Jihočeská univerzita v Českých Budějovicích

Přírodovědecká fakulta

Production and functional characterization of tick salivary protease inhibitors

Master Thesis in Molecular Biology

Jan Kotál

České Budějovice, 2013

Supervisor: Michail Kotsyfakis PhD.

Institute of Parasitology, Biology Centre, ASCR

Kotál J., 2013: Production and functional characterization of tick salivary protease inhibitors. Mgr. thesis in English, Faculty of Science, The University of South Bohemia, České Budějovice, Czech Republic.

Annotation:

Two cysteine and two serine protease inhibitors from a tick *Ixodes ricinus* saliva were overexpressed using a prokaryotic overexpression system and refolded to their native state. Both cysteine protease inhibitors were tested as potential antigens for an anti-tick vaccine showing no effect on tick feeding or reproduction. Various immunological methods were employed to test the potential immunomodulatory function of these proteins without success.

Prohlašuji, že svoji magisterskou práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své magisterské práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

....

Date, place Signature

Acknowledgement

First I would like to thank my supervisor Michail Kotsyfakis PhD. for offering me the opportunity to work in his laboratory and all the help and support he gave me. Big thanks also go to RNDr. Helena Langhansová PhD. for her good-will to assist and guide me through some experiments. I also want to thank RNDr. Jindřich Chmelař PhD., who guided me through all the experiments during my stay in the Technical University of Dresden. I would also like to thank all the other people I worked with, either in our lab or within the Biology Centre ASCR. You have made me feel like in a good team and among friends, which was really comfortable and important.

I am also very thankful to my family, my girlfriend and my friends that supported me throughout my whole studies.

Goals

The main goal of my work was to overexpress and refold *Ixodes ricinus* salivary proteins. This includes optimizing the overexpression in *Escherichia coli*, expression in a big scale, isolation of proteins from bacteria, refolding optimization and a big scale refolding.

The next task was to test some of the purified proteins as a vaccine against ticks.

The final part of my experiments was devoted in the familiarization with various immunological methods by testing the overexpressed proteins in various cellular assays and animal models of vertebrate host immunomodulation.

Table of contents

List of used abbreviations:

BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CystA	Cysteine protease inhibitor A
CystB	Cysteine protease inhibitor B
DTT	Dithiotreitol
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GSH	Reduced glutathione
GSSG	Oxidized glutathione
IB	Inclusion bodies
IFNγ	Interferon γ
LPS	Lipopolysaccharide
MW	Molecular weight
OPD	*o*-Phenylenediamine dihydrochloride
OVA	Ovalbumin
PBS	Phosphate buffer saline
PEG	Polyethyleneglycol
PMA	Phorbol Myristate Acetate
RT	Room temperature
SerA	Serine protease inhibitor A
SerB	Serine protease inhibitor B
SerC	Serine protease inhibitor C
TBS	Trisma base - salt
TNF	Tumor Necrosis Factor

1. Introduction

1.1. Ticks, *Ixodes ricinus*

1.1.1. Characterization, life cycle

Ticks are blood-sucking ectoparasites of mammals, birds or even reptiles. While soft ticks feed only for several hours, hard ticks are usually attached to their hosts for several days or even weeks (Brusca *et al*., 2003).

The tick *Ixodes ricinus* belongs to the order *Ixodida*, family *Ixodidae*, class *Arachnida*, phylum *Arthropoda*. The family *Ixodidae* is also known as "hard ticks" because of a sclerotized shield covering the entire dorsum. Ixodes ricinus is a European tick found also in the neighboring parts of Africa and the Middle East (Lindgren *et al*., 2000). Its habitats are woodlands, and other relatively humid areas where no dry summers or very cold winters occur.

I.ricinus has a three stage life cycle where each of the stages needs a specific host to feed on. Six-legged larvae hatch from eggs and seek for mainly small rodents. After a blood meal the larvae transform into nymphs. The eight-legged nymphs feed on mid-sized mammals, engorge for 3-5 days and they molt after they drop off. The resulting adult ticks (8 legs) attach to their hosts during the next year. Adult females feed on bigger mammals like deer, cattle or dogs for 7-10 days. Adult males do not suck blood, but they can also be found on hosts trying to copulate with females. After successful feeding and fertilization by the males, the females lay between 500 and 2000 eggs. New larvae hatch during late spring or fall and usually start feeding during the following period (Capinera *et al*., 2008).

1.1.2. Feeding

Since *I. ricinus* feeds strictly on blood, it must be able to process a large amount of blood in a relatively short time-period. Females increase their size up to 200 times (Capinera *et al*., 2008). A feeding starts with a slow phase where changes in tick physiology occur. The growth of cuticle follows in order to ensure enough space for the engorgement during the fast second phase of tick feeding when ticks suck a big amount of blood. There is a significant difference in the protein composition in saliva between these two phases. Ticks alternate between blood sucking and saliva release in 5-20min cycles. Water from the blood is pumped through the haemocoel back to saliva. Blood digestion takes place in the midgut, in the intracellular environment of the lumen of the midgut epithelial cells (Bowman *et al*., 2004; Waladde *et al*., 1979).

1.1.3. Disease vector

Ticks serve as the vectors for the transmission of important and dangerous diseases. Viruses and bacteria belong among the most common tick-borne pathogens, but fungi, protozoans or helminthes can also be transmitted (Melhorn *et al.*, 2008). The two major diseases transmitted by *I. ricinus* in central Europe are the Lyme disease and tick-borne encephalitis (TBE). TBE is caused by the tick-borne encephalitis virus and Lyme disease by spirochetes belonging to the *Borrelia burgdorferi sensu lato* complex in Europe and *Borrelia burgdorferi sensu stricto* in the USA (Hovius *et al.*, 2007).

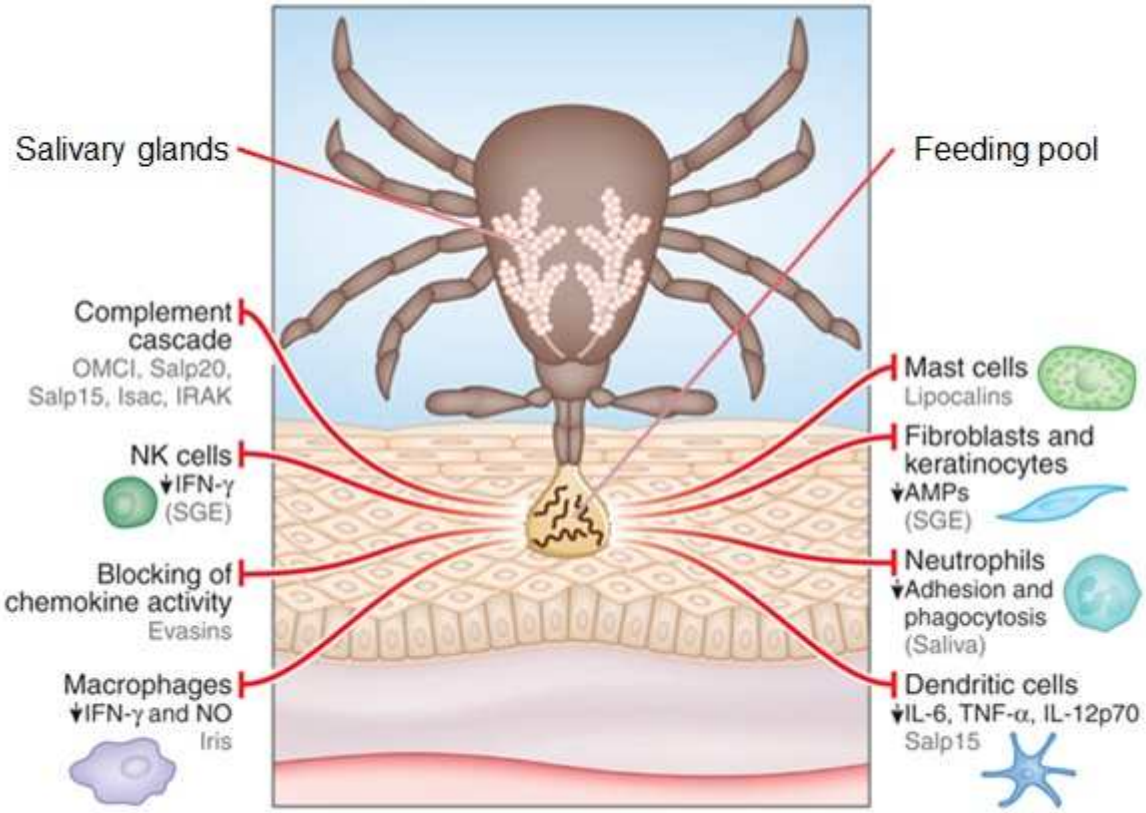
1.1.4. Tick saliva secretion and its role in tick feeding

It is important for a tick that blood is continuously delivered to the hypostome despite the injury caused to the host skin by the tick bite. Hemostasis triggers coagulation, vasoconstriction and also the platelet aggregation as the first host defenses to the caused injury by the tick mouthparts. These host physiological processes occur almost immediately after the injury. The vertebrate immune system may also respond to the intrusion of the tick mouthparts in the feeding site. Both cellular and humoral responses take place. The early response includes the complement reactions activated by an antigen/antibody complex. The infiltration of leukocytes occurs several hours after the injury (Francischetti *et al.*, 2009).

As an adaptation to blood feeding, ticks evolved a complex mixture of salivary components to minimize their host's response. These salivary compounds suppress innate and adaptive immune responses at the feeding site that they may cause pain and itching or disrupt blood flow. They also help to overcome their host's defenses against blood loss (hemostasis, Chmelar *et al.*, 2012). As a result the saliva of ticks contains anti-clotting, anti-platelet, vasodilatory, anti-inflammatory and immunomodulatory components (Hovius, 2009; Ribeiro *et al.*, 2002, Chmelar *et al.*, 2011). Proteins in the tick saliva inhibit complement (Ribeiro, 1987a), histamine binding (Mans, 2005), leukocyte function and proliferation (Ramachandra and Wikel, 1992), cytokine production (Kotsyfakis *et al.*, 2006) and natural killer (Kopecky and Kuthejlova, 1998) or dendritic cell function (Hovius *et al.*, 2008).

The effect of tick saliva on vertebrate innate immune response is shown in figure 1.

***Figure 1**: The effect of tick saliva on different immune mechanisms in skin. Proteins (or their group) causing the effect are noted in grey color; inhibition of several mediators is represented by an arrow. SGE = salivary gland extract; AMP = antimicrobial peptide; IFNγ =interferon γ; IL = interleukin; NO = nitric oxide; TNF = tumor necrosis factor. The picture was adapted from Hovius 2009.*



1.2. Ticks and vertebrate immunity

Ticks have to fight against vertebrate innate immunity during every infestation and in the case of secondary or subsequent infestations also against vertebrate adaptive immunity. Ticks face the immune response even before sucking blood. The mouthparts cause a wound in the host's skin. Resident leukocytes as well as mast cells, dendritic cells, eosinophils, macrophages or keratinocytes are in the contact with the saliva or hypostome immediately. Chemokines are released by these vertebrate cells in order to recruit neutrophils and other inflammatory cells in the area of tick infestation. Adaptive response by T or B cells can be activated during subsequent infestations by other ticks. The antibodies against tick saliva or mouthparts can activate complement or sensitize mast cells and basophils. The strength of this host immune response and the effect on the tick physiology depend on the species either of a host or a tick, on host's health and its genotype (Francischetti *et al.*, 2009)

1.2.1. Macrophages and monocytes

Most resident macrophages have their origin from blood marrow-derived monocytes. They can be found in many organs, including epidermis which is the most important one when dealing with ticks. Additional monocytes are recruited as an inflammatory response in the tick infestation site (Taylor *et al.,* 2005). These cells have a different phenotype than the resident macrophages. They can differ in cytokine production, expression of receptors or in their overall function as effector cells. The amount of macrophages and monocytes is increased in the tick feeding cavity but not in the surrounding tissues. Macrophages act as antigen presenting cells as well as cytokine and chemokine producers (Mantovani *et al*., 2004).

1.2.2. Mast cells

Mast cells are spread in the whole body; they are present especially under epithelial surfaces, such as skin. They have granules which contain a variety of different mediators like vasoactive compounds, serine proteases or cytokines. After activation, the mast cells are also able to produce new mediators to recruit more inflammatory cells (Metcalfe *et al*., 1997). The number of mast cells increases after secondary or subsequent tick infestations, but it is not increased after the primary tick infestation. Also the number of degranulated mast cells is significantly higher after repeated tick infestation (Steeves *et al.,* 1991; Gill, 1986).

The immunological importance of mast cells against ticks is however still unclear. Mast cell deficient mice developed some resistance against *Dermacentor variabillis* after repeated exposures, just like the wild type mice (Steeves *et al.,* 1991). On the other hand mast cell deficient mice did not create any resistance to *Haemaphysalis longicornis*. They regained the tick resistance after mast cell injection (Matsuda *et al*., 1985; 1987).

While the immunological importance of mast cells is still unclear, they for sure play a role in causing the itching feeling by releasing histamine. Therefore tick saliva contains histamine binding proteins to counteract this effect (Paesen *et al.,* 1999).

1.2.3. Dendritic cells

Dendritic cells initiate the host adaptive immunity by presenting the pathogenic antigens. We can find two states of dendritic cells – the immature form in skin or mucosa and the mature form in the lymphoid tissues. There is a specialized dendritic cell type in skin called Langerhans cells. The immature form has primarily an antigen uptake function. The mature dendritic cells effectively stimulate T cells but they have only a small phagocytic activity.

Several studies have proven that tick saliva interacts with dendritic cells. Langerhans cells incubated with tick saliva *in vitro* stimulated T cells proliferation (Nithiuthai *et al.*, 1985).Another study has shown a decrease in dendritic cells population at the tick feeding site which suggests that the cells move to the lymph nodes to activate T cells. Tick saliva has several effects on dendritic cells; it inhibits their maturation or reduces the amount of receptors on the surface of cells as well as their total surface (Sa-Nunes *et al.,* 2007; Oliveira *et al.,* 2008; Sa-Nunes *et al.,* 2009).

1.2.4. Granulocytes

Granulocytes are bone marrow derived cells with granules in their cytoplasm. Their nucleus is often partially divided into three segments and its shape is polymorphic. The granulocyte group consists of three major cell types – neutrophils, basophils and eosinophils. All of them are involved in the immune response to pathogens (Delves *et al.* 1998).

1.2.4.1. Neutrophils

Neutrophils are phagocytic cells of the innate immune system. They are highly motile with a relatively short lifetime. Their importance is in the early stages of vertebrate defense. Neutrophils phagocytose pathogenic microorganisms and produce cytokines to recruit other leukocytes during the early phases of inflammation response (Scapini *et al.*, 2000). Neutrophils are most abundant during the primary infestation, but not in the next infestations (Gill *et al.*, 1985). It is not known whether the absence of neutrophils would affect the resistance to ticks. Tick saliva (from *I.scapularis*) inhibits the granule release and neutrophil infiltration. It also has an inhibitory effect on the phagocytosis of *B.burgdorferi* (Ribeiro *et al.*, 1990). Neutrophils are not attracted by saliva itself but by the cleavage of C5 and the release of a chemotactic factor upon the encounter of tick saliva (Berenberg *et al.*, 1992).

1.2.4.2. Basophils

Basophils were characterized as a prevailing cell type in a tick feeding site and as important factors in tick rejection. They are an IgE-activated isotype of cells circulating throughout the body. After migration to the wounded tissues the basophils degranulate and they release mediators to reject a tick during a reaction which is known as cutaneous basophil hypersensitivity (Brown *et al.*, 1993). Ticks at the feeding site are then killed by a strong histamine release.

Guinea pigs are able to develop a protection against ticks already after the first infestation. The resistance can also be transferred from an immunized animal to a naive one by serum/antibodies transfusion (Brossard *et al.*, 1997).

1.2.4.3. Eosinophils

Eosinophils are present mainly at the body surfaces that interact with the outer environment. Their level in blood is quite low, especially when there is no allergic reaction or parasitic infection. Eosinophils produce cytokines, chemokines and other mediators; some of them (e.g. indoleamine 2,3deoxygenase IDO) inhibit Th1 lymphocyte population (Odemuyiwa *et al.*, 2004).Eosinophils are also rich in cytotoxic granules or they produce inflammation mediators and tissue repair molecules.

Repeated exposure to ticks leads to elevated levels of eosinophils in the feeding site among many animal species – including guinea pigs, cattle, dogs, mice (Francischetti *et al*., 2009).

1.3. Host-tick-pathogen interaction

The inhibition of host innate immunity is essential not only for tick feeding but also for the pathogen transmission by tick bites. Therefore some pathogens transmitted by ticks take advantage of this lack of appropriate host immune response in the presence of tick saliva to increase their infectivity. Tick saliva suppresses the production of antimicrobial peptides which play a role in the defense against *Borrelia* (Marchal *et al.*, 2009). Saliva also inhibits the migration of leukocytes towards the feeding site which helps tick-borne pathogens to survive and spread to the body (Hovius, 2009).

Salp15 is one of the tick salivary proteins that inhibits CD4+ T cells signaling and activation and it protects serum-sensitive *Borrelia* spirochetes against complement (Schuijt *et al*., 2008).

1.4. Cysteine protease inhibitors in tick saliva

Cysteine proteases are protein-cleaving enzymes that share a catalytic mechanism involving a cysteine residue in their catalytic site.

Cystatins are a big family or cysteine protease inhibitors acting mainly against papain and legumain protease families. They share a common tertiary structure with an α-helix placed on an anti-parallel β-sheet. Cystatins are found in many different organisms such as vertebrates, invertebrates, plants or protozoa (Vray *et al.*, 2002).

Among the other functions, tick cysteine protease inhibitors, like Sialostatin L, have been demonstrated to have a strong immuno-modulatory activity (Kotsyfakis *et al.*, 2006). Their

role involves the modification of antigen presentation, neutrophil chemotaxis during inflammation or apoptosis. As proteolysis regulators, they are associated with cell homeostasis and some pathological conditions (Reddy *et al.*, 1995). Tick cystatins show relatively small homology to all described cystatins from other species, just approximately 30-40% amino acid identity. Most of the studied cysteine protease inhibitors in ticks are secreted type-2cystatins with a molecular weight ranging between 13-15kDa. Currently 16 type-2cystatins of tick origin are described in the literature; the function of nine of them is also biochemically characterized (Schwarz *et al.*, 2012).

In this study, two novel *I.ricinus* cysteine protease inhibitors were produced and characterized. They are named as Cysteine protease inhibitor A and B (CystA, CystB) in this thesis to avoid a public disclosure of their names and functions. Both were tested at the biochemical level, showing a great specificity to cathepsins L, S and B (not part of this thesis). We also tested them as anti-tick vaccines and CystB as an inhibitor of *Borrelia* proliferation *in vitro* as well as *in vivo*. We further show the effect of CystB and Sialostatin L on inflammation and on the cells of the immune system.

1.5. Serine protease inhibitors in tick saliva

Serine proteases, like all the other proteases, cleave peptide bonds in proteins. Serine is involved in that process as the nucleophilic amino acid at the catalytic site of serine proteases. They play role in many physiological processes like immune response, blood coagulation cascade, fibrinolysis and food digestion in a wide variety of organisms, including hematophagous arthropods.

Since serine proteases are important for vertebrate host homeostasis, they are a good target to be inhibited by tick saliva. Serpins, a large subclass of serine protease inhibitors block or reduce the activity of serine proteases. Serpins share a 40kDa domain at the primary but also tertiary structure. This domain suggests that the molecular weight of serpins is at least 40kDa which differentiates them from other serine protease inhibitors. Similar to the regulation of other serine proteases, serpins have also regulatory properties for complement activation, blood coagulation, inflammation or fibrinolysis. (Mulenga, 2001; Imamura *et al.*, 2004)

Two *I.ricinus* salivary serine protease inhibitors were produced during the experimental work of this thesis. They are named as Serine protease inhibitor A and C (SerA, SerC) in this thesis to avoid a public disclosure of their names and functions. Both proteins were tested at the biochemical level, showing specificity to plasmin (SerA) and elastase (SerC). These data are not part of this thesis. The influence on inflammation and on the cells of the immune system

of another serine protease inhibitor (SerB) from *I.scapularis* was tested in the experimental work of this thesis.

1.6. Vaccines

Efforts have been made to minimize the impact of ticks on public health and the agriculture. Mainly acaricides (pesticides) are used to kill ticks because of their price and availability. Their disadvantage is the contamination of environment and also the development of resistance in ticks. Therefore, other tick control approaches need to be considered and developed.

Vaccination against ticks may be an alternative. It is a process of introducing an antigen to an organism to stimulate the immune system to adapt to the antigen and develop antibodies. It is widely used against infectious diseases (Mulenga *et al*., 2000) but still not very often against ectoparasites like ticks.

A vaccine against *Boophilus microplus* (Willadsen *et al.*, 1995) was the first practically and commercially working vaccine against ticks. However vaccines against many other tick species still need to be developed.

Tick antigens suitable for vaccines are categorized either as concealed or exposed.

1.6.1. Concealed antigens

Blood sucking parasites will ingest antibodies during blood feeding that may be targeted against their own antigens; e.g. against the midgut proteins. Such antibodies would damage or kill the tick tissues. Immunity gained in this way does not change the feeding environment of the tick and thus may not prevent from disease transmission (Mulenga *et al*., 2000). The first commercial vaccine against *B.microplus* (Willadsen *et al.*, 1995) is also based on a concealed antigen.

1.6.2. Exposed antigens

Exposed antigens are those which are injected to the host during tick feeding; these are mainly tick salivary constituents. Tick salivary gland extracts (SGE) were first used as a source of antigens for vaccination. The induction of protective immunity by SGE was problematic and not very successful which lead to testing single salivary proteins. Exposed antigens may have lower antigenicity because they evolved under the host immunity pressure (Mulenga *et al*., 2000).

Salivary proteins regulate tick feeding site and also pathogen transmission (Ribeiro, 1987b). This suggests that the immunity against crucial salivary proteins will prevent tick attachment and feeding as well as disease transmission. Other studies have shown an inhibition in *Borrelia* infection at animals with high anti-tick saliva antibody titers. These findings imply that tick saliva supports pathogen transmission and establishment of infection in the vertebrate host (Wikel *et al.,* 1980, 1997).

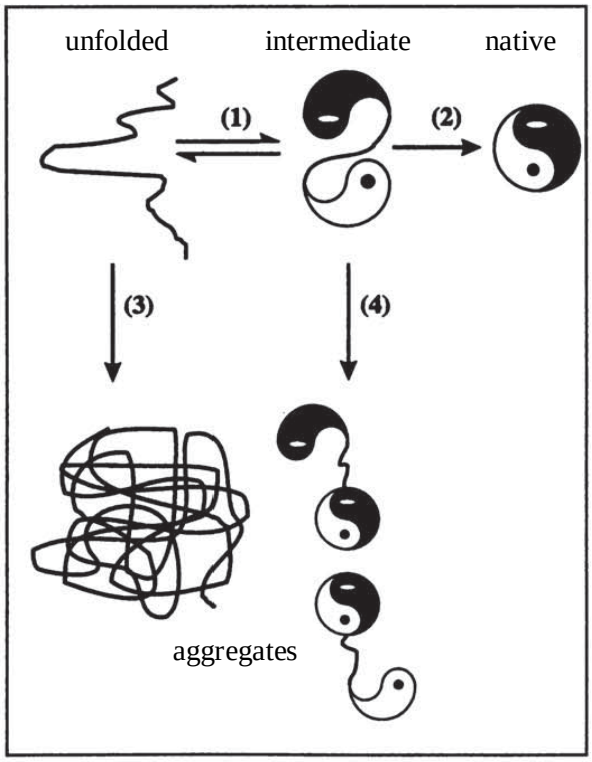
Here we test two salivary cysteine protease inhibitors from the tick *Ixodes ricinus* as antigens for a potential vaccine against ticks. The goal is to develop a vaccine that would prevent ticks from feeding on a host and thus a protection from tick-borne pathogens will be achieved– in other words a transmission blocking vaccine. We used guinea pigs as the vaccinated experimental animals and after reaching a sufficient titer of antibodies by repeated injections of the vaccination antigen, the guinea pigs were exposed to ticks for two times. We analyzed the mass of engorged ticks, the time necessary to complete feeding, the amount of eggs produced by the fed ticks and the molting efficiency from the eggs (production of larvae).
The shortcut GP is used for a guinea pig in this text.

1.7. Protein refolding

The most efficient way (amount-wise) to produce recombinant proteins is the expression in the form of inclusion bodies in bacteria. Refolding of these proteins from the inclusion bodies to an active state is a challenge of the specific overexpression system. Solubilization and unfolding of a recombinant protein from the inclusion bodies usually requires chaotropes like urea or guanidine hydrochloride. Reducing agents like dithiotreitol (DTT) or glutathione redox system (GSH/GSSG) reduce the disulfide bonds between cysteines in the process of protein unfolding. Protein is then refolded by removal of the chaotrope by dialysis or rapid dilution in a refolding buffer (Rudolph *et al.* 1996; Bird *et al.,* 2004).
Aggregation leads in many cases to decreased yields of refolded protein. While relatively simple proteins (that lack cysteine residues) are refolded quite easily, more complex proteins with disulfide bonds tend to misfold or aggregate. Aggregation, as an intermolecular process, is highly dependent on protein concentration. The best refolding yields can be expected at protein concentration of 10-50µg/ml. Such a low protein concentration would require excessive volumes of refolding buffer which would be too expensive and time demanding. The goal is refolding at a high protein concentration with a high yield (Rudolph *et al.* 1996; Hevehan *et al.,* 1997).

The early stages of the folding pathway are the most sensitive to aggregation. Folding intermediates with exposed hydrophobic regions are believed to play the major role in this process. These intermediates have a secondary structure similar to the native conformation, but they never reach the native-like tertiary structure. As a result, the exposed hydrophobic parts of single molecules interact with each other and cause the aggregation in protein multimers (Yamaguchi 2013).

Figure 2: *Schematic representation of the refolding and aggregation processes. 1 and 2 show the correct pathway to native protein. 3 and 4 are irreversible steps towards protein aggregation. The figure was adapted from Rudolph et al. 1996.*



One of the approaches to avoid aggregation is refolding at low temperature with enough time to form the intermediates. After this step, the temperature increases rapidly to initiate refolding of the intermediates to an active state. Another solution is a slow addition of denatured protein to the refolding buffer. The concentration of aggregates is low during the whole process because when new aggregates are formed upon addition of more protein, the other ones change conformation to the final native state (equilibrium shift).

The composition of the refolding buffer plays also an important role in protein folding. There is unfortunately no general rule for the refolding buffer composition; each protein is unique and the optimal refolding buffers often differ a lot even between closely related proteins. Physical parameters of the buffer such as pH, temperature or ionic strength have a great effect

on the folding process. Changing the composition by various additives is a simple strategy to further increase the final yields of native protein. The additives may either stabilize the native state or, more often, destabilize incorrectly folded molecules by favoring the intermediates or an unfolded state of the protein. Generally they do not affect much the folding rate but they inhibit the aggregation process (Rudolph *et al.* 1996).

A variety of additives is now known and used for improving the protein refolding efficiency. Chaotropic agents like arginine often significantly increase the folding efficiency by slowing down the aggregation process. Guanidine or urea at non-denaturing concentrations have the same effect. Other types of additives may be salts (NaCl, KCl, $CaCl_2$, ammonium sulphate), sugars (glucose, sucrose, glycerol), detergents (Tween, Triton X-100, SDS, Chaps) and other chemicals like EDTA or cyclohexanol (Bird *et al.*, 2004).

The major part of this thesis was the optimization of native protein production in a prokaryotic system of overexpression in both small scale and large scale (more than 10 mgrs of pure protein produced). Two produced *Ixodes ricinus* salivary cysteine protease inhibitors were tested as antigens for an anti-tick vaccine development. The role of cysteine protease inhibitor B on *Borrelia burgdorferi* transmission and proliferation was also analyzed. The effects of this protein and other proteins on vertebrate immunity were also investigated during my 20 days stay in the Technical University of Dresden in the laboratory for Vascular Inflammation, Diabetes and Kidney; Department of Internal Medicine III, University Hospital Carl Gustav Carus at the Technische Universität Dresden (Laboratory Head Professor Triantafyllos Chavakis, Local supervisor Jindřich Chmelař PhD.).

2. Materials and Methods

2.1. List of used chemicals and materials

The complete list of all chemicals and other consumables is shown in table 1. The list is alphabetically ordered according to the name of the consumable. The producer is listed next to each consumable.

Table 1: *List of used consumables, each with the corresponding company*

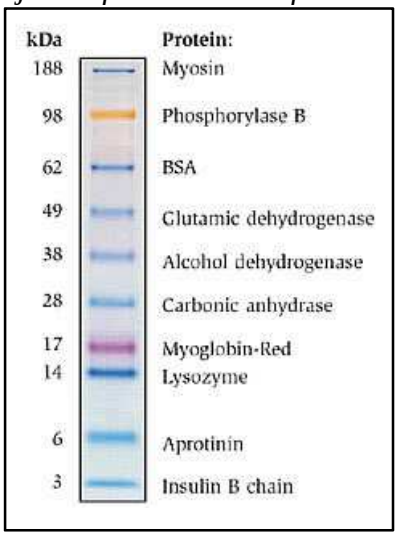
Consumable	**Company**
Acetic acid	Lach-Ner
Amicon Ultra-15 Centrifugal Filter Units	Millipore
Ampicillin	Sigma
Bovine Serum Albumin (BSA)	Sigma
BSK-H medium with 6% rabbit serum	Sigma
Calcium chloride ($CaCl_2$)	Lach-Ner
CAPS	Sigma
Cd11b antibodies	BD Pharmingen
Chloramphenicol	Sigma
Chloroform	Sigma
Citric acid	Lach-Ner
Complete Freund's Adjuvant (CFA)	Sigma
Coomassie Brilliant Blue	Merck
Disodium phosphate (Na_2HPO_4)	Lach-Ner
Dithiotreitol (DTT)	Sigma
E.coliBL21 Star™ (DE3)pLysS	Invitrogen
Enhanced Green Fluorescent Protein (EGFP)	BioVision
Ethanol	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Fast Universal master Mix ROX	Roche
Fetal Bovine Serum (FBS)	Sigma
Fc block solution	BD Pharmingen
FLAGELLIN primers and probe 69bp	Generi Biotech
F4/80 antibodies	BD Pharmingen
Guanidine	Sigma
Halothane	Sigma
HISTOPAQUE-1077	Sigma
Hydrochloric acid (HCl)	Lach-Ner
Incomplete Freund's Adjuvant (IFA)	Sigma
iScript	Bio-Rad
iScript reaction mix	Bio-Rad

Isopropanol	Sigma
Isopropyl thioglalactoside (IPTG)	Invitrogen
L-Arginine	Sigma
LB BROTH, MILLER (LURIA-BERTANI)	Amresco
Lipopolysaccharide (LPS)	Sigma
Ly-6C antibodies	BD Pharmingen
Ly-6G antibodies	BD Pharmingen
Magnesium chloride ($MgCl_2$)	Lach-Ner
Methanol	Lach-Ner
Mineral oil	Sigma
Monopotassium phosphate (KH_2PO_4)	Lach-Ner
MOPS	Sigma
Narkamon	Spofa
NucleoSpin® Tissue kit	Machery-Nagel
NuPAGE precast 4-12% gradient polyacrylamide gels	Invitrogen
NupaGE reducing agent	Invitrogen
NuPAGE running buffer	Invitrogen
NuPAGE sample buffer	Invitrogen
NuPAGE See Blue® Plus2 molecular weight marker	Invitrogen
o-Phenylenediamine dihydrochloride (OPD)	Sigma
Ovalbumin (OVA)	Sigma
PCR water	Top-Bio
PenStrep	Invitrogen
Percoll	GE Healthcare
Phorbol Myristate Acetate (PMA)	Sigma
Pierce® BCA Protein Assay Kit − Reducing Agent Compatible	Thermo Scientific
Potassium chloride (KCl)	Lach-Ner
Prep/Scale Spiral Wound TFF-6 Module PLBC filter	Millipore
ProteoStat® Protein aggregation assay kit	Enzo® Life Sciences
Red Blood Cells lysis Solution (RBC lysis solution)	Gibco
Rometar	Spofa
RPMI 1620 medium	Gibco
Sodium acetate (NaAc)	Lach-Ner
Sodium carbonate (Na_2CO_3)	Lach-Ner
Sodium chloride (NaCl)	Lach-Ner
Sodium hydrogencarbonate ($NaHCO_3$)	Lach-Ner
Sulfuric acid (H_2SO_4)	Lach-Ner
Thioglycolate Broth	Sigma
Tris	Sigma
Triton-X 100	Sigma
TRIZOL	mrcgene

2.2. NuPAGE

NuPAGE precast 4-12% gradient polyacrylamide gels were used for protein separation. Protein samples were mixed with NuPAGE sample buffer (4x) and NuPAGE reducing agent (10x). The mixture was then denatured by boiling for 10 minutes at 70°C. Electrophoresis was performed at constant voltage of 200V for 35-40 minutes in an electrophoretic tank filled with NuPAGE running buffer. After the run, gels were stained using Coomassie Brilliant Blue and the protein bands were visualized by washing the stained gel with Coomassie destaining solution (10% Acetic acid, 25% methanol, water). The molecular weight marker we used in all the experiments is shown in figure 3.

