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# DETOXIFICATION OF THE CROTOXIN COMPLEX BY GAMMA RADIATION

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1. Crotoxin, the major neurotoxin of the South American rattlesnake venom (2 mg protein/ml in 0.85% NaCl) was irradiated with a Co-60 gamma source at a dose rate of 1100 Gy/h and at doses of 250, 500, 1000, 1500 and 2000 Gy.

2. Irradiated crotoxin was analyzed for free SH-groups, protein concentration, electrophoretic profile (SDS-PAGE), 50% lethal dose ($LD_{50}$) in mice, and antigenicity against crotalic antiserum by diffusion immunoassay.

3. Irradiation led to the formation of protein aggregates and solubility was reduced at doses of 1000 Gy or higher.

4. The $LD_{50}$ increased about 2-fold for 1000 Gy and 3.5-fold for 1500 Gy. However, the antigenic response was not changed as judged by the capacity of irradiated protein receiving up to 1000 Gy to react with anti-*Crotalus durissus terrificus* venom horse serum.

5. The dose of 1000 Gy cleaved 0.95 disulfide bridges/mol and 1500 Gy cleaved 1.42 bridges/mol, indicating the importance of disulfide bond integrity for toxicity.

**Key words:** crotoxin, detoxification, snake venom, antigenicity, SH groups, gamma radiation.

## Introduction

Crotoxin, the major toxic component of *Crotalus durissus terrificus* venom, was first isolated by Slotta and Fraenkel-Conrat (1938). It is a heterodimeric protein composed of a basic subunit which displays weak toxicity but carries a high enzymatic activity and an acidic subunit which is devoid of both enzymatic and toxic activities. When the subunits are associated the complex presents toxicity about ten times higher than the isolated basic subunit and also shows neurotoxic activity (Hendon and Fraenkel-Conrat, 1971; Rubsamen et al., 1971). The enzymatic activity of the basic subunit, which presents both a hemolytic property and non-specific affinity for membranes, appears to be masked by the acidic component which serves as a "chaperone" to enhance the specificity of the basic subunit by guiding it to a specific binding site (Jeng et al., 1978; Hendon and Tu, 1979; Radvanyi et al., 1985). Some investigations have suggested that conformational changes occur when the

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subunits are associated, thus creating new structural regions in the molecule which contribute to the neurotoxic action of crotoxin (Hanley, 1979; Rogero, 1979).

The basic component is a single polypeptide chain of 122 amino acid residues and contains seven intramolecular disulfide bridges (Fraenkel-Conrat et al., 1980; Aird et al., 1986), while the acidic component consists of three polypeptide chains held together by seven disulfide bridges. The amino acid sequences of the two subunits are homologous to those of various segments of snake venom phospholipases (Aird et al., 1985). Recent investigations have demonstrated the existence of about ten isoforms of crotoxin, isolated from the venom when this is collected from a single specimen of *Crotalus durissus terrificus* (Faure and Bon, 1988).

Ionizing radiation can change the biological and antigenic properties of a protein through alterations in the molecular structure. The noxious action of ionizing radiation on biological macromolecules is more effective in aqueous systems than in dry samples due to the presence of free radicals and highly reactive substances formed by water radiolysis (Butler et al., 1984). The effects of radiation on proteins have been studied over the last two decades in order to determine the structural alterations provoked by radiation as well as the consequent changes in biological and biochemical properties (Dertinger and Jung, 1970; Skalka and Antoni, 1970; Marciani and Tolbert, 1974; Nayar and Srinivasan, 1975; Lynn and Raoult, 1976; Kempner and Schlegel, 1979; Duda, 1981). Furthermore, investigations of the effects of radiation on snake venoms have demonstrated that radiation can have biomedical applications since they attenuate toxicity and can provide important information on the structure-activity relationship in proteins (Tejasen and Ottolenghi, 1970; Purana nanda, 1972; Kankonkar et al., 1975; Baride et al., 1980; Hati et al., 1989).

