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BSc. Biochemistry

Expression, Purification and Stability Study of the Recombinant Human Interferon α-2b

Dissertation for obtaining the Master's Degree in Biotechnology

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Acknowledgments

“Deem graças ao Senhor porque ele é bom; o seu amor dura para sempre.”

Salmos 107:1

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Abstract

Recombinant human interferon α-2b (rhIFNα-2b) is a widely used therapeutic protein for the treatment of viral infections such as hepatitis. Being a therapeutic protein it is only active in its native conformation so that it is important to investigate possible pathways of degradation when producing it. In this work rhIFNα-2b was subjected to four different stress conditions and the resulting products characterized with fluorescence spectroscopy, fluorescence anisotropy, circular dichroism, dynamic light scattering and scanning electron microscopy. The results showed that rhIFNα-2b loses its native conformation in all conditions in which it was tested and there was formation of aggregates. It was also made a bioactivity assay where we saw that the protein had biological activity before and after the stress conditions.

Keywords: rhIFNα-2b, therapeutic protein, stress conditions, bioactivity assay

Resumo

O interferão recombinante humano α-2b (rhIFNα-2b) é uma proteína terapêutica utilizada para o tratamento de infeções virais, como a hepatite. Sendo uma proteína terapêutica é apenas ativa na sua conformação nativa, de modo que é importante investigar possíveis vias de degradação durante a sua produção. Neste trabalho o rhIFNα-2b foi sujeito a quatro condições de estresse diferentes e os produtos resultantes foram caracterizados por cinco diferentes técnicas: espectroscopia de fluorescência, anisotropia de fluorescência, dicroísmo circular, difusão dinâmica da luz e microscopia eletrônica de varredura. Os resultados mostraram que rhIFNα-2b perde a sua conformação nativa em todas as condições em que foi testado e observou-se formação de agregados. Também foi feito um ensaio de bioatividade onde vimos que a proteína tem atividade biológica antes e depois das condições de estresse.

Keywords: rhIFNα-2b, proteína terapêutica, condições de estresse, ensaio de bioatividade

List of abbreviations

CV – column volume

FDA – Food and Drug Administration

huIFN – human interferon

IFN – Interferon

IPTG - isopropyl β-D-1-thiogalactopyranoside

MCO – Metal Catalyzed Oxidation

RFU - relative fluorescence units

rpm - rotation per minute

SDS PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TB – Terrific Broth medium

VSV-GFP - vesicular stomatitis virus encoding green fluorescent protein

WST-1 - 4-[3-(4-Iodophényl)-2-(4-nitrophényl)-2H-5-tétrazolio]-1,3-benzene disulfonate

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Introduction

The use of therapeutic proteins for the treatment of human diseases and medical conditions has increased exponentially since human insulin, the first recombinant protein, created at Genentech, was approved by FDA in 1982. Since then, there was a great development of technologies, which allowed the production of innumerous therapeutic proteins such as antibody-based drugs, blood factors, enzymes, growth factors, hormones, thrombolytics, anticoagulants and interferons. These proteins have shown to be an effective solution to reduce the progression and morbidity of diseases such as diabetes and cancer. [1]

In order to have a good product quality, several parameters must be controlled in all stages of production when manufacturing these proteins. One of the most challenging tasks is being able to manage their physical and chemical instability. Usually they are stored under low temperature or lyophilized so that they may have a larger lifespan. Most therapeutic proteins are injected instead of being taken orally, thus avoiding extreme conditions of the stomach. A key factor to have in mind is the conformation of the protein which with time and certain conditions may start losing its conformation, meaning that secondary, tertiary and quaternary structure protein may change, resulting in misfolding or aggregation.

Aggregation can take place in various steps of its manufacturing process or during storage. It is a very important factor to be considered when developing therapeutic protein since aggregates have little or no activity, low solubility and it leads to immunogenicity. Aggregation can be the result of several factors such as protein concentration, ionic strength, temperature, light, pH, the type and concentration of the buffer and metal ions, and it can be caused during the various processing steps during the manufacture like expression, purification and storage.[2]

1.1. Aggregation Problem

Aggregation of proteins are typically accompanied by large changes in secondary and tertiary structures of the protein. These changes will jeopardize the safety, efficacy and toxicity of the final product formulation. In order to be active and not evoke immune reactions after parenteral injection to the patient, therapeutic proteins must be conserved in their native conformation. Aggregation leads to immunogenicity, i.e., the immune response from the human body against a particular substance and this response can be life threatening. Because of this effect, it is an important risk factor that must always be considered when assessing product quality. Therefore, to be able to stabilize protein formulations, possible pathways of degradation must be investigated and the resulting products characterized. [3, 4]

1.2. The Human Interferons

The chosen therapeutic protein for this project belongs to the class of human interferons (huIFN), which are a group of signaling proteins made by the cells of the immune system in response to the presence of pathogens, such as viruses and bacteria.

Interferons are divided in three different classes: type I, type II and type III. There are three different interferons in type I: IFN-α, IFN-β and IFN-ω. These interferons are produced by leukocytes, fibroblasts and monocytes. They have antiviral properties and are produced when the body recognizes the presence of viruses. This type of interferon interacts specifically with cell surface complex receptor, IFNAR, which has two subunits IFNAR-1 and IFNAR-2. The interaction between IFN-α and its receptors results in the activation of a number of signaling pathways that will trigger the protective defenses of the immune system.[5]

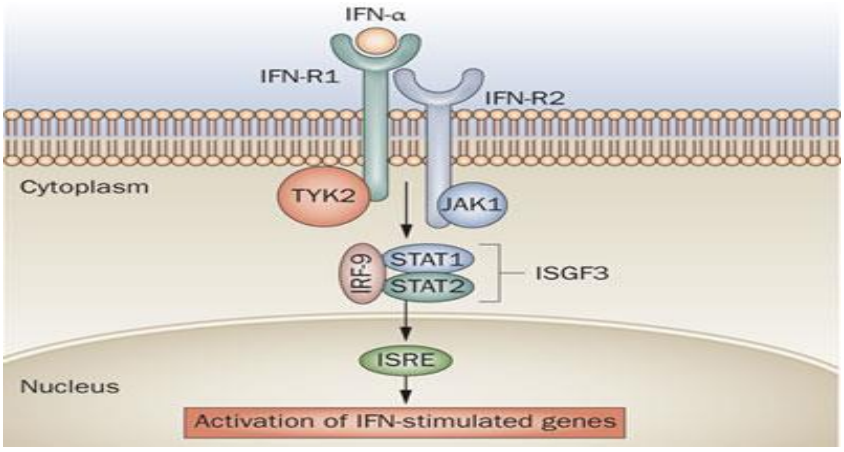

Figure 1. Production and Action of Type I Interferons. (Bekisz, Schmeisser et al. 2004)

Type II interferon comprises only INF-γ. It is produced by lymphocyte and are involved in the regulation of inflammatory and immune responses. This type of interferon interacts specifically with cell surface complex receptor, IFNGR, which has two subunits IFNGR1 and IFNGR2. [6]

Type III interferon comprises three IFN-λ: IFN-λ1, IFN-λ2 and IFN-λ3. They are produced by monocyte-derived dendritic cells and plasmacytoid dendritic cells and are also involved in antiviral response of the immune system. [7, 8]

1.3. Recombinant human Interferon alpha (rhIFN-α)

In this project I am going to work with a recombinant human interferon alpha, the rhIFN α-2b, which is a drug used in the treatment of diseases such as hepatitis and cancer, first introduced in 1986 by Merck Sharp and Dohme Corp with the branding name Intron A. It consists of 165 amino acids sequence and its secondary structure is composed by five α-helices with two disulfide bonds between Cys^{1}-Cys^{98} and Cys^{29}-Cys^{138} (Figure 2). [9]

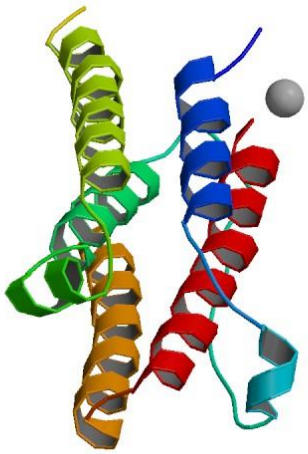

Figure 2. Structure of rhIFN **α** 2b (**DOI**: 10.2210/pdb1rh2/pdb)

1.4. Aim of the project

In this work I will study the stability of the rhIFNα-2b in different conditions and characterize the resulting products. It will be done using the following techniques: transfect *E. coli* strain with the plasmid containing the gene of the protein of interest. The expression will be done under specific growth conditions (medium, temperature, etc.), then the protein will be purified using various techniques such as affinity and size exclusion chromatography, SDS-PAGE, and Nanodrop to quantify the protein. The stability study of rhIFNα-2b in its native state and after various stress conditions will be done using four different methods: Dynamic light scattering to measure the size of native protein and aggregates, fluorescence spectroscopy to visualize the modifications occurred in the protein before and after stress conditions, circular dichroism to predict the secondary structure and scanning electron microscopy to visualize the native and aggregated protein. The last part of my work will involve a viability and a bioactivity assay.

