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Effects of ^{60}Co gamma radiation on crotamine

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Abstract

Ionizing radiation can change the molecular structure and affect the biological properties of biomolecules. This has been employed to attenuate animal toxins. Crotamine is a strongly basic polypeptide (pI 10.3) from *Crotalus durissus terrificus* venom composed of 42 amino acid residues. It induces skeletal muscle spasms leading to a spastic paralysis of hind limbs in mice. The objective of the present study was to carry out a biochemical study and a toxic activity assay on native and irradiated crotamine. Crotamine was purified from *C.d. terrificus* venom by Sephadex G-100 gel filtration followed by ion-exchange chromatography, and irradiated at 2 mg/ml in 0.15 M NaCl with 2.0 kGy gamma radiation emitted by a ^{60}Co source. The native and irradiated toxins were evaluated in terms of structure and toxic activity (LD_{50}). Irradiation did not change the protein concentration, the electrophoretic profile or the primary structure of the protein although differences were shown by spectroscopic techniques. Gamma radiation reduced crotamine toxicity by 48.3%, but did not eliminate it.

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- Gamma radiation
- Chromatography
- Toxicity
- Circular dichroism

Introduction

The vast majority of radiation effects on proteins in solution is due to the indirect action of radiation. When interacting with aqueous solutions, gamma radiation produces several reactive species like e^-_{aq}, O_2^-, $H^\bullet$ and $OH^\bullet$ as a result of radiolysis of water (1), which, in turn, can modify the biological activity of proteins and peptides by reacting with certain sites or groups in the molecules. These properties have been successfully employed to attenuate animal toxins (2,3), yielding suitable immunogens for antiserum production.

Crotamine is a strongly basic polypeptide (pI 10.3) from the venom of the South American rattlesnake *Crotalus durissus terrificus*. It is composed of 42 amino acid residues tightly reticulated by three disulfide bonds (4). This toxin was first reported by Gonçalves and Vieira (5). Crotamine produces skeletal muscle spasms leading to spastic paralysis of hind limbs in mice (6,7). It appears to affect the functioning of voltage-sensitive sodium channels of the skeletal muscle sarcolemma, inducing a sodium influx resulting in depolarization and contraction of skeletal muscle (7). In the present report, we investigated some of the effects of gamma radiation on

the structure and biological activity of this toxin.

Material and Methods

Venom, animals and chemicals

Crude air-dried venom from the South American rattlesnake *C.d. terrificus* was supplied by the Butantan Institute (São Paulo, SP, Brazil). Mice used in the experiment were obtained from the colony housed in the Department of Bioengineering, IPEN/CNEN (São Paulo, SP, Brazil). They were maintained on a 12-h light/12-h dark cycle (lights on at 7:00 h) in a temperature-controlled environment (22 ± 2ºC). Food and water were freely available. Salts and other chemicals used in this study were of the best quality available (ACS grade).

Crotamine purification

Crude venom was dissolved in 100 mM formic acid/ammonium formate, pH 3.0, to improve solubility (Rogero JR, unpublished results), centrifuged at 12,100 *g* for 10 min to remove insoluble material, and fractionated on a 2.5 x 85 cm Sephadex G-100 column (Pharmacia-LKB, Uppsala, Sweden) and equilibrated in the same buffer at a flow rate of 0.2 ml/min at 4ºC, and the absorbance of the eluate was monitored at 280 nm. The fraction corresponding to crotamine, as verified by electrophoresis, was pooled and refractionated on a 1-ml Resource S column (Pharmacia) connected to a dual pump FPLC system (Pharmacia) and equilibrated in buffer A (25 mM phosphoric acid/NaOH, pH 7.8). Buffer B was identical to buffer A, except that it contained 2 M NaCl. After an initial 5.0-ml wash with 5% buffer B, elution was started with a linear gradient for 30 ml (slope = 1%/ml). The column was then regenerated with 10 ml 100% buffer B followed by a 10-ml buffer A wash. The flow rate was 3.0 ml/min and the eluent was monitored as above. The fraction was then desalted by dialysis against water and lyophilized.

