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Production and purification of two allergens for the vaccination of horses against insect bite hypersensitivity

2nd year internship report of "DUT Génie Biologique option Industries Agroalimentaires et Biologiques"

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Finally, I would like to thank all my co-workers who made my internship and my stay in Iceland even much greater.

Thanks to all of those people.

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LIST OF ABBREVIATION

AP: Alkaline phosphatase
APCs: Antigen recognition cells
BCIP: 5-Bromo-4-chloro-3indolyl phosphate
bp: base pair
°C: Celsius degrees
ddH_20: distilled and deionized water
dH20: distilled water
E. coli: *Escherichia coli*
h: hour(s)
IBH: Insect bite hypersensitivity
IFN-γ: Interferon γ
Ig: Immunoglobulin
IL: Interleukin
kDa: kilodalton
MHC: Major histocompatibility complex
min: minutes
mL: milliliter
mM: millimolar
NBT: Nitro blue tetrazolium chloride
PBMCs: Peripheral blood mononuclear cells
PCR: Polymerase chain reaction
s: seconds
Th: T helper cells
U/mL: unit per milliliter
α-His: Anti-histidine
α-mouse: Anti-mouse
µg: microgram

[X] Webographic references
[(X)] Bibliographic references

LEXICON

*(Marked in the text with: *)*
Definitions from "English oxford living dictionary".

Dermatitis: A medical condition in which the skin becomes red, swollen, and sore, sometimes with small blisters, resulting from direct irritation of the skin by an external agent or an allergic reaction to it.

Phagocytosis: The ingestion of bacteria or other material by phagocytes.

Cytokines: Any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells.

Haematophagous: Animal/insect that feeds on blood.

Immunotherapy: The prevention or treatment of disease with substances that stimulate the immune response.

Adjuvant: Substance that enhances the body's immune response to an antigen.

Virus like Particles: Resemble viruses, but are non-infectious because they contain no viral genetic material.

Lipopolysaccharides: A complex molecule containing both lipid and polysaccharide parts, major constituent of the bacterial cell membrane.

Immunoassay: Procedure for detecting or measuring specific proteins or other substances through their properties as antigens or antibodies.

Post-translational modification: Occurring after the translation of a mRNA sequence into the amino-acid sequence it encodes.

Recombinant: Relating to or denoting an organism, cell, or genetic material formed by recombination.

FIGURE LIST

[X] Webographic references
[(X)] Bibliographic references

INTRODUCTION

Insect bite hypersensitivity (IBH), also called summer eczema, is an allergic reaction of horses due to bites of midges of the genus *Culicoides*. It is a recurrent seasonal dermatitis* (usually from spring to autumn), which causes severe itches, leading to a hair loss and various lesions of the skin (thickening, fibrosis, scaling, etc). Those symptoms can sometime also include secondary infection. The lesions are mainly situated on the preferential feeding sites of midges: the dorsal midline, the mane, the neck and the tail [(1)] (Figure 1).

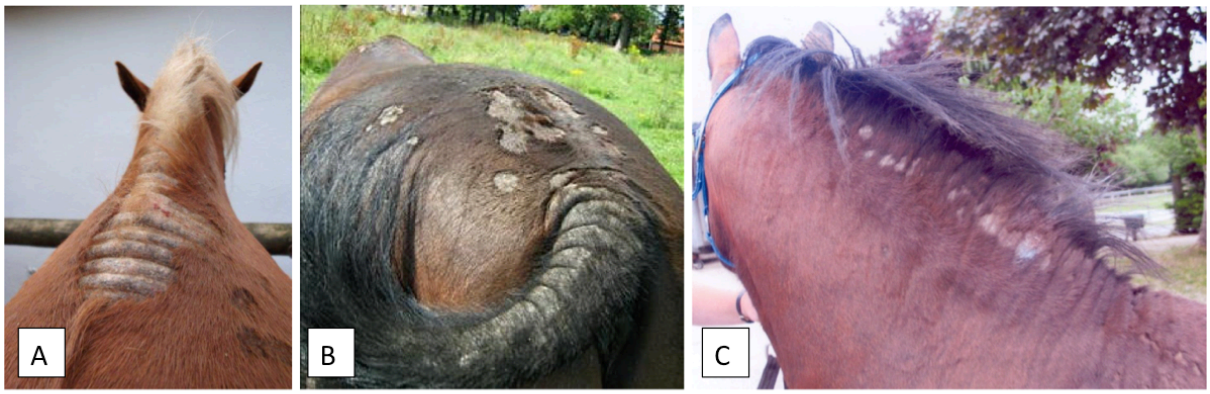

Figure 1: Clinical signs of the Insect Bite Hypersensitivity (IBH)
Sources: (A) http://keldur.is/node/421
(B) http://www.carterveterinaryservices.com/single-post/2017/04/20/Insect-Bite-Hypersensitivity
(C) http://www.deercreekequine.com/blog/

The disease can affect all breads of horses, although Icelandic horses born in Iceland and exported to the continent are the most affected. Indeed, the prevalence of IBH in Icelandic horses born on the continent is the same as for any other breeds (around 5 to 10%), whereas the prevalence of IBH for exported Icelandic horses is up to 50%. This difference is probably due to the absence of the midges in Iceland, preventing the horses from any contact with *Culicoides* in its early years [(1)].

There is no existing treatment to the IBH. To prevent horses from catching the disease, they can be covered with a special blanket (figure 2), or kept in stables during the main feeding times of *Culicoides* (dawn and dusk) [(2)].

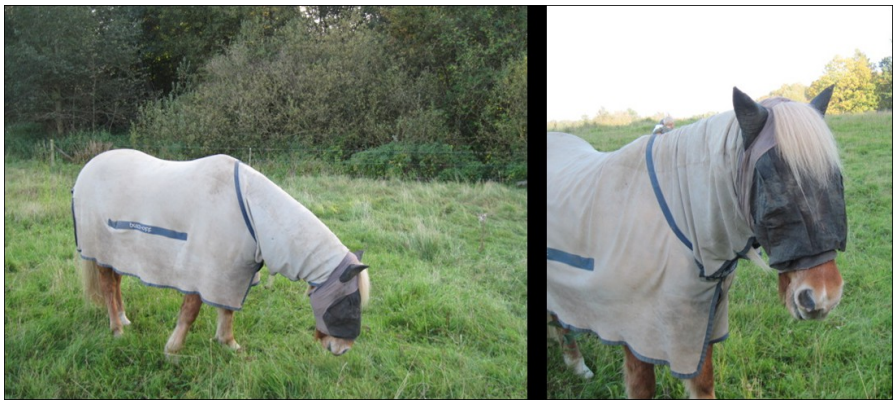

Figure 2: Horse wearing a blanket as a protection from *Culicoides* bites
Source: Þórunn Guðmundsdóttir

[X] Webographic references
[(X)] Bibliographic references

IBH is caused by an exaggerated immune response against a harmless protein. Allergens contained in *Culicoides* saliva penetrate into the skin through the bite. Proteins are phagocyted* by the antigen-presenting cell (APC) and presented to naïve $CD4^+$ T helper cells (Thn). In IBH, they differentiate into Th2 cells. Those cells release cytokines* (IL-4, IL-5, IL13), which induce the production of IgE by the B-cells. The IgE binds with a high affinity on the mast cells and basophils (Figure 3). This first step is called sensibilization. When the horse will come in contact with *Culicoides* saliva a second time, the allergens will cross binds IgE in the mast cells. Inflammatory mediators will be released, causing the allergic reaction (figure 3) [(3)].

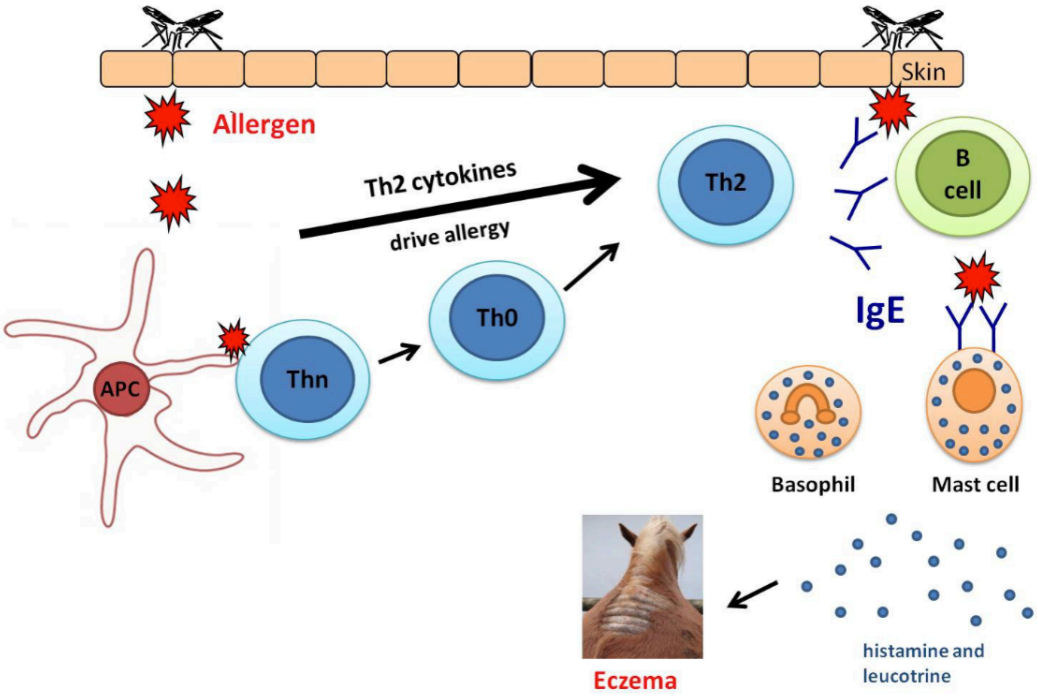

Figure 3: IgE mediated response in IBH
Source: Internship report of Juliette Depreaux

An allergen is a protein that causes an IgE antibody response in at least five allergic individuals. A major allergen is generally one that causes an IgE antibody response in at least 50% of a selected group of patient (clinically allergic to the allergen) [3]. Although what makes a protein an allergen is not yet clear, all of them induce a Th2 response: an IgE production and an IgE mediated allergic response [(1)].

Allergens responsible for the IBH are insect allergens from midges salivary glands. They have been found and isolated from three different *Culicoides* species: *C. nubeculosus* (Cul n), *C. obsoletus* (Cul o) and *C. sonorensis* (Cul s) [(2)]. A total of 22 allergic proteins have already been isolated.

