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Short communication

Quantification of crotamine, a small basic myotoxin, in South American rattlesnake (*Crotalus durissus terrificus*) venom by enzyme-linked immunosorbent assay with parallel-lines analysis

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Abstract

Intraspecific variation in *Crotalus durissus terrificus* venom composition was studied in relation to crotamine activity. Crotamine induces paralysis in extension of hind legs of mice and myonecrosis in skeletal muscle cells. To determine whether the venom of crotamine-negative rattlesnake contains a quantity of myotoxin incapable of inducing paralysis, we have developed a very sensitivity immunological assay method, an enzyme-linked immunoabsorbent assay (ELISA), capable of detecting 0.6 ng of purified crotamine. The parallel-lines analysis of ELISA data showed to be useful because it shows the reliability of the experimental conditions. A variation in the amount of myotoxin in the crotamine-positive venom was observed, but not less than 0.1 mg of crotamine per mg of venom. It was not possible to detect it in crotamine-negative venom even at high venom concentrations.

Keywords: Crotamine; Myotoxin; Rattlesnake; *Crotalus*; Venom

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Crotamine was first isolated from the South American rattlesnake *Crotalus durissus terrificus* venom by Gonçalves and Vieira (1950). Crotamine is a small basic 4.8 kDa myotoxin, isoelectric point 10.8. It is constituted by a single chain of 42 amino acid residues (Laure, 1975) containing three disulfide bonds (Beltran et al., 1990). It belongs to a family of small basic myotoxins found in the rattlesnake venoms, that are very similar to each other in amino acid sequence and also are structurally and antigenically related (Bieber and Nedelkov, 1997). Crotamine induces the paralysis and extension of hind legs and spontaneous and irregular contractions in the diaphragm of rats, mice and rabbits (Gonçalves, 1956). From this biological effect in individual venom samples, a geographical pattern of crotamine distribution was observed (Schenberg, 1959; Bicalho et al., 1989; Silveira et al., 1990).

Two methods have been described to estimate the crotamine concentration in the venom. Gonçalves and Arantes (1956) separated the components of the *C. durissus terrificus* venom by electrophoresis and quantified the crotamine using its distinct and strong affinity to aminoblack in relation to other venom toxins. Hampe and Belló (1997) estimated the amount of crotamine from the time required to observe the physiologic effect in mice after the intra-peritoneal injection of the venom. These methodologies require a large amount of venom and animals, and are not suitable for a large number of samples. An ELISA test has been established for carrying out a large number of tests for the quantification of crotamine in venom samples.

ELISA is the most frequently used method for identification and quantitation of venoms, due to its high sensitivity, specificity, simplicity and low cost (Theakston and Reid, 1979; Theakston, 1983). This method was used by Bober et al. (1988) to detect similar toxins to myotoxin *a*, in the venom from several rattlesnakes. In this work, the parallel-lines bioassay was used (Manclark et al., 1986).

Anti-crotamine serum was obtained from immunized female rabbits (White New Zealand), as described by Ownby et al. (1983), using crotamine purified using the Hidrazide Avidchrom Cartridge, according to the method described by Li et al. (1993), with some modifications.

The quantification test was standardized in Hemobag microtiter plates. The dilution of the venom and of the crotamine was done in phosphate-buffered saline (pH 7.4); 0.1 ml of the sample was placed in each well, and the plate was left at 4°C over night. Blocking with 0.2 ml of BSA 1%, first antibody (0.1 ml of anti-serum 1/32 000) and second antibody (0.1 ml of anti-rabbit IgG-peroxidase conjugate 1/3000), each application was done for 1 h at 37°C in humid conditions. Between each step, the plate was washed three times with PBS + 0.1% Tween 20. 0.1 ml of substrate solution of OPD/H_2O_2 was added for 20 min and the reaction terminated with 50 µl of 4 N H_2SO_4. Plates were read using a MultiScan Titertek filtered with a 492 nm filter.

