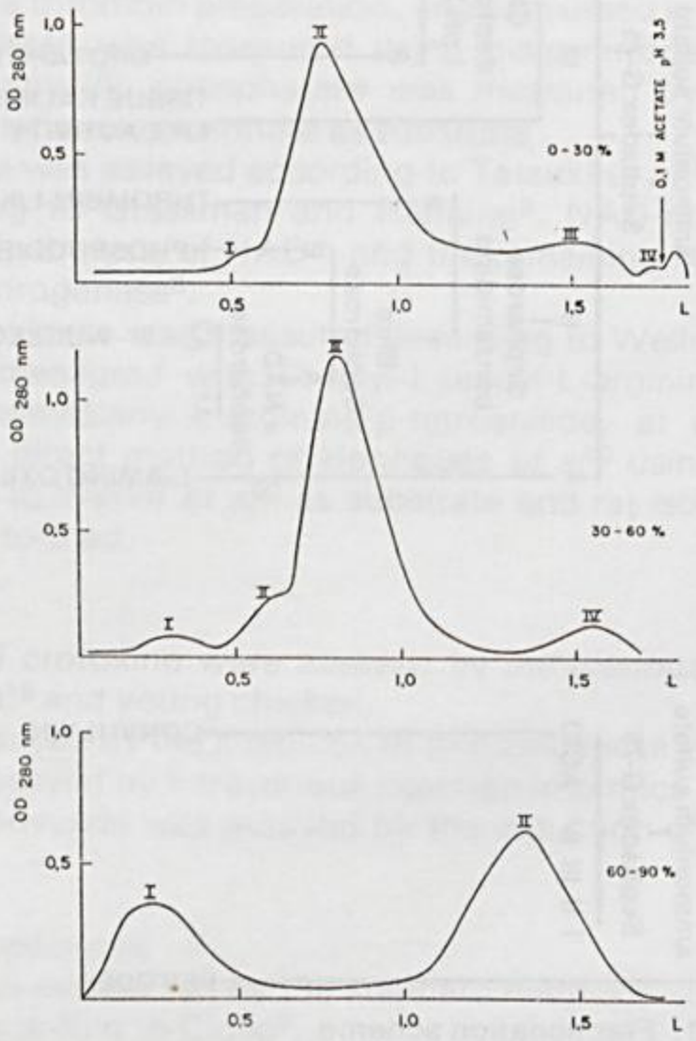

FIGURE 2. Sephadex G-75 chromatography of the 0-30, 30-60 and 60-90% ammonium sulfate fractions. The ammonium sulfate fractions obtained from 10g of venom were applied to a column (5 x 80cm) previously equilibrated with 0.1 M sodium chloride with 1 mM phosphate buffer pH 7.0 and eluted with the same buffer. In the 0-30% fraction the last peak was eluted with O.1M acetate buffer pH 3.5.

B. ENZYME PURIFICATION

1. *Thrombin-like enzyme*: Fraction II of the 30-60% ammonium sulfate fraction (30-60-II), obtained by chromatography on Sephadex G-75, was taken to pH 9.0 and introduced into a column (2.5 x 8cm) of Sepharose-1-4-butenediol-diglycil-p- aminobenzamidine. After 15 minutes, the column was washed with 5 mM Tris buffer, pH 9.0 containing 0.4M sodium chloride, to remove the proteins that do not bind to the column (fraction 30-60-II-1); the enzyme was eluted with the same buffer containing 0.1M benzamidine (fraction 30-60-II-2). As previously described[15], this fraction is homogeneous by SDS-PAGE, with a molecular weight of 34 kDaltons.

2. *Phosphodiesterase*: Fraction 30-60-II-1 from the above purification step was chromatographed in a blue-sepharose column (figure 2) as described by Oka, Ueda and Hayshi[11]. Fractions containing the enzyme were pooled and the residual 5'-nucleotidase was removed by heating for 3 hours at 37°C in 1% acetic acid[7], resulting in a preparation that was homogeneous by SDS-PAGE, with a molecular weight of 105 kDaltons. Table I shows the yield.