2. Materials and Methods

2.1. Competent cells

The Eppendorf containing the *E. coli* Origami 2 (DE3) pLysS (kindly donated by Dr. Remo Perozzo) was scratched with a sterilized pipette tip and then the tip was added to a Falcon tube containing 5 mL of TB medium and 5 µL of Chloramphenicol (34mg/mL) without touching its wall. The Falcon tube was then placed in a 37°C incubator with 250 rpm agitation for 7 hours. After 7 hours, it was taken 5 mL from the Falcon tube and added to a 500 mL flask containing 95 mL of TB medium and 200 µL of Chloramphenicol (34 mg/mL). The flask was placed overnight in a 37 °C incubator with 130 rpm agitation.

After one night, the volume (100 mL) of the flask was divided by 2 Falcon tubes (50 mL each) and centrifuged for 10 minutes at 3000 rpm and at a temperature of 4°C. The supernatant was removed and the pellet resuspended with 10 mL $CaCl_2$ 100 mM (kept at 4 °C). The resuspended solution was put on ice for 20 minutes. After 20 minutes the solution was centrifuged for 10 min at 3000 rpm and at a temperature of 4 °C. The supernatant was removed and the pellet was resuspended with 400 µL of 100 mM $CaCl_2$/15% Glycerol. The new solution was divided by Eppendorf tube and the frozen at -80 °C.

2.2. Transfection

An Eppendorf tube containing competent cells was taken from the freezer (-80°C) and put on ice for 5 minutes. When the cells were almost thawed it was added 2 µL of the plasmid containing the gene of interest (pET28b-IFNα2b) and then the tube was kept on ice for 5 minutes. After 5 minutes the cells were heat shocked at 42°C for 30 seconds and immediately put on ice for 2 minutes. After heat shocking the cells, it was added 200 µL of pre-warmed TB medium (37°C) the Eppendorf tube. The tube was then placed in a 37 °C incubator with 600 rpm agitation for 45 minutes.

Transformed bacteria were spread on a kanamycin and chloramphenicol selective plate and placed upside down in an incubator at 37°C overnight. After one night and under laminar flow, one isolated colony from the plate was picked with a pipette tip and placed inside a Falcon tube with 10 mL of TB medium, 10 µL of Kanamycin (50 mg/mL) and 20 µL of Chloramphenicol (34 mg/mL). The tube was put in the incubator at 37°C for 7 hours under 250 rpm orbital shaking.

2.3. Expression

After 7 hours, it was added 1 mL of the transformed culture to a 2L flask containing 1L of TB media complemented with 2 mM $MgSO_4$, 1mL of Kanamycin (50 mg/mL) and 1 mL of Chloramphenicol (34 mg/mL). The flask was placed in an incubator at 37°C under 170 rpm orbital shaking overnight. After one night, the incubator was cooled down from 37°C to 25°C. When 25 °C were reached it was made the induction with 1 mM IPTG and the flask were kept at 25°C with 170 rpm orbital shaking for 24 hours. 24 hours after being inducted, the bacterial culture was harvested by centrifugation at 5,000rpm for 20min at 4°C, the supernatant was discarded and the bacterial pellet was collected to a Falcon tube and frozen at -20 °C.

2.4. Purification

The Falcon tube containing the bacterial pellet was filled up to 40 mL with lysis buffer (solution containing 48 mL buffer A (20 mM Tris-HCl + 0.5 M NaCl, pH 8.0), 2 mL buffer B (buffer A + 0.5M imidazole, pH 8.0), 18 mg of Lysozyme (Sigma), 1 mL of Triton® X-100 (20 %) and with a spatula it was added a small amount of Deoxyribonuclease I (Sigma). The solution was kept in a rolling machine for 1hour at 4 °C.

After 1 hours, the lysed solution was centrifuged at 15,000 rpm for 40 minutes at 4°C. The supernatant was filtered on 0.45 µm nylon filter and the filtrate was applied to a His-Trap FF 5 mL column, previously equilibrate with buffer A using the Äkta protein purification system (GE Healthcare Life Sciences). Contaminants were washed away with 5 CV of 4% buffer B (20 mM

imidazole). Gradient elution was done from 4 to 100 % buffer B during 15 CV at a flow rate of 2 mL/min. The collected fractions were analyzed on 15% SDS-PAGE (200 Volts for 50 minutes) and the fractions that contained rhIFNα2b protein were concentrated using a Corning® spinX® UF concentrator (centrifugation at 6°C for 30 minutes at 4,000 rpm) until 1 mL remained. Concentrated protein was applied on Superdex 75 10/300 GL column (GE Healthcare Life Sciences), previously equilibrate with formulation buffer (130 mM NaCl + 10.8 mM NaH_2PO_4 + 12.6 mM Na_2HPO_4 + 0.1 mg/mL EDTA, pH 6.75). The collected fractions were analyzed on 15% SDS-PAGE (200 Volts for 50 minutes). The fractions that contained rhIFNα2b were kept and quantified with the Nanodrop spectrophotometer. To avoid aggregation Polysorbate 80 (0.1 mg/mL) were added to the protein solution and it was frozen at - 20 °C.

2.5. Thrombin Digestion

Since the protein being studied has a His-tag (see appendix page 22) and between the tag and the protein sequence there is a thrombin cleavage site, it was made this experiment to study the contaminants presents in the purified protein solution.

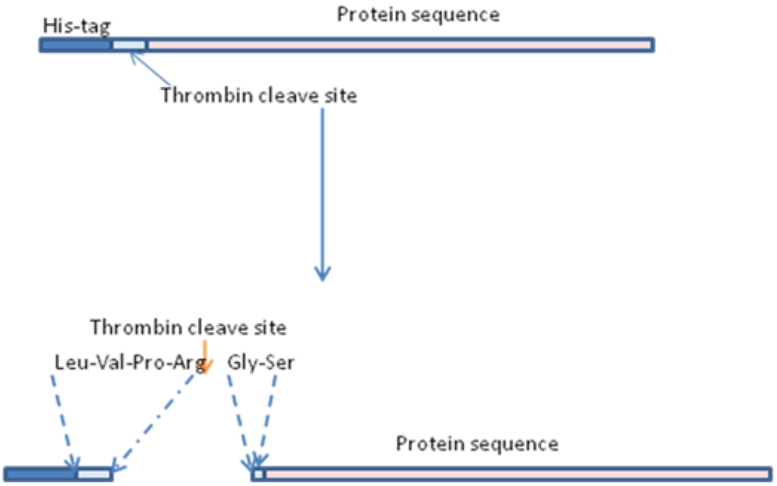

Figure 3. His - tagged Protein with Thrombin cleavage site. (Indian Institute of Science)

The sample was prepared by taking 485 µL of a rhIFNα-2b solution at 0.6 mg/mL and mixed in an Eppendorf tube with 5 µL of $CaCl_2$ 100 mM and 10 µL of the thrombin solution (1 U/mL). It was kept at 37 °C in a heating place with a 300 rpm agitation. It was also prepared in an Eppendorf tube a blank containing 485 µL of formulation buffer, with 5 µL of $CaCl_2$ 100 mM and 10 µL of the thrombin solution (1 U/mL). It was collected a first sample of 10 µL from the tube with the rhIFNα-2b immediately after finishing the preparation and added to another Eppendorf tube with 10 µL of 3X sample buffer and the solution was kept on room temperature. Samples were collected at T0, T1, T2, T3, t4 and T5. After 5 hours the samples were analyzed electrophoresis (SDS PAGE 15%, 200 Volts and 50 minutes). (Protocol developed by Dr. Remo Perozzo)

2.6. Degradation of rhIFNα-2b

For each stress condition it was used a protein solution of 0.1 mg/mL. The frozen protein solution (1 mg/mL) kept at - 20°C was thawed in a 37 °C bath for 3 minutes. Dilution was made by taking 0,1 mL of the protein solution at a concentration of 1 mg/mL and diluting it to 0.1 mg/mL with 0.9 mL of formulation buffer (130 mM NaCl + 10.8 mM NaH_2PO_4 + 12.6mM Na_2HPO_4 + 0.1 mg/mL EDTA, pH 6.75 + 0.1 mg/mL Polysorbate 80) in an Eppendorf tube.

