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Purification and Characterization of a Novel Factor of Crotoxin Inter-CRO (V-1), a New Phospholipase A_2 Isoform from *Crotalus durissus collilineatus* Snake Venom Using an In Vitro Neuromuscular Preparation

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Abstract: The fractionation of *Crotalus durissus collilineatus* whole venom through an HPLC chromatographic method enabled the purification of a new V-1 neurotoxin. Inter-CRO (V-1) presents similarity in its primary structure to crotoxin B (CB), suggesting another isoform of this toxin. The aim of this study was to compare V-1 to the crotoxin complex (CA/CB) and CB to elucidate aspects related to its functionality. The homogeneity of the purified protein was confirmed with a molecular mass of 1425.45 Da, further verified by mass spectrometry. The sequence of the protein showed high similarity to other viperid snake venom PLA_2 proteins. The results of this study report that V-1 is an uncharacterized novel toxin with different biological activities from CB. V-1 maintained catalytic activity but presented neurotoxic activity as observed by the 2.5-fold increase in twitch tension record compared to control values on isolated muscle cells.

Keywords: *Crotalus durissus collilineatus*; *Crotalus durissus terrificus*; neurotoxin; V-1; HPLC-RP

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1. Introduction

Crotoxin is a phospholipase A_2 (PLA_2) neurotoxin found in the rattlesnake *Crotalus durissus terrificus* [1–3] and *Crotalus durissus collilineatus* venom [4–8]. Crotoxin has exhibited anti-inflammatory [9,10], antimicrobial [11], immunomodulatory [12,13], antitumor [14,15], and analgesic actions [9,16]. Four neurotoxins have been described and well characterized in the rattlesnake *Crotalus durissus terrificus*: convulxin, crotoxin, crotamine, and gyroxin. Convulxin is a convulsion-inducing protein [17,18] that is a Lectin type C [19–21] of *Crotalus durissus collilineatus*. Gyroxin is a glycoprotein thrombin-like enzyme [22–27]. Crotamine is a neurotoxin that causes myonecrosis and skeletal muscle spastic paralysis of peripheral origin and neurotoxicity in mammals [28–33]. Crotamine is formed of two components: the basic toxic crotoxin B (CB) and the acidic non-toxic crotoxin A (CA, also known as crotapotin) [34–36]. Inter-CRO has been reported to elute

between crotamine and crotoxin chromatographic peaks, suggesting that it was not associated with the CA subunit in the *Crotalus durissus terrificus* venom [37]. However, since the initial characterization of Inter-CRO [37], no further isolation and characterization have been performed.

The similarities in the 3D structure of snake venom neurotoxins make them an attractive model to study the relationship between their structural differences and their potential beneficial physiological effects or toxicological intoxication interactions [34], which can lead to the development of novel pharmaceutical products. Furthermore, the improved analytical separation and characterization techniques as applied to proteins, including HPLC, mass spectrometry, and the use of recombinant DNA technology, raised additional questions about the homogeneity of several neurotoxins. For example, gel filtration chromatography methodology characterized crotamine as a single isoform, while recombinant DNA technology reported that crotamine is formed by several isoforms [38–41]. Another example is the recent purification of crotamine isoforms despite their high structural and pharmacological similarities to *Crotalus durissus terrificus* [35,36].

Therefore, the progression of more potent and specific analytical techniques can increase the knowledge of neurotoxins and their isoforms. The aims of this study were to first utilize a new HPLC method to characterize a novel neurotoxin, V-1, from the *Crotalus durissus collilineatus* and *Crotalus durissus terrificus* (yellow and white variation of rattlesnake) venom. The second aim was to evaluate the effect of V-1 on isolated muscle cells via myographic studies.

2. Materials and Methods

2.1. Venoms and Reagents

The *Crotalus durissus collilineatus* and *Crotalus durissus terrificus* rattlesnake yellow and white venom variety were provided by the Batatais serpentarium in Brazil. All the chemicals, solvents, and reagents were HPLC-grade and purchased from Merck, Sigma-Aldrich, and Bio Rad. Male Swiss white mice (26–32 g, N = 7 per experiment) were supplied by the State University of Campinas Animal Service, and the experiments were approved by the institutional Animal Ethics Committee.

