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Cathepsin B – a highly potent drug target in *T. brucei*

short oral communication

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(06/02/20-06/04/07)

introduction

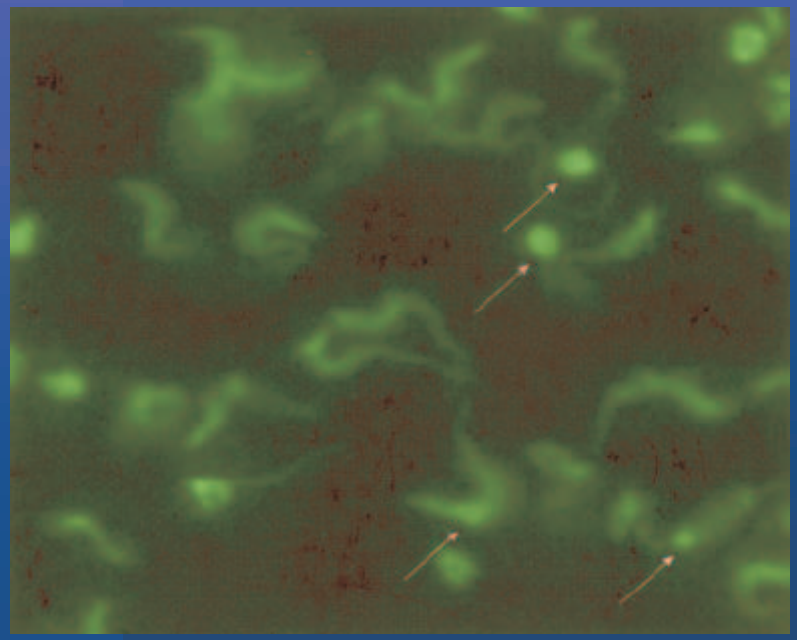
- ***Trypanosoma brucei* causes**

T. b. gambiense / rhodesiense	African Sleeping Sickness (human)
	400.000 cases / year
T. b. vivax	Nagana Epedemy (bovine)
	3.000.000 cases / year
T. b. congolese	Gambia Fever (bovine)
T. b. simiae	(swine)
T. b. brucei	(horses, mules, cats)

- **Mackey et al. described the cysteine protease Cathepsin B as a highly potent drug target. It was known that the parasite could be killed with cysteine protease inhibitors as is Z-Phe-Ala-CHN2.**
- **Whereas the presumed target of this inhibitor was rhodesain (the major CatL protease of *t. brucei)* Mackey found in comparative RNAi knockdowns, that silencing rhodesain did not change the phenotype.**

introduction

- **Instead, knocking down cathepsin B led to enlargement of the endosome, accumulation of transferrin, defective cytokinesis after completion of mitosis, and ultimately the death of the parasites.**



Screening for an specific cathepsin B inhibitor without side-effects gets necessary.

