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Development of vaccines aimed at controlling/down-regulating allergic reactions

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Title (English)

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Abstract

This report summarizes the development of a protocol for purifying three new allergy vaccine candidates. Such a protocol has now successfully been developed and this protocol can be used to purify relatively large amounts of the vaccine from one single fermentor batch.

Keywords

Recombinant protein, cloning, expression vector, vaccine, immune system, Ion Metal Affinity Chromatography (IMAC), Size Exclusion Chromatography (SEC), fermentation, fermentor, IgE, allergy

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Development aimed at controlling/down-regulating allergic reactions

Jonas Wamstad

Sammanfattning

Projektet Theravac Pharmaceuticals startades för omkring ett år sedan i syfte att utveckla ett nytt allergivaccin mot främst astma och atopisk dermatit. Vaccinet är ett nytt sätt att angripa allergiproblemet på och bygger på att det får mottagarens immunsystem att genom produktion av specifika antikroppar själv nedreglera förekomsten av de molekyler som orsakar allergireaktionen. Denna nedreglering kommer förhoppningsvis att leda till en långvarigt minskad allergenkänslighet hos allergipatienten. Förhoppningen är även att vaccinet skall visa sig vara många gånger effektivare på att nedreglera svåra allergier än vad idag förekommande allergiläkemedel är.

Detta examensarbete handlar om utvecklingen av ett protokoll för rening av tre potentiella vaccinkandidater. Ett sådant protokoll har nu framgångsrikt utvecklats och detta protokoll kan rena fram relativt stora mängder av vaccinet från en enda fermentorkörning. Produktionen sker i prokaryoten *Escherichia coli* vilken odlas i fermentor och för att därefter renas fram i tre reningssteg. Vissa mindre justeringar av protokollen återstår dock att göras innan vaccinet är redo att testas på försöksdjur för att utvärdera dess medicinska effekt.

Examensarbete 20p i Molekylär bioteknikprogrammet

Uppsala universitet augusti 2007

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1. Introduction

Allergies are becoming one of the serious medical challenges of our time. Over the last few decades, allergies have taken on almost epidemic proportions and according to some calculations as many as 20-30 % of the population in western countries is affected. It may not be deadly in most cases but nevertheless it costs the society billions of euros every year because of its negative effects on people's abilities to work. The existing drugs available today are in most cases not very efficient, and if they are they are so only for relatively mild cases. For more severe allergies there is no satisfactory solution as of today.

The Theravac Pharmaceuticals project was started about one year ago to try to develop a new approach to this problem by making the immune system itself shut down the overreaction to naturally occurring and harmless allergens, instead of just trying to control the symptoms of the disease. The project is trying to develop an allergy vaccine in the form of a recombinant protein that once injected in the body will make the immune system produce antibodies against those molecules responsible for the allergic reaction and thereby down-regulating them.

My involvement in this project has been to try to develop a purifying protocol and to improve an already existing production protocol for this recombinant protein. My work has been successful and there are today only minor refinements left to be done to the protocols before the project should be able to enter the first phase of in vivo tests in dogs or rats. This should be possible within the very not-too-distant future.

This report is a summary of my work in this project so far.

2. Background

Over the past two centuries, vaccination has been the most successful method in controlling most viral and many bacterial infections. Different vaccination programs have resulted in control and have in many cases completely eliminated important infectious diseases.

The dominating forms of allergies are the atopic or IgE-mediated allergies, and the question whether vaccines could be developed against this kind of diseases has been raised. Therapeutic vaccines aimed towards central molecules in our immune system could be one such possibility. One target molecule with great potential is therefore the IgE-molecule because of its function as a central mediator in a majority of allergies. Growth and differentiating factors are yet other important regulators of the allergic reaction and therefore they constitute other very interesting potential target molecules.

In experiments on rats, IgE vaccines have been shown to be efficient primarily in relatively low IgE levels. Only in animals with IgE levels below 100 nanograms per milliliter have allergy specific skin provocation proved to be successfully reduced (Hellman, 1994; Johansson et al., 2004; Hellman, 1996; Hellman and Carlsson, 1996; Vernersson et al., 2002; Hellman, 1999; Ledin et al., 2006; Johansson and Hellman, 2006). Unfortunately, most allergy patients are not found within this interval. Allergy vaccines aimed at the IgE molecule will therefore only be able to cure patients with relatively mild symptoms. In order to be able to find treatment for a large part of the world's allergic population we need considerably stronger alternatives, and this opens up the search for other target molecules.

Over the last decade research on growth factors that control the early phase in how the immune system chooses between cell-mediated defense and an immune system has lead to the identification of a number of very important regulators of the early phase in allergy development (Hellman, 2006). Three of these molecules, the cytokines IL-18, IL-33 and TSLP, are very interesting for the development of a new type of allergy vaccines. One of these three has for a few years been known as an important inducer of cell-mediated immunity or so called TH1-mediated inflammations. This molecule has later been shown also to induce strong humoral so called TH2-mediated inflammation in absence of another inflammatory cytokine, IL-12. These inflammations are also associated with high IgE levels. A study of over-expression of IL-18 in keratinocytes in mice proved to cause very strong atopic dermatitis (Tsutsui et al., 2004). Administration of IL-18 and antigen also in the bronchioles of mice proved to be causing severe asthma-like symptoms and high IgE levels.

This accumulated information indicates that IL-18 is an important regulator of both TH1- and TH2-mediated inflammations of the kind that can be seen in patients with asthma and atopic dermatitis. Then, is it possible to remove or lower the levels of this growth factor without noticing any strong side effects? An experiment of this kind was performed in mice where IL-18 was completely removed with knock-out technology and the results indicate a weak decrease in immune defense against some bacterial infections but showed apart from these mild effects very few recognizable symptoms (Takeda et al., 1998). These results indicate that it might be possible to without any major health risks remove the surplus IL-18 that is probably one of the main causes for the development of atopic allergy. However, further studies about

possible side effects and other drawbacks for the individual need to be done in order to safely rule out the possibility of down-setting any vital function of the immune system.

Two additional very interesting target molecules are IL-33 and TSLP. There are strong indications of these two cytokines being involved in the major increase of antigen specific IgE that can be seen in allergy patients with severe asthma or atopic dermatitis. It would therefore be interesting to develop vaccines based also on these two additional molecules, not only to increase our arsenal of treatments against severe asthma and atopic dermatitis but also as a potential complement to the IL-18 vaccine.

Atopic dermatitis is not only a big human problem but also a very big issue in veterinary medicine and vaccines aimed at these inflammatory cytokines can therefore turn out to be an important treatment for many pet animals. Atopic dermatitis is one of the most common reasons for dog owners visits to the veterinary clinic and the prevalence of atopic dermatitis has in some dog populations been assessed to as much as between 3 and 15 %. Today many of the dogs suffer from heavy side effects from their treatments which usually consist of massive cortisone doses, or the treatment is simply not enough to control the disease.