2.2. Molecular Exclusion HPLC

Whole venom (20 mg) was dissolved in 0.25 M ammonium bicarbonate and subjected to a novel molecular exclusion HPLC step using a Protein-Pak SW 300 (0.8 × 30 cm) equilibrated with the same solution. Elution was carried out with 0.25 M ammonium bicarbonate solution at 0.1 mL/min. The wavelength of 280 nm was used during the run, and all the obtained fractions were immediately freeze-dried and stored at −20 °C. The chromatographic system used to perform the molecular exclusion chromatography was the APPS LC 650E HPLC. The detection of the sample was conducted on a 490 Programmable Multiwavelength UV/Visible Detector. and each fraction was collected.

2.3. Reverse Phase HPLC (RP-HPLC)

Crotoxin V-1 (20 mg) was dissolved in 250 µL of 0.1% (*v/v*) trifluoroacetic acid (solvent A) and centrifuged; the supernatant was placed on a µ-Bondapack C-18 column (0.78 cm × 30 cm). The elution of proteins was carried out at a flow rate of 2.0 mL/min using a linear gradient (0–100%) of 66.5% (*v/v*) acetonitrile in 0.1% (*v/v*) trifluoroacetic acid (solvent B). The wavelength of 280 nm was used during the elution and all the obtained fractions were immediately freeze-dried and stored at −20 °C.

2.4. Electrophoresis

The molecular mass of the proteins was estimated using the SDS-PAGE-Tricine, as previously described [42].

2.5. Sequencing Procedure

The purified protein (2 mg) was dissolved, incubated, and reduced with DTT as previously described [43,44]. Furthermore, the reduced carboxymethylated PLA_2 (RC-PLA_2) protein was digested with *Staphylococcus aureus* protease SV8 as previously described [43,44], and the digested products were fractionated by reverse-phase HPLC.

The primary structure of the novel factor from crotoxin V-1 was the result of a composite of the purified peptides using a Procise automatic sequencer, and the phenylthiohyodantoin (PTH) amino acids were identified by comparing their retention times to standards [44].

2.6. MALDI-TOF Mass Spectrometric (MS) Analysis

The molecular mass of the isolated fraction V-1 was analyzed as previously described [45,46] under the following conditions: accelerated voltage 25 kV, laser fixation at 2890 mJ/cm^2, delay 300 ns, and linear analysis mode [46,47].

2.7. Molecular Mass Determination

The protein's aliquot (1 µL) was analyzed using a QExactive Orbitrap from Thermo Scientific Mass Spectrometry with electrospray as a source of ionization (ESI/MS), voltage in the capillary of 3700 V, temperature of 320 °C, and 5 µL/min as the flow rate. The accumulation of the spectra, processing, and deconvolution was performed as previously described [48,49]. The data were compared against the database of snake's protein.

2.8. Mouse Phrenic Nerve-Diaphragm Preparation

The phrenic nerve-diaphragm was removed from anesthetized mice (chloral hydrate, 3 mg/kg), which were euthanized via exsanguination. The left diaphragm was removed as previously described [50]. The toxin preparations were suspended and maintained as previously described [51]. The toxin preparations were stabilized for at least 20 min before adding a single dose (20 µg/mL). The supramaximal stimuli were delivered from a GRASS S4 electronic stimulator, and the isometric muscle tension was recorded following the conditions previously described [51].

2.9. Statistical Analysis

The results, expressed as the mean ± S.E.M., were compared statistically using the software Origin 6.0. The Student's unpaired *t*-test was performed with a p-value < 0.05 being considered statistically significant.

3. Results and Discussion

Purification of *Crotalus durissus collilineatus* and *Crotalus durissus terrificus* crude venom using the analytical molecular exclusion HPLC method demonstrated that crotoxin was subfractionated into three peaks (III, IV, and V). Peak IV (crotapotin + PLA_2) and peak V, a new uncharacterized neurotoxin from *Crotalus durissus collilineatus* (Figure 1a), were found to be significant in the fraction. The SDS-PAGE-Tricine revealed one electrophoretic band, indicating that the obtained toxin exhibited high molecular homogeneity and a molecular mass of 10 kDa. Fraction V was repurified using a µ-Bondapack C 18 column (0.78 cm × 30 cm) in reverse phase HPLC (Figure 1b) as an additional verification step. Two peaks were identified and named V-1 and V-2, respectively. Peak V-1 appeared to be the neurotoxic fraction of a high-level molecular homogeneity (95%) and was immediately followed by a single peak, V-2, with a minimum retention time difference (Figure 1b). The molecular mass value of the protein was determined by ESI mass spectrometry (Figure 2), showing that the protein carries a different number of protons because of the different characteristic peaks. The composition of amino acids revealed the fraction V-1 *Crotalus durissus collilineatus* was highly polar, sharing a basic load but with a slightly acidic character with a high proline content and the presence of two half cysteines. The amino acid sequence of V-1 was further compared with other sequences of myotoxic PLA_2 previously determined and recorded (Figure 3).