Data for each test and the reference samples were plotted as the $\log_{10}$ ($A_{492\ nm} \times 1000$) (y-axis) vs. the $\log_{10}$ (1/dilution) (x-axis) and the straight line was

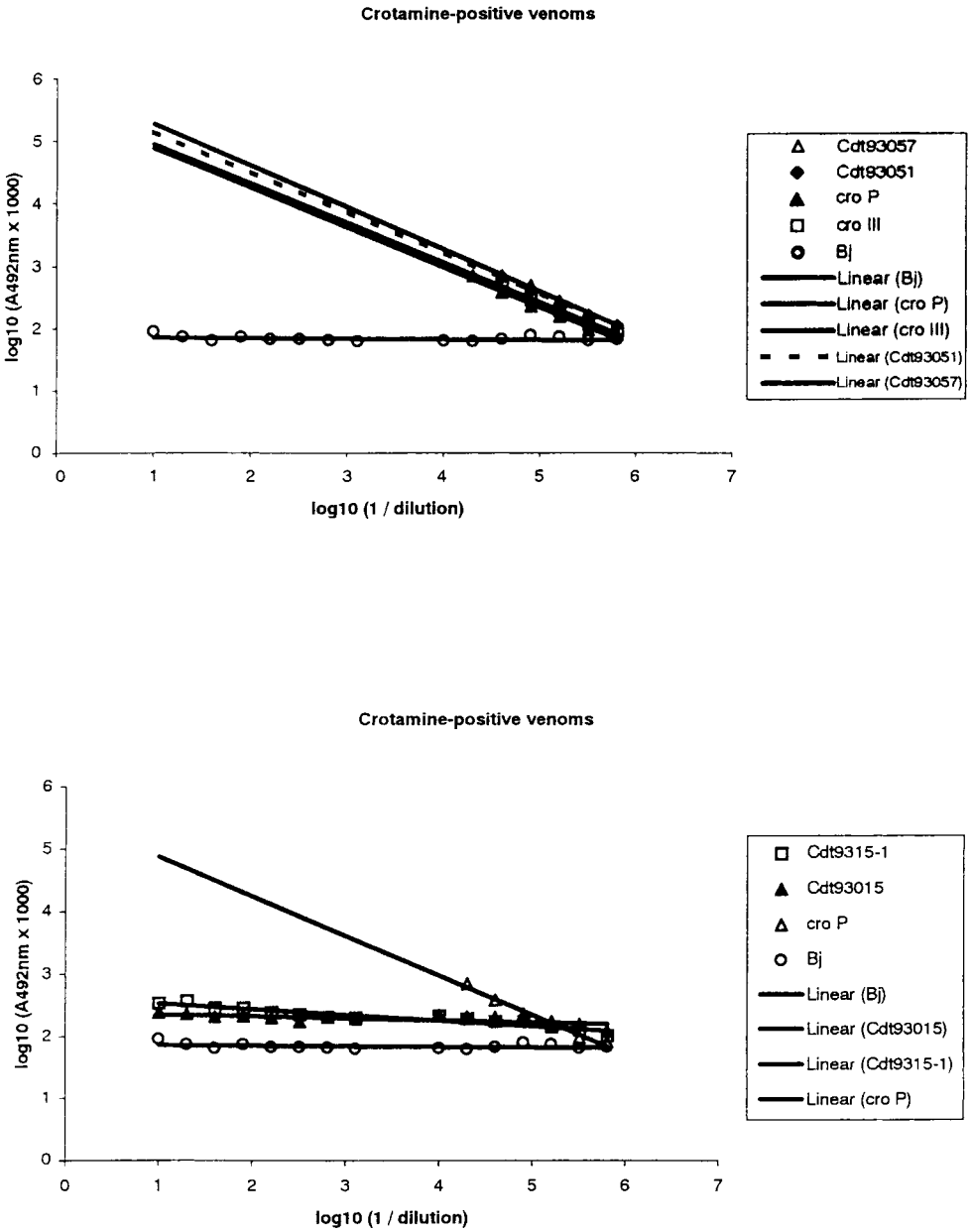

Fig. 1. ELISA plots of crotamine-positive and crotamine-negative venoms of *C. durissus terrificus*. Venom samples were lyophilized and diluted to 10 mg/ml in PBS. *Cro P* is the reference crotamine, quantity estimated by amino acid analysis and diluted to 1 mg/ml in PBS. *Bj* is the negative control, a *B. jararaca* venom. (a) *Cro III* is crotamine purified by Hidrazide Avidchrom Cartridge. Cdt 93051 and Cdt 93057 are venoms of rattlesnakes from Curitiba-PR. (b) Cdt 9315-1 is the venom of a rattlesnake from S.L. Paraitinga SP. Cdt 93015 is the venom of a rattlesnake from Itupeva-SP. The venoms were kindly provided by Dr. M.F.D. Furtado, from the Herpetology Department, Instituto Butantan. The *Cro P* was kindly provided by Dr. E.B. de Oliveira from the Biochemistry Laboratory in FMRP-USP.

traced. Fig. 1a shows two crotamine-positive venoms, and Fig. 1b exhibits two crotamine-negative venoms.

The crotamine concentration in venom was determined relative to the reference crotamine samples, and only the parallel lines to the reference crotamine were used. Thus, given one $A_{492\ nm}$, a line was drawn and the intersection point in straight lines gave the dilution value at the x-axis. Those values were used to quantify the crotamine in the venoms in the equation, where concentration of crotamine is known (1 mg/ml) and the values antilog dilution were obtained from Fig. 1 and used in the equation: crotamine concentration/crotamine antilog$_{10}$ dilution = crotamine concentration in venom/venom antilog$_{10}$ dilution.

Bothrops jararaca venom, used as negative control, showed the method to be specific because readings were similar to blank. Straight lines of crotamine-positive venoms were parallel to crotamine reference indicating that line displacement is related to different crotamine concentration (Fig. 1a). Low $A_{492\,nm}$ values observed in the crotamine-negative venom samples were not correlated to crotamine, once the straight line is not parallel to reference line. It indicated these readings could be consequence of cross-reaction with some antigens of rattlesnake venoms. The minimum quantity of purified crotamine detected was 0.6 ng, two times the blank value. Table 1 presents the crotamine concentration of *C. durissus terrificus* venoms.