TABLE 1. PURIFICACTION OF PHOSPHODIESTERASE BY AFFINITY CHROMATOGRAPHY

fraction	protein mg	phosphodiesterase specific activity*	% yield
fraction 30-60-II-1	1040	0.17	100
Blue-sepharose fractions			
30-60-II-1A	585	0.16	13.8
30-60-II-1B	24	3.49	46.8
30-60-II-1B after acid-heat treatment	22	1.21	22.8

*$U/A_{280}nm$

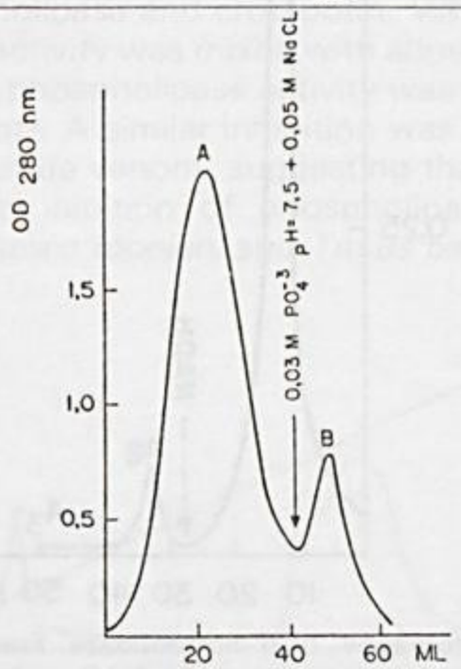

FIGURA 3. Separation of phosphodiesterase and 5-nucleotidase by blue-sepharose chromatography of fraction 30-60-II. 1.040mg of protein in 20ml was dialyzed against 5 mM phosphate buffer pH 7.0 in 0.05 M sodium chloride and introduced in a 1.5 x 15cm column previously equilibrated with the same buffer. The column was washed with the equilibrating buffer until all fraction A was removed and then fraction B, containing phosphodiesterase, was eluted with 0.03 M phosphate buffer pH 7.5 containing 0.05 M sodium chloride.

3. *5'-nucleotidase*: The fraction which was not retained by the blue-sepharose column (fraction 30-60-II-1A) was chromatographed on NAD-sepharose column (figure 3) as described by Tatsuki *et al*[16].

The yield is shown on table 2. The most active fractions (30-60-II 1A3) is homogeneous by SDS-PAGE with a molecular weight of 200 kDaltons. NAD-sepharose can be replaced by 5'-AMP-sepharose, but the purified enzyme has only half the specific activity.

TABLE 2. PURIFICATION OF 5'-NUCLEOTIDASE BY AFFINITY CHROMATOGRAPHY

fraction	protein mg	specific activity*	% yield
Fraction 30-60-II-1	1040	1.74	100
Blue-sepharose fractions			
30-60 II-1A	585	2.34	80
30-60 II 1B	24	0	0
NAD-sepharose fractions			
30-60-II-1A1	530	0.68	20
30-60-II-1A2	52	16.85	48
30-60-II-1A3	2	76.30	7