accumulation of flourescein marked transferrin in the endosome

overview

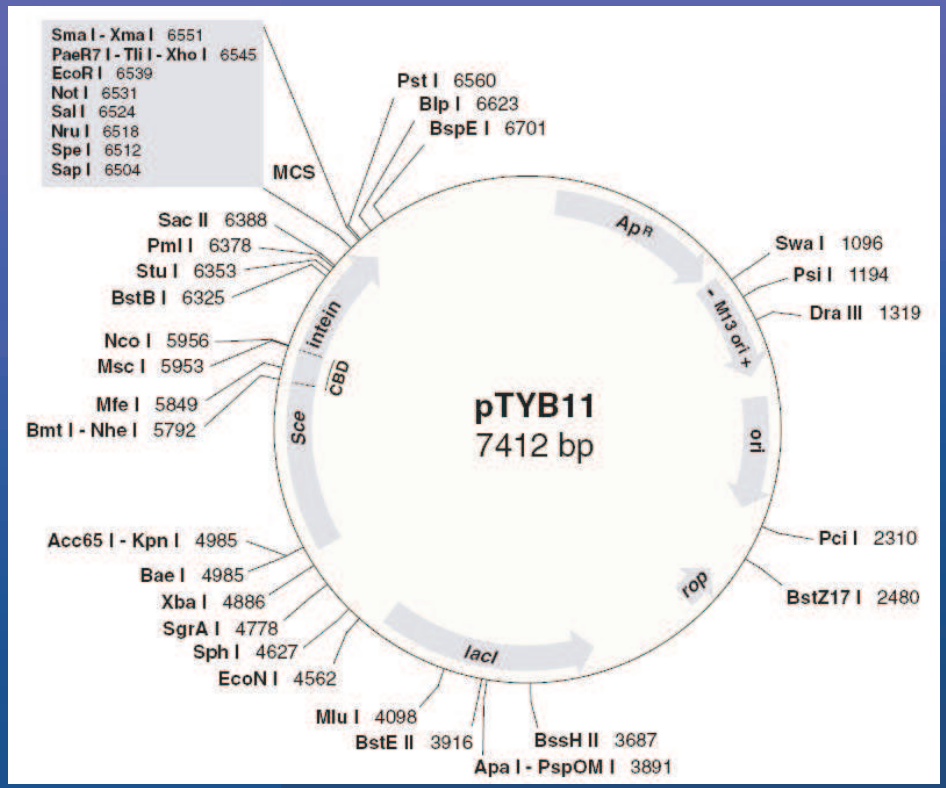
getting started:

- **cloning *t.b.* catb cDNA**
- **heterologous expression in *E.Coli***
- **isolation and purification**
- **establishing a cathepsin B activity assay**

outlook:

- **now the search for inhibitors is about to begin**

expression system



→ **cloning**
into MCS

↓

transformation
heat shock in competent cells

↓

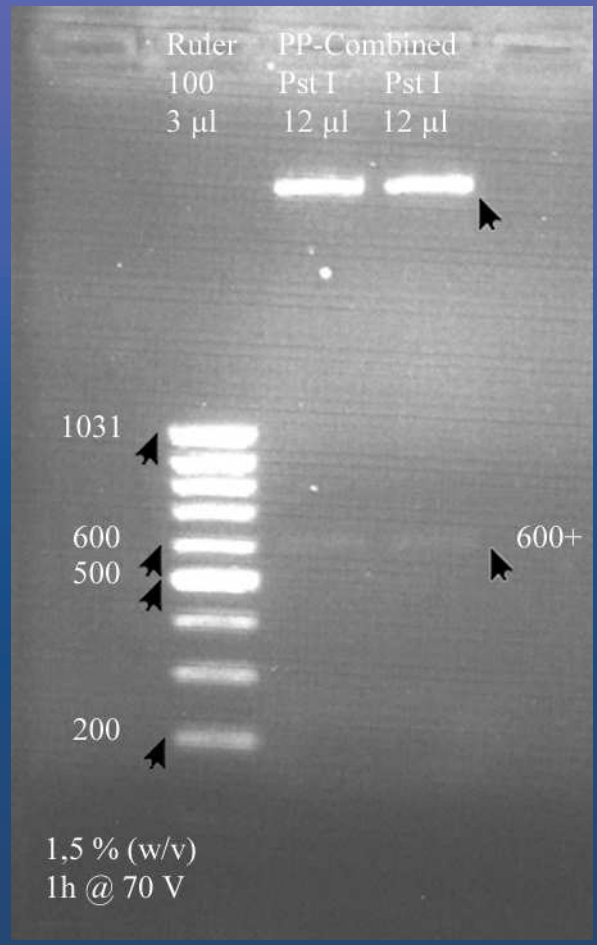
cultivation

expression system

Checking whether the insert is correctly positioned and orientated:

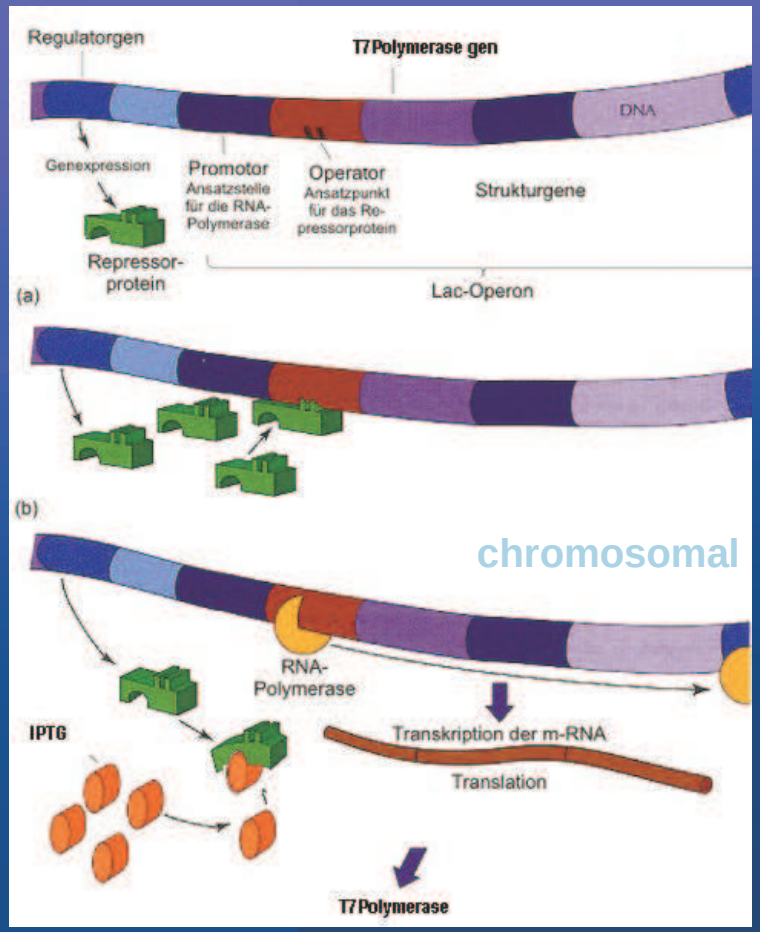
- **digestion with restriction enzymes and electrophoretic size control**
- **sequencing parts of the plasmide**

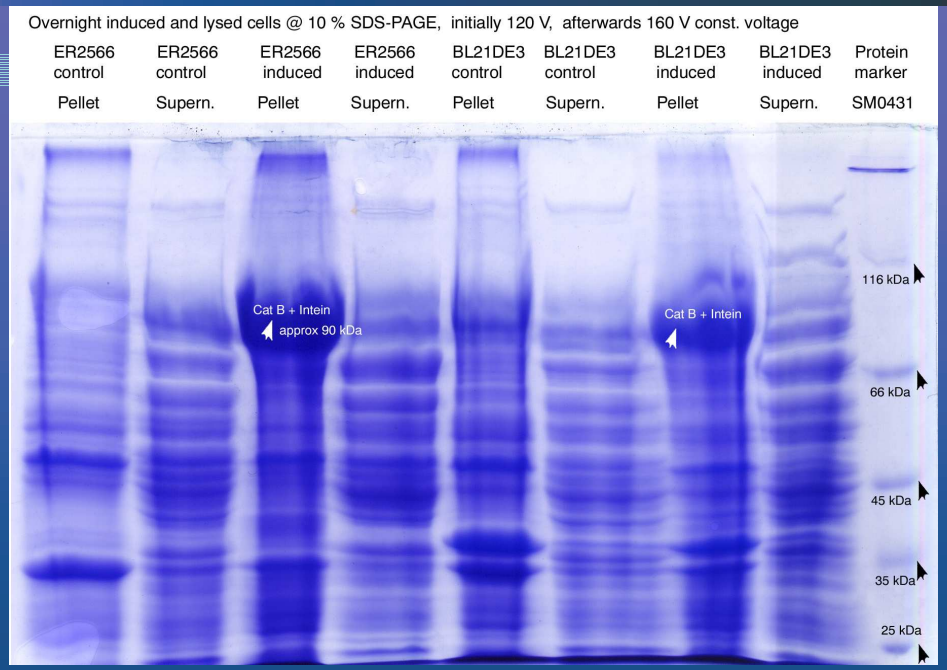
(MWG Biotech AG,
GATC Biotech AG)



