Tutor/s

Dr. Miquel Pons Vallès
Departament de Química Inorgànica i Orgànica. Secció de Química Orgànica.

Grau de Química

Treball Final de Grau

Autoproteolysis of the Unique domain of Src family proteins
Autoproteòlisi del domini Únic de proteïnes de la família Src

Carles Font Nomen

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M’agradaria agrair l’oportunitat d’haver pogut col·laborar en el grup d’investigació Biomolecular NMR del Dr. Miquel Pons de la UB

Agrair al Dr. Miquel Pons per estar sempre disposat a ajudar i haver-me introduït en el món de la bioquímica, el qual era bastant desconegut per a mi. A la Laareb per ajudar-me durant tot el treball i per la paciència que ha tingut amb mi i els meus múltiples dubtes. I finalment, a l’Hector i el Guille, la resta de companys del grup de recerca, pel bon ambient de treball que han aconseguit i per estar sempre disposats a donar-me un cop de mà quan els hi demanés.

REPORT

CONTENTS

1. SUMMARY

Src is a non-receptor tyrosine kinase which participates in many cell signalling pathways and its overactivation and overexpression are related to various types of human cancer. Its structure consists, from the N-terminus to the C-terminus, of a myristoyl group connected to the SH4 domain, a Unique domain, the SH3 domain, the SH2 domain, the SH1 domain and a regulatory segment. A domain is a section of the tertiary structure of a protein that has a defined function.

The BioNMR Group lead by Dr. Miquel Pons at the University of Barcelona has discovered that the intrinsically disordered region of the c-Src protein, called Unique domain, is a regulatory element: for example, changing one amino acid can decrease the invasive capacity of a type of colon cancer cells by 50%.

The proteolysis of the Src Unique domain after stroke converts this protein from neuroprotective to neurotoxic. This proteolysis is catalysed by the calpain protease. Protease-independent spontaneous proteolysis of the Unique domain in the same position has been observed in previous attempts to study the glycosylation of Src. It was hypothesized that this observation was related to environmental conditions that influence the interactions between the SH4 domain and the SH3 domain that cause conformational changes in the Unique domain.

In this work we optimized conditions to study glycosylation avoiding degradation and studied the interaction of $USH3^{Src}$ with the glycolysis enzyme OGT.

Keywords: Src, tyrosine kinase, proteolysis, expression and purification of proteins, O-GlcNAcylation.

2. RESUM

El Src és una tirosina-cinasa no receptora, relacionada amb diversos tipus de càncer humà degut a la seva sobreexpressió i sobreactivació, i que participa en moltes vies de senyalització cel·lular. La seva estructura consisteix en, des de l'extrem N-terminal fins al C-terminal, d'un grup miristoil unit al domini SH4, un domini Únic, el domini SH3, el domini SH2, el domini SH1 i un segment regulador. Un domini és una secció de l'estructura tridimensional d'una proteïna que té una funció concreta.

El Grup BioNMR dirigit pel Dr. Miquel Pons a la Universitat de Barcelona ha descobert que la regió intrínsecament desordenada de la proteïna Src, anomenada domini Únic, és un element de regulació: per exemple, el canvi d'un sol aminoàcid en aquesta regió pot disminuir en un 50% la capacitat invasiva d'un tipus de cèl·lules de càncer de colon.

La proteòlisi del domini Únic del Src al patir un ictus transforma aquesta proteïna de neuroprotectora a neurotòxica. Aquesta proteòlisi és catalitzada per la proteasa calpain. S'han observat proteòlisis espontànies independentment de la presència de proteases externes en la mateixa posició en els intents previs d'estudiar la glicosilació de Src. S'ha arribat a la hipòtesi que aquestes observacions semblen dependre de condicions ambientals que afecten les interaccions entre el domini SH4 i el domini SH3, que provoquen canvis conformacionals en el domini Únic.

En aquest treball s'han optimitzat les condicions per poder estudiar la glicosilació evitant la degradació de la proteïna i s'ha observat la interacció del $USH3^{Src}$ amb el OGT, l'enzim de la glicòlisis.

Paraules clau: Src, tirosina-cinasa, proteòlisi, expressió i purificació de proteïnes, O-GlcNAcetilació.

3. INTRODUCTION

In this section I introduce the c-Src, a human tyrosine kinase, and the related Src Family Kinases (SFKs). I also talk about the discovering of c-Src, its structure and regulation and its role in cancer and relevance as a drug target. Finally, I will describe the O-GlcNAcylation reaction and its interest.

3.1. A HISTORICAL OVERVIEW OF SRC

Src is a *hot topic* nowadays with ~2.5 publications per day over the past 30 years, mostly because of its implication in various types of cancer. However, Src history goes a century back in time.

Francis Peyton Roust, in 1911, proved that viruses can cause cancer when he worked with some chickens that grow a tumor called fibrosarcoma. Roust collected and ground up these sarcomas and then injected it into some chicks, that also developed fibrosarcoma. The causative agent of the infection was a virus, called the Rous Sarcoma Virus (RSV). Studies of RSV mutants in the early 1970s revealed its responsible oncogene, called v-Src or viral Src[1]. An oncogene is a malfunctioning gene which its uncontrollable cell growth leads to cancer. The cellular homologue of v-Src in humans and other species is known as c-Src or cellular Src, and it was the first proto-oncogen to be discovered[2]. Proto-oncogenes are normal gens that help cells grow.

3.2. C-SRC AND SFKS

A protein kinase is an enzyme that catalyze the transfer of a phosphate group from ATP to a protein (phosphorylation). Tyrosine kinases are a subclass of protein kinase that catalyze the phosphorylation of tyrosine residues in proteins, which causes a change in the function of the protein that they are contained in. The tyrosine kinases are divided into two main families: receptor tyrosine kinases, which are permanently attached to membranes by a transmembrane segment, and non-receptor tyrosine kinases, which are reversibly anchored to the inner membrane surface. SFKs are included in this last group[3].

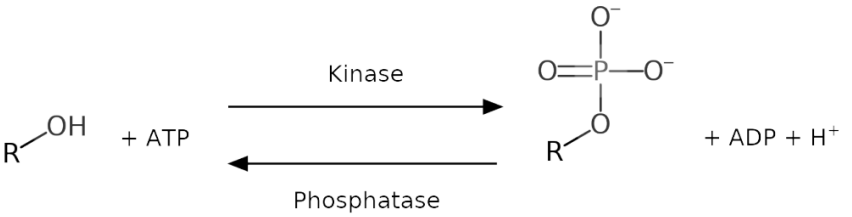

Figure 1. Phosphorylation/desphosphorylation reactions.

Src family kinases (SFKs) is a group of non-receptor tyrosine kinases composed by Src and nine additional variants that are closely related: Fyn, Yes, Blk, Yrk, Frk, Fgr, Srm, Lck and Lyn. While Src, Fyn and Yes are ubiquitously expressed, the rest is located in specific cells types[4].

These kinases play key roles in cell morphology, motility (ability of an organism to move independently), proliferation and survival, and also participate in many cell signalling pathways. The c-Src overexpression and overactivation are associated with several types of human cancer like colon, breast or prostate cancer, and several inhibitors of c-Src and SFKs, like Dasatinib, are being developed as a compounds for future anticancer drugs[5].

3.3. c-Src structure

Human c-Src is a non-receptor tyrosine kinase with a molecular mass of ~60 kDa and composed by 536 amino acids. From the N-terminus to C-terminus, it contains an SH4 domain, a Unique domain, an SH3 domain, an SH2 domain and an SH1 domain, also known as the kinase domain. The three last domains are homologous in all the SFKs members. Finally, c-Src also contains a 14-carbon myristoyl group attached to the SH4 domain and a C-terminal regulatory segment, which are not present in v-Src[6].

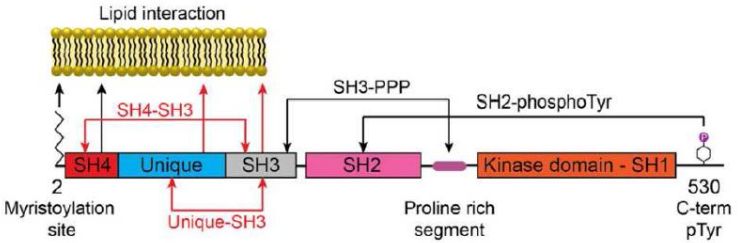

Figure 2. c-Src structure and interactions between domains *(image extracted from ref. 7).*

The function of the myristoylated N-terminal region is to allow the attachment of c-Src to the lipid membrane which is essential to carry out its chemical and physical functions[7]. Myristoylation is a modification that takes place directly in the protein nascent chain at the ribosome by the N-Myristoyl Transferase (NMT) enzyme, which cleaves the initial methionine to create an amide bond between a myristate group and the now N-terminal glycine[8].

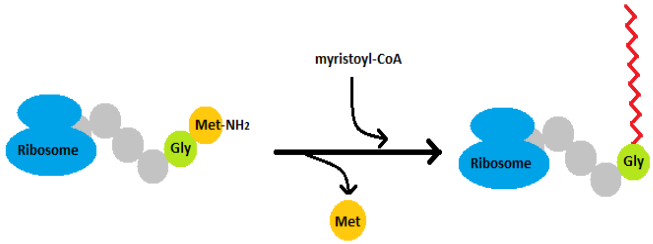

Figure 3. N-Myristoylation catalysed by N-Myristoyl Transferase (NMT).