Culicoides midges are small biting flies that can be found worldwide (except in Antarctica, New Zealand and Iceland). It is haematophagous*, although only females suck blood. The proteins found in the saliva of the midge enable them to overcome some of the host defenses (coagulation, immune response) and cause allergies in some individuals.

[X] Webographic references
[(X)] Bibliographic references

An immunotherapy* for IBH is currently being developed. The principle is to vaccine horses with the purified allergens in order to induce a Th1 response (instead of an allergic Th2 response), either by using allergens in adjuvant* or virus like particles*. Th1 cells will block the production of Th2 cells and hence, the allergy. Thereby, a vaccinated horse will have a Th1 response (non-allergic) to the allergen and won't develop IBH.

In the next experimental vaccination, three allergens will be tested in horses: Cul n 4, Cul o 1pet, Cul o 3. The antigens are going to be produced in *E. coli*, purified and injected to the horses as a vaccine along with a Th1 adjuvant or virus like particles. The immune response of the horses will be monitored to see whether the horses have Th1 or a Th2 response to the allergen after the vaccination.

The purification of Cul o 1pet has proven very difficult and the solubility of both Cul o 1pet and Cul o 3 into a non-toxic medium remains a challenge. The aim of my project was two fold. Improve the purification of Cul o 1pet and Cul o 3, and find a way to set them on a native form into a cell-friendly medium. Then, produce the two allergens in *E. coli*.

In my report, I will start by presenting you the laboratory in which I did my work. In a second part I will explain the aim of my project. I will then present the material and methods I used throughout my internship, and the results. I will finish my report with a discussion, followed by both a personal and a scientific conclusion.

1. Presentation of the institute

The Institute for Experimental Pathology (Keldur) was founded 1948 in connection with importation of sheep of the karakul breed. These sheep brought several unknown pathogens that infected the isolated Icelandic sheep breed. One of those pathogens was Visna- Maedi the first lentivirus to be isolated by Björn Sigurðsson and his team at Keldur.

Keldur operate according to law from 1990. The Institute is an academic establishment affiliated with the Faculty of Medicine University of Iceland, with a special governing board and an independent budget.

The predominant aspects of the activities are basic and service research in veterinary medicin. The expertise is on the following disciplines [1]:

- Prionology
- Virology
- Bacteriology
- Parasitology
- Pathology
- Immunology
- Biochemistry
- Molecular biology.

Applied veterinary research, animal health control, diagnostic services and expert advice on animal diseases are in collaboration with the Chief Veterinary Officer and the Icelandic Food and Veterinary Authority.

Services provided comprise a wide range of testing, screening, diagnostics and development of new methodology. Services also involve control of animal welfare and slaughter, autopsy and advanced pathological examination.

Production of vaccine and antiserum against sheep diseases is an important part of the Institute's work. The institute acts as a National reference laboratory on the following items: fish, mollusks, crustaceans, Campylobacter, parasites (in particular Trichinella, Echinococcus and Anisakis).

The predominant research activity is on animal diseases, their prevention and containment. This includes diseases of both terrestrial and aquatic animals. One of the research project at Keldur is on insect bite hypersensitivity in horses [1].

I did my internship with the IBH group. This team is led by Sigurbjörg Torsteinsdóttir and Vilhjálmur Svansson.

2. Aim of the project

The long-term aim of the study is to develop an immunotherapy against IBH. Three allergens are going to be used for experimental vaccination in horses this summer. Those allergens need to be produced and purified from *E. coli*. It is very difficult to completely purify the protein from bacteria and some lipopolysaccharide* (LPS) often remain. The presence of some LPS is not a problem for the vaccination; indeed, it will enhance the immune response of the horse. However, those proteins can't be used in immunoassays* for evaluating the immunotherapy. It is impossible to use them to stimulate *in vitro* the production of cytokines. The contaminant bacterial proteins will also trigger an immune response and create a background response. Therefore, it is essential to have the allergens also produced in another system such as insect cells, which are the closest match to the cells of *Culicoides* midges. Moreover, the use of eukaryote cells enables us to obtain the protein with the correct post-translational modification* (1). In order to be used in the in vitro stimulation for PBMCs, the purified proteins also need to be in a cell-friendly environnement.

The specific aims of my project were:

1. Purification of recombinant Cul o 1pet and Cul o 3 from insect cells with His-select nickel affinity gel and solubilization into a non-toxic buffer.

2. Transformation of *E. coli* strain BL21 (DE3) with plasmids containing codon optimized Cul o 1pet and Cul o 3 and production of the allergens.

In order to purify Cul o 1pet, several methods (native and denature purification), expression systems (SUMO and HBM vectors) and refolding buffers were tried. The native purification of Cul o 3 had already been proven possible, but its solubility into a non-toxic buffer remained difficult. The dialysis of both proteins (Cul o 1pet and Cul o 3) was tried with various buffers whose toxicity on horse cells was evaluated.

In order to produce Cul o 1pet and Cul o 3 in *E. coli*, bacteria were transformed with recombinant* plasmids and the transformation tested in PCR. The allergens were produced and tested in Western Blot. However, both methods for production and purification remain to be optimized.

3. Materials and methods

3.1. Cul o 1pet (Kunitz proteinase inhibitor)

Cul o 1pet is a 26 kDa proteinase inhibitor and a major allergen in IBH. It has been expressed with two different vectors: pl-secSUMOstar (figure 4) and pFastBac ™/HBM-TOPO® (figure 5) (1).

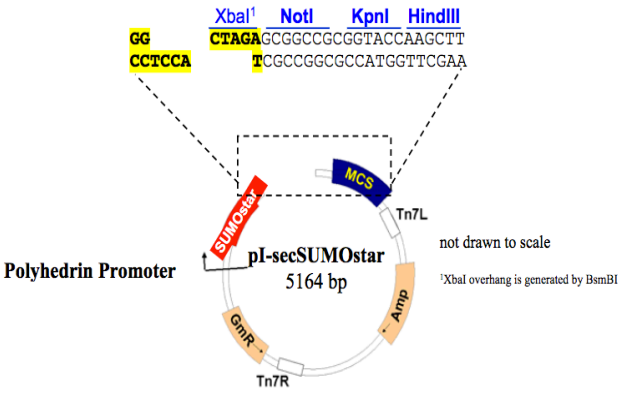

Figure 4: pl-secSUMOstar
Source: https://lifesensors.com/wp-content/uploads/2017/03/3105_3106_polylinker_pI-ecSUMOstar_pPolhAmp.pdf

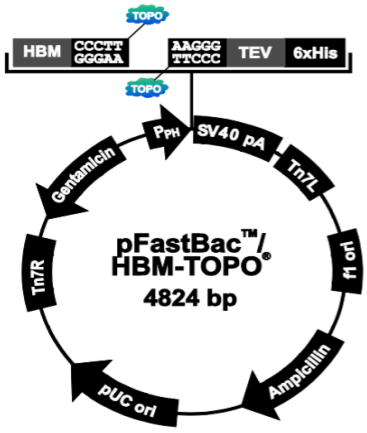

Figure 5: pFastBac ™/HBM-TOPO®
Source:
https://www.thermofisher.com/order/catalog/product/A11338

Allergens were expressed with the Bac-to-Bac baculovirus expression system. Bacmids are generated and recombinant Bac-viruses were produced in Sf-9 cells. The baculoviruses are cloned and used to infect High-5 cells (figure 7) for the production of the recombinant allergen.

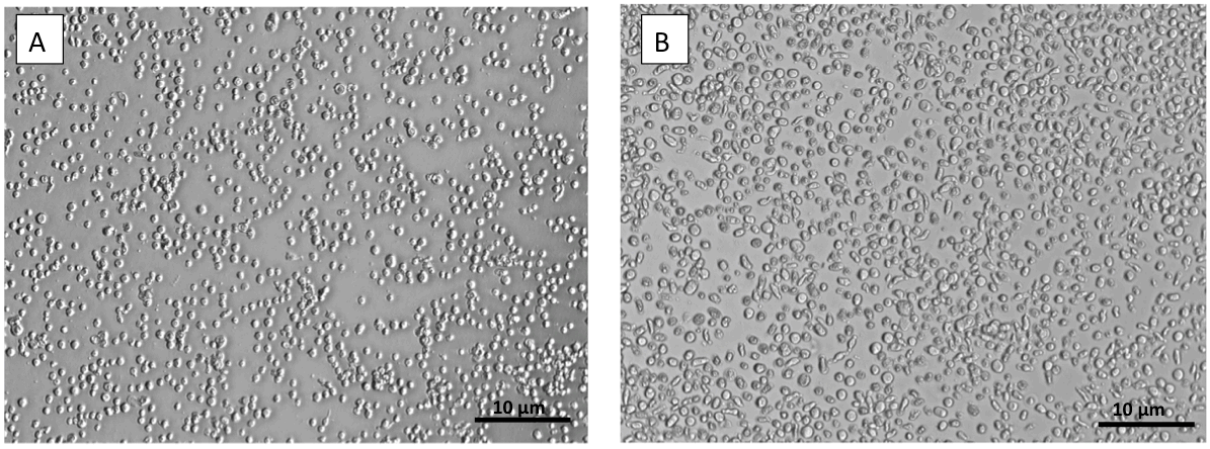

Figure 6: Healthy High 5 cells (a), High-5 cells infected with Cul o 1pet baculoviruses (b)
Source: Personal

3.2. Cul o 3 (Antigen 5)

Cul o 3 is a 41 kDa antigen 5 like protein and a major allergen in IBH. It has been expressed with the vector pl-secSUMOstar (figure 6) and the recombinant allergen produced the same way as Cul o 1 (paragraph 3.1).

[X] Webographic references
(X) Bibliographic references

3.3. Denatured purification

An HIS-select® HF Nickel Affinity Gel from Sigma-Aldrich was used. The gel was washed with ddH_20, centrifuged 5 min at 800 g, equilibrated in lysis buffer and centrifuged again 5 min at 800 g. The gel was washed and equilibrated with 10x its volume.

A cell pellet containing 100 millions cells was dissolved in the lysis buffer A (appendix A) and sonicated. The mix was centrifuged 15 min at 10 600 g and the supernatant collected. Thereafter, the supernatant was added to the gel in order to bind Cul o 1pet with the 6xHis-tag. The mixture was kept 1 h at room temperature and centrifuged 5 min at 800 g.

The gel was washed first with the wash buffer A and then B (appendix A) and centrifuged for 5 min at 800 g after each wash. It was then applied to a column and the protein eluted 3x first with elution buffer A and then elution buffer B (appendix A).

3.4. Dialysis and refolding of the protein

The dialysis is based on the principle of osmosis. There is a difference of concentration between inside of the dialysis bag and the buffer. Therefore, molecules (urea, imidazole, PBS, etc) smaller than the 13 kDa cutoff threshold of the membrane will diffuse through it to make equilibrium (figure 7). Cul o 1 and Cul o 3 are larger than 13 kDa and will stay trapped in the dialysis bag.