The economic potential of an allergy vaccine for dogs is very large, not least because of the fact that dogs are often considered to be a family member and therefore often receives rather expensive medical care when ill. If one also takes into account the distance-to-market being considerably shorter than the very same for humans, the strategy of focusing this market seems increasingly advantageous. Technically, the step between a vaccine developed for dogs and one for humans is very short due to the close biological relationship and we therefore share many immunological similarities. The project therefore intends to initially focus on the dog market and in the event of successful trials of this project it intends to start the development of a human version of this vaccine. The costs of such a vaccine would be considerably higher than a vaccine for dogs, but the reward is also considerably larger. The sales income from a dog vaccine might be able to finance such a project.

3. Theory

The majority of the therapeutic vaccines under development today use our immune system to regulate the levels of different self-proteins of medical interest. We are normally tolerant towards all self structures including protein, lipids and carbohydrates. Though, we do have auto-reactive B-lymphocytes in our circulation system. These B-cells however enters very rarely the proliferative phase and causes therefore no harm since they do not get any help from T-lymphocytes. This is because T-cells aimed towards different self-antigens are clonally deleted in thymus or anergized in peripheral tissues. By linking a self protein to a non-self protein, from viruses, bacteria, parasites, plants or some other animal species we can however recruit tolerated T-cell epitopes towards the self-protein. This makes it possible for T-cells that recognize these epitopes to not only help the B-cells aimed at destroying the non-self protein but also the circulating potentially auto-reactive B-cells. These auto-reactive B-cells can then expand clonally and give rise to an antibody response towards this self-antigen.

In this way we can lure the immune system to break its tolerance to a self-protein and start producing antibodies that can bind to the self-protein and thereby making it easier for the body's macrophages to remove it from the circulation system. A successful vaccination leads to lowered levels of the specific self-protein which in turn in the case of IgE makes the mast cells less sensitive for exposure of allergens.

In order to reduce the IL-18, IL-33 and TSLP levels respectively, we must therefore covalently link the target molecule of interest to a foreign non-self protein that contains non-tolerized T-cell epitopes. These epitopes stimulate the B-cell response (antigen-presenting and antibody-producing) towards not only the parasite protein but also our target protein when injected in the host. The foreign protein should be of approximately the same size as our target protein to get an optimal balance between self-epitopes stimulating the B-cell response and the number of non-tolerized T-cell epitopes stimulating the T-cell response. Figure 1 illustrates this recombinant protein.

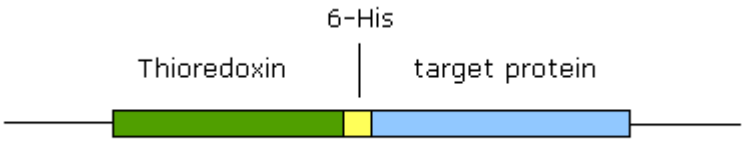


Figure 1. The recombinant protein.

3.1 Production

It was decided to produce recombinant proteins following this concept for dogs, rats and humans respectively with IL-18, IL-33 and TSLP as target molecules, respectively. The reason for adding rat to this group is its excellent ability to function as a test animal. Running experiments on rats is more expensive than on mice but rats

are a lot easier to take blood samples from. We decided that the latter fact was more important.

The first step in the production process is to design the recombinant protein from scratch and this was done by searching the internet databases for the correct gene sequences for the specific species. The sequences found were double checked against neighboring species in order to make sure that this was indeed this species' version of the specific protein IL-18, IL-33 or TSLP. Then we added six histidines in a row to create a histidine-tag that would enable us to later use IMAC for purification of this recombinant protein. The reason for placing of the His-tag at the center position of the construct was merely random. It could just as well have been place at the end of the construct but was placed in the middle as a clear marker of where the other parts began. Finally we added a Thioredoxin gene. This gene was chosen because of its similar size to our cytokine proteins which would give a rather equal balance between the two parts of the construct.

We now had the blueprints for seven new recombinant proteins. Two of the total nine recombinant proteins (IL-18 for dog and mouse) had already been produced prior to my introduction in the project. These blueprints were sent off to a lab in the US that produced it for us. We decided to outsource this production instead of making them ourselves mainly because of the time-consuming lab work this would save us.

The next step is to create expression vectors for these clones and E.coli is used as a host organism, mainly because of its ability to resist tough handling, ability to take up new vectors, its short generation time and its excellent ability to rapidly produce large amounts of protein. The following step is to produce the recombinant protein and this is done in a fermentor where the exact optimal conditions of the producing organism can be met regarding pH, temperature, oxygen and feed availability. Thereafter, the bacteria slurry containing the produced protein is centrifuged, sonicated and then run through the different purification steps of Immobilized Metal Ion Affinity Chromatography and Size Exclusion Chromatography to purify the protein.

Finally the produced and purified recombinant protein has to be tested in animals to try to assess the medical effects and possible side effects.

4. Methods and results

4.1 Development of analysis techniques

I would like to start the methods section by shortly describing an analysis technique that I developed by accident at an early stage of my involvement in the project. This technique became very useful to us and this project, not only as an analysis technique but also because it gave us indications of that something was wrong with the folding of our produced protein.

The technique is very simple in theory but still brilliantly efficient and easily interpreted. The protein samples are run on two parallel acryl amide gels with a denaturing agent (e.g. B-mercaptoethanol) in one of them. The denaturing agent will break all the cystein-bridges in the protein complexes and thereby letting all molecules enter the gel. This gel will show us the total amount of protein present in the solution. The samples applied to the other gel will not have any reducing agents present and will therefore still be in both aggregated and monomeric form. The monomers will be able to enter the gel because of their smaller size whereas the aggregates will not because of their bigger size and will therefore be left in the wells.

Now it is easy to compare the two gels and determine the percentage of monomers versus covalently bound aggregates and thereby also determining how successful the refolding process has been.

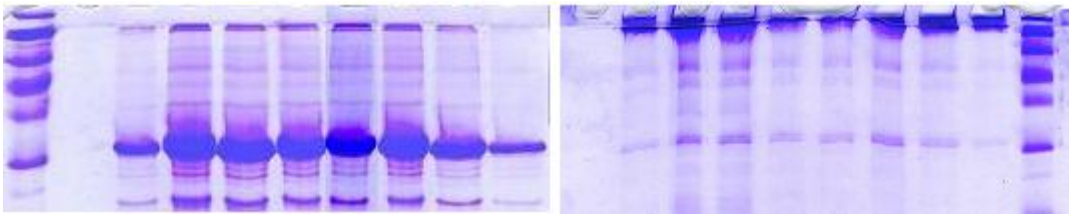


Figure 2 and 3. The same sample run with and without reducing agent, respectively. As can be seen, a large proportion of the protein is aggregated.

4.2 Gene design and cloning

4.2.1 Data base search

We wanted to create seven new fusion proteins and we knew what family of proteins we were looking for. But we did not know the specific gene sequences for IL-18, IL-33 and TSLP in dog, human and rat and therefore spent some time trying to identify them using the NCBI, ECB and SwissProt data bases. We then matched the sequence of each animal against neighboring species to check its accuracy before continuing. Thereafter, we added six histidines to form a His-tag before finally adding the Thioredoxin gene. We now had the coding sequences of the new fusion proteins.

4.2.2 Cloning

We decided to outsource the isolation of the new genes to a US company in order to save time. The coding region with purification tags were synthesized as overlapping oligonucleotides. The gain in time was considered of bigger importance than the cost of outsourcing the cloning.