expression system

using two cell lines (ER2566, BL21DE3)





Expression of T7 promoted t.b. catB with Intein-tag (on plasmid)

IPTG induction leads to expression of T7 polymerase

isolation and purification

ultrasonification

10 times for 30‘‘, cooling for 3‘

centrifugation

continue with pelleted inclusion bodies

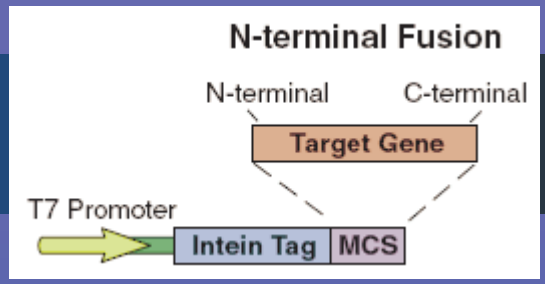
resolvation

guanidine as chaotropic agent

dialysis

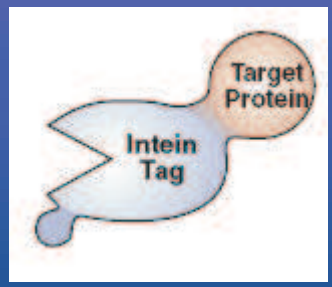
against urea

isolation and purification



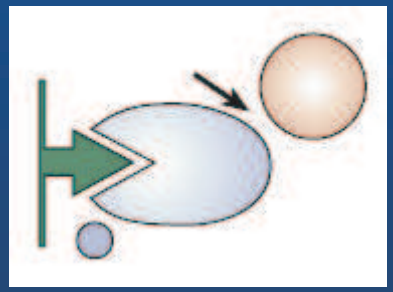