2.6.1. Metal Catalyzed Oxidation

1 mL of the diluted rhIFNα-2b solution was added to four 3 mL glass vials. The protein was oxidized by the addition of 5 µL $CuSO_4$ 0.1 M and 4 µL of ascorbic acid 0.1 M. The solution was incubated at 37 °C overnight. After one night, the reaction was stopped by the addition of 5 µL EDTA 0.1M. The oxidized protein was dialyzed overnight against the formulation buffer using a Float-A-Lyzer®G2 (MWCO 3.5-5kD) [9]

2.6.2. Stirring Stress

0.5 ml of the diluted rhIFNα-2b solution was added to four 3 mL glass vials containing one 1.1mm x 0.7mm magnetic stirrer. It was then stirred for 3 days under 1200 rpm agitation and at 4°C. [9]

2.6.3. Thermal Stress

1 mL of the diluted rhIFNα-2b solution was added to four 3 mL glass vials RhIFNa2b solutions. The vials were placed in a 64 °C bath for 1h and then incubated at room temperature for 1h. [10]

2.6.4. Cross-linking

Crosslinking was done with a glutaraldehyde concentration of 0.02% and with a protein concentration of 0.1 mg/mL (5.18 µM).**[11]**

Glutaraldehyde solution (1%) - 10 µL of Glutaraldehyde (25%) (Sigma Aldrich) were diluted with 240 µL miliq H_2O

Solution of 0.1M of sodium borohydride ($NaBH_4$) - 3.78 mg of $NaBH_4$ (Sigma Aldrich) were dissolved with 1 mL of miliq H_2O and the solution was kept on ice.

It was used 4 Eppendorf tubes with 1 mL of the protein solution (0.1 mg/mL). To each tube it was added 20 µL of glutaraldehyde (1%), quickly vortexed and incubated at room temperature for 2 minutes. After 2 minutes it was added 6.12 µL of $NaBH_4$ 0.1M**,** quickly vortexed and incubated at room temperature for 20 hours. The cross-linked protein was dialyzed overnight against the formulation buffer using a Float-A-Lyzer®G2 (MWCO 3.5-5kD). [9]

2.7. Characterization of rhIFNα-2b

2.7.1. Dynamic Light Scattering (DLS)

Dynamic light scattering is a non-invasive technique that allows the measurement of particles or molecules in suspension. The measurement is made possible due to the different intensities of the scattered light caused by the Brownian motion of particles and molecules in suspension. This technique allows us to know if it was obtained any aggregate after the stress conditions and their sizes. [12] All samples (120 µL) were analyzed using a Zetasizer Nano ZS (Malvern). After 10 seconds of equilibration, it was made 3 cycles of 11 measurements with 10 seconds each. The angle of detection was fixed at 173 ° and it was used a disposable UV- cuvette micro (Brand).

2.7.2. Intrinsic Fluorescence Spectroscopy

Fluorescence spectroscopy allows us to visualize any modification that may occur in the protein tertiary structure. The principle of this technique is based on the intensity of the fluorescence emitted by a substance after it has been excited by a beam of light (visible or UV). There are three amino acids with native fluorescence: Tryptophan, Tyrosine and phenylalanine. Of the three, Tryptophan is the most fluorescent and the only one present in the studied protein.

Its residues are excited at wavelengths between 275 and 305 nm and its emission are observed at 340 nm.[12] All measurements were done using a Fluoromax-4 (Horiba Jobin Yvon Inc.) at room temperature. Samples were measured with a 1 cm quartz cuvette (Hellma). Excitation was set to 295 nm, slits 4, emission from 300 to 380 nm, integration time 1s and accumulation 3. All samples were analyzed at a concentration of 2.85 µM.

2.7.3. Fluorescence Anisotropy

Fluorescence anisotropy is a useful tool to visualize changes in the protein size. Since it measures the rotational diffusion of a molecule, when there is a change in size, for example the aggregation, the larger product will have a lower rotational diffusion coefficient and thus a higher fluorescence anisotropy. [13] Fluorescence anisotropy was done using a Fluoromax-4 (Horiba Jobin Yvon Inc.) at room temperature. Samples were measured with a 1 cm quartz cuvette (Hellma). Excitation was set to 295 nm, slits 4, emission from 330 to 370 nm, integration time 10s and accumulation 1. All samples were analyzed at a concentration of 2.85 µM.

2.7.4. Circular Dichroism

Circular dichroism is a technique that measures the difference between the right and left polarized light. The different absorptions of light (near and far UV) give information about the secondary and tertiary structure of the proteins. The far-UV (180 – 250 nm) spectra give information about the secondary structure and the near-UV (250 nm – visible light) about the tertiary structure of the protein.

The rhIFNα-2b is mainly composed by 5 alpha helix [9] and the characteristic spectra for the alpha helix is given by 2 negative pics: 208 and 222 nm, approximately.[14] So, in this work it will be only used the far-UV (between 200 and 260 nm) to study the protein before and after the stress conditions.

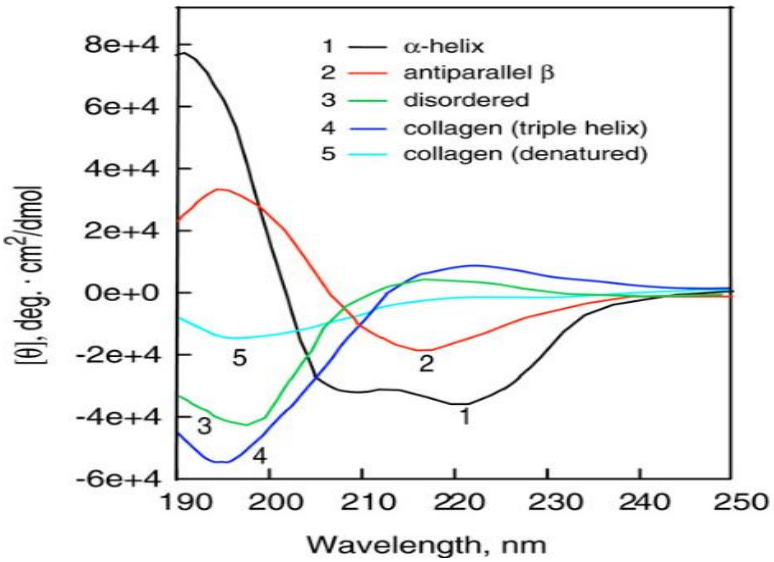

Figure 4. Circular Dichroism Spectra of different structures (Greenfield, N. J. (2006))

All spectra were recorded using a Jasco J-815 (Jasco Inc.) spectrophotometer, a 1mm quartz cuvette (Starna Gmbh, ref. 21-Q-1). The chosen parameters were: sensivity – standard; start- 260 nm; end – 200 nm; Data pitch – 1nm and accumulation – 3. All samples were analyzed at a concentration of 8 µM.

2.7.5. Scanning Electron Microscopy (SEM)

The SEM is a tools that allows us to see particles and molecules in a scale up to 1 micron. It uses a focused beam of electrons that interact with atoms in the sample and produces various signal which will be detected by specific detector and give information about the sample's composition and surface topography. Since nonconductive samples tend to charge, it is necessary to coat the sample with an electrically conductive material such as gold.
All samples were dried overnight using a desiccator and coated with a 20 nm layer of gold using a Leica EM SCD500 coating machine. The SEM used in this work it was a JEOL JSM-7001F.