4.2.3 Construction of expression vectors

After the cloning, the gene is inserted into an expression vector before transforming into a prokaryote organism, in this case E.coli p21ETa(+)Trx Rosetta bacteria, for expression. The construction of the expression vector for IL-18 dog and mouse respectively and their insertion in E.coli had been done prior to my introduction in the project and the seven other clones are still waiting to be inserted into host organisms. Unfortunately I did not get to do this before this report was due.

The reasons for choosing a bacterial production system are primarily its ability to very rapidly produce large quantities of protein to a relatively low cost. Bacteria is also often more tolerable towards tough handling than eukaryotic systems. The downside in using a bacterial system is its inability to control and to correct misfolded proteins, something that for example certain fungus species can do rather well. These funguses are however very slow growing and rather costly, which is why bacteria are often used as production system in these kinds of projects. Though, the winnings in time and cost has to be compensated for in developing purifying and refolding techniques that will yield the right configuration of the produced protein.

4.3 Development of the production protocol

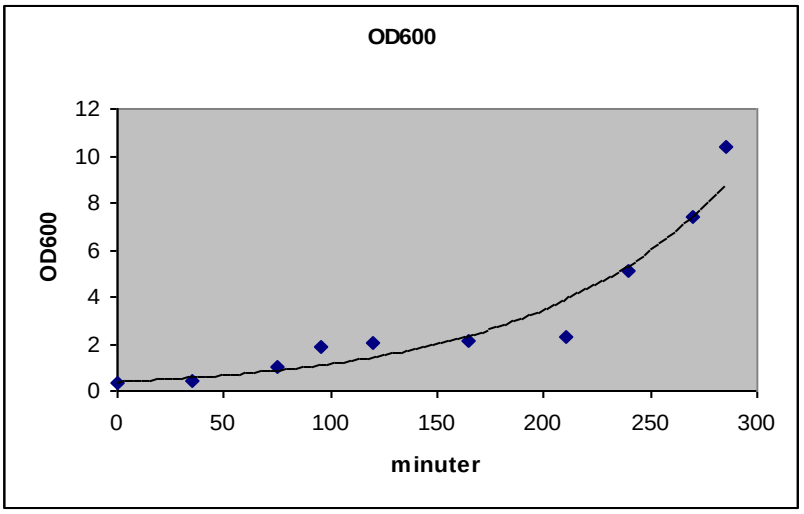
4.3.1 Optimization of the fermentation process

The fermentation process is one of the key steps in the production of a recombinant protein. The host organism often has its own preferences regarding temperature, pH, pO2, stirring speed and glucose density, and these preferences have to be carefully optimized in order to maximize the production of protein in the fermentor. A fermentation protocol for E.coli pET21a(+)Trx was put together successfully.

This protocol begins with inoculating a 200 ml cell culture and allowing the bacteria to grow over night in a one liter flask. The size of the flask is important for the bacteria to have enough access to oxygen. The cell culture medium consist of 200 ml 4x LB, 10 ml glucose (10 g/l) and 0,2 ml ampicillin (50 g/l). It was kept in 37 C over night. Preceding tests had found 37 C to be the optimal growth temperature for this organism and therefore we used this throughout the experiment. The following morning its optical density is checked (OD600) to see if the growth has been satisfactory.

The inoculation is added to an autoclaved 5-liter Infors Minifors fermentor in which has been prepared and sterilized a mix of 1 liter 4xLB, 700 ml H2O, 200 ml glucose (200 g/l), 200 ml MgSO4-solution (5 g/l), 2 ml Trace Element Solution and 2 ml ampicillin (50 g/l). The mix is well oxygenized (100%), the pH is set to 7.00, temperature to 37 C and the stirrer speed to 300 rpm to get a continuously good air-flow. The OD-value of the culture is measured every 30 minutes, and when OD600 reaches a value of 4, the addition of feed is started. The feed consists of (per liter) 200 ml 200 g/l glucose (final concentration 40 g/l), MgSO4 2 g/l, Trace Element Solution 2 ml/l and 800 ml 4xLB (Luria Broth, nourishment solution). When the OD-value reaches 10 the bacterial culture is ready for induction of protein expression and this is done by adding 7 ml 1 M IPTG.

The computer program Iris is used to closely monitor the oxygen and pH levels in the fermentor during the whole run. After three hours the fermentation run is stopped and the bacterial culture is harvested.



min	OD
0	0,3
35	0,428
75	1,023
95	1,868
120	2,004
165	2,092
210	2,27
240	5,12
270	7,39
285	10,35

Figure 4. The OD600 values increases exponentially with time.

4.4 Development of the purification protocol

4.4.1 Purification step 1

The culture of approximately 2.4 liter is poured into centrifuge bottles and spun down in 12000 rpm for 10 min in 4 C. The bacteria are concentrated to pellets at the bottom of the bottles. After discarding the supernatant the pellets are washed by being dissolved in PBS + 0.1% Tween and centrifuged once again.

Thereafter the pellets are dissolved in PBS + 0.1% Tween and the solutions are sonicated for 5x1 minute on ice and then centrifuged again. The proteins are able to resist being destroyed by the sonication because of their formation in insoluble inclusion bodies. The supernatant is discarded and the pellets are dissolved in PBS + 0.1% Tween. This procedure was repeated 4 times.

After the final sonication the solution is divided into ten equally large samples and spun down once more. This time the supernatant is discarded and the pellets (i.e. the protein) are stored in -20 C.

4.4.1.1 Protein concentration determination

One of the ten pellets from the fermentation was dissolved in 2 ml denaturing solution (X) from which 10 µl (micro symbol "µ" is hereafter referred to as "u") was taken (Y) and diluted ten times to a total volume of 100 ul (Z). From this dilution four different amounts were taken (A, B, C and D) and diluted up to a final volume of 20 ul, according to table 1. Out of the total volume of 20 ul, 10 ul was applied to an acryl amide gel (12.5 %) for detection. On the gel were also loaded three different concentrations (4, 1 and 0.25 ug) of BSA protein as reference markers. We could then easily compare the intensity of the different bands with the ones of the reference markers and thereby determine the total amount of produced protein that we had received from the fermentation.

	sample Z (ul)	water (ul)	SB (ul)	Total vol.(ul)	Tot. dilution (times)
A	10	0	10	20	20 (10x2)
B	5	5	10	20	40 (10x4)
C	2,5	7,5	10	20	80 (10x8)
D	1	9	10	20	200 (10x20)

Table 1. The table shows the calculation of the four sample concentrations (A-D). The total volume is at 20 ul of which 10 ul is sample buffer (SB). The other 10 ul is protein sample diluted with different amounts of water.

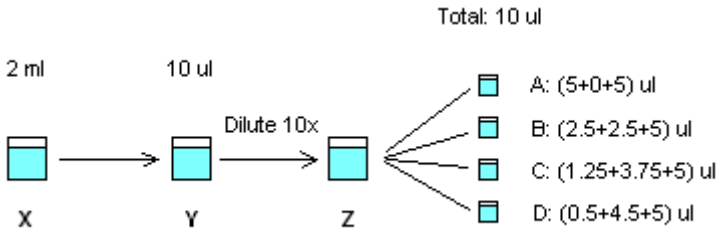


Figure 5. A schematic view of the sample dilution steps performed in the protein concentration determination procedure that is described above.