High-performance size exclusion chromatography

The purified crotamine was submitted to high-performance size exclusion chromatography (SE-HPLC) on a 7.5 mm x 60 cm Toso Haas TSK 2000 SW column equilibrated with 50 mM ammonium bicarbonate, pH 7.0, at 1 ml/min and 25ºC. The eluent was monitored at 280 nm.

Reversed phase high-performance liquid chromatography

Reversed phase (RP)-HPLC was performed for analytical purposes using a 3.9 x 300 mm C_{18} µBondapak column with a 0 to 60% linear gradient of acetonitrile (v/v) in 0.04% trifluoroacetic acid, at a flow rate of 1 ml/min and 25ºC. The eluent was monitored at 280 nm.

Crotamine irradiation

Purified crotamine was dissolved in 0.15 M NaCl to a concentration of 2 mg/ml and irradiated with 2.0 kGy using gamma rays emitted by a Gammacell 220 ^{60}Co source (Atomic Energy Agency of Canada Ltd.) in the presence of O_2, at room temperature and with a dose rate of 800 Gy/h.

Electrophoresis (SDS-PAGE)

Slab gel electrophoresis of native and irradiated crotamine in the presence of SDS was carried out following the method described by Schägger and von Jagow (8). The separation gel consisted of a 16.5% monomer with a 6% cross-linker. The marker kit for low molecular weight was obtained from Promega Corp., Madison, WI, USA. Protein bands were visualized by staining with Coomassie brilliant blue G-250.

Protein concentration

The protein content was estimated by the method of Lowry et al. (9) modified by Miller (10), using BSA as standard.

Ultraviolet absorption spectra

The ultraviolet (UV) absorption spectra between 230 and 350 nm were obtained using an Ultrospec III (Pharmacia Biotech, Uppsala, Sweden) spectrophotometer with automated recording and a 10-mm light path. Native and irradiated crotamine solutions were used at 1.0 mg/ml in 150 mM NaCl. The blank employed for baseline subtraction consisted of 150 mM NaCl.

Lethality assays

Lethality was evaluated after intraperitoneal (*ip*) injection, 0.25 ml/animal, of the solution into male Swiss mice (25 ± 2 g). Lethal dose values (LD_{50}) were calculated according to the method of Spermann-Kärber (11). Seven groups of 5 mice each were injected with serial dilutions of the native or irradiated crotamine dissolved in 150 mM NaCl. The starting concentrations for the native and irradiated toxin were 15.2 mg/kg (dilution factor 1.5) and 24.8 mg/kg (dilution factor 1.3), respectively.

Bioactivity tests

Forty micrograms of crotamine was injected *ip* into Swiss mice (18-22 g) to determine if it produced the characteristic paralytic signs of hyperextension of hind limbs associated with small basic myotoxins.

Amino acid sequencing

To confirm the amino acid sequence of native and irradiated crotamine, the compounds were subjected to N-terminal sequencing by automated Edman degradation for five cycles (12). The peptide sequences were determined using an Applied Biosystems 476A analyzer equipped with the standard sequencer program (13), and the detection of phenylthiohydantoin derivatives by HPLC.

Amino acid analysis

Samples were hydrolyzed in constant boiling 6 N HCl and phenol at 110ºC for 24 h (14). After hydrolysis, the samples were dissolved in buffer and submitted to amino acid analysis on a Beckman 7300 instrument using ninhydrin detection (15).

Circular dichroism

The circular dichroism (CD) spectra were obtained using a Jasco J-720 spectropolarimeter. The native and irradiated crotamine solutions were used at 125 µg/ml in 20 mM sodium acetate buffer, pH 4.0. Far UV CD scans were measured from 260 to 190 nm at a scanning rate of 20 nm/min, with a 2-s response in a 2-mm path cuvette. Spectra were corrected for background by subtracting a scan of buffer alone. Each spectrum is the result of two accumulations.