In the present study, the crotoxin complex was submitted to ionizing radiation using a Co-60 gamma source in order to evaluate the effect of radiation on the biological behavior of this toxin which is the most important neurotoxic complex of *C. d. terrificus* venom. The attenuated form obtained by gamma radiation may be of value for the production of anti-crotalic horse serum and for the understanding of the alterations provoked by radiation in the molecule and of the relationship between structural modifications and biological behavior. We determined the number of free sulfhydryl groups after radiation, as well as the solubility of the complex, its toxicity and its antigenic response to crotalic antiserum.

## Material and Methods

### *Venom*

The venom was collected from specimens of *Crotalus durissus terrificus* captured in Goias (Brazil), dried and kept at -20°C. About 300 mg of crude venom was dissolved in 10 ml 0.1 M acetic acid and centrifuged at 27,000 *g* for 15 min at 4°C using a model RC2-B Sorvall centrifuge. The supernatant was carefully removed and used to prepare crotoxin.

*Crotoxin preparation*

The crotoxin complex was obtained from the crude venom by gel filtration on Sephadex G-75 fine as described by Seki et al. (1980), followed by isoelectric precipitation.

Step 1 - Gel filtration was performed using a 2.5 x 80-cm column packed with Sephadex G-75 fine (Pharmacia), equilibrated with 0.1 M acetic acid at 4°C. Fractions of 5.7 ml were collected using an LKB Bromma 7000 collector at a flow rate of 13.8 ml/h and protein was detected spectrophotometrically at 280 nm. The 3rd peak (tubes 45 to 65) was separated, pooled, lyophilized and resubmitted to the same procedure.

Step 2 - The fraction obtained by chromatography was purified by isoelectric precipitation at pH 4.7 as follows: about 30 mg of the material isolated in step 1 was dissolved in 4 ml of twice-distilled water and 1 M formic acid was added until the pH reached pH 3.0; 0.1 M ammonium hydroxide was then carefully added up to pH 4.7. The precipitate was separated by centrifugation at 27,000 *g* for 15 min, resuspended in 100 mM ammonium formate buffer, pH 3.0, lyophilized and stored at -20°C.

*Purity criteria*

The crotoxin complex isolated by gel filtration chromatography and isoelectric precipitation was submitted to SDS-polyacrylamide gel electrophoresis on a discontinuous system as well as to diffusion immunoassay against crotalic antiserum kindly donated by Instituto Butantan (São Paulo, Brazil).

Method A: SDS-polyacrylamide gel electrophoresis was performed as described by Hames (1981) using 15% acrylamide as resolving gel and 25 mM Tris plus 0.192 M glycine, pH 8.3, as running buffer. After running (160 V, 3 h) the gel was treated with 12% (v/v) trichloroacetic acid for 15 h, stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) and destained with 7% (v/v) acetic acid.

Method B: Diffusion immunoassay as described by Ouchterlony (1958) was used to check the purity of the toxin. The diffusion time of the samples in 2% agar (1 g agar-agar (Difco) in 50 ml of 100 mM sodium phosphate buffer, pH 7.0) was 24 h. Afterwards, the plates were washed, stained with 0.4% (w/v) amido black (Merck) and destained with 5% (v/v) acetic acid.

*Sample preparation and irradiation*

The crotoxin sample, 20 mg toxin in 10 ml of 0.85% NaCl (w/v), was taken to pH 3.6 with acetic acid, filtered on Whatman No. 1 filter paper, aliquoted into 6 tubes, which were sealed in the presence of air and finally submitted to gamma radiation with a Co-60 GAMMACELL 220 source (produced by the Atomic Energy Commission of Canada, Ltd.) at a dose rate of 1100 Gy/h. (In the SI units of measurement, Gray is defined as the unit of radiation-absorbed dose. One Gray is equal to one joule of energy deposited in one kilogram of material by ionizing radiation. It is abreviated as Gy). Doses of 250, 500, 1000, 1500 and 2000 Gy were applied. One tube in the native form was used as control.