The results are shown in figure 6. The intensity of the sample D in well 8 seems to be 1.5 times the intensity of the 4 ug reference marker in well 1, and thereby indicating that there is 6 ug present in well 8. We loaded 0.5 ul sample in this well, and the concentration in solution Z (in figure 5) is therefore 12 mg/ml. This sample was in turn diluted 10 times from solution Y (in figure 5), which means its concentration is 120 mg/ml. The volume of the dissolved-pellet-solution X (in figure 5) is 2 ml and the total amount of protein in the sample X is therefore 240 mg. Since we have ten of these pellets, the total amount of protein produced in the fermentation is 2400 mg or approximately 2.4 g. This is a very good yield, considering that only about 10 mg is needed for each patient.

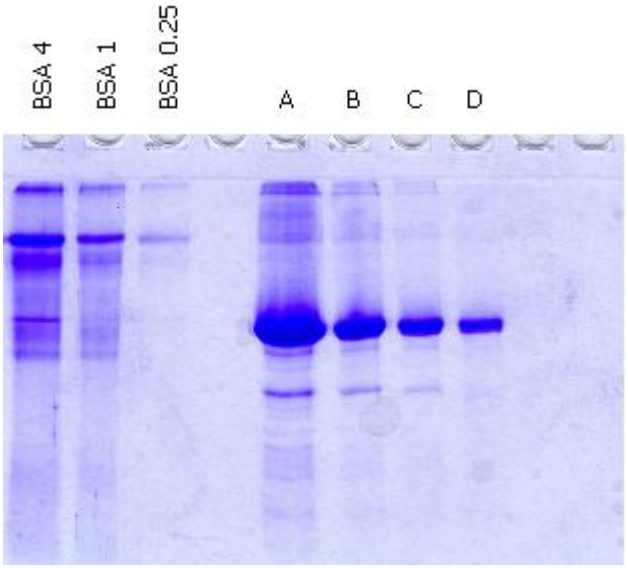


Figure 6. The figure shows concentration analysis against pre-determined concentrations. Sample D appears to be 1.5 times the strength of BSA 4, indicating the amount in D being 6 ug.

4.4.2 Denaturing of the protein

The absolute majority of the recombinant protein was found in the pellet as inclusion bodies and we knew that a large proportion of the produced protein therefore was misfolded. To be used as a vaccine the protein has to have the correct configuration and be soluble. We therefore had to develop a method for unfolding and refolding the recombinant protein in the correct configuration. We searched the internet and a wide range of data bases and managed to find a few interesting protocols for protein refolding. One of these protocols turned out to work very well for our particular recombinant protein.

The protocol we developed for this step did initially include EDTA but it was later omitted due to indications of it complicating the IMAC step by interfering with the nickel ions. The frozen protein pellet is dissolved in 6 M Guanidine-HCl, 0.1 M Tris-HCl pH 7.9 and 40 mM DTT. Distilled water is added to a final volume of 2 ml and the solution is kept in 37 C for 2 hours to let all of the protein molecules denature completely.

4.4.3 Refolding of the protein

4.4.3.1 Refolding gradient experiment

We experimented with the refolding protocol by first immobilizing the protein by attaching it to nickel ions on sepharose beads on a column (IMAC) before passing a Urea gradient over it and to let it refold slowly. The Urea gradient was created with a gradient mixer containing refolding solution A and B 20 ml each. Solution A consist

of 6 M Urea, 5mM DTT, 20 mM Tris-HCl, 0.5 mM NaCl and 20 mM Imidazol and solution B consist of 20 mM Tris-HCl, 0.5 mM NaCl and 20 mM Imidazol. Out of the mixer comes at first highly concentrated denaturing agents of solution A but this concentration is steadily decreasing due to the diluting effect of solution B on solution A. A schematic illustration of the gradient mixer is shown in figure 7.

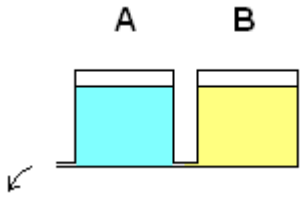


Figure 7. A gradient mixer that was used in this experiment.

After the refolding process is done, the immobilized and refolded protein is eluted from the column with 0.5 M Imidazol and the elution is collected in 0.5 ml fractions. These fractions are run on gel for detection and determination of the proportion of correctly refolded molecules versus aggregates. The results from our test run are shown below. The left gel was run without reducing agents and the right one with reducing agents allowing both monomeric and aggregated protein to enter the gel. This is a good comparison when measuring the percentage of what we think is correctly folded molecules.

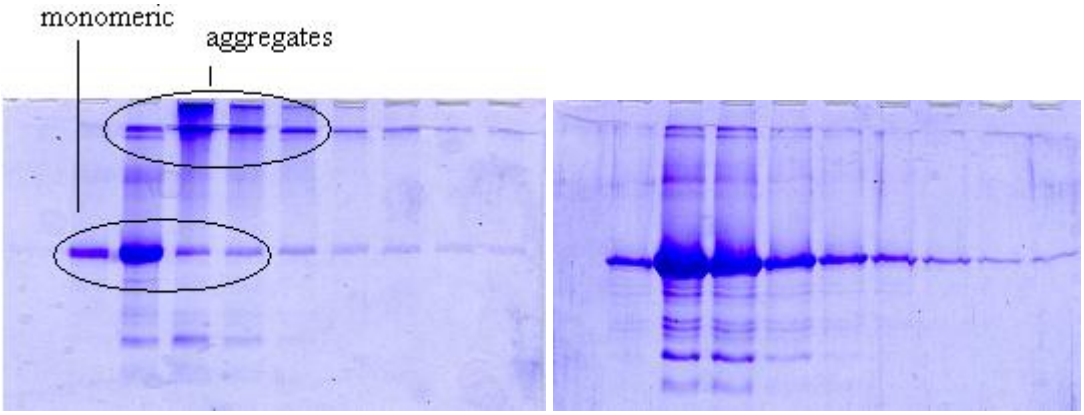


Figure 8 and 9, respectively. The left figure shows the proportion of protein with monomeric configuration vs aggregated configuration. The right figure shows the total amount of protein present.

The results show that the protein is present in monomeric form and we assume this indicates that the protein is correctly folded. The formation of the cystein bridges are very hard to control but one way of controlling them is to reduce the bonding options for every cystein, for example by reducing the number of cysteins present or creating steric hindrance between the cysteins. We considered this when designing the clones. We also made sure the number of cysteins in the recombinant protein was an even number in order to prevent highly reactive unbonded cysteins (“free radicals”) to interact with other molecules. However, we cannot be absolutely certain that the

folding of this new recombinant protein has preserved the correct folding of the cytokine. The Thioreoxin section could interfere in an undesired fashion.

There seems to be double bands of the monomeric protein present on the gel (somewhat difficult to see in the figure) and this would indicate that the protein might be present in two alternative configurations. This is not desirable and it would be difficult to try to separate the two molecules at a later stage of the purification process. We therefore continued the search for an alternative refolding protocol.