Fluorescence quenching

Fluorescence was measured with a Hitachi F-4500 spectrofluorimeter at 25ºC. Excitation and emission slit widths were set at 5 nm. The excitation wavelength was set at 295 nm and the emission was fixed at 355 nm. Measurements were performed on series of samples with a fixed toxin concentration and increasing amounts of KI (0-200 mM) with a 10-mm light path cuvette. To preserve ionic strength, KCl was added to the samples to give a final molarity of 200 mM. The crotamine concentration was 4×10^{-8} M in 100 mM phosphate buffer, pH 7. A blank consisting of 200 mM KI in phosphate buffer was substracted from each sample.

The fluorescence quenching data were analyzed using the classical Stern-Volmer equation (16).

Results

The gel filtration profile of the whole venom on Sephadex G-100 is shown in Figure 1A. Fraction 4 is crotamine, fraction 1 contains convulxin, fraction 2 contains gyroxin, and fraction 3 corresponds to crotoxin (17). Crotamine was rechromatographed by cation-exchange chromatography (Figure 1B). SE-HPLC (Figure 2A) and RP-HPLC (Figure 2B) showed only one peak with an area of 100 and 96.4%, respectively. The N-terminal analysis of five residues confirmed the amino acid sequences described in the literature, i.e., Tyr-Lys-Gln-Cys-Hys. The amino acid composition of the native toxin was the same as previously reported (4), and no alterations in composition were

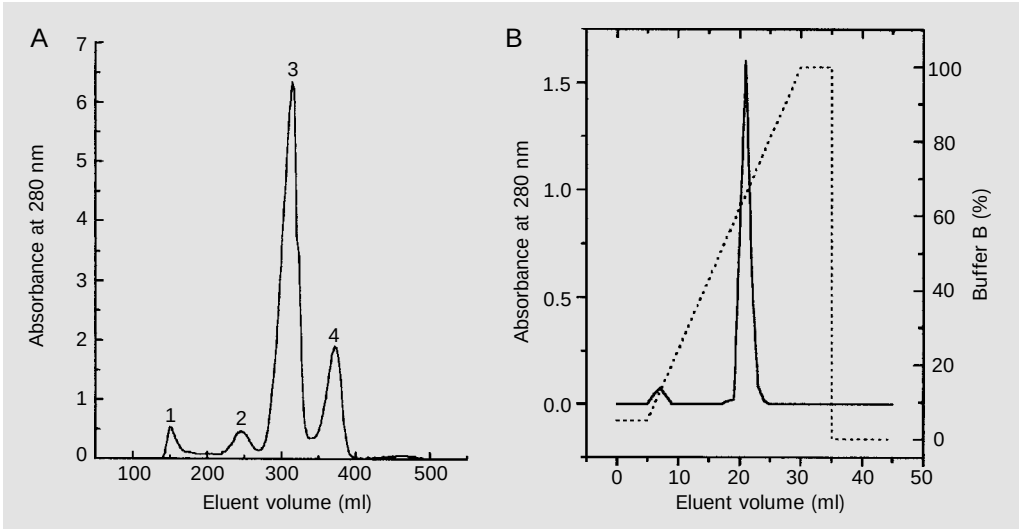

Figure 1. *A*, Crotamine purification. Size exclusion chromatography of 200 mg *Crotalus durissus terrificus* venom dissolved in 4.5 ml 100 mM ammonium formate buffer, pH 3.0, on a Sephadex G-100 (2.5 x 85 cm) column. Flow rate: 0.2 ml/min. The complete procedure was carried out at 4ºC. *B*, Ion-exchange chromatography of crotamine on a 1-ml Resource S column (6.4 x 30 mm, bead size: 15 µm). Buffer A: 25 mM sodium phosphate, pH 7.8. Buffer B: 25 mM sodium phosphate/2 M NaCl, pH 7.8. Flow rate: 3.0 ml/min. The gradient is indicated by the dashed line. The complete procedure was carried out at room temperature. Fraction 1 contains convulxin; fraction 2, gyroxin; fraction 3, crotoxin, and fraction 4, crotamine.