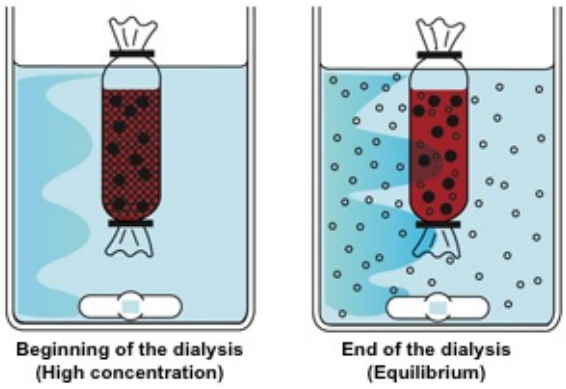

Figure 7: Diagram of the principle of the dialysis
Source: http://fr.spectrumlabs.com/dialysis/Fund.html

For the denatured protein, the purpose of the dialysis was to refold them into a non-toxic buffer. The samples were dialyzed twice: first into a refolding buffer (appendix B), then into PBS. Each time, the samples were dialyzed in 1000x their volumes, first for 3 h, then overnight after changing the buffer. The samples were centrifuged in order to verify whether or not a precipitation had occurred.

For the native proteins, the purpose of the dialysis was to transfer them into a non-toxic buffer. Two buffers were tried: elution buffer without imidazole and 2xPBS. PBS is a physiological buffer commonly used in handling cells. It is here used two times concentrated as high salt concentration helps proteins to refold without precipitation.

3.5. Native purification

Similar protocol was used as for the denatured purification (paragraph 3.3.). The differences were: 100 μL of PIC (protein inhibitor cocktail) was added to the cell pellet in lysis buffer, the binding was made at 4°C for 2h, four washes and three elutions with different buffers (appendix C). The samples were kept on ice for the entire procedure.

3.6. Bradford protein assay

The Bradford assay is a quick colorimetric method for measuring protein concentration. The Coomassie Blue, originally brown, binds to the basic amino acids of the proteins and becomes blue. Therefore, the blue color is proportional to the concentration of proteins [2].

The Coomassie Plus (Bradford) Assay Kit (Thermo Scientific) was used. 300 μL of the reagent was added to 10 μL of the sample in the non-adsorbent 96 wells-plates. The absorbance was read at 600 nm in a Perkin Elmer Victor 3 and compared to the ones of the standards.

3.7. Electrophoresis and Coomassie Blue staining

The principle of the Coomassie Blue staining is similar to the Bradford protein assay, except that it is combined to an electrophoresis. Proteins are separated on a SDS-PAGE gel with an electric current depending on their molecular weight. The SDS is a negatively charged molecule that denatures and fixes the proteins (around two molecules of SDS per amino acids). Therefore, larger proteins will bind to more SDS molecules and have more negative charges [3]. The original charge of the protein is negligible compared to the charge of the SDS. As the proteins are denatured, their structure doesn't impact their migration either. Therefore, only the molecular weight of the protein influences their movement in the gel [4].

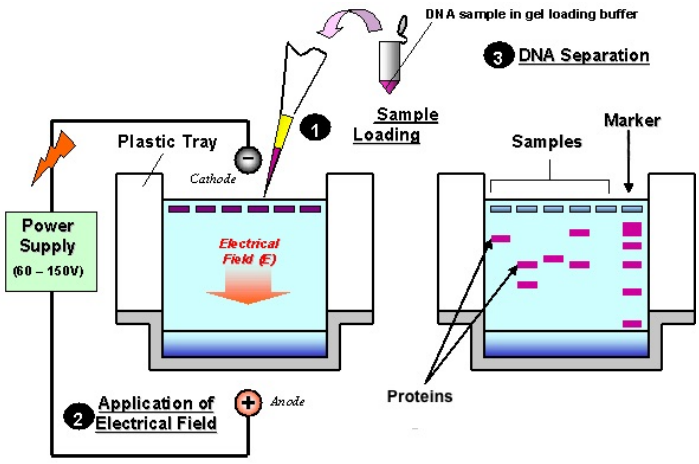

Figure 8: Diagram of the principle of the electrophoresis
Source: https://socratic.org/questions/what-is-gel-electrophoresis

1x volume of sample was added to 1x volume of sample buffer, the mix was then heated 5 min at 100 °C and spin for 2 min. The gel was loaded with 18 μL of sample and electrophoresed for 60 min at 200 V and 65 mA in electrophoresis buffer (appendix D). After

the electrophoresis the gel is dyed with Coomassie Blue. The dye enters the gel and binds to the amino acids of proteins, staining them in blue [6].

The protein samples were run on 12% SDS-PAGE gels (appendix E) and stained with Fairbanks solutions A, B, C and D (appendix F). Each time, the gel was heated with the solution for 90 s in a microwave and washed with ddH20. Fairbank A was left in contact with the gel for 5 min and Fairbank D for at least 15 min.

3.8. Western Blot

The Western Blot method is used to detect the presence of proteins with the use of specific antibodies. Proteins are separated by electrophoresis (paragraph 3.5). However, gels are fragile and antibodies can't bind to it. The proteins are therefore transferred to an Immobilon film with an electric current (figure 9) [5].

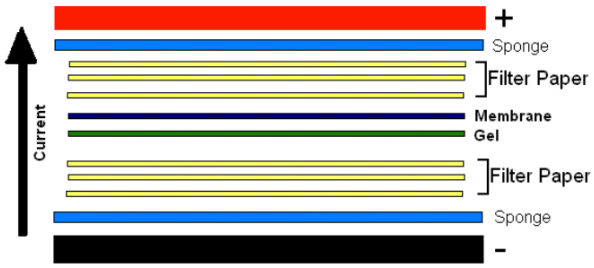

Figure 9: Diagram of the transfer
Source: http://docs.abcam.com/pdf/events/western-blot-introduction-et-optimisation.pdfelectrophoresis

Once the proteins are transferred to the membrane, the primary antibody α-His (BioRad MCA1396) diluted 1/1000 in TBS-T are added and incubated on the membrane at 4°C overnight. They will bind specifically to the His-tag of the protein. Second antibody Jackson AP α-mouse diluted 1/5000 are therefore added and incubated for 1 h at room temperature. The antibodies are linked to alkaline phosphatase. Substrate BCIP/NBT diluted 1/50 into alkaline phosphatase buffer is added to develop the color by reaction with the enzyme. It will highlight the protein of interest. The membrane is washed in TBS-T for 5 min 5x between each step.

3.9. Toxicity test

The blood of two horses was collected and the peripheral blood mononuclear cells (PBMCs) were isolated. The blood was left standing for 30 min at room temperature. The erythrocytes sink to the bottom and the plasma containing the white cells stays on top. The plasma is collected and overlaid 25 mL on 5 mL of Ficoll gradient, then centrifuged 20 min at 453 g. The plasma is discarded and the white band containing the PBMCs in the interface is collected. It is mixed with 45 mL of PBS and centrifuged at 290 xg for 10 min, brakes on 7. The cells are washed and centrifuged again. The cell pellet is resuspended in RPMI-1640 Medium GlutaMAX™ supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% horses serum and 50 μM 2-mercaptoethanol.

A 24 well plate was seeded with $2x10^6$ cells/wells: 500 μL of the cell suspension added to 500 μL of the dilutions of the buffers we wanted to test:

- Elution buffer with 250 mM imidazole (dilution 2x, 4x, 8x, without imidazole)
- Refolding buffer 3 (dilution 2x, 4x, 8x, without arginine)
- Refolding buffer 5 (dilution 2x, 4x, 8x, without arginine)
- Urea buffer (dilution 2x, 4x, 8x, without urea).

The cells were counted in the microscope after growing for 48 h at 37 °C and in an atmosphere of 5 % CO_2.

3.10. Transformation

Transformation is when plasmids containing the gene of interest are introduced into bacteria, in order to make them produce relevant proteins. *E. coli* (BL 21 DE3) was transformed with three different plasmids pet-42b(+) containing allergens genes which will be used for vaccinating the horses: Cul o 1pet, Cul o 3 and Cul n 4.

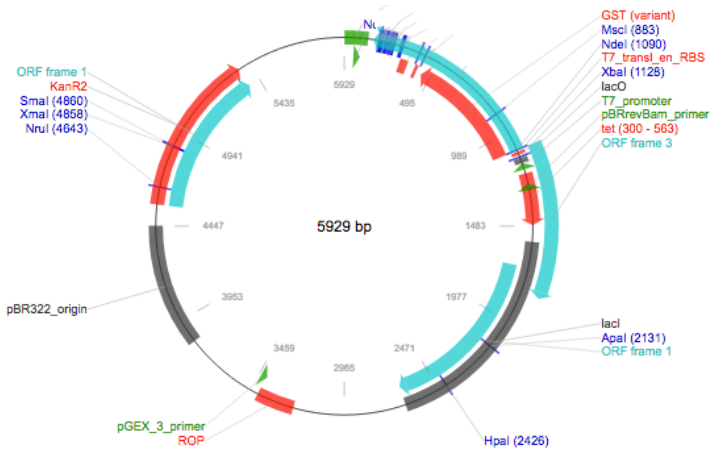

Figure 10: pet-42b(+)
Source: https://www.addgene.org/vector-database/2597/

The transformation was performed using heat chock. The mixture of bacteria and plasmid was kept on ice for 30 min, then heated at 42 °C for 30 s and put back on ice for 2 min. The thermal shock is used to destabilize the cell membrane and allow the plasmids DNA to penetrate into the bacteria. The transformed bacteria were selected on a LB medium plate containing kanamycin. The plasmid has a kanamycin resistance gene. Colonies picked from the plate are seeded into a liquid 2xYT medium with kanamycin (appendix G) and incubated at 37°C overnight on orbital shaker.

3.11. Production in *Escherichia coli*

When the *E. coli* has been transformed with the plasmid containing the gene coding for the allergens. The overnight culture, 100 μL in 2xYT medium is transferred into 100 mL of new medium and incubated at 37 °C on orbital shaker. The absorbance is read regularly

and 100 μL of IPTG (1 mol/L) is added when it reaches 0,6. The IPTG is going to bind on the inhibitor of the lactose operon and therefore, activate the production of the protein. The culture is incubated for 4 h at 37 °C on orbital shaker, then centrifuged at 2500 xg at 4 °C. The pellet is resuspended into 5 mL of lysis buffer (appendix A) and frozen at -80 °C.