The SH4 domain is composed of ~15 residues and interacts with acidic lipids (charged negatively) because it has three lysines and three arginines that are basic residues (charged positively). These also allow the interaction between the SH4 and the SH3 domains, that restricts the conformational space of the Unique domain[9].

The next ~65 residues form an intrinsically disordered region (IDR) that connects the SH4 and SH3 domains and is called Unique domain. Intrinsically disordered regions (IDRs) are important constituents of many protein complexes, play various structural, functional and regulatory roles, and define the ability of the protein to be involved in polyvalent interactions[10]. This disorder allows the Unique domain of c-Src acting as a regulatory element: for example, changing one amino acid in this domain can decrease the invasive capacity of a type of colon cancer cells in a 50%.

This domain presents unique sequences for each member of the SFKs and has an additionally lipid binding site known as Unique Lipid Binding Region (ULBR) which interacts with acidic lipids, as the SH4 domain, and allows the interactions between the Unique domain and the SH3 domain[7].

Adjacent to the Unique domain is the SH3 domain, the first folded domain forming the core shared by all SFKs. It is a folded domain composed by ~60 residues organized in a β sandwich model made of five antiparallel strands. Between strands β1 – β2 and β2 – β3 are two flexible loops, named RT and nSrc respectively, that bind to lipids in addition to the lipid binding regions

previously pointed out. These loops allows SH3 interactions with the SH4 and Unique domains, which modulate their flexible conformations. Furthermore, the binding of a polyproline peptide (a peptide with repeating proline residues) to the SH3 inhibits its interaction with lipids and the Unique domain but not with the SH4[11], as we can see in Figure 4. There is a segment rich in proline between SH2 and SH1, which is important in the c-Src regulation.

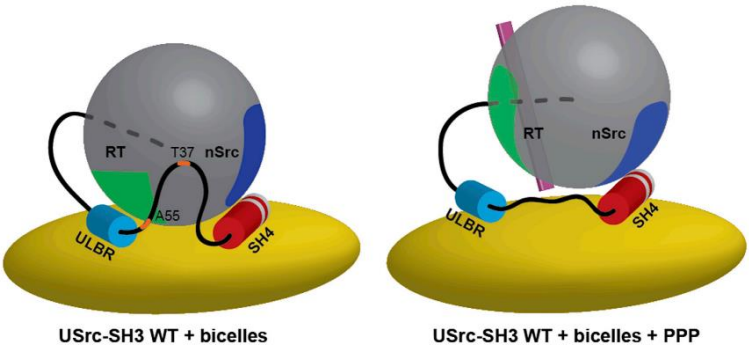

Figure 4. SH4-Unique-SH3 interactions in the presence of lipids *(image extracted from ref. 11).*

The gray sphere represents the SH3 domain and the black line corresponds to the Unique domain, which is connected to the back of the SH3 domain. Residues or regions detected to be involved in interactions are indicated and the yellow oval represents the lipid bicelle (not to scale). The picture on the left shows SH3, Unique and SH4 domains interactions with lipids: SH4, ULBR and the RT and nSrc loops of SH3 are involved in lipid contact, and ULBR – SH3 contacts are conserved. On the other hand, the picture on the right shows all these interactions in presence of polyproline peptides (pink stick): SH3 – lipids and SH3 – ULBR interactions are abolished, but ULBR and SH4 still interact with lipids and the SH4 – SH3 interaction is retained[11].

Towards the C-terminus, it follows the SH2 domain. It is composed of ~100 residues and is formed by two α-helix and seven β-strands. It interacts with the phosphorylated Tyrosine 530 of the C-terminal regulatory segment for the c-Src regulation[6].

The SH1 domain or Src kinase domain is ~250 residues long and is organized in two parts: a small N-terminal lobe which has an antiparallel β-sheet structure and is involved in anchoring and orienting ATP, and a large C-terminal lobe which is composed by α-helices and is responsible for binding the protein substrate. The catalytic site is situated between the two lobes[6].

Finally, at the C-terminal end of the SH1 domain is the regulatory segment, composed by ~15 residues, that contains the residue Tyrosine 530 whose phosphorylation and consequent interaction with the SH2 domain is the key in the regulation of c-Src.

3.3.1. c-Src regulation

Protein phosphorylation plays a key regulatory role in almost every aspect of eukaryotic cell biology. It is a reversible process catalyzed by protein kinases and phosphoprotein phosphatases. Protein kinases catalyze the phosphorylation:

$$MgATP^{1-} + \text{protein-OH} \rightarrow \text{protein-}OPO_3^{2-} + MgADP + H^+$$

Phosphoprotein phosphatases catalyze the reverse action, the dephosphorylation:

$$\text{Protein-}OPO_3^{2-} + H_2O \rightarrow \text{protein-OH} + HOPO_3^{2-}$$

The phosphorylation occurs in a serine, threonine or tyrosine hydroxyl group of the substrate protein. SFKs are tyrosine kinases because the phosphorylate residue is a tyrosine[12].

c-Src and all SFKs members have two important phosphorylation sites: phosphorylation of Tyr419 is stimulatory and phosphorylation of Tyr530 is inhibitory[6].

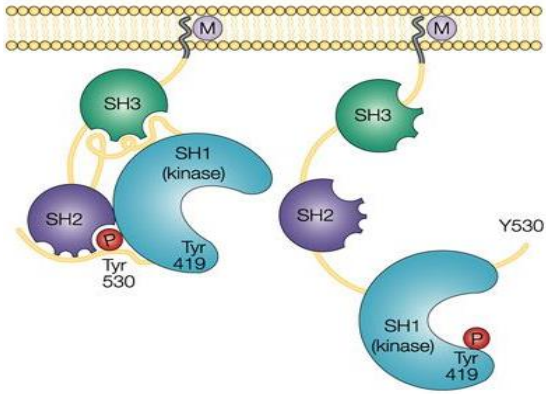

Figure 5. Inactivated and activated states of c-Src *(image extracted from ref. 13).*

Inactivation of c-Src occurs when the C-terminal Tyr530 is phosphorylated and interacts with the SH2 domain. This also allows the interaction between the SH3 domain and the segment rich in proline between SH2 and SH1 domains that results in a closed configuration which do not allow the substrates to reach the catalytic site localized in the kinase domain.

Activation of c-Src occurs with the removal of the phosphorylated Tyr530 and the consequent opening of the structure. To achieve its full activation, needed to start its catalytic activity, there must be an autophosphorylation of the Tyr419, localised on the catalytic site[13].

3.4. c-Src autoproteolysis

c-Src, besides being involved in many cellular signalling pathways and playing important roles in cell proliferation, is also involved in neuronal development by maintaining some of their transmissions. This tyrosine kinase is cleaved at a site in the Unique domain by the protease calpain generating a truncated c-Src fragment of ~52 kDa localized predominantly at the cytosol which expression induces neuronal death. Because c-Src maintains neuronal survival, calpain acts like a molecular switch converting this protein from neuroprotective to neurotoxic[14].

In previous studies realized by the BioNMR Group the same site is cleaved spontaneously in the absence of external proteases suggesting an autoproteolytic process triggered by conformational changes in the Unique domain, which seem to depend of the environmental conditions that influence the interactions between the SH4 domain and the SH3 domain. There are many hypothesis about how this proteolysis occurs (tension, groups that catalyze the process…), but is not known which one is correct.

The BioNMR suggests that, as the SH4 domain interacts with phospholipids, the presence of phosphate allows the interaction between it and the SH4 domain, releasing the interaction between the SH3 and the SH4 domains and reducing the proteolysis of the protein. This hypothesis is the one that we will try to prove in this work.

3.5. O-GlcNAcylation

A glycosylation is a reaction in which a carbohydrate is attached to a functional group (normally a hydroxyl group) of another molecule. O-GlcNAcylation consists in the addition of a uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is a derivate of glucose, to the Ser or Thr hydroxyl groups of nuclear and cytoplasmic proteins. This reactions follows a SN2 mechanism and regulates many cellular processes like DNA transcription or cell signalling, but if its optimal conditions are modified it can also contribute in many human diseases like cancer, diabetes or neurodegeneration[15].

This reaction has some differences with conventional glycosylation: it occurs in the nucleoplasm and cytoplasm and the substrate is not modified or elongated to form complex

structures[16]. O-GlcNAcylation is catalyzed by two enzymes: O-GlcNAC transferase or OGT, which catalyzes the addition of UDP-GlcNAc, and O-GlcNAcase or OGA, which catalyzes the hydrolysis of the glycosidic bond[17].

