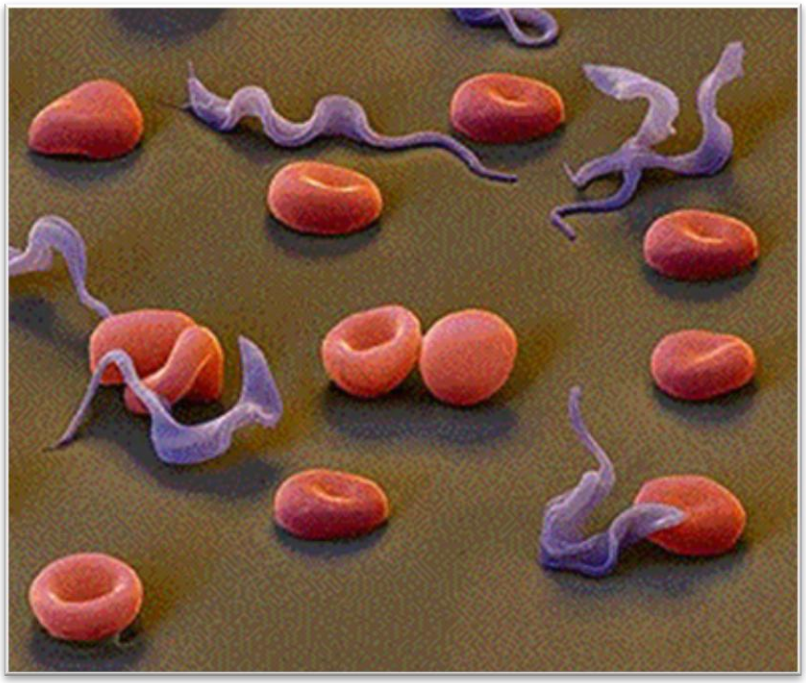
Cathepsin B-like Proteases

in *trypanosoma brucei*



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Cover picture: *trypanosoma brucei*

Trypanosoma brucei, the causative agent of African sleeping sickness, in the blood. The image is a false coloured scanning electron micrograph showing the trypanosome parasite, in purple, along with mammalian erythrocytes in red. At their most extreme, levels of parasite in the host blood can reach the levels indicated here, i.e. one parasite for about two erythrocytes. Image by Prof. Michael Duszenko, Tübingen, Germany.

Acknowledgements:

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My very special thanks go to *Rudolf Koopmann*, who was working on his doctoral thesis at this time, and who supervised me during my traineeship. Working with you was really fun.
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1 Introduction

Systematatic Classification		
	Domain:	Eukaryota
	Kingdom:	Protozoa
	Division:	Sarcomastigophorea
	Class:	Zoomastigophorea
	Order:	Kinetoplastida
	Family:	Tripanosomatida
	Genus:	*Trypanosoma*
	Species:	*T. brucei*
		T. congolese
		T. cruzi
		T. simiae
		T. vivax
	Subspecies:	*T. b. brucei*
		T. b. gambiense
		T. b. rhodesiense

Trypanosoma forms a notable genus of trypanosomes, which are a monophyletic group of unicellular parasitic protozoa. Their different species cause some severe diseases inside the tropical belt, such as human African sleeping sickness (*T.b.gambiense / T.b.rhodesiense*), Nagana pest or Gambia fever in cattle (*T.vivax / T.congolese*), pigs (*T.simiae*) as well as in horses, mules and cats (*T.b.brucei*). The World Health Organisation talks of about 400 thousand cases of human trypanosomiasis annually, and up to 3 million cases per year of bovine trypanosomiasis. As the dispersal of the disease is limited to the developing world, pharmaceutical industry is not interested in developing modern remedies in cost-intensive processes. Therefore, research on trypanosoma concentrates on academic facilities. As a result, there are only few medications available that often show low efficiency and strong side-effects. African sleeping sickness belongs to the group of the so-called most neglected diseases: only 10% of the world-wide research is being spent into the investigation of illnesses that cause about 90% of the global infections.

Trypanosomes undergo a very complex lifecycle in different hosts, changing their morphological form (Figure 1), especially the position of its flagellum. They may occur as **amastigotes** (lacking a flagellum), **promastigotes** (free flagellum anterior of the nucleus), **epimastigotes** (having the flagellum starting from anterior of the nucleus connected by a short undulating membrane) and **trypomastigotes** (posterior of the nucleus, connected by a long undulating membrane).

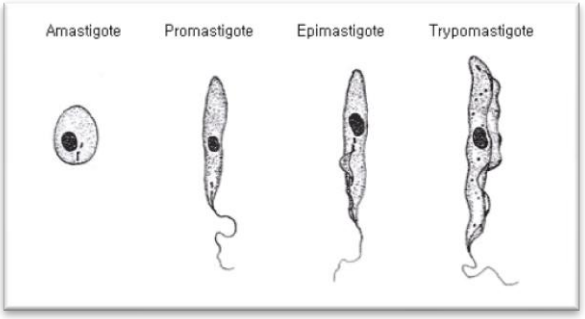

Figure 1: Morphological forms of trypanosome

Trypanosoma can appear in all five forms, with the trypomastigote stage occurring in the vertebrate host. The subspecies of *T.brucei* have two forms in the mammalian bloodstream, a rapidly-dividing **long-slender** form and a non-dividing **short-stumpy** form.

Figure 2 gives an overview of the *trypanosoma brucei* life cycle: The entire parasitic species has two hosts, an insect vector and a mammalian host. **(1)** At the beginning an infected tsetse fly takes a blood meal and injects metacyclic trypomastigotes into the human tissue. The parasites can enter the lymphatic system and pass into the bloodstream. **(2)** The bloodstream trypomastigotes spread throughout the body and accumulate for example in the spinal fluid. **(3)** The parasite multiplies by binary fission, which is a form of asexual replication. **(4,5)** Another tsetse fly may now ingest the trypomastigotes. **(6)** The procyclic trypomastigotes inside the flies midgut replicate, (7) leave the midgut and transform into epimastigotes. **(8)** The epimastigotes finally move into the salivary glands, replicate once again and wait for a mammalian host they can enter.

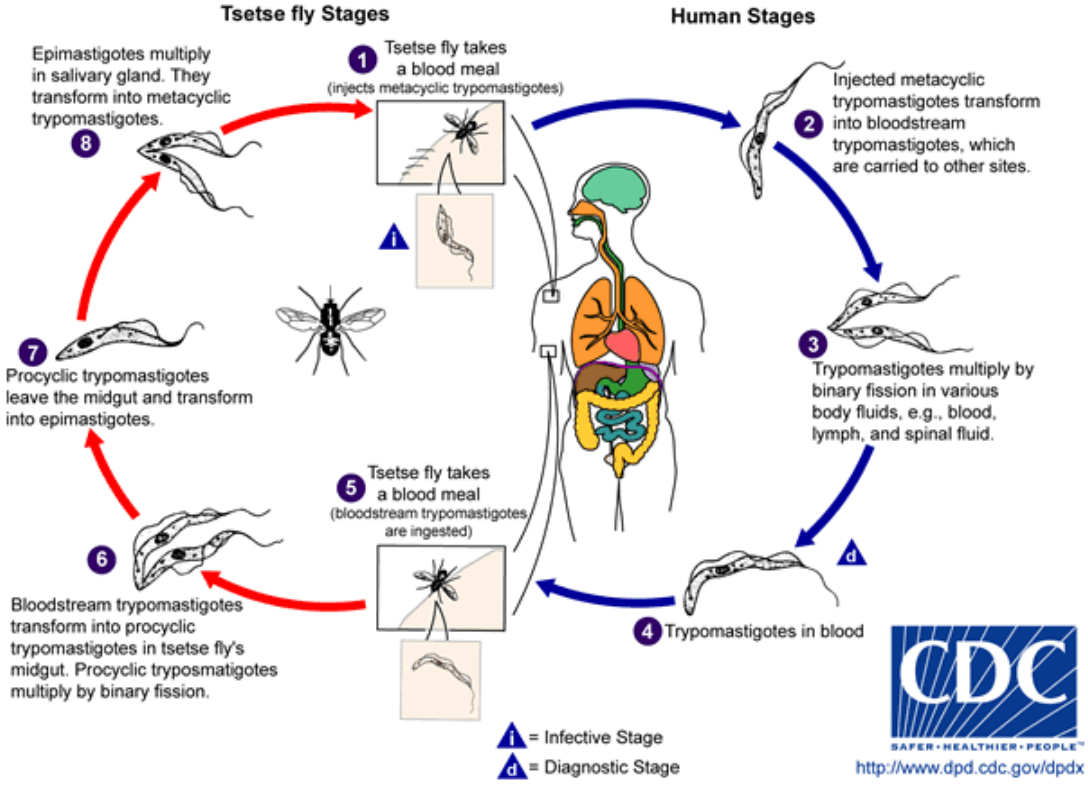

Figure 2: Lifecycle of trypanosoma *brucei*

Clinical symptoms follow the stage of the infection. Fever, headache and unspecific pain are followed by swollen lymph nodes which may reach tremendous size. In a later stage anaemia and secondary (endocrine, cardiac and kidney) disorders can be found. When the parasites

pass the blood-brain barrier, the disease enters the neurological phase: confusion, ataxia, tiredness during the day and insomnia at nights give the sleeping sickness its name.

Trypanosoma brucei protects itself from being attacked by its host's humeral immune defence. Its cell surface is coated with a variable surface glycoprotein (VSG), which changes from time to time in order to evade the produced specific antibodies. That surface works as a physical barrier hiding invariant surface proteins from immune recognition. It is estimated that many hundred copies of the VSG genes are coding for the vast diversity of the parasite. Any time the host has adapted to the new coat structure of the parasite, lowering the trypanosome titer, an alternative VSG will be expressed and repopulation begins. As a result of this boom and bust population cycle, there are distinct bouts of infection, each with a different coat.

The search for a therapeutic intervention showed that cysteine protease inhibitors as benzyloxycarbonyl-phenylalanine-alanine diazomethane (Z-Phe-Ala-CHN_2) were lethal to trypanosoma. The assumed targets of the inhibitor were rhodesain, (which is the major cathepsin L in *T.brucei rhodesiense*) brucipain, trypanopain or congopain (in other *Trypanosoma* species). However, Mackey et al. [(2)] found in comparative RNAi knockdowns, that silencing rhodesain did not change the trypanosomes' phenotype. Instead, knocking down tbcatB (a cathepsin B-like cysteine protease) led to distension of the posterior endosome/lysosome compartment within 12 hours. One day after RNAi induction the enlargement in the region of the endosome reached its maximum, and some parasites displayed multiple flagella that looked similar to uninduced control parasites undergoing cytokinesis. After 48 hours the cell cultures demonstrated a dramatic increase in the number of parasites containing two kinetoplasts and two nuclei. The parasites did not proliferate and died by 72 hours postinduction.

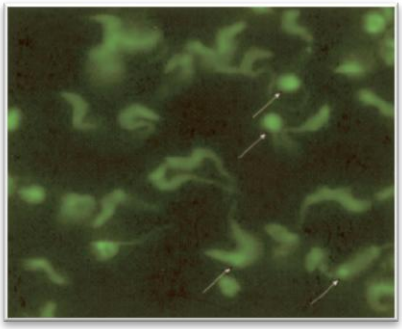

Figure 3: Accumulation of FITC-transferrin in the trypanosoma endosome [(2)]

The enlarged endosome results from accumulation of transferrin. Because trypanosomes lack cytochromes, the bloodstream form acquires iron from the host by internalizing transferrin through receptor-mediated endocytosis. Normally, the transferrin gets rapidly degraded in the endosome/lysosome located between the nucleus and the kinetoplast. Imaging of FITC-labeled transferrin showed that tbcatB-knockout parasites lost the ability to degrade transferrin (Figure 3). A similar accumulation of FITC-transferrin could be observed with the cysteine protease inhibitor Z-Phe-Ala-CHN_2. Therefore, diazomethane was proven to inhibit

not rhodesain, but cathepsin B. This makes cathepsin B a highly potent drug target in *t. brucei*.

Cathepsins are a family of intracellular digestive proteolytic enzymes. Among 500 proteases, which have been identified in the human genome, about 60 are lysosomal, and about a dozen are papain-like lysosomal proteases. However, there is no strict rule that links the reactive mechanism and localization of cathepsins with their name. All lysosomal cysteine proteases are cathepsins (including cathepsin B), but there are cathepsins that are not lysosomal (cathepsin E, G) or non-cysteine proteases (aspartic cathepsins D, E / serine cathepsins A, G) [(3)]. Exopeptidases (such as cathepsins B, C, H, X), in contrast to endopeptidases (cathepsins F, L, S, V), possess structural features that facilitate binding of N-C-terminal groups of substrates in the active-site cleft.

Papain-like lysosomal cysteine proteases have been found to be associated with a number of pathologies, i.e. cancer, inflammation, rheumatoid arthritis and osteoarthritis, Alzheimer's disease, multiple sclerosis, muscular dystrophy, pancreatitis, liver/lung/myocardial disorders and diabetes. Due to their ubiquity, it is necessary that drugs achieve appropriate specifities, only inhibiting the pathogen.

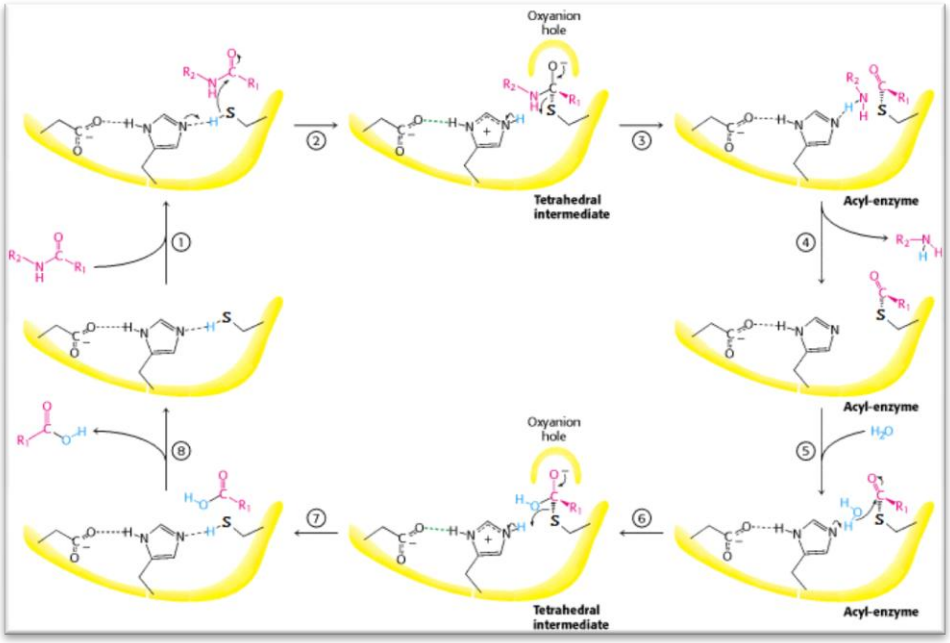

Figure 4: Mechanism of cysteine proteases [(1)]

The following experiments will focus on the heterologous expression of cathepsin B in *Escherichia Coli*, followed by purification and refolding steps. Furthermore, a zymographic method is developed to analyse the activity of cysteine proteases. *In silico* structure prediction and docking models are tested in order to provide the basic tools for future inhibition experiments.

2 Materials and Methods

Generally important biochemical means are listed below with detailed protocols which should make it possible to readily copy them without further lecture. As most methods needed to be optimized and performed with different parameters, we will advertence on differences in execution if necessary.

2.1 Working with DNA

2.1.1 Plasmid preparation

The QIAprep [51] miniprep procedure is based on alkaline lysis [6] of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt [7]. Plasmid yield varies depending on plasmid copy number per cell, the individual insert in a plasmid, factors that affect growth of the bacterial culture, the elution volume, and the elution incubating time.

Material:

QIAprep plasmid purification kit *Mini* (QIAGEN [51])	Kit contents Kits containing buffers of unknown composition: P1 (Resuspension buffer) P2 (Lysis buffer) N3 (Neutralization buffer) PB (Wash buffer to get rid of endonucleases) PE (Wash buffer to get rid of salts) EB (Elution buffer) QIAGEN column
QIAprep plasmid purification kit *Midi (tip-100)*	Kit contents Kits containing following buffers:
buffer P1	Resuspension buffer 50 mM Tris-HCl, pH 8.0 10 mM EDTA 100 µg/ml RNase A
buffer P2	Lysis buffer 200 mM NaOH 1% (w/v) SDS

buffer P3	Neutralization buffer 3.0 M potassium acetate, pH 5.0
buffer QBT	Equilibration buffer 750 mM sodium chloride 50 mM MOPS, pH 7.0 15 % (v/v) isopropanol
buffer QC	Wash buffer 1.0 M sodium chloride 50 mM MOPS, pH 7.0 15 % (v/v) isopropanol
buffer QF	Elution buffer 1.25 M sodium chloride 50 mM MOPS, pH 7.0 15 % (v/v) isopropanol

2.1.1.1 QIAprep mini

Method:

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from one to five milliliters overnight culture of *E. coli* in LB medium (chapter 2.2.1.2). A mini preparation of 1.5ml overnight culture can yield from 5 to 15 µg of plasmid DNA.

- Centrifugate 1 minute on a table-top centrifuge @ top speed
- Resuspend pelleted bacterial cells in 250 µl buffer P1 in a 1.5 ml eppendorf cup
  Hint: no cell clumps should be visible after resuspension of the pellet
- Add 250 µl buffer P2 and gently invert the tube 4-6 times to mix
  Hint: mix gently by inverting the tube; do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5'.
- Add 350 µl buffer N3 and invert the tube immediately but gently 4-6 times
  Hint: To avoid localized precipitation, mix the solution gently but thoroughly, immediately after addition of buffer N3. The solution should become cloudy.
- Centrifuge for 10 minutes @ 13000 revolutions per minute (18000 g) in a table-top micro centrifuge
  Hint: A compact white pellet will form.
- Apply the supernatants to the QIAprep spin column by decanting or pipetting

- Centrifuge for 30-60 seconds and discard the flow-through

- Wash the QIAprep spin column by adding 0.5 ml buffer PB, centrifuge for 30-60 seconds and discard the flow-through
  Hint: This step is necessary to remove trace nuclease activity when using endA strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue does not require this wash step.*

- Wash QIAprep spin column by adding 0.75 ml buffer PE and centrifuge for 30-60 seconds

- Discard the flow-through and centrifuge for an additional 1 minute to remove residual wash buffer

- Place the QIAprep column in a clean 1.5 ml eppendorf cup

- To elute DNA, add 50 µl (or another volume) buffer EB to the centre of the QIAprep spin column, let stand for 1 minute, and centrifuge for 1 minute

2.1.1.2 QIAprep midi

Method:

This protocol is designed for preparation of 75-100 µg of high-copy plasmid or cosmid DNA from 25 ml high-copy plasmid culture in LB medium.

- Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic. Incubate for 8 hours @ 37 °C with vigorous shaking [(2.2.1.2)].

- Dilute the starter culture 1/500 to 1/1000 into 25 ml selective LB medium. Grow @ 37°C for 12-16 hours with vigorous shaking [(2.2.1.3)].

- Harvest the bacterial cells by centrifugation for 15 minutes @ 6000 g and 4°C

- Resuspend the bacterial pellet in 4 ml or 10 ml buffer P1.

- Add 4 ml buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate for 5 minutes @ room temperature.

- Add 4 ml of chilled buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 minutes.
  Hint: The precipitated material contains genomic DNA, proteins, cell debris and SDS

- Centrifuge for 30 minutes @ 20000+ g and 4°C. Remove supernatant containing plasmid DNA promptly.
  Hint: Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes to prevent adsorption on the surface.

- Centrifuge the supernatant again for 15 minutes @ 20000+ g and 4°C. Remove supernatant containing plasmid DNA promptly.

