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INFLUENCE OF REACTIVE RADICALS $OH^{\bullet}$ AND E^{-}_{AQ} IN IRRADIATION OF PROTEIN IN AQUEOUS SOLUTIONS.

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ABSTRACT

Gamma irradiation of aqueous proteins solutions promotes structural modifications. This is mainly caused by $OH^{\bullet}$ and e^{-}_{aq} activity, reactive species produced by water radiolys. The objective of this study was to investigate the role of $OH^{\bullet}$ and e^{-}_{aq} in conformation changes observed in proteins irradiated in aqueous solutions.
Substances that react specifically with $OH^{\bullet}$ or e^{-}_{aq} producing stable products were used in order to study each reactive species' function: nitrate for e^{-}_{aq} and t-butilic alcohol for $OH^{\bullet}$.
Crotamine was used as test peptide. It is composed of 42 aminoacids, which are strongly reticulated by three disulfide bonds. This confers it a very compact conformation leading to high temperatures resistance. Crotamine also has a biological effect: mice hind limb distention.
Samples of crotamine in aqueous solution were irradiated in order to analyze the activity of $OH^{\bullet}$ and e^{-}_{aq}. Some contemned t-butilic alcohol and others, sodium nitrate; the results were compared to control samples with no neutralizing substances. To verify possible protein conformation change due to sodium nitrate or t-butilic alcohol addition, samples with different concentrations of these substances were prepared, kept without irradiation and their results were compared to only protein solution. Samples were also submitted to a biological test
Samples were submitted to SDS-PAGE, which did not show any alteration in those samples with t-butilic alcohol or sodium nitrate. In addition, the samples had their absorbance spectrum of 200 nm to 360 nm scanned which showed less conformation change in irradiated with t-butilic then only protein one. Irradiated samples had less biological effect than non-irradiated ones.

1. INTRODUCTION

Gamma ray incidence in proteins leads to physics and chemical alterations leading to structural, conformational and immunological alterations.

In aqueous solutions, gamma rays act in two ways: a direct way, where the protein itself is ionized and an indirect way in which highly reactive molecules produced in water radiolysis attack the protein causing its oxidation and aggregation. The water radiolysis equation is described below:

$$H_2O \rightarrow 2{,}7\ e^{-}_{aq.} + 0{,}45\ H_3O^{+} + 3{,}2\ OH^{\bullet} + 0{,}6\ H^{+} + 0{,}45\ H_2 + 0{,}7\ H_2O_2 \qquad (1)$$

Hydroxyl radical ($OH^{\bullet}$) removes hydrogen of alcohol and sulfydryl groups and produces highly reactive radicals in aromatic aminoacids residues. Aqueous electron (e^{-}_{aq}) acts in same way in aromatic residues, also reacts with amino groups and cleaves peptide bonds[1].

Substances called scavengers can be added before irradiation. They react with the high reactive species becoming stable molecules competing with the protein for the reaction.

Cisteines and alcohols donate their hydrogen to hydroxyl radicals; anions as nitrate will be reduced by $e^-_{aq.,}$ protecting the other molecules in the system .
Crotamine, a South American rattlesnake (*Crotalus durissus terrificus*) venom fraction, was used as test peptide. It is a small molecule with 42 aminoacids with strongly basic pI; it has three disulphide bonds what confers it a very compact conformation and high temperature resistance.

Crotamine aminoacid sequence has a great homology with miotoxins (attacks muscles damaging them) [2]. It has activity on sodium canals provoking influx and causing contraction of skeleton muscles [3], can lead to extreme activation of muscle cells causing mionecrosis [4]. Its macroscopic effect is characterized by the distention of the mice hind limbs, such specificity mechanism is not known yet.

Protein irradiation can be used in antivenom serum production. 2 kGy gamma radiation acts reducing biological activity. Anyway it maintains the native neutralizing antibody production capacity [5; 6; 7; 8].