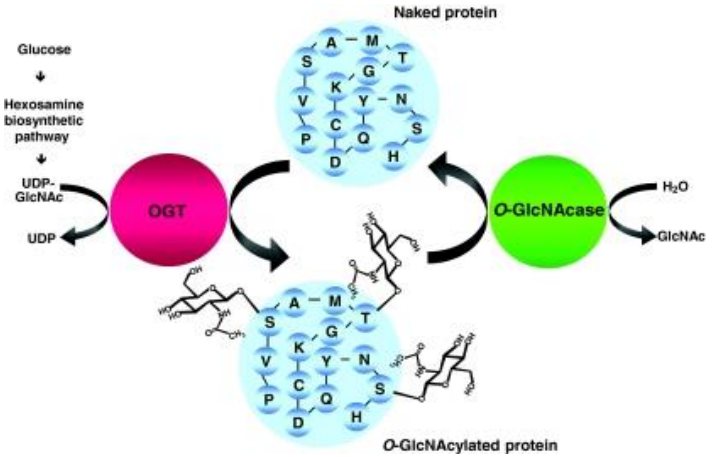

Figure 6. The O-GlcNAcylation cycle *(image extracted from ref. 16).*

3.5.1. The OGT enzyme

O-linked N-acetylglucosamine transferase is an enzyme that catalyzes the addition of UDP-GlcNAc to a serine or a threonine residues on proteins like c-Src following an SN2 mechanism. It shows an optimum catalytic activity at pH 6, but below pH 6 or above pH 7.5 its activity decreases significantly[18]. This enzyme contains an initial tail of 6 histidines, known as His-tag, and the tetratricopeptide repeat (TPR) which is a degenerate 34 amino acid sequence which can mediate its interactions with another proteins and contains the catalytic site[19]. The OGT that we have used contains 4.5 TPR.

About its mechanism, OGT follows an SN2 mechanism with inversion of stereochemistry as you can see in the following figure:

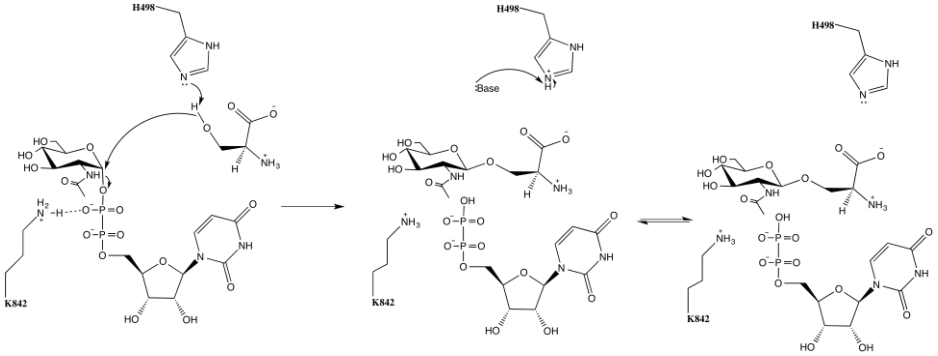

Figure 7. Addition of UDP-GlcNAc mechanism *(image extracted from Wikipedia Commons).*

3.6. $^{1}H - ^{15}N$ HSQC

Heteronuclear single quantum coherence (HSQC) is a highly sensitive 2D-NMR technique which is very significant in the field of protein NMR. It was first described in a $^{1}H - ^{15}N$ system but is also applicable to other atomic nucleus such as $^{1}H - ^{13}C$ system. This technique consists in the transfer of magnetization from a proton to the second nucleus (^{15}N or ^{13}C) and, after a time delay, the magnetization is transferred back to the proton and a signal is recorded. In $^{1}H - ^{15}N$ HSQC, isotopically labelled proteins are used, produced by expressing the protein in ^{15}N-labelled media.

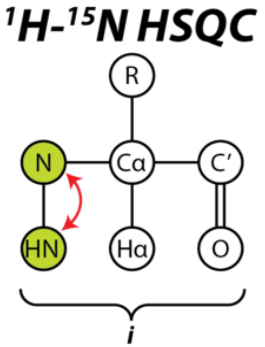

Figure 8. $^{1}H - ^{15}N$ HSQC polarization scheme for a protein/amino acid *(image extracted from Wikipedia Commons).*

Each residue of a protein has an amide proton attached to a nitrogen in the peptide bond, except the proline. This technique provides the correlation between the amide proton and the nitrogen, so we obtain an individually peak in the spectra for each residue. Also, the chemical shifts are very sensitive to perturbation so any change near the protein will be detected[20].

4. OBJECTIVES

The main objective of this research is optimizing conditions to study O-GlcNAcylation avoiding the protein degradation and observe the interactions of $USH3^{Src}$ with the glycolysis enzyme OGT. In order to answer these questions, we define the following objectives:

• Express and purify the protein construct of $USH3^{Src}$, and study its degradation in some solvents at different temperatures.

• Express and purify $USH3^{Src}$ with labelled ^{15}N and OGT enzyme, in order to perform the O-GlcNAcylation.

• Study the O-GlcNAcylation reaction using ^{1}H-^{15}N HSQC to observe if the protein has been glycosylated correctly and the interactions between $USH3^{Src}$ and OGT.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND METHODS

5.1.1. Materials

Uridine 5'-diphospho-N-acetylglucosamine acid sodium salt (U4375 - 100 mg) purchased from Sigma-Aldrich.

β-Nicotinamide adenine dinucleotide dipotassium salt (N4505 – 100 mg) purchased from Sigma-Aldrich.

The rest of reagents come from stocks stored at -80ºC.

The compositions of buffers are shown in Appendix 2.

5.1.1. Instrumental

Instrument	Model	Software
UV-VIS Spectrophotometer	Shimadzu UVmini-120	UV Probe 2.42
FPLC	AKTA FPLC W FRAC-950	Unicorn 5.20
Nickel Cartridge affinity column	HisTrap™ HP 1 mL	-
NMR	Bruker 600 Ultrashield Plus	CCP NMR

Table 1. Instruments used.

5.2. OBTAINING OF USH3^{Src}

Our USH3^{Src} protein is prepared from a precursor construct that contains:

- Histidine tail (His_6-tag) for purification purposes.
- Glutathione S-transferase (GST) protein to ensure solubility.
- TEV protease cleavage site.

• The nature SH4, SH3 and Unique domains.

The structure of our construction is the following:

His_6-tag --- GST --- TEV --- SH4 --- Unique --- SH3

The protocols followed for the expression and purification of proteins and enzymes have been provided by the BioNMR Research Group of the University of Barcelona[21].

5.2.1. Expression

The expression of the protein starts from glycerol stocks stored at -80ºC which contains *Escherichia coli* cells transformed by a plasmid that is provided with resistance to the antibiotics kanamycin and chloramphenicol. This resistance assure the accurate isolation of $USH3^{Src}$.

The procedure starts preparing the preculture media, 3g of Luria Broth (LB) in 100mL, and the culture media, 20g of LB in 1L. Luria Broth is a nutritionally rich medium primarily used for the growth of bacteria composed by NaCl, yeast extract and tryptone. In this part of the procedure sterile conditions are necessary, so both mediums are autoclaved. When the autoclave has finished, the antibiotics kanamycin and chloramphenicol (20 µL of 1M per antibiotic) are added to the preculture media along with 20 µL of glycerol stock, and we left it shaking at 37ºC for about 16 hours.

The following day, 20 mL of the preculture are transferred to the culture media and 0.5 mL of each of the previous antibiotics are added. These cultures are incubated at 130 rpm and 37ºC for 2-3 hours, until the optical density at 600 nm ($OD_{(600nm)}$) reaches between 0.6 and 0.8. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 1 mM is added to induce the protein expression. Once the induction starts, cultures are incubated at 130 rpm and 25ºC overnight but no longer than 18 hours. After the expression period, cultures are collected and centrifuged at 4000 rpm for 15 minutes. The supernatant is removed, and the remaining pellet resuspended with 20 mL of the corresponding lysis buffer in a sterile 50 mL falcon, which is stored at -80ºC until the start of the purification.

In the expression of $USH3^{Src}$ labelled with ^{15}N, there are two differences. The first one is the composition of the culture media: it contains 6 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl and 0.5 g of $^{15}NH_4Cl$ in 1L at a pH of 6.8. The second difference is that, aside from the preculture and both antibiotics, 1 mL of 1M $MgSO_4$, 1 mL of 1 mg/mL biotin, 1 mL of 1 mg/mL thiamine, 1

mL of Q solution (metal traces) and 20 mL of 10% w/v D-glucose are added to the culture media before its first incubation.

5.2.2. Purification

The resuspended pellet is thawed with water at 4ºC for about 30 minutes and then dithiothreitol (DTT) is added at a final concentration of 1 mM. DTT is a reducing agent used to avoid intramolecular and intermolecular disulphide bonds between cysteine residues. The pellet is sonicated in intervals of 30 seconds ON/OFF each for a total of 150 seconds and repeating it three times. Then DNase at a final concentration of 1mM is added to degrade the DNA released in the sonication and the sample is centrifuged at 20000 rpm for 45 minutes. The supernatant is collected and its pH is observed, which should be above 8 to ensure that the histidines are not protonated. If pH is lower than 8, a support buffer is added to ensure that it reaches the desired value. The supernatant is purified using the Nickel Cartridge affinity column connected to a peristaltic bomb to originate flux though the column in order to capture the protein of interest thanks to the presence of the histidine tag, which increases the affinity of the His-tagged protein with the column due to the formation of a metal complex with Ni (II). Then it is eluted with an elution buffer, which contains a high concentration of imidazole that competes with the interaction of the column. The sample is eluted with Lysis buffer though a PD-10 desalting column for the separation of high molecular weight substances, like $USH3^{Src}$, and low molecular weight substances, like imidazole, by desalting and exchanging the buffer. Then the eluted sample is submitted to the TEV cleavage by adding EDTA, DTT and TEV protease at a ratio of 1:50 and leaving it at 4ºC overnight rocking gently, to separate $USH3^{Src}$ from GST.