2.8. Viability Assay using WST-1

2.8.1. Principle

This is a colorimetric assay where the tetrazolium salt WST-1 is converted into a formazan dye by mitochondrial dehydrogenase enzymes present in the respiratory chain of the living cells.

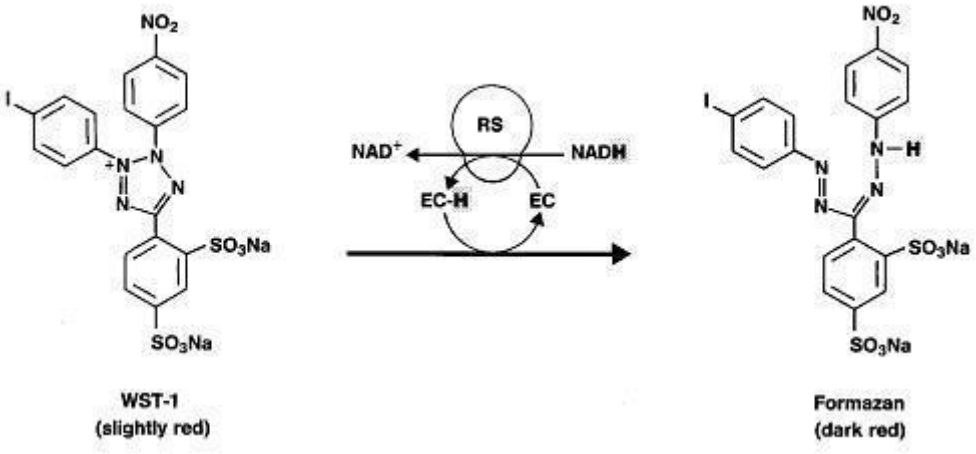

Figure 5. Cleavage of the WST-1 to formazan catalyzed by the NADH (Roche Applied Sciences)

This converted salt is then released into the media and it will be possible to observe a color change after a certain time. The formazan dye has a maximal absorbance at 450 nm and this absorbance is proportional to the number of viable cell in a given culture. The absorbance measured at 450 nm is subtracted to the one measured at 690 nm (wavelength in which the formazan has no absorbance).

This assay had the objective of studying a possible cytotoxic effect of glutaraldehyde on the cells during the bioactivity assay.

2.8.2. Protocol

The medium used was the F12 HEPES without phenol red (Gibco Life-Tech). With a final volume of 500 mL, the medium was supplemented with 4mM of glutamine, 10 % v/v of fetal calf serum (FCS), 1% v/v of penicillin and 1% v/v of streptomycin. The cell line used in the assay was the A549 (ATCC), which are adenocarcinomic human alveolar basal epithelial cells.

The cells were thawed in a 37°C bath for 2 min. it was taken 1 mL of the cells and added to a Falcon tube containing 9 mL of the supplemented medium. It was centrifuged and the supernatant discarded. The pellet was resuspended with 1 mL of the supplemented medium and then it was taken 1 mL of the solution and added to a T75 flask containing 15 mL of the supplemented medium. The flask was placed in a 37°C incubator for 1 week for cell proliferation.

The proliferated cells were attached to the flask so it was used trypsin to remove them. First the medium was aspirated and then it was added 5 ml of PBS to the flask with attached cells. The flask was placed in a 37 °C incubator for 3 minutes. After 3 minutes the PBS was aspirated and it was added 2 mL of trypsin (5X) to the flask. The flask was then placed in a 37° incubator for 5 minutes. After 5 minutes the cells were counted using a hemocytometer.

After counting the cells, they were diluted with supplemented medium to a cell concentration of $1x10^5$. It was then added 100 µL of the diluted cells to each one of the 96 wells in the plate except for the wells around the plate to avoid the border effect and three others to use as a blank. As positive control it was used the cells. As negative control it was added 1 µL of SDS (20%) in order to kill the cells and the blank was the medium.

The assay was started with a protein concentration of 288 ng/mL. First the dialyzed cross-linked protein was diluted with the medium to 576 ng/mL. Then it was taken 100 µL of the diluted cross-linked protein and added to the second row of well (V_T=200 µL; dilution 1:2). It was then made a serial dilution by taking 100 µL from the first addition (V_T=200 µL) and adding to the third row. The process was repeated until the row before the last. The plate was then placed in a 37 °C incubator for 24 hours. After 24 hours the medium was aspirated and then it was added 100 µL of WST-1 (1X) (Roche Applied Sciences) to each well. A first reading, using a ELISA micro-plate reader, was done immediately after adding the WST-1 and 1 hour after being placed in a 37 °C incubator.

2.9. Bioactivity Assay

The bioactivity assay was done in order to understand whether the purified protein had any activity before and after the stress conditions. Since the rhIFNα-2b has antiviral properties, it was used the vesicular stomatitis virus (VSV) encoding green fluorescent protein (GFP).

The proliferation of the virus leads to an increased concentration of the GFP and thus a higher intensity of the fluorescence which will be analyzed.

Following the previous protocol (viability assay) and after counting the cells, they were diluted with supplemented medium to a cell concentration of 1x105. It was then added 100 µL of the diluted cells to each one of the 96 wells except the first and last row that just had cells and three lines from the first row that had only the medium as a blank. We made triplicate for every sample, being them: blank, negative control (cells without protein), positive control (cell with commercial version of the protein), 2nd positive control (cell with purified protein) and protein after stress conditions.

The assay was started with a protein concentration of 19.25 ng/mL. It was made a serial dilution in several Eppendorf tubes containing 100 µL of medium. From the first tube (19.25 ng/mL with a volume of 200 µL) it was taken 100 µL to another tube with 100 µL of medium. The dilution was repeated 10 times.

It was added 100 µL of each protein solution to each well. The plate was then placed in a 37°C incubator for 10 hours. After 10 hours it was added 100 µL of the diluted virus to each well (3 µL of VSV-GFP at a concentration of 1011 particles/mL diluted with 30 ml of medium).

The plate was then placed overnight in a 33 °C incubator and 5 % CO2. After one night the plates were read using a ELISA micro-plate reader with the following parameters: excitation – 475 nm; emission – 509 nm; gain – 100; read – bottom.

3. Results and discussion

3.1. Expression and Purification

During the purification step it was possible to observe two unexpected contaminants in the SDS PAGE gel. After making a few change in the initial protocol, there was no modification in the final result and the two contaminants were always present.

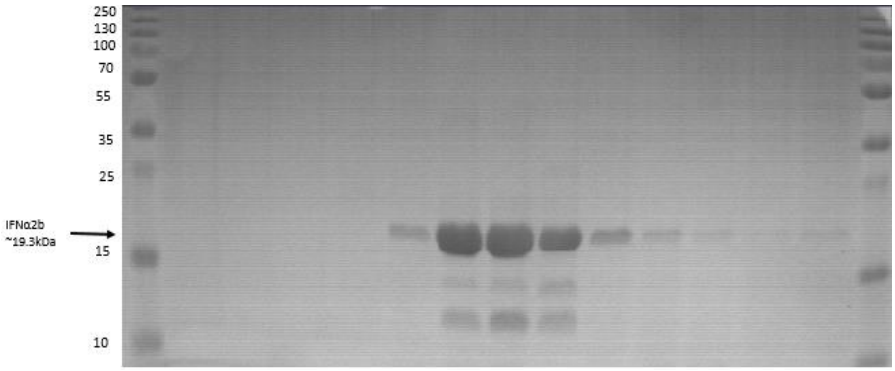

Figure 6. SDS PAGE gel with the purified protein at ~19.3 KDa and two contaminants after size exclusion chromatography.

It was decided that the problem was not coming from the purification step but it was occurring during the expression and that the two contaminants could be fragments of the protein being expressed. In order to know if these contaminants were in fact fragments of the protein, we did a thrombin digestion.