3.12. PCR

The purpose of a PCR is to amplify certain portion of DNA. The presence of the gene of interest in the transformed *E. coli* was verified with PCR. For each sample, 1 μL of bacterial culture, 2 μL of buffer, 2 μL of dNTP, 1 μL of forward primers, 1 μL of reverse primers, 0,2 μL of Taq polymerase and 12,8 μL of ddH20 were mixed together. Samples were then put in the thermocycler with the following program:

- 95 °C for 5 min
- 95 °C for 15 s ⎫
- 52 °C for 30 s ⎬ 30x
- 72 °C for 1 min ⎭
- 72 °C for 10min
- 4 °C forever

First, DNA is denatured at 95 °C. Then, at 52 °C the primers bind to the DNA and at 72 °C there is elongation of the DNA by the Taq polymerase. After the thermocycling, 10 μL of each sample is run on a 1 % agarose gel with 3 of drops of ethidium bromide for 1 h at 70 V.

4. Results

4.1. Purification of recombinant Cul o 1pet

4.1.1. Denatured purification of HBM-Cul o 1pet

It has been shown that HBM-Cul o 1 can't be purified native. Therefore, a denatured purification from High-5 insect cells followed by refolding was tried. The purpose was to test the efficiency of the denatured purification and see if the purified product could be transferred to a non-toxic buffer.

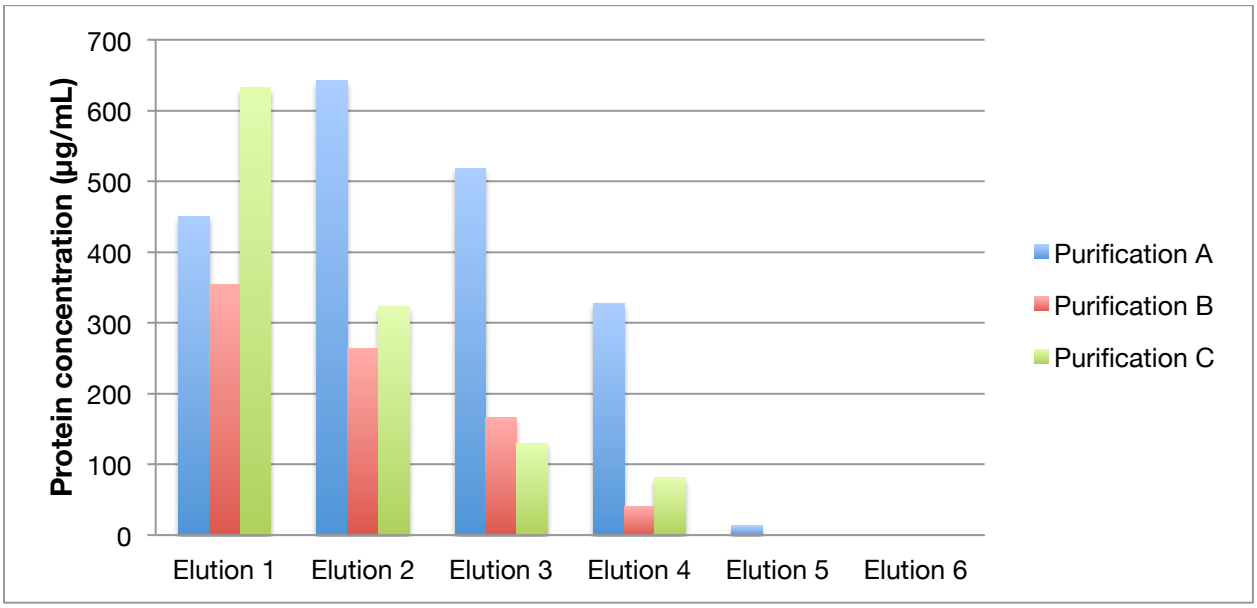

Figure 11: Three denature purifications A (blue), B (red) and C (green) of HBM-Cul o 1pet from High-5 cells; protein concentration (Bradford) in elution 1 to 6
Source: Personal

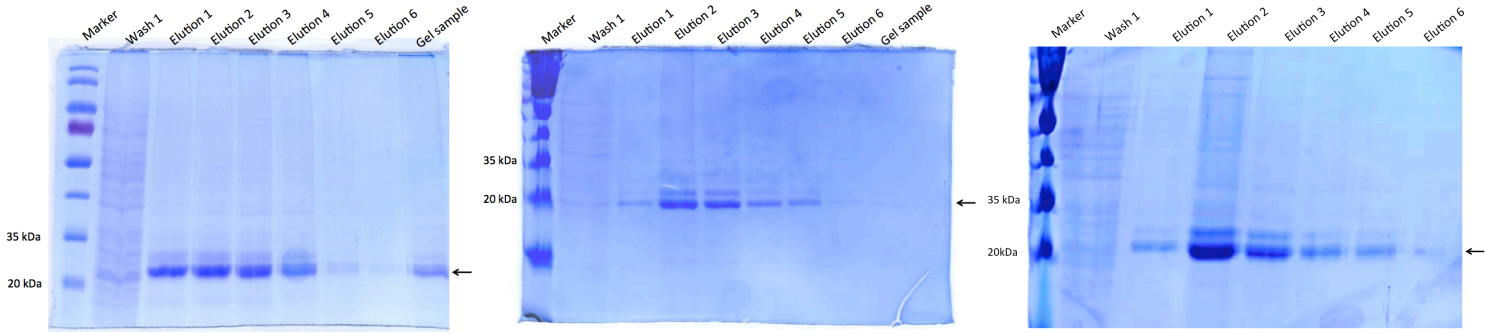

Figure 12: Coomassie staining of purification A, B and C of HBM-Cul o 1pet (26 kDa) from High-5 cells; Arrows indicates HBM-Cul o 1pet
Source: Personal

The samples from the elutions were tested in Bradford. The histogram (figure 11) shows that more proteins were purified in purification A and C than B. The three purifications were made following the same protocol and from the same batch of High-5 cells (paragraph 3.3). The only difference was that, for purification B, the gel was re-used whereas for purification A and C, it was new. Therefore, those results suggest that, to purify HBM-Cul o 1, it is

[X] Webographic references
[(X)] Bibliographic references

preferable to use a new gel each time. The three purifications were tested in Coomassie staining (Figure 12). The band that was detected was similar to the calculated size (26 kDa). The three purifications were successful, although some contamination protein bands can be seen, but they should not cause a problem.

4.1.2. Native purification of SUMO-Cul o 1pet

The gene was also express with as a SUMO construct. The SUMO vector contains a SUMOstar fusion protein at the N-terminus of the allergen [(1)]. It may prevent the 6xHis-tag to get folded inside the protein, making it inaccessible for binding to the His-gel. Therefore, a Bac-SUMO-Cul o 1pet construct was made, the protein produced and native purification tried.

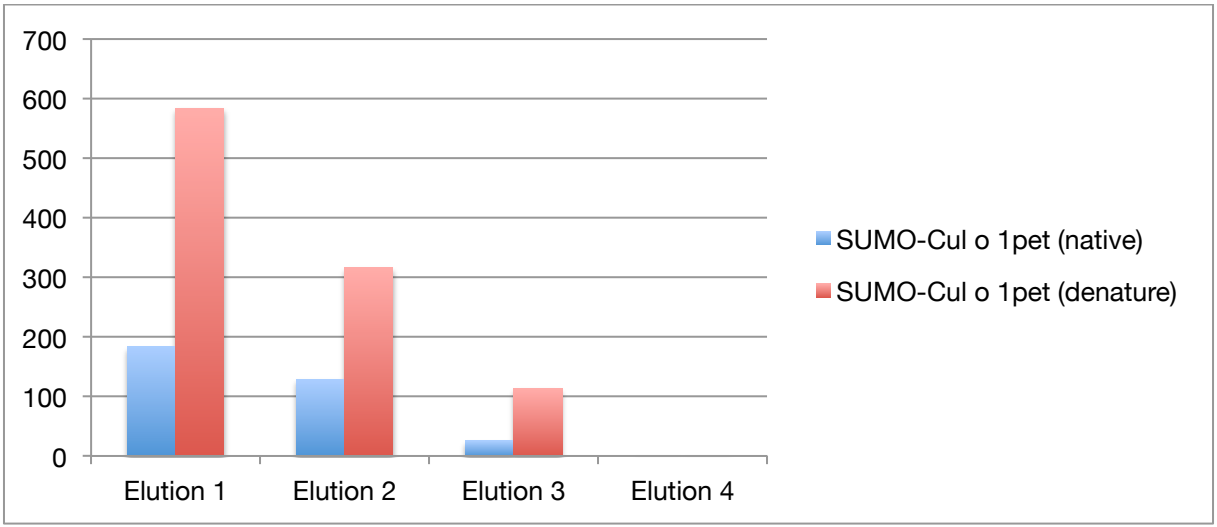

Figure 13: Native (blue) compared to denature purification (red) of SUMO-Cul o 1pet from High-5 cells; protein concentration (Bradford) in elution 1 to 4
Source: Personal

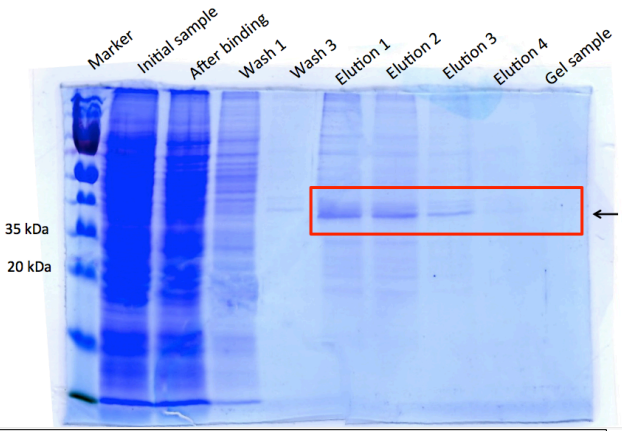

Figure 14: Coomassie staining of the native purification of SUMO-Cul o 1pet (38 kDa) from High-5 cells;
Arrow indicates SUMO-Cul o 1pet
Source: Personal

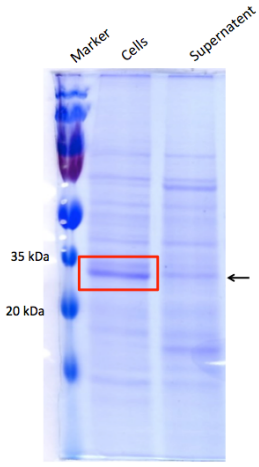

Figure 15: Coomassie staining of the production sample of SUMO-Cul o 1pet (38 kDa) from High-5 cells
Arrow indicates SUMO-Cul o 1pet
Source: Personal

The elutions were tested in Bradford. The blue band of the histogram (figure 13) represents the amount of SUMO-Cul o 1pet purified during the native purification. The results are compared with denature purification of SUMO-Cul o 1pet (paragraph 4.1.3). The performance of the denatured purification was three times better than the native one. The poor results of the native purification could be explained by either a problem with the production or by the fact that most of the protein stayed on the gel and was not eluted. In order to test this, a Coomassie staining was performed with samples from the production, the elutions and the gel (figure 14 and 15).