4.4.3.2 Refolding additives

We found an interesting protocol on the Internet that we decided to try (Hamada and Shiraki, 2007). This protocol was relatively simple in theory. It is based on a 40-fold dilution of the denaturing solution into a refolding solution containing 0.1 M PBS *or* Tris-buffer, 1 M L-Arginine *or* 1 M Guanidine-HCl, 5 mM oxidized Glutathione (GSSG) and 5 mM reduced glutathione (GSH). The concentrations of Guanidine and DTT from the denaturing solution then drop rapidly down to 150 mM and 1 mM, respectively. An additional 1 mM EDTA was, as mentioned earlier, also suggested to be present in the solution but this was omitted from the protocol since it proved to be interfering with the subsequent IMAC purification. Parallel experiments with these two versions of essentially the same protocol were run in order to asses which of L-Arginine and Guanidine was the more efficient additive in helping the protein to refold, and in what pH-environment the refolding was optimal. The experiments were run in parallel at pH 7.0, 7.5, 8.0 and 9.0. The setup of these is shown in table 2.

pH	7.0	7.5	8.0	9.0
L-Arg.	A1	B1	C1	D1
Guan.	A2	B2	C2	D2

Table 2. The table shows the codes for the different combinations of pH and refolding agents.

Addition of non-pH-adjusted L-Arginine turned out to result in a pH-value of around 11 while Guanidine had the opposite effect by lowering the pH to around 4. The pH was adjusted with HCl or NaOH to the desired values before adding the denatured protein.

The refolding mix was then left in room temperature (22 C) for over night (18 h) to allow the protein to refold slowly. The samples were then loaded onto two acrylamide gels according to Table 3. On gel A the reducing agent B-mercaptoethanol was added but not on gel B. This way we could determine the proportion of presumably non-covalently bound monomeric protein.

Well	1	2	3	4	5	6	7	8	9	10
Gel A	A1	A2	B1	B2	C1	C2	D1	D2	-	protein
Gel B	A1	A2	B1	B2	C1	C2	D1	D2	-	-

Table 3. The table shows the setup of the samples on acrylamide gel.

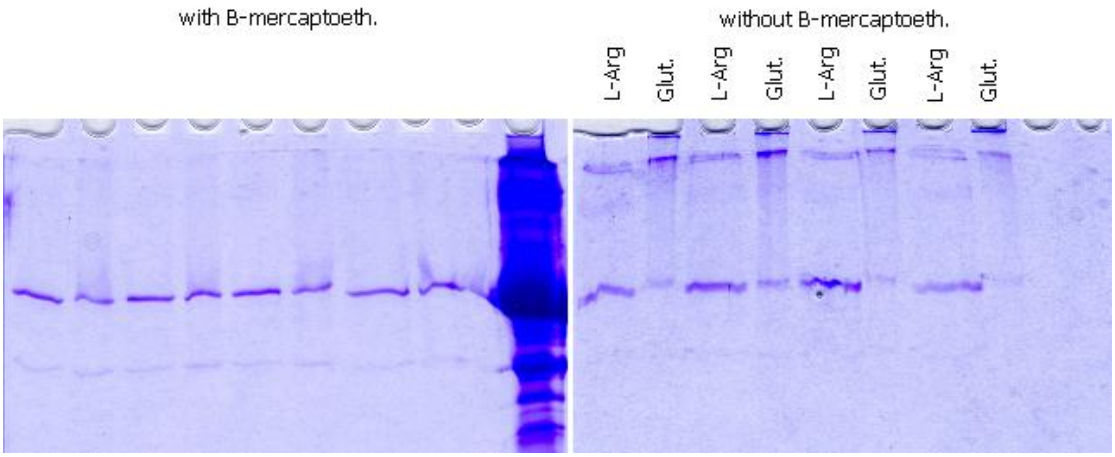


Figure 10 and 11. The figures show the eight test with (left) and without (right) B-mercaptoethanol. The right figure compares the refolding agents' different abilities to refold the recombinant protein.

The results showed that L-Arginine was superior to Guanidine in aiding the refolding process at all pH-values tested, and as much as 20-30 % of the total amount of protein was found to have monomeric form in the L-Arginine samples. A slightly higher percentage of monomeric protein seems to be present at pH 7.5 and 8.0, according to figure 11.

We decided to use L-Arginine at pH 8.0 and use a test refolding protocol of 0.1 M Tris-HCl pH 8.0, 1 M L-Arginine, 5 mM GSSG and 5 mM GSH. A test was run on an acrylamide gel without any denaturing agents in order to determine its efficiency and ability to refold the protein, and the results are shown in figure 12. The fractions 2 and 3 contain the bulk of the protein, and the results indicate that the percentage of correctly folded protein is about 20 %. The aggregated protein can be seen as a band right below the well. However, the monomers show a protein double band, which might be an indication of the presence of two alternative foldings, similarly to the refolding gradient experiment described earlier. This will have to be further examined, since it indicates that the protein may not have been folded in the specific configuration that we wanted.

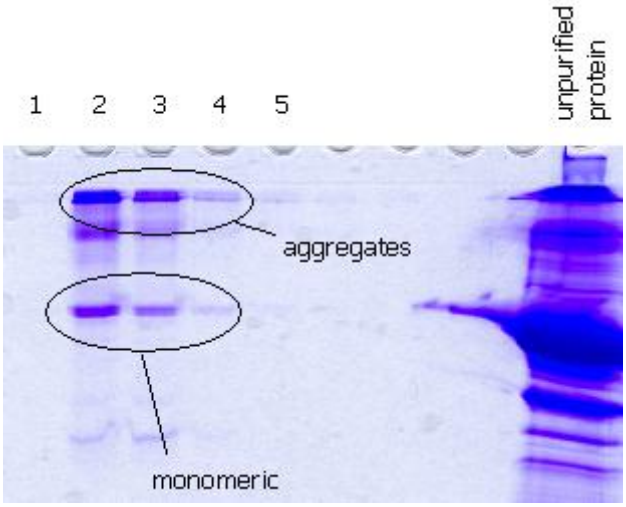


Figure 12. The refolding-aid abilities of 1 M L-Arginine.

4.4.3.3 What concentration is optimal for refolding?

We suspected that the percentage of monomeric protein is proportionally inverted to the concentration of protein in the solution because of the mathematical fact that a greater presence of other protein molecules around a refolding molecule might interfere with the molecule's refolding process. To determine this relationship we performed five parallel denature-refolding experiments with different additive concentrations. We then loaded different volumes of these solutions to the gel in order to get the same amount of protein in each well and to be able to easily compare the differences just by comparing the intensity of the bands. Table 4 shows in which well the different refolding concentrations were loaded. The results are shown in figure 13, and not surprisingly the sample with the highest concentration proved to have the lowest percentage of monomeric protein compared to the total amount of protein.

Well	1	2	3	4	5
Dilution (times)	1	2	4	8	16
Conc. (mg/ml)	4	2	1	0,5	0,25

Table 4. The table shows the concentration relative to the dilution of the sample.