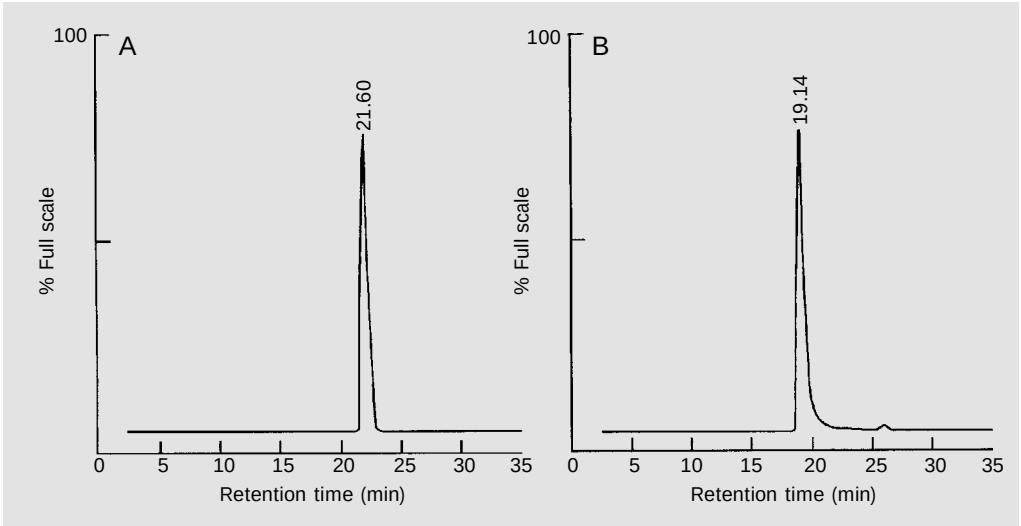

Figure 2. High-perfomance size exclusion chromatography of crotamine (A) on a 7.5 mm x 60 cm Toso Haas TSK 2000 SW column equilibrated with 50 mM ammonium bicarbonate buffer, pH 7.0, at 1 ml/min and 25ºC. Reversed phase high-performance liquid chromatography of crotamine (B) on a 3.9 x 300 mm C_{18} µBondapak-Waters column, with a linear gradient of buffer A [0.04% trifluoroacetic acid (v/v)] and eluted with 0-100% buffer B [60% acetonitrile/buffer A (v/v)] at 1 ml/min and 25ºC.

observed in irradiated crotamine.

The protein concentration of native crotamine was 1.91 ± 0.05 mg/ml and the protein concentration of irradiated crotamine was 1.90 ± 0.05 mg/ml (N = 8 samples). No differences in the electrophoretic profiles of native and irradiated crotamine were observed (Figure 3).

As shown in Figure 4A, the UV absorption spectra of crotamine samples ranged from 230 to 350 nm, a higher absorption being observed for irradiated crotamine.

The fluorescence quenching data suggest conformational changes within the molecule following irradiation, with a higher exposure of the tryptophans resulting in higher quenching constants (Figure 4A, inset).

The CD spectra of native and irradiated crotamine are shown in Figure 4B. A comparison of the spectra shows a discrete change from an apparently ordered conformation to a random coil.

The *ip* LD_{50} for mice was found to be 6.0 (4.2-8.5) mg/kg for native crotamine and 11.6 (9.1-14.7) mg/kg for irradiated crotamine, with the latter being about two-fold less toxic than the native toxin. However, both native and irradiated crotamine induced hind limb paralysis in mice at 2.0 mg/kg when injected *ip*. The onset of the effect was rapid, usually occurring within 15 min.