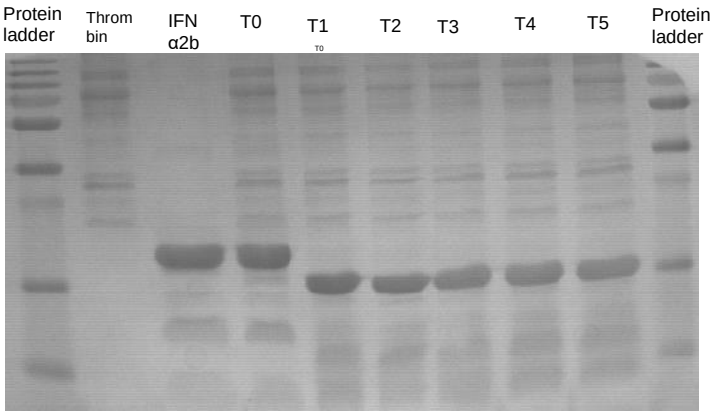

Figure 7. SDS PAGE gel after thrombin digestion. (T represents the time in hour after starting the digestion with thrombin)

After the digestion with thrombin it was possible to see that the molecular weight of the protein decreased as expected since it had the thrombin cleavage site but it was also possible to see that the molecular weight of one of the contaminants decreased. It can only be explained if these contaminants are fragments of the expressed protein and contain the cleavage site for the thrombin. By knowing that there was degradation of the protein during the expression, we started to use a protease inhibitor (P8849 – Sigma Aldrich) in order to avoid the degradation.

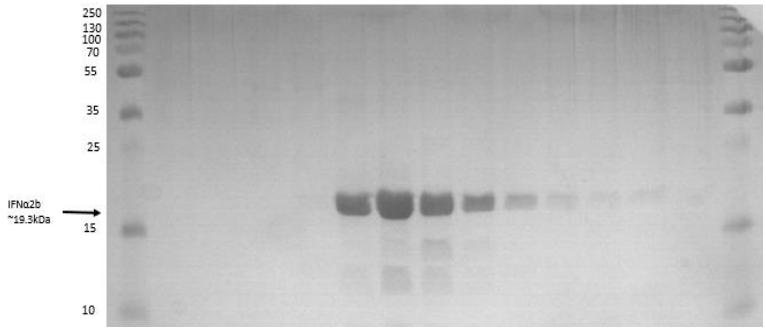

Figure 8. SDS PAGE gel after purification (size exclusion chromatography) using a protease inhibitor.

After purifying the rhIFNα-2b with a protease inhibitor it was possible to see that the two fragments were still present although they were less concentrated and less homogenous. Due to difficulties to choose another strain to express the protein, we preferred to carry on with the work and compare the bioactivity of the expressed protein with a commercial version of the same protein.

3.2. Circular Dichroism

The results obtained for this technique are shown in the following figures. All samples were analyzed at a concentration of 8μM.

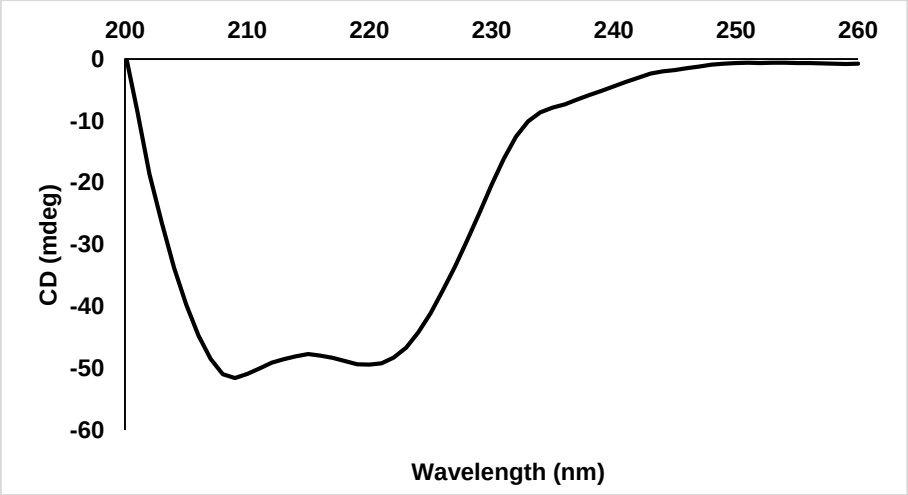

Figure 9. Circular dichroism spectrum of the purified huIFN α-2b 8μM in formulation buffer.

For the purified rhIFNα-2b (native) it was obtained a characteristic spectrum for an alpha-helix structure where we have two negative pics at 208 and 222 nm. This was expected since the protein is composed by 5 alpha-helix. [9]

3.2.1. Melting point

We used the circular dichroism the measure the melting point of the purified rhIFNα-2b.

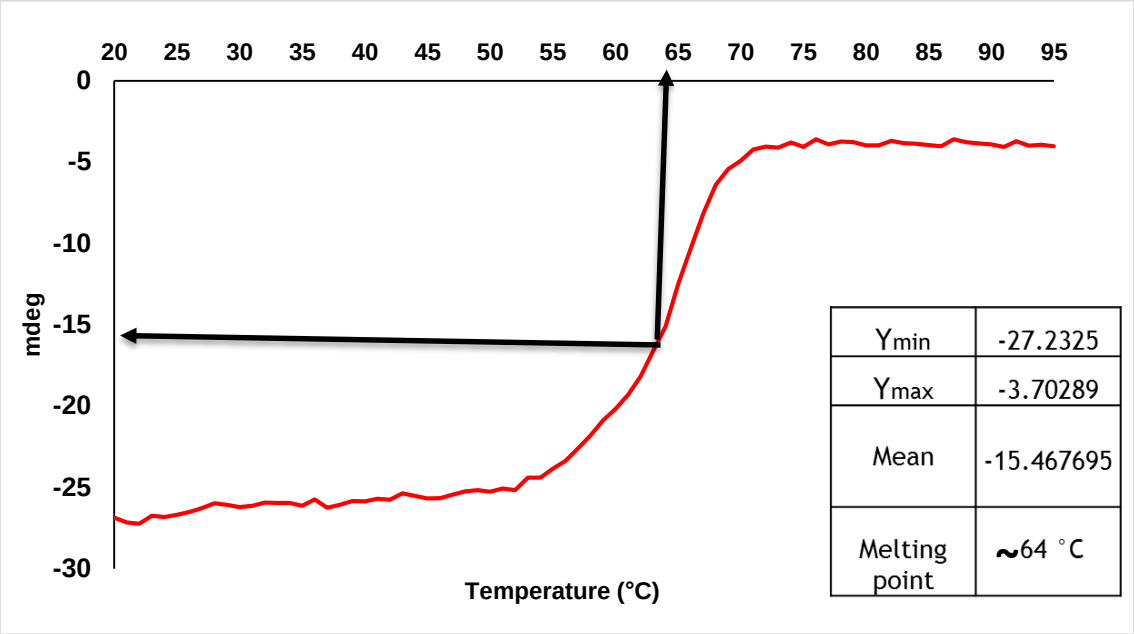

Figure 10. Measurement of the melting point using circular dichroism with huIFN α-2b 8μM in formulation buffer, λ=220 nm, ΔT (20 °C – 95 °C)

The melting point of the purified protein were approximately 64 °C, a value slight higher for the one found in the literature (61°C). (Beldarrain, A. et al., Biochemistry)

3.2.2. Stability study

The results obtained for the native protein (purified) and after the protein was exposed to stress conditions are presented in the following figure.