2. MATERIAL AND METHODS

2.1. Crotamine purification

2.1.1 Molecular exclusion chromatography

A hundred fifty milligrams of *Crotalus durissus terrificus* lyophilized total venom were dissolved in 2 mL of ammonium formiate buffer (100mM pH 3), which was centrifuged for 10 min at 201g. The over flow was applied in Superdex 75 gel filtration column in FPLC system, previously stabilized with same solution buffer. One mililiters fractions were collected with online absorbance measure on 280 nm. Crotamine peak fractions were mixed-up, congealed and lyophilized.

2.1.2 Ionic exchange chromatography

Pre-purified lyophilized crotamine was dissolved in sodium phosphate buffer (50mM pH7,8) and applied in resource S (anionic) column in FPLC system, already stabilized in same buffer. It was used a saline gradient from 0 until 2 M of sodium chloride for eluting. One mililiters fractions were collected with online absorbance measure on 280 nm.

Crotamine peak fractions were mixed-up and dialyzed against distilled water in a SIGMA membrane with capacity between 0 and 3000 Da. Sample was congealed, lyophilized and stored at –20ºC.

2.2 Mass spectrometry assay

A 2mg/ml native (non-irradiated) crotamine solution was analyzed by MALDI mass spectrometry.

2.3 Sample irradiation

Sample irradiations were carried out in a ^{60}CO gammacell 220 at Centro de Tecnologia das Radiações of IPEN-SP with tax dose of 4,34 x 10^3 per hour. Irradiation of 2 KGy produced 0,7 μmol of $OH^{\bullet}$ and 0,6 μmol of e^-_{aq}.

2.4 Biological activity

Swiss mice supplied by animal facility of IPEN-SP were used in assays.
Mice were divided in groups of two for each sample: Non-irradiated without scavenger, with $NaNO_3$ 0,7 μmolar and with t-butilic alcohol 0,7 μmolar; Irradiated without scavenger, with $NaNO_3$ 0,7 μmolar and with t-butilic alcohol 0,7 μmolar. Each animal was intra-peritoneal inoculated with 0,2 mg of crotamine. Activity was measured by the distention of hind limbs in at maximum 30 minutes; after that it was considered inactive.

2.5 SDS-PAGE gel assay

Samples of 40 μg of crotamine were applied in a 15% poliacrilamide gel. Bovine albumin with 66 kDa, carbonic anidrase with 29 kDa and C citocrome with 12,4 kDa were used as molecular weight markers.

2.6 Absorbance scanning assays

Absorbance scanning from 200 nm to 360 nm was made in spectrophotometer model Ultrospec (Pharmacia LKB – Bioteck).

2.6.1 Irradiated, non-irradiated samples scanning assay

Six samples were made: two in distilled water; two in sodium nitrate 0,7 μmolar; two in t-butilic alcohol 0,7 μmolar. One of each kind was irradiated. All samples were submitted to absorbance scanning.

2.6.2 Different scavenger concentration samples scanning assay

Six samples were made in different concentrations of scavenger: $NaNO_3$ 0,7 μmolar; $NaNO_3$ 1,4 μmolar; $NaNO_3$ 2,8 μmolar; t-butilic alcohol 0,7 μmolar; t-butilic alcohol 1,4 μmolar; t-butilic alcohol 2,8 μmolar. All samples were submitted to absorbance scanning.

3. RESULTS AND DISCUSSION

3.1 Crotamine purification

Purifications chromatograms are showed below (Fig.1; Fig.2)

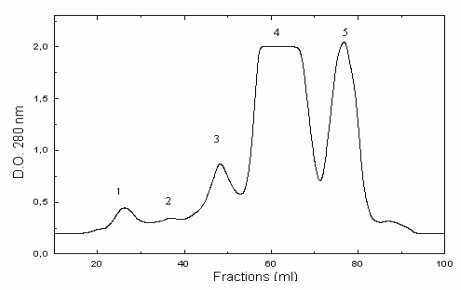

Figure 1. Molecular exclusion chromatography. Pick 5 corresponding

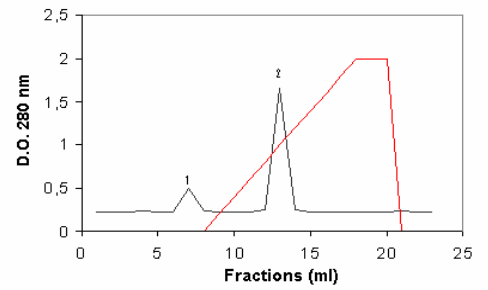

Figure 2. Ion exchange chromatography. Pick 2 corresponding to crotamine.

