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Single-step purification of crotapotin and crotactine from *Crotalus durissus terrificus* venom using preparative isoelectric focusing

A.S. Aguiar[1], A.R. Melgarejo[1], C.R. Alves[3] and S. Giovanni-De-Simone[2,3]

[1]Divisão de Animais Peçonhentos, Instituto Vital Brazil, 24230-340 Niterói, RJ, Brasil
[2]Departamento de Biologia Celular e Molecular, Universidade Federal Fluminense, 24001-970 Niterói, RJ, Brasil
[3]Laboratório de Microseqüenciamento de Proteínas, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, 21040-900 Rio de Janeiro, RJ, Brasil

Abstract

We describe the isolation of crotoxin, a presynaptic B-neurotoxin, as well as its subunits B (crotactine) and A (crotapotin) from lyophilized *Crotalus durissus terrificus* venom by a single-step preparative isoelectric focusing procedure. From 98 mg of dried venom protein 20.1 mg of crotactine and 13.1 mg of crotapotin were recovered in the first step of focalization and 4.2 mg in a second run. These values correspond to 35.7% of the total venom protein applied. Crotactine separated in the 9.3-7.0 pH range (tubes 1-6) and crotapotin in the 1.8-2.8 pH range (tubes 15-19) and both were homogeneous by SDS-PAGE and N-terminal amino acid analysis. Crotactine, a 12-kDa protein, presented hemolytic and phospholipase A_2 activity. Thus, using isoelectric focusing we simultaneously purified both toxins in high yields. This method can be used as an alternative for the purification and characterization of proteins from other snake venoms under conditions in which biological activity is retained.

Correspondence
S. Giovanni-De-Simone
Departamento de Bioquímica e Biologia Molecular
Instituto Oswaldo Cruz, FIOCRUZ
21040-900 Rio de Janeiro, RJ
Brasil
Fax: 55 (021) 590-3495
E-mail: dsimone@gene.dbbm.fiocruz.br

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- Isoelectrofocusing
- Presynaptic B-neurotoxin

Crotoxin is a presynaptic B-neurotoxin present in South American rattlesnake venoms (1). This toxin is a heterodimeric protein composed of noncovalently bound subunits: component B, or crotactine, a basic protein having phospholipase A_2 (PA_2) activity, and component A, or crotapotin, the acidic protein. Crotactine (B) presents moderate toxicity and crotapotin (A) is devoid of toxic or enzymatic activities. The addition of component A apparently increases the toxicity of component B (2-4). The dissociation of the subunits is reversible. It has been proposed that crotapotin functions as a chaperon molecule, preventing nonspecific binding of the phospholipase A_2 subunit of the toxin (5).

Although crotoxin was described in 1938 as the first rattlesnake venom protein to be isolated (6), its subunit composition was established only in 1978 (7) and the structure of the B-chain was only completed in 1990 (8,9). The toxin is present in variable concentrations in rattlesnake venom and accounts for nearly 80% of total protein of *Crotalus*

durissus collilineatus venom (1). Nevertheless its isolation as a homogeneous protein is tedious and time consuming and requires several chromatographic steps including gel filtration, ion-exchange chromatography and reverse-phase high performance liquid chromatography (HPLC) (8,9).

Since this toxin may be a useful tool in electrophysiological studies (10) and contribute to the understanding of the different biologial effects of the PA_2 enzyme subunit in different systems (11,12), we developed a simple procedure for the isolation of the A and B subunits by isoelectrofocusing of the lyophilized intact venom in a single step using a Rotofor® apparatus.

This method is simpler and more rapid than the multiple chromatographic steps described in the literature and is suitable for preparation of mg quantities of both subunits in the same run.

