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Purification and characterisation of a haemorrhagic fraction from the venom of the Uracoan rattlesnake *Crotalus vegrandis*

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Abstract

Uracoan rattlesnake (*Crotalus vegrandis*) venom was subjected to chromatographic, electrophoretic, biochemical and in vivo haemorrhagic analysis. A haemorrhagic toxin (Uracoina-1) active on skin at the site of inoculation in mice was purified by Mono Q2 anion-exchange chromatography and size exclusion (SE) high-performance liquid chromatography. The purified preparation was a protein of M_r 58 000 as revealed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis under denatured conditions and with silver staining. The use of EDTA, EGTA and 1,10-phenanthroline inhibited haemorrhagic and proteolytic activities. Inhibitors of serine proteinases such as PMSF and TCLK had no effect on the haemorrhagic fraction. Uracoina-1 hydrolyses casein, hide powder azure and fibrinogen have an optimal pH of 8.2. It rapidly digests the Aα-chain of fibrinogen. Thermal denaturation of Uracoina-1 after exposure at 60°C for 15 min led to inactivation of the haemorrhagic activity. In addition, Uracoina-1 is myotoxic, lacking haemolytic, defibrinating and lethal effects. The N-terminal amino acid sequence (20 residues) was determined.

Keywords: Crotalidae; Haemorrhagic fraction; Metalloprotease; Venom; *Crotalus vegrandis*

1. Introduction

Venoms of the same snake genus from different geographical regions often exhibit distinct pathological, pharmacological and antigenic characteristics [1]. The haemorrhagic manifestations of local tissue damage are among the best-documented effects of North American rattlesnake venom. However, haemorrhage had not been previously described with South American rattlesnakes [2]. Purification and characterisation of a specific haemorrhagic toxin in these rattlesnakes has not been previously published. Thus this is probably the first evidence of a haemorrhagic effect of this venom. Uracoan rattlesnake (*Crotalus vegrandis*) is known to be responsible for accidents in the eastern part of Venezuela and its venom caused severe pathophysiologic changes in the victims [3–5]. Although respiratory paralysis secondary to neurotoxic activities is the prevalent cause of deaths, local and systemic haemorrhages are the major signs of Uracoan rattlesnake envenomation [6]. This work shows the presence of haemorrhagic factors in the venom of these rattlesnakes. We have reported the purification and characterisation of a haemorrhagic fraction from *C. vegrandis* venom with an increased specific activity over crude venom.

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Here, we report the purification and characterisation of a haemorrhagic fraction from *C. vegrandis* venom having activity to skin at the site of injection in mice.

2. Materials and methods

Crude venom from *C. vegrandis* was milked from specimens captured near Uracoa, Distrito Sotillo, Monagas state, Venezuela and maintained in the Serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela. Venom was collected over ice, frozen, and lyophilised and maintained at −80°C. Outbreed male NIH Swiss albino mice weighing 20–22 g were used for all studies and obtained from the central animal house of this institute. MonoQ2 column, molecular mass protein standards were obtained from Bio-Rad. The Protein Pak 125 was from Waters and casein from Merck. Hide powder azure was purchased from Sigma Chemicals. All other chemicals were reagents grade or equivalent. Column operations were performed at 4°C with a Biologic HR from Bio-Rad.

2.1. Determination of protein concentration

The protein determination method followed the method of Lowry et al. [7] or by absorbance at 280 nm, standardised with bovine serum albumin.

2.2. Purification of haemorrhaging factors

Crude venom (20 mg by protein estimation) was diluted to 1.0 ml with 50 mM Tris–HCl buffer (pH 7.0) and exposed to a MonoQ2 column chromatography pre-equilibrated with the same buffer at a flow rate of 1.0 ml/min a 4°C. Venom proteins were eluted with a gradient of 0–1 M NaCl dissolved in 50 mM Tris–HCl buffer, pH 9. Fraction size was 0.5 ml. Elution of protein was monitored at 280 nm. The eluting peak tops were tested for haemorrhagic activity. The peak (P6) showing haemorrhagic activity was dialysed against water at 4°C and lyophilised. The P6 was further purified by size exclusion using a Waters Protein Pak 125 column (0.75×30 cm) equilibrated with 50 mM Tris–HCl buffer (pH 8.2) containing 125 mM NaCl. P6 was separated into major peak followed by peaks of minor intensity. The major peak had been found to possess haemorrhagic activity.