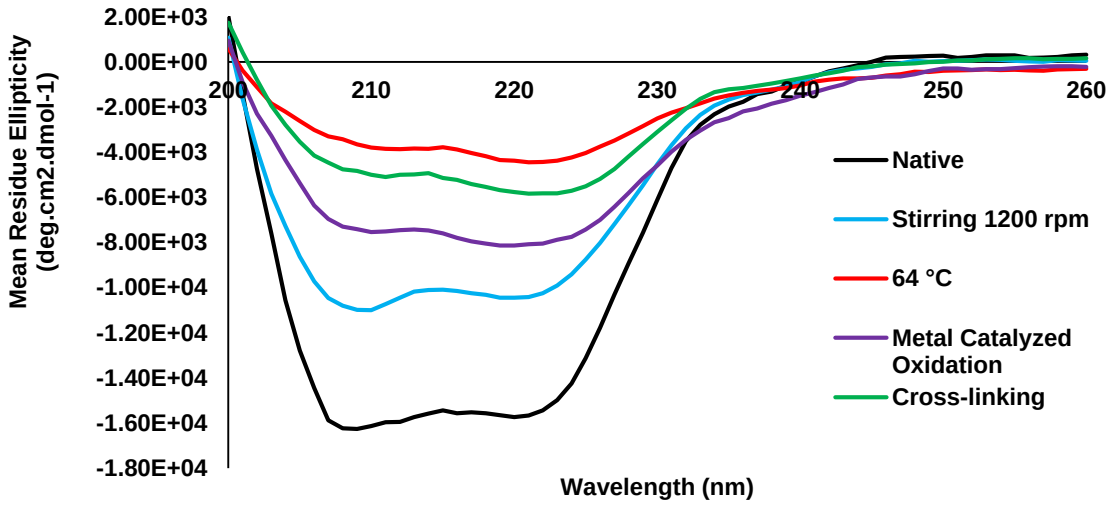

Figure 11. Circular dichroism spectra of huIFN α-2b 5.18 μM in formulation buffer after stress conditions **Black**- Native protein, Red – 64°C bath for 1h, Blue – 1200 rpm agitation for 3 days at 4 °C, Green – Cross-linking with 0.02% glutaraldehyde; Purple – metal catalyzed oxidation. (n=3)

It was possible to observe a great modification of the secondary structure of the protein in all stress conditions. We could see a decrease of the alpha helical content of the protein. Although there was a decrease in the helical content, all samples analyzed had the two negative pics characteristics of the alpha- helix, meaning that they were not completely degraded. The thermal stress was the one in which we could see the greater degradation of the protein. It could be explained by the temperature of the melting point of a protein which is a point where 50 % of all protein are completely denaturated.[15]

3.3. Fluorescence Spectroscopy

The results obtained for the native and the degraded huIFN α-2b are shown in the following figure. All samples were analyzed at a concentration of 2.85µM.

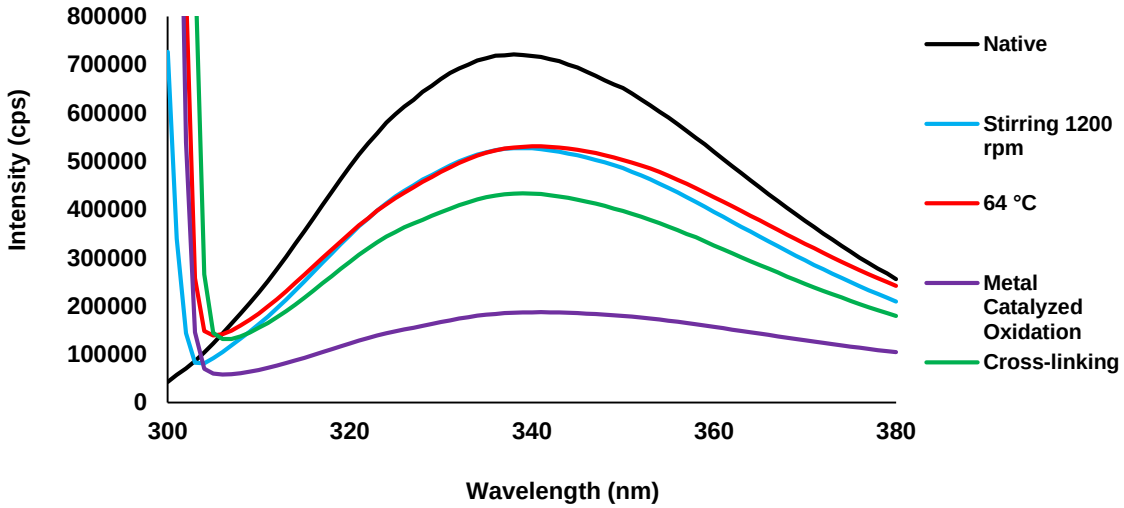

Figure 12. Fluorescence spectra of huIFN α-2b 2.85µM in PBS after stress conditions. **Black**- Native protein, Red – 64°C bath for 1h, Blue – 1200 rpm agitation for 3 days at 4 °C, Green – Cross-linking with 0.02% glutaraldehyde; Purple – metal catalyzed oxidation. (n=3)

The fluorescent spectrum of the huIFN α-2b are given by the two tryptophan residues in the positions 76 and 140. For the native protein, the spectrum obtained shown an intense pic with a maximal absorbance at 338 nm. For all the other conditions to which the protein was applied it was possible to see a decrease in the intensity for all four condition which indicates that there were also modifications in the tertiary structure of the protein leading to a change in the tryptophan environment. This change makes the tryptophan more hidden in the protein structure and it leads to a decrease in the intensity of the fluorescence. For the 64 °C bath, it was possible to see a red-shift (maximal absorbance at 341 nm) meaning that there was a change of the polarity in the environment of at least one of the tryptophan residues.

3.4. Fluorescence Anisotropy

The results obtained for the fluorescence anisotropy are shown in the following figure.

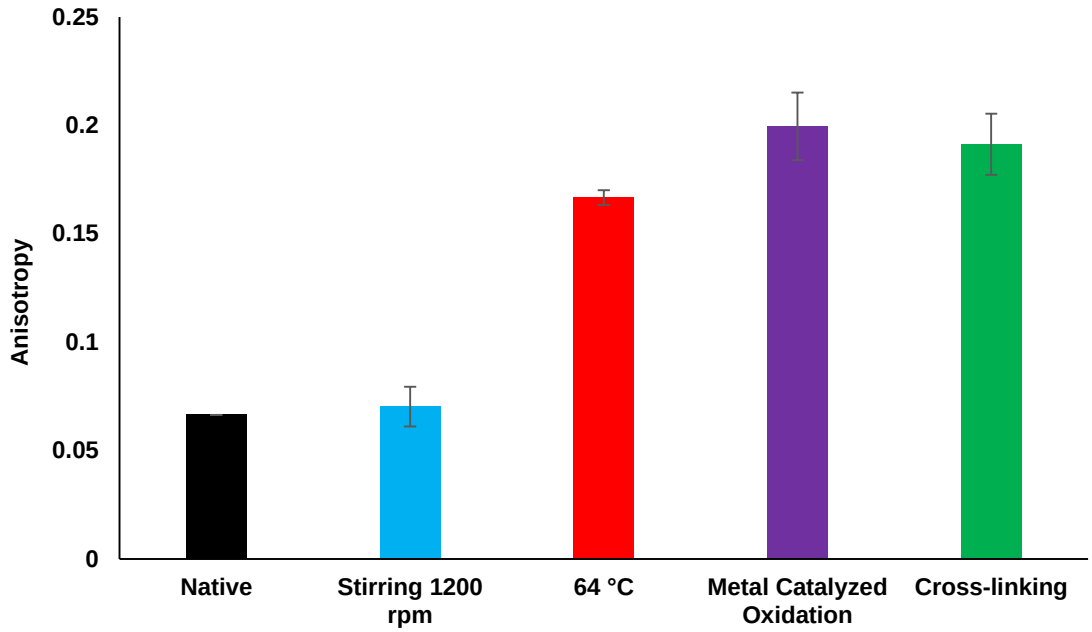

Figure 13. Fluorescence spectra of huIFN α-2b 2.85μM in PBS after stress conditions. **Black**- Native protein, Red – 64°C bath for 1h, Blue – 1200 rpm agitation for 3 days at 4 °C, Green – Cross-linking with 0.02% glutaraldehyde; Purple – metal catalyzed oxidation. (n=3)

The anisotropy increases with size due to a lower rotational diffusion of the molecules. The bigger the molecule the lower the rotation and the higher the anisotropy. By measuring the fluorescence anisotropy, it was possible to visualize that there were changes in the protein size after being applied to the stress conditions. For the metal catalyzed oxidation, cross-linking and thermal stress, it was possible to see a large anisotropy when compared to the native protein. For the stirring stress there was a small change when compared with the native protein, indicating that the aggregates that might had formed were not much bigger than the native protein.

3.5. Dynamic Light Scattering (DLS)

The results obtained for the DLS are shown in the following figure.