Lyophilized venom of *Crotalus durissus terrificus* (Lot 01/90-3) was obtained by "milking" snakes maintained in the serpentarium of the Vital Brazil Institute. Lyophilized venom (116 mg containing 93 mg of protein) was diluted to 20 ml with 10 mM Tris-HCl, pH 6.5, and dialyzed overnight against the same buffer solution at 4ºC. The insoluble material was removed by centrifugation (10,000 *g*, 30 min, 4ºC) and 200 µl of pH 3-10 ampholytes (Bio-Rad, Richmond, CA) was added to 20 ml of the supernatant solution. The final volume was made up to 45 ml with 10 mM Tris-HCl buffer providing a final concentration of ampholyte equal to 0.44% and the sample was focused in a Rotofor chamber (Bio-Rad, Richmond, CA) using 300 V for 1 h, 400 V for 1 h, and 500 V for 1 h, and a limit of 12 W. Twenty fractions were obtained after focusing, the pH of each was determined and the fractions were stored at 4ºC. Fractions 1-6 (pH 8.2 to 9.3) and 15-19 (pH 1.8 to 2.8) were pooled separately and 5.0 M NaCl was added to provide a final concentration of 1.0 M NaCl, necessary for the removal of ampholytes by dialysis using tubing with a 3,000 Mr cutoff, against 10 mM citrate buffer, pH 4.5, for 16 h at 4ºC, and stored frozen at -10ºC for further study. Protein concentration was determined by the method of Lowry et al. (13) using serum albumin as a standard.

Protein homogeneity was demonstrated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels in Laemmli buffers (14) under reducing conditions.

Figure 1a shows a Coomassie blue-stained SDS-PAGE gel of the *C. d. terrificus* venom proteins isolated by Rotofor-IEF electrophoresis. A single band of approximately 12 kDa was observed in fractions 1-6 and a 9-kDa band was observed in tubes 14-20. These fractions yielded 20.13 and 13.1 mg of protein, respectively, corresponding to 35.7% of total applied venom proteins. Band A (acidic protein or crotapotin) was distributed from pH 1.8 to pH 3.0 while the band B (basic protein or crotactine) from pH 7.0 to pH 9.3. The range of pH found for both proteins is within the range of pH (2.8-3.7 and 7.8-9.2) reported after IEF by others (5,7). The refractionation of the pooled tubes 7-9 permitted the recovery of more than 4.2 mg of pure 12-kDa protein, increasing the recovery of this protein to 24.33 mg. The spreading of the proteins during focusing may be attributed to different degrees of molecular glycosylation (15,16) or pI microheterogeneity due the presence of molecular isoforms as suggested for other venom proteins (17).

In order to identify and investigate the biological activity of the isolated proteins, aliquots from several tubes were analyzed for hemolytic activity (18). Tubes 1-7 contained hemolytic activity but no hydrolysis could be observed with the material from tubes 14-20 (data not shown), thus showing that the basic compound (crotactine) presented PA_2 activity and that the enzyme was still active after the isoelectrofocusing step. The identity of this basic 12-kDa protein was