Typically, some aggregates are formed during cleavage so after overnight incubation the TEV cleavage product is centrifuged at 2000 g for 10 minutes and the supernatant is recovered to remove TEV cleavage residues. Once more the Nickel Cartridge affinity column is used to purify the protein, but this time releasing the free $USH3^{Src}$ protein in the first flow and retaining the GST in the column because it is the construct fragment that contains the histidine tag. The TEV protease also has a histidine tag, so is also retained in the Nickel column. Once the separation has been carried out, the sample is introduced into the fast protein liquid chromatography (FPLC) to finish purifying and isolating the desired protein. When the chromatography has finished, those fractions that contain the desired protein (according to the relevant peak of the obtained chromatogram) are collected and mixed and will be determined by SDS-PAGE along with some

fractions collected during the purification process. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is an analytical method in biochemistry used for the separation of charged molecules in mixtures by their molecular masses in an electric field using sodium dodecyl sulfate (SDS) as a surfactant, covering the proteins' intrinsic charge and conferring them very similar charge-to-mass ratios. Finally, the protein is concentrated using a VIVASPIN centrifugal concentrator until reaching the desired concentration, measured with an UV spectrophotometer.

5.3. OBTAINING OF OGT ENZYME

Our OGT enzyme construct contain 4.5 TPR and an N-terminal His-tag, and has resistance to kanamycin due to the plasmid used.

5.3.1. Expression

The expression of the enzyme starts from glycerol stocks stored at -80ºC which contains *Escherichia coli* cells transformed by a plasmid that is provided with resistance to the antibiotic kanamycin.

The procedure starts preparing the preculture media, 3g of Luria Broth (LB) in 100mL, and the culture media, 1L of Terrific Broth. Terrific Broth is a nutritionally rich medium derived from LB that use additional sources of nutrients and an extra amount of buffer to further regulate the pH of culture allowing growths to larger ODs and is composed by 4 mL of glycerol, 24 g of yeast extract and 20 g of tryptone per litre. It is also composed by 100 mL of a phosphate buffer autoclaved (KH_2PO_4 0.17 M and K_2HPO_4 0.72 M). In this part of the procedure sterile conditions are necessary, so both mediums are autoclaved. When the autoclave has finished, the antibiotic kanamycin (20 µL of 1M per antibiotic) is added to the preculture media along with 20 µL of glycerol stock, and we left it shaking at 37ºC for about 16 hours.

The following day, 20 mL of the preculture are transferred to the culture media and 0.5 mL of the previous antibiotic are added. These cultures are incubated at 130 rpm and 37ºC for 4-5 hours, until the optical density at 600 nm ($OD_{(600nm)}$) reaches between 1.2 and 1.4. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 0.1 mM is added. Once the induction starts, cultures are incubated at 130 rpm and 16ºC overnight but no longer than 18 hours. After the expression period, cultures are collected and centrifuged at 4000 rpm for 15 minutes. The

supernatant is removed, and the remaining pellet resuspended with 20 mL of the corresponding lysis buffer in a sterile 50 mL falcon, which is stored at -80ºC until the start of the purification.

5.3.2. Purification

The resuspended pellet is thawed with water at 4ºC for about 30 minutes and then dithiothreitol (DTT) is added at a final concentration of 1 mM. The pellet is sonicated in intervals of 30 seconds ON/OFF each for a total of 150 seconds and repeating it three times. Then DNAse at a final concentration of 1mM is added and the sample is centrifuged at 20000 rpm for 20 minutes. The supernatant is collected and its pH is observed, which should be above 8 to ensure that the histidines are not protonated. If pH is lower than 8, a support buffer is added to ensure that it reaches the desired value. The supernatant is purified using the Nickel Cartridge affinity column connected to a peristaltic bomb. The His-tag present in the enzyme is bonded to the column. Then, a wash is made to remove protein residues of the column with the corresponding lysis buffer. Finally, the enzyme is eluted with elution buffer.

Once the separation has been carried out, the sample is introduced into the fast protein liquid chromatography (FPLC) to finish purifying and isolating the desired enzyme. When the chromatography has finished, those fractions that contain the desired protein (according to the relevant peak of the obtained chromatogram) are collected and mixed and will be determined by SDS-PAGE along with some fractions collected during the purification process. Finally, the protein is concentrated using a VIVASPIN centrifugal concentrator until reaching the desired concentration, measured with an UV spectrophotometer.

5.4. STUDYING OF USH3^{SRC} DEGRADATION

In previous studies realized by the BioNMR Group, a spontaneous autoproteolysis of USH3^{Src} is noticed. This process seems to depend on the environmental conditions, that influences the interactions between the SH4 domain and the SH3 domain. These interactions cause conformational changes in the Unique domain, resulting in the rupture and degradation of our protein. To study this effect, four different buffers are prepared and the degradation of USH3^{Src} is observed collecting fractions through the days and determining them by SDS-PAGE.

The BioNMR hypothesis for this degradation says that, as the SH4 domain interacts with phospholipids, the presence of phosphate allows the interaction between it and the SH4 domain,

releasing the interaction between the SH3 and the SH4 domains and reducing the proteolysis of the protein.

The first buffer (Buffer A) used is the one known as Tris Buffer, the most common in biochemistry due its buffer range (pH 7 – 9) coincides with the physiological pH of most living organisms, with the addition of some components to support the protein survival. The second buffer (Buffer B) includes the glycolysis conditions. The third buffer (Buffer C) includes phosphate to verify the proposed hypothesis. Finally, the fourth buffer (Buffer D) is the same as Buffer B but with the addition of some compounds needed for the correct performance of the O-GlcNAcylation reaction but without the OGT enzyme. The compositions of all these buffers are shown in Appendix 2.

5.5. STUDYING OF O-GLCNACYLATION REACTION

Three different samples were prepared for the studying of this reaction. The first one is the reaction sample, which includes 200 µL of $USH3^{Src}$ at 300 µM, 30 µL of Reaction Buffer, 4.6 µL of OGT at 120 µM, 6 µL of UDP-GlcNAc at 100 mM and 59.4 µL of Tris Buffer. The compositions of buffers are shown in Appendix 2. The second one is the control sample, which has the same composition of the reaction sample without UDP-GlcNAc. Finally, the no treated sample, which only includes the $USH3^{Src}$ construction in Tris Buffer. All three samples had a final volume of 300 µL and protein, enzyme and substrate concentrations were equal in all samples.

Both reaction and control sample were incubated for 90 minutes at 30ºC and a control of both reactions was done by collecting fractions and determining them by SDS-PAGE. When the incubation was finished, a PD-10 desalting column was used in order to exchange the buffer to Tris buffer and assure that all three samples were in the same conditions to improve the accuracy of the ^{1}H-^{15}N HSQC determination.

6. RESULTS AND DISCUSSION

6.1. EXPRESSION AND PURIFICATION RESULTS

SDS-PAGE is used to verify if the purification has been carried out correctly, using the fractions (about 10 µL) that have been collected during the purification.

6.1.1. Expression and purification of USH3^{Src}

The purification of USH3^{Src} consists in two Nickel affinity columns, separated by a TEV cleavage in order to isolate the desired protein. The purification results are shown in Figure 9 and Figure 10.

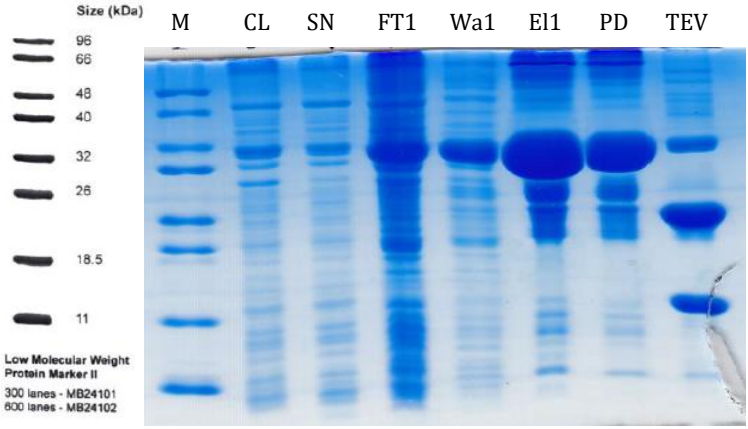

Figure 9. SDS-PAGE showing the first part of the USH3^{Src} protein purification.

The first sample is the protein marker (M), used to estimate the size of the proteins resolved. Cell lysis sample (CL) is after sonification and Supernatant sample (SN) after centrifugation and before the first Nickel affinity column. Both samples contain various compounds, including our construction among them. During the first Nickel column, everything that does not interact with the column is eluted in the flow-through sample (FT1) and our construction is cleaned up with

lysis buffer in the wash sample (Wa1). In the elution with imidazole (elution buffer), our protein with GST is eluted in the elution sample (El1). Fewer salts are observed after the elution of our sample though the PD-10 desalting column (PD). Finally, after TEV cleavage (TEV) the rupture between USH3^{Src} and GST is observed.