- Equilibrate QIAGEN-tip 100 by applying 4ml buffer QBT and allow the column to empty by gravity flow.

- Apply the plasmid solution to the column and allow it to enter the resin by gravity flow.

- Wash the column two times with 10ml buffer QC.

- Elute DNA with 5ml buffer QF.

- Precipitate DNA by adding 3.5ml room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately for 30 minutes @ 15000 g and 4 °C. Carefully decant the supernatant.
  Hint: All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample.

- Wash DNA pellet with 2ml of room-temperature 70 % (v/v) ethanol and centrifuge again for 10 minutes @ 15000 g. Carefully decant the supernatant without disturbing the pellet.

- Air-dry the pellet for 10 minutes and redissolve the DNA in a suitable volume of buffer EB.

2.1.1.3 EtOH precipitation

This method produces no analytical quality, but works at much lower costs, because it doesn't use the QIAprep columns, which are the limiting factor in plasmid extraction kits.

Method:

Start with a colony containing the DNA to be extracted. Pick a clone and inoculate in 3ml LB AMP medium overnight (chapter 2.2.1.2). Take 1.5 ml and perform the following steps:

Start @ room temperature

- centrifugate 1 minute on a table-top centrifuge @ top speed
- resuspend pellet in 150 µl of buffer P1 and incubate 5 minutes
- add 150 µl lysis buffer P2, invert 4-6 times and incubate 5 minutes
- neutralize with 210 µl buffer N3, invert and incubate 5 minutes on ice

Continue @ 4°C or on ice

- centrifugate 10 minutes on a table-top centrifuge @ top speed

- transfer the supernatant in a clear 1,5ml eppendorf cup
- add 900 µl ice-cold ethanol (-20 °C)
- centrifugate 10 minutes on a table-top centrifuge @ top speed
- discard supernatant and wash pellet with 1ml 70 % (v/v) ethanol
- centrifugate 10 minutes on a table-top centrifuge @ top speed

You may continue @ room temperature

- discard supernatant and dry pellet up to 45 minutes
- solve in 50 µl buffer EB

2.1.2 Quantification

Concentration and purity of nucleic acids can be controlled via measurement of the optical density [8]. Lambert-Beer law describes the relationship between the absorption of light to the properties of the material through which the light is travelling. The absorbance is $A = \log_{10}\left(\frac{I_0}{I}\right) = a \times c \times l$ with intensity of the incident light I_0, intensity after passing through the material I, extinction coefficient a, concentration c and path length l.

Method:

- For optimal results, the aqueous DNA sample solution should be diluted until the OD_{260nm} ranges between 0.1 und 1.0.
- Optical densities are measured spectrophotometrically at 260nm. Double stranded DNA concentrations can be calculated from $a_{dsDNA} = 20\frac{l}{g \times cm}$, single stranded DNA concentrations from $a_{ssDNA} = 30\frac{l}{g \times cm}$.
- Contamination with proteins or aromatic substances (i.e. phenol) can be detected via the absorbance at 280nm: Pure DNA gives a quotient $\mathbf{OD_{260nm}/OD_{280nm} \geq 1.8}$, $OD_{260nm}/OD_{280nm} > 2$ is an indication for RNA contamination.
  Hint: The above mentioned extinction coefficients may vary when nucleic acids are solved in other buffers or solutions than deionised water.

2.1.3 Enzymatic restriction

Restriction endonucleases scan the length of a DNA molecule until they encounter a specific recognition sequence. At this point they bind to the DNA and cut each of the two ribose-phosphate backbones of the double helix. Physiologically designed to protect prokaryotes against viral infections, restriction endonucleases have become indispensable for biochemical

work. Almost all endonuclease recognition sites are palindromes, what means both strands have the same sequence in 5'-3' direction. Symmetric cutting produces **blunt end**, asymmetric cutting cleaves the DNA backbone in positions that are not directly opposite to each other. Such protruding or **sticky ends** tend to pair with complementary sequences of bases by relatively weak hydrogen bonds.

Material:

Hind III	Properties	Buffer R (10x)
(Fermentas[48])	5'…A'AGCTT…3' 3'…TTCGA'A…5' dam/dcm/CpG: not sensitive	pH 8,5 10 mM Tris-HCl 10 mM magnesium chloride 100 mM potassium chloride 0,1 mg / ml BSA
Kpn I	**Properties**	**Buffer Kpn I (10x)**
(New England Biolabs[49])	5'…GGTAC'C…3' 3'…C'CATGG…5' dam/dcm/CpG: not sensitive	pH 7,0 10 mM bis-tris-propane-HCl 10 mM magnesium chloride 1 mM dithiothreitol
Sac II	**Properties**	**NEBuffer 4 (10x)**
(New England Biolabs[49])	5'…CCGC'GG…3' 3'…GG'CGCC…5' dam/dcm : not sensitive CpG: blocked	pH 7,9 20 mM tris-acetate 50 mM potassium acetate 10 mM magnesium acetate 1 mM dithiothreitol
Pst I	**Properties**	**Buffer O (10x)**
(Fermentas[48])	5'…CTGCA'G…3' 3'…G'ACGTC…5' dam/dcm/CpG: not sensitive	pH 7,5 50 mM Tris-HCl 10 mM magnesium chloride 100 mM sodium chloride 0,1 mg / ml BSA
Xho I	**Properties**	**Buffer R (10x)**
(Fermentas[48])	5'…C'TCGAG…3' 3'…GAGCT'C…5' dam/dcm: not sensitive CpG: cleavage impaired	pH 8,5 10 mM tris-HCl 10 mM magnesium chloride 100 mM potassium chloride 0,1 mg / ml BSA

Method:

- Solve 0.5-1 µg DNA in 17 µl ultrapure water (i.e. Ampuwa)
- Add 2 µl 10x Buffer solution
- Add 1 µl enzyme solution. Gently mix the solution by inversion.
  Hint: Always handle restriction enzymes at -20°C. Transport them in cooling blocks to avoid loss of activity.
- Incubate for 2 hours @ 37°C

2.1.4 Agarose Gel Electrophoresis

Small differences between related DNA molecules can be detected because their restriction fragments can be separated and displayed by gel electrophoresis. In many types of gels, the electrophoretic mobility of a DNA fragment is inversely proportional to the logarithm of the number of base pairs. Porous agarose gels can be used to resolve mixtures of fragments up to 20kb.

Material:

TAE buffer	Properties
(50-fold)	25 % (w/v) Tris pH 8 50 mM sodium EDTA 5 % (v/v) acetic acid
Gel sample buffer	**Properties**
(10-fold)	0.25 % (w/v) bromophenol blue 60 % (v/v) glycerol in 50 mM Tris pH 7.6
Miscellaneous	**Other material**
	agarose 1 % (w/v) ethidium bromide

Method:

- Prepare about 50 ml of a 0.8 % (w/v) agarose solution. Add up to 3 µl ethidium bromide and keep appropriate safety precautions in mind. Microwave heat it until a clear solution is obtained. Fill into gel chamber and allow to cool down for 30 minutes.
- Install the gel into the electrophoresis apparatus and add 1-fold TAE buffer.

- Mix 5 µl gel sample buffer with 20µl DNA restriction solution.
- Load the gel pockets with 12-20µl of each sample. As a comparative standard use 3µl of O'GeneRuler DNA Ladder from Fermentas [48].
- Run gel with 80 V constant voltage for approximately 1 hour.
- Visualize ethidium bromide intercalated DNA fragments via UV radiation.

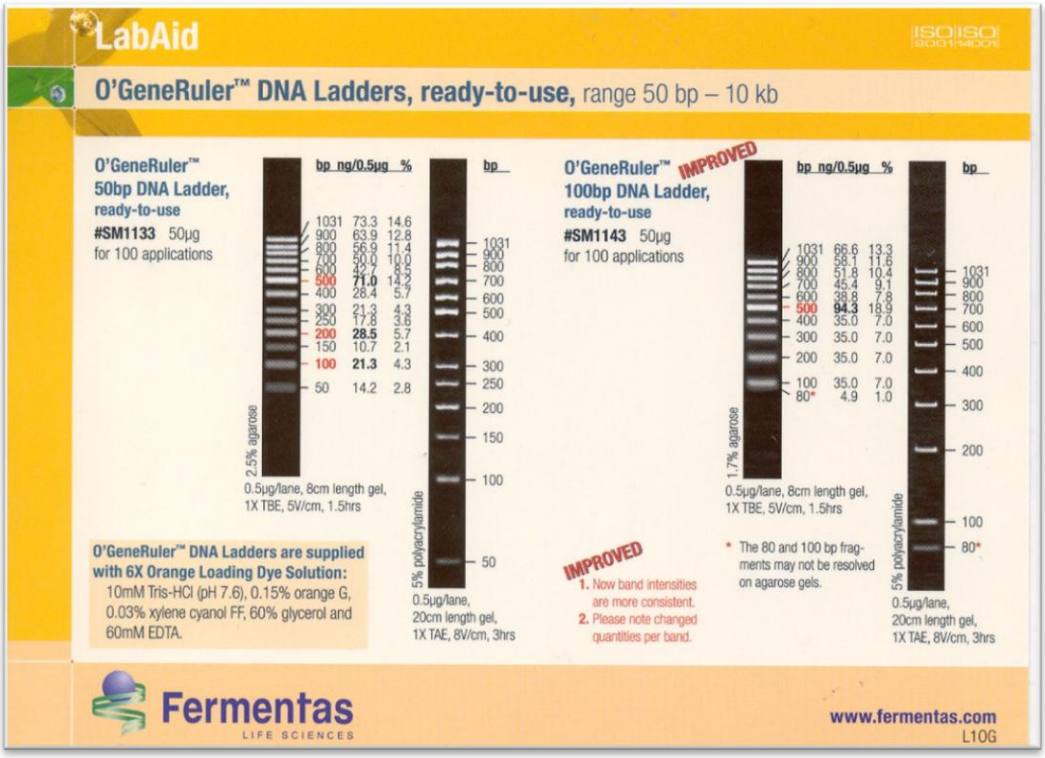

Figure 5: Standard DNA fragments for gel electrophoresis I

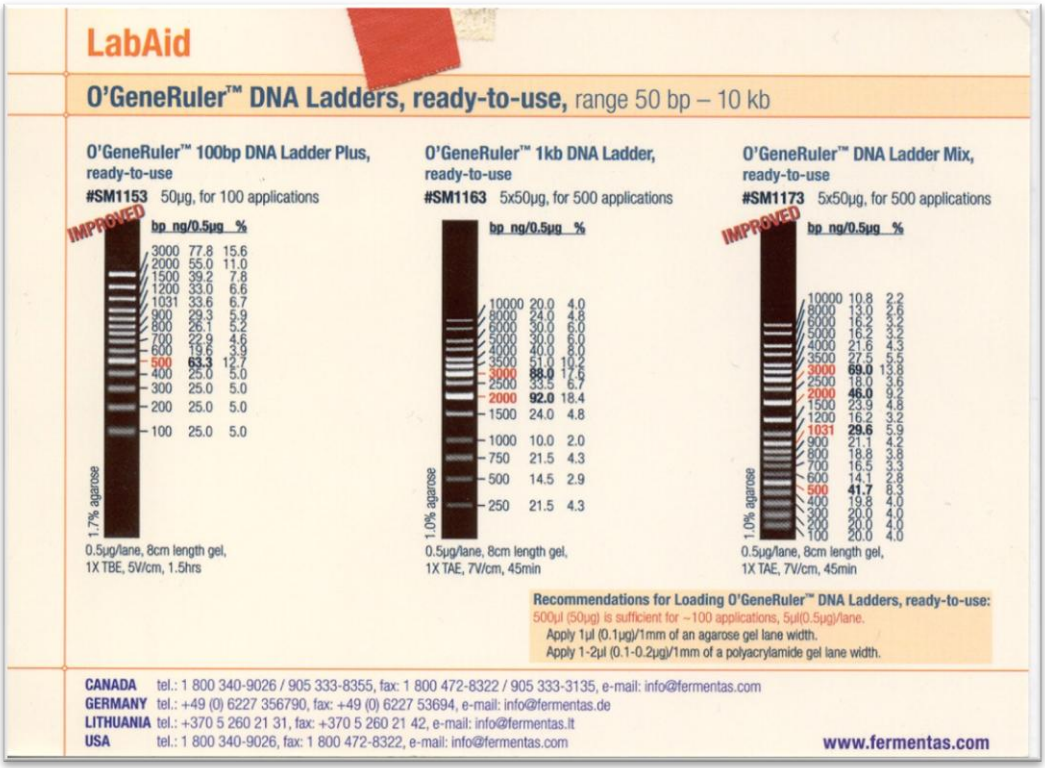

Figure 6: Standard DNA fragments for gel electrophoresis II

2.2 Working with cell lines

2.2.1 Cultivating and harvesting cells

Material:

Amp	Properties
(1000-fold)	50 mg/ml in EtOH, stored in darkness @ -20°C
X-Gal	**Properties**
(500-fold)	20 mg/ml in DMF, stored in darkness @ -20°C
LB	**Properties**
(Luria Bertani) (2-fold)	pH 7, autoclaved 2 % (w/v) bacto-tryptone [i] 1 % (w/v) bacto-yeast extract 2 % (w/v) sodium chloride
Agar	**Properties**
(2-fold)	4 % (w/v) agar-agar

2.2.1.1 On Petri plates

Note that all work has to be done under semi-sterile conditions. That implies the use of a clean bench with reduced germination number. Ensure to disinfect all materials with ethanol and heat. Especially bottlenecks and caps are sources of contamination. While an experienced scientist won't need to wear gloves, they may provide additional security.

Method:

- Plate some micro litres of a bacteria solution on agar plates. Sufficient dilution makes sure that distinct colonies will grow. 1-fold Amp and X-gal can be added if screening for transformed and recombined cells is requested. Incubate at 37°C or store at 4°C.

[i] **Bacto-tryptone:** Peptone from caseine, tryptically digested

2.2.1.2 Three millilitres starter culture

Method:

- Place 1.5ml LB (2x) and 1.5ml autoclaved water in a sterile Falcon tube.
- Optionally, add 6 µl Amp to prevent loss of antibiotic resistance. Final concentration should not exceed 100 µg/ml.
- Pick a clone from a Petri plate using a sterile Eppendorf pipette. Drop tip into the Falcon tube.
- Cultivate 8 hours at 37°C.

2.2.1.3 Main culture and cultivation to specific cell density

Method:

- Place 50ml LB (2x) and 50ml autoclaved water in a sterile chicane flask.
- Optionally, add 200µl Amp to prevent loss of antibiotic resistance.
- Add 200µl starter culture.
- Vigorously shake it at 37°C until the optical density at 600nm reaches 0.4 or 0.7, or any requested value.
  Hint: $8x10^8$ XL1-Blue cells give an optical density of 1, measured against a cell-free LB medium reference.

2.2.2 Transformation

Transformation is the genetic alteration of a cell, resulting from the introduction, uptake and expression of foreign genetic material. In order to transform bacteria, the cells have to be prepared in a way that allows them to accept extra-chromosomal DNA or plasmids. Such cells are called competent. Among different methods to make cells competent, the most well-known procedure involves cooling the bacteria in a Calcium Chloride bath at 0°C. Although the exact mechanism of the transformation progress is not yet fully understood, it is assumed that the calcium chloride ions neutralize the repulsion between the negatively charged phospholipid heads of the cell membrane and the negatively charged phosphate groups of the desoxynucleic acids. Quickly heating the cells to 47°C for approximately 90 seconds will create a thermal gradient which, in turn, creates a draft leading DNA into the cell. It is important not to extend the given times, as a too long heating period will denaturate the cell membrane, causing the bacteria to die. Until now, several attempts have been made to develop simpler protocols with high transformation efficiencies, and the absence of time-critical steps.

2.2.2.1 Creation of competent cells

We used a method by Chung et al. [16], which bases on the use of polyethylenglycole, dimethyl sulfoxide and divalent cations to make the cell membrane permeable for foreign DNA. It allows routine cloning in plasmids to be performed with ease. Preparation, transformation and storage of competent bacterial cells can be performed in the same solution. Beyond, a brief heat shock is not necessarily required for maximum uptake of plasmid DNA.

Material:

TSS	TSS buffer 10 % PEG 6000 5 % DMSO 50 mM magnesium chloride in LB medium

Methods:

Start with cells with or without antibiotic resistance episome, i.e. Stratagene XL1 Blue (tetracyclin resistance) or DH5α (no resistance). Then

- cultivate cells overnight on a Petri plate (with or without tetracycline, dependant on cell line) @ 37°C
- inoculate a picked clone in 3.5ml LB medium (+Tet/-Tet) overnight @ 37°C with gently agitation at 150 rpm
- transfer 1ml of the starting culture in 100ml LB medium and shake it until the optical density at 600nm reaches 0.4 or 0.7
  At this time, the bacteria are in a cell cycle state which will allow optimal results.
- harvest the cells by centrifugation @ 4°C and 4500rpm for about 5 minutes
- resuspend the pellet in 10ml TSS and split in 100µl portions
- freeze aliquots in liquid nitrogen and store @ -80°C

2.2.2.2 Transforming cells

Material:

SOB	SOB medium (per litre) 20 g tryptone 5 g yeast extract 0.5 g sodium chloride in 1 litre deionised water after autoclaving, add the following filter-sterilized solutions: 10ml 1M magnesium chloride 10ml 1M magnesium sulphate
SOC (prepare before use)	SOC medium (per 100ml) 2ml of filter-sterilized 20% (w/v) glucose in 98 ml SOB medium

Method (instructions by Stratagene [55]):

- Preheat SOC medium at 42°C. Pre-chill two 14ml Falcon polypropylene round-bottom tubes on ice.
  One tube is for the experimental transformation and one tube is for the pUC18 control.
- Thaw the cells on ice. When thawed, gently mix and aliquot 50µl of cells into each of the two pre-chilled tubes.
- Add 0.1-50ng of the experimental DNA to one aliquot of cells and add 1µl of the pUC18 control DNA to the other aliquot. Swirl the tubes gently.
- Incubate the tubes on ice for 20 minutes.
- Heat-pulse the tubes in a 42°C water bath for 45 seconds. The duration of the heat pulse is critical.
- Incubate the tubes on ice for 2 minutes.
- Add 0.9ml of preheated SOC medium and incubate the tubes at 37°C for 30minutes with shaking at 225-250rpm.
- Plate about 200µl or less of the transformation mixture on LB agar plates containing the appropriate antibiotic (and containing IPTG and X-gal if colour screening is desired).
  For the pUC18 control transformation, plate 100µl of the transformation mixture on LB-ampicillin agar plates.

- Incubate the plates at 37°C overnight. If performing blue-white colour screening, incubate the plates at 37°C for at least 17 hours to allow colour development.
  Colour can be enhanced by subsequent incubation of the plates for 2 hours at 4°C.
- For the experimental DNA, the number of colonies will vary according to the size and form of the transforming DNA, with larger and non-supercoiled DNA producing fewer colonies.
  For the pUC18 control, expect 10 colonies (that is $>1^{06}$ cfu/µg pUC18 DNA)

2.2.3 Cell Lysis [13] by ultra sonification

Choosing a pleasant method for cell lysis depends on the amount and the character of the tissue or bacteria cells. Common ways to disrupt or degrade cell walls include mechanical *(i.e. waring blenders, pearl mills, French Presses, Potter-Elvehjim homogenisators, nitrogen decompression, or ultra sonification)* as well as chemical protocols *(solvent-induced autolysis/dehydration, osmotic shock, lytic enzymes, or detergents).*
When we tried to isolate our expression products, namely the cathepsin B, we opened up the *Escherichia Coli* cells by ultra sonification.