The absence of a protein band in the gel sample (figure 14) shows that no protein was retained by in the gel. On the other hand, the second gel (figure 15) shows that the production of SUMO-Cul o 1pet seems very poor, which might explain the results of the native purification. However, the production sample came from the same batch as the denatured purified protein. Therefore, the problem might have only been in this batch and not in the other ones of the production. A denatured purification of the allergen was tried, in order to verify that the production was the problem.

4.1.3. Denatured purification of SUMO-Cul o 1pet

SUMO-Cul o 1 was purified denature. Elutions were tested in Bradford (figure 13). The amount of purified protein in the first elution was around 600 μg/mL; that quantity could not have been obtained if the production was low. Therefore, as the production was shown to be successful in the denature purification, native purifications were made from two other batches, but with the same results as in paragraph 4.1.2 (results in appendix H). Therefore, it appears that Cul o 1pet can't be purified native despite being express as a SUMOstar construct.

4.2. Solubilization of Cul o 1pet in non-toxic buffers

The elutions 1, 2 and 3 of purification A (paragraph 4.1.1) were combined into pool 1 (concentration of 493 μg/mL). The elutions 1, 2, 3 and 4 of purification B (paragraph 4.1.1) were combined into pool 2, which had a lower concentration of protein (260 μg/mL). The two samples were dialyzed first into refolding buffer 3 (appendix B), then into 2xPBS.

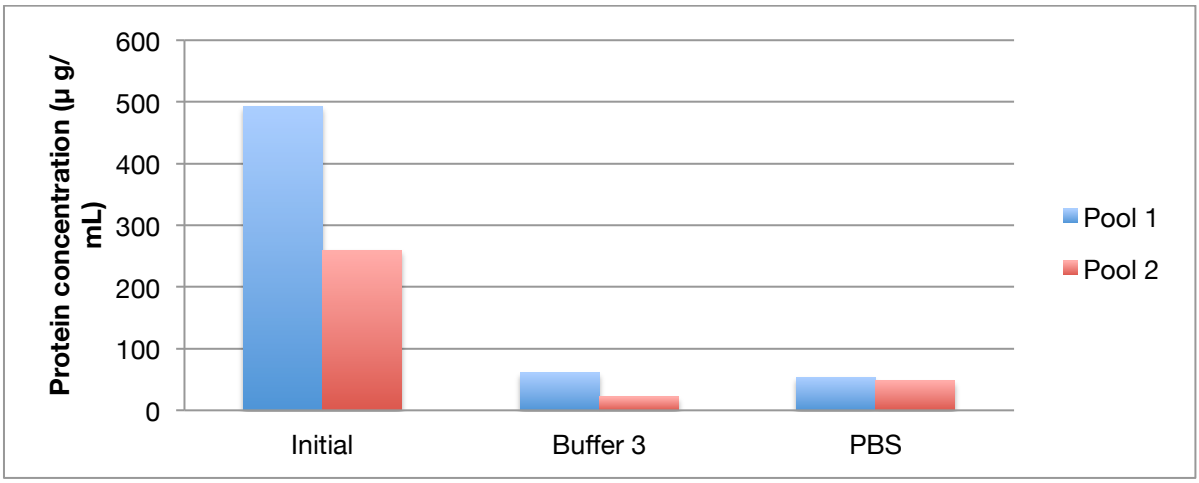

Figure 16: Pool 1 (blue, 493 μg/mL) and pool 2 (red, 260 μg/mL) of HBM-Cul o 1pet from purification A and B before and after dialysis in buffer 3 and PBS; protein concentration measured in Bradford
Source: Personal

The samples were tested in Bradford. The dialysis was performed two times with the same results. Almost all the proteins were lost by precipitation regardless of the original concentration of the pool (figure 16). The experiment enabled us to see whether or not reducing the concentration of protein in the samples could lessen their loss. In the first trial, the loss of protein was 89% for pool 1 and 81,5 % for pool 2. In the second, the loss was 80% for pool 1 and of 100 % for pool 2. The dialysis was unsuccessful regardless of the protein concentration.

Two more dialysis were tried with both HBM-Cul o 1 and SUMO-Cul o 1. One in refolding buffer 3 and one in refolding buffer 5, both followed by a dialysis in 2xPBS. For the HBM-Cul o 1pet pool, elutions 1, 2, 3 of purification C (paragraph 4.1.1) were combined. For the SUMO-Cul o 1pet pool, elutions 1, 2, 3 of the denatured purification (paragraph 4.1.3) were combined.

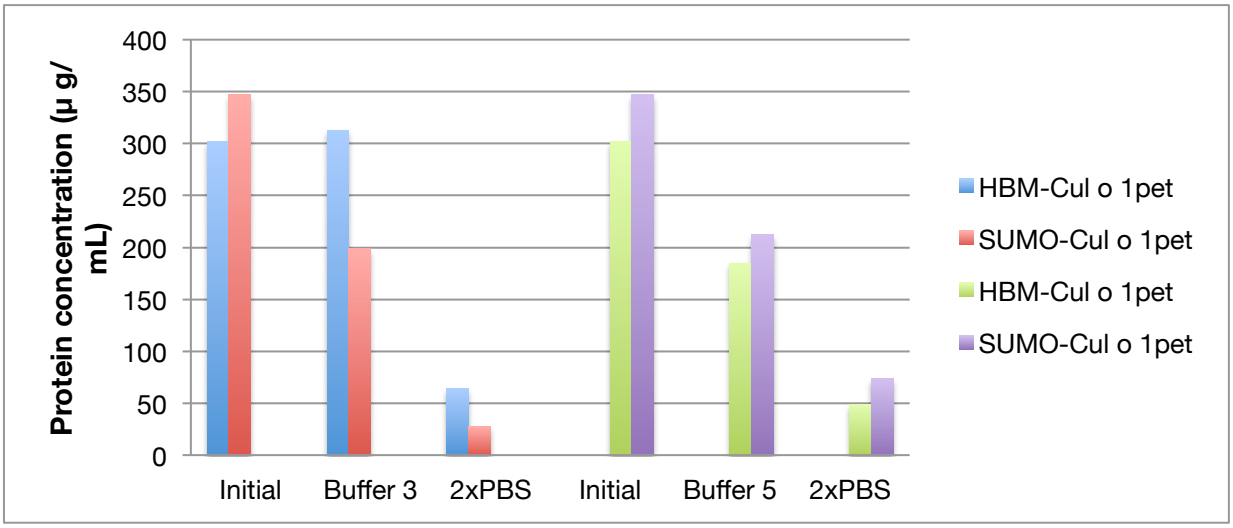

Figure 17: HBM-Cul o 1pet (blue and green) and SUMO-Cul o 1pet (red and purple) before and after dialysis in buffer 3, buffer 5 and PBS; protein concentration measured in Bradford
Source: Personal

Both proteins were successfully dialyzed in refolding buffer 3 and 5 (figure 17). The samples were tested in Bradford. HBM-Cul o 1pet seemed to solubilize better in buffer 3 (313 μg/mL) than buffer 5 (185 μg/mL), whereas the solubilization of SUMO-Cul o 1pet was similar in both buffers (around 200 μg/mL). The reason why the dialysis of HBM-Cul o 1 didn't work in buffer 3 in the first experiment is not known. However, dialysis from refolding buffer 3 and 5 into 2xPBS was unsuccessful.

4.3. Purification SUMO-Cul o 3

The native purification of SUMO-Cul o 3 had already been proven possible. For this purification, the protein was produced by infecting the High-5 cells and culturing them at 15°C for one week instead of 27°C for 3 days. Therefore, the goal was to test the impact of the 15 °C infection on the yield of the protein.

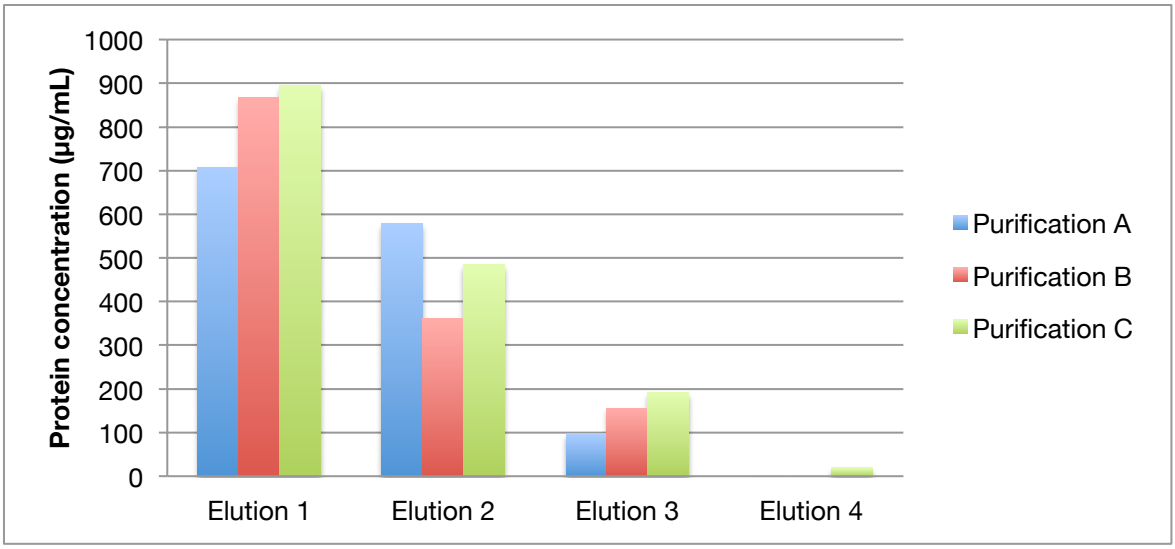

Figure 18: Three native purifications A (blue), B (red) and C (green) of SUMO-Cul o 3 from High-5 cells; protein concentration (Bradford) in elution 1 to 4
Source: Personal