Figure 3: *The molecular weight marker See Blue® Plus2. Picture was taken from www.lifetechnoligies.com and adjusted for the needs of this thesis.*



2.3. Protein expression

The gene of interest was first codon-optimized for overexpression in bacteria, subcloned in a pET-17b plasmid and transformed into an *E.coli*strainBL21 Star™ (DE3)pLysS.

We used in all the experiments antibiotics ampicillin at final concentration 100mg/l and chloramphenicol at final concentration 30mg/l.LB BROTH, MILLER (LURIA-BERTANI) was the growth medium used for all overexpressions; the term LB medium is used in the text.

The final concentration of IPTG in all the cultures induced for gene overexpression was 1mM.

A pilot gene-overexpression experiment was done in order to find the best time point that protein expression peaks in the bacterial cells. One day before the pilot experiment a bacterial culture in 5ml of LB medium in the presence of the antibiotics was prepared in order to have enough bacteria to start the culture for the pilot expression. 1ml of this overnight bacterial culture was added to 40 ml LB medium. After 2h of culture incubation at 37°C 1ml was

removed, centrifuged (5min, 10 000xg) and the pellet was stored at 5°C. After that, IPTG was added in the remaining culture, the sample was incubated in37°C and each hour 0.5 ml of culture was again removed and the bacterial pellet was stored. Totally 8 samples were collected - 0 to 6 and 24 hours after IPTG addition. Each of the 8 pellets was dissolved in 500µl 20mM Tris, pH8 and in four cycles cooled in liquid N_2 and heated at 55°C. After centrifugation (10min, 10 000xg), the pellet was dissolved in NuPAGE Sample buffer, NuPAGE Sample reducing agent and water. All 8 samples were analyzed using NuPAGE. The resulting supernatant from the centrifugation was also analyzed in the same way by using the NuPAGE gels.

The big scale protein expression was performed in LB medium with antibiotics, in 37°C under constant shaking (150 rpm). One day before the expression, the bacterial cells were grown in the same medium to increase their population before gene overexpression was induced. 15-20 ml of the overnight cell culture was added to 1L LB medium and optical density (OD) at 600nm was measured; ideal value should be up to 0.1. The OD of the bacterial culture was measured until reached 0.6-0.8 (ca 2.5h). After that, IPTG was added to induce protein expression and the culture was incubated at 37°C. Cells were harvested by centrifugation at the time point which was considered as optimal for protein overexpression based on the results of the pilot expression (please see above).

2.4. Inclusion bodies isolation

Isolated cells were dissolved in 20mM Tris, pH 8using a magnetic stirrer and disrupted by applying ultrasonic waves (maximum power, 3x30s). One liter of Tris was needed for a bacterial pellet from ca 4l of bacterial culture. The sample was centrifuged (10min, 15 000xg), the resulting pellet was dissolved in 1% Triton-X 100 and sonicated again followed by gentle stirring at room temperature for 1 hour. The consumption of Triton-X 100 was 1L for the pellet from 24L of bacterial culture. After another centrifugation, the pellet of inclusion bodies was washed 4x with 20mM Tris, pH 8 to remove all traces of Triton.

2.5. Protein refolding and concentration

The overexpressed protein, within the inclusion bodies, was dissolved in 6M guanidine hydrochloride, 20mM Tris, pH 8. We used 25ml of guanidine solution with DTT per 4l of refolding buffer. DTT was added (final conc. 10mM) followed by 1 hour of shaking at room temperature. DTT serves here as a reducing agent for the cysteine-cysteine bonds (Rudolph and Lilie 1996). Samples were centrifuged (10min, 10 000xg) to remove impurities contained

in the inclusion bodies and non dissolved inclusion bodies. Then the reduced and denatured protein was poured to the optimized refolding buffer with constant fast stirring for 3 hours at room temperature followed by an overnight incubation at 4°C.

After the overnight incubation the protein in refolding buffer was centrifuged (10min, 15 000xg) to get rid of all precipitates caused by improper refolding. After that the sample was concentrated using a Prep/Scale Spiral Wound TFF-6 Module PLBC filter followed by further concentration by Amicon Ultra-15 Centrifugal Filter Units (both Millipore). The pore size we used was 3kDa for cysteine protease inhibitors and 10kDa for serine protease inhibitors.

2.6. Refolding optimization

The first step was to roughly determine the best conditions for protein refolding (preferable pH and salt concentration in the refolding buffer). The following buffers were used:

1. 300mM NaCl, 20mM Na-Acetate, pH 5.5
2. 20mM Tris-HCl, 300mM NaCl, pH 8.5
3. 20mM Tris-HCl, 300mM NaCl, pH 6.8
4. 20mM Tris-HCl, 300mM NaCl, pH 8.0
5. 20mM Tris-HCl, 10mM NaCl, 0.5mM KCl, 1mM EDTA, pH 8.0
6. 20mM Tris-HCl, 10mM NaCl, 0.5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, pH 8.0
7. 20mM Tris-HCl, 240mM NaCl, 10mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, pH 8.0
8. 20mM Tris-HCl, 240mM NaCl, 10mM KCl, 1mM EDTA, pH 8.0

In some cases the first set of buffers was provided by the ProteoStat® Protein aggregation assay kit; the specific kit provides15 different buffers as the starting point for refolding optimization.

A small piece of the inclusion bodies pellet was dissolved in 5ml 6M guanidine, DTT was added (10mM final conc.) and the sample was shaken for 45min. After that, 312µl of the sample was added drop wise to 50ml of each refolding buffer. The tubes were stirred opened for 3h at RT and then stored at 4°C overnight. The next day the samples were centrifuged and the best condition was chosen based on the size of the pellet of protein precipitates, the better the refolding condition is the smaller the pellet should be. A “starting” buffer was found this way and further buffer composition optimization followed based on this buffer.

We tried afterwards different concentrations of:

- NaCl (0, 20, 50, 100, 150, 300mM)
- Arginine (0, 50, 100, 150, 250, 400mM) or guanidine (200, 400mM)
- Different pH values (6.8, 7.4, 8.0, 8.5; sometimes even 9.0, 9.5 or 10.0)
- One of the important parameters was also the inclusion bodies concentration in the refolding buffer (2, 1, 0.5, 0.25, 0.125 and 0.0625 g of wet inclusion bodies per liter of refolding buffer).

Each of these parameters was optimized in separate cycles of buffer optimization; the cycles followed after each other so the buffer composition was improved gradually/step-wise.

All 50ml samples were centrifuged (10min, 10 000xg) to remove precipitates. Then the samples were concentrated to 400-700µl final volume by using Amicon Ultra-15 Centrifugal Filter Units. The refolding success rate was determined after the concentration step in three ways:

I. Protein concentration was measured using a Pierce® BCA Protein Assay Kit − Reducing Agent Compatible according to the instructions provided by the manufacturer. A BCA method is based on reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium, known as the biuret reaction. Reduced Cu^{1+} ions then react with bicinchoninic acid (BCA) developing a colored reaction. The method was modified to minimize the effect of reducing agents such as DTT on the assay results. Measurements were done according to the manual using a microplate procedure.

II. The amount of aggregates in the sample was determined by the ProteoStat® Protein aggregation assay kit. Proteins in solutions tend to aggregate due to high concentration or simply natural instability (Rudolph *et al.* 1996). This method allows us to measure the amount of aggregates in the sample, thus indirectly determine the content of native, monomeric protein. The more aggregated protein we find, the less monomers are present. Protein aggregates are characterized by a cross-beta spine quaternary structure (Nelson *et al.,* 2005).The ProteoStat® Protein aggregation assay kit contains a fluorescent dye that shows almost no fluorescence in the presence of the monomeric form of a protein. The fluorescence intensity raises 20~90 times upon binding to the cross-beta spine quaternary structure. The whole procedure was performed according to the manual of the manufacturer.

III. Protein samples were analyzed using NuPAGE gel electrophoresis. Bands in the gel were compared between the different refolding conditions; also the relative abundance of the protein of interest compared to the other bands loaded to the same well was an important parameter. This step eliminated possible false positive results gained in the previous two experiments. We could also see the proportion of the overexpressed protein among the other proteins.

Results gained in all the three previously described steps were constructively compared and the best refolding condition for each protein was chosen.

2.7. Protein purification

All proteins were purified in two steps of gel filtration and one of ion exchange to assure high purity and separation of monomeric and multimeric fractions. All these steps were done in the USA by my supervisor. The purified monomeric fraction was then LPS decontaminated by an external contractor (Arvys Protein).

2.8. Quantitative PCR

Quantitative PCR (q-PCR)is a method that we used to determine the transcript accumulation of a chosen gene in a sample. We used it for flagellin gene from *Borrelia* during the Saliva assisted transport experiment to estimate the number of spirochetes in our samples and for pro-inflammatory genes after the activation of the cells with various effectors to estimate a potential immune-modulatory function of the tick salivary proteins.

The q-PCR for flagellin was done in triplicates using the Rotor Gene device and software. The total reaction volume was 20µl, where the volume of H_2O was 3.8µl, mastermix 10µl, primers 1+1µl, probe 0.2µl and tested DNA 4µl. We used Fast Universal master Mix ROX, PCR water and primers and probe FLAGELLIN 69bp. The cycler settings were 95°C/10min, (95°C/10s, 60°C/30s) x 45 cycles. Water was used as a non-template control.

The q-PCR run for pro-inflammatory genes was done in duplicates and for two housekeeping genes – 18S and RPS29. The reaction mixture contained 2µl of 5x diluted cDNA, and 8µl mix of (207µl Mastermix, 2.1µl each primer, 120.1µl H_2O). We used primers for 6 genes involved in inflammation – TNF, IL-1β, CXCL2, CCL2, IFNγ and iNOS.PCR was done in 384-wells plates and run for 40 cycles.

2.9. Vaccination of guinea pigs with cysteine protease inhibitors A and B

This experiment followed the animal approval protocol 165/2011.

Male white guinea pigs (200-250grams of average weight) were used in this experiment. They were kept in the animal facility at the institute of Parasitology, Biology Centre ASCR. The vaccination antigen was injected intradermally at the side of a guinea pig. All bleedings were done from the great saphenous vein *(Vena saphena magna)* after shaving the tight. The serum was isolated from the blood after 10min centrifugation at 10 000xg.

Ticks were collected in the Branišovský forest near České Budějovice and kept in the tick facility at the institute.

Ticks that died during the experiment were excluded from further analysis.

The shortcut GP is used for a guinea pig.

All ELISA experiments were done in duplicates or triplicates according to the following protocol:

We coated 50µl/well of 0.5µg pure monomeric protein (EGFP, CystA, CystB) in a sterile coating buffer (0.158g Na_2CO_3, 0.292g $NaHCO_3$ in 100ml H_2O, pH 9.6) at 4°C overnight. After three washing steps with PBS/0.1% Tween we blocked the unspecific binding sites by 200µl/well of4% BSA in PBS at 37°C for1 hour. After three washing steps with PBS/0.1% Tween, we added 100µl/well of sera diluted in 4% BSA and 0.1% Tween in PBS and incubated at 37°C for 1 hour. After washing with PBS/Tween we added 100µl/well of goat anti-GP secondary antibodies diluted in 4% BSA and 0.1% Tween in PBS and incubated at 37°C for 1 hour. The wells were washed 5x with PBS/Tween, 100µl/well of OPD solution (5ml phospho-citrate buffer, 2mg OPD, 2µl 30% H_2O_2, pH 5.0) was added and the plate was kept in dark at room temperature for 10min. The colored reaction was stopped by 2M H_2SO_4, 100µl/well. Absorbance was measured at 490nm using ELx800 Fluorescence microplate reader (BioTek).

Procedure:

Guinea pigs were divided into 4 groups. Two control groups were injected either just with PBS or with an Enhanced green fluorescent protein (EGFP) as an unspecific protein control; the other two groups were vaccinated with cysteine protease inhibitors A and B.

Pre-immunization sera were collected before the first immunization from the great saphenous vein of non-anesthetized animals.

The animals were first immunized on 29th March 2013. All guinea pigs were injected at their left side with 500µl of protein in PBS and 500µl Complete Freund's Adjuvant (CFA). The protein concentration was the following:
GPs 1, 2, 3: No protein, only PBS – control group
GPs 4, 5, 6: 20µg of EGFP in PBS – control group
GPs 7-11: 100µg of CystA multimers in PBS
GPs 12-16: 100µg of CystB multimers in PBS

The sera were again collected one or two days before the second immunization.

The second immunization was done on 11th April, 2 weeks after the first one at the other side of a GP. The amount of proteins was the same. We used 500µl of the Incomplete Freund's Adjuvant (IFA) in this injection.
Some GPs developed skin wounds at the site of the first injection, probably as a reaction to the administered adjuvant. These wounds healed during the next four weeks at the latest.

The sera were collected also one or two days before the third immunization. The titer of antibodies was estimated by ELISA to see how many additional immunizations will be needed.

Guinea pigs were immunized on 26th April for the 3rd time in the same way as during the second immunization.

Sera were collected again 12 days after the 3rd immunization and the titer of antibodies was estimated by ELISA (labeled as After 3rd vaccination).

The GPs were exposed to ticks for the first time on 9th and 10th May (13-14 days after the last vaccination). 30 female ticks and 25 males were put onto a GP's shaved back and covered to avoid their escape. The not-attached ticks were removed after 4 hours. If there were more than 25 attached ticks they were removed too, to have the final number of females 25 per one GP.

The guinea pigs were controlled every day (starting from the day 5 after exposure) and all detached ticks were collected, weighed and placed into a glass tube. These ticks were then

kept in the tick facility at the institute at the default conditions of the institute colony for tick molting.

One week after the last tick fell off from the GPs we collected the sera again and estimated the titer of antibodies by ELISA (labeled as After 1st exposure).

Guinea pigs were exposed to ticks for the second time on 11th and 12th June (one month after the first exposure on ticks). We followed the same procedure like during the first exposure to ticks. The caps did not stay glued very well so we lost many ticks; especially during the first night. This effect was caused probably by not fully recovered skin or some inflammation at the site of gluing the cap of the tick.

All removed ticks were again kept in the tick facility at the default conditions of the institute colony for tick molting.

One week after the last tick fell off we collected the sera again and estimated the titer of antibodies by ELISA (labeled as After 2nd exposure).

The amount of eggs was evaluated by scoring the egg production of each individual tick one month after the end of feeding. We used values from zero to four where:
0 = no eggs
1 = small load
2 = normal load
3 = big load

The molting efficiency was evaluated by scoring the percentage of molted eggs three months after the end of feeding.

Both scorings were done by Jan Erhart (the manager of the tick colony in the institute of Parasitology) and they were blinded.

2.10. Saliva-assisted transmission of *Borrelia* (SAT)

This experiment was done according to the animal approval protocol 165/2011 and followed all safety rules needed for work with *Borrelia*.

All mice used in this experiment were purchased from Anlab.

The experiment was repeated in three cycles; fifty mice were in total used for it. All of them were C57BL/6 females 8-10 weeks old. They were divided into groups as following:

15 mice – PBS control group

10 mice – Ovalbumin control group (OVA)

10 mice – CystA group

15 mice – CystB group

Borrelia species and strain used in this experiment was *Borrelia burgdorferi*, strain B31

Mice were anesthetized by intraperitoneal injection of 80µl of a mixture of 8ml 5% Narkamon, 2ml 2% Rometar and 10ml PBS. The hair on their back was cut. Then a mixture of 5 000 *Borrelia* in 20 µl medium and 10 µg protein in 20 µl PBS was injected subcutaneously. After 7 days the mice were euthanized and the skin from their back was dissected. DNA was isolated from the tissue using NucleoSpin® Tissue kit. The isolated DNA was further analyzed by q-PCR.

2.11. *In vitro Borrelia* proliferation

Borrelia burgdorferi, strain B31 were used for the experiment. All reactions were done in 100µl to reduce the amount of used protein. We did two separate experiments, each in triplicates. We tested three groups:

- TBS as a no protein control
- Ovalbumin as an unspecific protein control
- CystB

Borrelia spirochetes were first grown in a 15ml BSK-H medium with 6% rabbit serum for five days to increase their number and vitality. Then they were counted under a microscope and 10 000 cells in 100 µl were used. Proteins were added to a final concentration of 18µM. The amount of bacteria was counted for four days and the 7^{th} day and then compared to the control (proliferation of spirochetes in the absence of any protein).

All following animal experiments were approved by the Landesdirektion Dresden, Germany.

2.12. Cells activation and analysis

The goal of these experiments was to see whether some of the tick salivary proteins affect the activation of macrophages, neutrophils and monocytes in vitro. Mice bone marrow isolated cells were activated using LPS (lipopolysaccharide) or PMA (Phorbol Myristate Acetate). LPS is a component of bacterial cell wall and PMA is a Protein Kinase C activator derived from the oil of the seeds of the Croton plant.

We used RNA isolation and cDNA reverse transcription techniques which are common for all the following experiments:

2.12.1. RNA isolation

Samples were thawed (if frozen before). 80µl chloroform was added, vortexed for 15s and left at RT for 2-3min.Then the samples were centrifuged (12 000xg, 15min, 4°C); there were three phases visible after the centrifugation. The aqueous phase (top) was carefully transferred into a new tube. 200µl isopropanol was added and the samples were incubated for 10min at RT. After another centrifugation (same conditions) the supernatant was removed and the tubes were dried on a paper towel. The RNA pellet was washed with 1ml EtOH and vortexed. After centrifugation (7500xg, 5min, 4°C) the supernatant was removed and the pellet was air dried for approximately3min.The dry pellet was redissolved in 18µl DEPC water and the RNA concentration was measured.

2.12.2. Reverse transcription of cDNA

Based on the concentration of RNA, the samples were diluted in water. The final volume was 15µl and the maximum amount of RNA 1µg. The final amount of RNA was calculated to be the same in all the samples. The RNA samples were mixed with 1µl iScript and 4µl iScript reaction mix (both Bio-Rad).The cycler settings were 5min 25°C, 30min 42°C and 5min 85°C with only one cycle; as described in the iScript manual.

2.12.3. Macrophages

Isolation of macrophages:

Two legs from a mouse were cut and all muscles from the bones were removed by scissors and a blade. Bones were kept in PBS on ice.

Both ends of each bone (femur and tibia) were cut and the bone marrow was flushed into a 50ml tube using a 30G needle and a syringe with a RPMI medium. The bone marrow was

resuspended and filtered by a cell strainer into another tube. After centrifugation (230xg, 5min, 4°C) the pellet was washed with 0.5ml Red Blood Cells (RBC) lysis solution. After 1min incubation at room temperature we added 20ml PBS and the sample was centrifuged again (230xg, 5min, 4°C).The pellet was re-suspended in 50ml RPMI medium with 10% Fetal bovine serum (FBS) and 1% PenStrep (antibiotics).The solution was then divided into two 12-wells panels, 2ml/well and incubated for 7 days at 37°C, 5% CO_2, 92% humidity.

Activation of macrophages:

After 7 days, the medium from the cells was removed and the wells were washed 2x with 1ml PBS. Macrophages remained attached to the bottom of the well. We added 1ml RPMI with 1% BSA and 1% PenStrep into each well and the cells were incubated for 2h at 37°C, 5% CO_2, 92% humidity. Proteins (CystB, SerB, IRS2, SialoL) were added in the cell medium to achieve a final protein concentration of 1µM. 8 wells in each plate were used for proteins (2 wells for each protein) and the remaining wells were used as a no-treatment control (negative control) and LPS which served as the positive control (2 wells each). The plates were incubated for another 1 hour. The first plate was activated by adding LPS. The final LPS concentration was 100ng/ml. All wells except for the no-treatment control were activated. The second plate was activated by adding PMA at final concentration of 50mg/ml. Both plates were incubated for 4h under the same conditions. The medium was removed after the incubation and the cells were washed from the walls by 400µl TRIZOL. The samples were then stored in the freezer at -20°C.

RNA was then isolated from the samples, reverse transcribed to cDNA and analyzed by q-PCR.

2.12.4. Neutrophils

Isolation of neutrophils:

Two legs from a mouse were cut and all muscles from the bones were removed by scissors and a blade. Bones were kept in PBS on ice. Both ends of each bone were cut and the bone marrow was flushed into a 50ml tube using a 30G needle and a syringe with RPMI medium. The bone marrow was resuspended and filtered by a cell strainer into another tube. After centrifugation (230xg, 5min, 4°C) the pellet was washed with 0.5ml RBC lysis solution. After 1min at RT 20ml PBS was added and the sample was centrifuged again (230xg, 5min, 4°C).The pellet was resuspended in 6ml PBS. Neutrophils were isolated in two tubes with a

Percoll gradient: 3ml 81%Percoll, 3ml 68%Percoll, 2ml 55%Percoll and 3ml PBS containing the cells. The tubes were centrifuged for 30min at 230xg with a slow start and finish in a swinging rotor. The interface between 81% and 68% of Percoll was collected by a pipette into a new tube. The cells were washed with 25ml PBS, followed by centrifugation (600xg, 5min, 4°C). The pellet was resuspended in 9.5ml RPMI medium with 1% Bovine serum albumin (BSA) and 1% PenStrep (antibiotics). The samples was then divided into a 12-wells plate; 750µl of cell suspension into each well.

Activation of neutrophils:
Proteins (CystB, SerB, IRS2 and SialoL) were added to the cell medium to achieve final concentration of 1µM before the neutrophil activation. Eight wells in each plate were used for proteins (2 wells each protein) and the remaining wells were used as a no-treatment control (negative control) and to be treated with LPS which served as the positive control (2 wells each). Plates were incubated for 1 hour at 37°C, 5% CO_2, 92% humidity.LPS was added to achieve the final LPS concentration of 100ng/ml. All wells (except of the no-treatment control) were activated with LPS. The plate was incubated for 4h under the same conditions. The medium was removed after the incubation and the cells were washed from the wells with 400µl TRIZOL. The samples were then stored in a freezer at -20°C.

RNA was then isolated from the samples, reverse transcribed to cDNA and analyzed by q-PCR.

2.12.5. Monocytes

Isolation of monocytes:
Two legs from a mouse were cut and all muscles from the bones were removed by scissors and blade. Bones were kept in PBS on ice. Both ends of each bone were cut and the bone marrow was flushed into a 50ml tube using a 30G needle and a syringe with RPMI medium. The bone marrow was resuspended and filtered by a cell strainer into another tube. After centrifugation (230xg, 5min, 4°C) the pellet was dissolved in 8ml PBS. Cells in PBS were carefully transferred onto 3ml HISTOPAQUE-1077 (density gradient) in a 15ml tube. The tube was centrifuged at 400xg, 30min at RT with a slow start and finish in a swinging bucket rotor. After the centrifugation the lowest 5mm from the upper layer were transferred into a new tube and washed 2x with 15ml PBS (230xg, 5min, 4°C).The pellet was resuspended in

9.5ml RPMI medium with 1% BSA and 1% PenStrep. The samples was then divided into a 12-wells plate; 750µl into each well.

Activation of monocytes:
Proteins (CystB, SerB, IRS2, SialoL) were added to achieve a final concentration of 1µM. Eight wells in each plate were used for proteins (2 wells each protein) and the remaining wells were used as a no-treatment control (negative control) and to be treated with LPS which served as the positive control (2 wells each). The plates were incubated for 1 hour at 37°C, 5% CO_2, 92% humidity. After that, LPS was added to achieve the final concentration 100ng/ml. All wells except for the no-treatment control were activated. The plate was incubated for 4h under the same conditions. All medium was removed after the incubation and the cells were washed from the walls by 400µl TRIZOL. Samples were then stored in a freezer at -20°C.

RNA was then isolated from the samples, reverse transcribed to cDNA and analyzed by q-PCR.

2.13. LPS-induced lung inflammation

Introducing LPS to mice lungs causes a non-pathogenic inflammation. In this experiment we try to test the potential effect of tick salivary proteins in this animal model of inflammation.

Twenty8-10 weeks old mice females (C57BL/6) were divided into four groups; five mice in each group as following:

- Injected with PBS – control
- Injected with SialoL
- Injected with CystB
- Injected with SerB

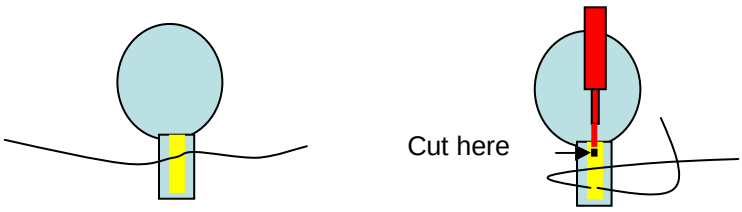
Mice were injected with proteins (or PBS) intraperitoneally (1µg of protein per 1g of a mouse).

The next day the mice were anesthetized for approximately15s in a jar with a paper towel soaked in 1ml of a mixture (1:1) of halothane and mineral oil. A mouse was laid on its back and 50µl of protein solution was dropped into a tip of its nose (while mouse is breathing). The protein concentration in the solution was 1µg per 1g of a mouse. Mice were put back in a

cage. One hour later 50µl of LPS solution (4µg/50µl) was introduced to the mice in the same way.

Mice were euthanized 24h after the LPS exposure. Each mouse was pinned to a polystyrene pad and its skin at the belly was cut from the legs to its head. Skin was then separated from the peritoneum. Peritoneum was opened and the diaphragm was cut from the ribs. Mouse salivary glands and muscles were removed from the trachea by forceps. A small hole was made in the trachea and PBS was injected by a needle (tip capped with tubing) with a syringe. The walls of the trachea were fixed to the needle inside using a thread Suture 2/0 as shown in the figure.

Figure 4: *Schematic representation of the needle injection into the trachea. Mouse head and neck are light blue, trachea is yellow and the needle is red. After the needle is injected into the hole in the trachea, it is fixed by the thread.*



Then 1ml of cold PBS was injected into the mouse lungs through the trachea. 500-600µl of cell suspension was collected into a tube and this lavage was repeated with another 1ml of PBS. The samples were centrifuged (5min, 500xg, 4°C). The supernatant can be used for protein detection and it was stored at -20°C. The pellet was re-suspended in 150µl RBC lysis solution. After 2min, 1.5ml FACS buffer (PBS (- Ca^{2+}, - Mg^{2+}, Gibco), 0.1% BSA, 0,1% Na_3N) was added to the solution. After centrifugation (5min, 500xg, 4°C) the pellet was resuspended in 250µl FACS buffer.

The cells were counted after trypan blue staining under a microscope.

The FACS analysis was done according to the following steps:

First 100µl of cells re-suspended in FACS buffer was mixed with 10µl Fc block solution (100x) and 10µl Ly-6G antibodies solution (20x) in FACS tubes. The mixture was incubated for 45min at 4°C in the darkness. After the incubation, 1 ml of FACS buffer was added to the solution to dilute Fc block and the antibodies. The samples were centrifuged (5min, 500xg,

4°C) and the supernatant was removed. The pellet was re-suspended in 500µl FACS buffer and the samples were analyzed by a flow cytometer BD FACS Canto II (BD). 10 000 events were measured for each sample. The data were analyzed by BD FACS Diva Software.
The total amount of neutrophils was then calculated as well as their percentage among the other cells.

2.14. Thioglycolate induced peritonitis

Introducing a thioglycolate medium to mice peritoneum causes a non-pathogenic inflammation.
In this experiment we try to test the potential effect of tick salivary proteins in this animal model of inflammation.

Thioglycolate medium (TGM) was prepared 2 weeks before use to enable enough time for its oxidation. Three grams of Thioglycolate broth were dissolved in 100ml H_2O, autoclaved and kept in dark with a slightly opened lid.
Twenty mice females (C57BL/6, 8-10 weeks old) were divided into 4 groups; 5 mice in each group:

- Injected with PBS – control
- Injected with SialoL
- Injected with CystB
- Injected with SerB

Mice were injected intraperitoneally (i.p.) with 200µl PBS or a protein in PBS (2µg protein / 1g mouse)
After 1h every mouse was injected i.p. with 1ml TGM. TGM induces inflammation and neutrophil infiltration.
Peritoneal lavage was done 4h later. The mouse was pinned to a polystyrene pad and its skin at the belly was cut from the legs to its head. The skin was then separated from the peritoneum. Two syringes with 6ml PBS and 24G needles were used. First, one of the syringes was injected into the peritoneum wall and the peritoneum was washed with PBS. Then the liquid was sucked back into the syringe. PBS from the other syringe was used to wash peritoneum further, but all the liquid was sucked into the first syringe. The yield was approximately 10ml.The samples were centrifuged (500xg, 5min, 4°C) and the supernatant was removed. The pellet was re-suspended in 500µl RBC lysis buffer and kept for 2-3min on ice. The RBC lysis solution was diluted with10ml PBS and the samples were centrifuged

(500xg, 5min, 4°C).The pellet was washed with 10ml FACS buffer and re-suspended in 0.5ml FACS buffer.

The cells were counted after staining with trypan blue under a microscope.

The FACS analysis was done in the following steps:

First 100µl of cells re-suspended in FACS buffer (PBS (- Ca^{2+}, - Mg^{2+}, Gibco), 0,1% BSA, 0,1% Na_3N) was mixed with 10µl Fc block solution (100x) and 10µl of each antibodies solution (F4/80, Cd11b, Ly-6G, Ly-6C; all 20x) in FACS tubes. The mixture was incubated for 45min at 4°C in the darkness. After the incubation, 1 ml of FACS buffer was added to the solution to dilute Fc block and the antibodies. The samples were centrifuged (5min, 500xg, 4°C) and the supernatant was removed. The pellet was re-suspended in 500µl FACS buffer and the samples were analyzed by a flow cytometer BD FACS Canto II (BD). 10 000 events were measured for each sample. The data were analyzed by BD FACS Diva Software.

The total amount of neutrophils, macrophages and monocytes was then calculated as well as their percentage among other cells.

3. Results

Error bars in all graphs represent the SEM (Standard error of the mean).

All data were statistically processed by *ANOVA* and statistical significance was considered when $p < 0.05$ after comparing the control and tested groups and marked with *. Statistical significance when $p < 0.01$ is marked with **.

3.1. Pilot expression and refolding optimization

3.1.1. Cysteine protease inhibitor A

All steps of CystA synthesis are shown here. This cysteine protease inhibitor was overexpessed in the *E.coli;* strain BL21-pLysS and it has a molecular weight approximately 14kDa.

3.1.1.1. Pilot expression

We can see in figure 5 that CystA is overexpressed already 1h after gene overexpression induction. This is a gel of the pellets of the bacterial cells after their breakage with freeze thawing and sonication which means that the protein was present within the inclusion bodies. The gel of the corresponding supernatants (soluble fraction) of the broken cells did not show any bands at the appropriate molecular weight (overexpressed protein) and it is not shown here. The best overexpression level of the protein is after 6 hours of gene overexpression induction where the 14kDa band is the strongest (marked with an arrow). The band at 40kDa (marked with an asterix) can be used as a loading control – the 40kDa band is the same in all the different samples which indicates that the experiment was carried out well in the sense that there was no loading effect during the gel run.