*$U/A_{280}nm$

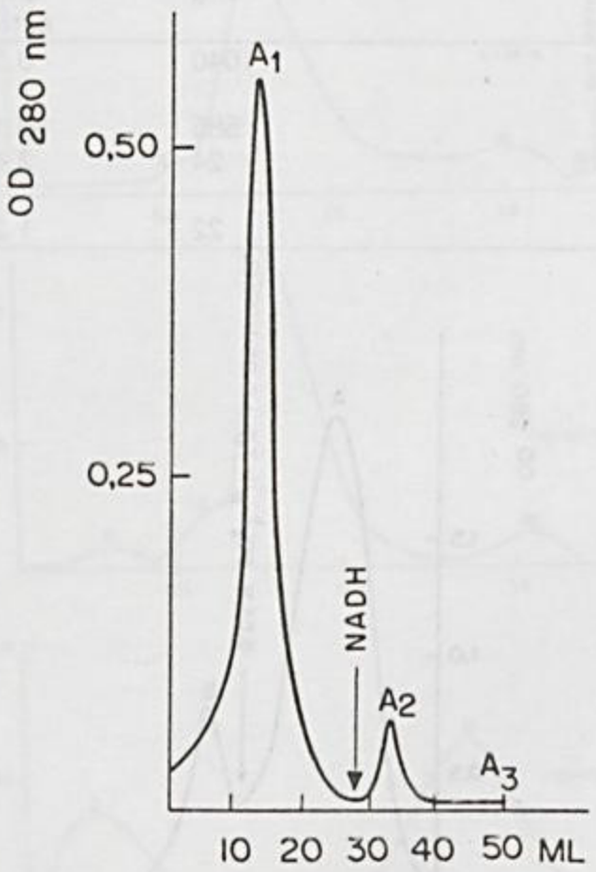

FIGURE 4. Affinity chromatography of 5'-nucleotidase. Fraction 30-60-II-1A containing 585mg of protein was introduced into NAD-sepharose column 1,5 x 5cm, previously equilibrated with 0.05M acetate buffer pH 7.0. The column was washed with the equilibrium buffer until all the first peak was eluted (A1), and then with 10ml of 0.2 M sodium bicarbonate buffer pH 8.6 containing 0.05 M NADH that eluted the enzyme (A2). After the collection of this peak, 6ml of effluent (A3), with very little protein (2mg) contained 5'-nucleotidase with the highest specific acvitity (76.3).

4. *Other enzymes*: The addition of crude venom to fibrinogen results in a transient clot, revealing the presence of a fibrinolytic and fibrinogenolytic activity, which is bound to sepharose-1-4-butenediol-d glycil-p-aminobenzamidine, but once eluted, is very unstable. This fraction has no thrombin-like activity. SDS-PAGE eletrophoresis of fibrinogen treated with this fraction[15] shows that it acts on the ß-fibrinogen chain. A similar enzyme was described in the venom of *Trimeresurus mucrosquamatus* by Ouyang and Teng[12].

Fraction 0-30-I contains a small NAD-hydrolase activity (0.024 µmol/mg/min at 25°C) acting on NAD^+, $NADP^+$ and NADH. A similar activity was described for the venom of *Agkistrodon halys blomhoffii*, but it does not hydrolvze NADH[17]. L-amino acid oxidase is present in the fraction 30-60-I, but was not further purified.

By biological assay, fractions 30-60-I, 30-60-II and 30-60-III present a weak kallikrein-like activity. Fraction 30-60-III hydrolyzes DL-valyl-L-leucyl-L- arginine-p-nitroanilide with a specific activity of 0.23 µmol/min/mg of protein at 25°C. It has only 1/30 the activity with benzyl-L- prolyl-L-phenylalanyl-L-arginine-p-nitroanilide or benzyl-L-phenyl-L-valyl-L-arginine-p-nitroanilide as substrates. This indicates a tissue kallikrein-like activity[2]. It is unstable at pH 3.3 and thus destroyed in the CM-sephadex chromatography used for the isolation of crotoxin components from this fraction.

C. TOXINS

1. *Phospholipase A2* and *crotapotin*: These were isolated from the fraction 30-60-III, by suspending in 0.1M formic acid, removal of the insoluble protein followed by chromatography on CM-sephadex at pH 3,3 using a linear gradient of ammonium formiate between 0.1M and 3.M (figure 4) which separates phospholipase and crotapotin. When the fraction containing phospholipase A2 activity was mixed with about 1.5 times the concentration of crotapotin, all phospholipase activity was inhibited, as previously described by Breinthaupt[3]. A similar inhibition was shown to occur by adding crotapotin to the crude venom, suggesting that there is an excess of free phospholipase. No inibition of phospholipase activity present in *Bothrops jararaca, Bothrops moojeni* and *Tityus bahiensis* venom was observed.