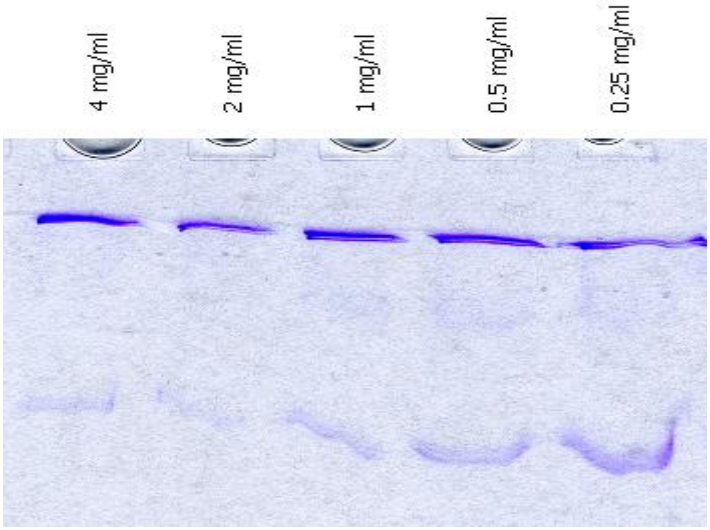


Figure 13. Higher concentrations to the left. The gel shows increasing proportion monomers when protein concentration drops.

From the results we can conclude that a higher concentration of protein leads to a lower percentage of correctly refolded protein. However, it might still be preferable to go with a lower percentage of correctly refolded protein because of the lower volumes that need to be handled. The more diluted the protein gets, the bigger is the amount of additives and ingredients that need to be used, and this increases the costs rather heavily. Since we are developing a protocol that should be able to be run on an industrial large scale production scale we need to consider the costs of producing the protein.

The most concentrated sample (4 mg/ml) shows an approximate 5 % successful refolding whereas the most diluted sample (0.25 mg/ml) reaches approximately 25-30 % of refolding. This gives a total of 0.2 mg/ml and 0.0625-0.075 mg/ml, for the higher concentration versus the lower concentration, respectively. This means that we might in fact end up with more protein in the end by choosing the less efficient highly concentrated protein and reduce the volume at the same time. The exact figures need

to be examined more extensively in order to draw any definitive conclusions. These results indicate that it might be preferable, both from an economic and a practical point of view, to use a more concentrated protein.

4.4.4 Dialysis

The refolded solution is dialyzed twice (1:100 times) in PBS for 3 and 18 hours respectively in room temperature in order to clear out the refolding agents which turned out to be interfering with the IMAC purification step. More detailed information about this can be found in section *4.4.5*. There are no indications that the protein does not stay folded and soluble throughout the dialysis, i.e. so far so good.

4.4.5 Immobilized Metal Ion Affinity Chromatography (IMAC)

4.4.5.1 Theory

The key step in the purification process of this protein is the Immobilized Metal Ion Affinity Chromatography (IMAC). Immobilized nickel ions on sepharose beads bind to our His-tagged protein. Our protein's six Histidines in a row gives it a very strong affinity for positively charged metal ions, and this strong affinity helps the protein compete for the nickel ions. Once attached to the nickel ions, the relatively large sized Sepharose beads make it impossible for the protein to escape through the filter at the bottom of the column and the protein-nickel-bead complex can therefore be retained while the column is washed with a low concentration salt buffer to get rid of other protein substances. When the column has been washed, the protein is eluted with a Imidazole containing buffer which makes the protein detach from the nickel beads. This method is not only an extremely potent purifying technique but also a very good volume reducer.

4.4.5.2 Experiment

In developing this protocol we set up several nickel columns with different dimensions in order to try to find the optimal characteristics. The optimal dimensions depend on the size of the protein sample, time factor, and of course cost efficiency since the nickel bead slurry used in packing the column is rather expensive.

After the mounting of the column, it is equilibrated with PBS buffer before the protein sample is loaded onto it. After loading the protein, double sample-volume washing buffer containing a low salt concentration of 20 mM Imidazol is applied to the column, in order to clear the column from remaining unwanted protein substances. The protein is thereafter eluted with elute buffer containing a high salt concentration of 500 mM Imidazol. We did experiment with different approaches in the elute buffer, such as different salt concentrations and increasing salt gradients. However, since we wanted to keep the elute volume down and the protein concentration at a relatively high level,

we decided to use a relatively high Imidazole concentration of 500 mM in the elute buffer.

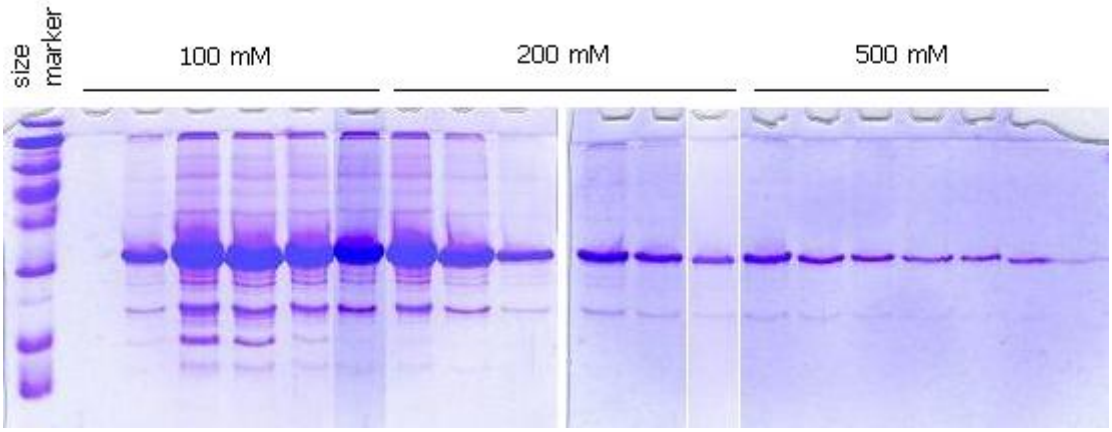


Figure 14. Gradient elute buffer with increasing salt concentration (100 mM to 500 mM). Denaturing agents are used.

4.4.5.3 Check for nickel-interfering agents in the refolding solution

When running the refolding solution on the nickel column we soon noticed that the blue color of the nickel disappeared and thereby also the nickel ions. This was later confirmed with a gel run of the eluted fractions from the column. Something had made the nickel ions being released from the sepharose beads. We immediately suspected EDTA to be the responsible component but when running the experiment again without EDTA, the nickel ions were still being washed out. We suspected one of the three remaining additives in the refolding solution (L-Arginine, GSSG and GSH) to be the reason for this phenomenon and we therefore needed to examine whether there was any crucial concentration of refolding solution at which the nickel stayed attached to the beads.

This study was performed by mixing four different concentrations of refolding solution with nickel-bead slurry and then determining how much protein that still clung on to the beads. The refolding solution was diluted with PBS to four different concentrations and then mixed with slurry containing nickel ions on sepharose beads. The mix was shaken for one hour in room temperature and then centrifuged at 10 000 rpm for ten minutes. The supernatant was then discarded and the pellet was washed with PBS. The mix was centrifuged again and the pellet of nickel beads (and hopefully protein attached) was thereafter mixed with sample buffer and loaded onto a gel for detection.

The results, shown in figure 15, showed proportionally stronger bands where the concentration of refolding solution was higher and vice versa, which was unexpected. We had expected equally strong bands because of a higher yield in the samples with weaker concentration of refolding solution and a lower yield in the samples with higher concentration. These results indicated that the concentration of refolding solution had little importance to the nickel ions ability to bind to the sepharose beads.