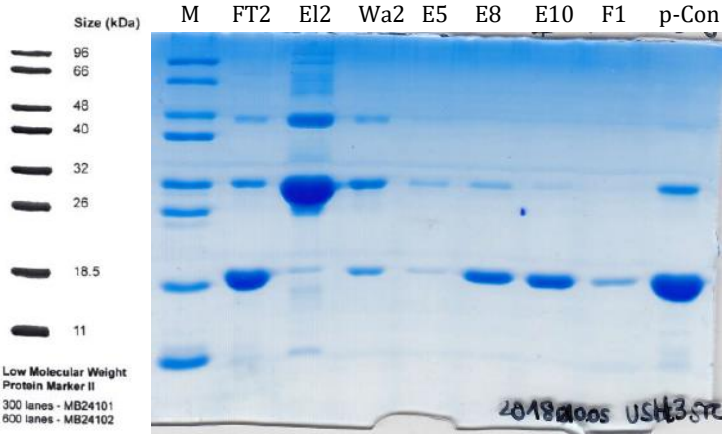

Figure 10. SDS-PAGE showing the second part of the USH3^{Src} protein purification.

After doing the second Nickel column, the protein of interest does not interact with the column and is eluted in the flow-through sample (FT2). GST remains in the column because of His-tag and is cleaned up in the wash sample (Wa2) and eluted by adding imidazole (elution buffer) in the elution sample 2 (El2).

Then, it is proceeded to purify completely the desired protein by means of the FPLC. The fractions where the main peak is found are collected according to the obtained chromatogram: in our case, from E5 to F1 fraction. Some of these fractions (E5, E8, E10 and F1) are determined by SDS-PAGE to assure that the protein has been purified correctly. Finally, a final sample is determined after the protein is concentrated using a VIVASPIN centrifugal concentrator. In this case we can observe our protein with some GST residues that will not affect our experiments due to its low concentration.

In ^{15}N USH3^{Src}, both expression and purification procedures are the same, so the results are equal as in USH3^{Src}. The fractions collected by FPLC are from E6 to F3 fractions, and the determined by SDS-PAGE are E6, E8, F1 and F3 fractions. The purification results of the labelled protein are shown in Figure 11.

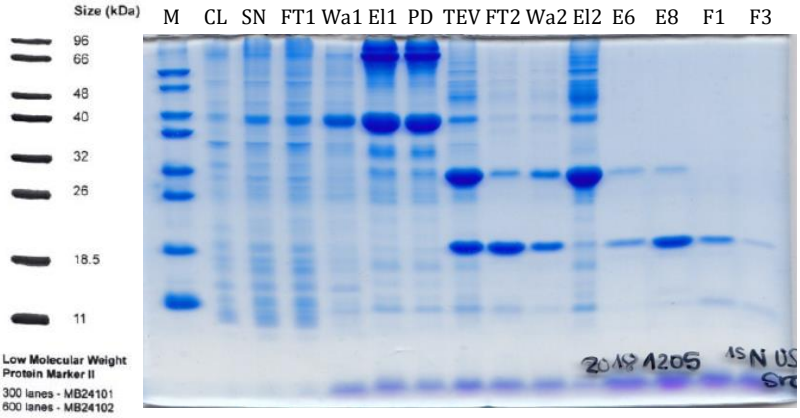

Figure 11. SDS-PAGE showing the ^{15}N USH3^{Src} protein purification.

6.1.2. Expression and purification of OGT

Only one purification by Nickel affinity column is carried out in the purification of OGT enzyme. The purification results are shown in Figure 12.

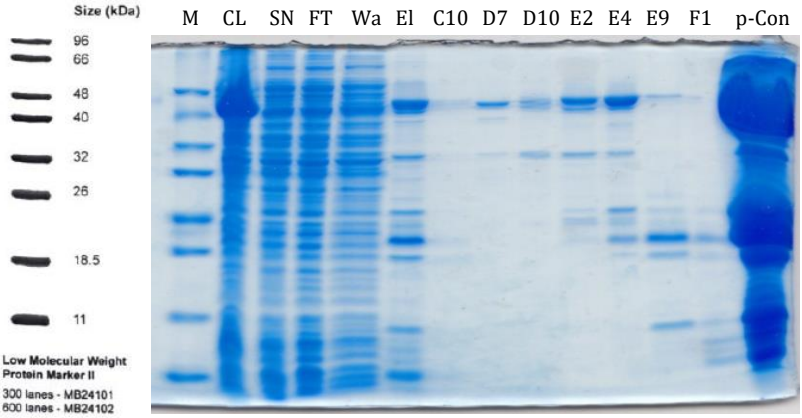

Figure 12. SDS-PAGE showing the OGT enzyme purification.

Both Cell lysis sample (CL) and Supernatant sample (SN) contain various compounds, including our construction among them. During the Nickel column, everything that does not interact with the column is eluted in the flow-through sample (FT) and our construction is cleaned

up with lysis buffer in the wash sample (Wa). In the elution with imidazole (elution buffer), our enzyme is eluted in the elution sample (El).

Then, it is proceeded to purify completely the enzyme by means of the FPLC. The fractions where the main peak is found are collected according to the obtained chromatogram: in our case, from C10 to F1 fraction. Some of these fractions (C10, D7, D10, E2, E4, E9 and F1) are determined by SDS-PAGE to assure that the protein has been purified correctly. Finally, a final sample is determined after the enzyme is concentrated using a VIVASPIN centrifugal concentrator. In this case we can observe the enzyme purified with some degradation residues that will not affect our reaction.

6.2. USH3^{SRC} DEGRADATION RESULTS

SDS-PAGE is used to verify the effect of the degradation in each buffer, using fractions (about 10 µL) that have been collected through some days.

6.2.1. Buffer A

This buffer is composed by Tris Buffer plus some compounds to improve the protein survival: EDTA to neutralize metal traces like Ni^{2+} from the Nickel column and NaN_3 as a preservative. One fraction per day were collected for seven consecutive days. The degradation was observed at 4ºC, 37ºC and at room temperature (RT). The degradation results are shown in Figure 13 and Figure 14.

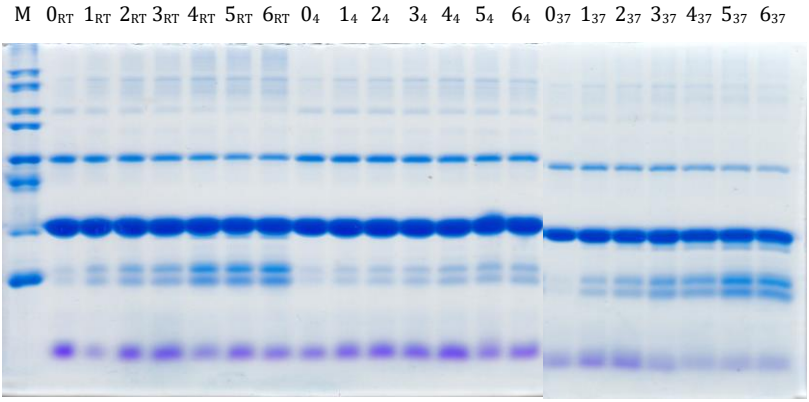

Figure 13. SDS-PAGE showing the USH3^{Src} degradation in Buffer A.

M is the protein marker. The number is the day when the fraction was collected and the subindices the temperature of the sample in the Celsius scale (ºC).

The information of the SDS-PAGE was converted into numeric data using the *ImageJ* software. This data was normalized and represented in the following graphics:

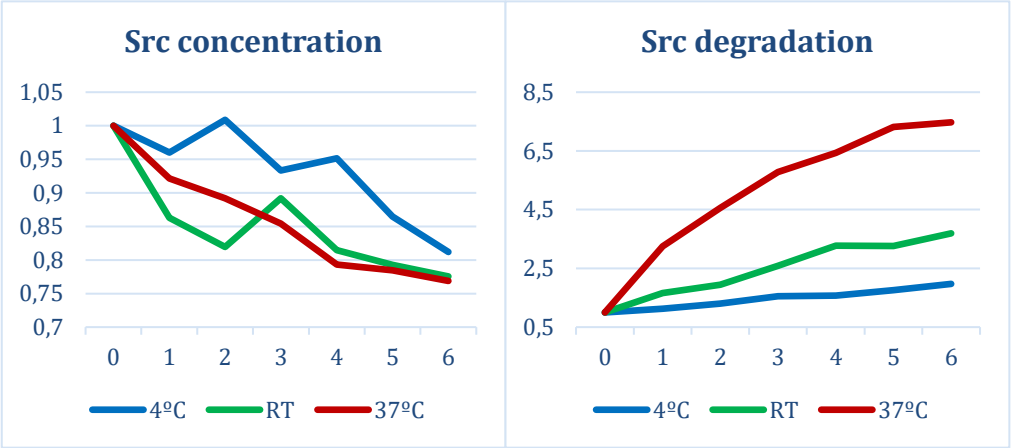

Figure 14. Representation of USH3^{Src} concentration and degradation through days at different temperatures in Buffer A. The x-axis represents the day and the y-axis the intensity.