*Determination of free SH groups*

The number of sulfhydryl groups was measured in native and irradiated samples immediately after irradiation by the method of Ellman (1959). Calculation of cysteine residues/mol was performed assuming a molecular weight of 24,000 for the complex and 28 half-cystine residues/mol.

*Protein concentration*

The protein concentration of the irradiated and non-irradiated samples was determined by the method of Lowry et al. (1951), modified by Miller (1959), using bovine serum albumin (Sigma) as standard. The determination was carried out after filtering the samples on Whatman No. 1 filter paper.

*Diffusion immunoassay*

The antigenicity of the samples was assayed against crotalic antiserum as described by Ouchterlony (1958).

*Toxicity*

The $LD_{50}$ of the samples was determined as described by Reed and Muench (1938). Six animals (female albino mice) weighing 24 ± 2 g were used to test each dilution. Each sample of the toxin was tested using six dilutions with a serial dilution factor of 1:1.33, except for the sample irradiated with 1500 Gy where the dilution factor was 1:1.5. Each mouse received 0.2 ml *ip*.

The number of dead and surviving animals was recorded 24 h after intraperitoneal inoculation.

## Results

*Fractionation procedure*

The gel filtration profile of crude venom extract on Sephadex G-75 fine and the re-filtration of the 3rd peak using the same procedure are shown in Figure 1. Four peaks were obtained after gel filtration. Peaks III and IV contained crotoxin and crotamine, respectively, and peaks I and II were isolated but not characterized in the present study. The gel re-filtered material (peak III) was pooled, lyophilized and submitted to isoelectric precipitation by acid-base titration for further toxin purification.

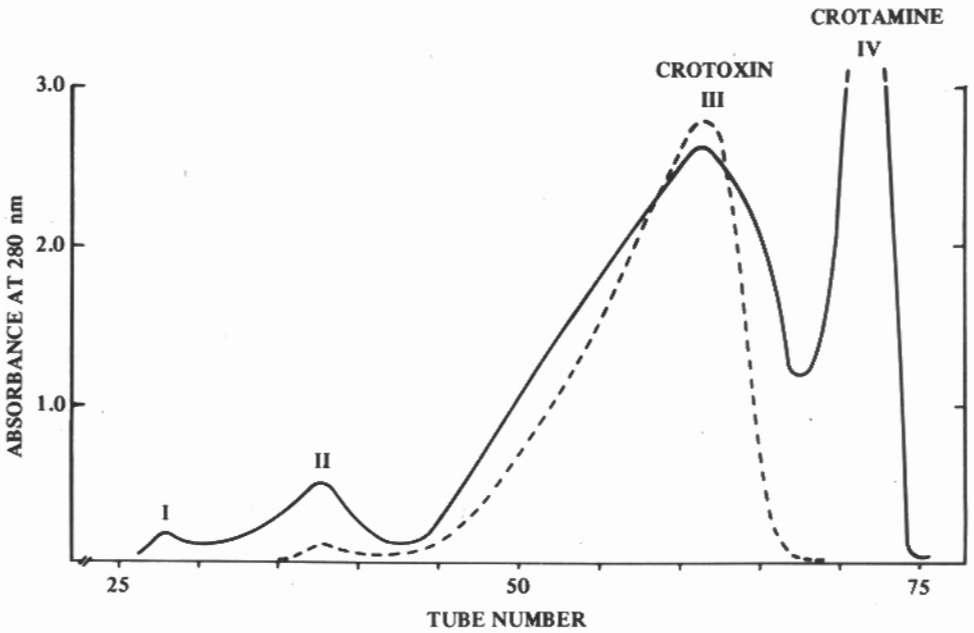


Figure 1 - Preparation of crotoxin complex from *Crotalus durissus terrificus* venom by gel filtration. The Sephadex G-75 column (2.5 x 80 cm) was eluted with 100 mM acetic acid at a flow rate of 13.8 ml/h at 4°C and fractions of 5.7 ml were collected. Protein was detected by absorbance at 280 nm. (——), 300 mg dried venom including salts in 10-ml sample volume. (- - -), Refiltration of fraction III, 120 mg protein in 2-ml sample volume.