2.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out conforming to the Laemmli method [8], using 12.5% gels under reducing conditions. Molecular mass markers (Bio-Rad) were run in parallel and gels were stained with Coomassie blue R-250 and Silver stain [9]. The molecular mass was determined by Multi-Analyst TM/PC version 1.1 software (Bio-Rad).

2.4. Determination of lethality

The lethality was determined by the Reed and Muench method [10]. Five mice per dose weighing 20–22 g were injected i.v. with 200 μl of serially diluted haemorrhagic fraction from 2 to 200 μg. Deaths were recorded during a 48-h period. Mice were observed up to 48 h after injection.

2.5. Fibrinogenolytic activity

Fibrinogenolytic activity was measured as described [11]. Specific cleavage of fibrinogen by the venom fraction (Uracoina-1) was shown by electrophoresis on 12.5% polyacrylamide gels. Fifty μl of human fibrinogen (Sigma) at a concentration of 5 mg/ml in 0.1 M Tris–HCl buffer (pH 8.0) was incubated with 9 μg (50 μl) of Uracoina-1. At different time intervals, aliquots of 20 μl were withdrawn from the digestion mixture. The samples were denatured and reduced by boiling for 5 min with 20 μl of denaturing solution before running on SDS–polyacrylamide gel. The gels were stained with a silver stain [9].

2.6. Defibrinating activity

The method used by Gené et al. [12] was followed. Four mice (20–22 g) were injected i.v. in the tail with 150 μg of the haemorrhagic fraction, in a volume of 200 μl of phosphate-buffered saline (PBS). Control mice were injected i.v. with 200 μl of PBS. After 1 h the animals were anaesthetised with ether and bled

by cardiac puncture. Blood was placed in tubes and kept at 22–25°C until clotting occurred.

2.7. Fibrinolytic activity

The Astrup and Müllertz method [13] modified by Franceschi [14] was followed. Two hundred μl of human thrombin (100 U/ml) was dispensed in Petri dishes. Then fibrinogen 0.2% solution was added and incubated until the formation of a clot. Different concentrations of Uracoina-1 or crude venom were applied to the clot and incubated at 37°C for 24 h. After that the diameter of the fibrinolytic halos was determined. The quantity of Uracoina-1 and crude venom that induced a 10-mm diameter fibrinolytic halo was determined.

2.8. Determination of haemorrhagic activity

Haemorrhagic activity was determined by the Kondo et al. method [15] modified by Gutierrez et al. [16]. Crude venom or purified toxins were tested. One hundred μl of Uracoina-1 or crude venom containing 5–50 μg were injected intradermal into the abdominal skin of four male NIH Swiss albino mice. The mice were killed after 2 h, and the inner surface was observed for haemorrhage. The minimum haemorrhagic dose (MHD) was defined as the amount of enzyme that induced a haemorrhagic area of 10 mm diameter.

2.9. Haemorrhagic activity inhibitors

Inhibition of haemorrhagic activity was measured by incubating Uracoina-1 for 30 min at 37°C in 50 mM Tris–HCl buffer (pH 8.2) containing the following inhibitors: ethylenediaminetetraacetic acid (EDTA, 2 mM), 1,10-phenanthroline (2 mM), phenylmethylsulphonylfluoride (PMSF, 2 mM), tosyl-L-lysine chloromethyl ketone (TCLK, 2 mM). Aliquots of 100 μl containing 10 MDH (47 μg) of Uracoina-1 were injected intradermally into the abdominal skin of four male NIH Swiss albino mice. Haemorrhagic activity was determined as described above.

2.10. Effect of temperature

Several samples of Uracoina-1 (4.7 μg in 0.1 ml) and crude venom (9.3 μg in 0.1 ml) in 50 mM Tris–HCl buffer (pH 8.2) solution were maintained for 15 min at 30°C, 40°C, 50°C, 60°C and 70°C. The samples were cooled in an ice bath and the haemorrhagic activity was determined as described above.

