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PHYSICO-CHEMICAL CHARACTERIZATION OF GAMMA RAYS IRRADIATED CROTAMINE

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ABSTRACT

Ionizing radiation can change the molecular structure and affect the biological properties of biomolecules. It has been employed to attenuate animal toxins. Crotamine, a toxin from *Crotalus durissus terrificus* (Cdt), is a highly basic polypeptide (pI - 10.3), with myotoxic activity and molecular weight of 4882 Da. It is composed of 42 amino acids residues and reticulated by three disulfide bonds. This study aimed the characterization of irradiated crotamine using Circular Dichroism (CD), Fluorescence Spectroscopy and Differential Scanning Calorimetry (DSC) techniques. We used size exclusion and ion-exchange chromatography to purify it from Cdt crude venom. The pure crotamine was irradiated with 2.0 kGy from a ^{60}Co source. Native and irradiated crotamine were analyzed in a fluorescence spectrophotometer (Hitachi F-4500), under excitation wavelength at 275 nm and the emission was scanned from 300 to 500 nm. The analysis of fluorescence quenching showed that the irradiated form displayed a lower quantum yield when compared to the native form. CD spectra, obtained from a Jasco, J-180 spectropolarimeter, of native and irradiated crotamine solutions, showed a discrete change between the samples, from apparently ordered conformation to a random coil. Finally, the thermodynamics analysis, realized in a calorimeter METTLER TOLEDO, DSC 822e, showed that irradiation promoted changes in the calorimetric profile. Our results indicate that irradiation leads to progressive changes in the structure of the toxin, which could explain the decrease in myotoxic activity.

1. INTRODUCTION

Venom in general are weakly immunogenic, yet fairly toxic. This causes problems because serotherapy is the treatment of choice in snakebite accidents, and horse antisera availability is dependent upon venom immunogenicity. To improve antisera production and extend the lifespan of immunized horses much effort has been devoted to decreasing chronic venom toxicity [1].

Ionizing radiation can change the molecular structure and the biological properties of biomolecules [2]. This can occur by two forms: direct process – ionizing radiation interacts directly on target biomolecules and indirect process – the products generated by water radiolysis, like e^-_{aq}, O_2^-, H• and OH•, interact with target molecules and can modify the biological activity of proteins and peptides by reacting with certain sites or groups in the molecule [2,3]. The vast majority of radiation effects on proteins in solution is due to the indirect action of radiation and it has been employed as a tool to attenuate animal toxins [2].

Snake venoms contain sets of proteins that are responsible for their neurotoxic, cardiotoxic, hemorrhagic and myotoxic activities. Among these toxins, myotoxins are of particular interest as they exhibit a broad spectrum of interesting biological responses [4]. Crotamine, from the South American rattlesnake *Crotalus durissus terrificus*, is a strongly basic polypeptide (pI – 10.3), with myotoxic activity and molecular weight of 4822 Da. It is

composed of 42 amino acid residues, without free sulfhydryl groups and reticulated by three disulfide bonds [5,6,7].

This toxin produces skeletal muscle spasms in mammals, leading to spastic paralysis of peripheral origin. These effects are mediated by the binding of crotamine to voltage-sensitive Na^+ channels in the skeletal muscle sarcolemma and results in an abnormal influx of Na^+ ions in skeletal muscle cells. This influx provokes cell membrane depolarization and strong contraction of the skeletal muscle, leading to necrosis of the muscle fibers characterized by extensive vacuolization of the sarcoplasmatic reticulum and disruption of actin and myosin filaments [6,8,9].

Despite a considerable amount of work about this subject, the relationships between structure-function of protein and ionizing radiation effects is still unclear. This study aimed the characterization of native and irradiated crotamine using Circular Dichroism (CD), Fluorescence Spectroscopy and Differential Calorimetry techniques.

2. MATERIAL AND METHODS

2.1. Venom

Crude air-dried venom from *Crotalus durissus terrificus* was supplied by the CEVAP (Botucatu - SP - Brazil).

2.2. Crotamine Purification

Crude venom was dissolved in 100 mM, pH 3.0 ammonium formate buffer, centrifuged at 14,000 g for 5 min to remove insoluble material, and fractionated on a 1.6 x 70 cm Superdex G-75 column (Pharmacia-Biotech), equilibrated in the same buffer at 0.5 ml/min. The absorbance of the eluate was monitored at 280 nm. The fraction corresponding to crotamine was pooled and refractionated on a Resource S column – 1 mL (Pharmacia Biotech AB), equilibrated in buffer A (50 mM phosphoric acid/NaOH, pH 7.8). Buffer B was identical to buffer A, except by 2 M NaCl. After an initial wash (5.0 mL) with 5% buffer B, elution was started with a linear gradient for 30% buffer B. The column was regenerated with 100% buffer B followed by buffer A to wash. The fraction was pooled and then desalted by dialysis against water and lyophilized.