Pick 5 in molecular exclusion correspond to crotamine [6] agreeing with its low molecular weight leading to lager time of retention. Pick 2 in ion exchange correspond to crotamine [7], agreeing with its strong basic composition.

Chromatograms show a succeeded purification obtaining crotamine with no contaminations (Fig. 2, pick 2).

3.2 Mass spectrometry assay

Graphic of mass spectrometry is showed below (Fig. 3).

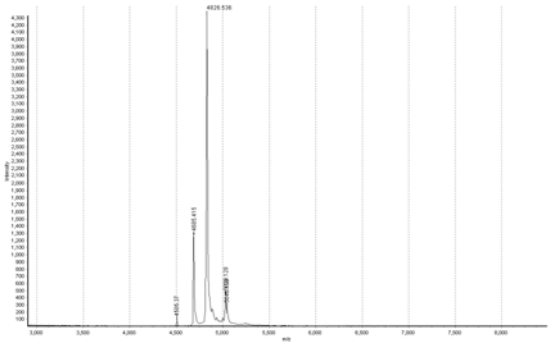

Figure 3. Mass spectrometry graphic.

Graphic indicates two major peck probably corresponding to crotamine isoforms [10]; the other minor pecks probably correspond to protein degradation and noise. Mass spectrometry assured sample's purity.

3.3 Biological activity

Biological activity showed inactive all irradiated samples, even those with scavengers. Non-irradiated samples with scavenger did not present activity lost.

Results confirm other similar experiments [7] [8] [9] showing lost of activity in irradiated samples. Scavengers did not protect those samples at this dose and concentration.

3.4 SDS-PAGE gel assay

Gel picture is showed below (Fig.4)

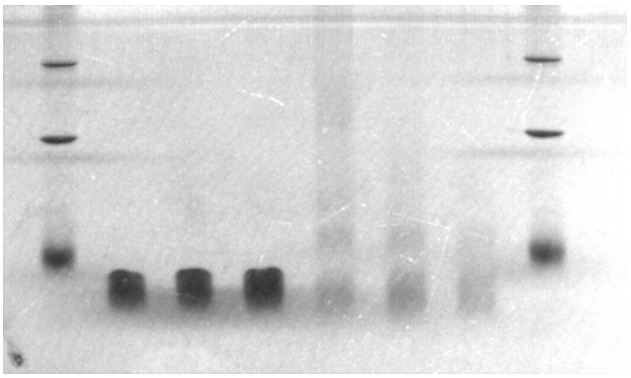

Figure 4. 1,8-marker; 2-native; 3-native in t-butilic alcohol solution; 4-native in $NaNO_3$ solution; 5-2kGy in distilled water; 6-2 kGy t-butilic alcohol solution; 7-2kGy

The SDS-PAGE gel points out no significant changes on crotamine run pattern because the scavengers addition in non-irradiated samples(Fig. 5; 1,2,3), in irradiated ones either (Fig.5; 4,5,6). Anyway, production of aggregates with lager molecular weight can be visualized in irradiated samples.

3.5 Absorbance scanning assay

3.5.1 Irradiated, non-irradiated samples scanning assay

Absorbance scanning graphic is showed below (Fig. 6, Fig.7). Changes on absorbance pattern can be noticed in irradiated samples, as well in samples with scavenger comparing to without scavengers ones.