*Purity criteria*

Figure 2 shows the results of the diffusion immunoassay (method B) for samples obtained before and after isoelectric precipitation. It is interesting to note the presence of two precipitation lines in the gel filtered material (3rd peak after re-filtration) and only one precipitin line after the isoelectric precipitation step.

*S-S bridge cleavage*

Table 1 shows the concentration of free SH residues in the non-irradiated and irradiated samples of crotoxin. It is interesting to note that free SH residues increase from 0 in the non-irradiated complex to 12 at a radiation dose of 2000 Gy.

*Protein solubility*

The final concentration of the native sample was overestimated by about 25% when compared to the "theoretical concentration" (2 mg/ml of 0.85% NaCl) according

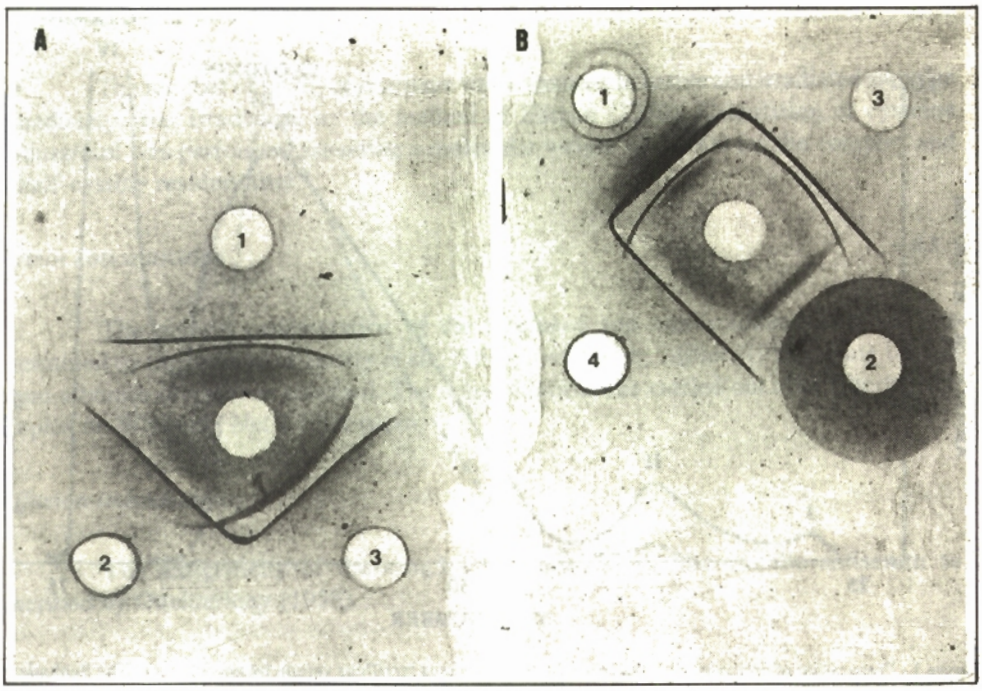


Figure 2 - Diffusion immunoassay of crotoxin and related fractions. The agar-agar gel, 2% w/v, was prepared in 100 mM sodium phosphate buffer, pH 7.0. The central well contained antiserum prepared against crude venom. *A*, 1 - Peak III obtained from first gel filtration (Figure 1, —); 2 - crotoxin obtained by two successive gel filtrations and soluble fraction obtained in step 2; 3 - mixture of precipitate and soluble fraction obtained in step 2. *B*, 1 - Crude *Crotalus durissus terrificus* venom; 2 - crotamine peak IV (Figure 1); 3 - peak III obtained from first gel filtration (Figure 1, - -); 4 - crotoxin obtained by two successive gel filtrations and isoelectric precipitation (step 2).

to Darke et al. (1980) who showed an overestimated value when the concentration of phospholipase A2 from *Naja naja naja* venom is determined by the method of Lowry using bovine serum albumin as standard.