A decrease of USH3^{Src} is observed along with an increase of its degradation through days but nothing exceptional. Degradation increases with the temperature. There are not evidences to assure that this buffer affects to USH3^{Src} degradation.

6.2.2. Buffer B

This buffer is the one used by the BioNMR Group in order to do the O-GlcNAcylation, composed by Tris and Magnesium. The last student who tried to realize this reaction using this buffer observed degradation of the protein even before the reaction began. One fraction per day were collected for seven consecutive days. The degradation was observed at 4ºC, 37ºC and at room temperature (RT). The degradation results are shown in Figure 15 and Figure 16.

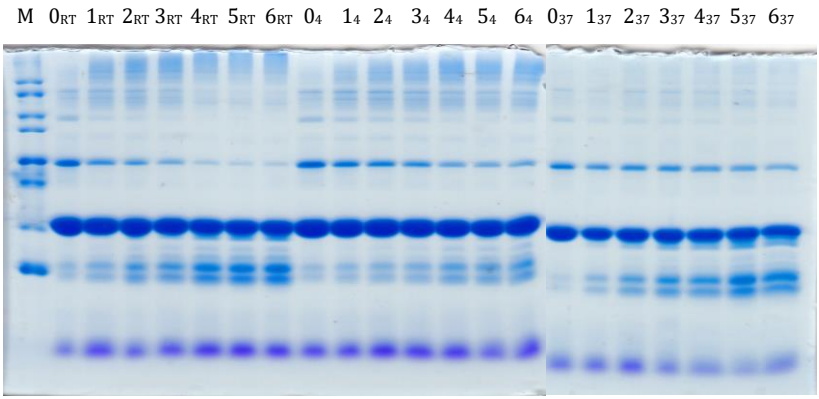

Figure 15. SDS-PAGE showing the USH3^{Src} degradation in Buffer B.

The information of the SDS-PAGE was converted into numeric data using the *ImageJ* software. This data was normalized and represented in the following graphics:

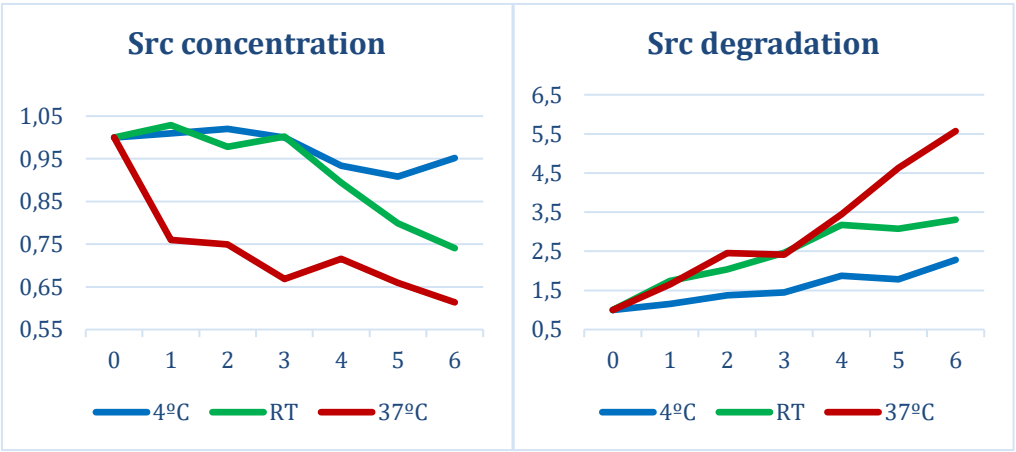

Figure 16. Representation of USH3^{Src} concentration and degradation through days at different temperatures in Buffer B. The x-axis represents the day and the y-axis the intensity.

A decrease of USH3^{Src} is observed along with an increase of its degradation through days but nothing exceptional. Degradation increases with the temperature. There are not evidences to assure that this buffer affects to USH3^{Src} degradation, unlike the last student who tried this and observed an intense degradation.

6.2.3. Buffer C

This buffer has the same composition as Buffer A but using NaH_2PO_4 and Na_2HPO_4 instead of Tris for the pH regulation. According to the BioNMR Group hypothesis, phosphate interacts with the SH4 domain and the interactions between it and the SH3 domain release, decreasing the conformational changes on the Unique domain and reducing the protein autoproteolysis. One fraction per day were collected for seven consecutive days. The degradation was observed at 4ºC, 37ºC and at room temperature (RT). The degradation results are shown in Figure 17 and Figure 18.

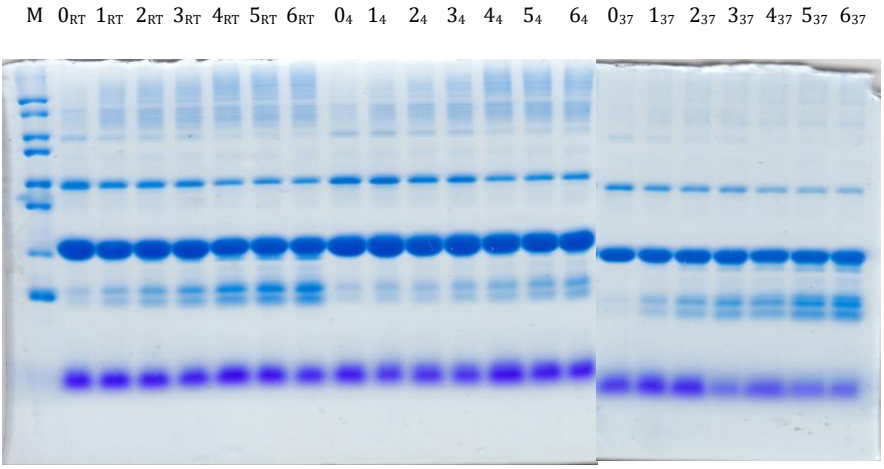

Figure 17. SDS-PAGE showing the $USH3^{Src}$ degradation in Buffer C.

The information of the SDS-PAGE was converted into numeric data using the *ImageJ* software. This data was normalized and represented in the following graphics:

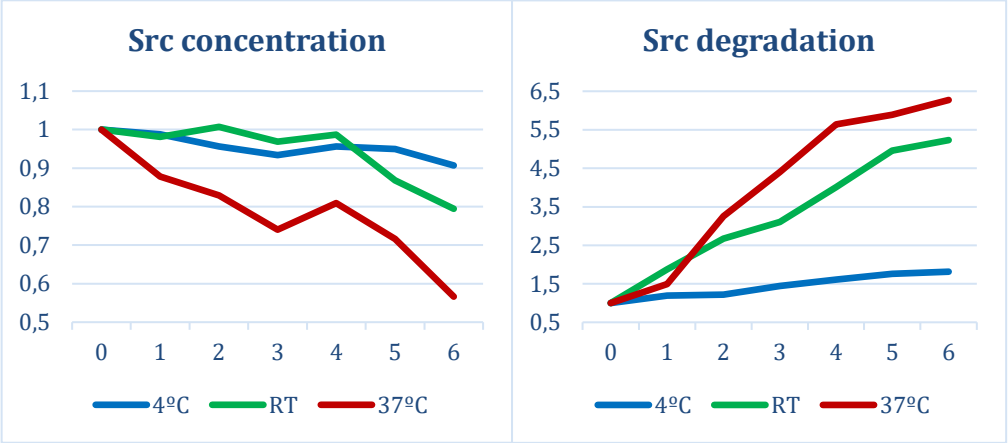

Figure 18. Representation of USH3^{Src} concentration and degradation through days at different temperatures in Buffer C. The x-axis represents the day and the y-axis the intensity.

A decrease of USH3^{Src} is observed along with an increase of its degradation through days but nothing exceptional. Degradation increases with the temperature. There are not evidences to assure that this buffer affects to USH3^{Src} degradation, so we cannot make certain the truthfulness of the hypothesis proposed by the BioNMR Group.

6.2.4. Buffer D

This buffer has the same composition as Buffer B but with the addition of the compounds needed for the correct performance the O-GlcNAcylation reaction (without OGT). These compounds are DTT to avoid intramolecular and intermolecular disulphide bonds between cysteine residues and allow the O-GlcNAcylation in these residues, and UDP-GlcNAc which is the substrate of the reaction. One fraction per day were collected for just four consecutive days, as DTT degrades very quickly. The degradation was observed at 4ºC, 37ºC and at room temperature (RT). The degradation results are shown in Figure 19 and Figure 20.

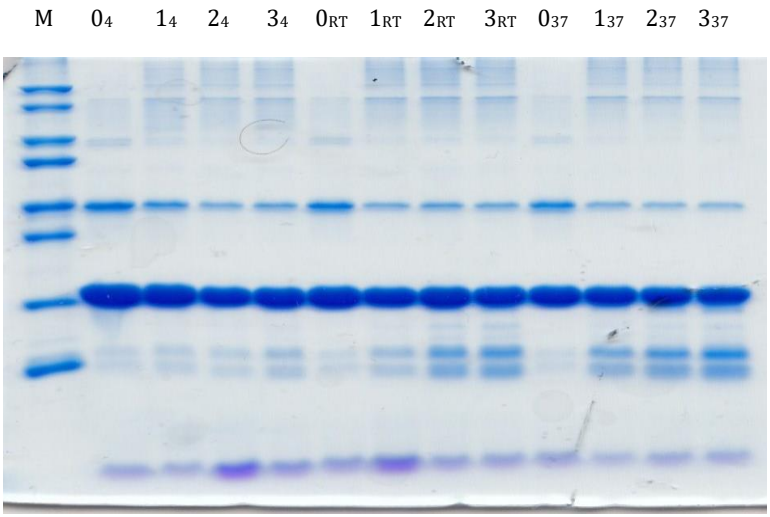

Figure 19. SDS-PAGE showing the USH3^{Src} degradation in Buffer D.