Material:

Devices	Type and manufacturer
UltraSonifier	Ultra Sonifier Cell Disruptor B-30 (Branson Sonic Power Corporation [56])
Lysis Buffer	**Properties**
	20 mM Na-HEPES (ph 6.0 - 9.0) or Tris-HCl or Na-Phosphate 500 mM sodium chloride (optional 50-1000 mM) 1 mM EDTA (optional) 0,1 % (w/v) Triton X-100 (optional) Protease inhibitors (optional) (for details refer to 2.2.4)

Method:

Perform all work @ 4°C or on ice

- spin down LB culture and resuspend pellet in 2.5ml Lysis Buffer
- lyse 30s with ultra sonifier @ output control level 5
- cool for 3min in ice water
- repeat ultra sonification 10 times

2.2.4 Inhibit protease activity [12]

Proteases can be divided into four families. They are named after the most important amino acid in their catalytic centre. There are serine proteases *(which include trypsin, chymotrypsin and elastase)*, cysteine proteases *(cathepsin B, papain, calpain)*, aspartic proteases *(pepsin)*, and metalloproteases *(carboxypeptidase a/b, leu-aminopeptidase)*.
Often misunderstood, we want to point out, that the name of the protease family has nothing to do with at which amino acid residue in a given substrate the cleavage will take place. In fact, trypsin cleaves specifically at positively charged amino acids, whereas chymotrypsin prefers aromatic and big aliphatic amino acids. Each protease family can be specifically inhibited. Figure 7 [12] shows which enzymes might indicate the use of which inhibitors.

Protease Inhibitor	General inhibitors for: Serine proteases[a]	Cysteine proteases[b]	Metallo-proteases[c]	Aspartic proteases[d]
Aprotinin		E-64	Phosphoramidon	Pepstatin
Pefabloc SC and Pefabloc SC PLUS			Bestatin (aminopeptidases)	
Leupeptin *(inhibits serine and cysteine proteases with trypsin-like specificity)*				
PMSF				
c*O*mplete, EDTA-free Protease Inhibitor Cocktail Tablets*				
c*O*mplete Protease Inhibitor Cocktail Tablets*				
α_2-Macroglobulin				

* When extractions or single-step isolations are necessary in the acidic pH range, include Pepstatin along with c*O*mplete tablets to ensure aspartic (acid) protease inhibition.

a) Contain serine and histidine in the active center
b) Contain cysteine (thiol, SH-) in the active center
c) Contain metal ions (*e.g.*, Zn^{2+}, Ca^{2+}, Mn^{2+}) in the active center
d) Contain aspartic (acidic) group in the active center

Protease-specific inhibitors	for the inhibition of:
Antipain dihydrochloride	Papain, Trypsin (Plasmin)
Calpain Inhibitor I	Calpain I > Calpain II
Calpain Inhibitor II	Calpain II > Calpain I
Chymostatin	Chymotrypsin
TLCK	Trypsin, other serine and cysteine proteases (*e.g.*, Bromelain, Ficin, Papain)
Trypsin-Inhibitor (chicken egg white, soybean)	Trypsin

Figure 7: Protease Inhibitors

2.3 Working with protein

2.3.1 Bradford

Bradford's protein quantification method [25] uses a phosphoric acid solution with *Coomassie Brilliant Blue G250*, a triphenylmethane dye, to complex cationic (basic) or non-polar, hydrophobic amino acid side chains. The complex stabilizes the dye in its anionic sulphate form, which leads to an absorbance shift from 470nm to 595nm.

The method had been calibrated with well-defined amounts of bovine serum albumin.

Table 1: Calibration of Bradford Assay

BSA (140 mg/l) V [µl]	**Aqua dest.** V [µl]	**Bradford Reagent** V [µl]	**Cuvette conc.** c [µg/ml]	**Extinction** E_{595} [ ]
0	800	200	0	0,000
14,29	785,71	200	2	0,111
28,57	771,43	200	4	0,215
42,86	757,14	200	6	0,299
57,14	742,86	200	8	0,390
71,43	728,57	200	10	0,474
Linear regression gives:		E = 0,0488 (ml/µg) * c		

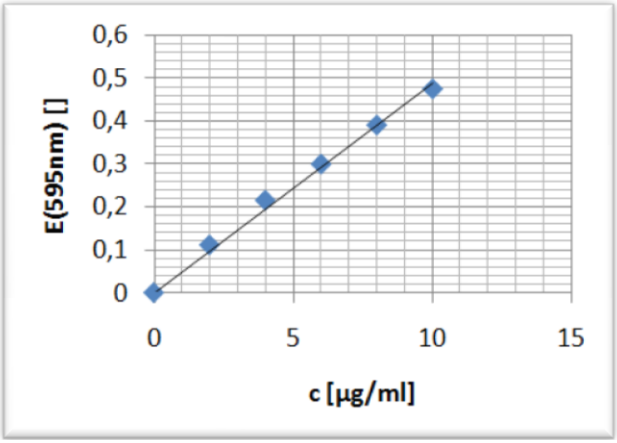

Figure 8: Calibration of Bradford Assay

Method:

- Dilute 1-2µl of protein sample with deionised water to a final volume of 800µl.
- Add 200µl Bradford Reagent.
- Incubate 5min and measure optical density @ 595nm.

2.3.2 SDS-PAGE

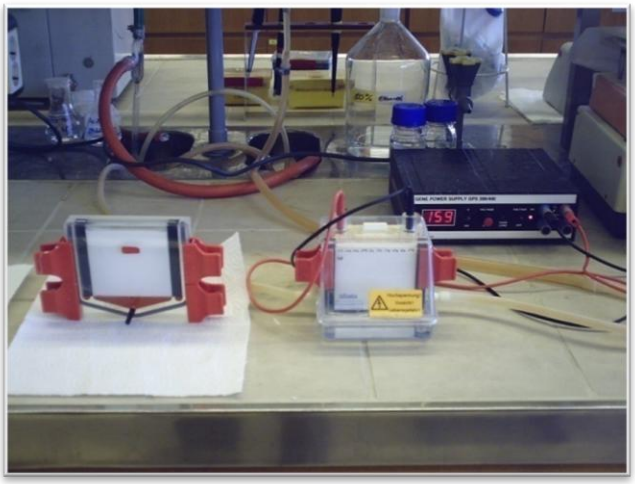

Figure 9: SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis is a technique to separate proteins according to their electrophoretic mobility, which makes it the companion piece to DNA gel electrophoresis in the protein world: SDS, an anionic detergent, is used to denature secondary and non-disulfate-linked tertiary protein structures, and to apply negative charges to each protein in proportion to its mass. Tertiary disulfate-cross links are reduced by dithiothreitol or 2-mercaptoethanol. The resulting protein has a linearized structure and an approximately uniform mass to charge ratio. It is applied to an acryamide gel and an electric current causes the negatively charged sulphate groups to migrate towards the cathode.

Material:

Devices	Type and manufacturer
PowerSupply	Pharmacia, GenePower Supply GPS 200/400
Roti Load 1 (Roth [52])	Roti reducing loading buffer (4x) *Proprietary composition unknown*
Roti Load 2 (Roth [52])	Roti non-reducing loading buffer (4x) *Proprietary composition unknown*
Non-red. Load. B	Non-reducing loading buffer (2x) 5 % (w/v) SDS 25 % (v/v) glycerol 0.02 % (w/v) bromophenol blue in 500 mM Tris/HCl pH 6,8
Running Buffer	Laemmli [17] Running Buffer (10x) 1 % (w/v) SDS 14 % (w/v) glycine 250 mM Tris pH 8.3-9.0

Staining Buffer	Coomassie Blue staining solution (1x) 0.25 % (w/v) Coomassie Brilliant Blue R-250 45 % (v/v) methanol 10 % (v/v) acetic acid in deionised water
Destaining Buffer	Coomassie Blue destaining solution (1x) 50 % (v/v) methanol 10 % (v/v) acetic acid in deionised water
Gel drying buffer	Gel drying buffer 25 % ethanol 2 % (v/v) glycerol in deionised water
Stock solutions APS Buffer A Buffer B SDS	Solutions for gel polymerisation 10 % (w/v) ammoniumperoxidisulfate 1 M Tris-HCl, pH 8,8 1 M Tris-HCl, pH 6,8 10 % (w/v) sodium dodecylsulfate

Table 2: Pipetting scheme for 0.75mm gels *(polymerisation time is approx 45 min)*

	2 gels	**3 gels**	**4 gels**	**5 gels**	**6 gels**	**8 gels**
separating gel (10,3 % T, 2,6 % C)						
buffer A	4,3 ml	6,5 ml	8,6 ml	10,75 ml	12,9 ml	17,2 ml
aqua dest.	3,3 ml	5,0 ml	6,6 ml	8,25 ml	9,9 ml	13,2 ml
acrylamide	3,8 ml	5,7 ml	7,6 ml	9,5 ml	11,4 ml	15,2 ml
SDS	114 µl	171 µl	228 µl	285 µl	342 µl	456 µl
APS	39 µl	58,5 µl	78 µl	97,5 µl	117 µl	156 µl
TEMED	12 µl	18 µl	24 µl	30 µl	36 µl	48 µl
stacking gel (5,1 % T, 2,6 % C)						
buffer B	0,75 ml	1,1 ml	1,5 ml	1,9 ml	2,25 ml	3,0 µl
aqua dest.	4,2 ml	6,3 ml	8,4 ml	10,5 ml	12,6 ml	16,8 ml
acrylamide	1 ml	1,5 ml	2,0 ml	2,5 ml	3 ml	4,0 ml
SDS	60 µl	90 µl	120 µl	150 µl	180 µl	240 µl
APS	30 µl	45 µl	60 µl	75 µl	90 µl	120 µl
TEMED	6 µl	9 µl	12 µl	15 µl	18 µl	24 µl

Method:

- Mix buffer, water, acrylamide and SDS according to Table 2 (*Separating gel*). Add APS and TEMED, then pour gel quickly. Cover with 1ml water or isopropanol and let set for 30-45min.
- Drain the water or isopropanol. If necessary, rinse with water.
- Prepare and pour *Stacking gel* according to Table 2. Insert Comb and let set.
- Remove comb, install gel in SDS-PAGE running device and fill buffer chambers with Running Buffer.
- Prepare samples of 5-10µg protein in volumes of 15µl. Add 5µl Loading Buffer (4x) and optionally cook samples for some minutes.
- Apply 20µl sample solution per pocket. As comparative protein standard, use Fermentas Protein Molecular Weight Marker (Table 3). Run gel with 120V-160V at constant voltage.
- Stain and destain gel for 1 hour, or as long as necessary.
- Gels can be stored for some days in aqueous solutions, or permanently after washing in Drying Buffer and fixing between two cellophane foils.

Table 3: Fermentas [48], Protein Molecular Weight Marker

Protein	Source and Reference	
β-galactosidase	*E. coli* [18]	
bovine serum albumin	bovine plasma [19]	
ovalbumin	chicken egg white [20]	
lactate dehydrogenase	porcine muscle [21]	
REase Bsp98I	*E. coli*	
beta-lactoglobulin	bovine milk [22]	
lysozyme	chicken egg white [23]	

2.3.3 Resolving inclusion bodies

Material:

Devices	Type and manufacturer
Centrifuge	Heraeus Sepatech, Suprafuge 22
Washing Buffer (Roth [52])	Washing Buffer pH 8 0.5% (v/v) Triton X-100 10 mM EDTA
Solving Buffer (Roth [52])	Solving Buffer pH 8 6M guanidin-HCl 20mM Tris pH 8 100mM sodium dihydrogen phosphate
Dialysis Buffer	Dialysis Buffer pH 8 3M (or without) urea 25 % (v/v) glycerol 500mM sodium chloride 20mM HEPES pH 8

Method:

- Vigorously shake inclusion body containing pellet in 10ml washing buffer for 15min at room temperature
- Centrifuge 15min @13000 g and 4°C. Store supernatant for acetone precipitation.
  (Respectively 10.5k revolutions per minute with rotor radius of 109mm.)
- Resuspend pellet in 500 µl deionised water and pour suspension into 10 ml solving buffer. Incubate 2h at room temperature.
- Centrifuge for 15min @ 13000 g and 4°C. No pellet should remain.
- Dialyse overnight against 1 litre of Dialyses Buffer (3M urea) at 4°C. Take 6ml sample.
- Dialyse 3.5h against 1 litre of Dialyses Buffer without urea. Take 6ml sample.
- Add 20ml of -20°C pre-chilled acetone to all samples for acetone precipitation. Spin down 15min @ 13000 g and less than -10°C
- Resuspend in 1ml Lysis Buffer and check with SDS-PAGE.

2.3.4 Zymography

Material:

Gelatin Stock	Gelatin Stock Solution (10x) 10 % (w/v) gelatin in deionised water
Renaturing Buffer	Renaturing Buffer 2.5 % (v/v) Triton X-100 in deionised water 1.1 mM EDTA (optional)
Development I	Development Buffer I pH 5.0 50 mM sodium acetate 10mM calcium chloride 100mM sodium chloride
Development II (optimized)	Development Buffer II pH 6.5 50mM phosphate buffer 1.1mM EDTA 0.067mM mercaptoethanol 5.5mM cysteine

Method:

- Prepare gels as described in 2.3.2. Add gelatin stock to get a gelatine concentration of 0.5 % (w/v).
- Combine 20µl protein sample and 10µl non-reducing sample buffer (2x).
- Incubate 10min at room temperature. DO NOT HEAT.
- Apply samples (20 µl) and start SDS-PAGE.
- Incubate gel in Renaturing Buffer with gentle agitation for 30min at room temperature.
- Incubate gel in Development Buffer for an additional 30min.
- Refresh development buffer. Incubate 4-8 h @ 37 °C.
- Stain and destain for 30min. with Staining Solution for 30min.

Figure 10: Zymogram

2.4 Molecular Modelling

2.4.1 Web based structure prediction

Nowadays many protein structures have been experimentally determined by X-Ray crystallography or NMR spectroscopy. These are time-consuming and relative expensive methods, which are lacking far behind the output of protein sequences. Known crystal structures are published in web-accessible databases [43, 44], unknown structures can sometimes be predicted *in silico*. In general, there are two prediction methods: First, *ab initio* (or *de novo*) protein modelling methods seek to build three-dimensional protein models from scratch, only based on physical principles, i.e. by optimization of a suitable energy function. Yet, these procedures have only been successful for tiny proteins. Second, comparative protein modelling uses previously solved structures as templates, which is effective, because there is only a limited set of conserved tertiary structure motifs. Based on a known primary structure, protein threading tools [45, 46] scan the amino acid sequence against a database of solved structures, score the compatibility of the sequence to the structure, thus yielding a possible three-dimensional model. To visualize the results, there are a number of software programs available [38, 39, 40].

2.4.2 Docking simulation

Specific protein-ligand, protein-protein, antibody-antigen, enzyme-substrate or enzyme-inhibitor docking can be studied or predicted by *in silico* simulation. If the structures of both substances are known, **Hex Protein Docking** [37] and **PatchDock** [47] can be used to predict possible interaction sites.

For our studies on cathepsin B (structure from protein threading) and Z-Phe-Ala-CHN_2 (unknown three-dimensional structure), we tried to visualize possible conformations of the small molecule by calculating the lowest energy conformation with InSight II [41] and the modules Modeler and ZDockPro.

3 Experimental part

3.1 Strategy and status quo

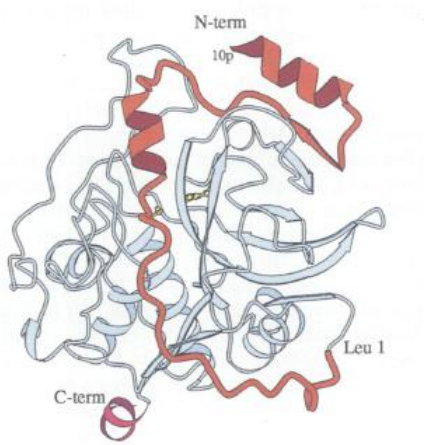

Figure 11: Zymogenic pro-form of cathepsin B [4]

We used an **Impact CN Protein Purification** Kit from New England Biolabs [13] in order to clone, express and purify trypanosomal cathepsin B heterologously in *E. Coli*. Impact stands for Intein Mediated Purification with an Affinity Chitin-binding Tag. Its vector pTYB11 (Figure 12) provides ampicillin resistance, an IPTG-inducable T7 expression system, a multiple cloning site linked to an intein tag with chitin-binding domain for easy purification on a chitin column. In the presence of thiols as dithiothreitol, 2-mercaptoethanol or cysteine, the intein tag itself may be excised by peptide bond cleavage ("protein splicing") coupled to succinimide formation involving Asn 454 at the intein C-terminus. This self-cleavage releases the target protein from the chitin-bound intein tag resulting in a single-column purification of the target protein (Figure 13, Figure 14).

So far, the mRNA coding for cathepsin B (tbcatB) had been isolated and reversely transcribed by RT-PCR, cloned into pTYB11 and transformed into Escherichia Coli cells (**T4-150-6II**).

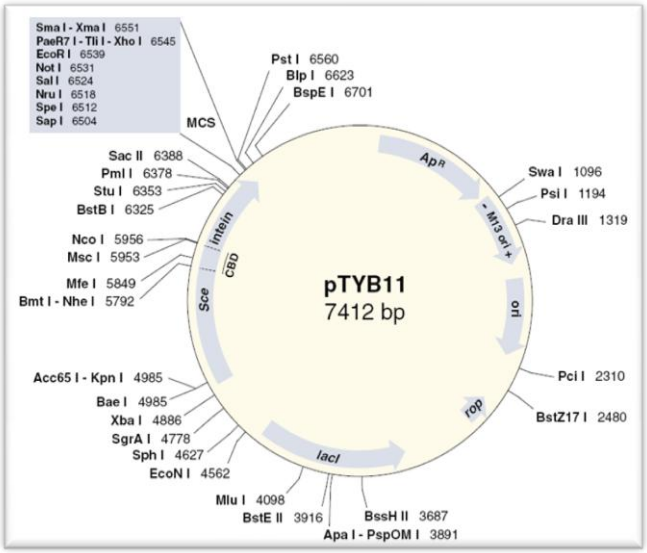

Figure 12: Plasmid vector pTYB11 [13]