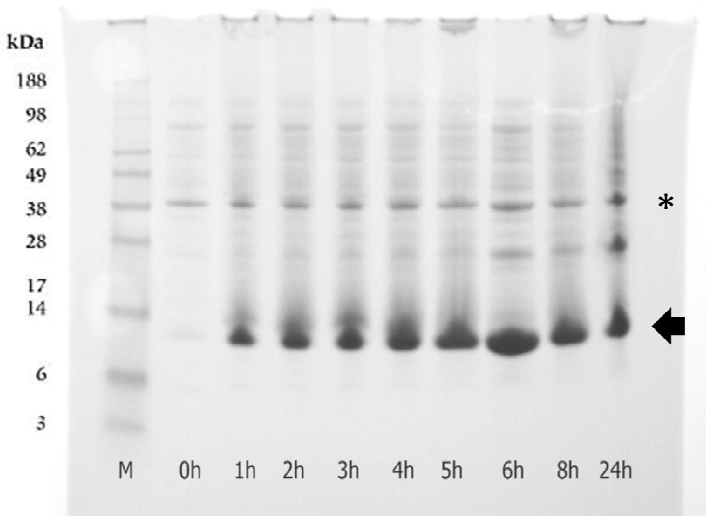


***Figure 5**: Pilot expression of CystA. The molecular weight marker is labeled as M; 0h-24h represent the time points after induction of overexpression by IPTG. Over-expressed CystA is marked by an arrow, the loading control band with an asterix.*

3.1.1.2. Isolation of protein from the bacterial inclusion bodies

The presence of protein in the inclusion bodies can be proven again in figure 6 where the inclusion body preparation in large scale is shown on a coomassie stained NuPAGE gel. There is a strong band marked with an arrow at the appropriate molecular weight in the inclusion bodies while nothing in the other two fractions.

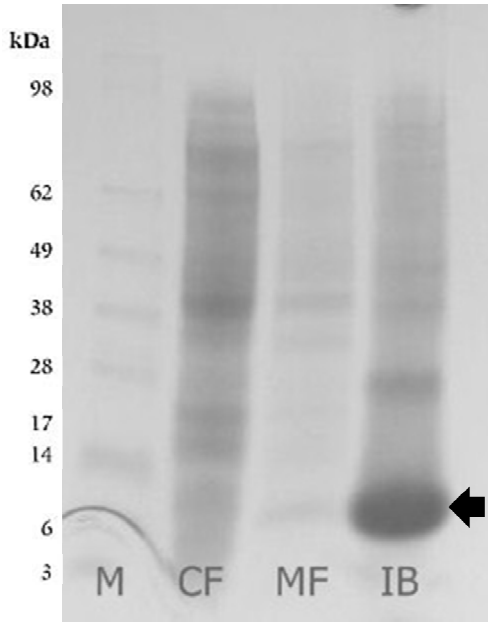


Figure 6: *Fractions after isolation of CystA from bacteria. M = marker, CF = cytosolic fraction (disrupting cells in Tris), MF = membrane fraction (disrupting cells in Triton), IB = inclusion bodies. CystA band is marked with an arrow.*

3.1.1.3. Pilot refolding

Refolding in buffers 1-8 (please see Materials and Methods) was evaluated just by comparing the size of the resulting pellets of aggregated protein post refolding in the various buffers; buffer No.2 was chosen as the best condition (20mM Tris, 300mM NaCl, pH 8.5).

3.1.1.4. Optimization of pH upon protein refolding

The next step was the optimization of pH in the refolding buffer. We show in figure 7 a picture of a gel with the refolded samples in various pH (8.0, 8.5, 9.0, 10.0) after concentration using first a 30kDa cutoff filter device and then a 3kDa cutoff. The 30kDa cutoff filter device was supposed to remove the high molecular weight impurities and aggregates while the 3kDa filter concentrated monomeric CystA. Based on the final volume of each sample, the more concentrated samples were diluted to achieve the same starting/final volume ratio in all samples. This step made the comparison of protein bands in the gel not affected by concentrating each sample to a different final volume. The samples preparation was done according to the Materials and Methods section and 40μl of each sample was loaded

to the gel. The fraction collected by 30kDa cutoff probably contained protein aggregates which were denatured during NuPAGE sample preparation and can be seen at 14kDa. We found out that pH 10.0 is the best pH condition because there is a strong band in the 3kDa fraction marked by an arrow. We also discovered that the pre-fractionation of the proteins using a high MW (30 kDa) cutoff does not work well. There is still a lot of protein trapped in the supernatant fraction of the 30 kDa filter.

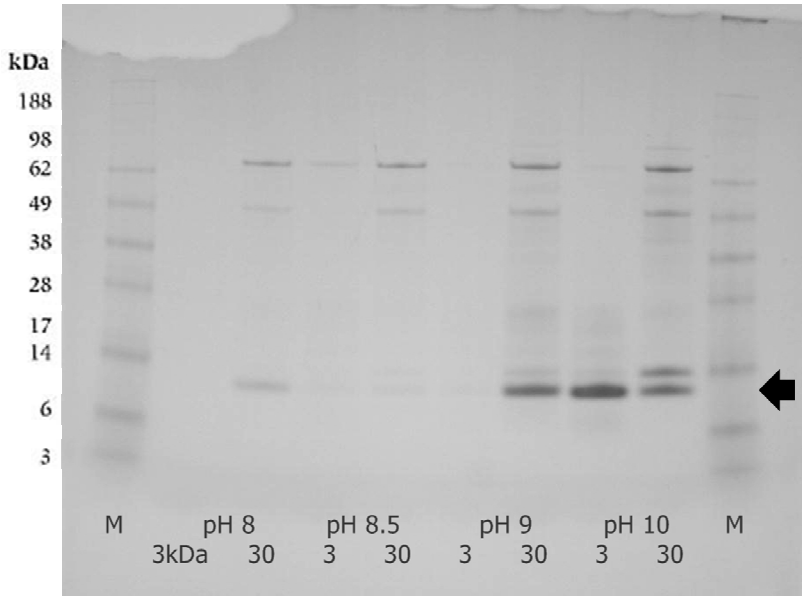


Figure 7: *Optimization of pH in the refolding buffer for CystA.*
M = marker, pH values are always valid for two samples above, 3 and 30kDa label the cutoff used for the fractionation of all the samples. There is always a 3kDa fraction first followed by a 30kDa fraction for each sample. The arrow shows the bands of CystA at the correct molecular weight.

3.1.1.5. First optimization of inclusion bodies concentration

Refolding of CystA at different concentrations of inclusion bodies gives us the information about the concentration which gives the better outcome of refolded protein since the success of the refolding methodology we followed is known as depending on the protein concentration of the protein to be refolded. We run all the samples on a gel in the same way as described in chapter 2.1.1.4, and the concentration of 1mg/ml was chosen as the best. The reason for this is the best ratio between bands in the 30 and 3kDa fractions (marked by an arrow in Fig. 8). The bands in 3kDa fractions are visible only for samples with IB concentrations 1 or 2mg/ml. This means that the amount of refolded protein increases with increasing the initial IB concentration. On the other hand the 30kDa band at 2mg/ml is a lot stronger than the 30kDa band at 1mg/ml. This means that the increased IB amount resulted increased amount of protein aggregates which got trapped by the 30kDa filter resulting in the stronger band of 14kDa in the 30kDa sample. Since we want to avoid aggregates, the concentration 1mg/ml was chosen.

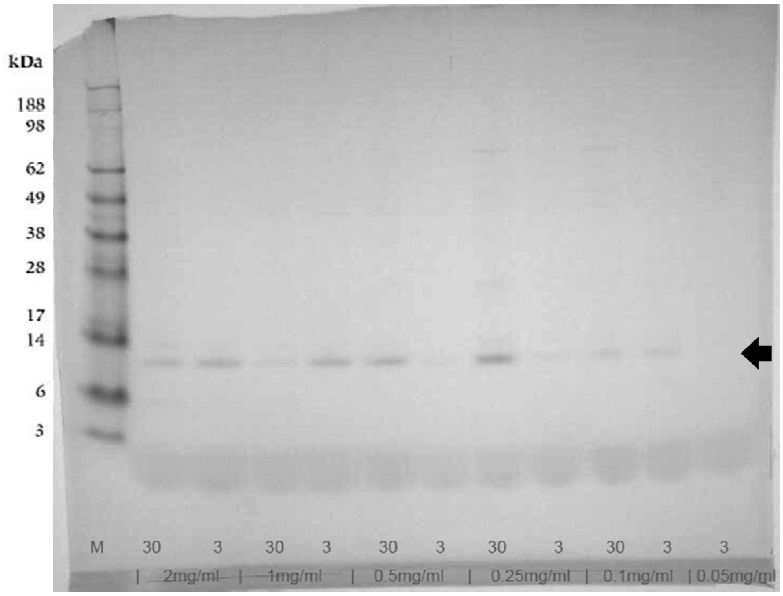


Figure 8: *Optimization of the ideal IB concentration in the refolding buffer. M = marker, the concentration values are always valid for two samples above, 3 and 30 label the cutoff in kDa used to get these samples. There is always a 30kDa fraction first followed by a 3kDa fraction for each sample. Bands of CystA are marked by an arrow.*

3.1.1.6. Optimization of pH in a narrow pH range

It was clear from the previous optimization that pH around 10 is ideal for CystA refolding. At such an alkaline environment, we wanted to find this pH precisely. Increasing pH too much could lead to damaging the protein. The folding kinetics is strongly pH dependent and we wanted to increase the refolding efficiency as much as possible by finding the best pH value. In figure 9a we can see the protein refolding efficiency in four different pH values (9.5, 9.7, 10.0, 10.2). The gel was run in the same way as described in chapter 2.1.1.4. The best was obviously pH 10.0 with the strongest band in the 3kDa fraction (arrow).

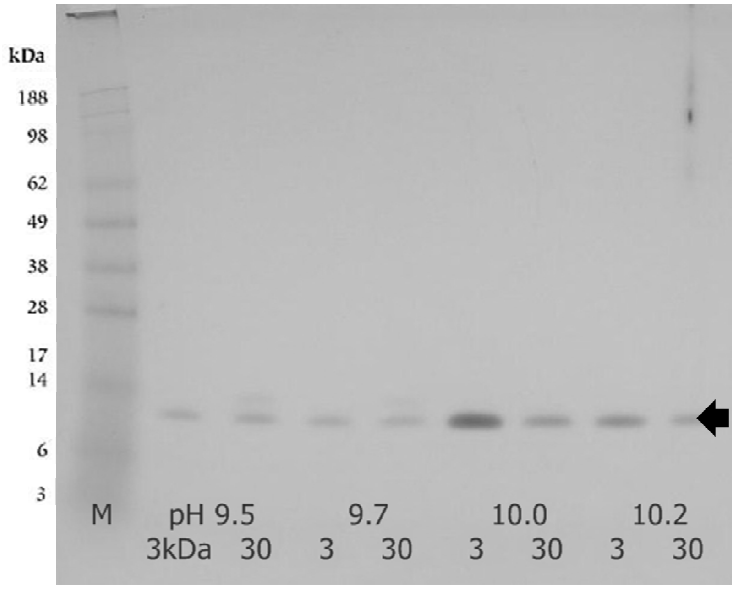


Figure 9a: *pH optimization in the refolding buffer in a narrow pH range. M = marker, pH values are always valid for two samples above, 3 and 30kDa label the cutoff used to get these samples. There is always a 3kDa fraction first followed by a 30kDa fraction for each sample. CystA bands are marked by an arrow.*

3.1.1.7. Purification of CystA

After refolding of CystA in the best buffer (20mM CAPS, 300mM NaCl, pH 10.0; IB concentration 1mg/ml) and its concentration, it was purified with a yield of 5mg of pure protein. Figure 9b shows a chromatogram from the purification. We can see that CystA was eluted from the column after passing 16-19ml of 50mM Tris pH 8, 150mM NaCl (TBS). We found out during the purification process that the fraction captured by the 30kDa cutoff filter contains not only protein aggregates but also much monomeric CystA. Therefore only 3kDa cutoff filter device was used in the next applications.

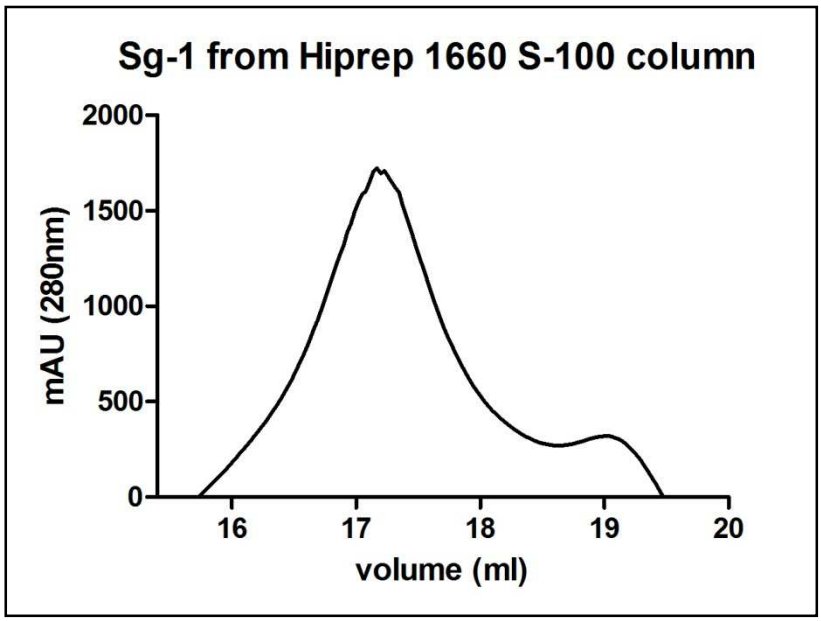


Figure 9b: *Chromatogram from the CystA purification. The amount of protein is shown as the absorbance at 280nm.*

3.1.1.8. Optimization of the chaotropic agents concentration

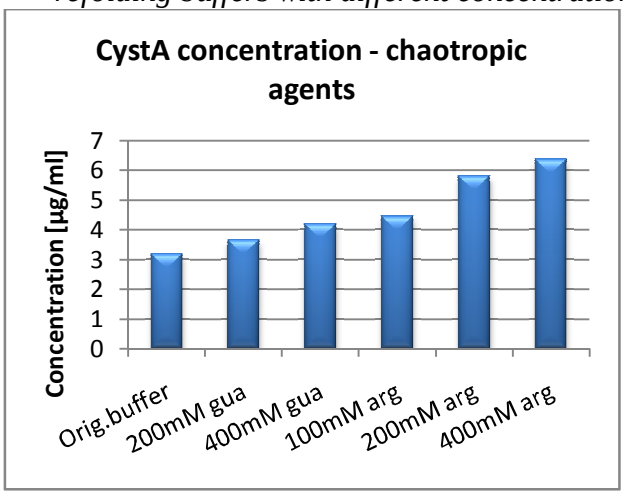
After getting such a poor yield of protein (we target for more than 10 mgrs of protein) we started with the optimizations almost from the beginning.

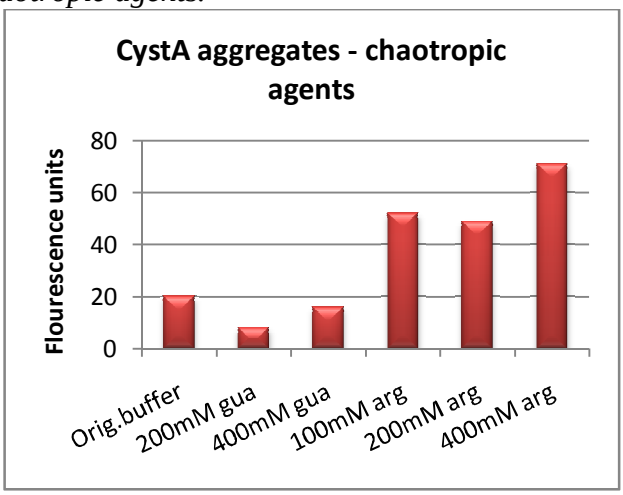
The effect of chaotropic agents such as arginine (100, 200, 400mM) and guanidine (200, 400mM) was tested and compared to the buffer which was previously found as the best.

After centrifugation the precipitates, samples were concentrated using a 3kDa cutoff filter device to 500-1000µl and the buffer was exchanged for TBS. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The graph of refolded protein concentration in the refolding buffer (Fig. 10) shows that both guanidine and arginine increased the amount of refolded protein in comparison to the original buffer. The strongest effect was caused by arginine at concentrations 200 and 400mM.

The other graph (Fig. 11) shows the amount of protein aggregates upon refolding. According to the instructions of the kit we used, the amount of aggregates is directly proportional to measured fluorescence units. The original buffer and buffers with guanidine have significantly less aggregates than the samples with arginine. The higher amount of protein aggregates can partially be caused by the higher protein concentration in the samples that arginine was used in the refolding buffer.

Figures 10 (left), 11 (right): *Concentration of CystA and the amount of CystA aggregates in refolding buffers with different concentration of chaotropic agents.*





The gel (run in the same way as described in chapter 2.1.1.4.) with all six samples confirms the measurement of the concentration. We can see that the 14kDa band is getting stronger with the increasing amount of arginine (marked with an arrow). The bands at 65 and 50kDa (marked with *, #) are also getting stronger which may indicate either using different inclusion bodies concentrations by chance or (more likely) that refolding of these proteins was also enhanced.

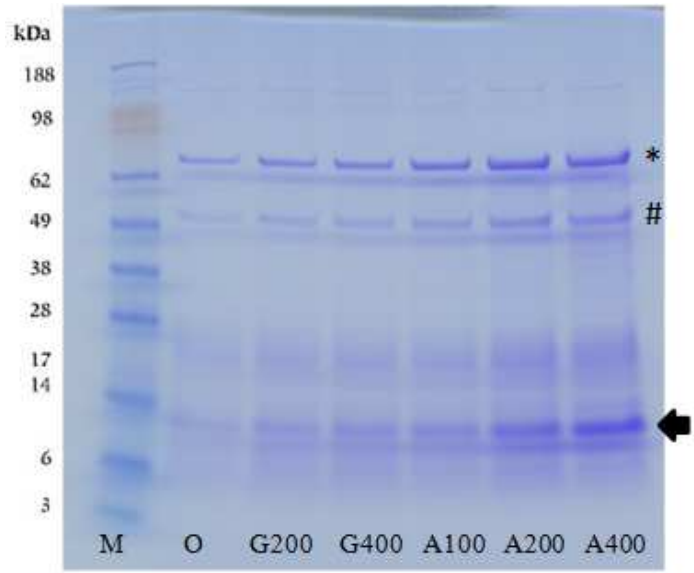


Figure 12: *Samples in refolding buffers with different concentration of chaotropic agents. M = marker; O = original buffer; G200 and G400 = original buffer with 200 or 400mM guanidine; A100, A200 and A400 = original buffer with 100, 200 or 400mM arginine.*
** marks a 65kDa band, # marks a 50kDa band and an arrow a 14kDa band.*

Arginine concentration 300mM was estimated as the best by comparing the gel bands (Fig. 12), the refolded protein concentration (Fig. 10) and the aggregates results (Fig. 11). Overall our conclusion is that 300mM of Arginine provides the highest yield of CystA as well as a little lower amount of aggregates than in the case of 400mM arginine.

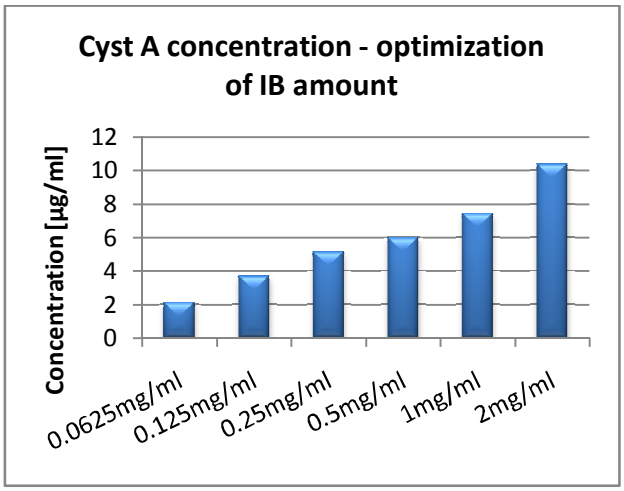
3.1.1.9. Second optimization of inclusion bodies concentration

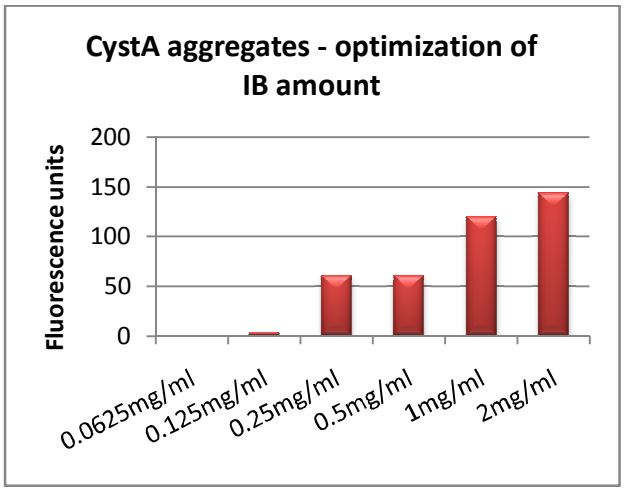
Refolding of CystA at different concentrations of inclusion bodies was repeated since the refolding buffer composition is now changed (20mM CAPS, 300mM NaCl, 300mM L-arginine, pH 10.0). Figure 13 shows the concentration of CystA in the refolding buffer. After centrifugation the precipitates, samples were concentrated using a 3kDa cutoff filter device to 500-1000µl and the buffer was exchanged for TBS. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. Protein concentration in the refolding buffer increases with increasing initial inclusion bodies concentration. The initial IB concentration starts at 0.0625mg/ml and increases geometrically in the following samples by the factor of two (x-axis in the graphs). The increase in the refolded protein concentration is slower which suggest forming more aggregates or incorrectly folded intermediates.

The amount of aggregates in the refolding was also measured and is shown in figure 14. According to the instructions of the kit we used, the amount of aggregates is directly proportional to measured fluorescence units.

The samples with the two lowest IB concentrations are almost aggregate free. Then the amount of aggregates rises with increasing IB concentration.

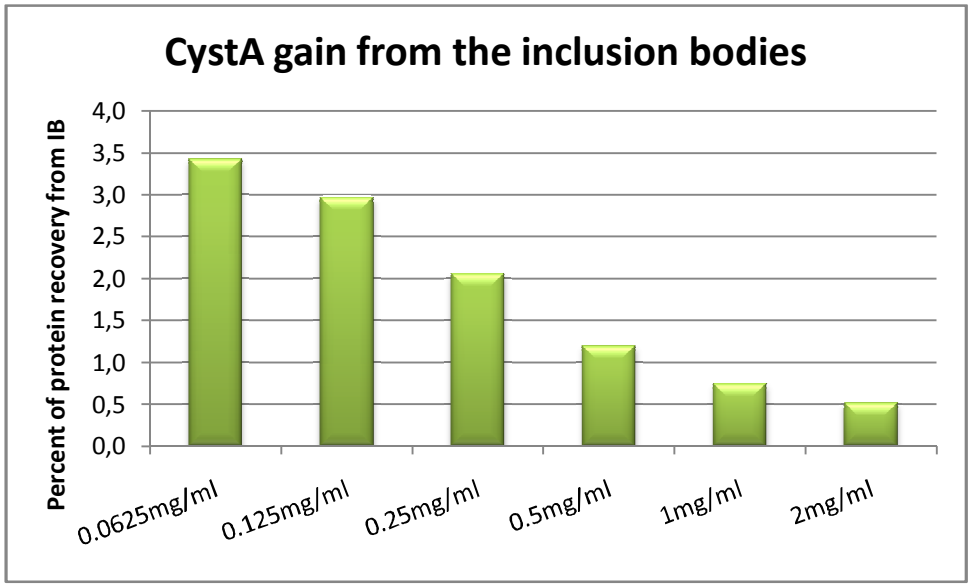
Figures 13 (left), 14 (right): *Concentration of CystA and the amount of CystA aggregates in refolding buffers with different concentration of inclusion bodies. The initial IB concentration is at the x-axis.*





The refolded protein concentration in the refolding buffer was strongly affected by the amount of IB used in each sample (Fig. 13). Next we calculated the yield of the refolded protein from the inclusion bodies as the percentage of the weight of the inclusion bodies used that resulted in refolded protein. Figure 15 shows that the protein refolding efficiency is the highest at low IB concentrations and decreases from 3.5% (0.0625mg/ml) to less than 1% (1 and 2mg/ml).

Figure 15: *The yield of CystA in % from the inclusion bodies in refolding buffers with their different concentration. The initial IB concentration is at the x-axis.*



The gel (run in the same way as described in chapter 2.1.1.4.) shows again the same result as the concentration measurement in Figure 13. Samples with higher initial IB concentration

have stronger bands of CystA (arrow). We can see that the band of CystA does not faint so rapidly as two high molecular weight bands (marked with * and #). This suggests that these bands are protein multimers formed upon precipitation at high protein concentration.

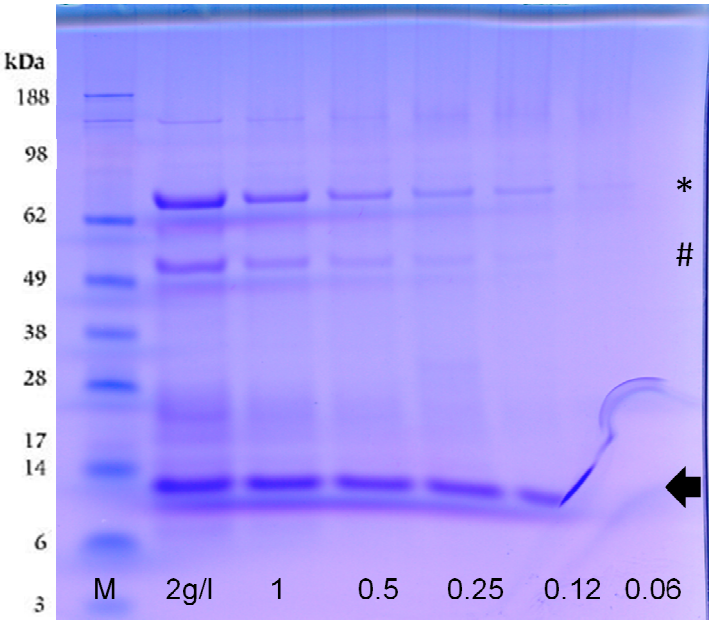


Figure 16: *Samples in the refolding buffer with different concentration of inclusion bodies. M = marker; values below the lines show the inclusion bodies concentration in grams per 1l of refolding buffer. The * and # mark high MW bands and an arrow a 14kDa band.*

After evaluating and comparing all the data, the concentration 0.125mg/ml was chosen as the best for refolding the IB in high scale. It gives a good yield in % from the inclusion bodies, has almost no aggregates and still looks good in the gel.

3.1.1.10. Optimization of NaCl concentration

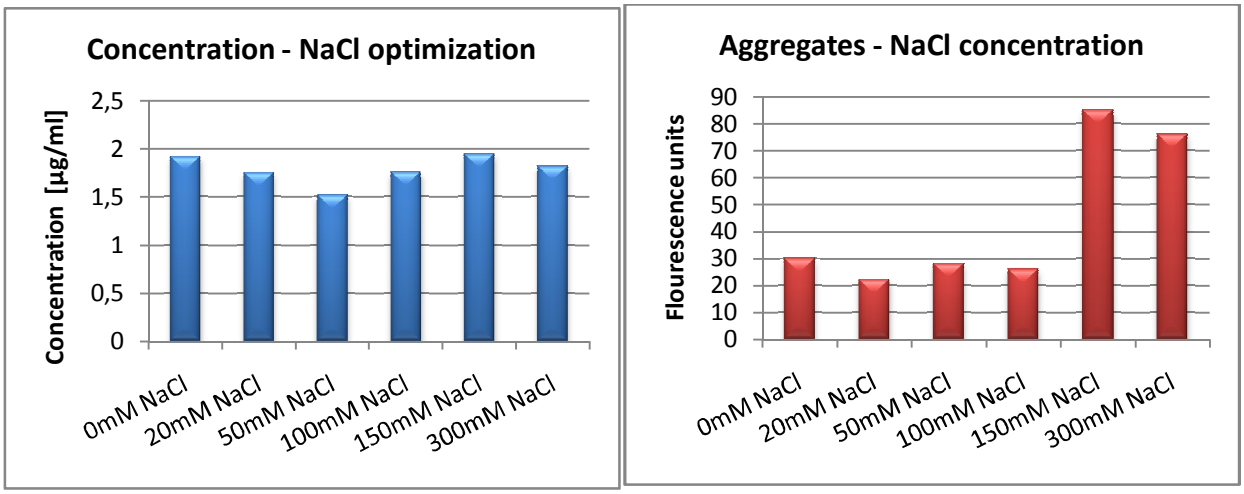
CystA was further refolded in the best buffer up to date (20mM CAPS, 300mM L-arginine, pH 10.0) with various NaCl concentration (0, 20, 50, 100, 150 and 300mM) at IB concentration 0.125mg/ml.

Figure 17 shows the concentration of CystA in the refolding buffer. After centrifugation the precipitates, samples were concentrated using a 3kDa cutoff filter device to 500-1000µl and the buffer was exchanged for TBS. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The concentration of CystA in the refolding buffer after refolding was approximately the same in all six concentrations of NaCl. It reaches values between 1.5 and 2µg/ml.

The measurement of the amount of aggregates (Fig. 18) gives us a better idea about the ideal condition as far as it concerns the NaCl concentration. The values of fluorescence units in the

samples with NaCl concentration from 0 to 100mM are the same – between 23 and 30 units. Fluorescence in samples with NaCl at concentrations 150 and 300mM reaches 80 units which means more aggregates and thus these conditions are not favorable.

Figures 17 (left), 18 (right): *Concentration of CystA and the amount of aggregates in refolding buffers with a different concentration of NaCl. NaCl concentration is given at the x-axis.*



The gel was run in the same way as described in chapter 2.1.1.4. and we can see that all the CystA bands in the gel are weak (Fig 19; arrow). The band at IB concentration 0.125mg/ml was much stronger in the previous figure (16) than here. Since the same IB concentration was used, we can infer that the piece of IB used for this optimization was composed of more water and less protein.

It seems that the strongest band is at 100mM NaCl. This observation complies with the aggregates measurement which shows very little aggregation at 100mM NaCl and therefore this condition was chosen as the best condition for big scale refolding.

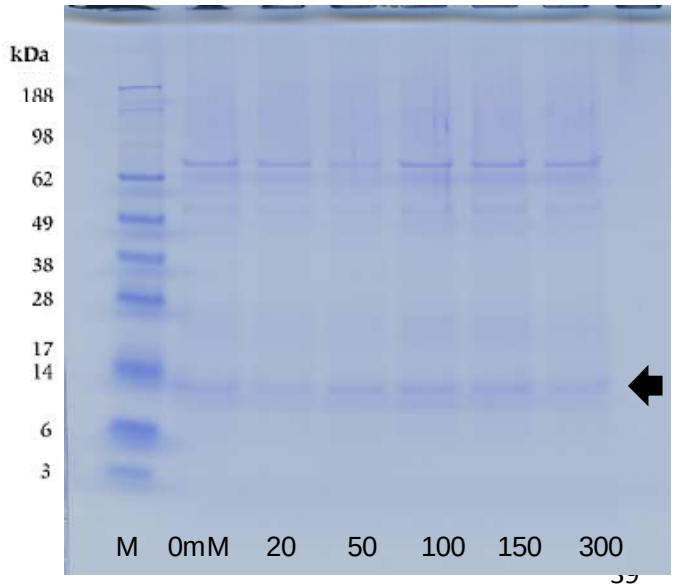


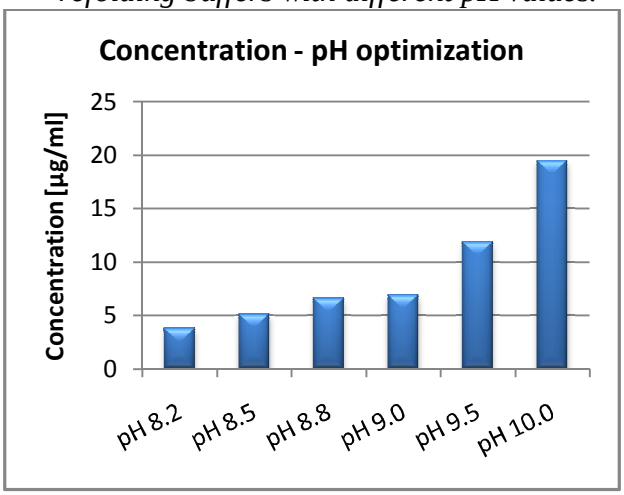
Figure 19: *CystA in the refolding buffers with different concentration of NaCl. M = marker; values below the lines show the concentration of NaCl in mM. CystA band is marked by an arrow.*

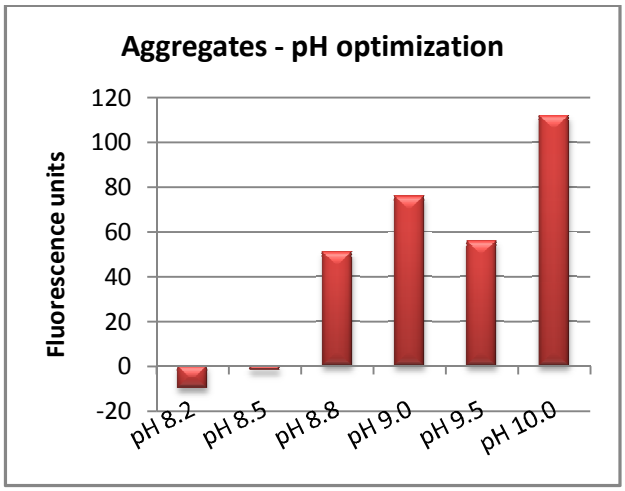
3.1.1.11. Second pH optimization

The pH optimization was also repeated for the new refolding conditions (20mM CAPS, 100mM NaCl, 300mM L-arginine; at IB concentration 0.125mg/ml). The values of pH ranged from 8.2 to 10.0. We can see in the gel (Fig. 22) and in the graph of protein concentration in the refolding buffer (Fig. 20) that pH 10 gives the highest CystA yield followed by pH 9.5. The aggregates measurement (Fig. 21) shows that pH 9.5 seems to be a better option than pH 10.0 because of a lower amount of aggregated protein.