The solubility of a solution containing 2.589 mg/ml crotoxin complex was unchanged after irradiation with 250 and 500 Gy but decreased by 14%, 38% and 59% with 1000, 1500 and 2000 Gy, respectively.

*SDS-polyacrylamide slab gel electrophoresis*

Figure 3 shows the resolving gel stained with Coomassie Brilliant Blue R 250 for native and irradiated samples. The native sample was found to be of satisfactory purity by both methods. At least four distinct bands were observed in the crude venom

extract profile but only two of these were isolated, i.e., the crotoxin complex and crotamine (a basic protein composed of a single polypeptide chain of 42 amino acid residues and with a molecular weight of 4,880, and containing 6 half-cystine residues (Laure, 1975).

The irradiated sample showed a diffuse zone and well-defined smaller bands whose intensity increased with radiation dose. However, the great loss of protein in solution and the other types of damage to the macromolecule induced by the higher dose used (2000 Gy) caused a significant reduction in protein content after SDS-PAGE. In addition, a diffuse zone could still be observed in this sample.

Table 1 - *Cleavage of the disulfide bridges of the crotoxin complex by gamma irradiation.*

Free SH groups were determined by the method of Ellman (1959). The number of bridges cleaved was taken to be half of the number of SH groups detected per molecule after correction for the amount of soluble protein.

| Radiation dose (Gy) | Soluble cysteine ($10^{-4}$ M) | S-S bridges cleaved/ molecule |
|---|---|---|
| 0 (native) | 0 | 0 |
| 250 | 0.23 ± 0.06 | 0.10 |
| 500 | 0.52 ± 0.09 | 0.22 |
| 1000 | 1.78 ± 0.13 | 0.95 |
| 1500 | 1.91 ± 0.13 | 1.42 |
| 2000 | 5.39 | 6.13 |

*Diffusion immunoassay*

The native and irradiated crotoxin samples were assayed against crotalic antiserum. The antigenic response for the samples irradiated with 1500 and 2000 Gy and the appearance of a second precipitin line should be emphasized (see Figure 4). The antigenic response of the samples irradiated with doses up to 1000 Gy did not change (results not shown), indicating that the response was fully maintained, at least for doses up to 1000 Gy.

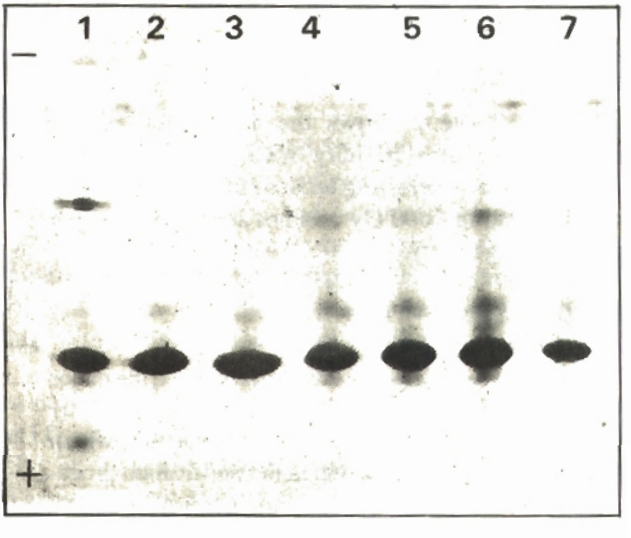


Figure 3 - SDS-PAGE of crotoxin and related fractions. Electrophoresis was carried out in 15% acrylamide with 25 mM Tris, pH 8.3, and 192 mM glycine as running buffer. Electrophoresis was carried out for 3 h at 160 V and the gel was treated with 12% trichloroacetic acid (w/v) and stained with Coomassie Brilliant Blue R 250. *1* - Crude venom; *2* - crotoxin complex; *3-7* - crotoxin complex irradiated with 250, 500, 1000, 1500 and 2000 Gy, respectively.