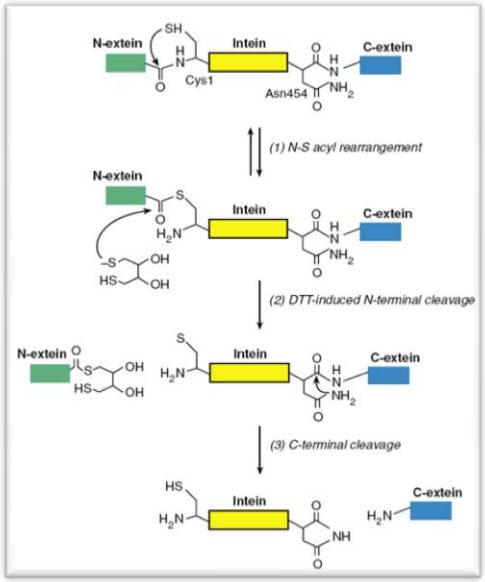

Figure 13: Excision of intein by peptide bond cleavage

The complete sequence of pTYB11 with the gene for *t.brucei* cathepsin b is listed below:
(sequence as found on 5'-3'-coding strand)

```
1       AGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT beta-
91      GAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTT lactamase
181     TGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTA  signal
271     CATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTCTCCAATGATGAGCACTTTTAAAGTTCTGCT  peptide
361     ATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTC
451     ACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAA  (for amp
541     CTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGA  resist.)
631     ACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGG
721     CGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC
811     GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTC
901     CCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAA
991     GCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTACCCCGGTTGATAATCAGAAAAGCCCCAAAAACAGGAAG
1081    ATTGTATAAGCAAATATTTAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAA M13
1171    TAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTA  origin
1261    TTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCAAATCAAGTTTT  of
1351    TTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCG  replic.
1441    AGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCG Sac II
1531    CTTAATGCGCCGCTACAGGGCGCGTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTC
1621    GTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAA
1711    AAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGAT pMB1
1801    ACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCT  origin
1891    GTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGG  of
1981    CTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGC  replic.
2071    CACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAA
2161    CGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATG
2251    GAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTC
2341    TGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGC
2431    TATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGC
2521    GCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGG
2611    GAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGCACGCTTC rop CDS
2701    ACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAG  ON COMPLEM.
2791    GGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGAT  STRAND
2881    GCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCA
2971    GAGAAAAATCACTCAGGGTCAATGCCAGCCGAACGCCAGCAAGACGTAGCCCAGCGCGTCGGCCGCCATGCCGGCGATAATGGCCTGCTT
3061    CTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCAT
3151    CGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGT
3241    CATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGG
3331    TGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATG
3421    AATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGC
3511    CCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACG lacI CDS
3601    GCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGC  ON COMPLEM.
3691    GCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAAC  STRAND
3781    CGGACATGGCACTCCAGTCGCCTTCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGAC
3871    GCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGT
3961    CTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAG
4051    CAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTT
4141    CGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCG
4231    CGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCA
4321    GCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGA
4411    CACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATAC
4501    CGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGG
4591    TTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATA
4681    CCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCA
4771    CCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGG T7 Prom.
4861    AATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACTTATGAAAATCGAAGAAGGTAAACT LacOp/RBS
4951    GGTAATCGGTTCCCTGGAGGGTTGCTTTGCCAAGGTACCAATGTTTTAATGGCGGATGGGTCTATTGAATGTATTGAAAACATTGAGGT malE KpnI
5041    TGGTAATAAGGTCATGGGTAAAGATGGCAGACCTCGTGAGGTAATTAAATTGCCCAGAGGAAGAGAAACTATGTACAGCGTCGTGCAGAA
5131    AAGTCAGCACAGAGCCCACAAAAGTGACTCAAGTCGTGAAGTGCCAGAATTACTCAAGTTTACGTGTAATGCGACCCATGAGTTGGTTGT Intein 5'
5221    TAGAACACCTCGTAGTGTCCGCCGTTTGTCTCGTACCATTAAGGGTGTCGAATATTTTGAAGTTATTACTTTTGAGATGGGCCAAAAGAA  region
5311    AGCCCCCGACGGTAGAATTGTTGAGCTTGTCAAGGAAGTTTCAAAGAGCTACCCAATATCTGAGGGGCCTGAGAGAGCCAACGAATTAGT
5401    AGAATCCTATAGAAAGGCTTCAAATAAAGCTTATTTTGAGTGGACTATTGAGGCCAGAGATCTTTCTCTGTTGGGTTCCCATGTTCGTAA Hind III
5491    AGCTACCTACCAGACTTACGCTCCAATTCTTTATGAGAATGACCACTTTTTCGACTACATGCAAAAAAGTAAGTTTCATCTCACCATTGA
5581    AGGTCCAAAAGTACTTGCTTATTTACTTGGTTTATGGATTGGTGATGGATTGTCTGACAGGGCAACTTTTTCGGTTGATTCCAGAGATAC
5671    TTCTTTGATGGAACGTGTTACTGAATATGCTGAAAAGTTGAATTTGTGCGCCGAGTATAAGGACAGAAAAGAACCACAAGTTGCCAAAAC
5761    TGTTAATTTGTACTCTAAAGTTGTCAGAGGTGCTAGCACAAATCCTGGTGTATCCGCTTGGCAGGTCAACACAGCTTATACTGCGGGACA  Chitin
5851    ATTGGTCACATATAACGGCAAGACGTATAAATGTTTGCAGCCCCACACCTCCTTGGCAGGATGGGAACCATCCAACGTTCCTGCCTTGTG  binding
5941    GCAGCTTCAAGGTGGCCATGGTGGTATTCGCAATAATCTTAATACTGAGAATCCATTATGGGACGCTATTGTTGGCTTAGGATTCTTGAA  domain
6031    GGACGGTGTCAAAAATATTCCTTCTTTCTTGTCTACGGACAATATCGGTACTCGTGAAACATTTCTTGCTGGTCTAATTGATTCTGATGG
6121    CTATGTTACTGATGAGCATGGTATTAAAGCAACAATAAAGACAATTCATACTTCTGTCAGAGATGGTTTGGTTTCCCTTGCTCGTTCTTT Intein 3'
6211    AGGCTTAGTAGTCTCGGTTAACGCAGAACCTGCTAAGGTTGACATGAATGTCACCAAACATAAAATTAGTTATGCTATTTATATGTCTGG  region
6301    TGGAGATGTTTTGCTTAACGTTCTTTCGAAGTGTGCCGGCTCTAAAAAATTCAGGCCTGCTCCCGCCGCTGCTTTTGCACGTGAGTGCCG
6391    CGGATTTTATTTCGAGTTACAAGAATTGAAGGAAGACGATTATTATGGGATTACTTTATCTGATGATTCTGATCATCAGTTTTTGCTTGG former
6481    ATCCCAGGTTGTTGTACAGAACATGcatCTCatgCGTgccTGCatcACAtttTGTatcGCTtcgACGgctGTAgtcGCCgtaAACgccGC  Sap I
        Cathepsin B --> M H L M R A C I T F C I A S T A V V A V N A A
6571    CctcGTTgctGAAgacGCGcccGTCctcTCCaagGCTttcGTCgacCGCgtgAACcgcCTCaacCGCgggATTtggAAGgcgAAGtacGA Sac II
          L V A E D A P V L S K A F V D R V N R L N R G I W K A K Y D
6661    TggcGTCatgCAAaacATAacaTTGcgcGAGgcgAAGcggCTCaacGGTgttATTaaaAAGaatAATaacGCCagcATCcttCCGaagAG
          G V M Q N I T L R E A K R L N G V I K K N N N A S I L P K R
6751    GcgaTTCaccGAGgaaGAGgcaCGTgccCCGcttCCCagcAGCttcGATtccGCTgaaGCTtggCCCaacTGCcccACCatcCCAcaaAT Hind III
          R F T E E E A R A P L P S S F D S A E A W P N C P T I P Q I
6841    TgcaGATcagTCCgccTGTggcTCCtgcTGGgcaGTCgctGCGgccTCAgccATGtccGACcgaTTTtgtACGatgGGGggtGTGcagGA
          A D Q S A C G S C W A V A A A S A M S D R F C T M G G V Q D
```

```
6931   CgtcCATatcTCAgcaGGTgacTTGcttGCGtgcTGCagtGATtgtGGCgatGGCtgtAATggtGGCgatCCGgatAGGgcgTGGgcgTA  Pst I
         V  H  I  S  A  G  D  L  L  A  C  C  S  D  C  G  D  G  C  N  G  G  D  P  D  R  A  W  A  Y
7021   CtttAGCtcaACAggcCTCgttTCGgacTACtgcCAAcccTACccaTTTcccCATtgcTCAcacCACagcAAAagcAAAaatGGGtacCC  Kpn I
         F  S  S  T  G  L  V  S  D  Y  C  Q  P  Y  P  F  P  H  C  S  H  H  S  K  S  K  N  G  Y  P
7111   TcccTGTtctCAGttcAACtttGACacgCCCaagTGCaatTACacgTGCgatGACccgACAatcCCCgttGTCaacTACcgtTCCtggAC
         P  C  S  Q  F  N  F  D  T  P  K  C  N  Y  T  C  D  D  P  T  I  P  V  V  N  Y  R  S  W  T
7201   GagcTATgcgCTTcaaGGAgagGATgacTACatgCGTgagTTGttcTTCcgcGGTccaTTTgagGTGgcgTTTgatGTCtacGAAgacTT  Sac II
         S  Y  A  L  Q  G  E  D  D  Y  M  R  E  L  F  F  R  G  P  F  E  V  A  F  D  V  Y  E  D  F
7291   CatcGCCtacAACagcGGCgtcTACcacCACgttTCAggaCAAtatCTCggaGGAcatGCGgtgAGGctcGTTgggTGGggcACAtcaAA
         I  A  Y  N  S  G  V  Y  H  H  V  S  G  Q  Y  L  G  G  H  A  V  R  L  V  G  W  G  T  S  N
7381   CggaGTGccgTACtggAAGattGCCaacAGCtggAACaccGAGtggGGAatgGACggtTACtttCTCatcCGCcgcGGAagtAGCgaaTG  Sac II
         G  V  P  Y  W  K  I  A  N  S  W  N  T  E  W  G  M  D  G  Y  F  L  I  R  R  G  S  S  E  C
7471   CggcATCgagGATggcGGTtctGCTggcATTcctCTTgcaCCCaacACGgcgTAGCTCGAGCCCGGGTGACTGCAGGAAGGGGATCCGGC  XhoI PstI
         G  I  E  D  G  G  S  A  G  I  P  L  A  P  N  T  A  *        <-- Cathepsin B
7561   TGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTT  T7 transk
7651   GAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCCGCAAGAGGCCCGGCAGTACCGGCATAACCAAGCCTATGCCTACAGCA   termin.
7741   TCCAGGGTGACGGTGCCGAGGATGACGATGAGCGCATTGTTAGATTTCATACACGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAA
7831   CTACCGCATTAAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCA  Hind III
7921   CATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCAG
8011   CTTGGCTGTTTTGGCGGATGAGATAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCT
8101   GGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCAT
8191   GCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAA
8281   CGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATA
8371   AACTGCCAGGCATCAAATTAAGC
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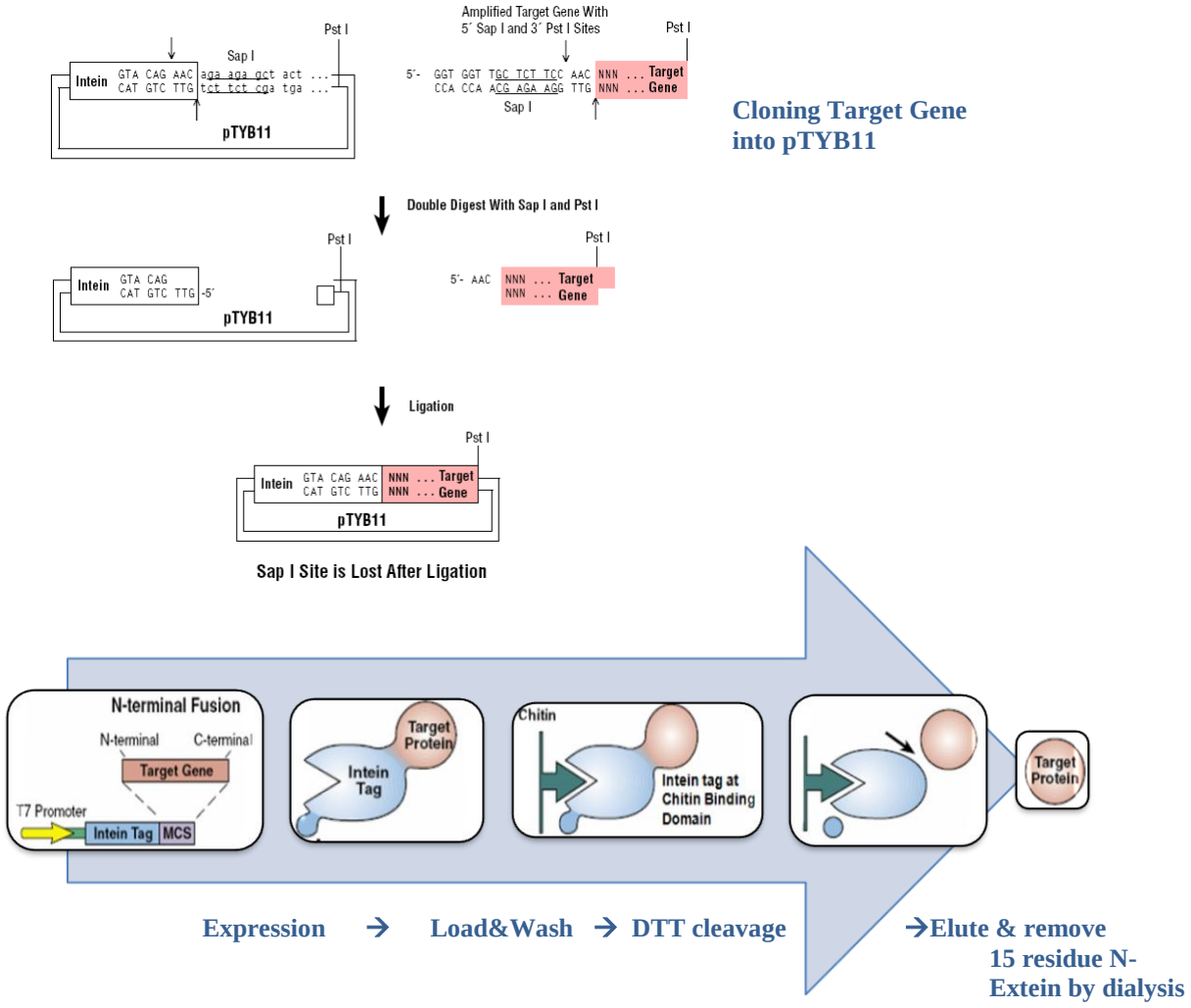