This was one of the reasons to choose pH 9.5 as an ideal condition for our next refolding attempt for Cyst A. The other reason was that the protein might be damaged more at high pH like 10 by the effect of OH- ions, consequent H+ loss from proteins, changing the charge of amino acids and possibly also the protein conformation.

Figures 20 (left), 21(right): *Concentration of CystA and the amount of CystA aggregates in refolding buffers with different pH values.*





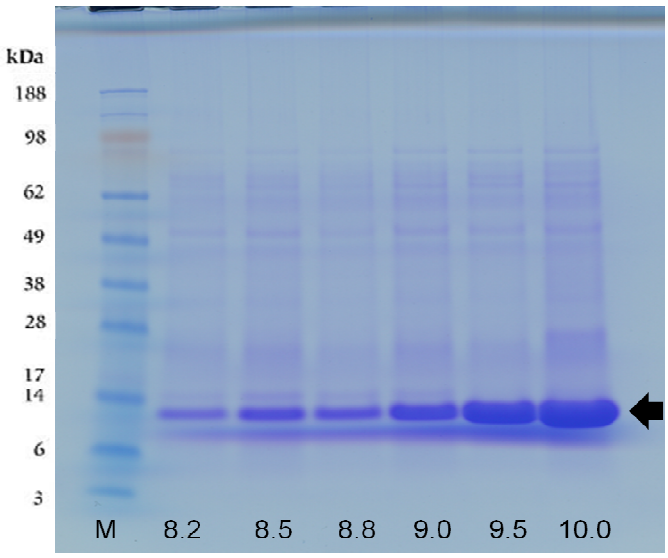


Figure 22: *Samples in the refolding buffers with different pH.*
M = marker; values below the lines show pH of the refolding buffer., CystA band is marked by an arrow.

After comparing all data from all the different optimization steps, the ideal buffer composition was found to be: 20mM CAPS, 300mM L-Arginine and 100mM NaCl, pH 9.5; with 0.125g of IB per 1 liter of refolding buffer.

3.1.2. Cysteine protease inhibitor B

All steps of CystB synthesis are shown here. This cysteine protease inhibitor was overexpessed in *E.coli*; strain BL21-pLysS and it has a molecular weight of approximately 14kDa.

3.1.2.1. Pilot expression

Figure 23 shows a gel of the pellets of the bacterial cells after their breakage with freeze thawing and sonication which means that the protein was present within the inclusion bodies. The gel of the corresponding supernatants (soluble fraction) of the broken cells did not show any bands at the appropriate molecular weight (overexpressed protein) and it is not shown here. The protein is overexpressed well already 2 hours after gene overexpression induction and its amount does not change with an extended incubation time. Therefore the time point of 2 hours after IPTG induction was chosen.

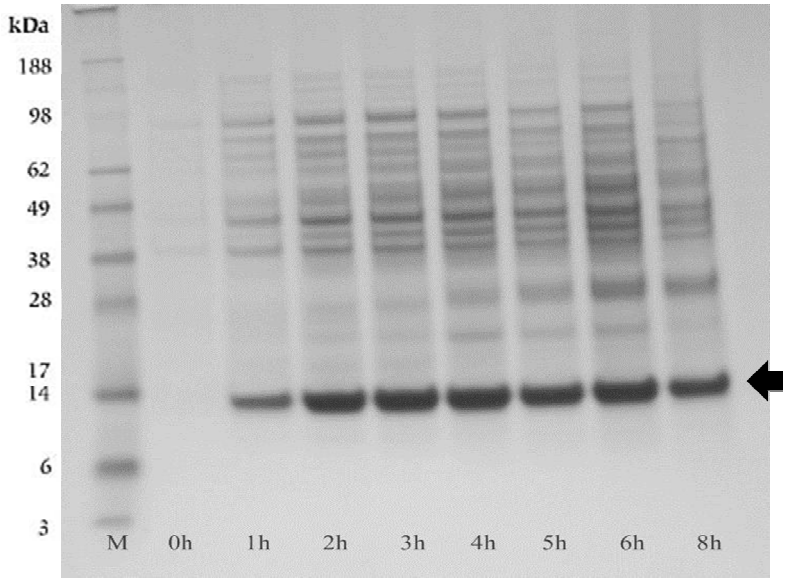


Figure 23: *Pilot expression of CystB. The molecular weight marker is labeled as M; 0h-8h represent the time points after induction of overexpression by IPTG. The CystB band is marked by an arrow.*

3.1.2.2. Isolation of protein from bacteria

The presence of protein in the inclusion bodies can be proven again in figure 24 where the inclusion body preparation in large scale is shown on a coomassie stained NuPAGE gel. Although the picture was taken at a bad quality and it is not very sharp we can see a strong band at the appropriate molecular weight in the inclusion bodies while nothing in the other two fractions. The CystB band is marked by an arrow in the figure.

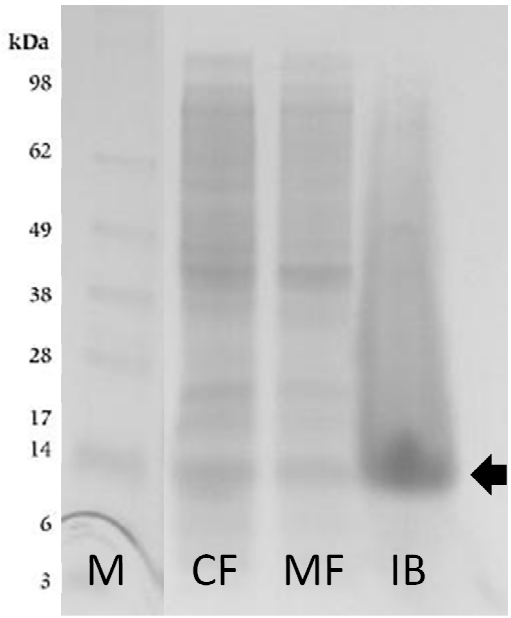


Figure 24: *Fractions after isolation of CystB from bacteria. M = marker, CF = cytosolic fraction (disrupting cells in Tris), MF = membrane fraction (disrupting cells in triton), IB = inclusion bodies. The CystB band is marked by an arrow. The picture of the protein marker is the same as in Figure 6 because the samples were run on the same gel.*

3.1.2.3. Pilot refolding

Refolding in buffers 1-8 (please see Materials and methods) was evaluated just by comparing the resulting pellets of aggregated protein post refolding in the various buffers before and also after high speed centrifugation; No.2 and No.4 were chosen as the best starting refolding conditions.

(No.2: 20mM Tris, 300mM NaCl, pH 8.5)

(No.4: 20mM Tris, 300mM NaCl, pH 8.0)

3.1.2.4. Optimization of pH upon protein refolding

The only difference between the best two buffers was their pH and therefore, the next step was the pH optimization of the refolding buffer. The tested pH values were 7.4, 8.0, 8.5 and 8.8. We show a picture of a gel (run in the same way as described in chapter 2.1.1.4.) with tested samples after concentration through 30 and 3kDa cutoff filter devices. The 30kDa filter

was supposed to remove the high molecular weight impurities and aggregates while the 3kDa filter concentrated monomeric CystB. We found out that pH 8.5 is the best condition. There were no high MW impurities in the 30kDa fraction and a big band at the 3kDa fraction (marked by an arrow in Fig. 25). Other samples also show a strong band in the 3kDa fraction but all of them contain impurities trapped in the 30kDa fraction.

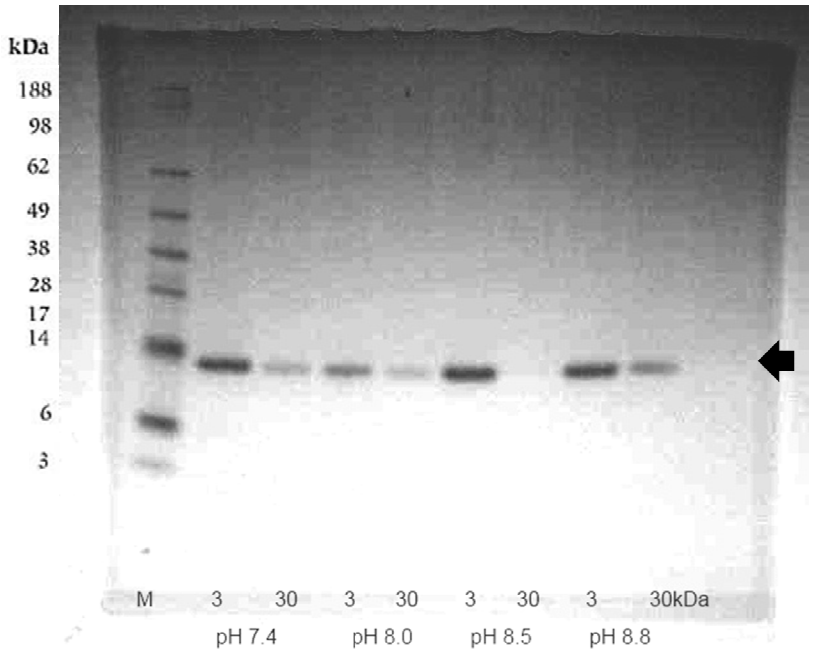


Figure 25: *Optimization of pH in the refolding buffer. M = marker, pH values are always valid for two samples above, 3 and 30kDa label the cutoff used to get these samples. There is always a 3kDa fraction first followed by a 30kDa fraction for each sample. The CystB band is marked by an arrow.*

3.1.2.5. Optimization of inclusion bodies concentration

The last optimization step of CystB refolding was to estimate the best concentration of inclusion bodies in the refolding buffer to avoid protein precipitation on one side and excessive refolding buffer volume at the other side. We ran all samples at IB concentration 2, 1, 0.5 and 0.25mg/ml in a gel (in the same way as described in chapter 2.1.1.4.) and the concentration of 2mg/ml was chosen as the best. The reason for this is a strong band in the 3kDa fraction while a relatively small band of protein aggregates in the 30kDa fraction (marked by an arrow in Fig. 26).

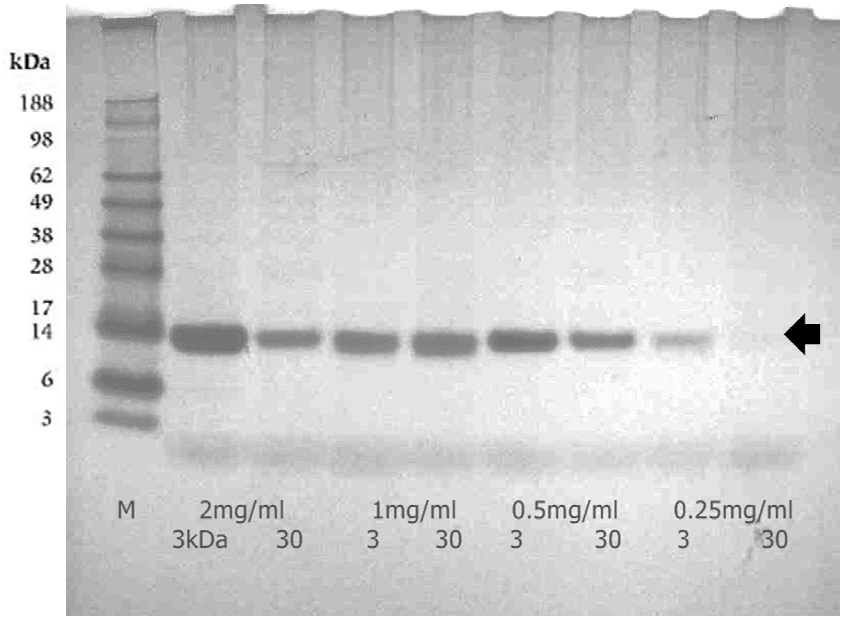


Figure 26: *Optimization of the ideal IB concentration in the refolding buffer.*
M = marker, the concentration values are always valid for two samples above, 3 and 30 label the cutoff in kDa used to get these samples. There is always a 3kDa fraction first, followed by a 30kDa fraction for each sample. The CystB band is marked by an arrow.

We found out that CystB is refolded quite easily. The 14kDa CystB band was strong during all refolding optimization steps. No further optimization was needed and the big scale refolding was carried out in 20mM Tris, 300mM NaCl, pH 8.5 with a yield approximately 60mg of pure monomeric protein.

3.1.3. Serine protease inhibitor A

All steps of SerA synthesis are shown here. This serine protease inhibitor was overexpessed in *E.coli;* strain BL21-pLysS and has a molecular weight 44kDa.

3.1.3.1. Pilot expression

We can see that the overexpression of SerA is strong in this bacterial strain. Figure 27 shows a gel of the pellets of the bacterial cells after their breakage with freeze thawing and sonication which means that the protein was present within the inclusion bodies. The gel of the corresponding supernatants (soluble fraction) of the broken cells did not show any bands at the appropriate molecular weight (overexpressed protein) and it is not shown here. The protein is overexpressed best 5-6 hours after gene overexpression induction and the band is marked by an arrow.

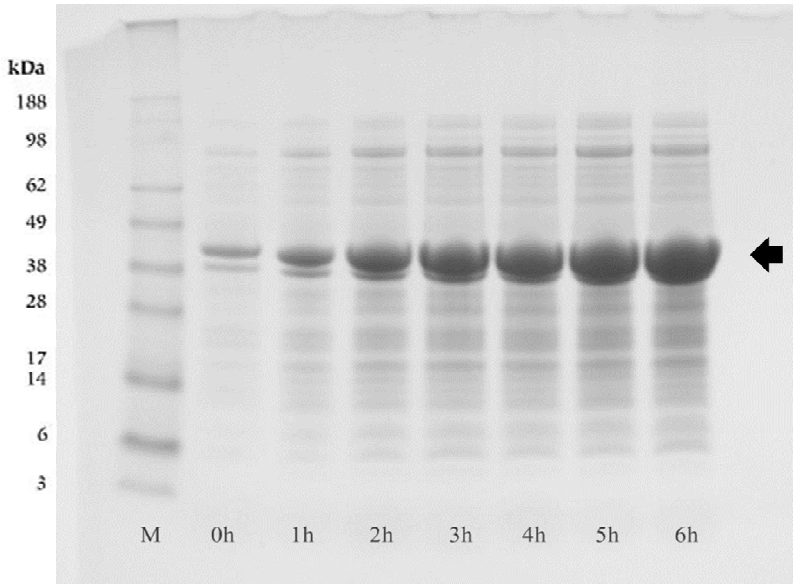


Figure 27: *Pilot expression of SerA. The molecular weight marker is labeled as M; 0h-6h represent the time points after induction of overexpression by IPTG. The SerA band is marked by an arrow.*

The presence of protein in the insoluble fraction (= inclusion bodies) was proven here and was not confirmed again in another experiment.

3.1.3.2. Pilot refolding

Pilot refolding in was done in refolding buffers 1-8 (please see Materials and methods). Samples were analyzed by concentration through 100kDa and 10kDa cutoff filter devices. The 100kDa filter was supposed to remove the high molecular weight impurities and aggregates while the 10kDa filter concentrated monomeric SerA. Then the samples were compared using NuPAGE gels in the same way as described in chapter 2.1.1.4. The bands were weak but the best ones were buffer 2, 3 and 4 (All 20mM Tris, 300mM NaCl, differ in pH only). Pictures of the gels are not shown because of a poor quality. Comparison was done mainly using the gels, not the pictures of them.

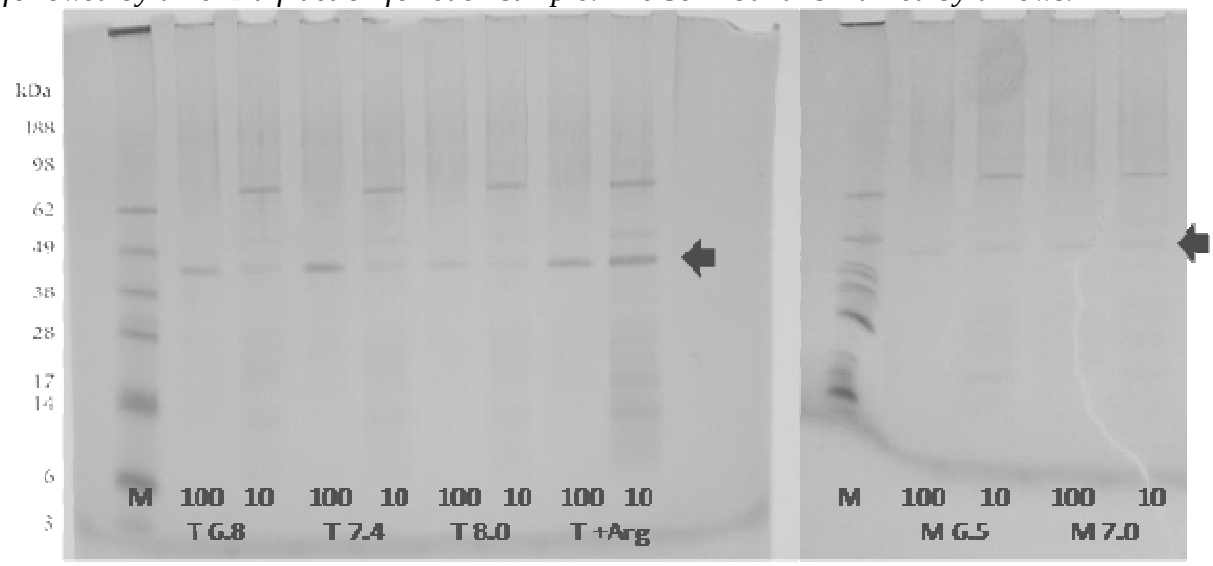
3.1.3.3. Optimization of pH

The next step was the optimization of pH in the refolding buffer. We show a picture of two gels with tested samples after concentration using a 100 and 10kDa cutoff filter devices (Fig. 28). The 100kDa filter was supposed to remove the high molecular weight impurities. Three 20mM Tris buffers were used (pH 6.8, 7.4, 8.0), two 20mM MOPS buffers with lower pH (6.5 and 7) and one 20mM Tris buffer with 250mM Arginine, pH 8. All six buffers contained 300mM NaCl, which was found before to be important during the first optimization step.

We were again searching for the strongest band in the fraction purified from aggregates; 10kDa in this case.

Buffer labeled as "T+Arg" with composition 20mM Tris, 300mM NaCl, 250mM arginine, pH 8.0 looks the best. There is the strongest band (arrows in figure 28) in the 10kDa fraction among all others suggesting the highest amount of refolded protein. The band in the 100kDa fraction for this buffer is also quite strong; comparable to those in Tris buffers with pH 6.8 and 7.4.

Figure 28: *Optimization of pH in the refolding buffer. M = marker, T = Tris buffer with the corresponding pH or arginine at pH 8.0, M = MOPS buffer with the corresponding pH; 100 and 10kDa label the cutoff used to get these samples. There is always a 100kDa fraction first followed by a 10kDa fraction for each sample. The SerA band is marked by arrows.*



3.1.3.4. Optimization of inclusion bodies concentration in the refolding buffer

Refolding of SerA at three different concentrations of inclusion bodies (1.5, 3, 5mg/ml) gives us the information about the concentration which provides the best outcome of refolded protein. We show a picture of two gels (run in the same way as described in chapter 2.1.1.4.) with tested samples after concentration using a 100 and 10kDa cutoff filters (Fig. 29). The 100kDa filter was again supposed to remove aggregates and the 10kDa filter concentrated the sample.

The concentration of 3mg/ml was estimated to be the best. The arrow-marked protein band in the 10kDa fraction is significantly stronger at 3mg/ml than in the concentration 1.5mg/ml which allows going to such a high concentration. On the other hand there is no further increase in intensity when going to 5mg/ml which suggests that the excess of the protein precipitated.

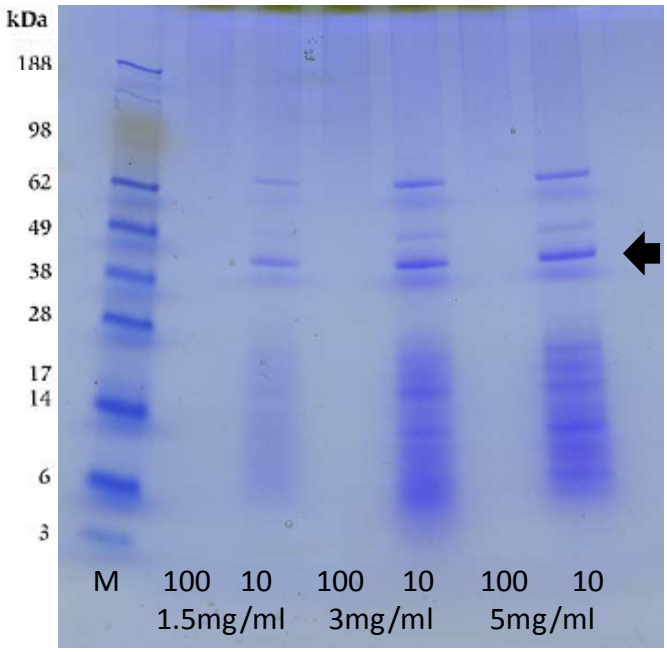


Figure 29: *Optimization of the ideal IB concentration in the refolding buffer. M = marker, the concentration values at the bottom line are always valid for two samples above, 100 and 10 label the cutoff in kDa used to get these samples. There is always a 100kDa fraction first followed by a 10kDa fraction for each sample. The SerA band is marked with an arrow.*

Serine protease A was then refolded in a big scale (8l refolding buffer) with a yield 5mg.

Further optimizations starting with the ProteoStat® Protein aggregation assay kit (Enzo® Life Sciences) were conducted to increase the yield of pure protein.

Due to our findings during the CystA purification (Chap. 2.1.1.7.), we stopped to use the high molecular filter (100kDa) to capture the precipitates. Only 10kDa cutoff filter device was used in the next applications.

3.1.3.5. Optimization using a kit – 1st round

The ProteoStat® Protein aggregation assay kit provides 15 different buffers as the starting point for refolding optimization. We tested all of them, each with 2 different reducing environments (A = DTT and B = GSH/GSSG). The protocol described in this kit requires only very small volume of samples and therefore only the amount of aggregates was estimated (the amount of refolded protein was too small to be further analyzed; protein aggregates can be analyzed in the samples due to the highly sensitive fluorogenic assay of the specific kit). After fluorescence was measured, the three best conditions with the lowest fluorescence (and thus lowest amount of protein aggregates and the most monomeric protein) were picked and optimized further. Data from the first measurement are shown in table 2 with highlighted best values.

Table 2: *Fluorescence measurement of aggregates after the 1st kit optimization. A1-A15 are buffers from the ProteoStat® Protein aggregation assay kit where DTT was used as a reducing agent; B1-B15 are samples with glutathione as a reducing agent. The three lowest values are highlighted in yellow. All the values of this table correspond to arbitrary fluorescence units measured according to the instructions of the kit.*

A1	11086
A2	266
A3	740
A4	419
A5	329
A6	1150
A7	7481
A8	647
A9	2255
A10	1234
A11	1478
A12	2011
A13	1457
A14	1194
A15	4141

B1	857
B2	847
B3	1433
B4	947
B5	706
B6	787
B7	1267
B8	351
B9	1446
B10	720
B11	2981
B12	1345
B13	391
B14	584
B15	849

The composition of three best buffers was:

A2 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 5mM DTT, pH 7.6

A5 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 5mM DTT, 0.5M Guanidine-HCl, pH 7.6

B8 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, 0.4M L-Arginine, pH 7.6

3.1.3.6. Optimization using a kit – 2nd round

The three best buffers from the previous step and the one found previously using the 100/10kDa cutoff filter devices were further optimized by various additives from the ProteoStat® Protein aggregation assay kit. We used DTT as a reducing agent for buffers A2, A5 and the one from 100/10kDa cutoff filters and GSH/GSSG for B8. Buffers were numbered as: 1=A2, 2=A5, 3=B8 and 4=buffer from 100/10kDa cutoff, to shorten the labeling. The labeling of additives is in table 3.

Table 3: *Additives in the refolding buffers at their final concentration.*

A	no additive
B	1mM PEG
C	5mM EDTA
D	5mM $CaCl_2$
E	5mM $MgCl_2$
F	100mM NaCl
G	0.05% Tween
H	120mM Sucrose
I	40mg/ml α-Cyclodextrin

Fluorescence was measured again using the ProteoStat® Protein aggregation assay kit. The fluorescence values of protein aggregates after this step are shown in table 4. Samples 3B and 4A showed the smallest fluorescence of aggregates and are highlighted in the table.

Table 4: *Fluorescence of aggregates in refolding buffer after changes in their composition by additives. Buffer compositions are described above. The best values are highlighted in yellow. All the values of this table correspond to arbitrary fluorescence units measured according to the instructions of the kit.*

1A	1B	1C	1D	1E	1F	1G	1H	1I
280	212	292	291	279	269	243	296	386

2A	2B	2C	2D	2E	2F	2G	2H	2I
242	211	285	210	268	385	222	302	OVER

3A	3B	3C	3D	3E	3F	3G	3H	3I
309	208	290	356	274	301	381	283	815

4A	4B	4C	4D	4E	4F	4G	4H	4I
199	238	315	227	261	442	254	307	535

The composition of best two buffers was:

3B: 50mM Tris, 20mM NaCl, 0.8mM KCl, 400mM L-arginine, 2mM GSH, 0.4mM GSSG, 1mM PEG, pH 7.6

4A: 10mM Tris, 150mM NaCl, 125mM L-arginine, pH 6.8

The concentration of all buffer components in buffer 4A was by calculation mistake ½ of the original buffer. The original buffer was included into further optimization steps and labeled as “Ori”.

We continued with buffers 3B and 4A which were found as giving the best refolding results when using the ProteoStat® Protein aggregation assay kit. We also used the “Ori” buffer which was previously found by the 100/10kDa cutoff filter approach.

3.1.3.7. Optimization of the inclusion bodies concentration in the best three buffers

Refolding efficiency was tested in 50ml of buffers 3B, 4A and Ori at three different inclusion bodies concentrations – 0.3, 0.6 and 1.3mg/ml. DTT was used as a reducing agent for buffers 4A and Ori, GSH/GSSG for the buffer 3B. After concentration to 500-1000µl using 10kDa cutoff filter device and buffer exchange for TBS, samples were run in a gel. Protein concentration in the concentrate was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the

measured values. The amount of aggregates was estimated by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 30-32; an arrow marks the SerA band in a gel. The gel was run in the same way as described in chapter 2.1.1.4.

We can see in the gel (Fig. 30) that buffers 4A and Ori have the strongest protein band at IB concentration 0.6mg/ml. The band is much stronger in buffer Ori than in 4A. Buffer 3B was difficult to concentrate (possibly because of PEG) and it also looks bad at the gel – the band is not sharp and can only be seen in the sample with 1.3mg/ml IB concentration.

Buffers Ori and 3B show higher protein concentration (Fig. 31) than 4A. The amount of aggregated protein upon refolding is the lowest in buffers 4A and Ori at IB concentration 0.6mg/ml (Fig. 32).

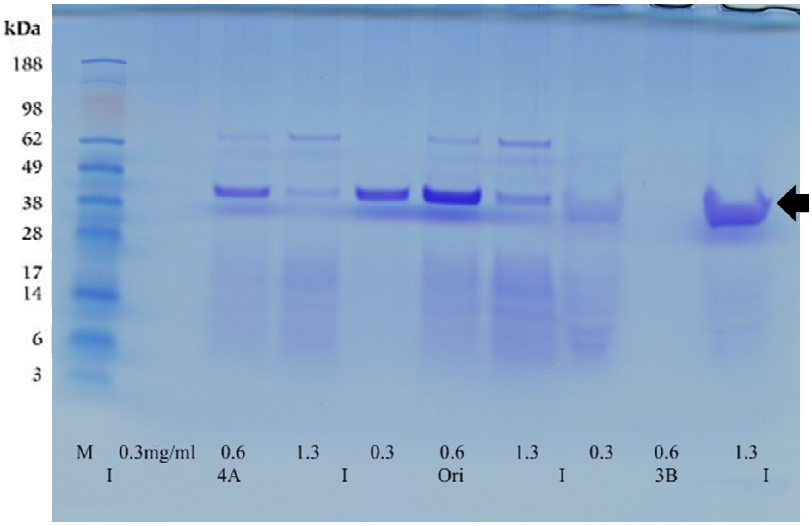
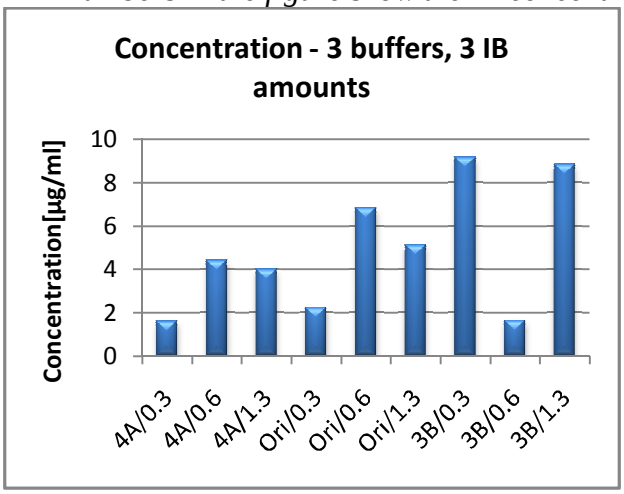
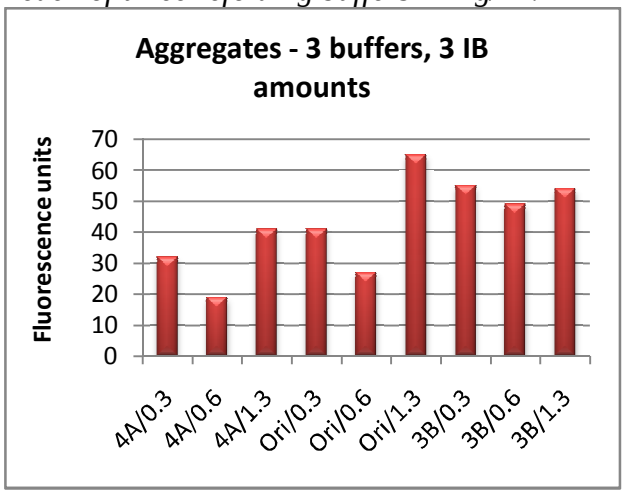


Figure 30: *SerA in refolding buffers 4A, Ori and 3B at different concentrations of IB in the refolding buffer. Numbers in the figure show the IB concentration in each of three refolding buffers in mg/ml. First three lanes are SerA in buffer 4A, second three lanes SerA in Ori and the last three lanes SerA in 3B. The arrow marks the SerA band.*

Figures 31 (left), 32 (right): *Concentration and the amount of aggregates of SerA in refolding buffers 4A, Ori and 3B at different concentrations of IB in the refolding buffer. Numbers in the figure show the IB concentration in each of three refolding buffers in mg/ml.*





After comparing all the results, the buffer Ori at IB concentration 0.6mg/ml was chosen as the best condition for big scale refolding.

3.1.3.8. Optimization of the arginine concentration

The effect of arginine at concentrations 50, 100, 150, 250 and 400mM was tested and compared to the Ori buffer (where 250mM arginine is present). The initial IB concentration was 0.6mg/ml in all samples. After concentration to 500-1000µl using a 10kDa cutoff filter device and buffer exchange for TBS, samples were run in a NuPAGE gel (in the same way as described in chapter 2.1.1.4.). Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The amount of aggregates was measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 33-35; an arrow marks the SerA band in a gel.