Well	1	2	3	4	10
Dilution (times)	1	2	4	8	protein

Table 5. The different dilutions loaded on the gel.

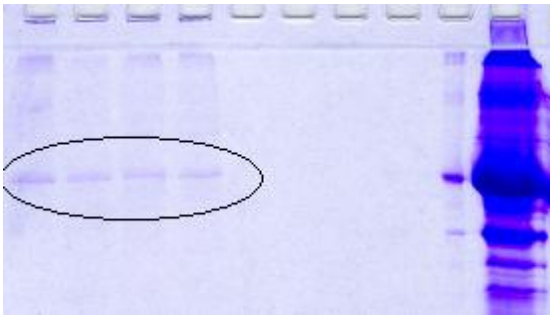


Figure 15. The different dilutions of the protein run "through a nickel column".

4.4.6 Size Exclusion Chromatography (SEC)

4.4.6.1 Theory

As the next step in the process of purifying the protein we use Size Exclusion Chromatography. This technique allows us to separate differently sized molecules by letting them take different paths through the matrix created by the gel in the column. Larger molecules cannot enter the matrix as efficiently as smaller molecules and therefore have to take the by-pass road on the outside, while smaller molecules enter the matrix and therefore get a longer journey through the column, with a longer elution time as result. The bigger molecules get eluted first and last come the smallest ones. These size-based separation characteristics of Size Exclusion Chromatography allow us to separate the biologically inactive polymeric aggregates of our protein from the monomers as well as all of the other remaining substances. These characteristics make SEC a very good compliment to the IMAC separation in purifying this protein.

4.4.6.2 Setting of the gel filtration column

We decided to prepare and set up the column ourselves. As packing material we used Sepharose S-200 HR Light Molecular Weight (GE Healthcare) with a resolution window of between 10 kDa and 170 kDa, characteristics that suited our 30 000 kDa protein well. We washed the gel and packed the column with it under pressure created from a pump connected to the inlet of the column.

After packing the column it was equilibrated with PBS over night. Dextrane was used to determine the void volume of the column by measuring its elution time. Dextrane gives a good indication of a columns void volume since it is too large to enter the matrix. Thereafter a calibration test was run on the column and we used GE Healthcare Low Molecular Weight Calibration Kit. The calibration kit consisted of Aprotinin (6500 kDa), Ribonuclease (13700 kDa), Carbonic Anhydrase (29000 kDa), Ovalbumin (43000 kDa) and Conalbumin (75000 kDa), five proteins with different

molecular weights and sizes and therefore give a good indication of the columns properties.

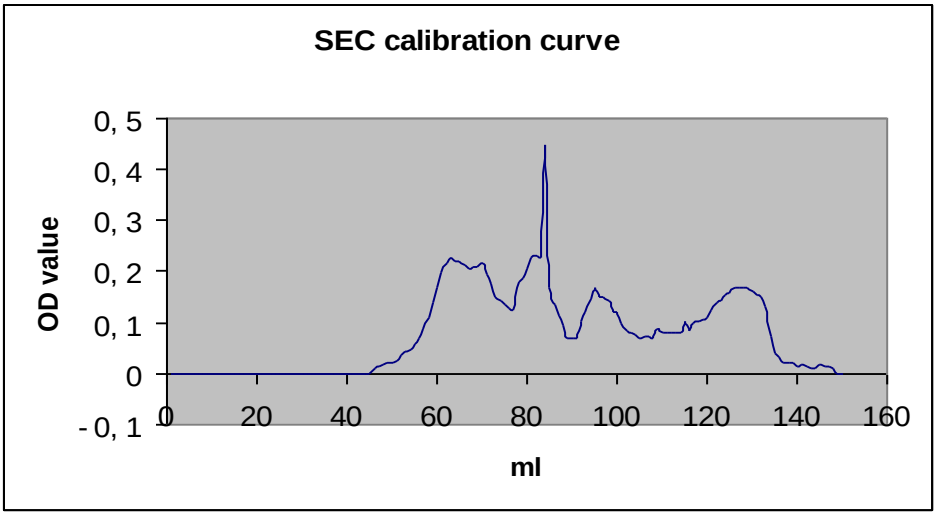


Figure 16. The diagram indicates that our protein should have an elute volume (Ve) of around 83 ml.

Unfortunately, I did not manage to get any results from this purification step before this report's deadline. However, this purification step will probably not have to be tested and optimized as carefully as the other purification steps because of its relatively simple theory. Hopefully, this step will not render us that much trouble.

5. Funding applications

I decided to make an interesting twist on this thesis by involving a seemingly different but not all too irrelevant issue in the biotech industry, namely the issue of funding. All biotech projects need money, and often quite a lot of it, to be able to run. Every new drug project that has ever made it to the market has started with a new invention or an idea of how to refine an already existing invention, and the aim is of course to profit from the idea financially. But to be able to take the first steps on this journey you will need funding. I have learned that nothing is free in this business, everything costs and it is not cheap.

So far this project has been funded by own investments from the owners, but sooner or later the project will need to take in more money than people normally can or are willing to venture themselves because of the high risk involved. I had the opportunity to be involved with the funding applications for this project to different government bureaus commissioned to support grass-root biotech projects like this one financially, and decided to seize this opportunity. The reason for us concentrating primarily on government money is that the company wants to avoid venture capitalists at this early stage of the project. Venture capitalists are known to be eager to go in at an early stage in the life cycle of small biotech projects if they recognize any economic potential. However, a small company's need for cash makes it vulnerable to these kinds of investors that might take advantage of the situation by chewing up a big part

of the company for less than its actual or potential worth. This might lead to a rapid loss of control of the company for the founders.

I spent about three weeks assisting in application writings and this gave me a good, and I think very healthy, insight to how this part of the business works. It is very tough. There are a lot of companies out there competing for the same money and in order to get it you will need to not only prove the project's potential by showing good results but also be able to "sell" the project to the investors. Selling a project is truly an art worth practicing, because marketing is much harder than it sounds.

Unfortunately, the project did not manage to get any fundings in this round of investments because of the project's lack of proof-of-concept. The company will therefore have to continue looking for other investment alternatives.

6. Conclusions

The project has come a long way in creating a potential drug candidate and a base for a future pharmaceutical company. We have designed two recombinant protein and clones of seven others by searching for the gene sequences of the respective proteins in different data bases and editing missing parts by comparing them to neighboring species.

After the expression vectors have been constructed we have developed protocols for the different steps in bringing these recombinant proteins "to life". We have developed a protocol for the production process in a fermentor and optimized the protocol for our specific host organism E.coli. We chose this organism because it is relatively easy to work with. It is also fast-growing and can rapidly produce relatively large amounts of protein. A big draw back of E.coli however is its lack of correct-fold control machinery for many eukaryotic proteins.

This production protocol includes not only the optimal preferences known for protein production in E.coli, but also guidelines as to when addition of the different substances and additives should be added and in what amounts in order to get the maximum protein harvest.