2.3. Crotamine Irradiation

Purified toxin was dissolved in a 0.15 M NaCl solution to a final concentration of 400 μg/mL and irradiated with 2.0 kGy using gamma rays from a ^{60}Co source (Gammacell 220 Canada), in the presence of O_2, at room temperature and with a dose rate of 1.2 kGy/h.

2.4. Fluorescence Quenching

Fluorescence was measured with a Hitachi F-4500 Fluorescence Spectrophotometer at 25 ºC. The excitation wavelength was set at 275 nm and scanning of emission from 300 to 500 nm. The crotamine concentration was 400 μg/mL, in 150 mM sodium chloride buffer. A blank consisting of 150 mM NaCl was subtracted from each sample. The fluorescence quenching data were analyzed using the Origin program.

2.5. Circular Dichroism

The Circular Dichroism (CD) spectra were obtained using a Jasco, J-180 spectropolarimeter. The native and irradiated crotamine were analyzed at 400 μg/mL in 25 mM, pH 7.6 phosphate buffer. Spectra were calculated after three accumulations and subtraction of a blank consisting of buffer alone.

2.6. Differential Scanning Calorimetry

The Differential Scanning Calorimetry (DSC) spectra were performed on a METTLER TOLEDO, DSC 822e. The native and irradiated crotamine samples were previously diluted to 400 μg/mL in 25 mM phosphate buffer, pH 7.2. The thermodynamics analysis were obtained by gradual heating (1 ºC/minute), with an initial temperature of 20 ºC and a final of 90 ºC. The signal was registered each 0.5 minute. The signal was corrected against a spectrum of buffer.

3. RESULTS

The fluorescence quenching spectra of native and irradiated crotamine can be observed in the **Figure 1**. In the graphic, the native form of crotamine is represented by red line and the irradiated form by blue one.

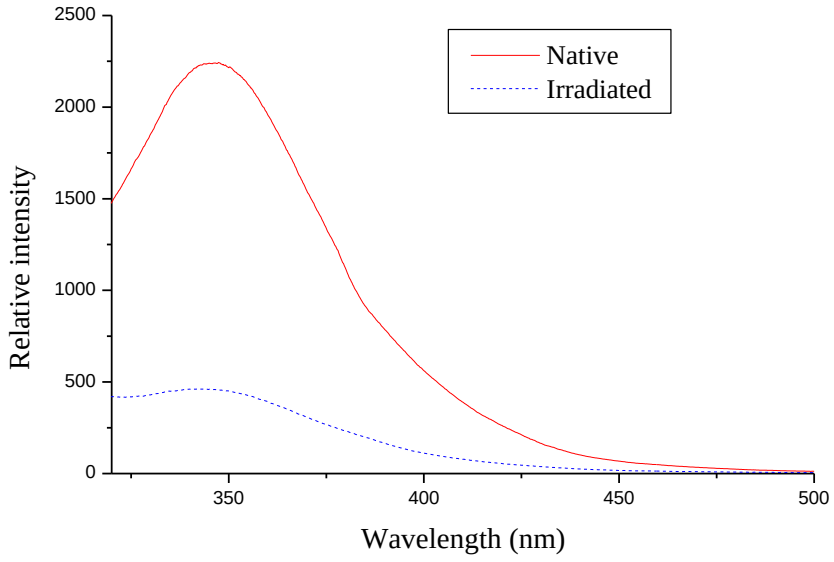

Figure 1: Analysis of fluorescence quenching of native and irradiated crotamine.

Figure 2 shows the CD spectra of native and irradiated crotamine. It is possible observe a change between the spectra of the native (red line) and irradiated (blue line) form.

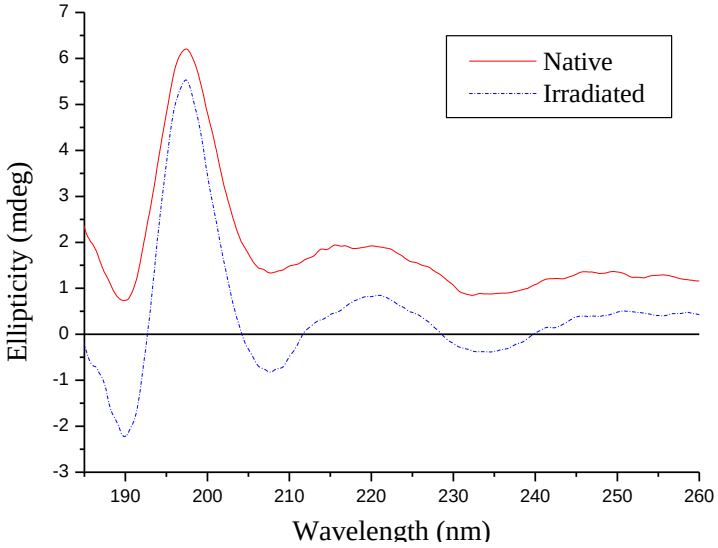

Figure 2: Circular Dichroism spectra of native and irradiated crotamine.