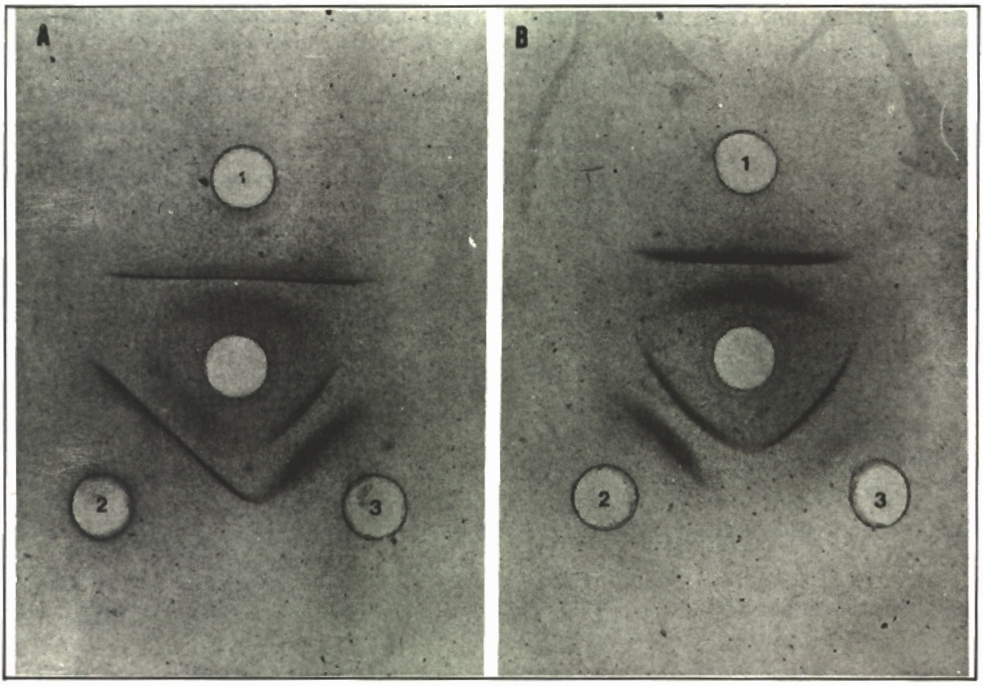


Figure 4 - Diffusion immunoassay of crotoxin complex before and after radiation. The gel was prepared as described in the legend to Figure 2. The central well contained antiserum prepared against crude venom. *A*, 1 - Crotoxin complex; 2 and 3 - crotoxin complex after irradiation with 1000 and 1500 Gy, respectively. *B*, 1 - Crotoxin complex; 2 and 3 - crotoxin complex after irradiation with 1500 and 2000 Gy, respectively.

*Toxicity*

Table 2 shows the attenuation of the crotoxin complex toxicity with radiation doses. These data are interesting because the loss of toxicity for doses up to 1000 Gy was not accompanied by a loss of antigenicity.

## Discussion

We isolated the crotoxin complex, the major toxin present in South American rattlesnake venom, by gel filtration followed by isoelectric precipitation, which proved to be an efficient step in the purification procedure. Ouchterlony's diffusion immunoassay, used as a

Table 2 - *Effect of irradiation on the toxicity of the crotoxin complex.*

Toxicity was determined on 24-g mice as described in the text and mortality was determined after 24 h.

| Radiation dose (Gy) | $LD_{50}$ (μg/kg) | Decrease of toxicity (%) |
|---|---|---|
| 0 (native) | 147 ± 4 | - |
| 250 | 193 ± 6 | 31 |
| 500 | 203 ± 4 | 38 |
| 1000 | 309 ± 13 | 110 |
| 1500 | 523 ± 41 | 255 |

criterion for purity, showed that the gel filtered material presented two precipitation lines, suggesting the existence of at least one contaminant fraction which was eliminated by isoelectric precipitation.