We can see in figure 33 that the protein band is stronger with increasing arginine concentration. It means that the more arginine we have in the buffer, the better refolding conditions are achieved. Figure 34 also shows an increase of SerA concentration with increasing arginine amount. The concentration of SerA in the sample with 400mM arginine is almost twice higher than in all the other buffers. There is not much difference in the aggregates amount among refolding buffers (Fig.35). Fluorescence reaches values between 170 and 270 units in all cases.

The L-arginine concentration 400mM shows the best protein concentration, strongest band in a gel and not so high amount of aggregates. 400mM arginine was then chosen as the best condition for a big scale refolding.

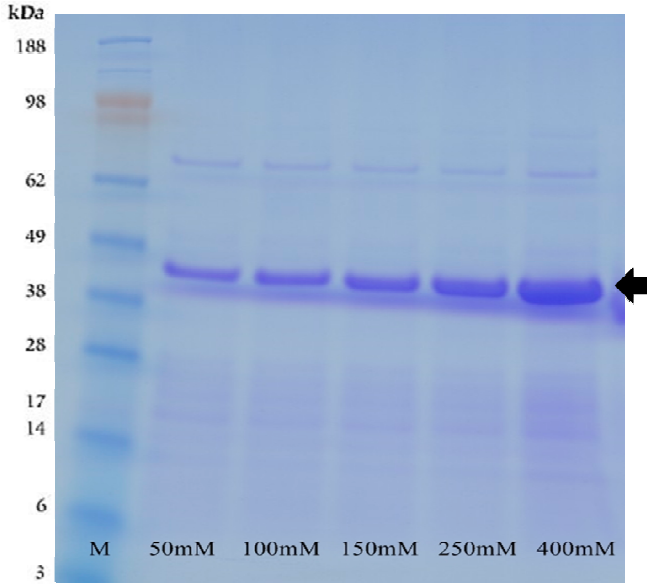
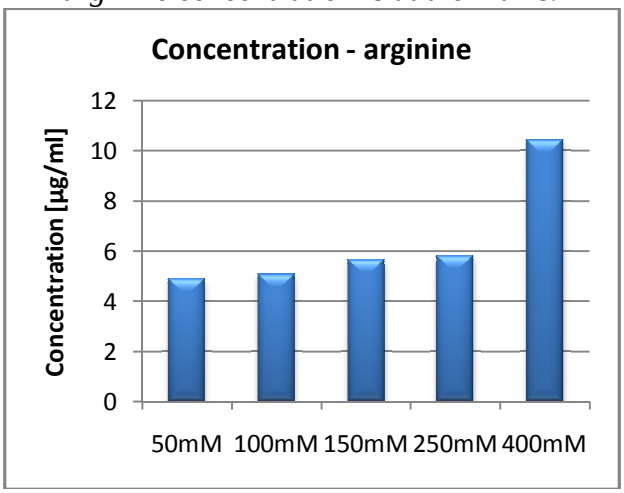
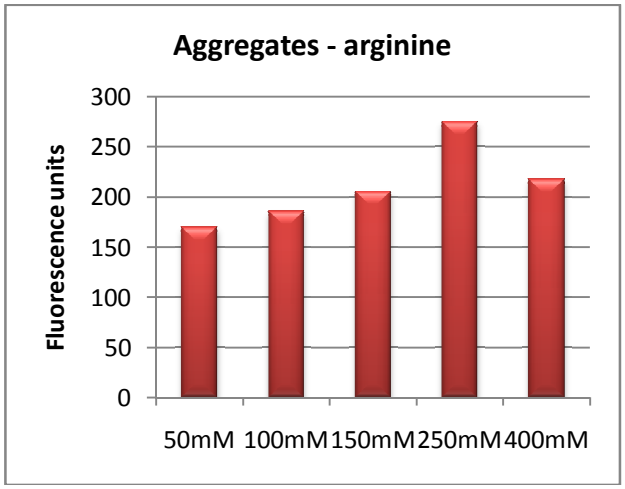


Figure 33: *Optimization of L-arginine in the SerA refolding buffer. The arginine concentration is given at the bottom of the figure. The SerA band is marked with an arrow.*

Figures 34 (left), 35 (right): *Concentration and the amount of aggregates of SerA in refolding buffer Ori with different concentrations of L-arginine in the refolding buffer. The arginine concentration is at the x-axis.*





3.1.3.9. Optimization of NaCl concentration

The NaCl concentration optimization was the next step. SerA was refolded in 20mM Tris, 400mM L-arginine, pH 6.8 with NaCl at concentrations 0, 20, 50, 100, 150 and 300mM. After concentration to 500-1000µl using a 10kDa cutoff filter device and buffer exchange for TBS, samples were run in a gel in the same way as described in chapter 2.1.1.4. Protein concentration in the concentrate was estimated using the Pierce® BCA Protein Assay Kit and aggregates were measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 36-38; an arrow marks the SerA band in a gel.

NaCl does not seem to have any big effect on the refolding efficiency. Protein bands in the gel look all the same (Fig. 36).The protein concentration measurement does not provide much information about the right condition either (Fig. 37). The protein concentration in all 6 buffers varies between 8.5 and 10µg/ml. The amount of aggregated protein differs a lot among these six buffers (Fig. 38). The buffer with 50mM NaCl shows 2.5 times lower aggregate fluorescence than the one with 100mM NaCl.

Finally, the concentration 50mM NaCl was picked as the best based mainly on the smallest aggregates amount and also on yielding tightly the best protein concentration.

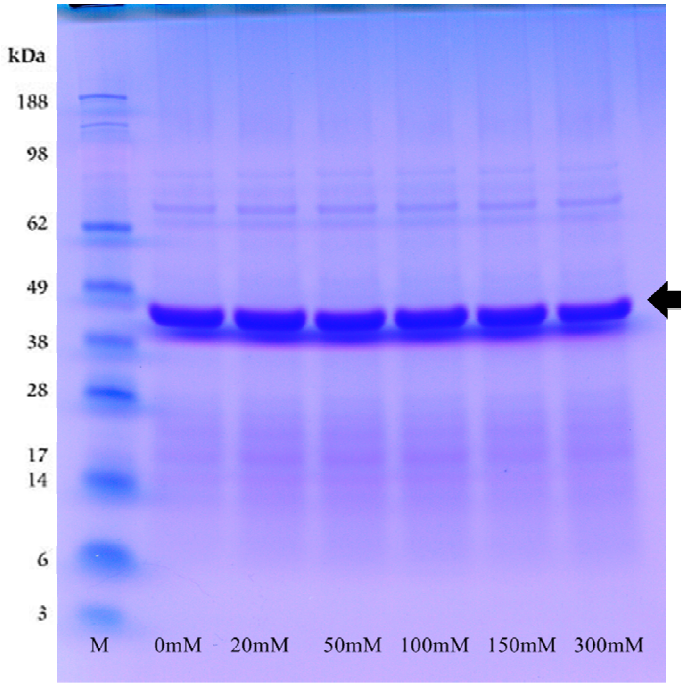
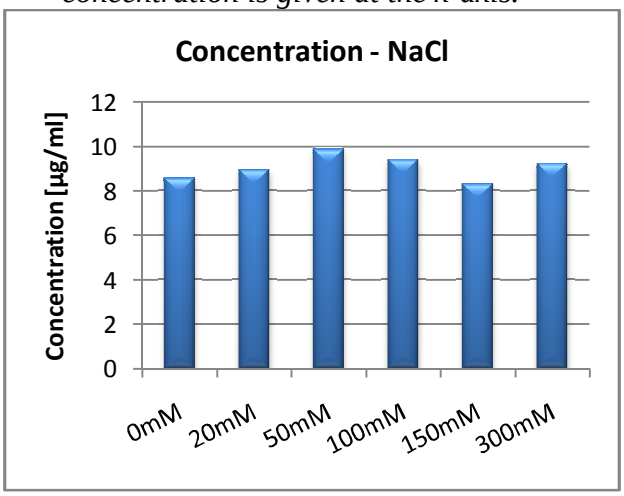
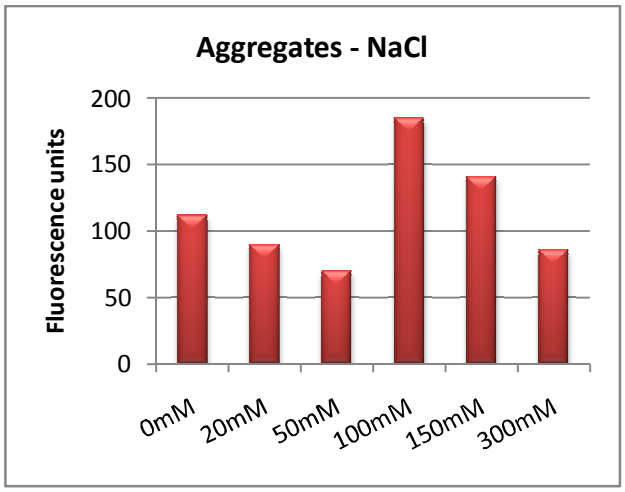


Figure 36: *Optimization of NaCl concentration in the refolding buffer for SerA. The concentration of NaCl is given at the bottom of the figure. The SerA band is marked with an arrow.*

Figures 37 (left), 38 (right): *Concentration of SerA and the amount of aggregates of SerA in the refolding buffer with different concentrations of NaCl in the refolding buffer. The NaCl concentration is given at the x-axis.*





3.1.3.10. Optimization of the refolding buffer pH

The last optimization step was to find the best pH. The buffer composition was in all cases 20mM Tris, 400mM L-arginine and 50mM NaCl. The IB concentration was 0.6mg/ml. Four pH values were tested: pH 6.8, 7.4, 8.0 and 8.5. After concentration to 500-1000µl using 10kDa cutoff filter devices and buffer exchange for TBS, samples were run in a gel in the same way as described in chapter 2.1.1.4. Protein concentration in the concentrate was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The

aggregates were measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 39-41; an arrow marks the SerA band in a gel.

SerA, which was refolded in buffers with pH 6.8 and 7.4, shows the strongest band in the gel (Fig. 39). The protein concentration in these two pH environments is also higher than in buffer with pH 8.0 or 8.5 (Fig 40). The difference is however quite small. The lowest amount of aggregates in the buffer with pH 7.4, compared to all other buffers (Fig. 41), has shown that this pH is the most suitable for SerA refolding.

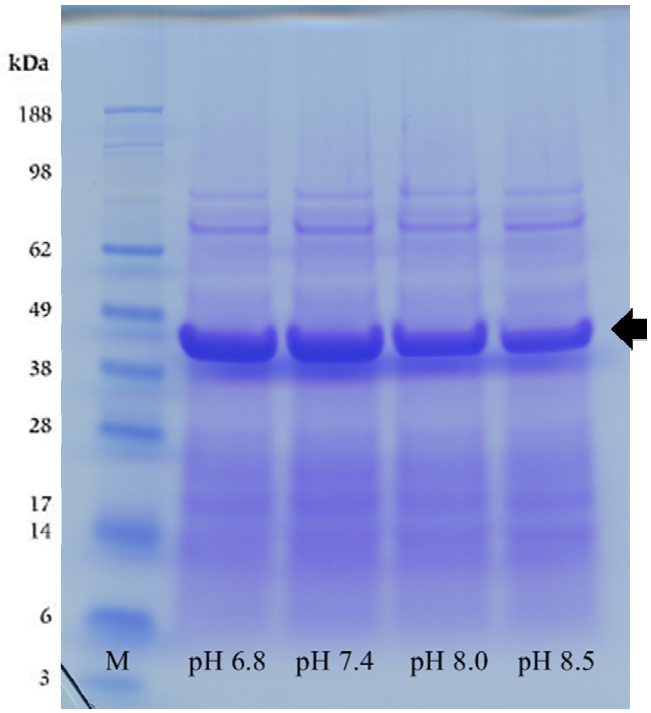
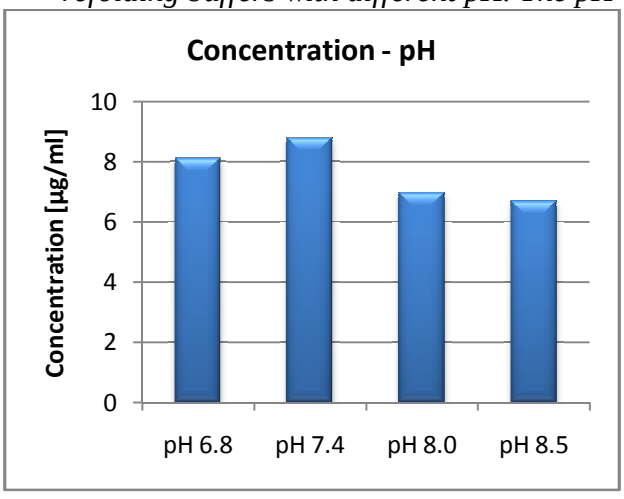
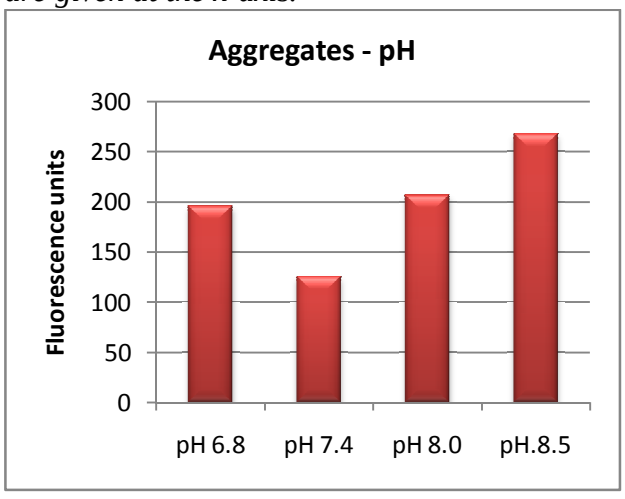


Figure 39: *Optimization of pH of the refolding buffer for SerA. The pH values are given at the bottom of the figure. The SerA band is marked with an arrow.*

Figures 40 (left), 41 (right): *Concentration and the amount of aggregates of SerA in refolding buffers with different pH. The pH values are given at the x-axis.*





The final buffer composition was then:

20mM Tris, 400mM L-arginine, 50mM NaCl, pH 7.4 with the concentration of inclusion bodies 0.6mg/ml.

Even after all optimization steps, the yield of SerA was only 12mg after purification.

3.1.4. Serine protease inhibitor C

All steps of SerC synthesis are shown here. This serine protease inhibitor was overexpessed in *E.coli,* strainBL21-pLysS and has a molecular weight 42kDa.

3.1.4.1. Pilot expression

We can see an extremely strong overexpression of SerC in this bacterial strain. Figure 42 shows a gel of the pellets of the bacterial cells after their breakage with freeze thawing and sonication which means that the protein was present within the inclusion bodies. The gel of the corresponding supernatants (soluble fraction) of the broken cells did not show any bands at the appropriate molecular weight (overexpressed protein) and it is not shown here. SerC is overexpressed well already 3 hours after the IPTG induction. The strength of the band (Fig. 42; marked with an arrow) does not increase further over time.

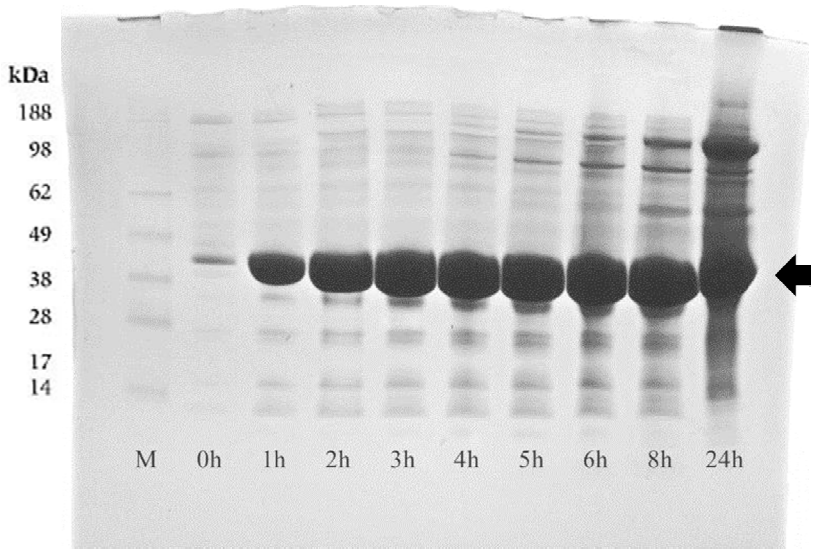


Figure 42: *Pilot expression of SerC. The molecular weight marker is labeled as M; 0h-24h represent the time points after induction of overexpression by IPTG. An arrow marks the SerC band.*

The presence of SerC in the insoluble fraction (= inclusion bodies) was proven here and was not confirmed again in another experiment.

3.1.4.2. Refolding optimization using a kit – 1st round

The ProteoStat® Protein aggregation assay kit provides 15 different buffers as the starting point for refolding optimization. We tested all of them, each with 2 different reducing environments (C = DTT and D = GSH/GSSG). The protocol described in this kit requires only very small volume of samples and therefore only the amount of aggregates was estimated (the amount of refolded protein was too small to be further analyzed; protein aggregates can be analyzed in the samples due to the highly sensitive fluorogenic assay of the specific kit). After fluorescence was measured, the three best conditions with the lowest fluorescence (and thus lowest amount of protein aggregates and the most monomeric protein) were picked and optimized further. Data from the first measurement are shown in table 5 with highlighted best results.

Table 5: *Fluorescence measurement of aggregates after the 1st kit optimization. C1-C15 are buffers where DTT was used as a reducing agent; D1-D15 are samples with glutathione as a reducing agent. The best three values are highlighted in yellow. All the values of this table correspond to arbitrary fluorescence units measured according to the instructions of the kit.*

C1	952
C2	311
C3	382
C4	393
C5	502
C6	671
C7	598
C8	653
C9	432
C10	1292
C11	892
C12	752
C13	4945
C14	925
C15	537

D1	175
D2	342
D3	2092
D4	341
D5	327
D6	436
D7	610
D8	837
D9	752
D10	1707
D11	1245
D12	1027
D13	1364
D14	1486
D15	1393

The composition of three best buffers was:

C2 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 5mM DTT, pH 7.6

D1 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, pH 6.8

D4 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, 500mM Guanidine-HCl, pH 6.8

D5 – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, 500mM Guanidine-HCl, pH 7.6

3.1.4.3. Optimization using a kit – 2nd round

Four best buffers found previously were further optimized by various additives from the ProteoStat® Protein aggregation assay kit. We used DTT as a reducing agent for buffer C2, and GSH/GSSG for D1, D4 and D5. Buffers were numbered: 5=C2, 6=D1, 7=D4 and 8=D5 to shorten the labeling. The labeling of additives is in the same as at the optimization of SerA (please see above in table 3).

The fluorescence values of protein aggregates measured in this optimization step are shown in table 6. Samples 5F, 6A, 6C and 6D show the lowest fluorescence and are highlighted in the table.

Table 6: *Fluorescence of aggregates in refolding buffer after changes in their composition by additives. Buffer compositions are described above. The best values are highlighted in yellow. All the values of this table correspond to arbitrary fluorescence units measured according to the instructions of the kit.*

5A	5B	5C	5D	5E	5F	5G	5H	5I
350	345	357	337	344	329	361	365	523

6A	6B	6C	6D	6E	6F	6G	6H	6I
294	321	305	300	380	326	335	375	485

7A	7B	7C	7D	7E	7F	7G	7H	7I
430	342	449	404	411	392	411	468	776

8A	8B	8C	8D	8E	8F	8G	8H	8I
439	368	547	422	454	509	412	571	796

The composition of best four buffers was:

6A – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, pH 6.8

6C – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, 5mM EDTA, pH 6.8

6D – 50mM Tris, 20mM NaCl, 0.8mM KCl, 2mM GSH, 0.4mM GSSG, 5mM $CaCl_2$, pH 6.8

5F – 50mM Tris, 120mM NaCl, 0.8mM KCl, 5mM DTT, pH 7.6

Because buffer 6 (with various additives) shows three best fluorescence values, it was modified by varying the reducing agent composition in the buffer. Two more buffers were prepared based on buffer 6A, where glutathione was replaced by DTT in one case or not used at all in the other case.

Names and composition of the new buffers were:

6T – 50mM Tris, 20mM NaCl, 0.8mM KCl, 5mM DTT, pH 6.8 (glutathione replaced by DTT)

6X – 50mM Tris, 20mM NaCl, 0.8mM KCl, pH 6.8 (no reducing agent in the buffer)

3.1.4.4. Optimization of the reducing agent in the refolding buffer

50ml of each of 6 buffers (5F, 6A, 6C, 6D, 6T, 6X) was used for testing the refolding efficiency. SerC was refolded at the concentration of IB 0.5mg/ml. After concentration to 500-1000µl using a 10kDa cutoff filter device and buffer exchange for TBS, samples were run in a NuPAGE gel as described in chapter 2.1.1.4. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The amount of aggregates was measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 43-45; an arrow marks the SerC band in a gel.

Buffers 6X and 5F have the strongest band in a gel (Fig. 43, marked with an arrow). Buffers 6A, 6C and 6T show bands similar to each other and there is nothing in buffer 6D. The concentration of protein in buffer 6D is also very low which probably means that the sample was lost during concentration. On the other hand buffers 5F and 6X show the highest protein concentration in the refolding buffer (Fig. 44).

Buffer 6X was finally chosen to be better than 5F because of a five times lower amount of aggregates (Fig 45).

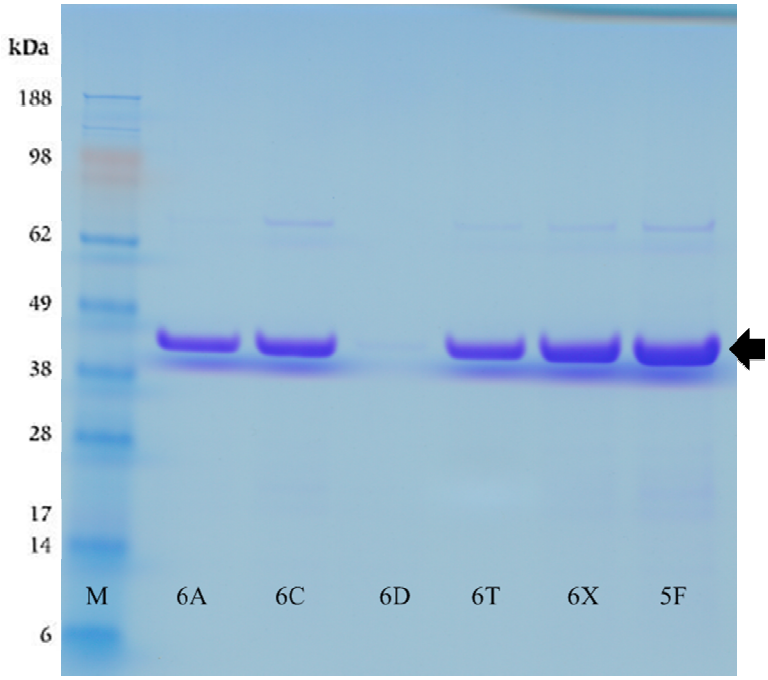
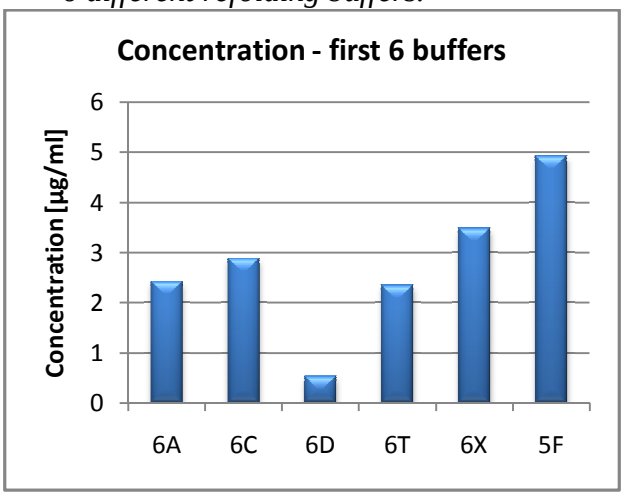
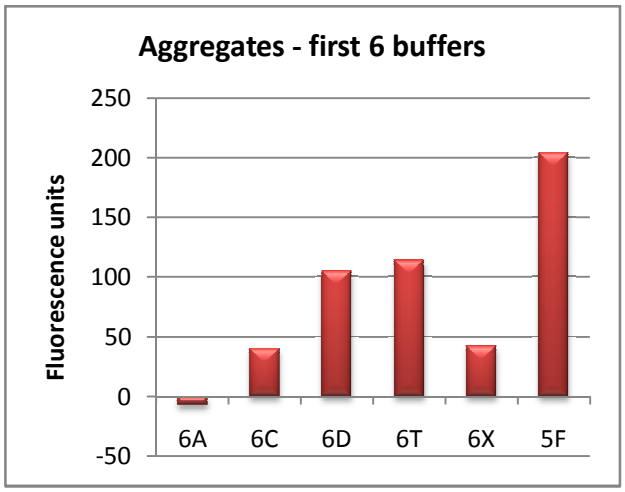


Figure 43: *The gel of SerC refolded in 6 different buffers. The buffer names are at the bottom of the figure. The SerC band is marked with an arrow.*

Figures 44 (left), 45 (right): *Concentration and the amount of aggregates of SerC in 6 different refolding buffers.*





3.1.4.5. Optimization of NaCl concentration

The optimization of NaCl concentration in the refolding buffer was the next step. SerC was refolded in 50mM Tris, 0.8mM KCl, pH 6.8 with NaCl at concentrations 0, 20, 50, 100, 150 and 300mM at the IB concentration 0.5mg/ml. After protein concentration to 500-1000µl using a 10kDa cutoff filter device and buffer exchange for TBS, samples were run in a gel in the same way as described in chapter 2.1.1.4. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The amount of aggregates was measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 46-48; an arrow marks the SerC band in a gel.

We can see in figure 46 that SerC refolded in buffers with NaCl concentration 150 and 300mM has the strongest band. 100mM NaCl seems to be the worst option with a weak protein band. The protein concentration measurement in figure 47 shows the same result as the NuPAGE gel. The protein concentration is almost twice higher in buffers with 150 and 300mM NaCl than in the others. Lower amount of aggregated protein (Fig. 48) in buffer with 300mM NaCl decided that 300mM NaCl is a better condition. It shows the least aggregate fluorescence among all buffers and 3x lower than the buffer with 150mM NaCl.

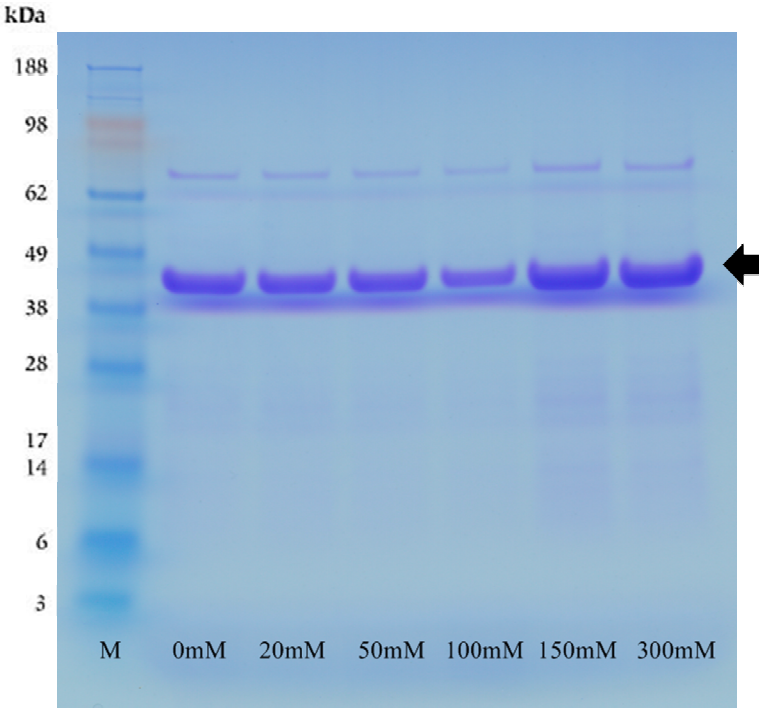
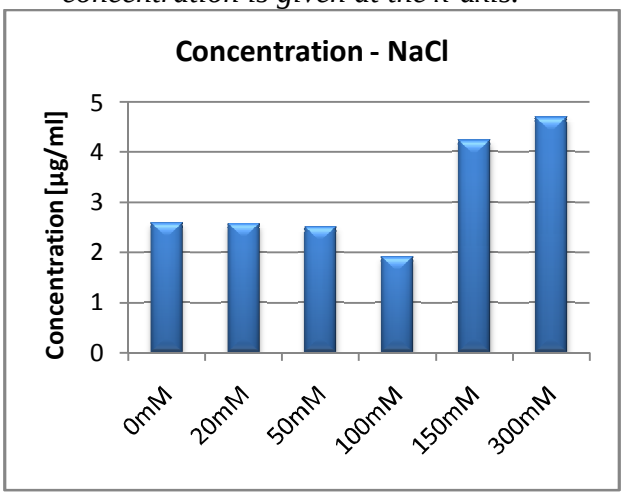
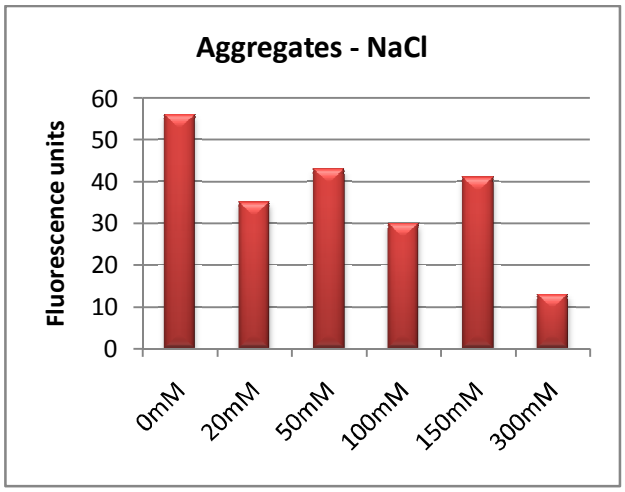


Figure 46: *Optimization of NaCl concentration in the refolding buffer for SerC. The concentration of NaCl is given at the bottom of the figure. SerC band is marked with an arrow.*

Figures 47 (left), 48 (right): *Concentration and the amount of aggregates of SerC in refolding buffer with different concentrations of NaCl in the refolding buffer. The NaCl concentration is given at the x-axis.*





3.1.4.6. Optimization of IB concentration in the refolding buffer

We further optimized the concentration of inclusion bodies in the refolding buffer. The inclusion bodies concentrations were: 2, 1, 0.5, 0.25, 0.125 and 0.0625mg/ml. Refolding of SerC at each IB concentration was done in 50ml in 50mM Tris, 0.8mM KCl, 300mM NaCl, pH 6.8. After concentration to 500-1000µl using a 10kDa cutoff filter device and buffer exchange for TBS, samples were run in a gel in the same way as described in chapter 2.1.1.4. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was

calculated using the measured values. The aggregates were measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 49-51; an arrow marks the SerC band in a gel.

We can see in figure 49 that the protein band is the strongest in buffer with IB concentration 0.25mg/ml. Bands in samples with IB concentration 0.5 and 0.125mg/ml are also quite strong, the others cannot be seen at all. The protein BCA concentration measurement also shows the highest value in the buffer with IB concentration 0.25mg/ml (Fig. 50). The amount of precipitated protein surprisingly increases with decreasing IB concentration. The trend is not strong and the lowest and the highest value differ only by 25% (Fig. 51).

Based on the gel and the SerC concentration measurement the best IB concentration is 0.25mg/ml. The amount of refolded protein decreases with increasing IB concentration which means that SerC probably precipitates at higher concentrations. Going to lower concentrations makes no sense. It would significantly increase the volume of refolding buffer and has no positive effect. The sample at IB concentration 1mg/ml was probably lost during the concentration but the result at 0.25mg/ml is clearly the best so there is no need to repeat this step.