Figure 14: Protein Purification Flowchart [13]

3.2 Cloning t. brucei cathepsin B into E. Coli

We began our experimental work with *Escherichia Coli* **T4-150-6II** cells that had been previously transformed with a pTYB11-tbcatB construct. At this stage we worked with bacteria without the T7-RNA-polymerase expression system, as the cells were mainly used for amplification and screening purposes. In general, the insertion of a gene into a plasmid vector may lead to different (by-) products: First of all, the plasmid might re-ligate without any insert. Second, the gene of interest might be inserted in wrong direction (*anti-reverse*). The Impact CN Protein Purification system uses Sap I and Pst I as restriction endonucleases whereby the multiple cloning site and the Sap I recognition site are completely cut out. Different sticky ends remain, making simple re-ligation or wrong directed insertion impossible. In theory it would be possible that a small amount of plasmid is going to re-insert the just cut-off fragment instead of tbcatB. Further, there is always a risk of mutations during amplification and processing. This is why recombination and cloning should be thoroughly confirmed by restriction analysis and DNA sequencing before proceeding to the cell culture and protein expression.

Correct transformation, namely the uptake of plasmid DNA into the cell, is ensured by antibiotic selection on agar plates. pTYB11 contains a gene coding for beta-lactamase, which allows the bacteria to grow on ampicillin. However, blue/white screening for correct recombination on bases of X-gal is neither necessary, nor possible with pTYB11.

To check whether the transformed **T4-150-6II** cells contained the pTYB11-tbcatB construct, the cells were grown on Petri plates (2.2.1.1). We picked two clones, cultivated them in three milliliter starter cultures (2.2.1.2), isolated plasmid DNA by alkaline lysis and ethanol precipitation (2.1.1.3). Enzymatic restriction (2.1.3) was used to create fragments of a specific length that can be visualized by agarose gel electrophoresis (2.1.4). There are many commercially available restriction enzymes. For logical reasons we needed at least two restriction enzymes, one with a single recognition site on the vector, and one with a recognition site on the insert. Comparison with *in silico* [42] restriction helped to interpret the results.

Software emulation identified one recognition site for **Xho I**, which is located in direct neighborhood of the insert, and four recognition sites for **Sac II**, three of them located on the insert. Single digestion with one enzyme should lead to four XhoI-fragments sized 204, 246, 615, 7328 base pairs, and one 8393 bp long SacII-fragment respectively (Figure 15).

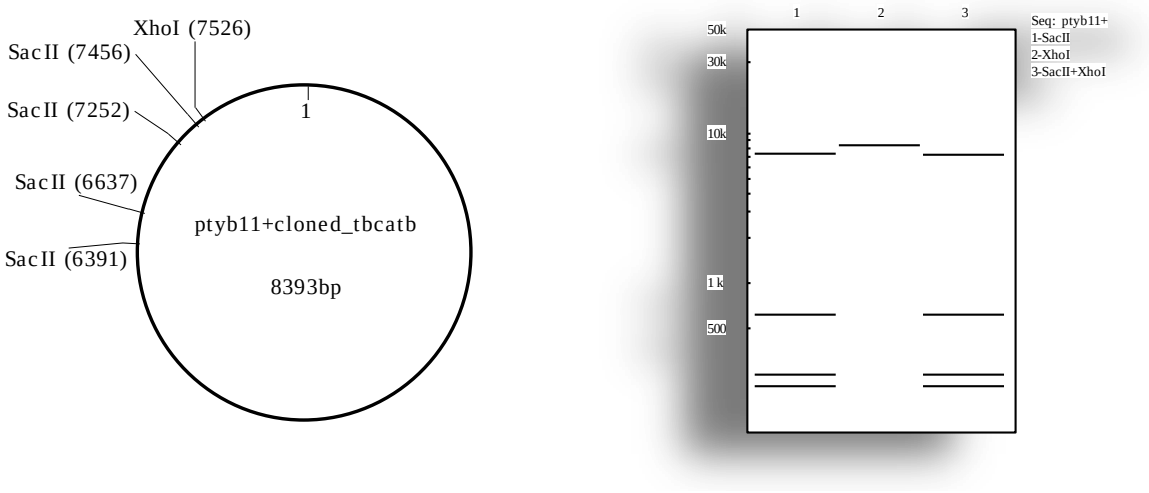


Figure 15: SacII / XhoI restriction sites and fragment sizes

As discussed, wrong directed insertion is not possible when using two different sticky ends. However, Figure 16 shows exemplarily how such faulty plasmids could be detected by restriction analysis. An anti-reversely inserted gene would lead to a different SacII-pattern of fragments (180, 204, 615, 7394 base pairs).

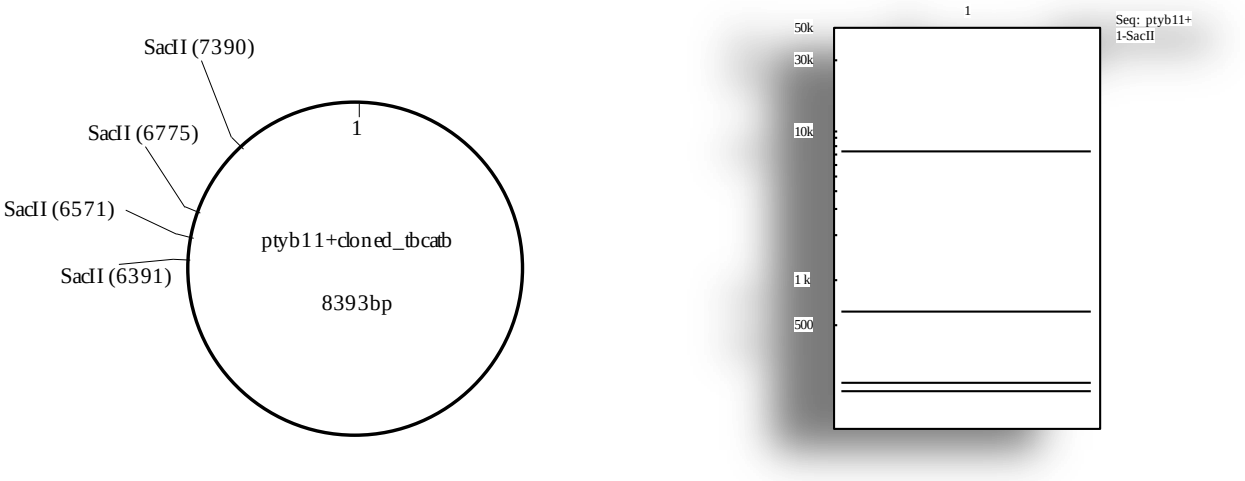


Figure 16: tbcatB *anti-reversely* inserted. SacII restriction sites and fragment sizes.

We separated the DNA fragments electrophoretically on a 0.8% (w/v) agarose gel, whose resolution ranges from 0.7 up to 11 kilo base pairs (Figure 17). The big fragment could be easily detected, but the smaller ones showed only very weak fluorescence under UV light.

For an additional test we chose **Pst I** as restriction endonuclease, which has one recognition site on the insert, as well as one recognition site on the plasmid vector (Figure 18, Figure 19). The fragments of 577 and 7816 bp length could be visualized on a 1.5% (w/v) gel with a resolution from 0.2 up to 3 kbp.

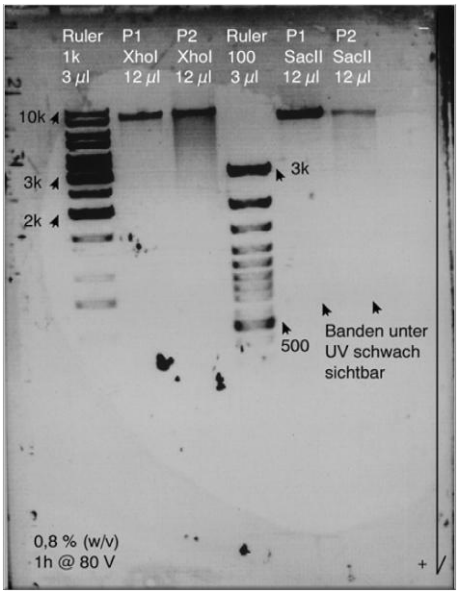


Figure 17: Enzymatic restriction with XhoI/SacII

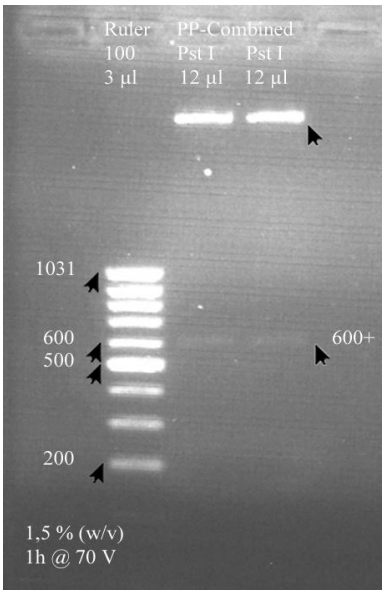


Figure 18: Enzymatic restriction with PstI

Figure 19: PstI restriction sites and fragment sizes

Although the fragments are not very intense and the digestion therefore may not be quantitative, fragment sizes seemed to be correct.

At this stage we focused on the expression system and transformed (2.2.2.2) the pTYB11-tbcatB construct into bacteria cells containing T7 RNA polymerase. Initially we worked with two *Escherichia coli* strains named **ER2566** and **BL21DE3**. Each transformed strain was cultivated overnight on LB Agar plates (2.2.1.1) to get distinct clones. One clone per strain was picked and proliferated in 3ml cultures (2.2.1.2) without antibiotics.

To verify the plasmid had been taken up into the new strains, we repeated experimental control by DNA extraction and restriction analysis. Endonuclease **Pst I** was used once again to give two fragments of 577 and 7816 bp. Additionally, we tried **Hind III**, that hydrolyses our circular plasmid into fragments of 1035, 1380 and 5978 bp. All results were obtained as expected (data shown in Figure 21 and Figure 22).

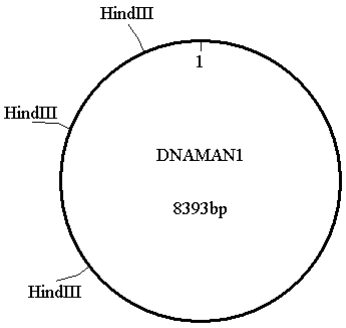


Figure 20: HindIII restriction sites

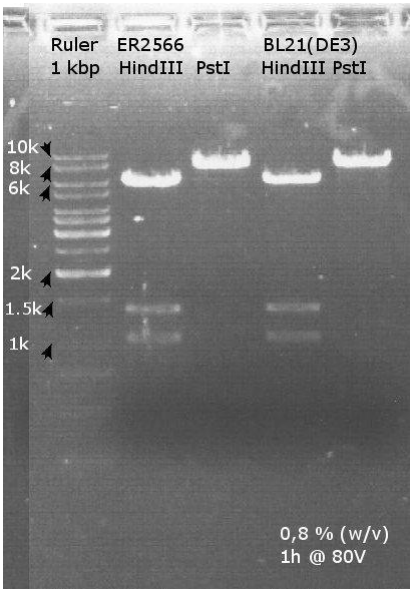


Figure 21: Enzymatic restriction with HindIII / PstI

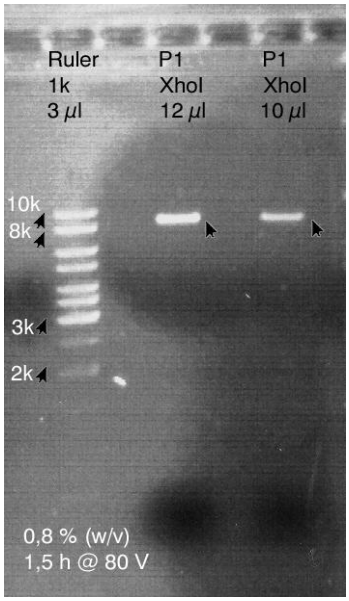


Figure 22: Enzymatic restriction with XhoI (BL21DE3)

Because the correct transformation and recombination is crucially important for all continuous work, we decided to check the plasmid for correct insertion and the absence of mutations by DNA sequencing. We isolated **BL21DE3** plasmid DNA using a QIAgen preparation kit (2.1.1.1) and charged MWG [(53)] and GATC [(54)] with the sequencing process (Sanger's didesoxy method).

The ascertained base sequence was compared to the theoretically wanted sequence. Computer-based alignment tools (BLAST [(43)]) revealed four differences that had to be scrutinized:

I.) At forward position 595 (which corresponds to position 7126 of pTYB11-tbcatB) a thymine has been sequenced instead of cytosine. Reverse sequencing confirms the mutation at position 386. As TTT and TTC are wobble bases (Figure 23), there should be no effect on protein level.

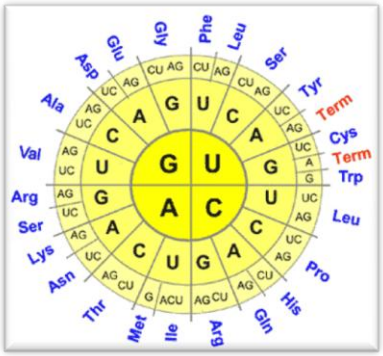
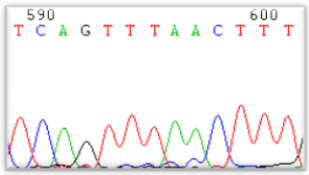
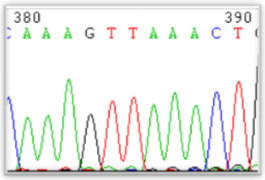


Figure 23: Wobble bases

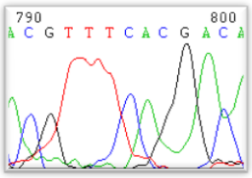


forward primer

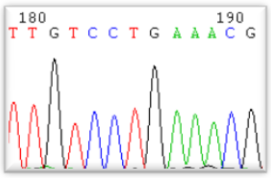


reverse primer

II.) At forward position 797 (which corresponds to position 7328 of pTYB11-tbcatB) a cytosine has been sequenced automatically instead of guanine. A view on the plots shows that there might be a detection error. The corresponding codon CGA should have rather been detected as GGA. The reverse run gives the correct codon at position 184.

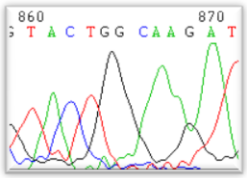


forward primer

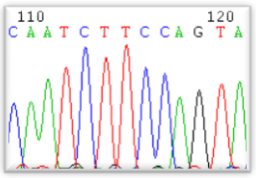


reverse primer

III.) At forward position 866 (which corresponds to position 7397 of pTYB11-tbcatB) a cytosine has been inserted, that is not present in the original sequence. One-base insertions are critical, as the resulting frame shift would change the entire primary sequence of the translation product. However, the reverse run does not show any insertion between 115/116.

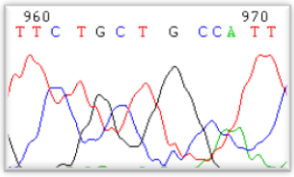


forward primer

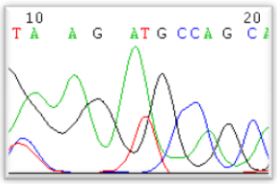


reverse primer

IV.) At position 967 (which corresponds to position 7497 of pTYBB11-tbcatB) a cytosine has been sequenced instead of guanine. Manual correction shows that this is a detection error. The reverse run at position 16 gives the correct sequence.



forward primer



reverse primer

3.3 Expressing t. brucei cathepsin B

3.3.1 Induction and lysis. Localisation of intein-tagged cathepsin B.

The pTYB11 vector is designed to transcribe the gene of interest after isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Therefore it implements the T7 polymerase expression system: T7 is a viral DNA-dependant RNA polymerase, that has been cloned into some *E. Coli* strains, i.e. **ER2566** and **BL21DE3**. It recognizes the T7 promoter region, that is located upstream of the pTYB11 multiple cloning site. If is not hindered by any repressor, it transcribes the target gene until it reaches the T7 termination sequence.

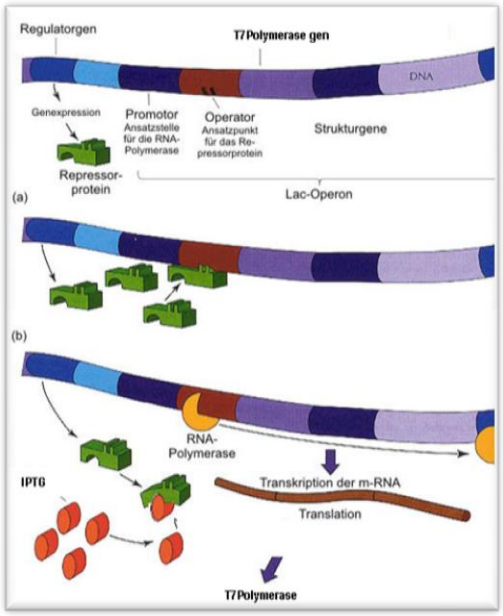


Figure 24: IPTG Induction

The construct uses a *lac* operon as kind of a biological "switch". In a physiological state, the cells synthesize *lac* repressor which binds on an operator region between promoter and gene. Induction with IPTG changes the *lac* repressor conformation, which in turn releases the operator. As a result, T7 polymerase gets able to start transcription. Once the expression has been induced, the cells will stop proliferation and spend all their energy into synthesis of the foreign protein.

Both pTYB11-tbcatB transformed *E.Coli* strains were cultivated as described (3ml culture overnight, 2.2.1.2, then 100ml culture with 200µl ampicillin, 2.2.1.3) to an optical density of 0.55. Both cultures were divided into 50ml portions, one left uninduced as negative control, one inoculated with 0.5mM IPTG for several hours at 37°C. The cells were lysed by ultra sonification (2.2.3). To prevent autoproteolysis, we added Roche [(12)] Complete Protease