2.11. Effect of pH

Uracoina-1 was dissolved in 50 mM Tris–HCl (pH 7–11) and incubated for 2 h at room temperature, and then the pH of the solutions was neutralised.

2.12. Assay of proteolytic activity

The procedure of Lomonte and Gutierrez [17] was followed. Uracoina-1 (5–40 μg) and crude venom (10–80 μg) were incubated with 1 ml of the casein substrate (1% in 0.1 M Tris–HCl, pH 8.0) for 20 min at 35°C. The reaction was stopped by adding 3.0 ml of 5% trichloroacetic acid. After 30 min, tubes were centrifuged at $3000 \times g$ and the absorbance of the supernatants at 280 nm was recorded. Control tubes containing substrates without Uracoina-1 were included, and the absorbances of these samples were subtracted from the rest of the experimental values. Proteolytic activity was expressed in units/mg protein.

Proteolytic activity was also studied with hide power azure. A modified method of Rinderknech et al. [18] was used. Hydrolysis of hide powder azure was determined by incubating different amounts of Uracoina-1, dissolved in 0.01 M Tris–HCl buffer (pH 8.0), with 8 mg of hide power azure for 1 h at 37°C and agitated at 5-min intervals. After incubation, each vial was centrifuged at $4000 \times g$ for 5 min. The supernatant was transferred into a different vial and the absorbance measured at 595 nm. The specific activity was expressed in U/mg protein, calculated as follows: change in absorbance in 1 h divided by mg of protein used.

The temperature dependency of proteolytic activity was studied by incubating aliquots of the enzyme for 15 min at 30°C, 40°C, 50°C, 60°C or 70°C.

2.13. Effect of inhibitors on proteolytic activity

Inhibition of proteolytic activity incubating Ura-

coina-1 for 30 min at 22–25°C with ethylenediaminetetraacetic acid (EDTA, 2 mM) was measured. Control samples had the same amount of enzyme without inhibitor. After incubation, proteolytic activity was tested on casein as described.

2.14. Assay for creatine kinase activity

Myotoxicity was tested measuring the plasma creatine kinase (CK) levels in a group of six mice (20–22 g) that were injected intramuscularly with 75 μg of the enzyme in a volume of 0.1 ml in 0.9% saline. Control experiment using uninjected mice and saline-injected mice for each injection group were performed. Blood was removed by cardiac puncture 3 h after injection and plasma CK level using a Creatine kinase kit (Sigma) was determined.

2.15. Determination of amino acid sequence

The amino acid sequence using an automated Edman degradation in an Applied Biosystem Procise 494 protein Sequencer (Perkin Elmer Corp.) was determined.

2.16. Esterase activity

The method of Tu et al. [19] to measure the esterase activity was used. Several samples of Uracoina-1 (2–43 μg) or crude venom of *C. vegrandis* (0.2 mg in 0.1 ml) on *N*-benzoyl-L-arginine ethyl ester (BAEE) at 25°C with 2.9 ml of 0.00025 M BAEE in 0.0667 M Phosphate buffer (pH 7.0) were tested. The change in absorbance at 253 nm was determined.

2.17. Indirect haemolytic assay

Indirect haemolytic assay was carried out according to the method of Franceschi et al. [14].

3. Results

3.1. Purification of the haemorrhagic fraction

As reported in Section 2, the crude venom of *C. vegrandis* was initially fractionated in a column Mono Q2 of anionic exchange and six peaks were obtained (Fig. 1). Peak 6 (P6) produced haemor-