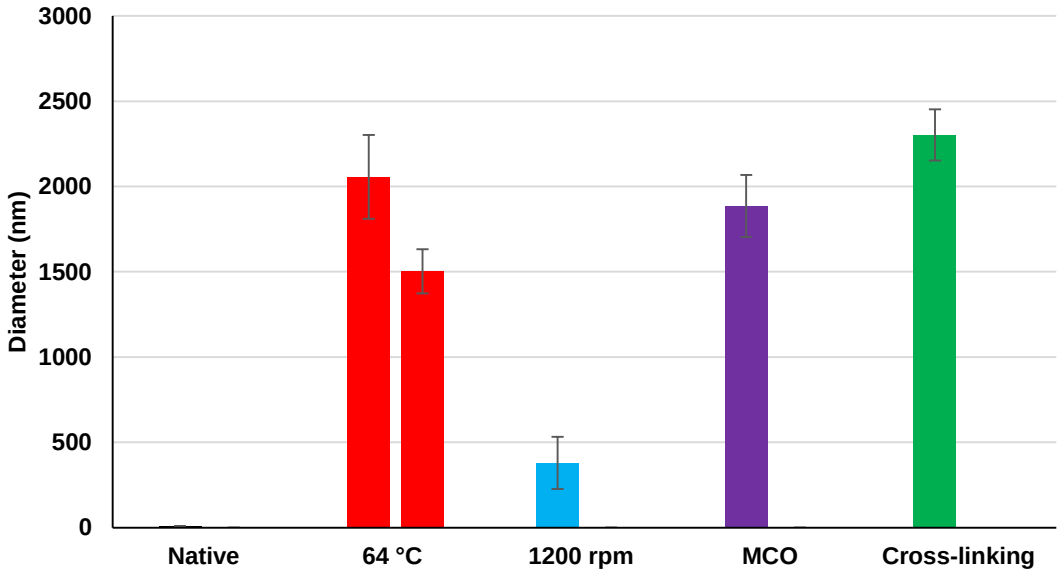

Figure 14. Diameter of huIFN α-2b in PBS obtained using dynamic light scattering after stress conditions. **Black**- Native protein, Red – 64°C bath for 1h, Blue – 1200 rpm agitation for 3 days at 4 °C, Green – Cross-linking with 0.02% glutaraldehyde, Purple – metal catalyzed oxidation. (n=3)

The DLS makes it possible to have an approximated diameter of the studied protein. For the native huIFN α-2b it was obtained an average value of 10 nm. For the thermal stress it was obtained two different populations of molecules with different sizes and being both much bigger than the native protein. For the other three conditions tested, the stirring study gave the smaller molecules. These results are comparable with the ones obtained for the fluorescence anisotropy where it was possible to say that there was a change in size of the protein after being exposed to different stress conditions.

3.6. Scanning Electron Microscopy (SEM)

The results obtained for the SEM are shown in the following figure. Bar scale represents 1 µm.

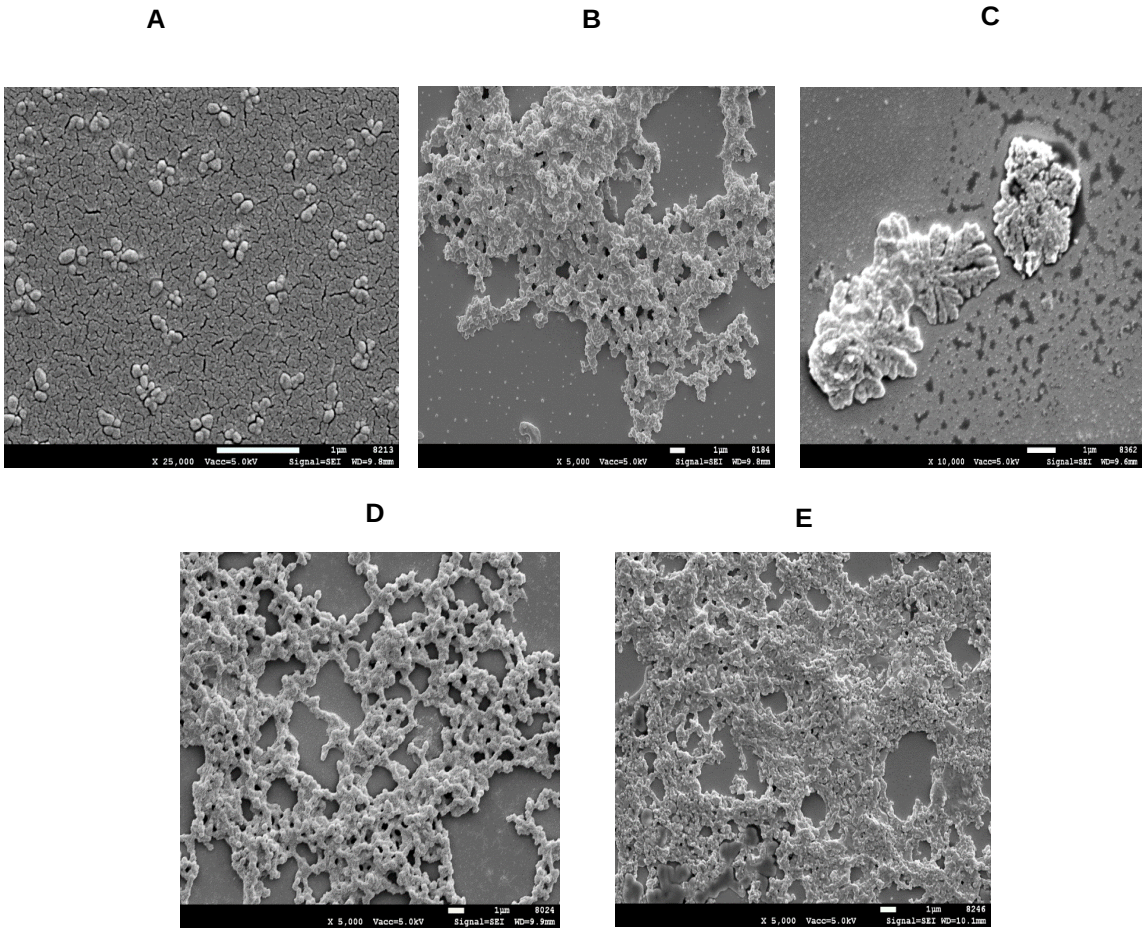

Figure 15. SEM images for the native huIFN α-2b and after stress conditions. **A**- Native protein, **B** – 64°C bath for 1h, **C** – 1200 rpm agitation for 3 days at 4 °C, **D** – Cross-linking with 0.02% glutaraldehyde, **E** – metal catalyzed oxidation.

The results obtained by this technique were once again comparable with the previous results. It was possible to visualize the different shapes and sizes of the protein before and after the stress conditions to which it was exposed to. In figure A we have the native protein and it was possible to see how much smaller it was when compared with the other samples. The thermal and cross-linked protein formed aggregates with similar shapes, while the stirring and metal oxidation formed aggregates with different shapes. This technique also gives an idea of the molecules sizes. The size is an approximated value since the sample preparation is also a stress condition to the protein.

3.7. Viability Assay

The results obtained for the viability assay are shown in the following figure.

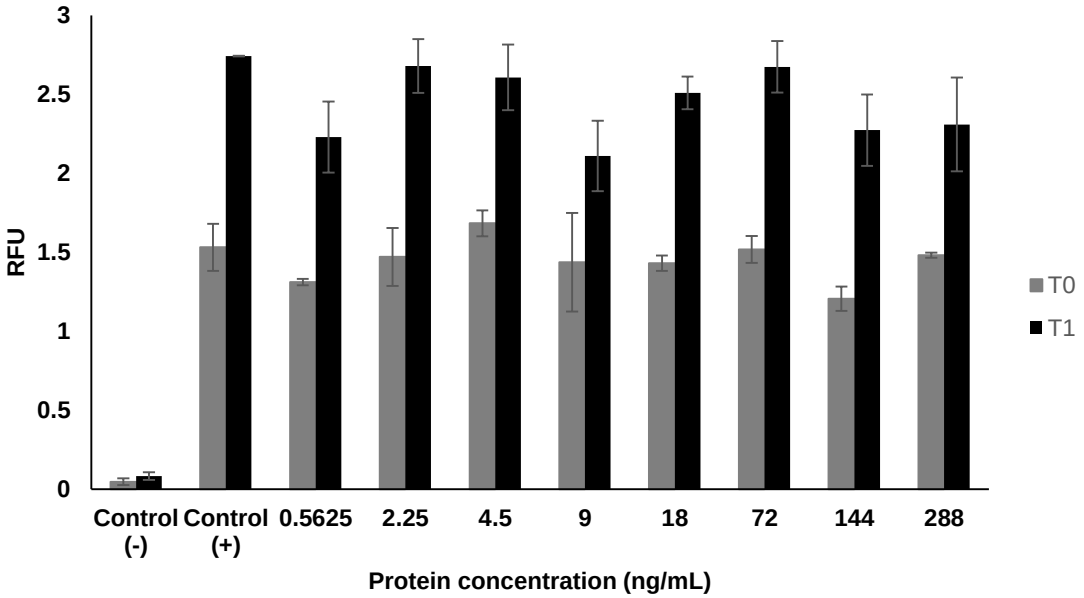

Figure 16. Viability assay of the cross-linked protein with glutaraldehyde measured immediately and after 1 hour. (n=3)

This assay was done in order to control the toxicity of glutaraldehyde to the cell. As said before (materials and methods) the salt WST-1 is converted into a formazan dye by mitochondrial dehydrogenase enzymes present in the respiratory chain of the living cells.