Immediately after irradiation, we measured the amount of free SH groups which indicates cleavage of S-S bridges provoked by radiation. The crotoxin complex contains 28 half-cystine residues in disulfide bridges (Breithaupt et al., 1974; Aird et al., 1985, 1986) and no free SH groups. Although we did not detect SH groups in the control non-irradiated protein, there was an increase to 12 SH/mol complex for 2000 Gy. Approximately one disulfide bridge per molecule was cleaved by radiation with 1000 Gy, approximately one and a half S-S bridges per molecule with 1500 Gy, and six bridges with 2000 Gy. Doses of 250 and 500 Gy had little effect. However, even at lower doses, the conformation of the toxin molecule may have been changed. Since irradiation was carried out under aerobic conditions, the actual number of S-S bridges cleaved by radiation may have been underestimated due to reoxidation.

The present data are consistent with those reported by Yang (1967) who showed that the integrity of all disulfide bonds is essential for the toxicity of crobotoxin (a potent toxin of *Naja naja atra* venom that contains 62 amino acid residues/mol and 4 disulfide bonds), showing that re-formation of all the disulfide bonds is necessary for the recovery of full lethality after reduction and reoxidation of the bridges.

Protein solubility was reduced by gamma irradiation starting from an irradiation dose of 1000 Gy. It is known that ionizing radiation can promote intermolecular binding by structural alterations that may lead to a partial loss of biological activity (Butler et al., 1984) due to formation of protein aggregates. The samples irradiated with doses of 250 and 500 Gy did not show any significant alterations in solubility when compared to the native sample. The highest radiation dose used (2000 Gy) produced a decrease in protein solubility of about 59%, indicating a great protein loss probably due to aggregation and precipitation.

The electrophoretic SDS-PAGE profile of the native and irradiated samples and crude venom extract of *C. d. terrificus* showed the formation of a diffuse zone and defined smaller bands whose intensity increased with radiation dose. This suggests the formation of intermolecular covalent bonds provoked by radiation.

These results show that the toxin was affected by gamma radiation with consequent molecular damage. The effect of the toxin on the antigenic response as measured by the Ouchterlony method indicated that no alterations had occurred with doses up to 1000 Gy, but doses of 1500 and 2000 Gy altered the antigenic determinants of the crotoxin complex, promoting a diffuse precipitin line that tended to disappear with higher doses. At radiation levels of 1500 and 2000 Gy, however, the diffusion immunoassay indicated also a second precipitin line increasing with radiation dose, thus confirming a change in antigenic behavior.

The toxicity of the native and irradiated samples showed that the crotoxin complex irradiated with 1000 Gy presented an $LD_{50}$ of 309 µg/kg, indicating an attenuation level of about two-fold when compared to the $LD_{50}$ of the native form (147 µg/kg), whereas the sample irradiated with 1500 Gy showed an approximately 3.5-fold higher attenuation level.

These results suggest that the "neurotoxic region" of the molecule was affected by radiation. In fact, we observed that the toxin sample irradiated with 1000 Gy appeared to maintain its antigenic response whereas its toxicity was attenuated about two-fold. The neurotoxic site and antigenic determinants are apparently located in different regions of the molecule. It is interesting to note that the attenuation level was significant at radiation doses of 1000 Gy or more. This fact seems to be associated with the rupture of disulfide bridges occurring at the same doses.

The present data showing the possible attenuation of the toxicity of the crotoxin complex by action of gamma radiation and the maintenance of its antigenic behavior indicate that ionizing radiation can be used to produce toxoids of *Crotalus durissus terrificus* venom for biomedical applications. Further studies are required to determine the specific chemical modifications caused by ionizing radiation.

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