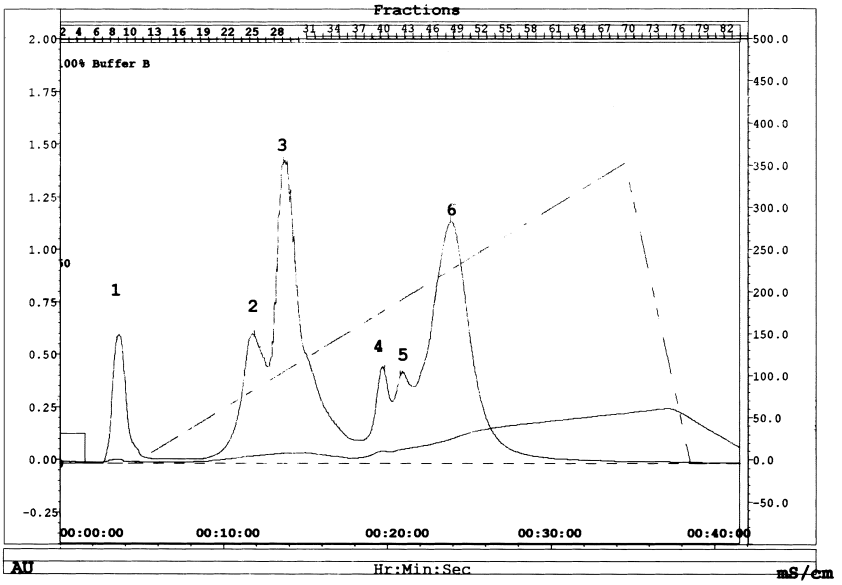

Fig. 1. Fractionation of *C. vegrandis* venom by ion-exchange chromatography on a MonoQ2 column. Venom (20 mg) was dissolved in 1.0 ml of 50 mM Tris–HCl buffer (pH 7.0) and loaded on a column equilibrated with the same buffer. After elution of unbound material, a linear NaCl gradient from 0 to 1 M (pH 9) was developed. Fractions of 0.5 ml were collected. Peak 6 shows caseinolytic and haemorrhagic activities.

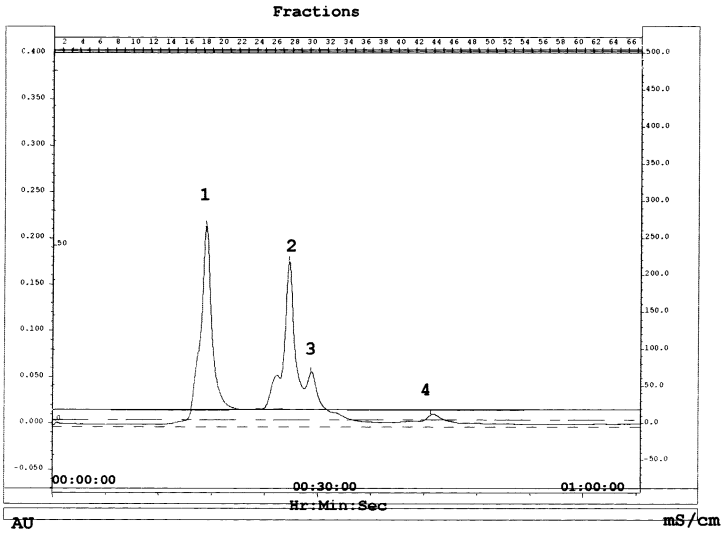

Fig. 2. Fractionation of peak 6 from a MonoQ2 column by size exclusion on a Protein Pak 125 column. The first peak had caseinolytic and haemorrhagic activities and was homogeneous by SDS–PAGE. This protein was named Uracoina-1.

rhagic activity when it was injected in the mouse skin. The yield in relation with the crude venom was 5–6 mg. When peak 6 was SDS–PAGE analysed a M_r 58 000 band was observed next to four bands of less intensity and smaller molecular masses. P6 was then subjected to molecular exclusion purification in a Protein Pak 125 column. From this fractionation four peaks were obtained, one prominent followed by three smaller peaks (Fig. 2). This main peak presented haemorrhagic activity and its yield was 0.5–1 mg. The SDS–PAGE analysis showed a single band M_r 58 000 that was named Uracoina-1. The haemorrhagic specific activity of crude venom, P6 and Uracoina-1 were 83.3, 125 and 212.8 U/mg, respectively.

3.2. Molecular mass

Uracoina-1 migrates as a homogeneous single band (M_r 58 000) in SDS–PAGE under reducing conditions (Fig. 3).

3.3. Lethality

Uracoina-1 was not lethal to mice even when dose of 7.5 μg/g was administered i.v., while crude venom was lethal to a 0.26564 mg/kg dose.