Discussion

Crotamine was purified from *C.d. terrificus* venom by gel filtration on Sephadex

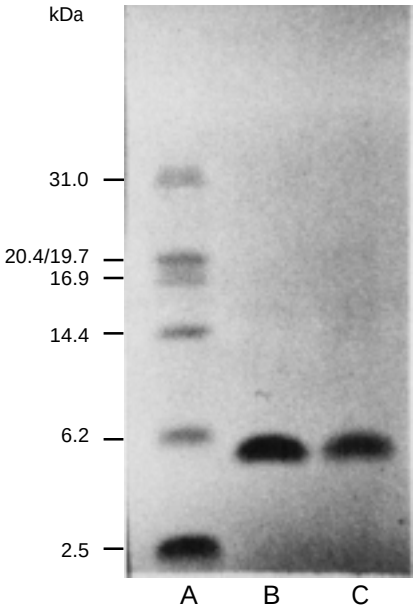

Figure 3. SDS-PAGE of native and irradiated crotamine. *A*, Molecular weight markers (carbonic anhydrase: 31 kDa, soybean trypsin inhibitor: 20.4/19.7 kDa, horse heart myoglobin: 16.9 kDa, lysozyme: 14.4 kDa, myoglobin fragment (F2): 6.2 kDa, myoglobin (F3): 2.5 kDa). *B*, Native crotamine and *C*, irradiated crotamine.

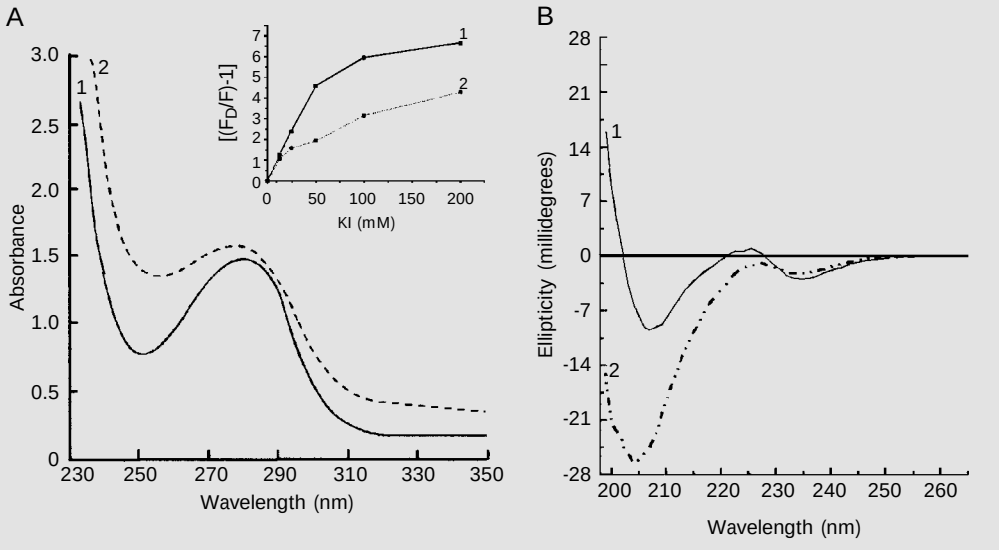

Figure 4. Spectroscopic characterization of native and irradiated crotamine (A). UV absorption spectra of native crotamine (1) and irradiated crotamine (2). Concentration was 1.0 mg/ml in 150 mM NaCl. *Inset*, Stern-Volmer plot of the native (1) and irradiated (2) crotamine fluorescence quenching by iodide. Crotamine concentration was 4 x 10^{-8} M in 100 mM phosphate buffer, pH 7, at 25ºC (B). Circular dichroism spectra of native crotamine (1) and irradiated crotamine (2). Concentration was 125 µg/ml in 20 mM sodium acetate buffer, pH 4.0.

G-100 (Figure 1A) followed by cation-exchange chromatography (Figure 1B). As judged from the SE-HPLC, RP-HPLC, SDS-PAGE profiles (Figures 2A,B, and 3), and N-terminal analysis, the toxin was pure. Crotamine is composed of 42 amino acid residues and lacks alanine, valine and threonine. In the present study, protein composition data were found to be similar to those described in the literature (4). An elution profile closely similar to that illustrated in Figure 1A was obtained in all cases except for the crotamine-negative *C.d. terrificus* venoms, which did not show peak 4 at 280 nm. The protein concentration of native crotamine was 1.91 mg/ml, while the protein concentration of irradiated crotamine was 1.90 mg/ml. These findings show that the protein content of the sample submitted to radiation was maintained. These data show good agreement with those obtained by Costa and Rogero (18). As shown in Figure 3, there were no differences in the electrophoretic profiles of native and irradiated crotamine. This suggests that there was no aggregation or rupture of the polypeptide chain-producing fragments, in contrast to data observed for crotoxin (3), the major neurotoxin of this venom.