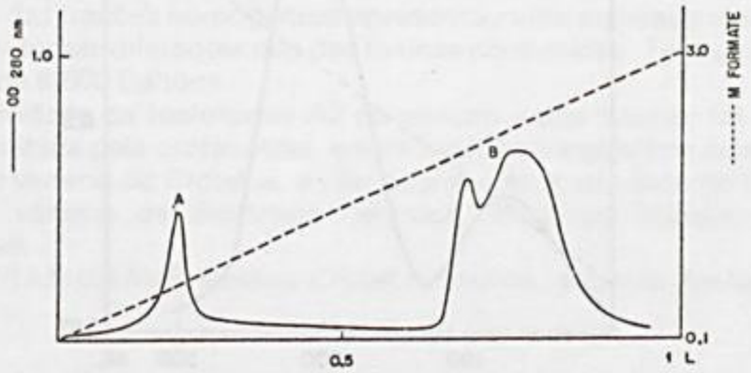

FIGURE 5. Purification of crotapotin and phospholipase A2 by chromatography on CM-sephadex. Fraction 30-60-III from 10g of venom in 0.1M formic acid was centrifuged to remove the insoluble protein, taken to pH 3.3 and introduced in a column (5 x 12cm), previously equilibrated with 0.1 M formate and eluted with a linear gradient between 0.1M and 3.0 M for 4 hours, at a rate of 80 ml/hour. Crotapotin is the peak A and phospholipase A2 is the peak B.

2. *Crotamine:* Crótamine was isolated from the 30-60-IV fraction by chromatography on CM-sephadex, at pH 7.0 (figure 5). Some crotamine can also be recovered by a similar chromatography of the 60-90-II fraction (figure 6). The fractions containing crotamine were pooled and precipitated with picric acid (Giglio, 1975). The preparation migrates as a single band on PAGE pH 4,0 and is crotoxin-free when assayed on young chickens. The yield was 380mg of protein.

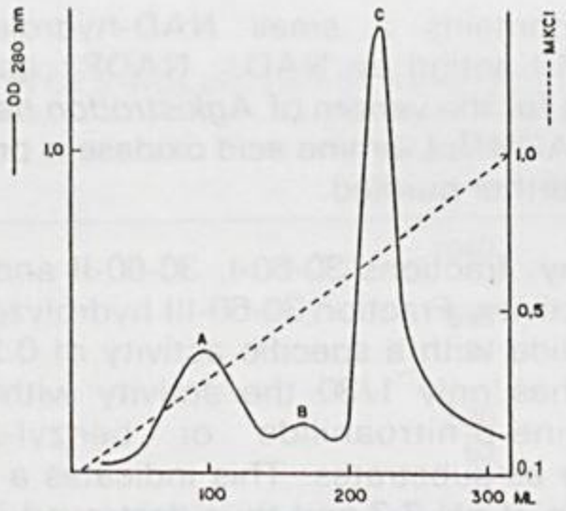

FIGURE 6. Purification of crotamine (C) and PM-toxin (B) by chromatography on CM-Sephadex. Fraction 30-60-IV from 30g of venom, previously dialyzed against 0.01 M phosphate buffer pH 7.0, was introduced in a column (5x 12cm), previously equilibrated with 0.1 M potassium chloride in 0.01 M Tris buffer pH 7.0. The column was eluted for 4 hours with a linear gradient between 0.1 and 1.0 M potassium chloride, at a rate of 80 ml/hour.

3. *Gyroxin*: Gyroxin activity was detected in the 60-90-I and 60-90-II fractions. In the CM-Sephadex chromatography of the fraction 60-90-II gyroxin is eluted in the first peak, while crotamine in the second (figure 5). This gyroxin fraction is homogeneous by SDS-PAGE, with a molecular weight of 35 kDaltons, which agrees with that found by Barrabin *et al*[1]. The yield was 45mg of protein.