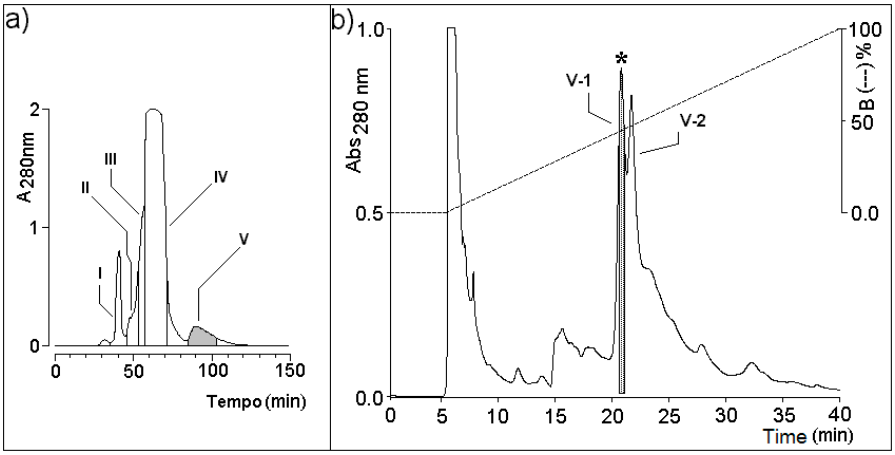

Figure 1. (**a**,**b**) Outcomes using HPLC method. * Denoted the peak of the V-1 fraction.

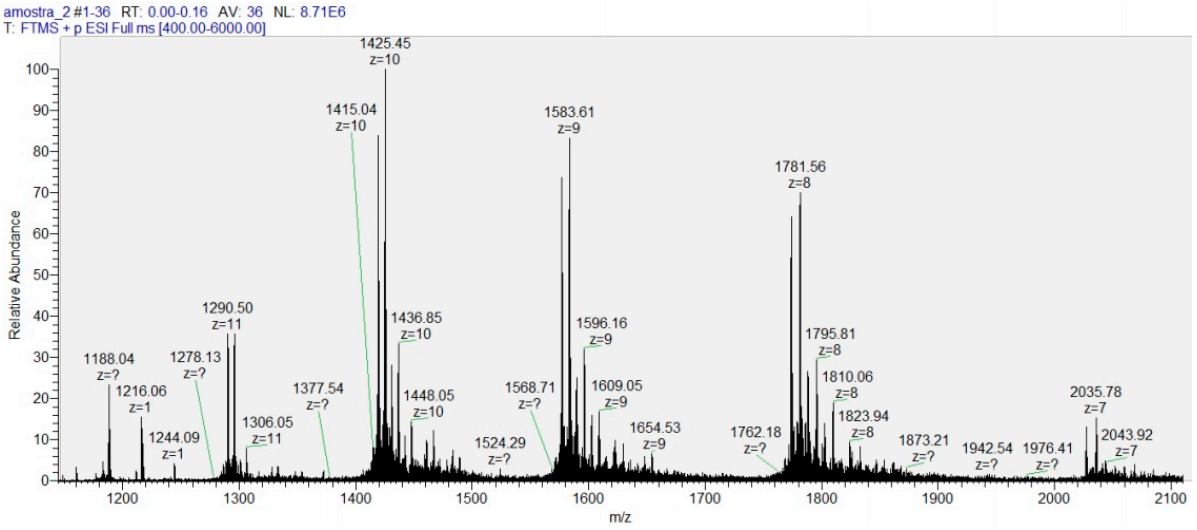

Figure 2. Chromatograms of Venom Extracts using ESI mass spectrometry.