The information of the SDS-PAGE was converted into numeric data using the *ImageJ* software. This data was normalized and represented in the following graphics:

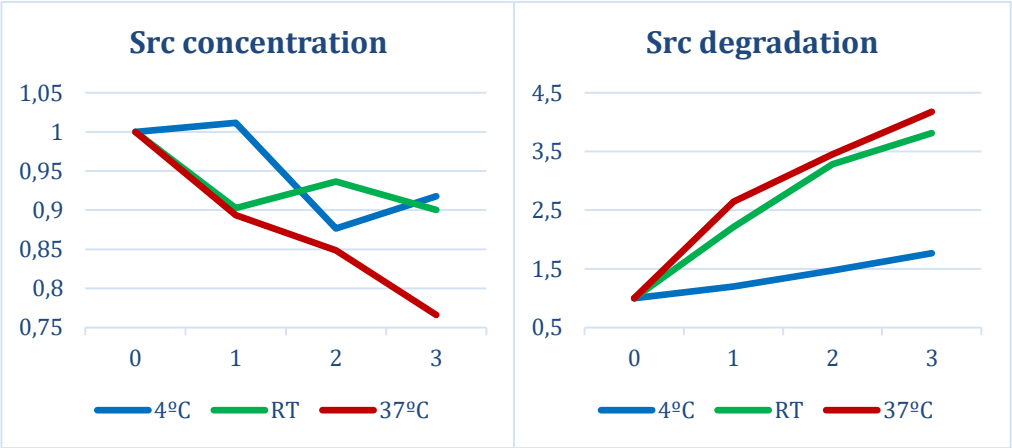

Figure 20. Representation of USH3^{Src} concentration and degradation through days at different temperatures in Buffer D. The x-axis represents the day and the y-axis the intensity.

A decrease of USH3^{Src} is observed along with an increase of its degradation through days but nothing exceptional. Degradation increases with the temperature. There are not evidences to assure that the presence of DTT and UDP-GlcNAc affects to USH3^{Src} degradation.

6.2.5. Degradation comparison

To ensure that none of these buffers affects significantly to USH3^{Src} degradation, we compared all the data obtained in the same graphic. The 4ºC data is used in this comparison, as RT can oscillate significantly and at 37ºC the degradation is so fast. The comparison results are shown in the Figure 21.

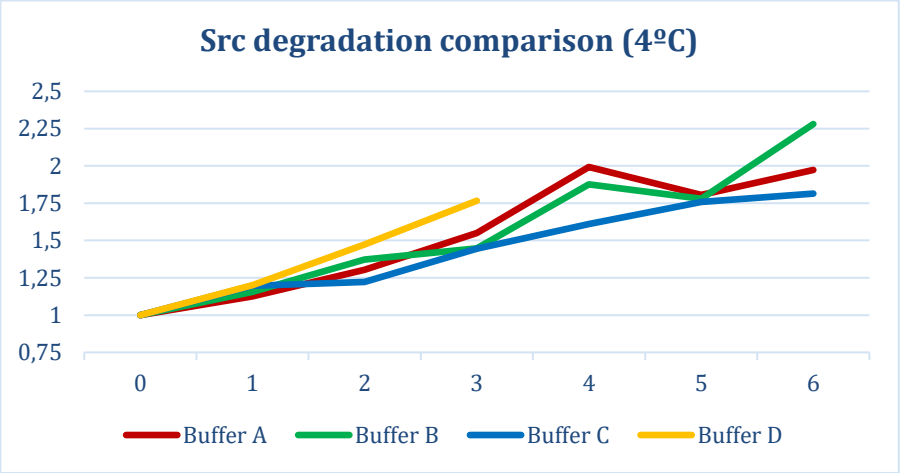

Figure 21. Representation of USH3^{Src} degradation through days in different buffers at 4ºC. The x-axis represents the day and the y-axis the intensity.

There are not significant differences in the degradation of USH3^{Src} between buffers. The degradation seen is the habitual for all proteins through time. None spontaneous proteolysis depending on the environmental conditions has been observed.

The reason why I didn't observe an important degradation of the protein like the other students who tried this before me is unknown. It can depend of many variables, like the way of working with the samples as proteins are very delicate.

6.3. O-GlcNAcylation control

To assure that the reaction has been carried out without protein degradation, a fraction off reaction and control samples were collected at the beginning of the reaction, after 45 minutes, and after 90 minutes (end of the reaction). Fractions of three samples after doing the ^{1}H – ^{15}N HSQC were also collected. All fractions were determined by SDS-Page. The results are shown in Figure 22.

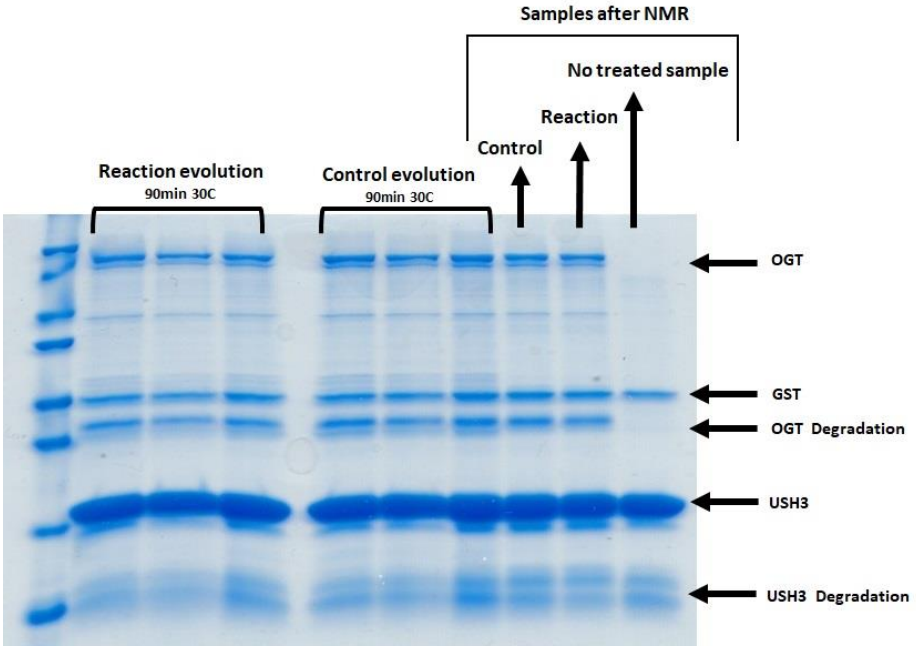

Figure 22. SDS-PAGE showing the control of the O-GlcNAcylation reaction and the samples after RMN.

Non USH3^{Src} degradation has been observed, so the O-GlcNAcylation reaction seems to be carried out correctly.

6.4. ^{1}H – ^{15}N HSQC RESULTS

Our objective using this technique is to identify if there are interactions of USH3^{Src} with the OGT enzyme and try to identify if the O-GlcNAcylation reaction has been accomplished and which are the serines and threonines that contains the glycosylation sites. In order to study that, the spectra of the Control sample in front of the no treated sample was studied. The NMR results are shown in Figure 23.

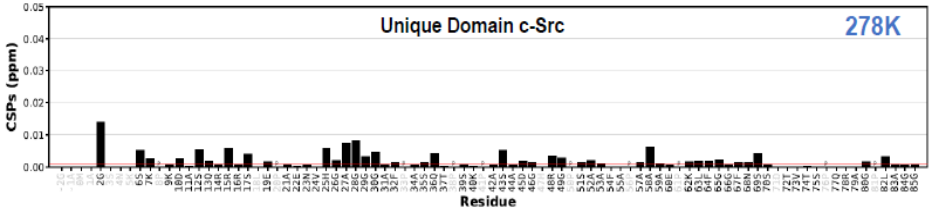

Figure 23. Comparison of control sample in front of no treated sample using ^{1}H – ^{15}N HSQC.

The red line corresponds to 3 times the standard deviation of the 10 peaks with less perturbations, and it's used to determine which peaks are significative. Despite that, the scale of this spectra is very small (normally is about 0.2 – 0.3) so these perturbations are very low. The peaks indicate the chemical shift perturbations (CSP) of each residue. These perturbations are caused by a physical change of the Unique domain or by an alteration of the interactions between this domain and the OGT enzyme.

The peaks in the extremes of the spectra are not considered, because they are close to the following domains and can be affected by more effects. A region containing three glycines (28G, 29G and 30G) that have been quite affected by perturbations is found, and this is because glycines are very flexible and make this region easier to be disturbed. Many of the perturbed residues are serines, which are potential glycosylation sites. In order to confirm that, the spectra of the reaction sample in front of the control sample was studied. The NMR results are shown in Figure 24.