It was two analyzes, one immediately after adding the WTS-1 to the cell and another after 1h at a 37 °C incubator. The results shown that if there was still any glutaraldehyde in solution after the dialyses, it was not toxic to the cell. In the negative control we saw no change in the intensity of the signal, which was expected since the cell were killed after the addition of SDS. For the positive control (only cells) and the other samples (cross-linked protein) the results obtained were similar, showing an increased intensity after 1 hour which means that the cells were alive and converting the WST-1 in the formazan dye.

3.8. Bioactivity Assay

The results obtained for the bioactivity assay are shown in the following figure.

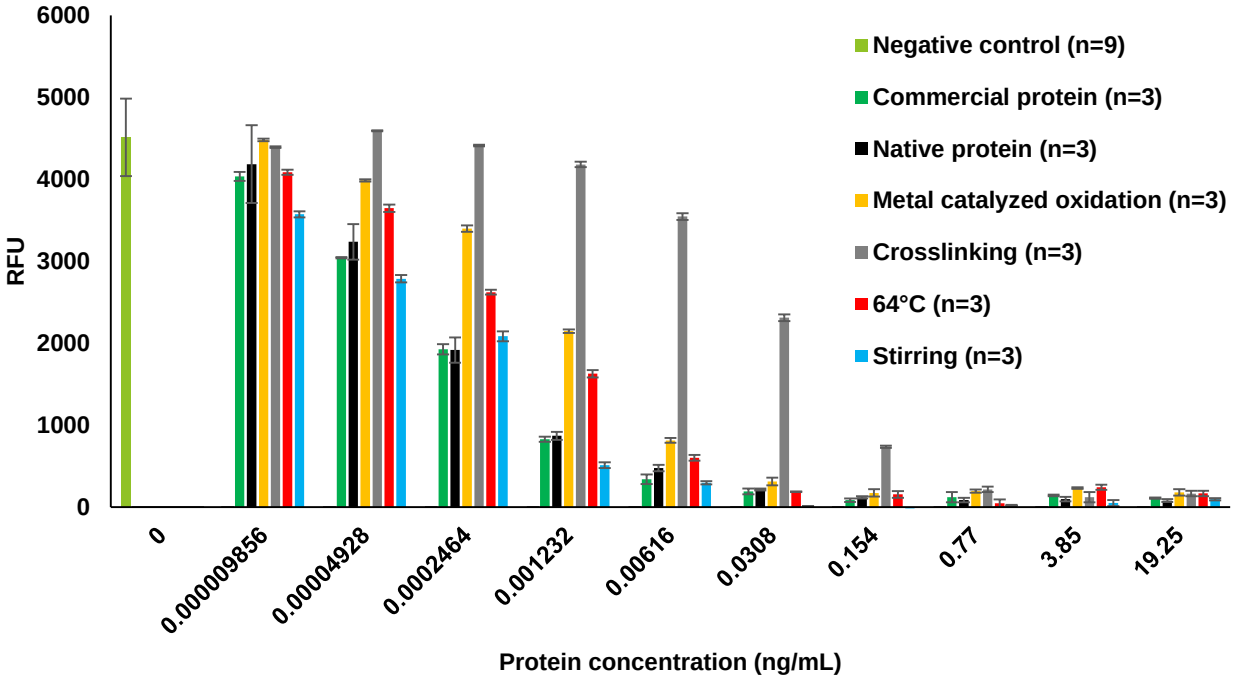

Figure 17. Bioactivity assay of purified huIFN α-2b and the protein after stress conditions and the commercial version of the huIFN α-2b. (n=3)

The bioactivity was made in order to compare the activity of the purified huIFN α-2b and the commercial version of the huIFN α-2b. It was also possible to study the activity of the huIFN α-2b after it was exposed to different stress conditions.

The results obtained showed that the purified and commercial version of the huIFN α-2b had a similar bioactivity and thus protecting the cells against the infection with the virus. It was also possible to see that the protein exposed to stress conditions also kept some of its activity being the cross-linked protein the less active and with a higher rate of cell infection.

4. Conclusion and outlook

After making all these experiments it is possible to conclude that:

- the rhIFNα-2b loses its stability in all tested conditions and all samples had aggregates.
- it was possible to observe changes in both secondary and tertiary structure.
- We also saw that the purified protein had a similar activity with its commercial version even if it had some fragments of degraded protein.
- The stress conditions lead to the formation of aggregates and some loss of activity.
- At a higher concentration of protein even the protein exposed to stress conditions were able to protect the cell against the viral infection.

This results showed that aggregation of proteins is an important factor to be controlled during the production of therapeutic proteins since it leads to changes in the structure and activity of proteins.

For future works it would be interesting to study other conditions and also study the immune reactions caused by the aggregates when injected in patients.

5. Bibliography

1. Carter, P.J., *Introduction to current and future protein therapeutics: a protein engineering perspective.* Exp Cell Res, 2011. **317**(9): p. 1261-9.
2. Roberts, W.W., *Aggregation of Therapeutic Proteins*. 2010, New Jersey, USA: Wiley. 515.
3. Rosenberg, A.S., *Effects of Protein Aggregates: An Immunologic Perspective.* The AAPS Journal, 2006: p. 7.
4. Tyedmers, J., Mogk, A., and Bukau, B., *Cellular strategies for controlling protein aggregation.* Nat Rev Mol Cell Biol, 2010. **11**(11): p. 777-88.
5. Bekisz, J., et al., *Human interferons alpha, beta and omega.* Growth Factors, 2004. **22**(4): p. 243-51.
6. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
7. Ank, N., et al., *Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo.* J Virol, 2006. **80**(9): p. 4501-9.
8. Yin, Z., et al., *Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells.* J Immunol, 2012. **189**(6): p. 2735-45.
9. Asmana, R., *Human interferon alpha-2b: a therapeutic protein for cancer treatment.* Scientifica (Cairo), 2014. **2014**: p. 970315.
10. Diress, A., et al., *Study of aggregation, denaturation and reduction of interferon alpha-2 products by size-exclusion high-performance liquid chromatography with fluorescence detection and biological assays.* J Chromatogr A, 2010. **1217**(19): p. 3297-306.
11. Hermeling, S., Mirjam, L., Damen, A., Monique, H., Jiskoot, W., *Structural characterization and immunogenicity in wildtype and immunetolerant mice of degraded recombinanthuman interferon alpha2b.* Pharmaceutical Research,, 2005.
12. Kamerzell, T.J., et al., *Protein–excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development.* Advanced Drug Delivery Reviews, 2011. **63**(13): p. 1118-1159.
13. Pollard, T.D., *A guide to simple and informative binding assays.* Mol Biol Cell, 2010. **21**(23): p. 4061-7.
14. Greenfield, N.J., *Using circular dichroism spectra to estimate protein secondary structure.* Nat Protoc, 2006. **1**(6): p. 2876-90.
15. Ku, T., et al., *Predicting melting temperature directly from protein sequences.* Comput Biol Chem, 2009. **33**(6): p. 445-50.

6. Appendix

Appendix 1. pET28b + IFNa2b plasmidic card