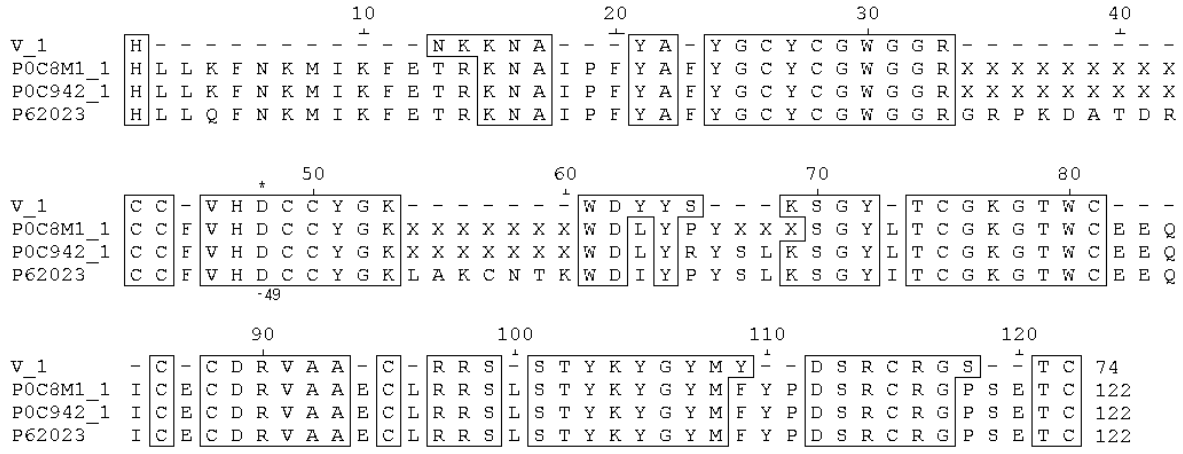

Figure 3. PLA_2 comparison with sequences of myotoxic PLA_2.

The sequence alignment of the deduced amino acid of the novel V-1 was compared with PLA_2 present in other venoms such as the ones coming from *Lachesis muta muta* (Accession Number P0C8M_1 and P0C942_1) [52] and *Crotalus scutulatus scutulatus* (Mojave rattlesnake) (Accession Number P62023). As shown in Figure 3, non-determined amino

acid residues were marked by (X), and boxed amino acid residues were identical. The highlighted amino acid residues belong to the PLA_2 conserved domain Ca^{2+}-binding loop, the β-wing region, and the catalytic site.

The effect of V-1 on isolated muscle cells was also investigated (Figure 4). Fraction V-1 exhibited a 2.5-fold increase in twitch tension record compared to control values, whereas the crotamine exhibited a 2.3-fold increase.

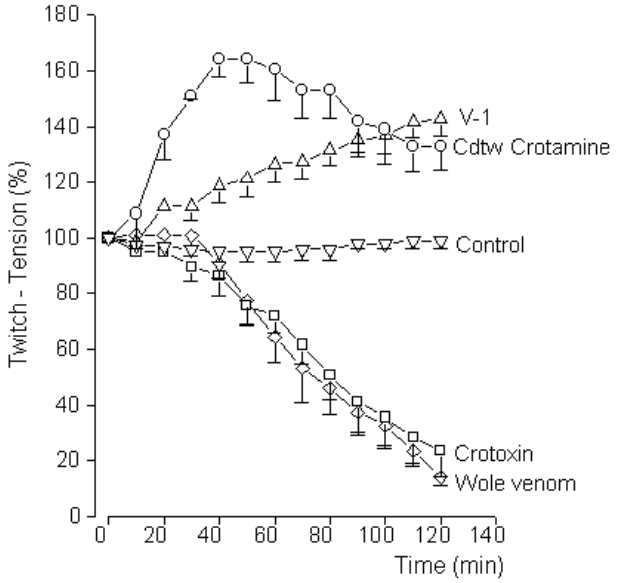

Figure 4. Effect of V-1 on isolated muscle cells.

The myographic analysis demonstrated crotoxin exhibited an irreversible blockage effect, and that V-1 increased in a linear fashion over time whereas Cdtw crotamine had a peak at 40 min then declined. The molecular exclusion HPLC analysis showed that V-1 is also found in other rattlesnake varieties, specifically the venom of *Crotalus durissus terrificus*, the yellow (Cdty) and white (Cdtw) rattlesnake varieties. The *Crotalus durissus terrificus* yellow and white venom analyses were performed using the same experimental methodological conditions described for the *Crotalus durissus collilineatus* sample analysis, and the results are summarized in Figure 5.