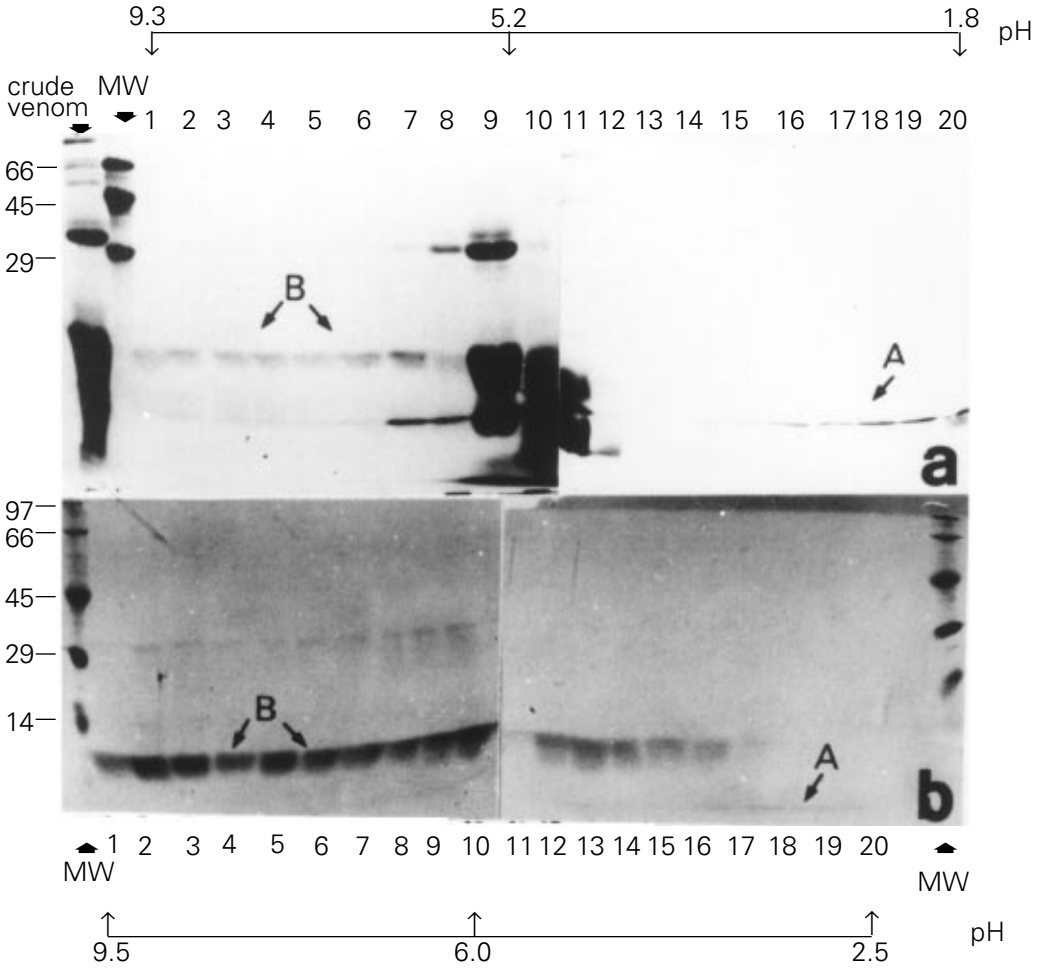

Figure 1 - SDS-polyacrylamide (15%) gel electrophoresis of the *Crotalus durissus terrificus* venom proteins fractionated in a Rotofor cell (fractions 1 to 20) without (panel a) and with (panel b) 1 M urea. In Figure 1a, 16-26 µg of protein was applied to each lane except intact venom to which 25 µg was applied. In Figure 1b, 20-28 µg of protein was applied to each lane. The gels were stained with Coomassie blue. On the left side of the figures the Mr values of the standard proteins [phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000) and lysozyme (14,500)] are shown and the pH values determined after isoelectrofocusing are indicated above and below the figure. In the top panel, fractions 1 to 6 were combined to provide crotactine and fractions 15 to 19 contained crotapotin. A, The acidic protein crotapotin; B, the basic protein crotactine.

established by partial amino N-terminal sequencing using the automatic Edman degradation method and a Shimadzu PSQ-1 microsequencer (initial amount loaded, 100 pmol; initial yield, 32%; repetitive yield, 92.3%). The sequence of 10 aminoterminal residues, HLLQFNKMIK, is identical to that described for PA_2 from *C. d. terrificus* (9).

Since the crotoxin chains are dissociated in the presence of urea, an attempt at fractionation was made using the same buffer containing 1 M urea under the conditions described above (Figure 1, panel b). In this case, the amount of recovered protein (bands A and B) was 1.2-1.8-fold higher. However, a spreading of band B (crotactine) over pI 9.3-4.2 with traces of the 29-kDa protein could be observed (Figure 1, panel b). The purification of both proteins in urea required more than two electrophoresis steps, making the method laborious and time consuming.

In comparison to methods commonly used for purification of crotactine (basic subunit) and crotapotin (acid subunit) proteins, the present method is simpler and requires less reagents and time. Only the Rotofor chamber equipment and ampholines are required. Using this method, which requires only dialysis, highly purified crotactine and crotapotin can be obtained in mg amounts from crude venom of *C. durissus terrificus* within about 4 h. It should be possible to scale up the method to fractionate about 1 g of protein in each run.

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