Inhibitor. The crude extracts were centrifuged and comparable amounts (2.3.1) of protein of all fractions (pellet and supernatant) were loaded on a SDS-PAGE (2.3.2, Figure 25).

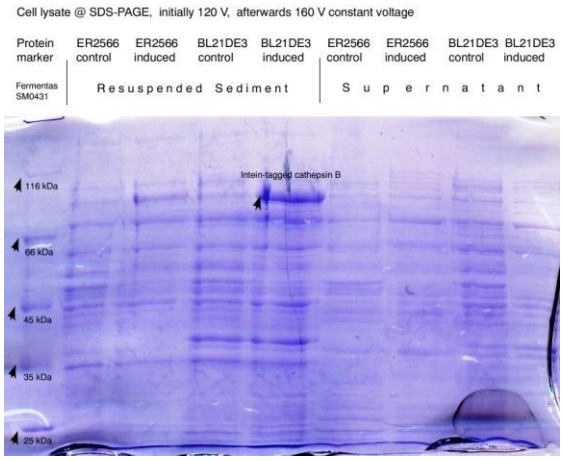


Figure 25: SDS-PAGE of cell lysates

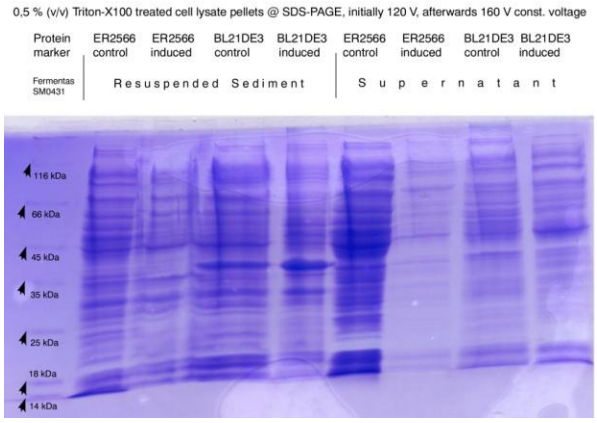


Figure 26: Detergent extraction of cell debris

Only **BL21DE3** cells show a significant accumulation of intein-tagged cathepsin B, which has got a molecular weight of 90kDa (cathepsin B: 35 kDa). Unfortunately, it is located in the resuspended sediment. Efforts to get the protein back into solution with Triton-X100 were not satisfactory. In order to increase the yield, we repeated the experiment with overnight induction at 20°C. This time 0.1% (w/v) Triton-X100 were directly added to the Cell Lysis Buffer. Triton-X100 is a non-ionic detergent that is capable of solving proteins out of membrane bilayers. In contrast to SDS, it does not denature proteins. This time, both strains expressed large amounts of intein-tagged cathepsin B, however still as solid. This was when we had to accept that our expression system was not able to create the protein in native conformation. Instead, it conglomerated in form of *inclusion bodies* (Figure 27).

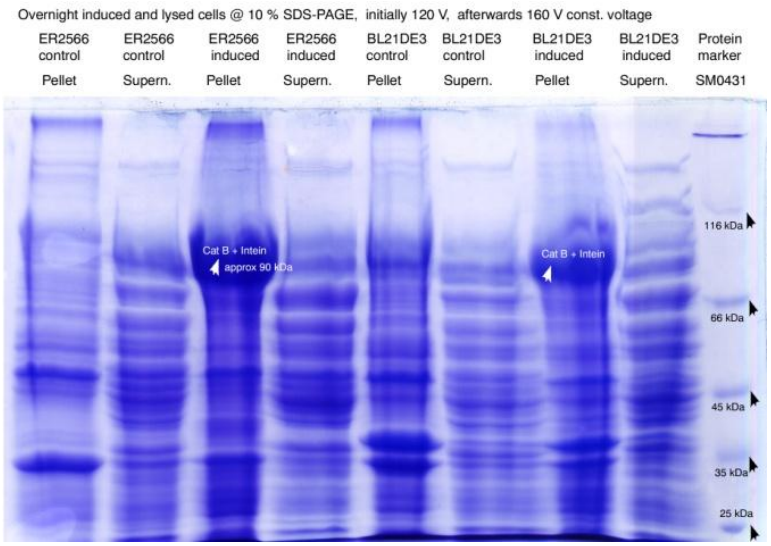


Figure 27: SDS-PAGE of cell lysates after overnight induction at 20°C

3.3.2 Protein isolation from inclusion bodies

Recombinant protein overexpression in *E. Coli* often leads to formation of large, spherical particles, due to the lack of host chaperones. Kuhelj et al [(5)] wrote: „The probability that polypeptide chains possessing many cysteine residues would fold properly within the reducing milieu of *E. Coli* cytoplasm is relatively low and insoluble inclusion bodies usually result". Cathepsin B possesses not less than fourteen cysteine residues, its tertiary structure is hold together by twelve disulfide bonds and one cysteine is situated within its active centre. For disulfide bond formation, the number of possible combinations increases dramatically with the number of cysteine residues present in the polypeptide chain. Based on pure statistics, an exceedingly low yield of correct folding would be expected for the random cysteine pairing. However, correct disulfide bond formation is biased by the free energy gained upon correct formation.

Overproduction by itself, that means the increase in concentration of nascent polypeptide chain, can be sufficient to induce the formation of aggregates, as the yield of native protein depends on the rate of folding, the rate of aggregation and the rate of protein synthesis [(15)]. This model implies that the yield of native protein increases with a decreasing rate of protein expression. Cells cultivated under reduced growth conditions and limited induction might produce more soluble protein. Anyhow, our experimental work at 20°C showed no enrichment of solved cathepsin B. Hence, we needed a method to refold the enzyme into its physiological structure.

In a first attempt we combined Triton X-100 washing steps described by Sambrook, Fritsch, Maniatis [(9, Chapter 17.39)] and the use of strong chaotrophic agents described by Mackey et al. [(2)]: We lysed IPTG-induced **ER2566** cells, centrifuged the suspension for 15min at 13000g and discarded the supernatant. The inclusion body-containing pellet was washed with 0.5% (v/v) Triton X-100, 10mM EDTA, pH8 and centrifuged again. The pellet was resuspended in 6M guanidin-HCl, 20mM Tris pH 8, and 100mM sodium dihydrogen phosphate. After two hours, the solid had not solved, but became vitreous and could not even mechanically been crushed. SDS-PAGE showed no cathepsin B in solution (Figure 28).

In fact, for certain proteins, an initial solubilisation can lead to a new species of folding intermediate aggregates, which cannot be brought into solution again. Higher dilution does not let folding intermediates accumulate and precipitate. We repeated the experiment with **BE21DE3** cells in bigger volumes, as described in chapter 2.3.3. This time, almost all protein was resolved. Guanidin-HCl was removed via two-step dialysis against 3M urea and

chaotroph-free medium. SDS-PAGE (Figure 29) was normalised to 6.8 µg protein per pocket. Although there was some protein loss, the refolding process seemed to work quite well. Anyhow the question remains, if the cathepsin could be brought into its enzymatically active form.

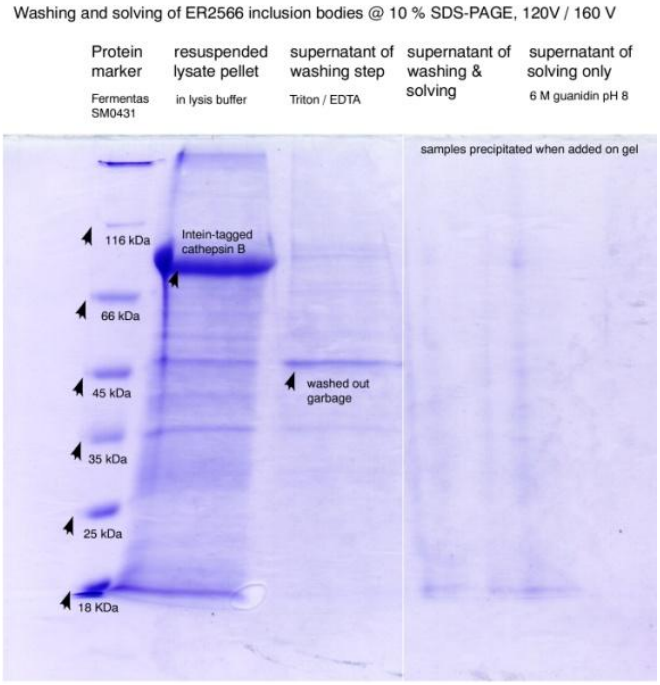


Figure 28: Inclusion body extraction of intein-tagged cathepsin B

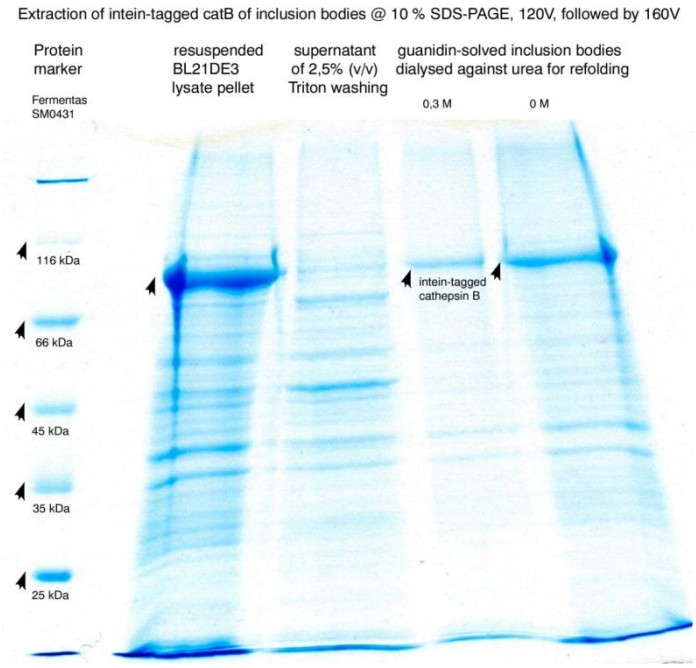


Figure 29: Inclusion body extraction of intein-tagged cathepsin B

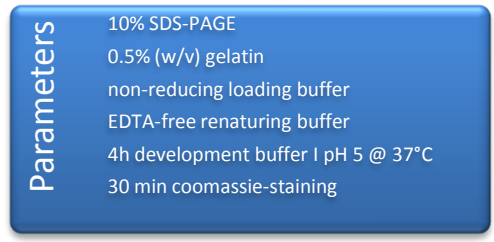
3.3.3 Establishment of enzyme assays (Zymography)

Proteolytic activity can be visualized on slightly modified SDS polyacrylamide gels without the need of blotting the separated bands on nitrocellulose membranes or agar plates. Zymography has been reported [(26)] to be an extremely sensitive method which shows even picogram amounts of gelatinase [(27)]. Therefore gelatin is copolymerized with a conventional SDS gel. All isolation, purification and separation procedures are performed at 4°C to prevent autoproteolysis. After running the gelatin-SDS-PAGE, sodium dodecyl sulphate is replaced by detergents like Triton X-100 in an intermitting washing step. Afterwards, incubation in development buffer lets proteins, which are still inside the gel, refold into their physiological and active structure. Enzymatic active proteases will digest gelatin and appear as white bands in a blue coomassie-stained gel.

However, most zymographic methods described are based upon tryptic activity, as well as other serine proteases capable of digesting gelatin or casein. As cathepsin B uses cysteine in its catalytic triade, we had to modify recently described protocols. First, papain-like cathepsins seem to be rather non-specific [(3)] enzymes with no clear substrate-recognition site. This does not imply that specific inhibitors cannot be designed, but it encouraged us to use gelatin as a substrate. Gelatin represents the denaturated form of fibrilic collagen type I, II and/or III. It is built up of peptide chains, which unusually wind to a left-handed helix. Those peptides consist of repetitive motives of proline, hydroxyproline and glycine. Second, cathepsins B, C, H and X are exopeptidases [(3)], which have an insertion of about 20 residues, which build a so-called occluding loop, that reduces the number of substrate-binding sites. This would convert any digestion assay to an extremely time-intensive experiment. Anyhow, due to the flexibility of its occluding loop, cathepsin B is able to move it aside and to permit full access to the active site cleft. In that way, endopeptidase activity is also made possible. Third, cathepsin B is a member of the family of papain-like molecules. Papain has its optimum at a pH of 6-7 and cleaves bonds of basic amino acids, leucine and glycine [(50)]. We decided to use papain as a standard for our work on zymography. There would have also been available cathepsin B out of bovine spleen or human liver, but at comparatively high costs. Last, we decided to process refolding and digestion at pH 5, which is found in lysosomes. Although that could lower the activity of our papain standard, we hoped to generate better reaction conditions for the extracted (in *t. brucei* lysosomal) cathepsin B. As found in (3) all known lysosomal cysteine proteases are cathepsins, but not all cathepsins are lysosomal or cysteine proteases. However, cathepsin B is.

When we had lysed transformed and induced *E. coli* cultures, we were really enthusiastic as we found an intense band at the expected size of approximately 90 kDa. But it was clear that an enzyme assay got more and more necessary to validate whether our protein of interest had been synthesized correctly and was still functioning.

When Khalid Muhammed [29] told us of his experience on zymography, we started a first attempt with a papain standard and aliquots of the lysation steps. All steps were performed as described in section 2.3.4. The papain turned itself out as not completely soluble. For further experiments we ordered lyophylized, water soluble enzyme.



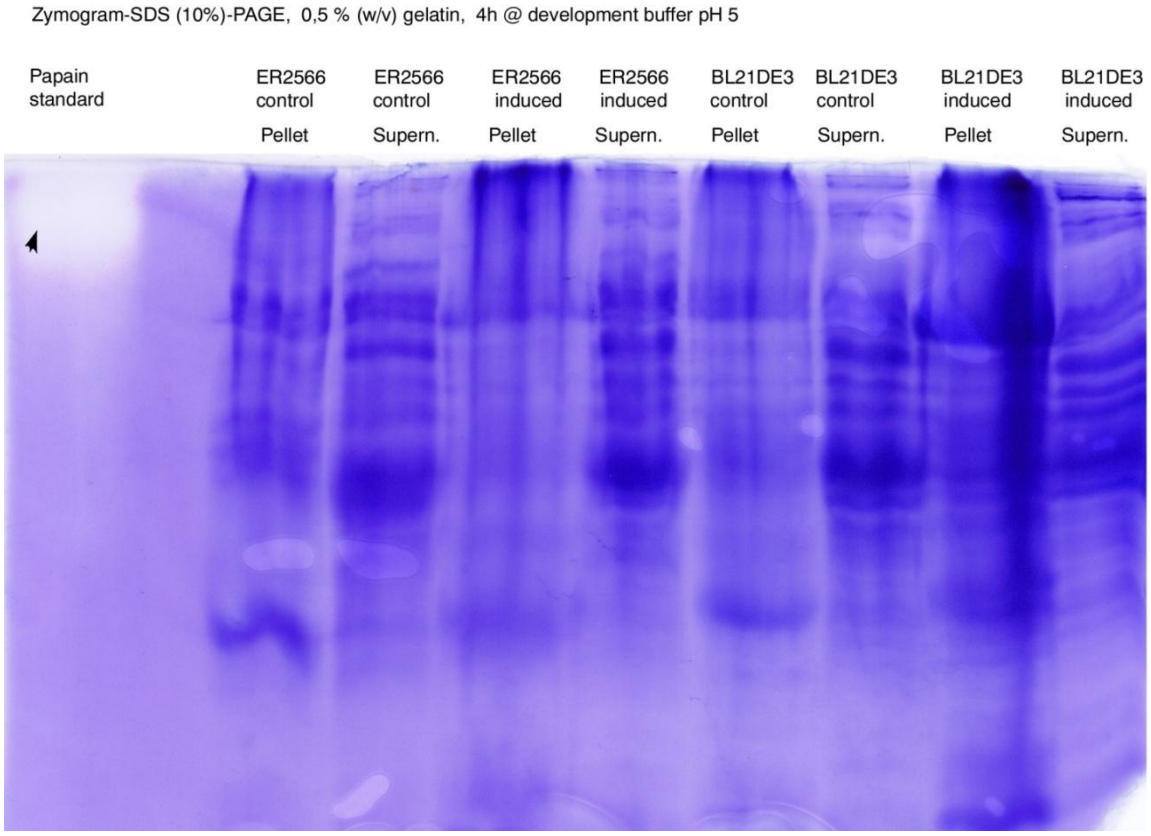
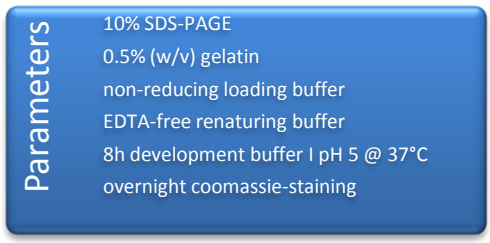


Figure 30: First experiments on zymography

The papain standard retained its activity and digested the gelatine to a bright uncoloured spot. Due to its insolubility it did not move correctly through the gel, generating extensive bands without proper definition. It is evident, that it occurs at a very early running position, compared to its size of only 21 kDa. Isolation samples showed no activity, which might result of the short development time. The next assay is to be incubated for 8h.