Figure 3A and 3B shows the calorimetric degradation of native and irradiated crotamine.

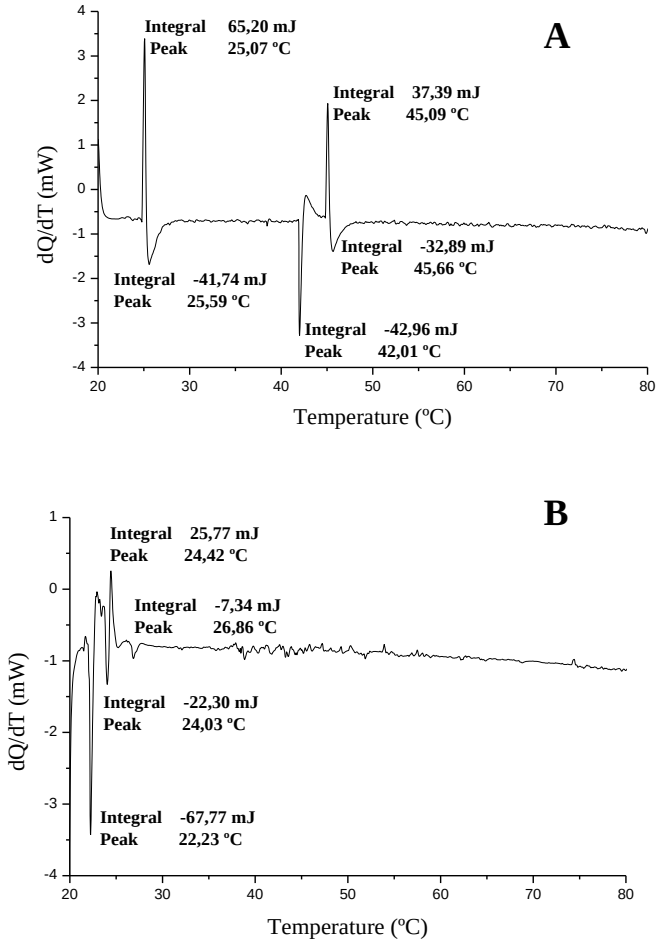

Figure 3: Differential Calorimetry (DSC) analysis of native (A) and irradiated (B) crotamine.

4. DISCUSSION AND CONCLUSIONS

Irradiation of proteins, in aqueous solution, induces chemical and structural alterations in proteins and peptides. These changes are related to loss of biological activity in addition to interfering in immunological properties after irradiation [10].

It is known that the environment of tryptophans can greatly modify the intensity of their fluorescence. So, changes in protein conformation can result in different spectrum data. The analysis of fluorescence quenching (**Figure 1**) showed that irradiated crotamine quantum yield decreases significantly if compared with the native form. This suggests an increase of the solvent accessibility to the tryptophan, possibly due to unfolding of the polypeptide chain, since the ionizing radiation can change the molecular structure. These results are in agreement with other studies about characterization of native and irradiated crotamine [2,6,11].

Although fluorescence can be very helpful in following molecular changes, such measurements are difficult to interpret directly in terms of changes of secondary structure (α-helix and β-sheet). When a native structure of a protein, containing α-helical and β-sheet regions, is denatured, it undergoes an unfolding process. Crotamine is considered a myotoxin *a* [12] because both the peptides have a very similar primary structure. The analysis of CD spectra from native and irradiated crotamine (**Figure 2**) showed a discrete change between the samples, apparently from ordered conformation towards a random coil. This data corroborate with Boni-Mitake, 2001 [2].

Finally, the thermodynamics analysis showed that irradiation modified the molecules enthalpy. Endothermic transitions are down and exothermic transitions are up. In the **Figure 3A**, the native crotamine presents in two moments, at approximately 25 ºC and 45 ºC, a peak up followed by a peak down. This suggests the formation of a structure and its subsequent melting. On the other hand, around 42 °C, there is an indication of another state transition, probably an intermediate. Also these data suggest that above 46 ºC there is a complete denaturation of the toxin. **Figure 3B** shows an irregular spectrum with a first component indicating melting at 22.23 ºC, thus suggesting a lower enthalpy. In other words, the energy required to denaturate the toxin after irradiation was much lower, showing that the structural stability was significantly affected by the irradiation process.

Our results indicate that irradiation process leads to progressive changes of the secondary and tertiary structure of the toxin, which could explain its myotoxic activity loss. This data are in agreement with the other observations related in literature [3,6,10,13].

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