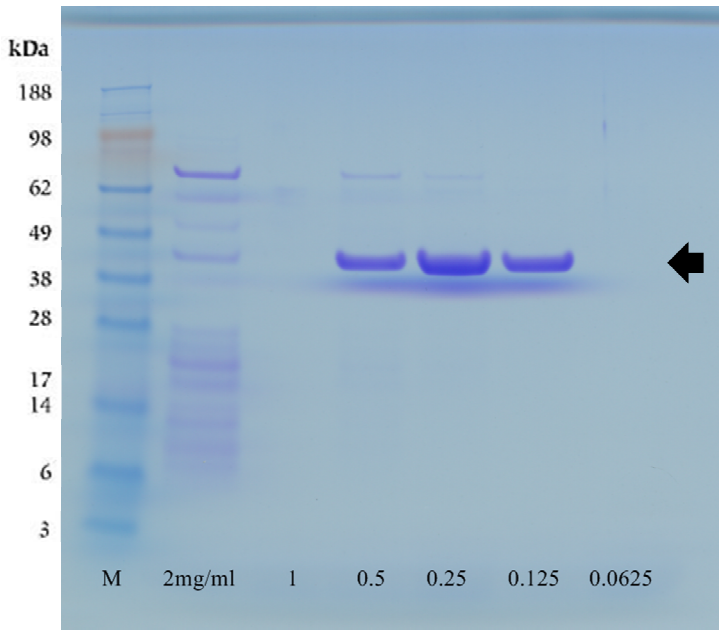
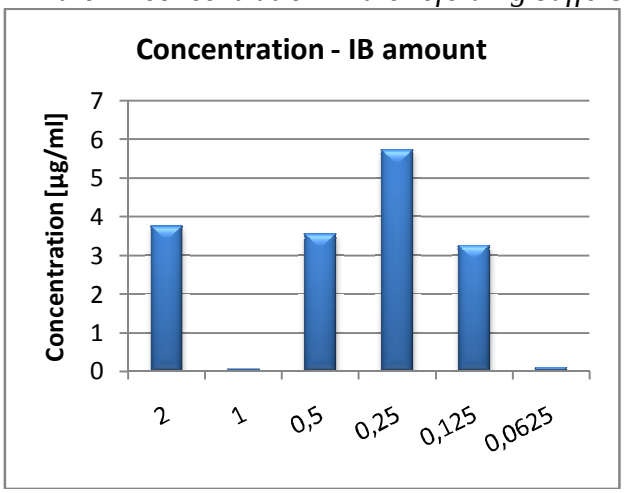
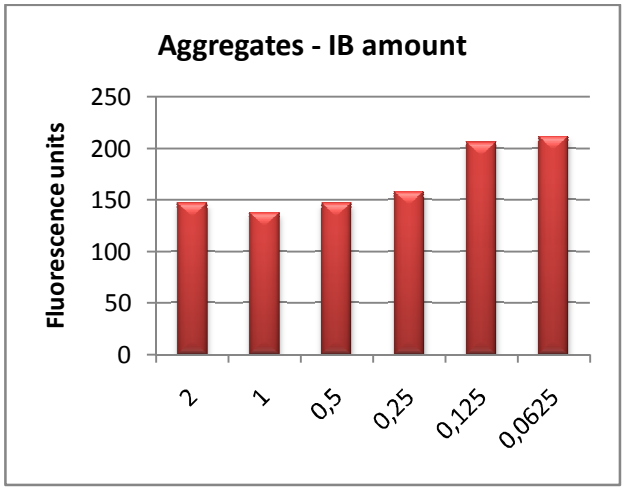


Figure 49: *Concentration of SerC in the refolding buffer at different concentrations if IB in the refolding buffer. Numbers in the figure show the IB concentration in mg/ml. The SerC band is marked with an arrow.*

Figures 50 (left), 51 (right): *Concentration and the amount of aggregates of SerC after refolding at different concentrations of IB in the refolding buffer. Numbers in the figure show the IB concentration in the refolding buffers in mg/ml.*





3.1.4.7. Optimization of the arginine concentration

Refolding buffer (50mM Tris, 300mM NaCl, 0.8mM KCl, pH6.8) was further improved by varying the L-arginine concentration – 0, 50, 100, 150, 250 and 400mM. The IB concentration was 0.25mg/ml in all samples, as found to be the best in the previous step. After concentration to 500-1000µl using a 10kDa cutoff and buffer exchange for TBS, samples were run in a NuPAGE gel as described in chapter 2.1.1.4. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The aggregates were measured by the ProteoStat® Protein aggregation assay kit. The results are presented in figures 52-54; an arrow marks the SerC band in a gel.

Figure 52 shows that the SerC band gets stronger with the increasing arginine concentration. Especially the protein band in buffer with 400mM arginine is stronger than the others. The SerC concentration measurement also shows an increase of refolded SerC amount with increasing arginine molarity (Fig. 53). Just like in the gel, buffer with 400mM arginine shows again the best result. The amount of aggregates is similar in all buffers except the one with 400mM arginine. There are at least twice more aggregates in the buffer with 400mM arginine (Fig. 54).

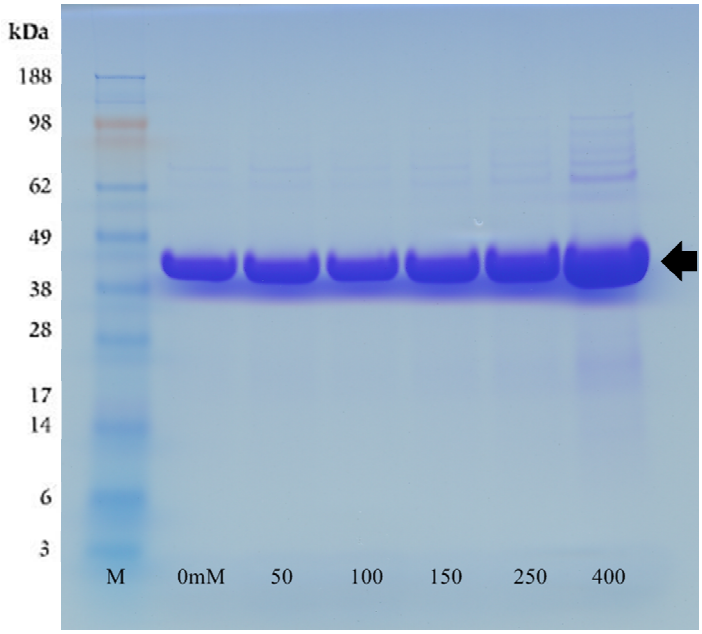
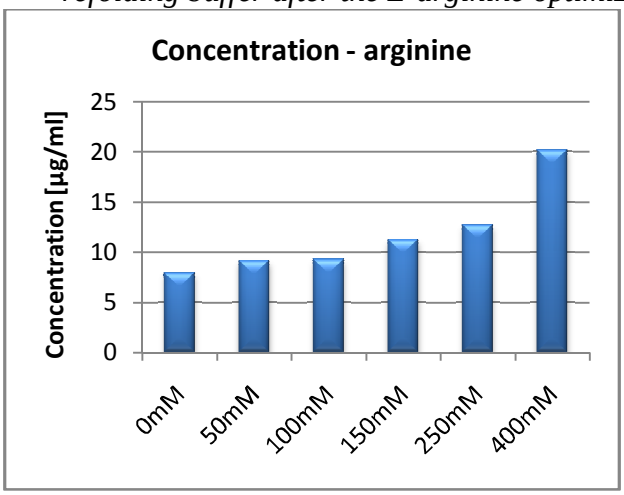
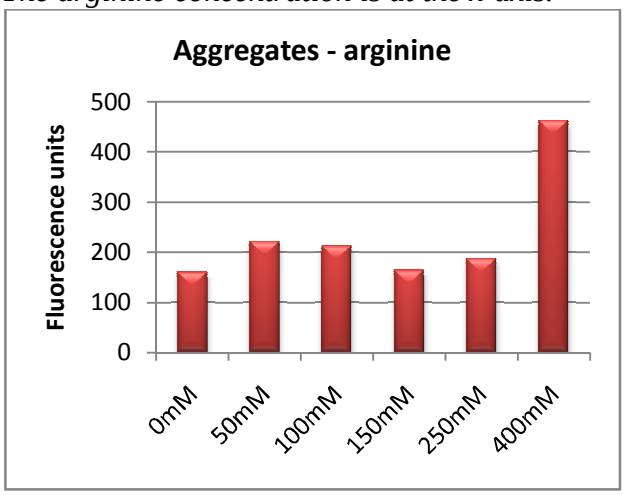


Figure 52: *Optimization of L-arginine in the SerC refolding buffer. The arginine concentration is given at the bottom of the figure. The arrow marks the SerC band.*

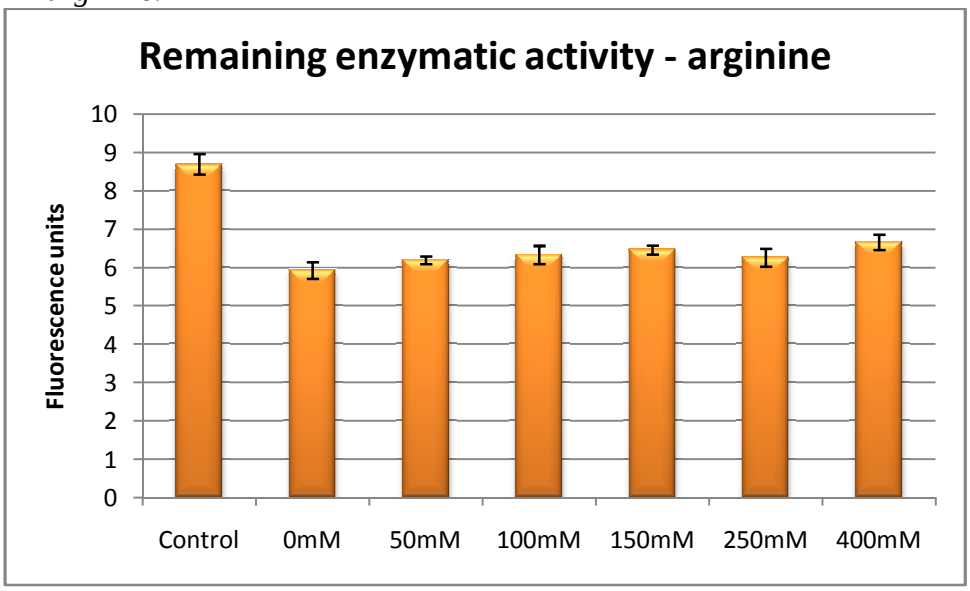
Figures 53 (left), 54 (right): *Concentration and the amount of aggregates of SerC in refolding buffer after the L-arginine optimization. The arginine concentration is at the x-axis.*





The quality of refolding was further tested by my supervisor. SerC has an inhibitory activity against elastase. We show the graph of remaining enzymatic activity of 70pM elastase after incubation with 5nM SerC in figure 55. We can see that SerC refolded in a buffer without arginine has the strongest inhibitory activity (= least remaining activity). The effect is however similar for all samples.

Figure 55: *Remaining enzymatic activity of elastase after incubation with SerC. Control is the effect of elastase with no inhibitors; 0mM - 400mM is SerC refolded in buffer with 0-400mM L-arginine.*



After comparison of all data, L-arginine at 250mM concentration was chosen as the best condition. It provides the 2nd best protein yield (after 400mM) but has significantly less aggregates. The inhibitory activity is the same in both buffers.

3.1.4.8. Optimization of the refolding buffer pH

The last optimization step was to find the best pH for SerC refolding. The buffer composition was in all cases 50mM Tris, 300mM NaCl, 0.8mM KCl and 250mM L-arginine. The IB concentration was 0.25mg/ml. Four pH values were tested: pH 6.8, 7.4, 8.0 and 8.5. After concentration to 500-1000µl using a 10kDa cutoff filter device and buffer exchange for TBS and the samples were run in a gel as described in chapter 2.1.1.4. Protein concentration (in the 500-1000µl volume) was estimated using the Pierce® BCA Protein Assay Kit and then the refolded protein concentration in the refolding buffer was calculated using the measured values. The amount of aggregates was measured by the ProteoStat® Protein aggregation assay kit. The inhibitory enzymatic activity against elastase was tested for 7nM SerC under the same conditions as previously. The results are presented in figures 56-59; an arrow marks the SerC band in a gel.

The gel bands get a little stronger with an increasing pH of the refolding buffer (Fig. 56). This trend can be more easily seen in the graph of SerC concentration in the refolding buffer. The protein concentration in buffers with pH 8.0 and 8.5 is almost twice higher than in the other two buffers (Fig. 57). Figure 58 shows that there are the least aggregates in the buffer

with pH 6.8. Buffers with pH 7.4 and 8.5 have the same amount of aggregated SerC; the highest amount of aggregates is in the buffer with pH 8.0 (Fig. 58). The activity (and the remaining activity) of SerC is almost the same after refolding in any of these pH environments (Fig. 59).

Based on all these measurements pH 8.5 was chosen as the best condition. This buffer provides a little better result in all tests than the second best buffer – pH 8.0.

We can see from the gel and the protein concentration measurement that pH 8.0 and 8.5 are better for SerC refolding than the buffers with lower pH. The lower amount of aggregates, as well as the activity tests show that pH 8.5 will be the most suitable environment for big scale SerC refolding.

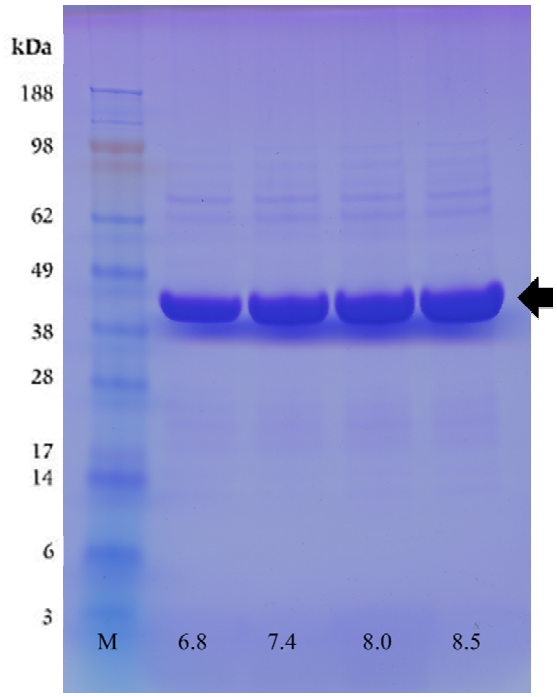
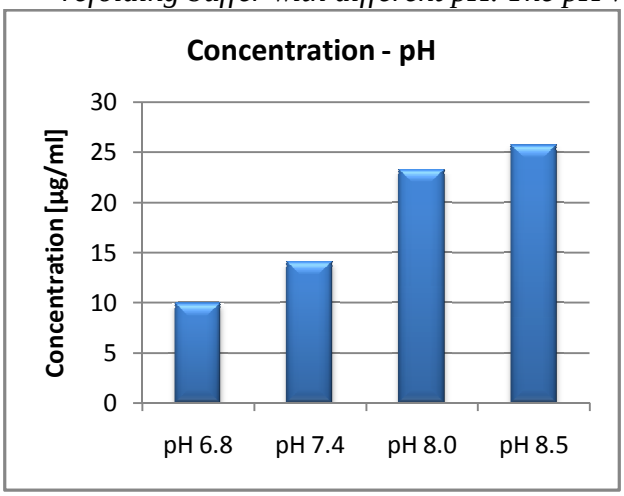


Figure 56: *Optimization of pH of the refolding buffer for SerC. The pH values are given at the bottom of the figure. The arrow marks the SerC band.*

Figures 57 (left), 58 (right): *Concentration and the amount of aggregates of SerC in refolding buffer with different pH. The pH values are given at the x-axis.*



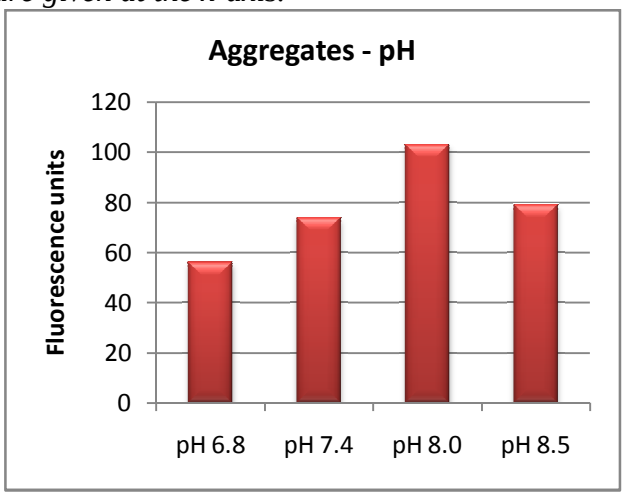
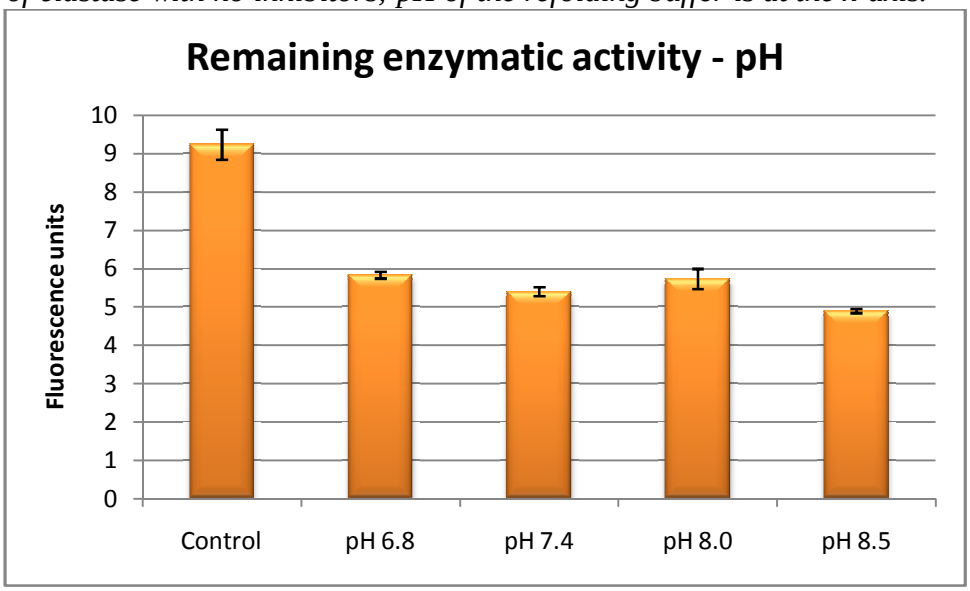


Figure 59: *Remaining enzymatic activity of elastase after incubation with SerC. Control is the effect of elastase with no inhibitors; pH of the refolding buffer is at the x-axis.*



The final buffer composition is:

50mM Tris, 300mM NaCl, 0.8mM KCl, 250mM L-arginine, pH 8.5 with the inclusion bodies concentration 0.25mg/ml.

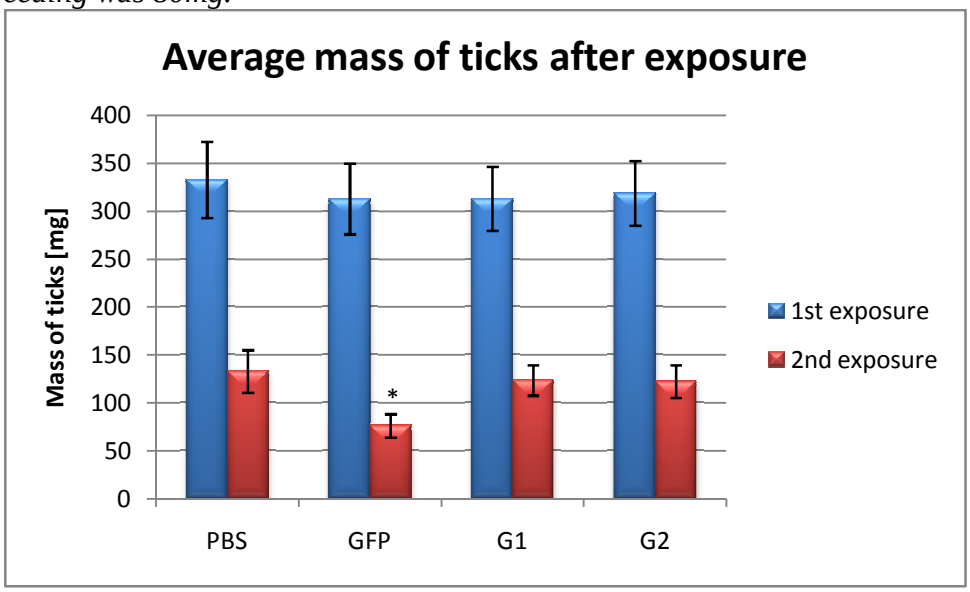
SerC was overexpressed and refolded with a final yield 20mg.

3.2. Vaccination of guinea pigs with cysteine protease inhibitors A and B

Blue bars represent the 1st exposure, red bars the 2nd exposure in all graphs.

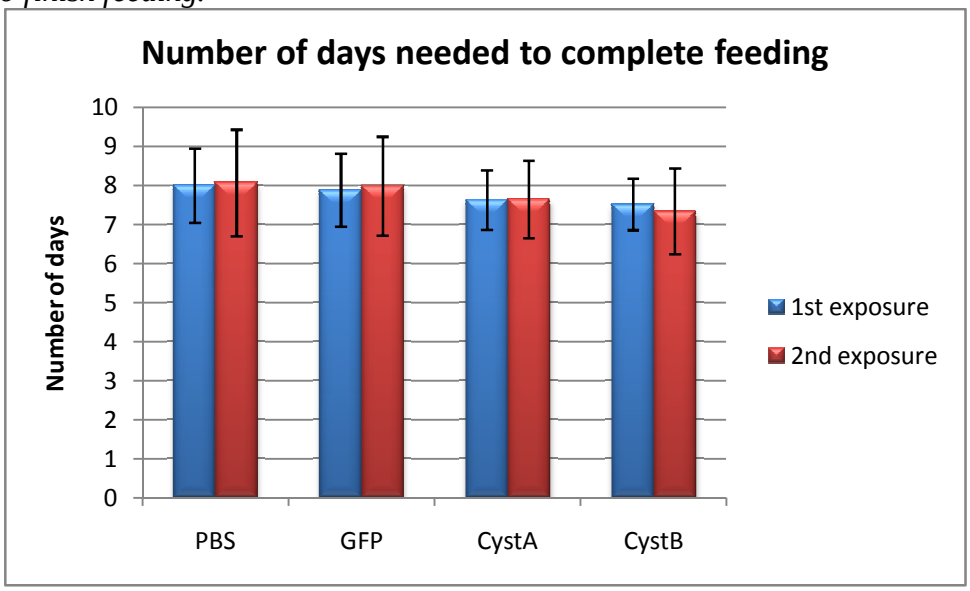
Figure 60 shows the average mass of ticks after they finished feeding. We can see that the mass of ticks was almost three times higher after the first exposure (320mg) than after the second exposure (120mg). There are no differences among the tested groups. The only exception is a lower mass of ticks in the GFP group after the second exposure. It reaches 80mg, while all other ticks from groups after the second exposure weighed 120mg in average. Such a difference seems not to have any biological meaning.

***Figure 60**: The mass of ticks after finished feeding. The mass after the 1st exposure was ca 320mg in all cases while 120mg after the 2nd feeding. The average mass in the GFP group after 2nd feeding was 80mg.*



The number of days needed for ticks to complete feeding is shown in figure61. All ticks (no matter what group) needed in average the same time to finish feeding, varying from seven to eight days.

Figure 61: *The number of days needed to finish feeding. All ticks needed 7.5-8 days in average to finish feeding.*



The amount of eggs was determined by scoring from 0 to 3and is shown in the next figure. No eggs were scored as 0, small amount as 1, normal amount as 2 and a big amount of eggs as 3. The amount of eggs was slightly higher after the first exposure than after the second one. It reached ca 2.2 points in all groups after the first exposure while only ca 1.8 points after the second exposure. There are no differences among the different experimental groups as far as it concerns the egg laying ability of the ticks.

Figure 62: *The egg laying of ticks from different groups after both tick exposures. The amount of eggs was scored from 0 (no eggs) to 3 (lots of eggs). The amount of eggs was slightly higher after the 1st exposure. There are no differences among tested groups.*

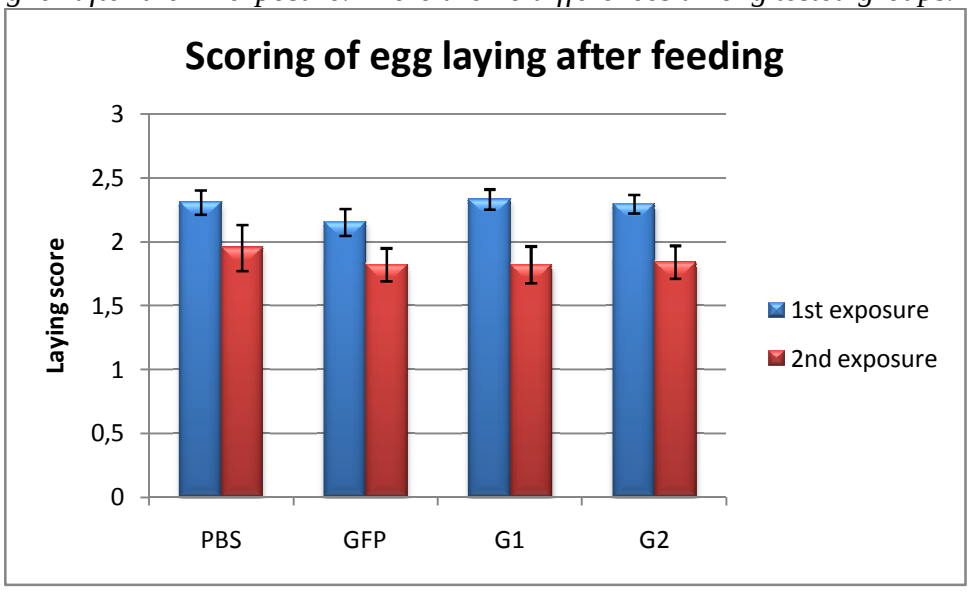
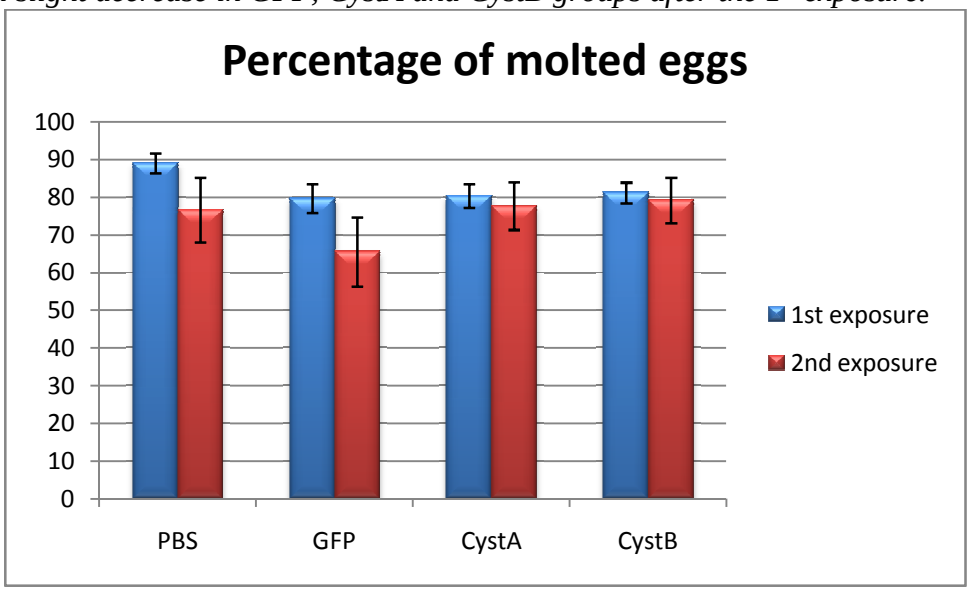


Figure 63 demonstrates the proportion of molted eggs. The value after the first exposure in the PBS control group reaches almost 90% while in the others groups ca 80%. The values after the second exposure reach ca 75% except for the GFP group where the value is 65%. None of these differences is statistically significant.

Figure 63: *The egg molting efficiency from different groups after both exposures. Molting was scored by the percentage of molted eggs and was not dependent on the eggs amount. There is a slight decrease in GFP, CystA and CystB groups after the 1st exposure.*



The following three graphs (Fig. 64-66) show how the titer of antibodies changed during the experiment.

The titer measured after the 2nd vaccination is not shown. The dilution of sera during ELISA was too low to reach the linear trend of absorbance and thus it was not possible to find the exact value of the titer. We estimated the titer to be greater than 200 000 in most of the cases.

The shown values in the graphs are coming:

a) After the 3rd immunization with the various antigens

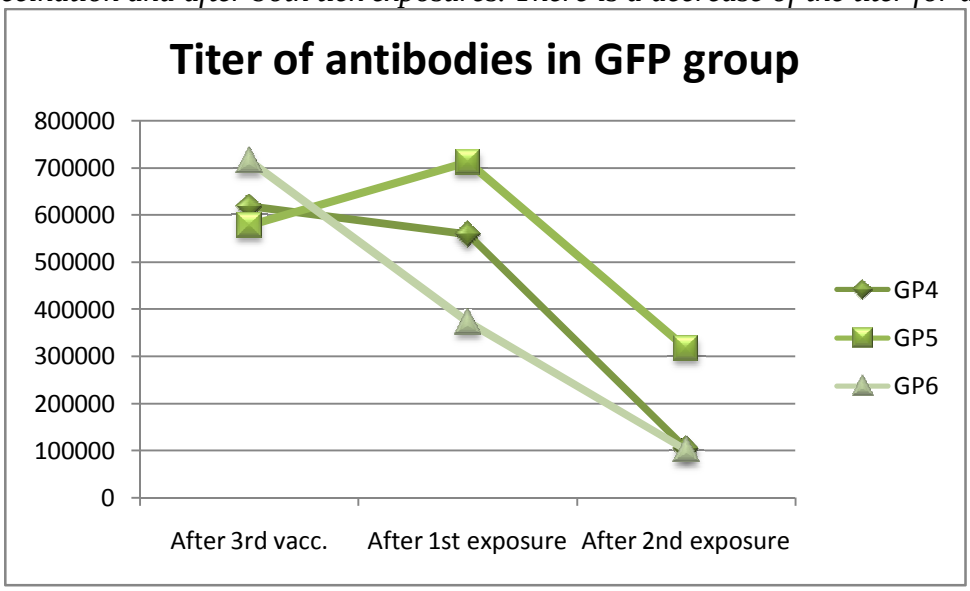
b) After the 1st exposure to ticks

c) After the 2nd exposure to ticks

Values of the antibody titer for the GPs from the PBS group were the same as the values of the pre-immunization sera and are not shown in the graphs.

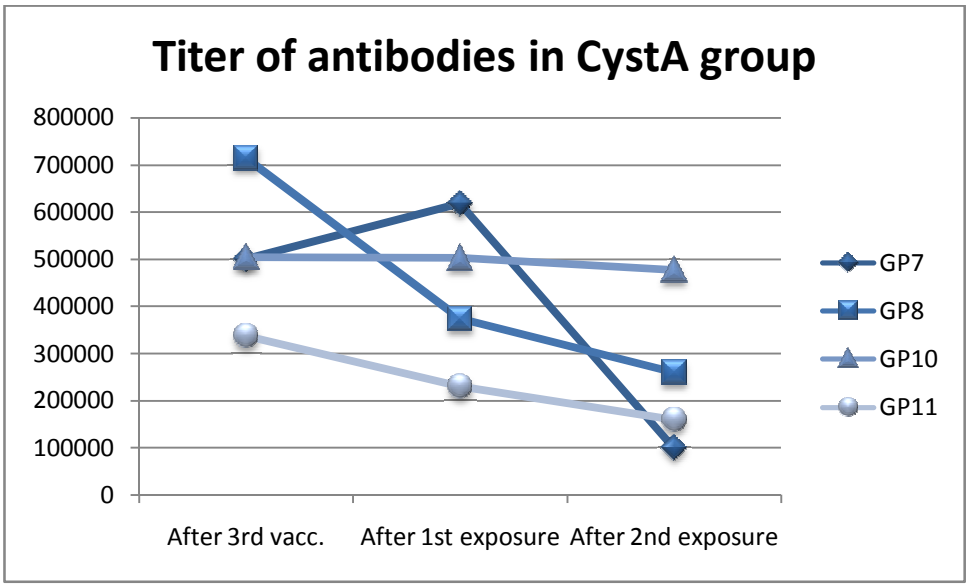
We can see a decrease of the anti-GFP antibodies in all 3 GPs. The titer decreased from 600-700 000 to 100 000 in GPs 4 and 6 or to 300 000 in GP 5. The antibody titer values are shown in figure 64. One exception is the course of titer in GP5 where it increased first and then decreased following the same slope as in the other GPs.