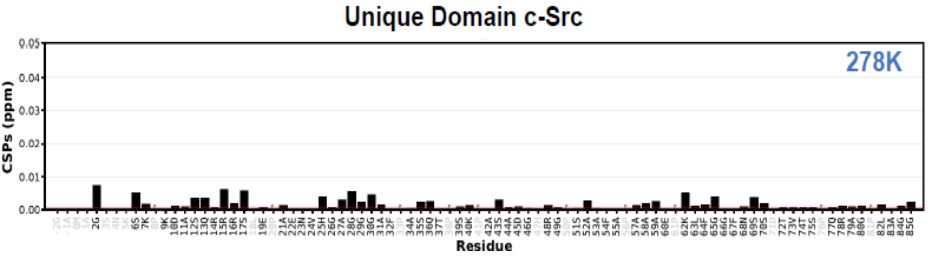

Figure 24. Comparison of reaction sample in front of control sample using ^{1}H – ^{15}N HSQC.

The scale of this spectra is also very small, so these perturbations are also very low. These perturbations are caused by a physical change of the Unique domain, by an alteration of the interactions between this domain and the OGT enzyme or by the glycosylation of the OGT enzyme (some studies have demonstrated that OGT can be glycosylated) that changes the way it interacts with the Unique domain.

None substantial changes are found in comparison with the previous spectra, so perturbations are practically the same in both cases. There are few variations in quantification due to the buffer exchange before the analysis of the sample, that modified a little the protein concentration. These results mean that the O-GlcNAcylation reaction has not been accomplished and the perturbations in our protein are due to its interactions with the OGT enzyme. In order to check this, we observed our reaction sample using mass spectrometry (MS). The MS results are shown in Figure 25.

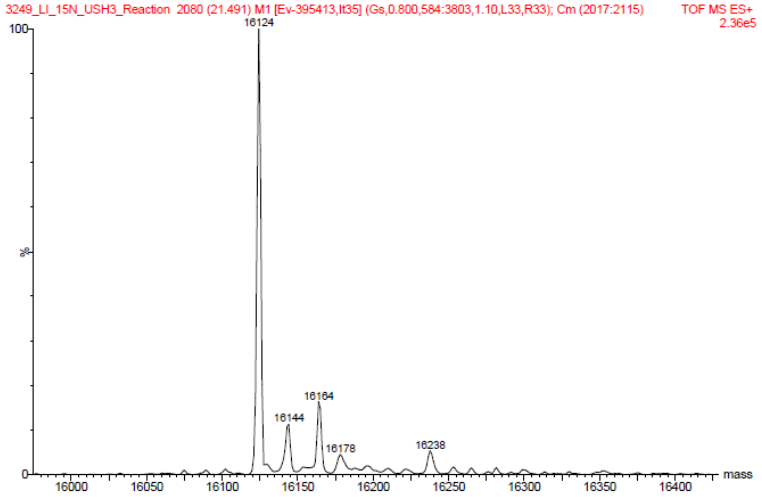

Figure 25. Mass spectrometry spectra of the reaction sample.

The isotopically averaged molecular weight of our protein is 16125 Da. There are not found relevant peaks at higher molecular weight, so we can ensure that the O-GlcNAcylation reaction has not been carried out.

7. CONCLUSIONS

The main conclusions obtained from this research are:

• The $USH3^{Src}$ protein and the ^{15}N $USH3^{Src}$ protein, along with the OGT enzyme, have been expressed and purified successfully.

• No evidences have been found to assure that the environmental conditions influence in the $USH3^{Src}$ degradation.

• In both spectrums obtained by ^{1}H – ^{15}N HSQC and by MS is observed that the O-GlcNAcylation reaction has not been carried out.

• Very low interactions between $USH3^{Src}$ and the OGT enzyme have been found using the ^{1}H – ^{15}N HSQC technique.

8. REFERENCES AND NOTES

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9. ACRONYMS

Ser	Serine
Thr	Threonine
OGT	O-linked-β-D-N-acetylglucosamine transferase
$USH3^{Src}$	Unique domain and Src Homologous 3 domain of c-Src
O-GlcNAc	β-linked N-acetylglucosamine
TPR	Tetratricopeptide repeat
$OD_{(600\ nm)}$	Optical density at 600 nm
IPTG	Isopropyl β-D-1-thiogalactopyranoside
DTT	Dithiothreitol
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
TEV	Tobacco Etch Virus
EDTA	Ethylenediaminetetraacetic acid
GST	Glutathione S-transferase
FPLC	Fast Protein Liquid Chromatography
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
HSQC	Heteronuclear Single Quantum Coherence
MS	Mass Spectrometry
UV	Ultraviolet

APPENDICES

APPENDIX 1: PROTEIN SEQUENCES

USH3^{Src} sequence:

G A M G S N K S K P K D A S Q R R R S L E P A E N V H G A G G G A F P A S Q
T P S K P A S A D G H R G P S A A F A P A A A E P K L F G G F N S S D T V T S
P Q R A G P L A G G V T T F V A L Y D Y E S R T E T D L S F K K G E R L Q I V N
N T E G D W W L A H S L S T G Q T G Y I P S N Y V A P S D S I Q A E E

OGT enzyme sequence:

The initial histidine (H) tail is observed in first amino acids.

Each colour corresponds to a different TPR, and the grey one corresponds to half TPR.

M G S S H H H H H H S S G L E V L F Q G P A M C P T H A D S L N N L A N I K R
E Q G N I E E A V R L Y R K A L E V F P E F A A A H S N L A S V L Q Q Q G K L Q
E A L M H Y K E A I R I S P T F A D A Y S N M G N T L K E M Q D V Q G A L Q C
Y T R A I Q I N P A F A D A H S N L A S I H K D S G N I P E A I A S Y R T A L K L
K P D F P D A Y C N L A H C L Q I V C D W T D Y D E R M K K L V S I V A D Q L E
K N R L P S V H P H H S M L Y P L S H G F R K A I A E R H G N L C L D K I N V L
H K P P Y E H P K D L K L S D G R L R V G Y V S S D F G N H P T S H L M Q S I
P G M H N P D K F E V F C Y A L S P D D G T N F R V K V M A E A N H F I D L S
Q I P C N G K A A D R I H Q D G I H I L V N M N G Y T K G A R N E L F A L R P A
P I Q A M W L G Y P G T S G A L F M D Y I I T D Q E T S P A E V A E Q Y S E K L
A Y M P H T F F I G D H A N M F P H L K K K A V I D F K S N G H I Y D N R I V L
N G I D L K A F L D S L P D V K I V K M K C P D G G D N A D S S N T A L N M P V
I P M N T I A E A V I E M I N R G Q I Q I T I N G F S I S N G L A T T Q I N N K A A
T G E E V P R T I I V T T R S Q Y G L P E D A I V Y C N F N Q L Y K I D P S T L Q
M W A N I L K R V P N S V L W L L R F P A V G E P N I Q Q Y A Q N M G L P Q N
R I I F S P V A P K E E H V R R G Q L A D V C L D T P L C N G H T T G M D V L W
A G T P M V T M P G E T L A S R V A A S Q L T C L G C L E L I A K N R Q E Y E
D I A V K L G T D L E Y L K K V R G K V W K Q R I S S P L F N T K Q Y T M E L E
R L Y L Q M W E H Y A A G N K P D H M I K P V E

APPENDIX 2: BUFFERS

- Lysis buffer Src (pH 8):

Trizma® base 20 mM

NaCl 300 mM

Imidazole 10 mM

NaN_3 0.01 %

DTT 1 mM

- Lysis buffer OGT (pH 8):

Trizma® base 50 mM

NaCl 500 mM

Imidazole 10 mM

Glycerol 2 %

DTT 1 mM

- Support buffer (pH 8):

This buffer consists in the corresponding lysis buffer and adding Trizma® base at a final concentration between 0.5 – 1 M.

- Elution buffer (pH 8):

This buffer consists in the corresponding lysis buffer and adding imidazole at a final concentration of 400 mM.

- FPLC buffer Src (pH 7.6):

NaH_2PO_4 8 mM

$Na_2HPO_4 \cdot 7H_2O$ 42 mM

NaCl 150 mM

EDTA 0.2 mM

NaN_3 0.01 %

- FPLC buffer OGT (pH 7.6):

Trizma® base 50 mM

NaCl 300 mM

EDTA 0.1 mM

Glycerol 1 %

DTT 5 mM

- Buffer A (pH 7.2):

Trizma® base 50 mM

NaCl 150 mM

EDTA 0.2 mM

NaN_3 0.01 %

- Buffer B (pH 7.2):

Trizma® base 75 mM

$MgCl_2$ 0.5 mM

- Buffer C (pH 7.5):

This buffer consists in the FPLC buffer for Src.

- Buffer D (pH 7.2):

This buffer consists in buffer B and adding DTT at a final concentration of 0.2 mM and UDP-GlcNAc at a final concentration of 150 mM.

- Reaction buffer (pH 7.3):

Trizma® base 75 mM

NaCl 1.5 M

EDTA 6 mM

$MgCl_2$ 75 mM

DTT 3 mM