3.4. Haemorrhagic activity

Uracoina-1 had haemorrhagic activity when tested by intradermal injections in mice. The minimum haemorrhagic dose was 4.7 μg, indicating that Uracoina-1 is more active than crude venom (MHD = 12 μg).

3.5. Effect of enzyme inhibitors

The proteolytic and haemorrhagic activities of

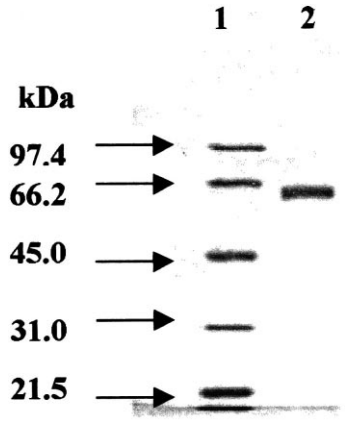

Fig. 3. SDS–PAGE of Uracoina-1 run under reducing conditions in 12.5% gels. (1) Molecular masses. (2) Uracoina-1 (10 μg).

Table 1
Effects of haemorrhagic activity inhibitors on Uracoina-1

Inhibitor	Concentration (mM)	Relative activity (%)
Uracoina-1 (control)	2	100
EDTA	2	0
1,10-Phenanthroline	2	0
PMSF	2	100 ± 2
TLCK	2	100 ± 2

The haemorrhagic fraction of Uracoina-1 (47 μg corresponds to 10 MDH in 0.1 ml) was treated with each reagent at the indicated concentration in saline at 37°C for 30 min. The reaction mixtures were used for haemorrhagic test according to Section 2.

Uracoina-1 were completely abolished by 2 mM EDTA and 2 mM 1,10-phenanthroline. TCLK or PMSF did not inhibit either activity (Table 1).

3.6. pH and heat stability of haemorrhagic activity

Uracoina-1 was stable at temperature ranges from 30°C to 40°C (Fig. 4A). The haemorrhagic activity was completely lost at 60°C. The optimal pH for Uracoina-1 haemorrhagic activity was between pH 7 and 9 (Fig. 4B).

3.7. Assay of proteolytic activity

Uracoina-1 degraded casein, hide power azure and fibrinogen. When tested on casein, the activity was 156 U/mg protein, as compared to 257 U/mg with crude venom and 207 U/mg with *Bothrops lanceolatus* venom, whereas when tested on hide power azure the activity was 6.46 U/mg protein. The optimal pH for Uracoina-1 caseinolytic activity was between pH 7 and 9. Incubation of Uracoina-1 over pH 9 resulted in a decrease of proteolytic activity. Enzymatic activity of Uracoina-1 was stable in the temperature range from 20°C to 40°C gradually losing the activity over 50°C and totally abolished at 70°C.

The haemorrhagic fraction (Uracoina-1) was incubated for 1 min with fibrinogen. The SDS–PAGE revealed a smaller molecular mass component that coincided with the disappearance of the Aα chain. In Fig. 5 it is observed that the haemorrhagic fraction (Uracoina-1) band was found in the place of the Aα chain. In relation to the Bβ chain, at 120 min degradation products of smaller molecular mass were also observed. The γ chain was not affected (Fig. 5).

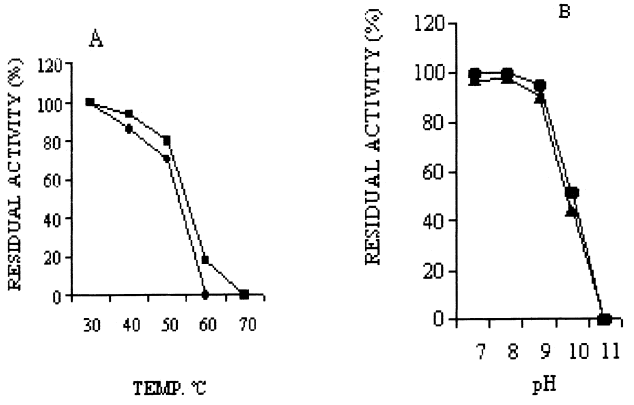

Fig. 4. Effect of temperature and pH on the haemorrhagic activity of Uracoina-1. (A) Temperature: ■, Uracoina-1; ●, crude venom. (B) pH: ■, Uracoina-1; ▲, crude venom.