We had expected the gelatin gel would be completely blue except of the bands with protease activity. However, protein without activity can easily be distinguished from the coomassie-stained gelatin. This would make an additional use of molecular weight markers possible.

When we got new papain (11.4 U/mg) from Sigma Aldrich [(50)], we retried to optimize our zymography protocol. This time we focused on several concentrations of papain to find out how sensitive our assay would be. Additionally we applied SDS molecular weight marker [(chapter 2.3.2, selected readings 48)], as well as the guanidinium-resolved and dialysed inclusion bodies. Development time was set to eight hours.



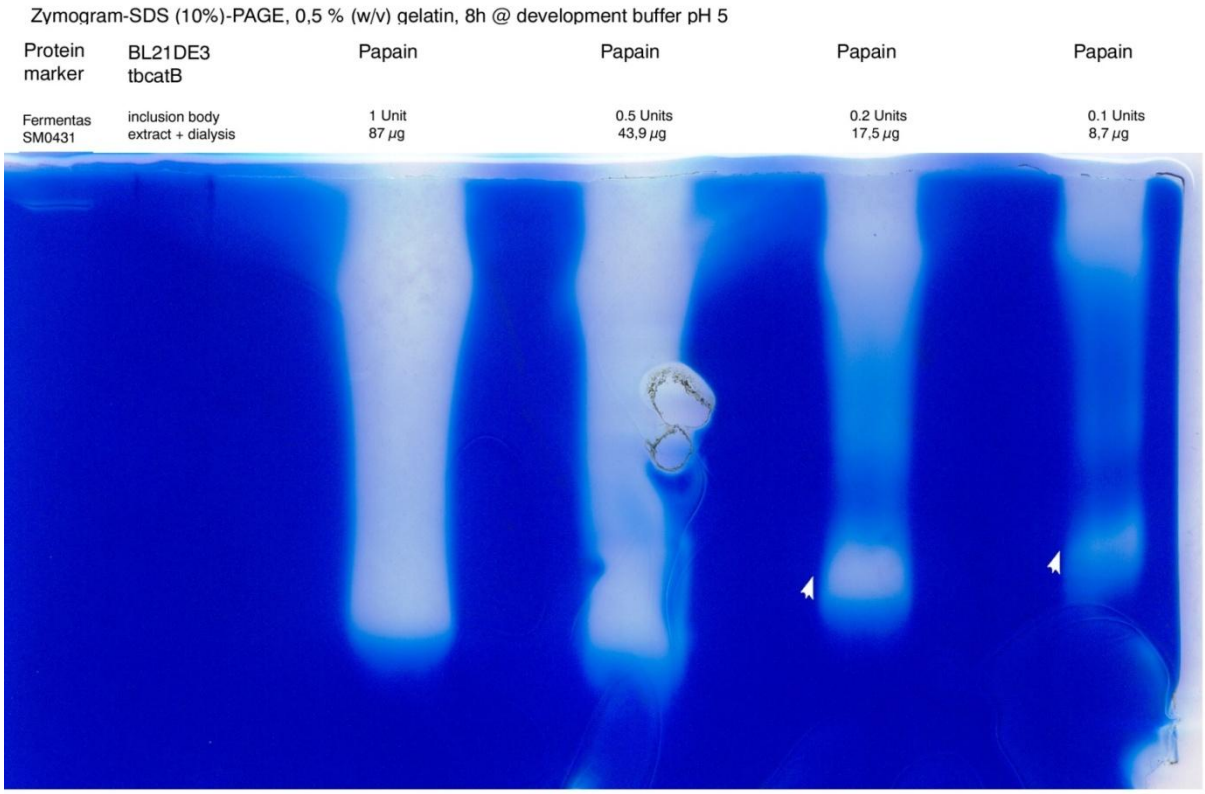
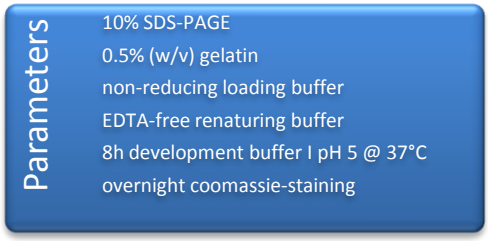


Figure 31: Finding out papain standard concentrations for zymographic SDS-PAGE gels (1 - 0.1 U)

Every lain that was loaded with papain showed very strong digestion. Enzymatic activities as well as applied concentration (which of course correlate, but are responsible for slightly different effects) seemed to be much too high. The gelatine cleavage seemed to have taken place during electrophoresis leaving a trace of white and uncolorable gel. This time no activity-free protein could be visualized on the dark background staining. This is why we couldn't do any comparison with the applied molecular weight marker. Our protein of interest showed no gelatinase activity at all. We should mention that it is still attached to a

55kDa huge intein tag which hinders the cathepsin B from refolding correctly. Beyond that, we realized that we are working with the pre-pro-protein form containing a signal peptide that blocks any access to the active site cleft. For further work we would be only in need of activities below 0.1 units. At this point we prepared aliquots with 20 µl of 0.1 units papain (8,8 µg) out of a 11.4 U/ml stock solution. We proceeded the optimization by applying lower amounts of papain. Planned were 50, 10, 5, 1 and 0.5 mU.



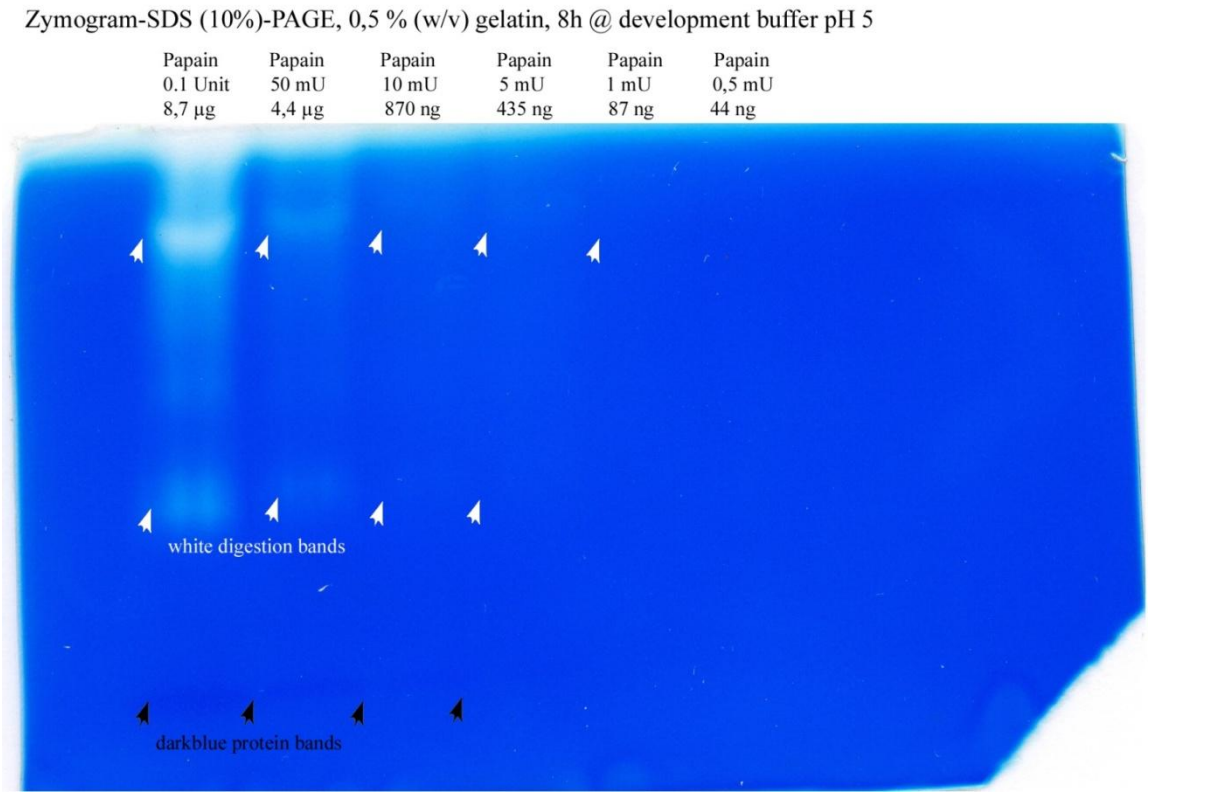


Figure 32: Finding out papain standard concentrations for zymographic SDS-PAGE gels (0.1 U - 0,5 mU)

This time, digestion of 0.1 units papain seemed a little bit weaker than in the previous experiment. This loss of activity may be the result of freezing the aliquoted aqueous enzyme solutions, what is not recommended according to (33). So far, 100 mU (or 8,7 µg papain) showed to be the best choice for carrying out zymography at the applied conditions.

However, the results were very confusing. There have always been two digestive bands, and both of them are at positions of high molecular weights. But we know that papain is a rather small monomeric enzyme with about 21 kDa. In fact there is a dark blue protein band with the estimated molecular weight, but it does not show any gelatinase activity at all. So what could have caused such effects ?

Unfortunately there are a number of possibilities what could have gone wrong. Therefore it was indicated to systematically determine sources of errors and to start specific tests in order to eliminate them. Our papain charge (Sigma-Aldrich [50], product no 76218) came along with a certificate of analysis, which described it as a faintly beige and hygroscopic lyophilisate, soluble up to 5 mg/ml water, giving a brown solution of clear turbidity. Its specific activity was 11.4 U/mg, where a unit was defined as the amount of enzyme which hydrolyzes 1µmol N-benzoyl-L-arginine ethyl ester (BAEE) per minute at pH 6.2 and 25 °C. It should be handled in the dark due to its photosensitivity and under argon atmosphere.

I.) Papain's instability might lead to fragmentation, or more possible according to recent results, to aggregation of enzyme molecules. This could explain why there were digestive bands at high molecular weights. If this theory was true, it would be remarkable that even a papain conglomerate remained active. Literature lookups revealed that papain can indeed build dimers if there is mercury (anorganic or organic) or zinc available [34]. Furthermore, we should consider that our self-made non-reducing loading buffer might cause some strange effects. This is why we wanted to compare how our and Roth's non-reducing loading buffer would behave, as well as Roth's reducing loading buffer (Roti load 1). All samples were applied on a normal 10 % SDS-PAGE as described on section 2.3.2 and stained for 30 minutes with coomassie brilliant blue R-250 (Figure 33a). We found that our non-reducing sample buffer produced at least three bands, one of them slightly bigger than 25 kDa, the others smaller than 25 kDa. The smallest band was also the most intensive. Non-reducing Roti Load 2 gave no clear protein band at all, while Roth's reducing buffer confirmed the band at 25 kDa, but also showed another one below. The fact that Roth's ready-to-use chemical didn't work was really astonishing and made us think of an application error. We decided to repeat this experiment.

Parameters	
	10% SDS-PAGE
	0.5% (w/v) gelatin
	several loading buffers
	30 min coomassie-staining

II.) We had guessed that digestion might even start in the cold room during electrophoresis. This could explain why there were enzymatically active bands (or even digestion over the complete lane), although at those positions was no protein localized in traditional SDS-PAGE. However, the papain sample (Roti Load 2) that was applied on a gelatin copolymerized polyacrylamide gel without incubating it at 37 °C (Figure 33b) showed that there was no unwanted digestion during electrophoresis in the cold-room. All digestive bands must have occurred during renaturation and development of the protease.

Parameters
10% SDS-PAGE
0.5% (w/v) gelatin
Roti Load 2
no renaturing
no development
30 min coomassie-staining

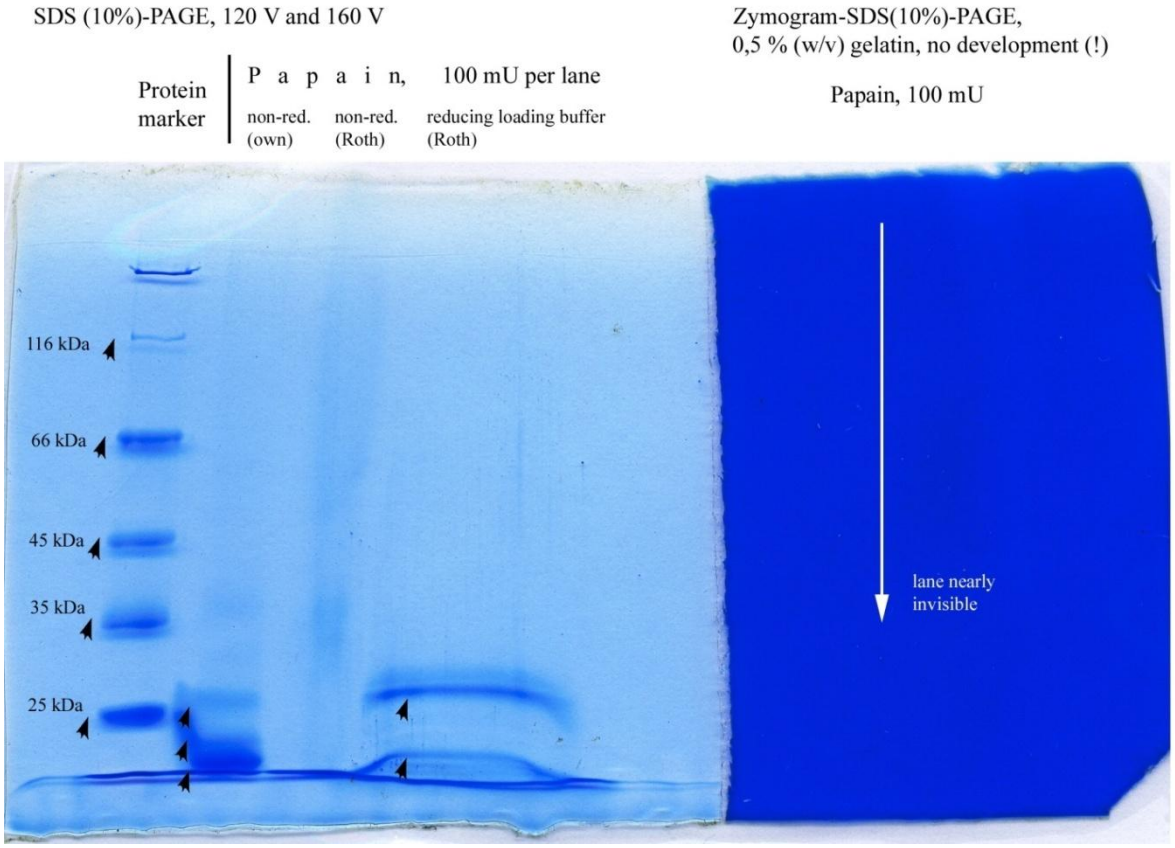


Figure 33: a) Comparison of different loading buffers with papain as a sample. b) Test for unwanted enzymatic activity during electrophoresis

The appearance of several bands with the self-made non-reducing loading buffer, as well as the abundance of any bands with Roti Load 2 pointed to a pollution of our buffer or to an error in using Roth's buffer. To verify our results we retried that part of the experiment:

Parameters
10% SDS-PAGE
several loading buffers
30 min coomassie-staining

Molecular Weight Marker and papain samples (with own loading buffer / Roti Load 2 / Roti Load 1 heated to 95 °C for 5 minutes) were run on a SDS-PAGE (Figure 34a).

Additionally we loaded the gel with our own non-reducing as well as Roth's buffers only, that means without any protein sample. This negative control (Figure 34b) should show whether any of these buffers was contaminated with protein, leading to false bands.

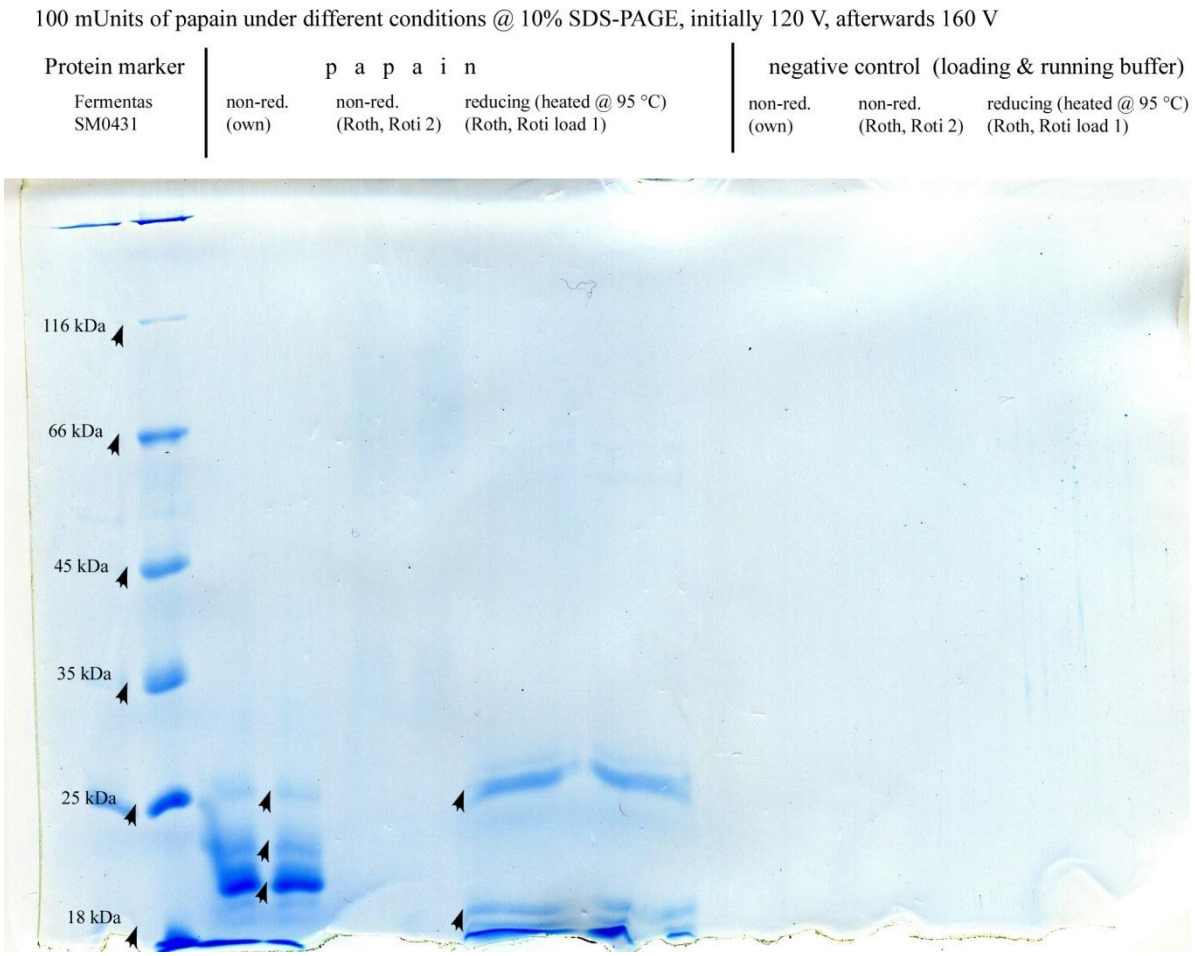


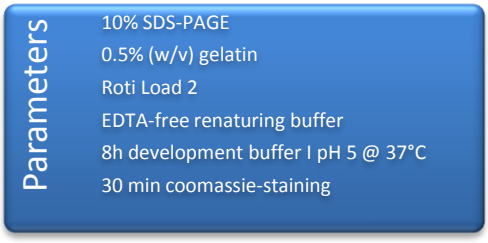
Figure 34: a) Comparison of different loading buffers with papain as a sample. b) Testing loading buffers for purity

This experiment completely confirmed what we already knew: at least three bands could be seen with our non-reducing buffer; Roti Load 2 did not work, and Roti Load 1 produced two distinct bands at approximately 25 kDa and 18 kDa. The loading buffers themselves contained no protein contamination.

This leads to the conclusion that non-reducing conditions promote the fragmentation of the papain sample. This fragmentation is definitely not caused by the loss of subunits as papain is known to be a monomer. Further, non-reducing buffers should stabilize disulfide bonds, which usually fixate tertiary and quaternary structures. But as we remember is papain

recommended to be handled on argon. It is possible that it gets oxidized and fragmented by atmosphere oxygen when not steadily reduced by mercaptoethanol or dithiothreitol.
We would suggest that papain itself can be visualized at a 25 kDa band, while the smaller molecules are oxidation products. Anyhow, this does not explain why activity was found at apparently much higher molecular weights.

III.) Zymography is based on gels which include copolymerized gelatin. This will surely change the pore size of the polyacrylamide web. We thought that this would cause a sample to behave completely different on a zymogram compared to a SDS-PAGE. It was necessary to check how a molecular weight marker would run, and to determine the molecule sizes that caused digestive bands. We run a zymogram with protein marker and papain plus Roti Load 2 (at this time we were not yet informed of its bad running properties, as we carried out several experiments in parallel). Coomassie staining was limited to 30 minutes to maintain a contrast between dark blue protein bands and blue gelatin background. As before, we saw two bright bands where digestion had taken place. This was the first time we could assign them to molecular weights of 35 kDa and 125+ kDa. After all we know, they must have been caused by papain (or any non-protein compound of the papain charge), but their size was totally wrong (Figure 35).



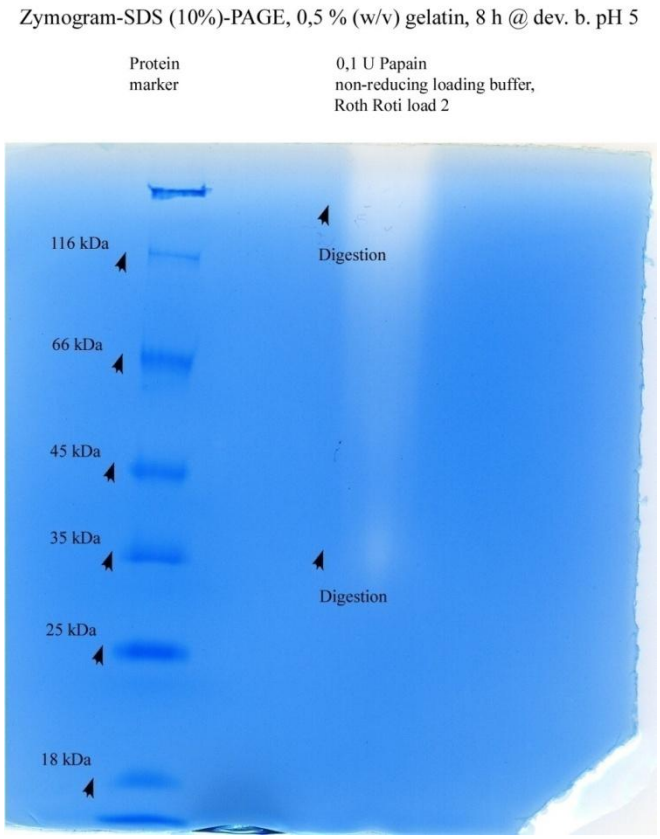


Figure 35: Checking papain movability compared to a molecular weight marker in an gelatine-polyacrylamide-gel