After the protein production in the fermentor is finished it is time for purification of the protein. We developed and optimized a purification protocol involving three steps that efficiently purify our recombinant protein and give a high yield of the total amount produced. We always sought cost efficiency when developing the protocols and tried at all times to develop strategies that would be suitable for industrial large-scale production.

We soon discovered that the protein did aggregate in inclusion bodies and that these configurations have no biological activity. We therefore had to develop a protocol for correctly folding the protein and came up with one in which we first denature the protein before slowly letting it refold with the aid of additives. However, the additives

turned out to be interfering with the nickel beads in the nickel column used in one of the purification steps and we therefore had to clear these out by dialysis.

Hopefully, these protocols will turn out to be compatible with the remaining and yet untested recombinant proteins. The characteristics and looks of these proteins are very similar to those already tested. However, to determine this will need further studies..

7. Discussion

It is probable that the protein concentration of the refolding process described in this report and used in this project could be optimized further. Also, we are not yet completely sure what refolding technique to use in the refolding process. Both the gradient refolding protocol and the dilution refolding protocol seemed to be working satisfactorily with rather similar results. But what is more important, and worrying, is that both showed signs of a protein double band when run on gel. This indicates, as mentioned earlier, the presence of two different protein configurations and because of their equal size and apparently equal ability to bind to the nickel ions on the Sepharose beads in the IMAC, they might be virtually impossible to separate.

The reason for the appearance of alternative configurations might be the existence of an uneven number of the highly reactive cysteins. If the number is even, the cysteins can form strong cystein bonds to each other and be neutral on the outside towards other molecules, but an odd number might leave one cystein "unattended" and thereby turning it into a "free radical", able to interfere with other molecules. The number of cysteins can differ between different species but are often even in number to prevent the just mentioned fact from happening. A solution to this problem could be to redesign the gene into having an even number of cysteins or simply hoping for it not to have any visible effects in the clinical testing. This is a problem that is going to need further investigation.

Another issue that could use additional attention is to determine what concentrations of the additives used in our protocol are the optimal ones. L-Arginine for example proved its ability to aid the refolding process of the protein in a satisfactory manor, but the optimal concentration of L-Arginine has yet to be determined, especially since the additives turned out to be interfering with the nickel ions in the IMAC step. What if we can receive an equally good protein refolding percentage with only half the additives? A reduction of the additives might make the refolding process's incompatibility with the IMAC disappear, who knows.

Further studies of the optimal amounts of ingredients in every step would also be interesting from a financial point of view. Since we are trying to develop a protocol for large-scale industrial production we must try to keep as many costs down as possible. Every Euro will count, especially when multiplied by a thousand or a million. However, this is not of primary importance at this stage of the project. We could spend months and hundreds of thousands of euros at trying to optimize every step of the production if we wanted to, but we would still not be certain that the investment would be worth the cost since we do not know if the vaccine works or not. Before the

vaccine has proven its efficiency in treating asthma and atopic dermatitis the project will not spend more money on the different production and purification steps than necessary in order to produce the milligrams we need for the clinical tests.

The project needs proof-of-concept, and to be able to prove this vaccine to work in vivo. Once the project has got there it will be a lot easier attracting venture capital and other investments, which in turn will make it possible for the company to expand its activities.

8. Remaining challenges in the project

So far the project has managed to design, produce and purify recombinant IL-18 protein for dog and mouse. The outcome of one fermentation run is very high, approximating between 2 and 3 grams of recombinant protein and the purification steps that have been developed seem to be working well enough for larger scale production. With all the pieces coming together in the vaccine production puzzle, it seems as if entering the phase of clinical testing is not too far away now, perhaps only a few months.

The clinical testing will truly be the decisive step for the existence of this project which has been underway for almost a year now. The in vivo testing will give the necessary indications of whether the vaccine seems to be having the effect we want it to have and if there are any visible side effects. Also, before entering clinical testing the strategy decision of whether targeting dog or man in the first clinical tests will have to be made.

Preceding experiments of the vaccine on rats will show whether there are any unwanted side effects or not and it will indicate if the vaccine seems to be having the desired effect. This approach is cheaper and it is also faster than running the tests on dogs, but with the down side that we might still have difficulties in attracting investors and venture capital with experiments based only on rats. Positive results on rats do not necessarily mean positive results on dogs.

Phase one testing on dogs would give the company the information about unwanted side effects needed to be able to enter phase two of clinical testing, in which the search for medical and biological activity is performed more extensively. This is however very expensive and it would be a very heavy burden for the company financially. Successful results from this testing would heavily increase the company’s chances to attract investors, but the financial risk being taken by the owners might be too great to bear. The likelihood that the results will not be positive is unfortunately substantial.

9. Acknowledgements

I would like to thank Professor Lars Hellman for excellent guidance in this project, for his patience and for letting me experience first hand what an up-start of a biotech company from scratch is like. I would also like to thank all the people at the Uppsala University Department of Immunology for their support, and especially Jenny Reimer, Mattias Andersson and Jun Yin. Finally, I would like to thank Magnus Åbrink for helping me reviewing this report.

10. References

Litterature

1 Hellman, L. Profound reduction in allergen sensitivity following treatment with a novel allergy vaccine. *Eur J Immunol* 1994, 24(2), 415-420.

2 Johansson, J., Ledin, A., Vernersson, M., Lovgren-Bengtsson, K. & Hellman, L. Identification of adjuvants that enhance the therapeutic antibody response to host IgE. *Vaccine* 2004, 22(21-22), 2873-2880.

3 Hellman, L. Is vaccination against IgE possible? *Adv Exp Med Biol* 1996, 409, 337-342.

4 Hellman, L. & Carlsson, M. Allergy vaccines: A review of developments. *Clin Immunotherapeutics* 1996, 6(2), 130-142.

5 Vernersson, M., Ledin, A., Johansson, J. & Hellman, L. Generation of therapeutic antibody responses against IgE through vaccination. *Faseb J* 2002, 16(8), 875-877.

6 Hellman, L. Vaccines against allergies. In *Handbook of Experimental Pharmacology, Vol.133, Vaccines*, Vol. 133 (Eds. Perlmann, P. & Wigzell, H.) Springer-Verlag, Berlin, 1999. 499-526.

7 Ledin, A., Bergvall, K., Salmon-Hillbertz, N. *et al.* Generation of therapeutic antibody responses against IgE in dogs, an animal species with exceptionally high plasma IgE levels. *Vaccine* 24, 2006, 66-74.

8 Johansson J. and Hellman L. Modifications increasing the efficacy of recombinant vaccines; marked increase in antibody titers with moderately repetitive variants of a therapeutic allergy vaccine. Submitted to *Vaccine* May 2006.

9 Hellman, L. Regulation of IgE homeostasis, and the identification of potential targets for therapeutic intervention. *Biomed Pharmacother* 2006, 1-16.

10 Tsutsui, H., Yoshimoto, T., Hayashi, N., Mizutani, H. & Nakanishi, K. Induction of allergic inflammation by interleukin-18 in experimental animal models. *Immunol Rev* 2004, 202, 115-138.

11 Takeda, K., Tsutsui, H., Yoshimoto, T., Adashi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8 (1998), 383-390.

12 Hamada, H. and Shiraki, K. L-Argininamide improves the refolding more effectively that L-arginine. *J. of Biotechnology* 2007.