3.8. Defibrination activity

Uracoina-1 did not induce a defibrination effect; the whole blood clotting times in samples collected from mice injected i.v. with haemorrhagic fraction

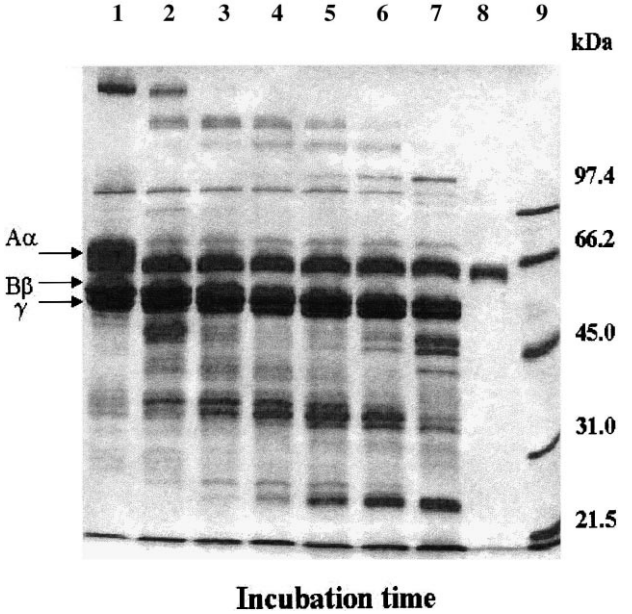

Fig. 5. SDS–PAGE gel electrophoresis of reduced human fibrinogen after digestion by Uracoina-1. Fibrinogen consists of three polypeptide chains Aα, Bβ and γ. Lane 1, fibrinogen without enzyme. Other lanes (from 2 to 7) correspond to fibrinogen incubated with Uracoina-1 for 1, 5, 10, 30, 60 and 120 min. Lane 8, Uracoina-1 (0.7 μg/5 μl). Lane 9 contains molecular mass markers.

did not differ from clotting times of samples from mice injected with saline solution. The blood of mice i.v.-injected with 150 μg of Uracoina-1 coagulated at 300 ± 5 s, and with saline solution it coagulated at 240 ± 5 s. A minimal defribination dose (MDD) of 1 μg crude venom kept the blood incoagulable for 24 h.

3.9. Creatine kinase activity

CK activity in mouse blood was assayed. The haemorrhagic fraction induced myotoxicity, since i.m. injection of a dose of 7.5 μg/g in mice was followed by a significant increment in plasma CK levels, which reached values of 313 U/l compared to saline injection (control) at a dose of 80.3 U/l.

3.10. N-Terminal sequence of Uracoina-1

Automated Edman degradation determined partial N-terminal amino acid sequence of Uracoina-1 is as follows: H-Q-K-Y-N-P-F-R-F-V-E-L-V-L-V-V-D-K-A-M.

3.11. Other assays

Uracoina-1 neither presented esterase activity on BAEE nor haemolytic or fibrinolytic activities, while crude venom had a 352.5 U/mg specific esterase activity.

4. Discussion

The venom of Uracoan rattlesnake (*C. vegrandis*) has proteolytic, haemorrhagic and esterasic activities [20,21]. Crotoxin, one of these main venom components, has also been studied [22]. Haemorrhage is one of the important effects of this snake envenomation since South American rattlesnakes are not often haemorrhagic. Previously, Amaral et al. [23] demonstrated a fibrinolytic activity of *Crotalus durissus terrificus* venom in an envenomed patient.

A haemorrhagic agent, Uracoina-1, has now been extracted to a homogeneous form from Uracoan rattlesnake venom by a combination of ion exchange chromatography (MonoQ2 column) and size exclusion (Waters Protein Pak 125 column). Uracoina-1 was eluted from an anionic exchange column at pH 8.2 with a high salt gradient and therefore the p*I* of the protein must be less than 8, indicating an acidic protein. The purification procedure had 0.5–1% yield.