The UV absorption spectrum of irradiated crotamine between 230 and 350 nm showed higher absorption (Figure 4A), indicating exposure of the chromophore groups, possibly due to unfolding of the polypeptide chain. These results are in agreement with data reported previously about crotalic venom and Indian snake venoms (19,20). The fluorescence spectroscopy assays further indicate structural modification of the crotamine molecule after irradiation (Figure 4A, inset). Indeed, the quenching constants show an increase of the solvent accessibility to the fluorophores (Trp 32 and 34), suggesting unfolding of the molecule.

The LD_{50} of native crotamine administered *ip* to mice was 6.0 mg/kg. These results differ from the LD_{50} values reported in the literature which were 3.4 (6) and 1.5 mg/kg (21) by the intravenous route, 0.07 mg/kg (22,23), 0.80 mg/kg (Rogero JR, unpublished results) and 0.70 mg/kg (Nakazone AK, unpublished results) by the *ip* route, and 0.46 mg/kg by the subcutaneous (*sc*) route (24). Allen et al. (25) injected 6.3 mg/kg of crotamine in H_2O by the *sc* route in C_3H mice and observed that this dose was not lethal. However, when crotamine was dissolved in 0.4 ml and 0.6 ml sodium acetate, the LD_{50} values reported were 1.26 and 0.69 mg/kg, respectively.

Some explanations are available for the various LD_{50} values reported for crotamine and other toxins, although no details are available about how the LD_{50} values were obtained (26). Moreover, these different values also reflect an improvement in methods for toxin purification.

In the present study, the minimum dose of crotamine that caused death was 4.5 mg/kg, which is in agreement with the literature (25). Our experiments have shown that the LD_{50} value obtained for irradiated crotamine was 11.6 mg/kg, corresponding to a two-fold decrease in toxicity. Similar results were reported by Souza-Filho et al. (27) and Nascimento et al. (3) using crotoxin. In recent work from our laboratory the toxicity of bee venom decreased when the venom was exposed to gamma radiation (28). These results indicate that gamma radiation reduces the toxicity of animal venoms and toxins. The native and irradiated crotamine induced hind limb paralysis in mice at 2.0 mg/kg when injected *ip*, showing that gamma radiation did not abolish the bioactivity of crotamine.

The lack of detailed structural information about the rattlesnake venom myotoxins is a major deficiency in the literature pertaining to these unique proteins. None of the rattlesnake myotoxins has been crystallized. Data from Raman spectroscopy studies of crotamine led to a prediction that crotamine is predominantly formed of ß sheet (29).

Myotoxin *a* has significant amounts of ß sheet and ß turn with small amounts of random coil (30). A molecular model based on two lobes was proposed by SAXS methodology for crotamine (31).

The CD spectrum of native crotamine (Figure 4B) was closely similar to that obtained by Cameron and Tu (32) for myotoxin *a*. These two proteins appear to be very similar as suggested by amino acid analysis and primary structure (33); however, small structural differences were reflected in CD spectra. These small differences observed may be ascribed to slight differences in the primary structure or conformational changes. With respect to irradiated crotamine, a change was observed suggesting an equilibrium transition towards a random coil conformation. The fluorescence data corroborate these findings, showing that the fluorophores which were partially buried in the native crotamine are further exposed after irradiation of the toxin. All our data indicate that although not affecting the peptide primary structure, which is reported to be affected by radiation (1,34), the secondary and tertiary structures of the toxin were modified. These data are in agreement with the observations of Stadtman (34) who reported changes in hydrophobicity, viscosity and CD spectra of irradiated proteins. The decrease in toxicity may be ascribed to irreversible conformational changes which can affect the interaction of the toxin with its target tissue and/or decrease its ability to modify sodium influx.

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