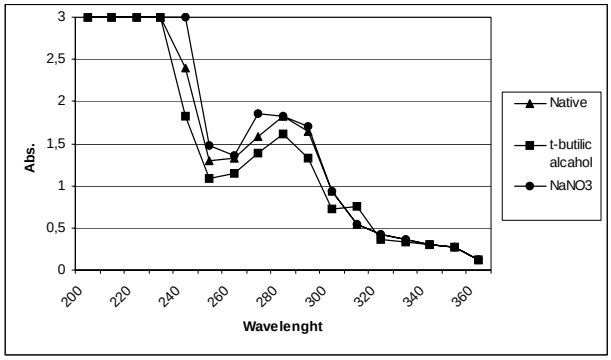

Figure 5. Non-irradiated samples absorbance scanning.

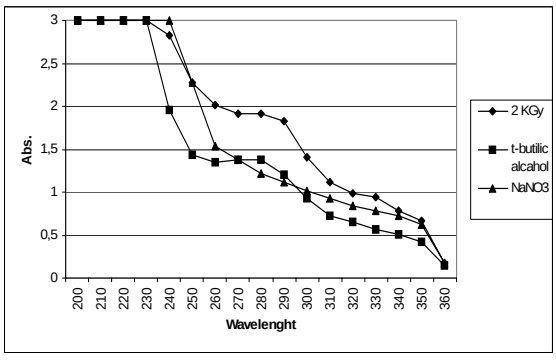

Figure 6. Irradiated samples absorbance scanning.

Perceptive changes on absorbance pattern are visualized around 260nm and 280nm. Two hundred sixty nanometers spectrum zone correspond to non-aromatics aminoacids residues;

280nm zone correspond to aromatics aminoacids residues. Higher absorbance denotes higher residues exposition; lower denotes aminoacid degradation or hiding.

Addiction of t-butilic alcohol in native samples (Fig.5) leads to an aromatic residue hiding. Irradiation probably causes a native protein linearization since is noticed high absorbance in almost all spectrum zones (Fig. 6). T-butilic alcohol irradiated sample show a small difference in 280nm comparing to non-irradiated ones, what denotes a kind of protection. That is probably because $OH^{\bullet}$ radicals are neutralized in this solution preventing them from oxiding the aromatic residues.

3.5.2 Different scavenger concentration samples scanning assay

Absorbance scanning graphic is showed below (Fig. 8, Fig.9).

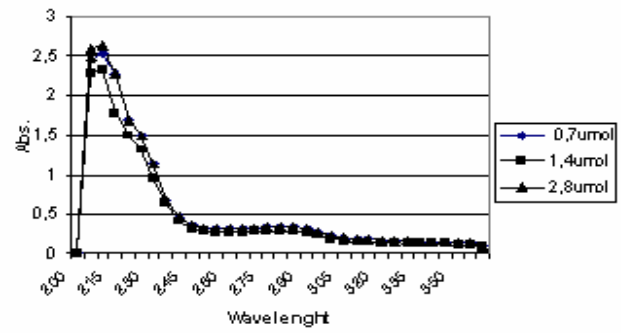

Figure 7. Different $NaNO_3$ sample's concentration.

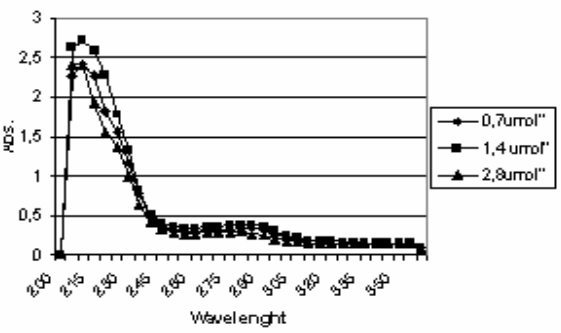

Figure 8. Different t-butilic alcohol sample's concentration.

Graphics do not show any change in protein absorbance pattern until triple scavenger necessary concentration.

4. CONCLUSIONS

Chromatography methods demonstrated a good level of purity. Gamma radiation promotes structure modifications in crotamine. Scavengers promote a little protection against radiation. Scavenger concentration does not induce significantly differences when analyzed in absorbance scanning. The irradiated crotamine presented loose of toxic activity, demonstrated by biological activity.

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