Figure 64: *The titer of antibodies in GFP group. The graph shows three time points – after the 3rd vaccination and after both tick exposures. There is a decrease of the titer for all 3 GPs.*



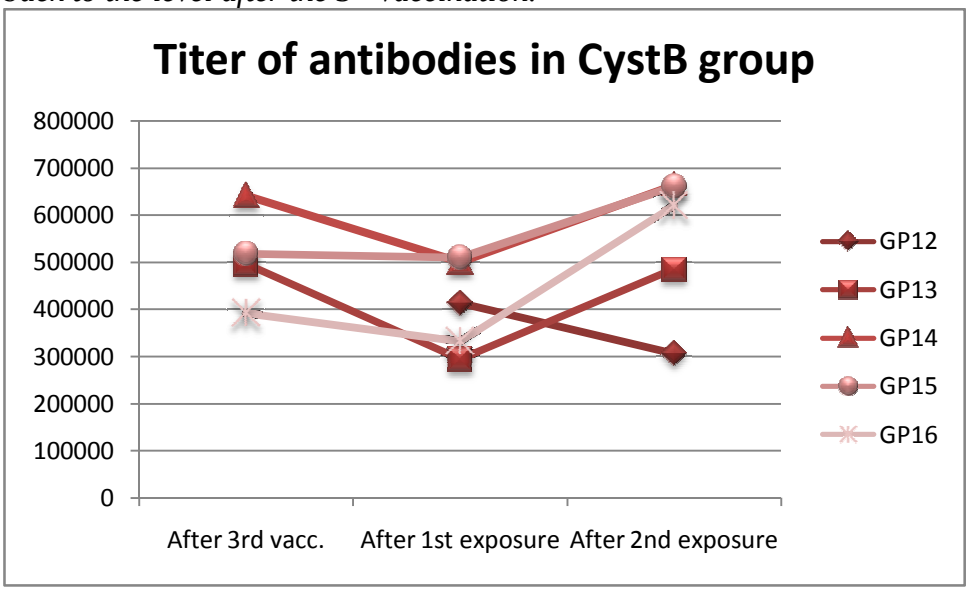
Values for the antibodies against CystA tend to decrease over time in all four tested animals. The titer of anti-CystA antibodies started at values between 300 and 700 000 (after the 3rd immunization) and dropped to 100-500 000 after the last exposure. One exception is the course of titer in GP7 where it increased first and then decreased rapidly. The values are shown in figure 65.

Figure 65: *The titer of antibodies in the CystA group. The graph shows three time points – after the 3rd vaccination and after both tick exposures. There is a decrease of the titer for all 4 GPs.*



Values for the antibodies against CystB decreased after the 1st exposure in comparison to the values after the last immunization. They increased again after the 2nd exposure, approximately to the same level as they were after the last immunization. The value for GP 12 after the 3rd vaccination is missing because we were not able to take blood from this GP. CystB shows the highest antibodies titer of all three tested proteins – it never dropped below 300 000. The results are shown in figure 66.

Figure 66: *The titer of antibodies in CystB group. The graph shows three time points – after the 3rd vaccination and after both tick exposures. The titer decreases first but then returns back to the level after the 3rd vaccination.*



3.3. Saliva assisted transmission of *Borrelia*

We run q-PCR to find the amount of *Borrelia* (flagellin DNA) in mouse skin. DNA isolated from mouse skin was used as a template. The amount of flagellin gene copies was proportional to the amount of *Borrelia* spirochetes. The amount of flagellin gene copies was compared to mouse actin gene copies as well as to the mass of skin (in mg) where DNA was isolated from.

The data show that the ratio between *Borrelia* flagellin genes and mouse actin genes varies from 40 to 110 (Fig. 67). We can see almost no difference between TBS, OVA and CystA samples after comparison to actin (Fig. 67). CystB sample shows higher *Borrelia* numbers but with a high SEM and no statistical significance at $p < 0.05$.

***Figure 67**: The comparison of Borrelia per the amount of mouse actin genes. TBS is the no protein control group, OVA the unspecific protein control group, CystA and CystB are the tested samples with inhibitors.*

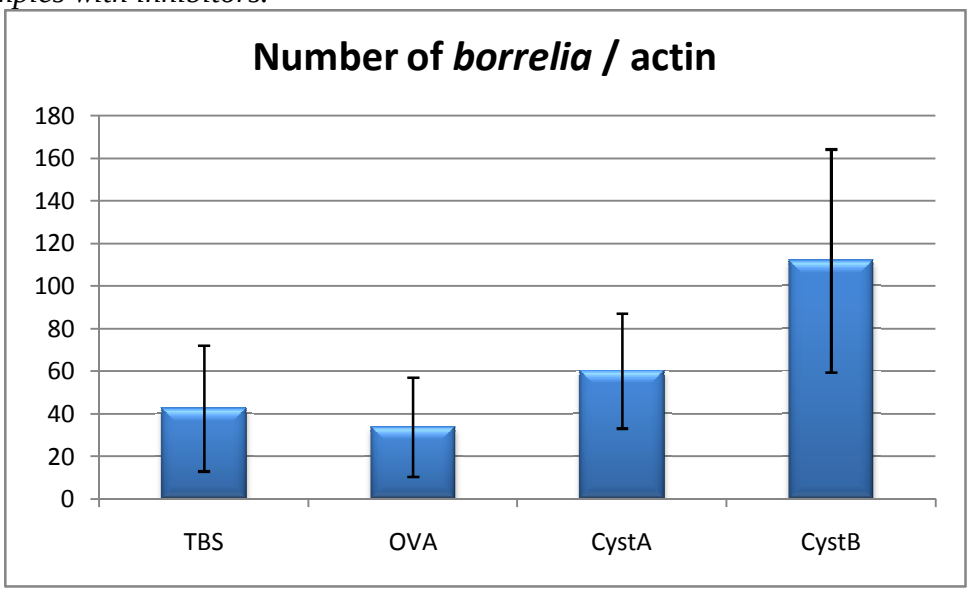
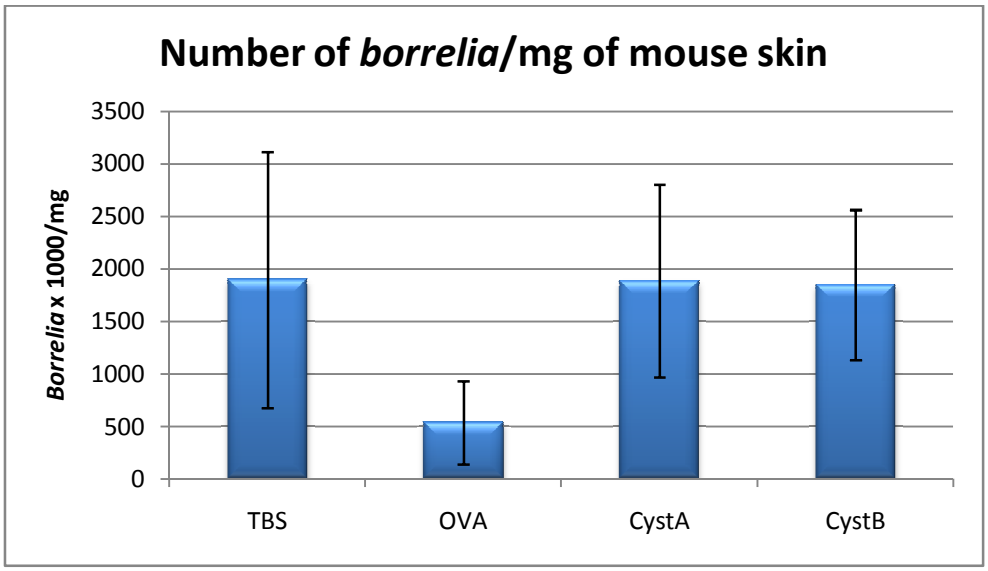


Figure 68 shows that the average amount of *Borrelia* was ca 1.8 million per 1mg of skin in mice injected with TBS, CystA or CystB. There was a significant reduction of *Borrelia* in mice treated with the control protein – ovalbumin; the amount of *Borrelia* is only 500 000 per one milligram of tissue. None of these results is statistically significant at $p < 0.05$.

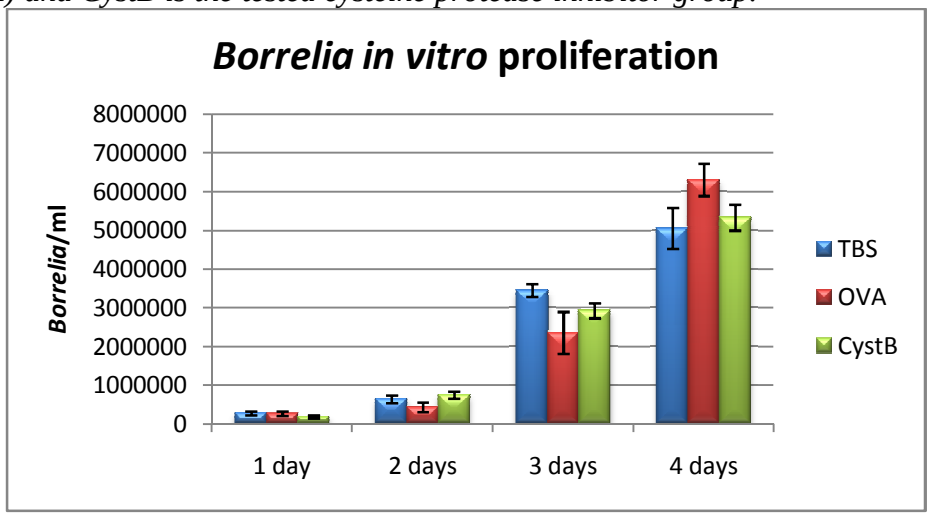
Figure 68: *The comparison of Borrelia per milligrams of mouse skin. TBS is the no protein control group, OVA the unspecific protein control group, CystA and CystB are the tested samples with inhibitors.*



3.4. *In vitro Borrelia* proliferation

We next tested in vitro for a potential effect of the CystB cysteine protease inhibitor in *Borrelia* proliferation as the time passes (Fig. 69). In all the experimental groups, the number of spirochetes increased from less than 200 000 / ml to 5-6 million / ml between days 1 and 4. The number of living *Borrelia* decreased almost to zero 7 days after the initiation of the experiment and it is not shown in the graph. There is no statistically significant effect of the tested protease inhibitor CystB after any of analyzed days. *Borrelia* numbers in the CystB group reach the same values during all day as both control groups.

Figure 69: *The amount of Borrelia per ml after an in vitro cultivation during four days after inoculation. TBS is the no protein control group, OVA is the unspecific protein control group (ovalbumin) and CystB is the tested cysteine protease inhibitor group.*



3.5. Cells activation

The results for macrophages, neutrophils and monocytes activation are presented as a ratio of the amount of pro-inflammatory gene transcripts between a non-activated group of cells shown as “No treatment control” (NT) and the amount of transcripts in tested groups after cell activation. The value for this NT was set to 1 in all cases (negative control) to make the comparison easier and it is not shown in the graphs. Bars in the graphs labeled as “LPS only” or “PMA only” serve as a positive control of activation. Bars CystB, SialoL, SerB and IRS2 represent activated cells treated with the tested proteins as described in the Materials and Methods. The values in the graph show the amount of transcripts in an activated sample normalized for the NT.

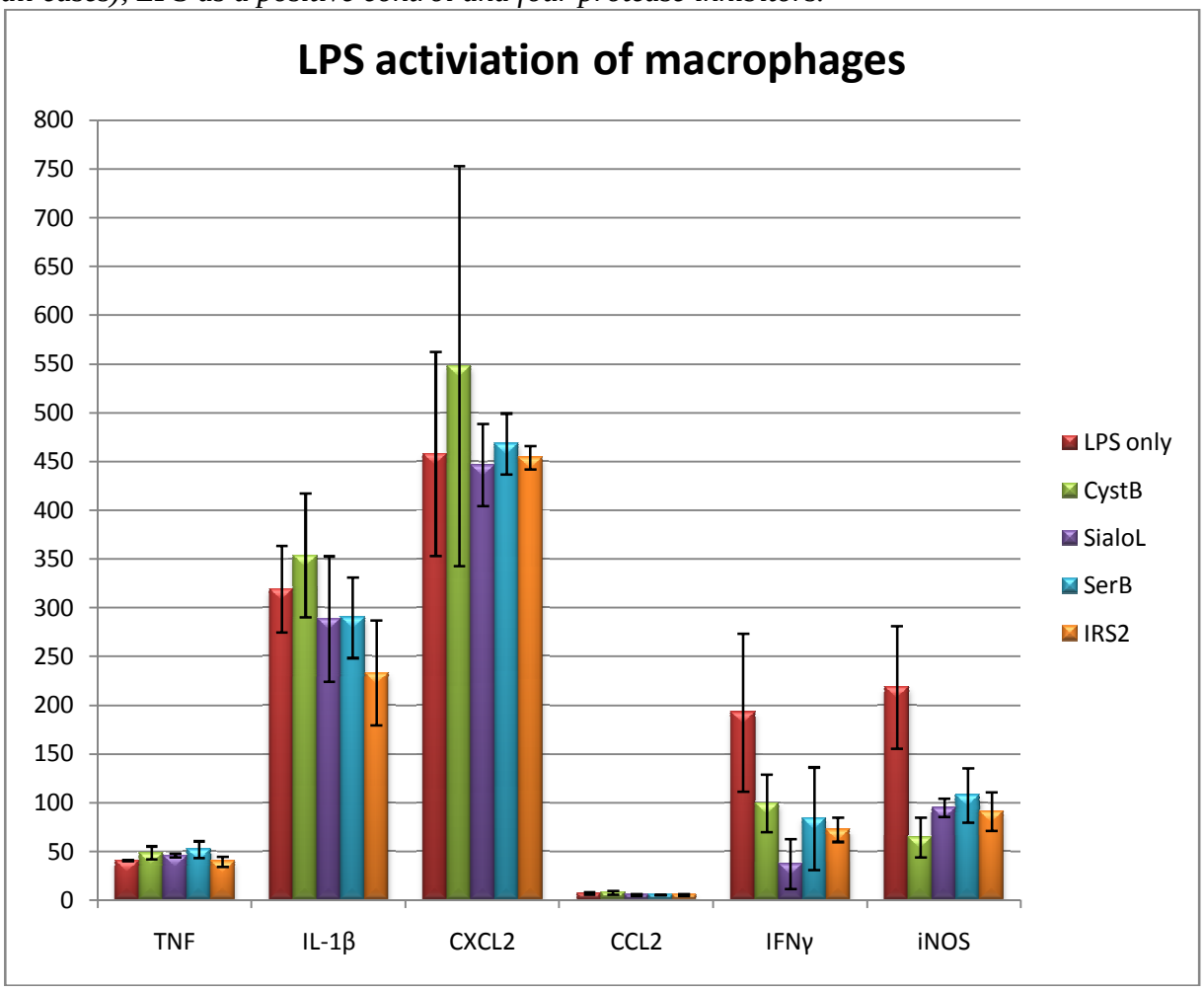
All values were calculated twice – using two different housekeeping genes (18S and RPS29) for equalizing the RNA isolation quality. Both housekeeping equalizations provide the same results; only one housekeeping gene graph is presented in this thesis.

3.5.1. Macrophages

Macrophages were activated by two different ways - using either LPS or PMA.

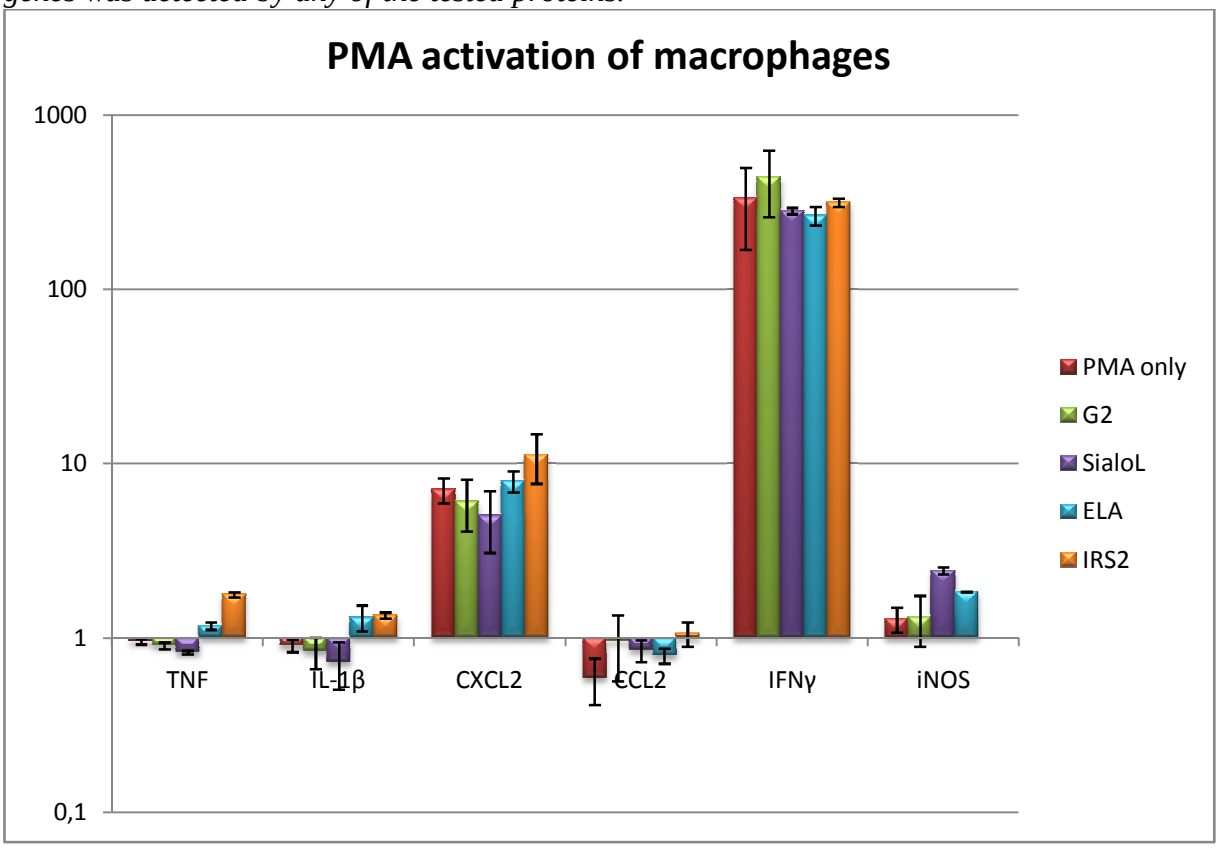
LPS activated all tested genes except CCL2. There was no significant inhibition in samples where TNF, IL-1β or CXCL2 were measured. Transcription of TNF was enhanced 50x, IFNγ and iNOS 200x, IL-1β 300x and CXCL2 450x in positive controls. The transcription of IFNγ and iNOS was inhibited to 50-25% by all tested proteins. The results are however not statistically significant, probably because only duplicate repeats for each tested sample. The results are shown in figure 70.

Figure 70: *The LPS activation of six different mice pro-inflammatory genes in macrophages. Six groups were tested: No treatment as a negative control (not shown, value set to 1 in all cases), LPS as a positive control and four protease inhibitors.*



PMA activated only the genes CXCL2 and IFNγ. Transcription of CXCL2 was enhanced 10x, transcription of IFNγ330x in positive controls. In all the other cases there is almost no difference between a negative control, positive control and the samples. Unfortunately no inhibitory effects can be seen, the amount of transcripts is the same in all groups including the positive control. The results are shown if figure 71.

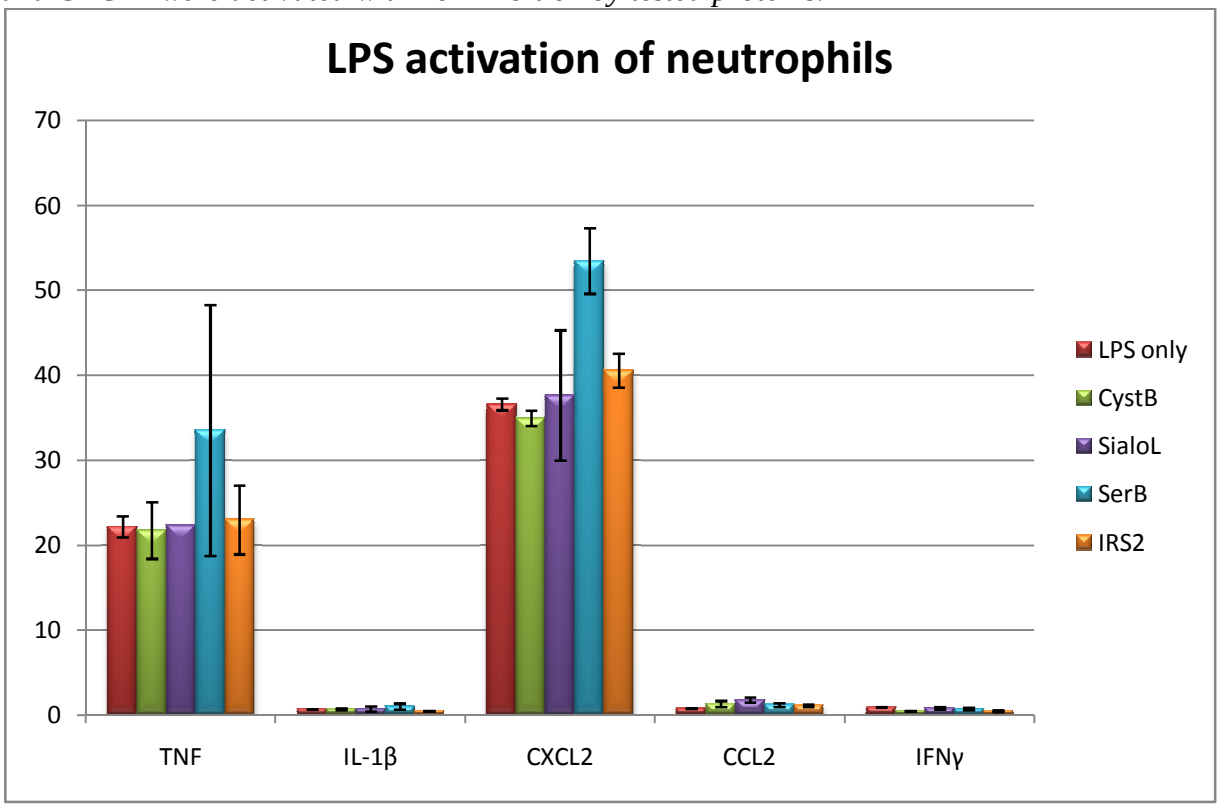
Figure 71: *The PMA activation of six different mice pro-inflammatory genes in macrophages. Six groups were tested: No treatment as a negative control (not shown, value set to 1 in all cases), LPS as a positive control and four protease inhibitors. Only genes CXCL2 and IFNγ were activated in the positive control and no inhibition in the activation of both these genes was detected by any of the tested proteins.*



3.5.2. Neutrophils

LPS activated only TNF and CXCL2 genes in neutrophils. Transcription of TNF was enhanced more than 20x ant the transcription of CXCL2 almost 40x in positive controls. We can see no inhibitory effect of the tested proteins on the transcription of these two neutrophil genes – there is no difference between the positive control and tested samples. SerB even seems to enhance the CXCL2 transcription by ca 25% but not significantly according to *ANOVA*. Transcription of IL-1β, CCL2 and IFNγ was not triggered by LPS. Results are presented in figure 72.

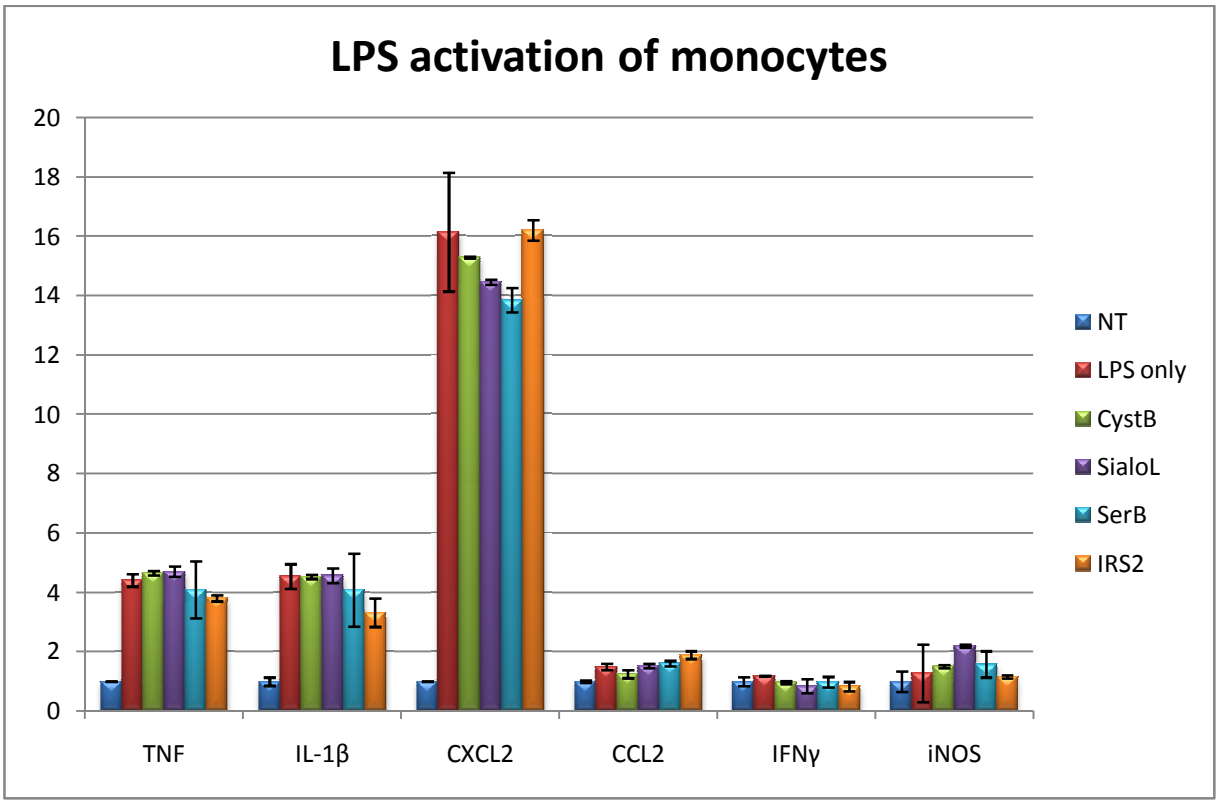
***Figure 72**: The LPS activation of five different mice pro-inflammatory genes in neutrophils. Six groups were tested: No treatment as a negative control (not shown, value set to 1 in all cases), LPS as a positive control and four groups with protease inhibitors. Only genes TNF and CXCL2 were activated with no inhibition by tested proteins.*



3.5.3. Monocytes

LPS activated TNF, IL-1β and CXCL2 genes in monocytes. Transcription of TNF and IL-1β increased 4x, transcription of CXCL2 16x in comparison to the NT group. CCL2, IFNγ and iNOS genes were not activated. There is no statistically significant inhibition apparent in the expression of any of these genes by any of tested proteins (Fig. 73).

Figure 73: *The LPS activation of six different mice pro-inflammatory genes in monocytes. Six groups were tested: No treatment as a negative control (value set to 1 in all cases), LPS as a positive control and four protease inhibitors. Only genes TNF, IL-1βand CXCL2 were activated with no inhibition by tested proteins.*

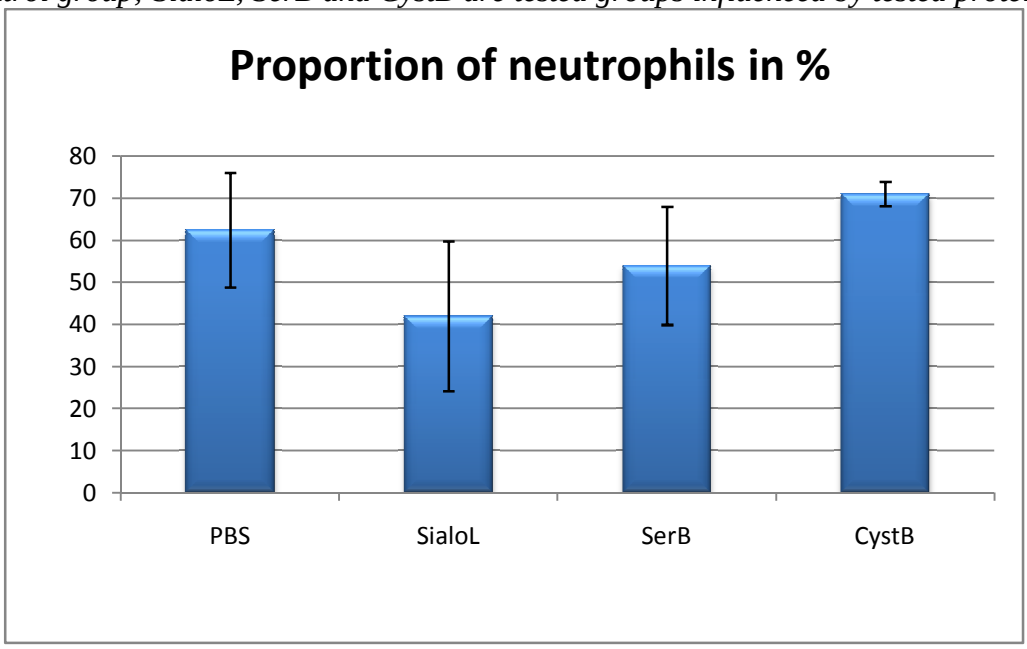


3.6. LPS induced lung inflammation and collecting BAL

Neutrophils were the only isotype analyzed in this experiment. The PBS group is a positive control with no protein inhibiting the inflammatory reaction; the other samples are with tested proteins (SialoL, SerB, CystB).

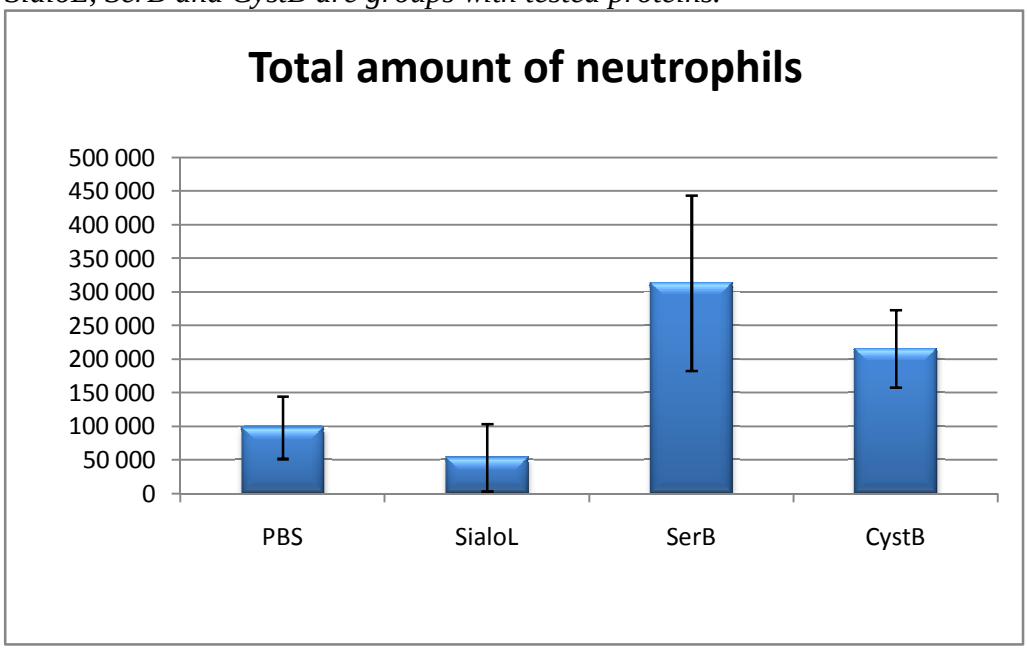
Based on the FACS data we show the graph of percentage of neutrophils among the other cells of the immune system in lungs. There are no significant differences among groups. We can see that the proportion of neutrophils is 40-70% in all the cases with big error bars. We did not find any impact of the tested proteins on the neutrophil proportion among other cells. The results are shown in figure 74.