IV.) Finally, we deliberated whether the long process of development could falsify the electrophoresis separation, maybe by diffusion. This was more an idea than a serious consideration, because diffusion or any similar process would take place in all directions and never produce well-defined bands. To be sure, we started a SDS-PAGE of papain, renatured and developed it, exactly as we would do with a zymogram. As expected, there was no significant difference to an immediately coomassie-stained SDS-PAGE *(image not shown)*.

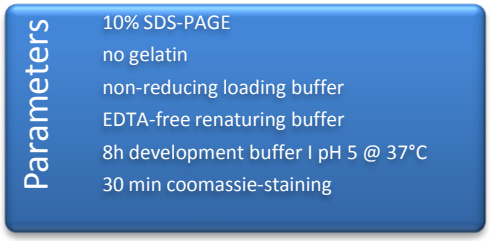


Figure 36 summarizes the workflow that was performed to check which errors had been made and to find out, why there were two digestive bands of wrong sizes and one papain-sized protein band, which showed no activity.

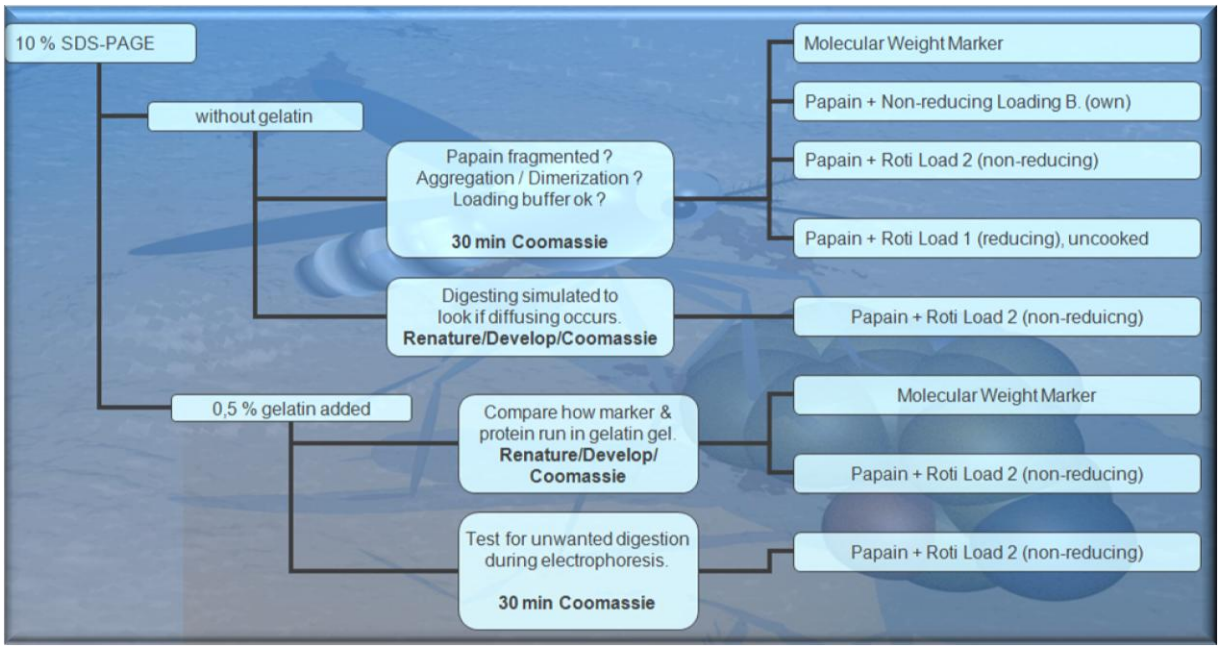


Figure 36: Flowchart to identify and eliminate possible errors

When we found additional information on papain [57] we decided to change some very basic parameters of our essay. It was reported that the enzyme should be fully activated before use in a solution containing 1.1 mM EDTA, 0.067 mM mercaptoethanol and 5.5 mM cysteine for 30 minutes. Optimal activity would be obtained at a pH range from 6.0 to 7.0. Stabilizing agents are EDTA, cysteine and dimercaptoethanol.

Creating a development buffer of this composition would make it possible to use Roti load 1, although its reducing properties might cleave intramolecular disulfide bonds. As papain is monomeric it would not break into parts, but its three disulfide bonds will be destroyed [57]

and will have to be rebuild while the protein is refolded. That catalytic amounts of thiols can manage this rebuilding has been shown by Anfinsen in 1957 [57].

We tried to run a zymography with 100 mU of freshly solved papain in 1.1 mM EDTA, kept it on ice and in the dark whenever possible, renatured it with 2,5 % (v/v) Triton X-100 and 1.1 mM EDTA and incubated it in our new development buffer II for only 4 hours at 37 °C. Finally, the gel was stained with coomassie brilliant blue R-250 for 30 minutes. As loading buffers we tested both non-reducing and reducing buffers, cooked and uncooked (Figure 37).

Parameters	
	10% SDS-PAGE
	0.5% (w/v) gelatin
	several loading buffers
	EDTA-containing renaturing buffer
	4h development buffer II pH 6.5 @ 37°C
	30 min coomassie-staining

Although our previous problems concerning the band sizes persisted, we were delighted of the improvement in sensitivity. Only 4 hours development of 0.1 U papain produced an enormous digestion, so that further dilutions could be tried out. We hope that the new pH of 6.5 will be low enough to allow lysosomal cathepsins to work efficiently.

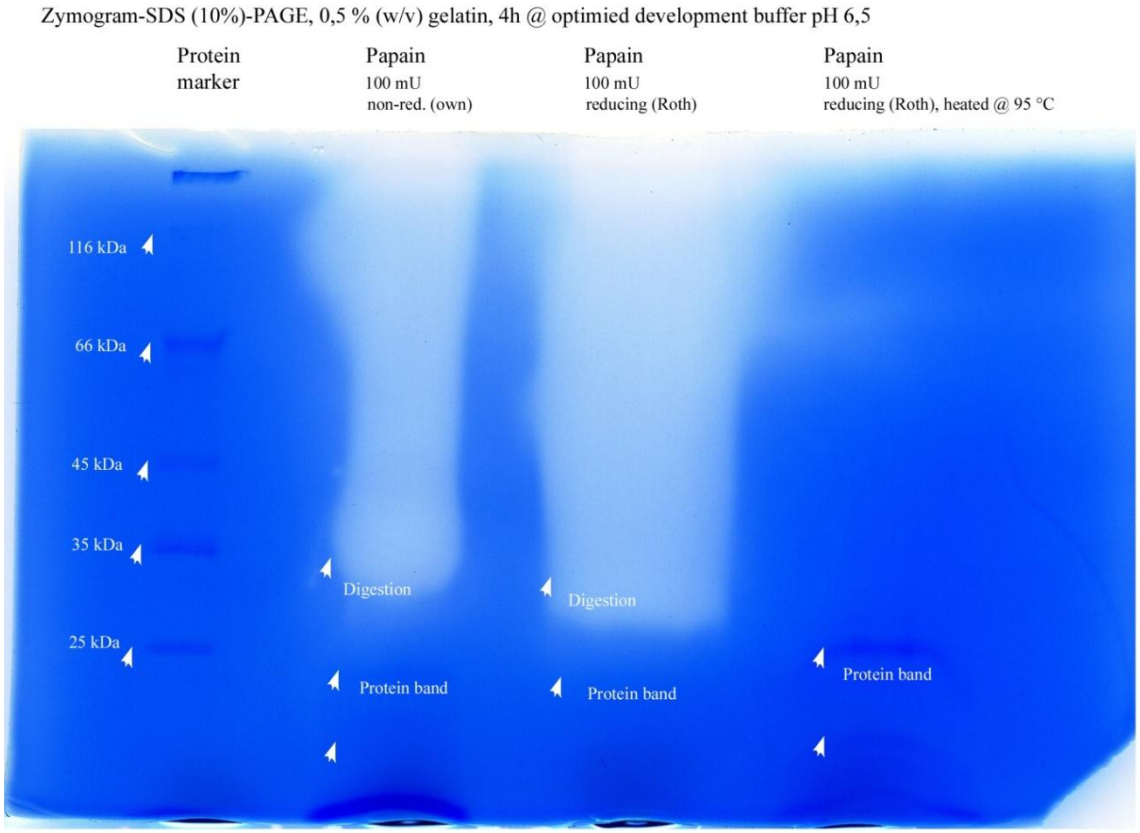


Figure 37: Testing optimized Development Buffer II pH 6.5

Final adjustments of the papain concentration revealed that under EDTA complexing conditions an activity of 5 to 10 mU was sufficient to be detected within four hours (Figure 38). Although digestion and protein bands are still not congruent, the assay can be considered ready to use for comparative activity assays.

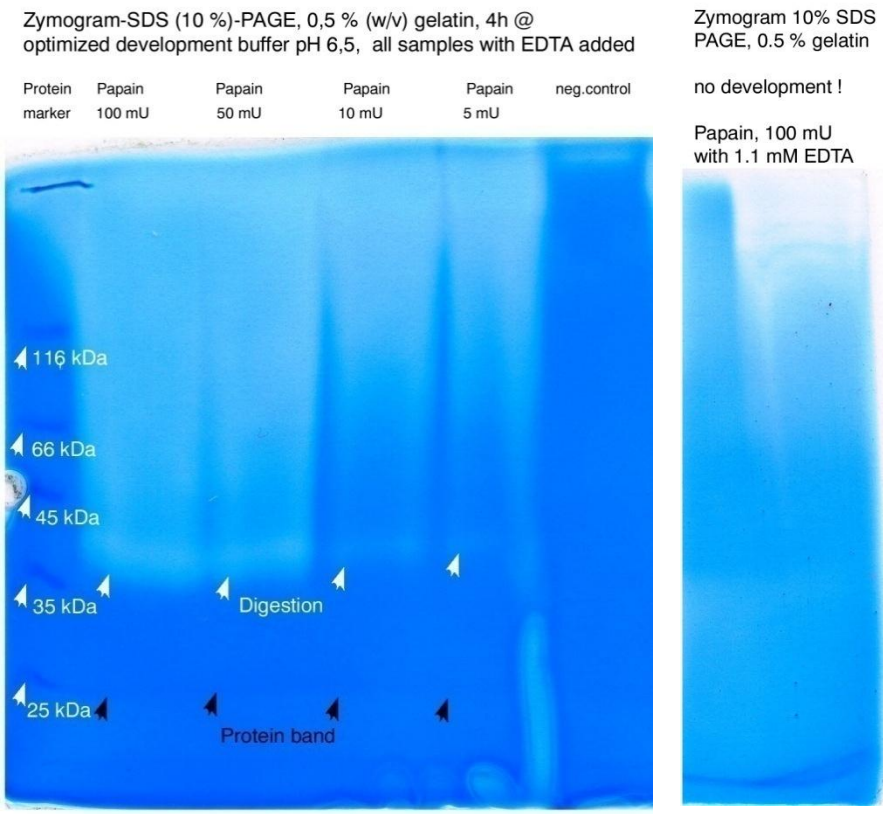


Figure 38: Different dilutions with optimized Development Buffer II pH 6.5

Having established a working zymographic assay, it would be indicated to test if pure cathepsin B (from Sigma-Aldrich) can be detected with our protocol. If this is the case, the method can be used to check the correct expression, purification and refolding of cathepsin B with *E.Coli* cells. Therefore, it is necessary to remove the intein-tag with DTT and to cleave the propeptide with pepsin [(5)].

3.4 Molecular modelling

In vivo, Cathepsin B is synthesized as a pre-proenzyme. After being glycosylated, it is targeted to the lysosomes by the mannose-6-phosphate signal. In the acidic lysosomal environment, it undergoes proteolytic processing into the single-chain from of the mature enzyme. Further processing may also lead to a two-chain cathepsin B, consisting of a heavy and light chain linked together by a disulfide bond.

All cathepsins share the common fold of a papain-like structure: There are two domains, which separate at the top in a V-shaped active site cleft, in the middle of which the residues cysteine, histidine and aspartic acid form the catalytic triade (Figure 40) of the enzyme. The most prominent feature of the left domain is a central α-helix of about thirty residues in length, whereas the right domain forms a kind of β-barrel, which includes a shorter α-helical motif (Figure 39).

Cathepsin B has an insertion of about twenty amino acid residues, termed the occluding loop, which can block the active-site cleft to provide only exopeptidase activity. Anyhow, due to its flexibility, the occluding loop can be displaced from the active site cleft, allowing cathepsin B to also exhibit endopeptidase activity.

The structure of the mature (single-chain) enzyme is already formed in the zymogenic proform. Propeptide chain builds a α-helical domain, which continues along the active-site cleft towards the N-terminus of the mature enzyme in a predominantly extended conformation in the direction opposite to substrate binding. As a result, any access to the active site is blocked (Figure 41).

X-Ray crystallography of rat Cathepsin B has shown the perfect fitting of an inhibitor into the active site cleft (Figure 42). *In silico* docking models might be used to visualize at which sites other known inhibitors could interact with the enzyme, or to investigate which structural motifs of an inhibitor are needed for optimal docking results. The amount of possible conformations even a small ligand can take, makes it very difficult to simulate three-dimensional models. Figure 43 gives only a few exemplarily conformations, benzyloxycarbonyl-phenylalanine-alanine-diazomethane can have. Finding the lowest energy conformation would be a good start for docking simulations.

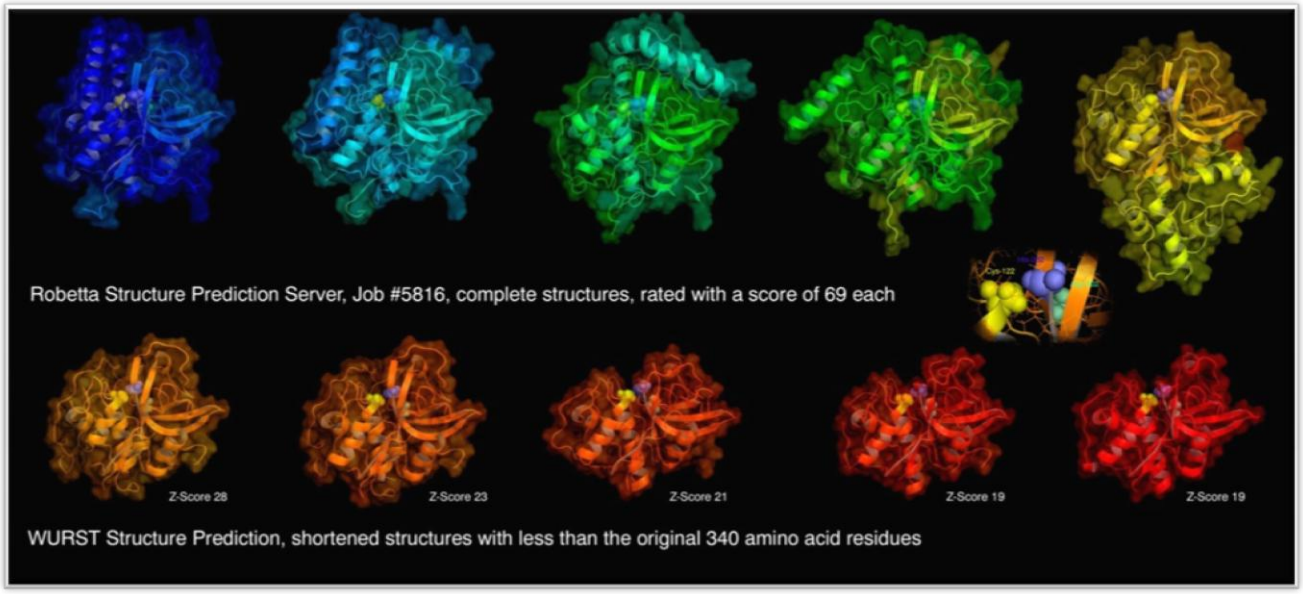


Figure 39: Comparison of different structure predictions

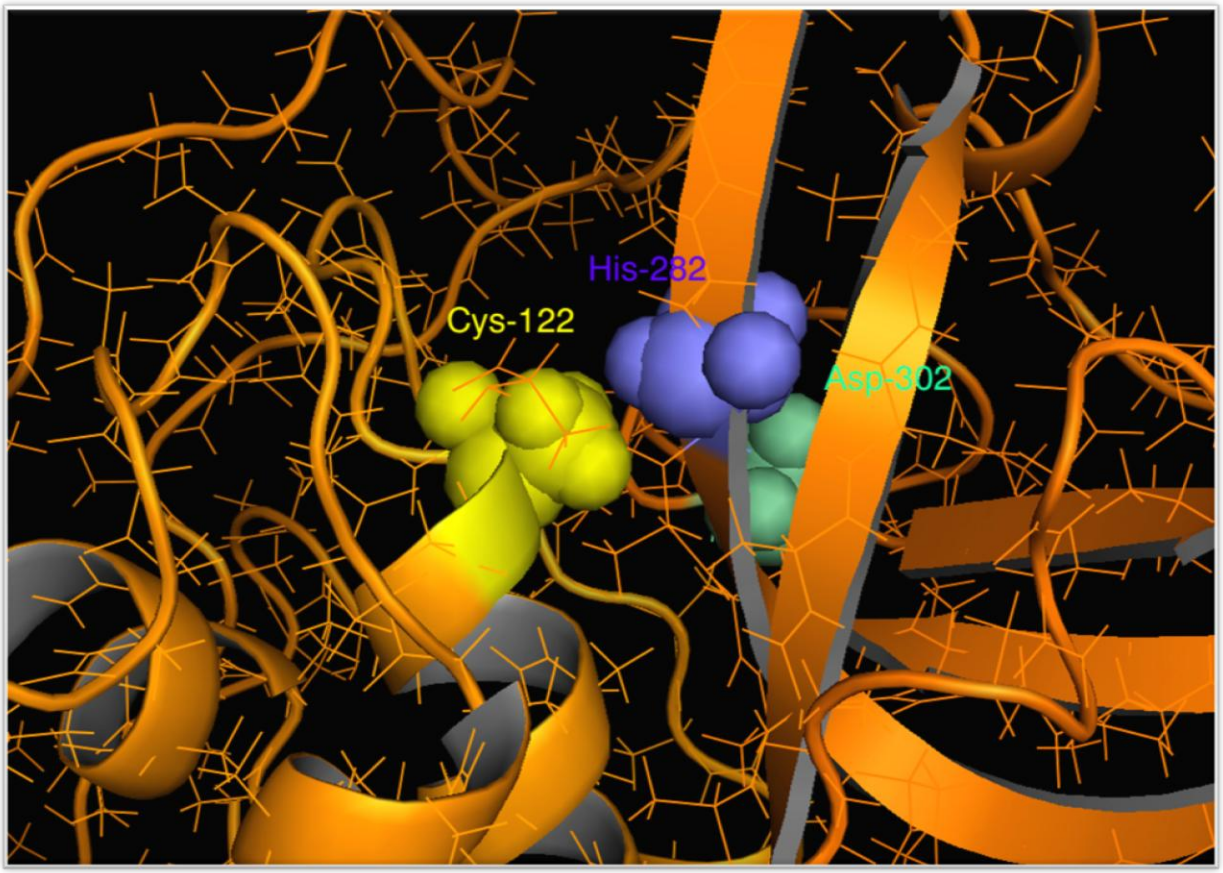


Figure 40: Active center of *T. brucei* cathepsin B with its catalytic triade

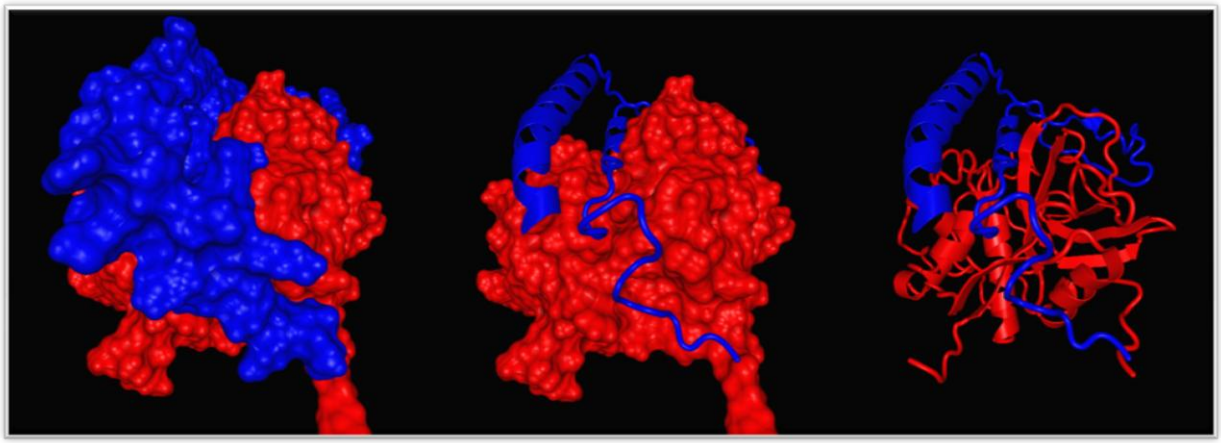


Figure 41: Robetta structure prediction of cathepsin B (red) with pre-pro-signal peptide (blue)

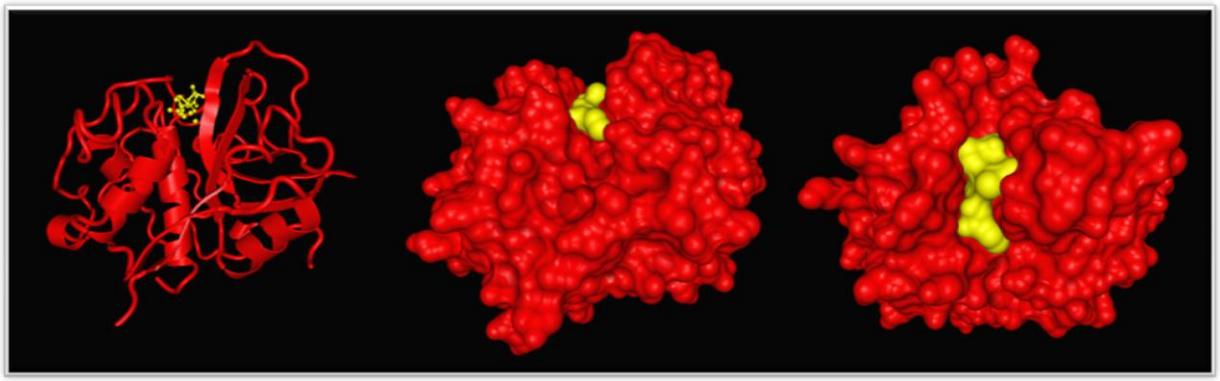


Figure 42: Protein database [44] structure of rat cathepsin B with specific inhibitor

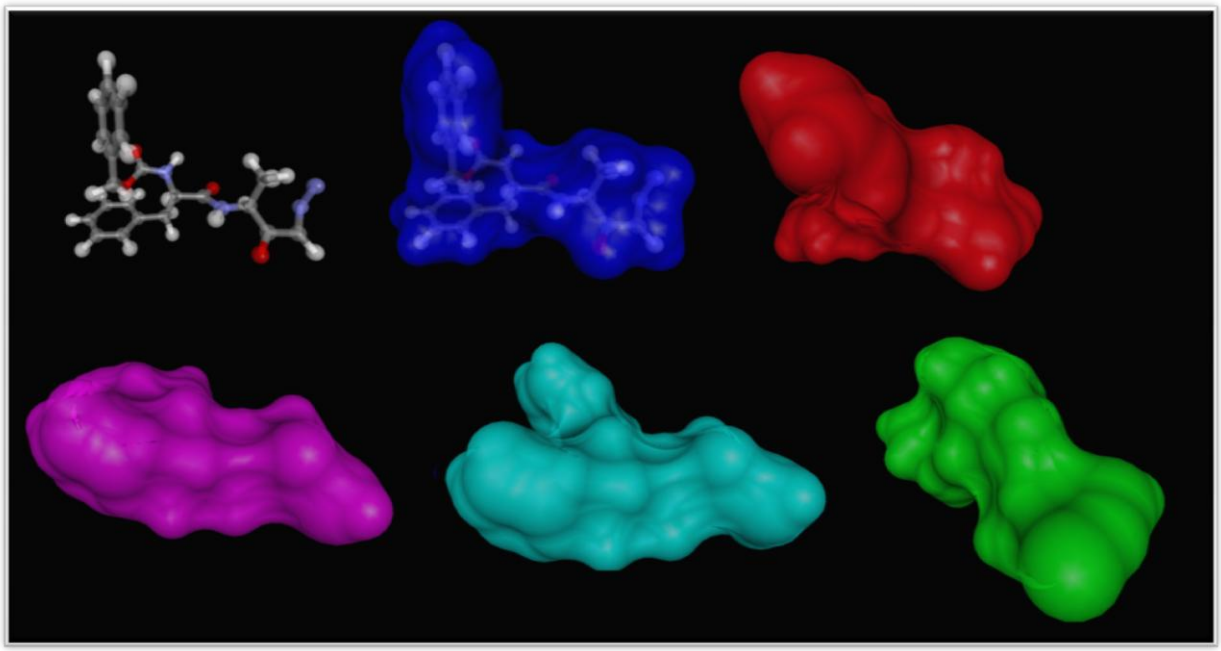


Figure 43: Calculation of different Z-Phe-Ala-CHN_2 energy conformations with Insight II [41]

4 Summary and Outlook

Cathepsin B is a potential drug target in *t. brucei*, the causative agent of African sleeping sickness. In order to develop specific inhibitors, the pure lysosomal papain-like cysteine protease has to be readily available.

The gene coding for tbcatB has been successfully cloned and heterologously expressed in *Escherichia coli.* As the protein builds up several disulfide bonds, it tends to accumulate in inclusion bodies rather than to fold into its physiological conformation. It has been isolated and subsequently refolded using chaotropic reagents. The next step would be its purification via chitin-beated affinity chromatography, and conversion of the zymogen into the active form. To verify its activity a zymographic method has been established, using papain as a standard. On our studies of cathepsin's structure we had to rely on predictions, since there was no x-ray crystallography available yet. It would be great if the protein could be precipitated in a highly organized crystal structure. X-ray diffraction could be used to add the trypanosomal subtype of cathepsin B to the protein data base [(44)]. Computer-based docking analysis will then lead to potential inhibitors which would eventually have no side-effects *in vivo*. Those molecules might play an important role in drug design against African sleeping sickness. In fact, there are a number of ideas on randomly building up such agents and screening for their effectiveness. Such an effort needs a reliable quantitative enzymatic assay. Protease activities are commonly measured photometrically: so-called chromogene substrates are hydrolysed by the enzyme. The cleavage leaves a fragment with an extended delocalized pi electron system. This results in a shift of the spectral absorbance properties.

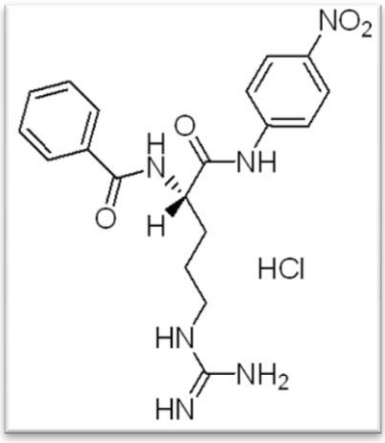


Figure 45: BAPNA

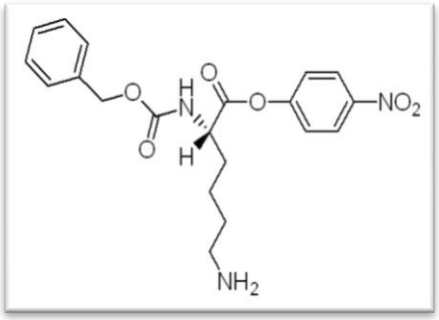


Figure 44: Z-Lys-ONp

Well-known chromogene substrates are any forms of para-nitro-anilides, linked to one or more amino acids, which are a preferred target for the protease that is to be analysed. Their synthesis [(36)] is often based upon liquid-phase peptide chemistry. Commercially available are BAPNA (benzoyl-arginine-p-nitro-anilide) which has been used by Thangam and Rajkumar [(32)]. For cathepsin B another chromogene substrate should be even more interesting: Z-Lys-ONp, or long-termed benzyloxycarbonyl-lysine-p-nitrophenyleester which is commercially available from Sigma Aldrich [(50)].

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http://www.zbh.uni-hamburg.de/wurst/index.php
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