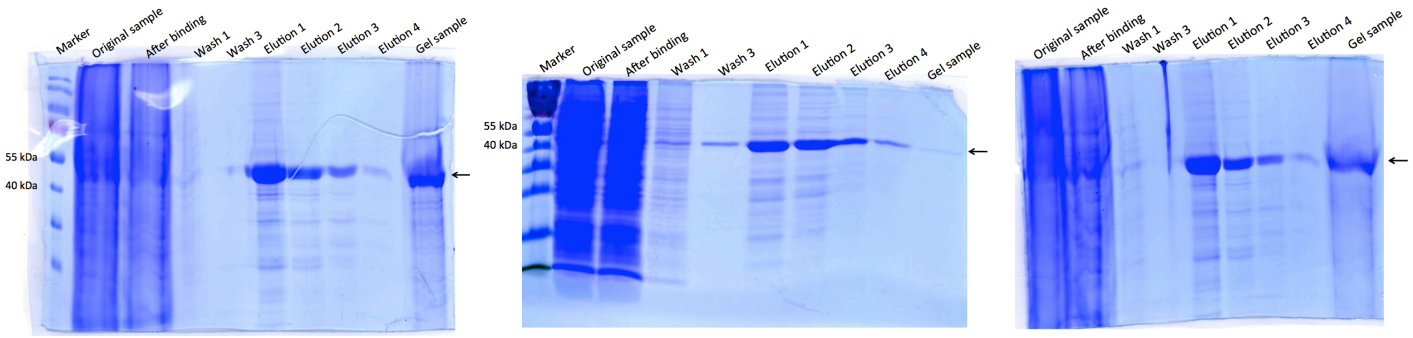

Figure 19: Coomassie staining of purification A, B and C of SUMO-Cul o 3 (41 kDa) from High-5 cells; Arrow indicates SUMO-Cul o 3
Source: Personal

The samples from the elutions were tested in Bradford. The histogram (figure 18) shows that the three natives purifications of SUMO-Cul o 3 give similar results, and that a large amount of protein was purified (700 to 900 μg/mL in the first elutions). This was much higher yield than obtained after infection at 27 °C where the amount of purified protein was around 250 μg/mL [(1)]. Moreover, the results of the Coomassie staining (figure 19) show us that Cul o 3 is quite pure. Despite the good yield, the gels retained some proteins that seemed to be firmly bound to the gel as nothing came out of the fourth elution.

4.4. Solubilization of SUMO-Cul o 3 in non-toxic buffers

After the native purification, allergens are in elution buffer containing 250 mM imidazole, which is toxic for cells. Therefore, it is necessary to dialyze them into a non-toxic buffers.

Elutions 1, 2 and 3 of natives purifications A, B and C (paragraph 4.3) of SUMO-Cul o 3 were combined into one pool (concentration of 503 μg/mL). Beside 2x PBS, dialysis was also tried against the elution buffer without imidazole (pH=8,0). It was thought that dialyzing

[X] Webographic references
[(X)] Bibliographic references

the protein in a buffer more similar to the one it was in might reduce the precipitation. The samples were measured in Bradford.

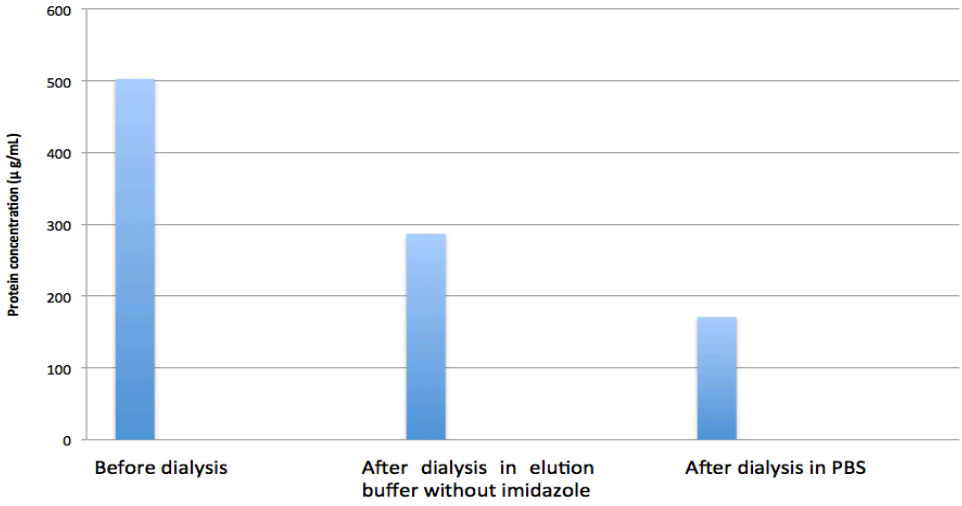

Figure 20: Pool of SUMO-Cul o 3 made of the elution of natives purifications A, B and C, before and after dialysis in elution buffer without imidazole and PBS; protein concentration measured in Bradford
Source: Personal

After the dialysis into the elution buffer without imidazole, the concentration of allergens was 280 μg/mL and at 170 μg/mL into PBS (figure 20). Therefore, 55 % of the proteins were retained in elution buffer without imidazole and 34 % in PBS. Those results are very encouraging. Both concentrations should be enough to use it in the in-vitro stimulation of PBMCs for measurement of cytokines following the vaccination.

4.5. Toxicity of the buffers used in protein purification

The toxicity of several buffers used in both native and denature purifications (paragraph 3.9) was tested by adding these buffers in several dilution into cell cultures with PBMCs from two horses. Live cells were counted on a neubauer cell counting chamber in light microscope (Leitz Diavert) after 48h incubation.

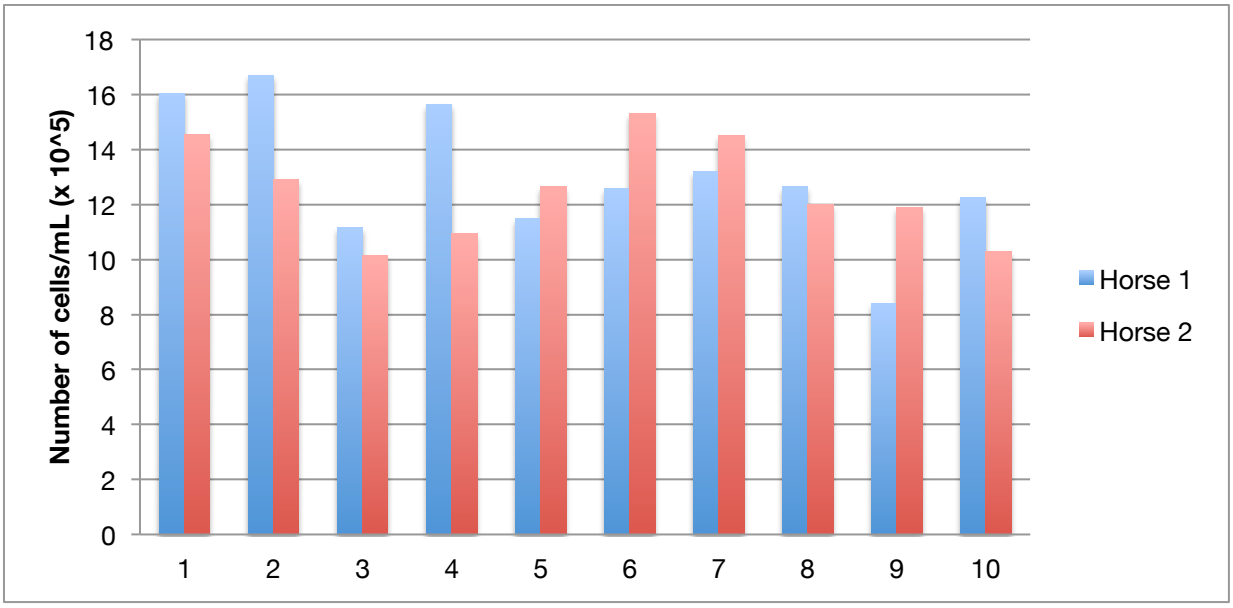

Figure 21: PBMCs in cell culture medium supplemented with the buffers;

1) buffer with imidazole; 2) refolding buffer 3; 3) refolding buffer 5; 4) urea buffer; 5) buffer without imidazole; 6) refolding buffer 3 without arginine; 7) refolding buffer 5 without arginine; 8) buffer without urea; 9 and 10) Medium

Source: Personal

The highest concentration of the buffers was 40x diluted from the concentration used in the purification process, as that would be the minimal dilution of the proteins when used for the stimulation of PBMCs from vaccinated horses. The cell count was not lower in any of the buffers in the highest concentration than in medium alone (figure 21). Therefore, no toxic effect on the cells was observed.

4.5. Production of Cul o 1pet and Cul o 3 in *E. coli* BL21 (DE3)

4.5.1. Efficacy of the transformation

E. coli was transformed using heat shock with the codon optimized gene on a pet-42b(+) plasmid (paragraph 3.10). For each allergen, one recombinant colony was selected to produce the protein and tested in PCR. The purpose being to verify the presence of the gene of interest, part of the Cul o 3 gene was amplified (341 bp), whereas the entire size of the gene is 810 bp. Cul o 1pet was amplified at full length.

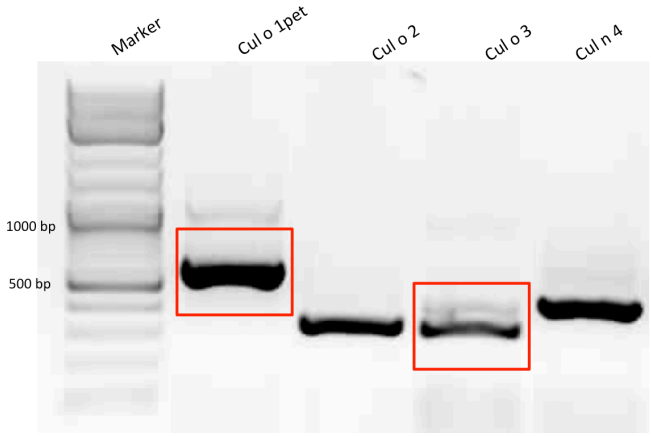

Figure 22: Amplification of Cul o 1pet (615 bp) and Cul o 3 (341 bp) in *E. coli* BL 21 (DE3) for testing the bacterial culture
Source: Personal

A strong band of DNA is present in each column (figure 22). The presence of the genes for Cul o 1pet and Cul o 3 were verified with amplification in PCR.

4.5.2. Production of Cul o 1pet and Cul o 3 in *E. coli*

The production was induced by addition of IPTG in the culture. After 4 h the cells were harvested and the samples tested on Western Blot.