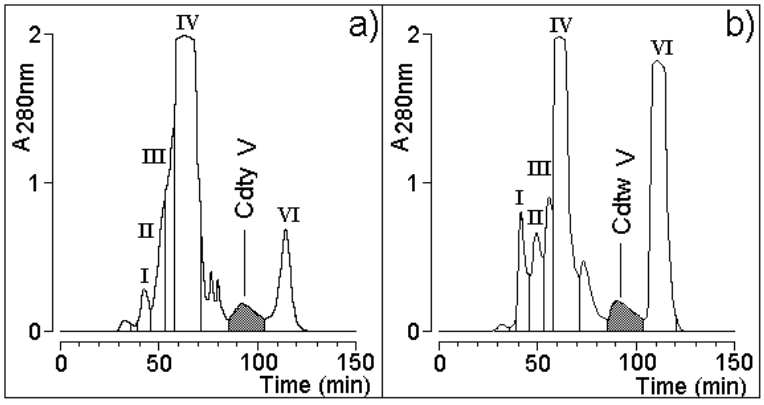

Figure 5. *Crotalus durissus terrificus*, eluents from the yellow (Cdty) Panel (**a**) and white (Cdtw) Panel (**b**) rattlesnake varieties.

The increase in ionic strength of the elution buffer from 0.1 M (Sephadex G75) to 0.25 M and the use of analytical molecular exclusion HPLC allowed the elution of the various fractions to be separated and identified from the native crotoxin. The use of ammonium bicarbonate solution allowed the most optimal recovery of these fractions

because ammonium bicarbonate is very quickly lyophilizable, and dialysis is not required. These findings suggested that the new crotoxin fraction described in this study from *Crotalus durissus collilineatus*, V-1, plays a vital role in this venom's action.

For years, crotoxin was considered to be composed only of PLA_2 and crotapotin subunits [53]. Nevertheless, improved techniques for the separation and characterization of proteins coupled with improvements in molecular biology analysis have raised scientific questions about the homogeneity of crotoxin [54]. The current study results reveal that native crotoxin is composed of at least three different neurotoxins that have not been previously described in the literature. The composition of crotoxin provides a plausible explanation for the multiple neurotoxic and biological effects observed in previous studies, including pre- and post-synaptic neurotoxicity, myotoxicity, platelet aggregation properties, and hemolytic activity [55–58].

The mass spectrometry [59] of the dissociated V-1 subunit revealed an MZ value of 14,150 (Figure 2) while free V-1 showed two peaks with MZ values of 14,254 (Figure 2), which are proposed to correspond to the two predominant V-1 isoforms. The presence of multiple PLA_2 isoforms from the *Crotalus* genus has been reported previously [59,60]. The characterized amino acid sequence of V-1 and its sequential identity with other PLA_2 enzymes from the snake is shown in Figure 2a. Because of the highly conserved residues in disulfides bridges and the presence in the catalytic site (D_{42} XCCXXH D_{49}) and Ca^{2+} binding site (X), V-1 could be classified as a PLA_2 biochemical and functional superfamily member, while CB and V-1 also demonstrated a PLA_2 group II [60].

The functional myographical analysis of V-1, with or without crotoxin A (CA), did not appear to block neuromuscular transmission, which is a common effect of the crotoxin complex. The efficiency of the crotoxin complex in producing neurotoxic effects has been reported to be linked with the ability of CA to locate crotoxin B (CB) to the terminal of the nerve [61,62], and V-1 does not appear to act at the nerve terminal. Chromatographic experiments revealed that at pH 3.5 (and in the range of pH 4.5 to 8.0), V-1 was obtained as a single molecular entity and not associated with CA. This finding was observed for the crotoxin complex, which was found in a stable complex form in the pH range between 3.5 at 8.0. This could be due to the similarity of V-1 with the two CB isoforms that were previously structurally characterized: CBa and CBb [63]. These results showed that native Crotoxin comprises four different neurotoxins that are not described in the literature. Despite the variation of all the venoms analyzed, the common distribution of this neurotoxin among the *Crotalus durissus terrificus* variants (yellow and white venom) indicates that this toxin plays a vital role in the envenoming effect caused by the bite of the venomous snake Crotalus.

4. Conclusions

Inter-CRO (V-1) is a protein from rattlesnake venom, with similarity in its primary structure to crotoxin B (CB) but with different biological activities to CB. Our results are consistent with CB not being able to form an active neurotoxic complex with crotoxin A as observed for CB. Furthermore, the common distribution of this neurotoxin among the *Crotalus durissus terrificus* variants (yellow and white venom) indicates that this toxin plays a vital role in the envenoming effect caused by toxins in the bite of the venomous snake Crotalus. Further research and characterization of this novel phospholipase A2 isoform are warranted.

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Informed Consent Statement: The animal study protocol was approved by the Institutional Animal Ethics Committee of Universidade Estadual de Campinas.

Data Availability Statement: The data presented in this study are available on request.

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Conflicts of Interest: The authors declare no conflict of interest.

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