Figure 74: *The ratio of neutrophils among other cells in lungs after the inflammation. PBS is the control group; SialoL, SerB and CystB are tested groups influenced by tested proteins.*



The second graph (Fig. 75) shows the total amount of neutrophils isolated from the lungs. Cells were counted under a microscope and the results were then adjusted based on the neutrophils proportion in each group. The total amount of neutrophils differs a lot. SerB and CystB groups show a higher amount than SialoL and a control group.

Figure 75: *The amount of neutrophils in lungs after the inflammation.PBS is the control group; SialoL, SerB and CystB are groups with tested proteins.*



3.7. Thioglycolate induced peritonitis

We estimated the effect of SialoL, SerB and CystB on the proportion of neutrophils, monocytes and macrophages after causing an inflammation in peritoneum. We can see that neutrophils form the main part of all cells of the immune system in this animal model of acute inflammation (40-60% of cells in all tested groups). The percentage of neutrophils compared to PBS group is a little higher in all groups treated with a protein (Fig. 76). Monocytes and macrophages only contribute to approximately the 15% of the total population (each of them). The abundance of macrophages in SialoL and SerB groups is more than 2x lower than in PBS and CystB groups. The presence of monocytes is not affected by the tested proteins at all. Unfortunately none of these results is statistically significant; mainly because of the high variance among the different experimental samples.

Figure 76: *Ratio of neutrophils, monocytes and macrophages in peritoneum after the peritonitis. All three isotypes were tested in four groups – PBS as a control and groups treated with SialoL, SerB and CystB.*

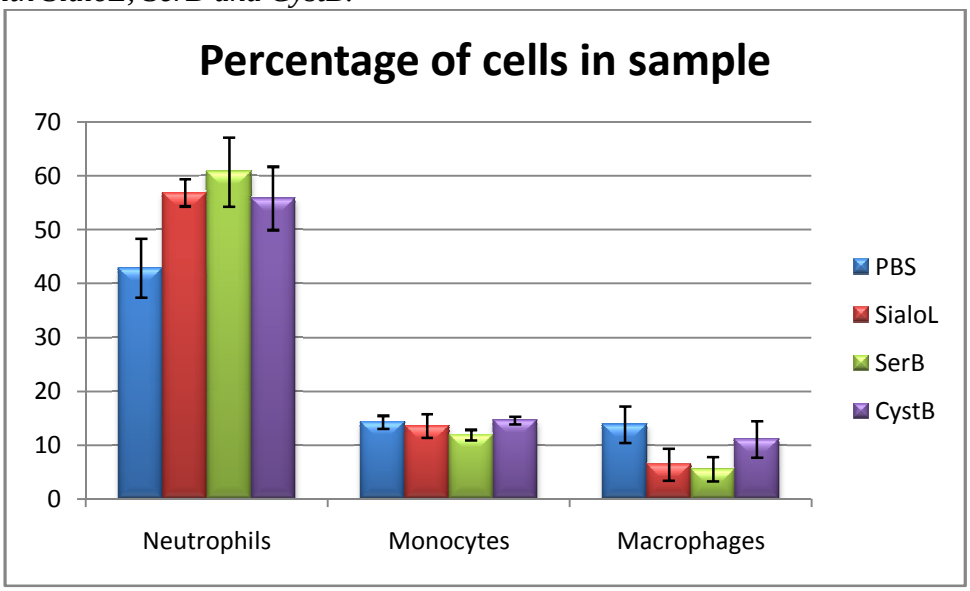
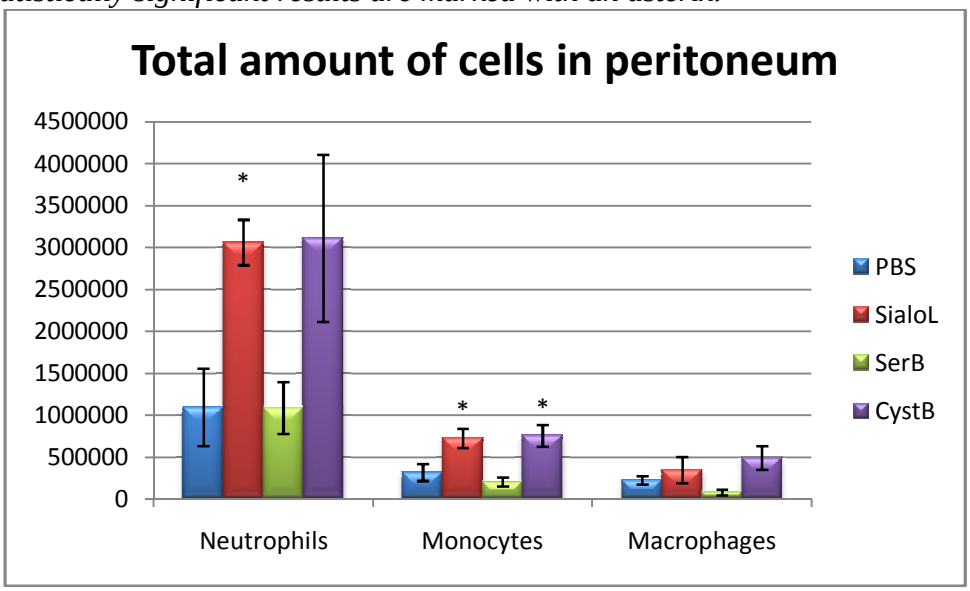


Figure 77 shows the total amount of neutrophils, monocytes and macrophages present in the peritoneum. The total amount of all cells was higher in SialoL and CystB groups; no matter what type of cells it was. Neutrophils are the most abundant isotype of all, followed by monocytes and macrophages (as shown also in Fig. 76). We found a statistically significant increase in the amount of neutrophils after treatment with SialoL and of monocytes after treatment with SialoL and CystB.

Figure 77: *The amount of different isotypes in peritoneum after peritonitis. All three isotypes were tested in four groups – PBS as a control and groups treated with SialoL, SerB and CystB. Statistically significant results are marked with an asterix.*



4. Conclusion

4.1. Protein expression

We have seen that all four produced proteins were overexpressed well in the *E.coli* bacterial strain BL21(DE3)pLysS. This was the first strain tried in all cases and no others were used because of a good result with this one. Especially the overexpression of SerC was very strong.

4.2. Refolding

As mentioned above, optimization of protein refolding was the main part of this thesis. It has been confirmed that even two proteins from the same protein family from the same organism are refolded in a very different way as described by Rudolph (1996) or Bird (2004). Various approaches were used to identify best refolding conditions for each protein since all four proteins were overexpressed as aggregates in bacterial inclusion bodies. Every buffer component was analyzed by running samples of the refolded proteins for each different refolding condition in a gel, by measuring the total protein concentration in the refolded protein solution and by measuring the amount of aggregated protein in the same solution. Protein activity measurements were also employed in the final stages of optimization. However we were not able to achieve a yield of refolded protein in the range of ten percent refolding efficiency like Hevehan and Clarke (1997) or Katoh *et al.* (2000).

Using disulfide shuffling reagents like GSH/GSSG at low concentration might help in the future. GSSG promotes the disulfide bond formation between two cysteins. GSH can break incorrect disulfide bonds and allows the cysteins to pair again correctly (Yamaguchi *et al.*, 2013). This approach might be effective especially for the cysteine protease inhibitors which possess four cysteins each.

4.2.1. Cysteine protease inhibitors

Both refolded cysteine protease inhibitors have four cysteins forming two disulphide bonds (Bulaj 2005).This could mean that the observed difference in the refolding efficiency of the two cysteine protease inhibitors of this study has another reason than the number of cysteins. CystA needs only 100mM NaCl (Fig. 19) in the refolding buffer while CystB 300mM. The ideal pH also differs – 9.5 for CystA (Fig. 22) and 8.5 for CystB (Fig 25). It actually means that the concentration of OH^- ions is 10x lower! Based on these differences in the refolding buffers of the two proteins we can conclude that ionic interactions play a different role in their refolding.

The initial inclusion bodies concentration upon refolding was also very different for the two proteins. CystB was successfully refolded at 2mg/ml (weight of wet inclusion bodies per ml of refolding solution) (Fig. 26). CystA was refolded at 16x lower concentration (Fig. 16) to reduce the precipitation; unfortunately without a big success. The CystA concentration (125µg/ml) almost reached the optimal value suggested by Rudolph *et al*. (1996) or Hevehan and Clarke (1997) which lies between 10 and 50µg/ml. This difference suggests that intramolecular interactions upon refolding leads to the aggregation observed in the case of CystA which is not the case for Cyst B.

Furthermore CystB did not require any L-arginine in the refolding buffer which suggests that the refolding intermediates were stable and changed their conformation correctly to the native state. CystA formed many precipitates even after the addition of 300mMarginine (Fig. 12) and was never refolded with a good yield.

Refolding buffers of CystA and CystB share one important fact. Their pH was higher than the calculated protein pI. Protein molecules were negatively charged which helped them to fold (Coutard *et al.,* 2012). Opposite charges or partial charges on the molecule apparently attracted each other and shaped the correct final conformation. Coutard *et al.* also claim that the protein refolding efficiency increases with the difference between protein pI and the pH of refolding buffers. The ideal difference is at least 1. This rule is valid also for refolding of proteins in buffers with pH lower than their pI.

Refolding of CystA is a strongly pH dependent process. Three pH optimizations were done in total all showing that CystA is refolded best at pH 9.5-10 but the efficiency drops to less than $^1/_3$ when going down to pH 9.0 or even 8.5 (Fig. 7, 9, 11).From all this we can claim that the refolding intermediates of CystA were very unstable with tendencies to misfold or aggregate. Even much effort to optimize the conditions did not help to refold this protein at a high yield.

4.2.2. Serine protease inhibitors

Unlike the cysteine protease inhibitors, SerA and SerC only have one and none cysteine in their amino acid sequence. Therefore there are no disulfide bonds in their molecules (Bulaj 2005).

Their refolding buffers share high arginine content and pH near neutral 7 (Fig. 39, 56). Even buffers with quite low pH charge the proteins negatively; the pI of both serine protease inhibitors is lower by at least 1 than pH of the corresponding refolding buffer which favors good refolding (Coutard *et al.,* 2012). The pI of SerA is 5.87; pI of SerC is 5.63 according to the server expasy.org/protparam.

The buffers differ one from another in the NaCl concentration. Where SerA needs only 50mM NaCl (Fig. 36), SerC needs a bigger ionic strength in a high salt buffer with 300mM NaCl (Fig. 46).This difference suggests a difference in the role of ionic interactions upon refolding of both proteins. They were both refolded at a moderate to low initial IB concentration – 0.6 and 0.25mg/ml (Rudolph *et al.*, 1996).

Arginine was important for both serine protease inhibitors to slow down the refolding kinetics (Fig. 33 and 52) and helped the intermediates to fold to the native state as described by Bird *et al*. (2003).

Refolding of SerA is significantly dependent on the initial IB concentration. The IB concentration 0.6mg/ml gives much better protein yield than 1.3mg/ml or 0.3mg/ml (Fig. 30). In the case of cysteine protease inhibitors the amount of refolded protein did not drop with increasing initial IB concentration which was the case for SerA. It is possible that when going to concentrations like 1.3mg/ml, SerA forms dimmers by formation of a disulfide bond between cysteins in each molecule (Bulaj 2005). Refolding of SerC is also strongly IB concentration dependent (Fig. 49). The trend is similar to SerA but the best initial IB concentration is lower.

Other refolding conditions, except arginine and protein concentration, did not play any big role in the refolding kinetics as shown in figures 36, 39, 46 and 56.

The extensive optimization of refolding conditions lead to an increased refolding efficiency for both serine protease inhibitors.

In all the cases the results gained from the optimizations in small scale promised significantly better result than what was the real yield after the big scale refolding. Some of the protein was definitely lost during the concentration from liters of refolding buffer to 10-20ml of the final protein solution. This step was time consuming so proteolysis could have been a reason. Another one was most likely the additional precipitation of proteins at the filtration/concentration membrane, although the sample was always stirred or under continuous flux to avoid protein overconcentration in the vicinity of the concentration filter.

4.3. Vaccination

We did not find any effect on the tick feeding or reproducing ability when vaccinating guinea pigs with CystA or CystB.

The ticks mass after feeding differed a lot between the first and second exposure. Ticks after the second exposure were three times smaller even in the control group. This effect was most likely caused by wounds and inflammation at the feeding site caused by the first exposure and preventing ticks from normal feeding. GPs could have also evolved an adaptive immune response after the first exposure to ticks as described by Brossard *et al.*, (1997). This reaction would prevent ticks from proper feeding.
No differences between the tested groups were found except the GFP group after 2nd exposure where the mass of ticks was lower (Fig. 60). This decrease in mass can originate in as a coincidence and obviously it does not have biological significance.

There was absolutely no difference in the time needed to finish feeding. In all cases ticks needed ca 8 days to engorge. We also found that the amount of eggs was lower after the second exposure. The mass of ticks plays most likely a big role here. Smaller ticks were not able to produce so many eggs. The difference is however much smaller than in the case of the mass comparison. Again there were no differences between the tested groups.

Molting of eggs was also a little better after the first exposure; especially in the control groups. The value after the first exposure in the PBS control group is ca 10% higher than in the other groups. This may be caused by an unspecific reaction of adjuvant and a protein which somehow affected the GPs. The 10% decrease in GFP group after the 2nd exposure can be caused by an inaccuracy of scoring or by very few ticks attached to GP6 which were all also very small and poor egg layers.

The titer measurements show that anti-GFP and anti-CystA antibodies vanish over time. The anti-CystB antibodies level remained the same until the end of experiment. It is possible that CystB protein was recognized by GPs in tick saliva and the production of antibodies continued. The titer increased almost twice in the study done by Kotsyfakis *et al.* (2008) which shows that the effect of CystB is still weak. The antibody titer results suggest that these cysteine protease inhibitors might be expressed in different experimental conditions such as in the salivary glands from nymphal ticks or adult ticks feeding on a rabbit or a mouse. It could be also that their expression is higher in the tick midgut. Further protein expression studies in tick tissues (preferably with antibodies against the proteins) are necessary for us to conclude about the observed effect of tick feeding in the antibody titers for these two cysteine protease inhibitors.

Indeed another strategy to investigate the effect of CystA and B could be an exposure of vaccinated animals to nymphs of *I.ricinus*. Salat *et al*. (2010) found out that vaccination of mice with Om-cystatin 2 does not affect feeding of adult ticks but significantly increases the post-engorgement mortality of nymphs. Another tick salivary cysteine protease inhibitor - Sialostatin L2 from *I.scapularis* decreased the body weight of engorged nymphs feeding on guinea pigs (Kotsyfakis *et al*., 2008).

4.4. Saliva assisted transmission

We did not find any effect of either of the tested proteins (CystA and CystB) on *Borrelia* transmission and *in vivo* proliferation when compared to two control groups. The comparison of flagellin DNA to mouse actin genes did not confirm any decrease or increase in *Borrelia* numbers. CystB seems to enhance *Borrelia* proliferation but with no statistical significance. Unlike Salp15 (Schuijt *et al*., 2008) it does not protect *Borrelia* from the serum effectors. Overall the comparison of our data to the effect of the tick saliva in the same experimental set-up (Machackova *et al*., 2006) shows that these proteins do not play any significant role in *Borrelia* transmission.

The comparison of flagellin DNA to milligrams of mouse tissue shows that CystA and CystB were comparable to the no protein control (TBS). The big decrease in *Borrelia* numbers by ovalbumin could have theoretically been caused by contamination of ovalbumin which would evoke a stronger immune response in mice. Another explanation could be the worse quality of DNA isolation or simply a coincidence. This difference cannot be considered significant also due to a big variance among samples and thus a big SEM.

4.5. *In vitro Borrelia* proliferation

We have proven that CystB does not affect *Borrelia in vitro* proliferation in any way. Other experiments would be needed to really prove that CystB does not alter *Borrelia* surface; for example employing serum co-incubation (Schuijt *et al*., 2008; Kenedy and Atkins, 2011)

We also confirmed the fact that *Borrelia* do not grow well in the small volume we used in our experiments since there were no living bacteria after 7 days of incubation in any of the tested samples.

Visit in an Institute abroad during my Master thesis:

All the following experiments were done during my stay in Dresden under the supervision of Jindra Chmelař. Some of them provided results which need further verification. This was unfortunately not possible due to a lack of time since the aim of my visit in Dresden was to familiarize myself with as many different animal models and immune cell populations so that I continue working with the specific methodology during my potential PhD studies in the same field.

4.6. *In vitro* activation of macrophages

LPS was shown to be a stronger activator of inflammatory genes in macrophages than PMA. LPS (Fig. 70) activated five of the tested genes while PMA (Fig. 71) only two. Only the activation of IFNγ gene is at a similar level with both stimuli.

Although we achieved *in vitro* activation of macrophages, we found only two anti-inflammatory effects of the tested proteins – in the IFNγ and iNOS production by LPS-activated macrophages. This inhibition is however caused by all tested proteins which may indicate a mistake in a positive control or an unspecific protein inhibition. However according to *ANOVA* test this result cannot be considered significant. Further experimental optimization such as testing the effect of cell pre-incubation time with the protease inhibitors in the observed effect would be needed to verify these data.

Macrophages were activated by LPS much stronger than neutrophils or monocytes. This could show that they are the main defense cells against bacterial infection. According to the data we can imply that IL-1β and CXCL2 are the main genes activated by LPS. PMA is a strong activator of IFNγ gene.

The next steps could involve testing for an effect in the activation of more genes involved in the immune function of the specific cell types, other ways of MF activation, isolating RNA at a different time points (e.g. 24 and 48h) or testing the effect of a protein in different concentrations.

4.7. *In vitro* activation of neutrophils

Although we achieved *in vitro* activation of neutrophils, we did not find any anti-inflammatory effects of the tested proteins (Fig. 72). Only two of the five tested genes were successfully activated by LPS (TNF, CXCL2). Testing more genes which are activated by LPS might give us a better understanding of the potential effect of these proteins. Stronger neutrophil activation by LPS in the presence of SerB can be explained by better quality RNA

isolation from SerB treated sample or by LPS contamination of SerB which would enhance the immune response stronger than in the samples treated with other proteins.

Next approaches to this experimental model could involve: Testing the activation of other genes in neutrophils in the presence of LPS, trying another way of neutrophil activation or different concentrations of LPS, incubation with the protease inhibitors for 24 or 48h after the neutrophil activation instead of 4h or testing different protein concentrations and pre-incubation of neutrophils for various time points with the protease inhibitors before the addition of LPS.

4.8. *In vitro* activation of monocytes

We found that neither of the tested proteins affects the transcription of six chosen pro-inflammatory genes in monocytes (Fig. 73).

The level of activation by LPS was much lower than in macrophages, which suggests that monocytes are less important in the immune response to bacteria.

Only transcription of CXCL2 was activated considerably by the presence of LPS, TNF and IL-1β were activated weakly and the other genes not at all.

Next approaches could involve: Testing the activation of other genes in monocytes in the presence of LPS, trying another way of monocyte activation or different concentrations of LPS, incubation with the protease inhibitors for 24 or 48h after the monocyte activation instead of 4h or testing different protein concentrations and pre-incubation of monocytes for various time points with the protease inhibitors before the addition of LPS.

4.9. LPS induced lung inflammation

We have found that the percentage of neutrophils recruited in the inflamed lungs does not differ among the different experimental groups (Fig. 74). The total amount of neutrophils (Fig. 75) was significantly affected by the total amount of cells counted using a microscope because the proportion of neutrophils is quite the same in all four cases. It seems that SerB and CystB have even pro-inflammatory effect – the total amount of neutrophils is significantly higher in these two samples. I believe that this effect was mainly caused by really big differences in an amount of cells (and thus neutrophils) after counting under a microscope; even within a group. In general no anti-inflammatory effect of CystB, SialoL or SerB was found.

The experiment could be expanded by using various approaches. A negative control of inflammation is one of them. Also testing other cell populations like monocytes or

macrophages would give more information, as well as collecting the cells at different time points.

4.10. Thioglycolate induced peritonitis

We have confirmed that neutrophils are the first isotype that infiltrates the inflammation sites (after 4h in this experiment). Sialostatin L and SerB may have an effect on macrophage recruitment by lowering their proportion to ½ in comparison to no treatment control (Fig. 76). We have also found significantly higher total amount of cells in mice treated with Sialostatin L or CystB (Fig. 77). The general conclusion is that the tested proteins either do not have anti-inflammatory properties on this mouse model or our experimental design due to time limitations did not reveal them.

Although no scientifically important discovery was done during the years of working on this thesis now we have produced four novel protease inhibitors from *I.ricinus* which are ready to be tested in the future in many different assays. I have also learnt much about the host immunomodulatory assays that I can do with these protease inhibitors and I have produced the first data that suggest their function at the cellular level as potential immunomodulators.

5. References

Berenberg JL, Ward PA, Sonenshine DE. *Tick-bite injury: mediation by a complement-derived chemotactic factor.* J Immunol. 1972; 109:451–456

Bird PI, Pak SC, Worrall DM, Bottomley SP. Production of recombinant serpins in *Escherichia coli*. Methods. 2004; 32:169–176.

Bowman AS, Sauer JR. *Tick salivary glands: function, physiology and future*. Parasitology. 2004; 129(Suppl):S67–81

Brossard M, Wikel SK. *Immunology of interactions between ticks and hosts*. Med Vet Entomol.1997; 11:270–276.

Brown SJ, Askenase PW. *Immune rejection of ectoparasites (ticks) by T cell and IgG1 antibody recruitment of basophils and eosinophils*. Fed Proc. 1983; 42:1744–1749

Brusca R.C., Brusca G. J. *Invertebrates*. Sunderland: Sinauer Associates. 2003; chap. 19, p. 1-7

BulajG.*Formation of disulfide bonds in proteins and peptides*. Biotechnology Advances. 2005; Volume 23, Issue 1, Pages 87-92

CapineraJ.L.. *"Ticks (Acari: Ixodida)". Encyclopedia of Entomology, Volume 3 (2nd ed.)*. Springer. 2008; p. 3733–3802

Chmelar J, Oliveira CJ, Rezacova P, Francischetti IM, Kovarova Z, *et al*. *A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation.* Blood. 2011; 117: 736–44

Chmelar J, Calvo E, Pedra JH, Francischetti IM, Kotsyfakis M. *Tick salivary secretion as a source of antihemostatics*. J Proteomics. 2012; 75: 3842–3854

Delves PJ *et al*. *Encyclopedia of Immunology*. Academic Press. 1998; p. 332-34, 822-25, 1854-58

Francischetti I. M. B., Sa-Nunes A., Mans B. J., Santos I. M., Ribeiro J. M. C. *The role of saliva in tick feeding*. Front. Biosci. 2009; 14, 2051–2088.10.2741/3363

Gill HS, Walker AR. *Differential cellular responses at Hyalomma anatolicum anatolicum feeding sites on susceptible and tick-resistant rabbits*. Parasitology. 1985; 91(Pt 3):591–607.

Gill HS. *Kinetics of mast cell, basophil and eosinophil populations at Hyalomma anatolicum anatolicum feeding sites on cattle and the acquisition of resistance*. Parasitology. 1986; 93(Pt 2):305–315

Hevehan D.L., De Bernardez Clark E. *Oxidative renaturation of lysozyme at high concentrations*. Biotechnol.Bioeng. 1997; 54:221–230.

Hovius, J. W., van Dam, A. P., Fikrig E. *Tick-host-pathogen interactions in Lyme borreliosis.* Trends Parasitol.2007; 23(9), 434-8.

Hovius JW, de Jong MA, den Dunnen J, LitjensM, Fikrig E, van der PT *et al*. *Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization*. PLoS Pathog. 2008; 4:e31

Hovius JW. *Spitting Image: Tick Saliva Assists the Causative Agent of Lyme Disease in Evading Host Skin's Innate Immune Response*. Journal of Investigative Dermatology.2009; 129,2337–2339

Imamura S, Silva Vaz JI, Sugino M, Ohashi K, Onuma M; *A serine protease inhibitor (serpin) from Haemaphysalis longicornisas an anti-tick vaccine.* Vaccine. 2004; 23 1301–1311

Katoh, Y., Farshbaf, M., Kurooka, N., Nohara, D. *et al*., *High yield refolding of lysozyme and carbonic anhydrase at high protein concentrations*. J.Chem. Eng. Japan.2000; *33*, 773–777.

Kenedy M. R., Akins D. R. *The OspE-related proteins inhibit complement deposition and enhance serum resistance of Borrelia burgdorferi, the Lyme disease spirochete*. Infect. Immun. 2011;**79**, 1451–1457

Kopecky J, Kuthejlova M. *Suppressive effect of Ixodes ricinus salivary gland extract on mechanisms of natural immunity in vitro*. Parasite Immunol. *1998;* 20:169–74

Kotsyfakis M, Sa-Nunes A, Francischetti IM, Mather TN, Andersen JF, Ribeiro JM. *Antiinflammatory and immunosuppressive activity of Sialostatin L, a salivary cystatins from the tick Ixodes scapularis*.JBiol Chem. *2006;* 281:26298–307

Kotsyfakis M., Anderson J. M., Andersen J. F., Calvo E., Francischetti I. M., Mather T. N., *et al*. *Cutting edge: immunity against a "silent" salivary antigen of the Lyme vector Ixodes scapularis impairs its ability to feed*. J. Immunol. (2008); 181, 5209–5212.

Lindgren E., Tälleklint L., Polfeldt T. *Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick Ixodes ricinus*. Environmental Health Perspectives. 2000; 108 (2): 119–123.

Machackova M, Obornik M, Kopecky J. *Effect of salivary gland extract from Ixodes ricinus ticks on the proliferation of Borrelia burgdorferi sensu stricto in vivo*. Folia Parasitol.2006; 53:153–158

MansBJ.*Tick histamine-binding proteins and related lipocalins: potential as therapeutic agents*. Curr Opin Investig Drugs. 2005;6:1131–5

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. *The chemokine system indiverse forms of macrophage activation and polarization.* Trends Immunol.2004; 25:677–686.

Marchal CMP, Luft BJ, Yang X, Sibilia J, Jaulhac B, Boulanger NM. *Defensin is suppressed by tick salivary gland extract during the in vitro interaction of resident skin cells with Borrelia burgdorferi.* J Invest Dermatol; (2009)129:2515–7

Matsuda H, Fukui K, Kiso Y, Kitamura Y. *Inability of genetically mast cell-deficient W/Wv mice to acquire resistance against larval Haemaphysalis longicornis ticks*.J Parasitol.1985; 71:443–448.

Matsuda H, Nakano T, Kiso Y, Kitamura Y. *Normalization of anti-tick response of mast cell deficient W/Wv mice by intracutaneous injection of cultured mast cells*. JParasitol. 1987; 73:155–160

Mehlhorn F. *Encyclopedia of Parasitology 3rd ed.*Springer. 2008; p. 1378-1383

Metcalfe DD, Baram D, MekoriYA. *Mast cells*. Physiol Rev. 1997; 77:1033–1079.

Mulenga A, Sugimoto C, Onuma M; *Issues in tick vaccine development: identification and characterization of potential candidate vaccine antigens.* Microbes and Infection. 2000; 1353–1361

Nelson R, Sawaya MR, Balbirnie M, Madsen AØ, Riekel C, Grothe R, Eisenberg D. *Structure of the cross-beta spine of amyloid-like fibrils*. Nature. 2005; 435(7043):773-778.

Nithiuthai S, Allen JR. Langerhans cells present tick antigens to lymph node cells from tick sensitized guinea-pigs. Immunology.1985; 55:157–163.

Odemuyiwa SO, Ghahary A, Li Y, Puttagunta L, Lee JE, Musat-Marcu S, Ghahary A, Moqbel R. *Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase*. J Immunol. 2004; 173:5909–5913

Oliveira CJ, Cavassani KA, More DD, Garlet GP, Aliberti JC, Silva JS, Ferreira BR. *Tick saliva inhibits the chemotactic function of MIP-1alpha and selectively impairs chemotaxis of immature dendritic cells by down-regulating cell-surface CCR5*. Int J Parasitol. 2008; 38, 705–716

Paesen GC, Adams PL, Harlos K, Nuttall PA, Stuart DI. *Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure*. Mol Cell.1999; 3:661–671.

Ramachandra RN, Wikel SK. *Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production*. J Med Entomol. 1992; 29:818–26

Reddy, V.Y., Zhang, Q.Y., Weiss, S.J. *Pericellular mobilization of the tissue destructive cysteine proteinases, cathepsins B, L, and S, by human monocytes derived macrophages*. Proc. Natl. Acad. Sci. U. S. A. 1995; 92, 3849–3853.

Ribeiro J.M.C. *Role of saliva in blood-feeding by arthropods.* Ann. Rev. Entomol. 1987a; 463–478.

Ribeiro JM. *Ixodes dammini: salivary anticomplement activity*. Exp Parasitol. 1987b; 64:347–53

Ribeiro JMC, Weis JJ, Telford SR III. *Saliva of the tick Ixodes dammini inhibits neutrophil function.* ExpParasitol. 1990; 70:382–388

Ribeiro JM, Francischetti IM. *Role of arthropod saliva in blood feeding: Sialome and post-sialome perspectives.* Annu Rev Entomol.2002; 19:19.

Rudolph R, Lilie H. *In vitro folding of inclusion body proteins*. FASEB J. 1996; Jan;10(1):49-56.

Sa-Nunes A, Bafica A, Lucas DA, Conrads TP, Veenstra TD, Andersen JF, Mather TN, Ribeiro JM, Francischetti IM. *Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in Ixodes scapularis saliva*. J Immunol.2007; 179:1497–1505.

Sa-Nunes A., Bafica A., Antonelli L. R., Choi E. Y., Francischetti I. M. B., Andersen J. F. *The immunomodulatory action of Sialostatin L on dendritic cells reveals its potential to interfere with autoimmunity*. J. Immunol. 2009; 182 7422–7429

Salát, J., Paesen, G.C., Rezácová, P., Kotsyfakis, M., Kovárová, Z., Sanda, M., Majtán, J.,Grunclová, L., Horká, H., Andersen, J.F., Brynda, J., Horn, M., Nunn, M.A., Kopácek,P., Kopecky´ , J., Mares, M.,. *Crystal structure and functional characterization of an immunomodulatory salivary cystatin from the soft tick Ornithodoros moubata*. Biochem. J. 2010; 429, 103–112

Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA. *The neutrophil as a cellular source of chemokines.* Immunol Rev. 2000; 177:195–203.

Schuijt T. J., Hovius J. W., Van Burgel N. D., Ramamoorthi N.,Fikrig E., Van Dam A. P. *The tick salivary protein Salp15 inhibits the killing of serum-sensitive Borrelia burgdorferi sensu lato isolates*. Infect. Immun. 2008; 76, 2888–2894

Schwarz A, Valdés JJ, Kotsyfakis M, *The role of cystatins in tick physiology and blood feeding*. Ticks Tick Borne Dis. 2012; Jun;3(3):117-27

Steeves EB, Allen JR. *Tick resistance in mast cell-deficient mice: histological studies*. Int JParasitol.1991; 21:265–268.

Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. *Macrophage receptors and immune recognition*. Annu Rev Immunol.2005; 23:901–944.

Vray, B., Hartmann, S., Hoebeke, J. *Immunomodulatory properties of cystatins*. Cell. Mol. Life Sci. 2002; 59, 1503–1512.

Waladde SM, Kemp DH, Rice MJ. *Feeding electrograms and fluid uptake measurements of cattle tick Boophilus microplus attached on artificial membranes.* Int J Parasitol. 1979; 9:89–95.

Wikel S.K. *Host resistance to tick-borne pathogen by virtue of resistance to tick infestation*. Ann. Trop. Med. Parasitol.1980; 103–104.

Wikel S.K., Ramanchandra R.N., Bergman D.K., Burkot T.R., PiesmanJ. *Infestation with pathogen-free nymphs of the tick Ixodes scapularis induces host resistance to transmission of Borrelia burgdorferi ticks.* Infect. Immun. 1997; 65:335–338.

Willadsen P., Bird J., Cobon G.S., Hungerford J. *Commercialization of a recombinant vaccine against Boophilus microplus*, Parasitology.1995; 110 (suppl) S43–S50.

Yamaguchi S, Yamamoto E, Mannen T and Nagamune T. *Protein refolding using chemical refolding additives.* Biotechnol. J. 2013; 8, 17–31