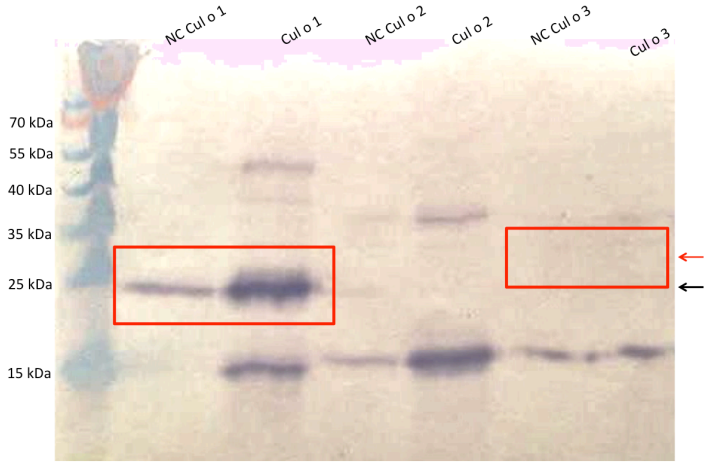

Figure 23: Western Blot of the production of Cul o 1pet (24 kDa) and Cul o 3 (31 kDa) in *E. coli* (BL21 DE3)
NC: Negative control
Black arrow indicates Cul o 1pet
Red arrow indicates where Cul o 3 should be
Source: Personal

The blot (figure 23) shows that the bacterial production of Cul o 1pet was successful. The band is detected at the calculated size. A smaller one is present at 15 kDa and probably corresponds to some fragment of Cul o 1pet. The band corresponding to the protein is much stronger after induction than before.

For Cul o 3, no protein band is detected around 31 kDa. Both in the negative control and in the sample after production, a band at 15 kDa is present. The protein could have broken into smaller fragments. Nonetheless, no difference can be seen between the negative control and the sample after induction, therefore, the production of Cul o 3 by *E. coli* was not successful.

5. Discussion

In order to develop a vaccine against IBH, purified allergens are needed both to vaccinate and to monitor the immune response of vaccinated horses. *E. coli* produced proteins will be used for the vaccination but they are not suitable for some of the immunological test. Therefore the allergens need also to be purified from other expression systems, like insect cells.

The aim of the project was twofold. First, express and produce Cul o 1 and Cul o 3 in *E. coli* for vaccination of horses. Then, purify the two allergens from insect cells and solubilize them in non-toxic buffers. Those proteins will be used to stimulate *in vitro* PMBCs from experimentally vaccinated horses for production of cytokines.

Cul o 1pet and Cul o 3 were successfully transformed into *E. coli*. However, the Cul o 3 protein was not produced (figure 23). The reason why the production of Cul o 3 didn't work is not known, but it will be tried again picking other colonies. On the other hand, Cul o 1pet was produced successfully and seemed to have the correct size. In order to produce a sufficient amount of protein for purification, the production of Cul o 1pet will have to be up-scaled. Due to the inclusion bodies, bacterial proteins can't be purified following the same protocol as proteins from insect cells, a different protocol will be followed and will have to be optimized.

The Cul o 1pet protein was expressed as HBM-Cul o 1pet and SUMO-Cul o 1pet. It had been shown that native purification of HBM-Cul o 1pet was not possible [(1)] and that of SUMO-Cul o 1pet did not give much yield (figure 13). Therefore, the SUMO vector doesn't seem to have much influence on the amount of native purified Cul o 1pet. Hence, it was tried to purify Cul o 1pet denatured and refold it. Two buffers were tested in order to try to refold the protein from urea: refolding buffer 3 and 5. Solubilization of HBM-Cul o 1pet seemed to be better in buffer 3 but that of SUMO-Cul o 1pet was the same in both buffer (figure 17). Nonetheless, both buffers seemed to be working. However, almost all the protein was lost when dialyzed into 2xPBS. Reducing the concentration of protein before dialyzes did not seem to minimize the precipitation. Neither refolding buffer 3 nor 5 seemed to be toxic for PBMCs at the tested concentrations (figure 21). Therefore, it might not be necessary to dialyze the protein into PBS. However we do not know if the proteins precipitate at this given concentration from the refolding buffers when they are mixed with cell culture medium.

It was shown that SUMO-Cul o 3 could be purified native. The purification seemed to be about three times more efficient when the protein is produced in High-5 insect cells infected for a week at 15 °C, rather than 3 days at 27 °C [(1)]. SUMO-Cul o 3 could be dialyzed both in elution buffer without imidazole and in PBS, without too much loss and with a better efficiency in the first one. Elution buffer without imidazole was shown not toxic for the PBMCs at the tested concentration, but solubilization of SUMO-Cul o 3, dialysis in 2xPBS remains an option.

[X] Webographic references
[(X)] Bibliographic references

6. Conclusion

6.1. Scientific conclusion

During my internship, I had to improve the purification and solubilization of Cul o 1pet and Cul o 3. It was shown that Cul o 1pet couldn't be purified native despite the SUMO construct. The denature purification of HBM-Cul o 1pet and SUMO-Cul o 1pet is successful and gives similar results. Their solubilization has been proven possible in both refolding buffer 3 and 5. However, dialysis in 2xPBS remain unsuccessful.

It has been shown that the production of Cul o 3 is more efficient when the cells are infected and cultured one week at 15 °C instead of three days at 27 °C. The native purification of SUMO-Cul o 3 is successful, and its solubilization has been proven possible in both elution buffer without imidazole and 2xPBS.

The toxicity of the buffers of both native and denature purification on PBMCs was tested and no toxic effect was observed at those dilutions. Therefore, solubilization in PBS might not be necessary.

The production of Cul o 1pet in E. coli BL21 (DE3) was successful, but the one of Cul o 3 remains a challenge. The protein Cul o 3 has been ordered online for the vaccination experiment. Therefore, the next step will be to try to purify bacterial Cul o 1pet.

6.2. Personal conclusion

I am truly grateful to have had the opportunity to do my internship in this laboratory, in Iceland. Indeed, doing my internship abroad was a real opportunity for me to discover another country, another culture while learning biology and improving my technical skills. Working in English all day long was great and enabled me to be more comfortable with the scientific vocabulary in this language. Being alone in a new country and working in a lab also made me gain a lot in autonomy, both in the lab and in my personal life. During my internship, I worked a lot with techniques seen in class. It was very interesting to see their application in the research world. I also loved working in a team, seeing what the results were used for, understanding the next steps, etc. Being able to contribute to the IBH project at my scale was very rewarding. I also acquired a new theoretical knowledge in immunology and in molecular biology and it was very interesting to use them to understand the background of the project.

Webography

(Indicated in the text by a number like: [X])

[1] http://keldur.is/node/421 (consulted on April the 11th)

[2] https://www.thermofisher.com/order/catalog/product/23236 (consulted on April the 25th)

[3] https://planet-vie.ens.fr/article/1500/electrophorese#l-electrophorese-en-conditions-denaturantes (consulted on April the 25th)

[4] http://www8.umoncton.ca/umcm-gauthier_didier/siitub/elepgpa.html (consulted on April the 25th)

[5] https://www.anticorps-enligne.fr/resources/17/1224/test-de-western-blot-immunotransfert-electrophorese-de-proteines-sur-gel/ (consulted on May the 2nd)

[X] Webographic references

(X) Bibliographic references

Bibliography

(Indicated in the text by a number like: [X])

[1] Sara Björk Stefánsdóttir, (2015) Development of methods for expressing and purifying recombinant allergens in insect cells and their application for evaluating immunotherapy, *Thesis for degree of Master of Science*.

[2] Sigríður Jónsdóttir, (March 2017), Development of immunotherapy for Insect Bite Hypersensitivity in Horses, *Thesis for the degree of Philosophiæ Doctor*.

[3] Juliette Depreaux, (2016), Production and purification of Culicoides hyaluronidase, an allergen in Insect Bite Hypersensitivity in horses (IBH*), DUT internship report*.

[X] Webographic references
[X] Bibliographic references

Appendixes table

[X] Webographic references

[(X)] Bibliographic references

APPENDIX A

Composition of the buffers for the denatured purification

Lysis buffer A *pH = 8,0*	Guanidin-HCl (6M)	573,2 g/L
	$NaH_2PO_4xH_20$ (0,1M)	13,8 g/L

Wash buffer A *pH = 8,0*	Urea (8M)	480,5 g/L
	$NaH_2PO_4xH_2O$	13,8 g/L
	Tris-base (0,01M)	1,6 g/L
	NH_4Cl (0,1M)	5,4 g/L

Wash buffer B *pH = 6,3*	Urea (8M)	480,5 g/L
	$NaH_2PO_4xH_2O$	13,8 g/L
	Tris-base (0,01M)	1,6 g/L
	NH_4Cl (0,1M)	5,4 g/L

Elution buffer A *pH = 4,4*	Urea (8M)	480,5 g/L
	$NaH_2PO_4xH_2O$	13,8 g/L
	Tris-base (0,01M)	1,6 g/L
	NH_4Cl (0,1M)	5,4 g/L

Wash buffer B *pH = 4,0*	Urea (8M)	480,5 g/L
	$NaH_2PO_4xH_2O$	13,8 g/L
	Tris-base (0,01M)	1,6 g/L
	NH_4Cl (0,1M)	5,4 g/L

[X] Webographic references
(X) Bibliographic references

APPENDIX B

Composition of the refolding buffer

Refolding buffer 3	L-arginine	153,3 g/L
	Tris	6,7 g/L
	NaCl	1,2 g/L
	KCl	0,07 g/L
	EDTA (200mM)	500 µL

Refolding buffer 5	Guanidine	32,4 g/L
	L-arginine	76,7 g/L
	Tris	6,7 g/L
	NaCl	1,2 g/L
	KCl	0,07 g/L
	EDTA (200 mM)	500 µL

APPENDIX C

Composition of the buffers for the native purification

Lysis buffer *pH = 8,0*	NaCl (150 mM)	6,90 g/L
	$NaH_2PO_4xH_20$ (50 mM)	8,77 g/L
	IGEPAL (1 %)	10 mL

Wash buffer A *pH = 8,0*	NaCl (300 mM)	17,5 g/L
	$NaH_2PO_4xH_20$ (50 mM)	6,90 g/L
	Imidazole (10 mM)	0,68 g/L

Wash buffer B *pH = 8,0*	NaCl (300 mM)	17,5 g/L
	$NaH_2PO_4xH_20$ (50 mM)	6,90 g/L
	Imidazole (20 mM)	1,36 g/L

Elution buffer A *pH = 8,0*	NaCl (300 mM)	17,5 g/L
	$NaH_2PO_4xH_20$ (50 mM)	6,90 g/L
	Imidazole (250 mM)	17,02 g/L

Elution buffer B *pH = 6,5*	NaCl (300 mM)	17,5 g/L
	$NaH_2PO_4xH_20$ (50 mM)	6,90 g/L
	Imidazole (250 mM)	17,02 g/L

APPENDIX D

Composition of the electrophoresis and transfer buffer

Electrophoresis buffer:

Tris	25 mM
Glycine	192 mM
SDS	0,1%

Transfer buffer:

Tris	25 mM
Glycine	192 mM
Methanol	20%

[X] Webographic references
(X) Bibliographic references

APPENDIX E

Composition of the stacking gel and 12% separating gel

Stacking gel (per gel):

dH20	1,55 mL
1,5 M Tris (pH=8,8)	625 µL
40% bis-acrylamid	325 µL
10% SDS	25 µL
10% APS	25 µL
TEMED	10 µL
Bromophenol Blue	5 µL

12% separating gel (per gel):

dH20	2,15 mL
1,5 M Tris (pH=8,8)	1,25 mL
40% bis-acrylamid	1,50 mL
10% SDS	50 µL
10% APS	50 µL
TEMED	10 µL

[X] Webographic references
(X) Bibliographic references

APPENDIX F

Composition of the Fairbanks solutions

	Solution A	Solution B	Solution C	Solution D
0,2% Coomassie stock	25 mL	2,5 mL	1 mL	0 mL
Isopropanol	10 mL	10 mL	0 mL	0 mL
Acetic acid	25 mL	10 mL	10 mL	10 mL
dH20	40 mL	77,5 mL	89 mL	90 mL

[X] Webographic references
[(X)] Bibliographic references

APPENDIX G

Composition of the 2xYT medium with kanamycin

Per 1 L:

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g

[X] Webographic references
(X) Bibliographic references

APPENDIX H

Results of the second native purification of SUMO-Cul o 1pet

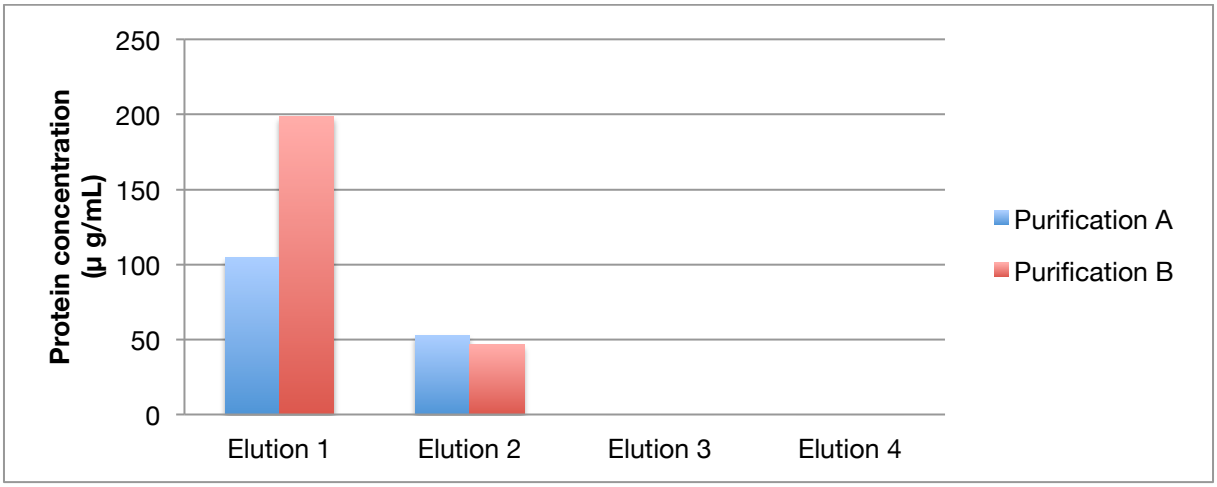

Figure 17: Two native purifications A (blue), B (red) of SUMO-Cul o 1 from High-5 cells; protein concentration (Bradford) in elution 1 to 4
Source: Personal

APPENDIX I

PPP, compétences et connaissances

Connaissances, compétences / IUT

Quelles sont les connaissances acquises à l'IUT que vous avez été amené à mobiliser pendant votre stage ?

J'ai principalement été amenée à mobiliser les connaissances acquises en cours d'immunologie (1ere année), de biologie moléculaire (1ere année), de purification de biomolécules (2e année) et de transformation bactérienne (2e année).

Quelles sont les compétences acquises à l'IUT que vous avez été amené à mobiliser pendant votre stage ?

J'ai bien entendue été amenée à mobiliser toutes les compétences de base de manipulation en laboratoire (BOPLA...), mais j'ai également été amenée à réaliser des manipulations similaires à celles effectuées en cours d'immunologie (ELISA, Western Blot...) et de transformation bactérienne. J'ai également dû utiliser quelques techniques de manipulation sous hotte stérile apprises à la fois lors des séances de TP de culture cellulaire animale et lors de mon stage de 1ere année.

Quelles sont les connaissances qui vous ont fait défaut pour réaliser au mieux votre sujet de stage ?

Aucune connaissance ne m'a réellement fait défaut lors de mon stage. En effet, je pense avoir acquis toutes les bases nécessaires à la compréhension des problématiques et des méthodes utilisées lors de mon stage. Cependant, mon stage se déroulant dans une unité d'immunologie, je pense que des cours théoriques plus poussés dans ce domaine auraient pu m'aider à être plus rapidement à l'aise avec le vocabulaire utilisé au laboratoire, bien que cela n'ait pas posé de problème majeur.

Quelles sont les compétences qui vous ont fait défaut pour réaliser au mieux votre sujet de stage ?

Ayant déjà réalisé la majorité des manipulations de mon stage à l'IUT, je ne pense pas qu'il m'ait manqué des compétences particulières pour la réalisation de mon sujet de stage.

Connaissances, compétences / stage

Quelles sont les connaissances majeures acquises pendant le stage ? Lesquelles pensez-vous mobiliser dans votre PPP, pourquoi ?

Je pense avoir principalement acquis des connaissances en immunologie, notamment les médiations des réactions allergiques dans l'organisme. J'ai également pu approfondir mes connaissances théoriques sur la purification des protéines. Cependant, je ne pense pas mobiliser ces connaissances dans mon PPP, en effet, je souhaiterai, l'année prochaine,

intégrer une école d'ingénieur en biomédical. Il s'agit là d'une formation comportant une plus grande part de physique que de biologie pure. De plus, les métiers que je vise après cette école sont plus orientés vers la conception de prothèses (ou d'appareillages de ce type) que vers le travail en laboratoire de biologie.

Quelles sont les compétences majeures acquises pendant le stage ? Lesquelles pensez-vous mobiliser dans votre PPP, pourquoi ?

Comme je l'ai décrit plus haut, les manipulations que j'ai été amenée à réaliser au cours de mon stage étaient des méthodes que j'avais déjà pu mettre en œuvre une première fois à l'IUT ou bien au cours de mon stage de 1ere année. Ainsi, je pense qu'il serait plus juste de parler d'un renforcement plutôt que d'une acquisition de compétences. Je pense avoir principalement été amenée à renforcer mes compétences en purification de biomolécules, ainsi que mes compétences dans le dosage et l'analyse d'échantillons de protéines via différentes méthodes (ELISA, western Blot, Bradford...). Cependant, je ne pense pas particulièrement mobiliser ces compétences dans mon PPP pour les mêmes raisons que celles décrites dans la question précédente.

Et si c'était à refaire :

Quelles propositions de changement feriez vous au niveau de votre formation à l'IUT ?

Je pense que la formation dispensée à l'IUT était très complète. Cependant, je pense que pour une option IAB (Industries Agroalimentaires et Biologiques), la partie « biologique » est trop souvent négligée en faveur de la partie « agroalimentaire ». Avec le recul, je pense que l'option ABB aurait mieux convenu à mes attentes. Néanmoins, au moins la moitié des étudiants choisissant l'option IAB sont plus attirés par le coté biotechnologie que le coté agroalimentaire et je pense que la formation pourrait être améliorée en équilibrant la formation à part égale sur ces deux domaines.

Quelles propositions de changement feriez vous au niveau de votre stage (recherche, préparation, réalisation…) ?

Bien que mon stage ne corresponde pas directement à mon projet professionnel, je n'y changerai absolument rien. J'avais commencé mes recherches durant l'été 2017 en ciblant les laboratoires dont la thématique de recherche m'intéressait le plus, dans un pays étranger, en particulier l'Islande ou l'Irlande, car l'expérience à l'international me semblait très enrichissante. Keldur (mon lieu de stage) était au sommet de ma liste et je suis toujours très heureuse d'y réaliser mon stage actuellement. Je suis également très contente d'effectuer mon stage à l'étranger et ne pourrai que le conseiller à de prochains étudiants de 2^{e} année. De même, mon stage s'étant toujours très bien déroulé, je ne vois pas ce que j'aurais pu améliorer, ni au niveau de la préparation, ni au niveau de la réalisation.

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Production and purification of two allergens for the vaccination of horses against insect bite hypersensitivity

Insect bite hypersensitivity (IBH) is an allergic reaction of horses due to the bite of *Culicoides* midges. It is a recurrent seasonal dermatitis, which causes severe itches, leading to a hair loss, various lesions of the skin and even to an infection. Icelandic horses born in Iceland and exported to the continent are the most affected by this pathology. For that reason, Keldur is trying to develop a vaccine to prevent this allergy. Cul o 1pet and Cul o 3 are two major allergens originated from the salivary gland of *Culicoides* midges and responsible for IBH. The two allergens also had to be produced in *E. coli* for the vaccination. However, Cul o 1pet and Cul o 3 were also produced in insect cells. They had to be both purified and solubilized in a non-toxic environment, in order to be used in *in vitro* stimulation to monitor the production of cytokines of experimentally vaccinated horses.

L'eczéma d'été du cheval est une réaction allergique due à la morsure des moucherons hématophages du genre *Culicoides*. Il s'agit d'une dermatite saisonnière causant de fortes démangeaison menant à une perte de poil, diverses lésions dermatologique et parfois même à une infection. Les chevaux islandais née en Islande et exportés vers le continent sont le plus touchés par cette pathologie. Pour cette raison, Keldur s'est lancé dans la mise au point d'un vaccin pour prévenir cette allergie. Cul o 1 pet et Cul o 3 sont deux allergènes majeurs issus des glandes salivaires des moucherons *Culicoides* et responsable de l'eczéma d'été. Les deux allergènes ont due être produit dans *E. coli* pour la mise au point du vaccin. Cependant, Cul o 1pet et Cul o 3 ont également due être produit dans des cellules d'insecte. Ils ont ensuite été purifié et solubilisé dans un environnement non toxique, afin de pouvoir être utilisés dans des stimulations *in vitro* destinées à surveiller la production de cytokines des chevaux expérimentalement vaccinés.

Key words: insect bite hypersensitivity (IBH), summer eczema, vaccine, allergens, *Culicoides*, horses, purification, production.

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