Uracoina-1 showed strong haemorrhagic and proteolytic activities. As more than one sort of activity was found to be related with Uracoan rattlesnake, homogeneity of the sample is an important enquiry. Uracoina-1 has been demonstrated by silver staining to be a single 58 000 band in SDS–PAGE. Uracoina-1 protein was eluted from a Water Protein Pak 125 column with a volume (3 ml) near the void volume. The protein ran like a simple band in presence or absence of 2-β-mercaptoethanol, indicating that it is a simple polypeptidic chain. That the activities related with Uracoan rattlesnake venom are not from a contaminant could also be estimated from the high specific activity of its role. It is probably not possible for an imperceptible contaminant to show such a high degree of specific activity.

Uracoina-1 presented proteolytic activity on casein and hide powder azure. Its activity on casein was less than crude and *B. lanceolatus* venoms, leading us to believe that there is more than one haemorrhagic factor in *C. vegrandis* crude venom. It was higher than *Crotalus durissus durissus* and *C. durissus terrificus* crude venoms [24].

Treatment of this haemorrhagic factor with 1,10-phenanthroline and EDTA caused inhibition of haemorrhagic activity. Its sensitivity to these chelating metal agents confirmed its metalloprotease character. Several authors [25,26] have proposed that these venom snake haemorrhagic toxins can be classified into three main classes according to their molecular mass. Uracoina-1 is located in the medium size toxins with molecular mass of 30–60 kDa. Its enzymatic activity has an optimal alkaline pH and it is thermolabile for both proteolytic and haemorrhagic activities. Ownby et al. [27], studying 28 different snake venoms for the presence of heat-stable haemorrhagic toxins, found that certain snake venoms maintain some haemorrhagic activities after exposition to exceeding heat (100°C, 5 min), suggesting that some haemorrhagic toxins may be relatively heat-stable and retained substantial activity.

To determine if the haemorrhagic toxin was able to interfere with the coagulation system, its proteo-

lytic effect on fibrinogen and fibrin was assayed. Regarding fibrinogen, the proteolytic Uracoina-1 activity was mainly on Aα chain which it quickly digested. Uracoina-1 did not coagulate fibrinogen, indicating that it was different from the thrombin; this makes sense because the fibrin clot is formed for the action of the thrombin on the fibrinogen which removes fibrinopeptides A and B from the fibrinogen Aα and Bβ chains [28].

Most of the fibrinogenolytic enzymes are metalloproteinases and fibrinolytic with specificity preferentially directed to the fibrinogen Aα chain and with low activity on the Bβ chain. Serine proteinases with fibrinogenolytic activity is directed preferentially to the fibrinogen Bβ chain and has low activity on Aα chain. Several serine proteinases have both fibrinogenolytic and fibrinolytic activity, but others only have fibrinolytic activity. The venom defibrinating activity is a consequence of its clotting components, particularly of thrombin-like enzymes.

According to Kamiguti [29], South American rattlesnake venoms produce blood incoagulability due to fibrinogen consumption. Thrombin-like enzymes in this venom probably cause this. The crude venom of *C. vegrandis* is anticoagulant in vivo. Uracoina-1 showed neither defibrinant activity nor proteolytic activity on the fibrin.

Uracoina-1 did not hydrolyse synthetic substrata TAME or BAEE although crude venom did. These findings indicated that Uracoina-1 is different from trypsin and it does not have arginine esters activity. Prasad et al. [30] proposed that the venom esterase activity (BAEE) could be used as an indicator to determine the presence of factor V activator enzymes. The clotting activity of *Bothrops jararaca* venom has been associated with esterase activity (BAEE) but not with caseinolytic activity. The caseinolytic activity of venoms is attributed to the factor X-activating enzymes [30].

Uracoina-1 have a MHD of 4.7 μg. It was not lethal when 7.5 μg/g of mice were injected i.v. into mice. When the injection was i.p. rupture of the peritoneum was observed. When the injection was i.m. an intense haemorrhage was observed. To quantify the CK activity in plasma the myotoxic activity was determined. Their values were smaller than those obtained with crude venom, where they were dramatically increased.

Creatinase activity was low for *C. durissus durissus* and high for *C. durissus terrificus* [24]. To determine if Uracoina-1 presented phospholipase activity the indirect haemolytic method was used, and Uracoina-1 did not present this activity.

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