This treatment of data was generally used for the quantification of antibodies but can be used with success to estimate the amount of crotamine or another antigen. The method described to quantify the crotamine is simple, specific and can be used in population studies. One major advantage is that no animals are required. The crotamine, in spite of its small molecular weight, was highly

Table 1
Qualitative and quantitative assay of crotamine in *C. durissus terrificus* venoms from Brazil

Proceeding[a]	Test in vivo[b]	Crotamine (mg of crotamine/mg of venom)[d]
Curitiba-PR	+	0.118
Curitiba-PR	+	0.151
Itatiba-SP	−	ND[e]
Itu-SP	−	ND
Itupeva-SP	−	ND
Martinópolis-SP	+[c]	0.135
Martinópolis-SP	+[c]	0.240
Ribeirão Preto-SP	−	ND
São Luis do Paraitinga-SP	−	ND
São Luis do Paraitinga-SP	−	ND
São Roque-SP	+	0.222

[a] City of origin of South American rattlesnakes, provided by Instituto Butantan.
[b] I.m. injection of 5 μg of venom/g Swiss Female mice and observation of paralysis of hind legs.
[d] Quantity of crotamine estimated by ELISA using the parallel-line bioassay.
[e] ND: not detected.
[c] Venoms tested by ELISA using 5 μg/well.

antigenic; this was demonstrated even using high serum dilution (1/32 000). We concluded that the absence of paralysis in extension in hind legs of mice is due to the absence of crotamine in venom.

In agreement with the observation made by Gonçalves (1956), our method of analysis also indicated a great variation in the amount of crotamine in the venom. It can not categorically be stated that crotamine is completely absent in the crotamine-negative samples, because the sensitivity limit is 0.6 ng/mg of venom. It is curious that no *C. durissus terrificus* venom with crotamine concentration of less than 0.1 mg/mg of venom was found. A plausible explanation would be the multigene hypotheses, by which these genes would be organized in a locus that could be functional or not. Functional alleles in homozygote could result in the maximum expression and consequently increase the amount of crotamine in the venom, while functional alleles in the heterozygote could represent the intermediary proportion and finally, both unfunctional alleles would produce no crotamine. These data reinforce the Mendelian character of crotamine suggested by Schenberg (1959).

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