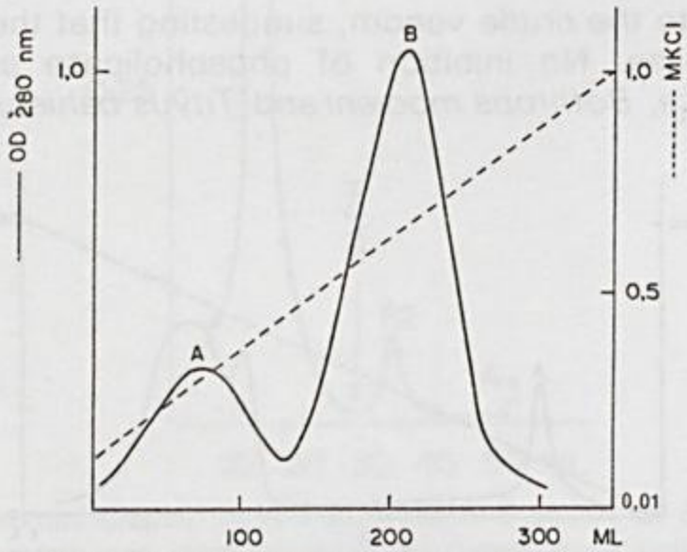

FIGURE 7. Purification of gyroxin (A) and crotamine (B) by chromatography on CM-Sephadex. Fraction 60-90-II from 30g of venom was dialysed against 0.01M phosphate buffer pH 7.0 and introduced in a column (5 x 12cm), previously equilibrated with 0.1 M potassium chloride in 0.01 M Tris buffer pH 7.0. The column was eluted for 4 hours with a linear gradient between 0.1 and 1.0 M potassium chloride, at a rate of 80 ml/hour.

4. Convulxin: Convulxin is present in the 0-30 fraction and in Sephadex G-75 chromatography some is eluted in the fourth peak, but most of the activity remains bound to the Sephadex, being eluted by acid[13] as a PAGE-SDS homogeneous fraction with a molecular weight of 68 kDaltons.

5. *PM-toxin*: In the chromatography of the 30-60-IV fraction on CM-Sephadex, a fraction was found (figure 5) that injected intravenously in 25g mice in sub-lethal dosis (less than 100 μg) produced a temporary excitation, with erratic walking. After that, and for about 1 hour, the mice jumped high and far when touched, and if placed inside a cage, would bump into the walls. At lethal dosis this fraction does not cause the typical muscular effects of crotoxin or crotamine in mice. PAGE of this fraction at pH 4 shows a single band and in 15% SDS-PAGE it migrated with an apparent molecular weight of 8,600 Daltons.

D. PEPTIDE FRACTION

The peptide fraction was assayed for endorphine activity in mice, employing the hot place method of Woolfe *et al*[23], being negative up to 24 μg dry power/kg, whereas the effective dose of morphine under these conditions was 6 μg/kg. No activity was found on the motility of rabbit duodenum or jejunum, up to a concentration of 30 μg dry power/ml. On the electric stimulated ductus deferens of mouse[14] it shows an inhibitory effect at a concentration of 200 μg dry power/ml, which, however, was not reverted by 0.1 μM naloxone.

It is interesting to note that crotamine and the PM-toxin do not appear in the 10 kDalton filtrate. The distribution of proteins of the fraction 30-60% in the Sephadex G-75 column is also not as expected for their molecular weights. It is possible that, at least in the high concentrations used these proteins aggregate.

RESUMO; 1. O veneno de *C.d. terrificus* foi fracionado de forma a obter da mesma preparação: 5'- nucleotidase, fosfodiesterase, enzima tipo trombínico, fosfolipase A2, crotapotina, convulxina e giroxina. Todas estas proteínas foram purificadas até a homogêneidade a eletroforese em acrilamida na presença de dodecil-sulfato de sódio.

2. Em outras frações foram identificadas a presença de L-amino oxidase, atividade de kalikreina tipo tissular e NAD-hidrolase.

3. Uma das frações homogeneas apresentou uma atividade tóxica produzindo sintomas diferentes dos das toxinas conhecidas. Tem um peso molecular de 8.600 Daltons.

4. A atividade da fosfolipase A2 do veneno e das frações foi completamente inibida pela crotapotina, e esta inibição é específica para a fosfolipase do veneno do Crotalus, e não ocorre com a atividade de fosfolipase A2 do veneno da *Bothrops jararaca, Bothrops moojeni* e *Tityus bahiensis*.

PALAVRAS-CHAVE: veneno *Crotalus*, toxinas, enzimas, fosfolipase A2

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