Expression

Intein tag with Chitin – Binding Domain



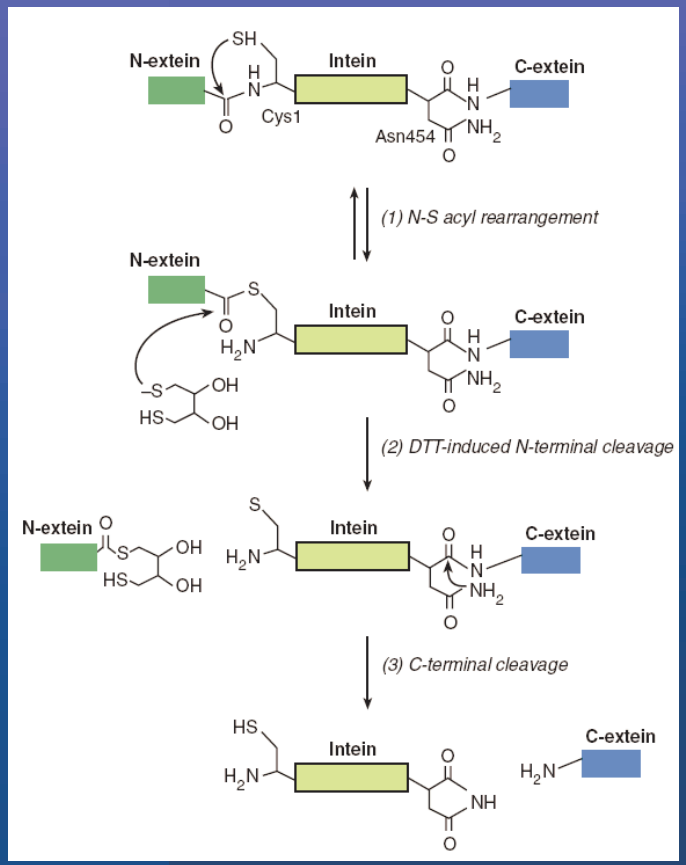
Load & Wash

Induce DTT cleavage @ 4 ℃



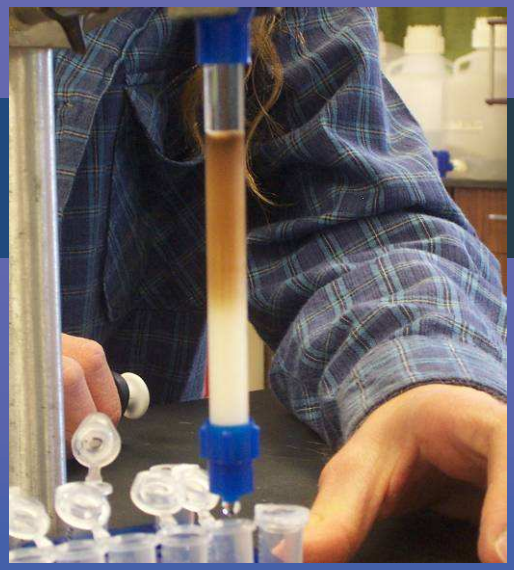
Elute & remove 15 residue N-extein by dialysis

isolation and purification

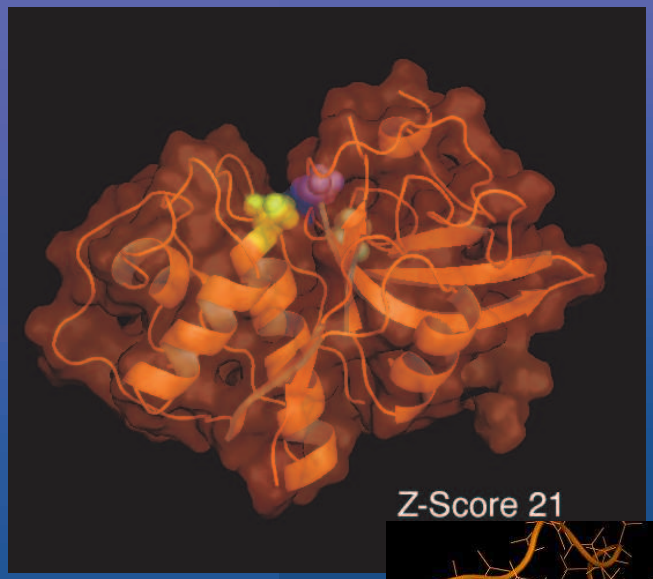


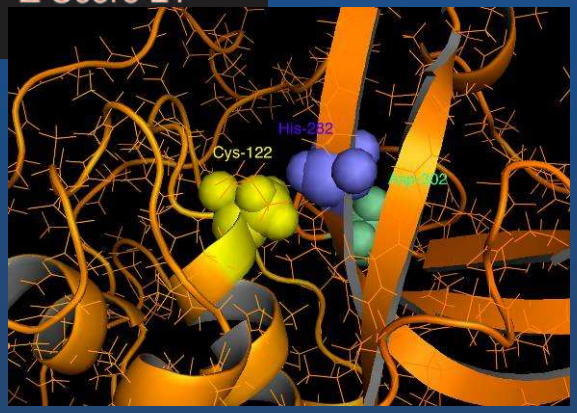
Excision of the intein by peptide bond cleavage coupled to succinimide formation involving Asn 454 at the C-terminus of the intein.

need for activity assays



What do we get when we purified Cathepsin B ?



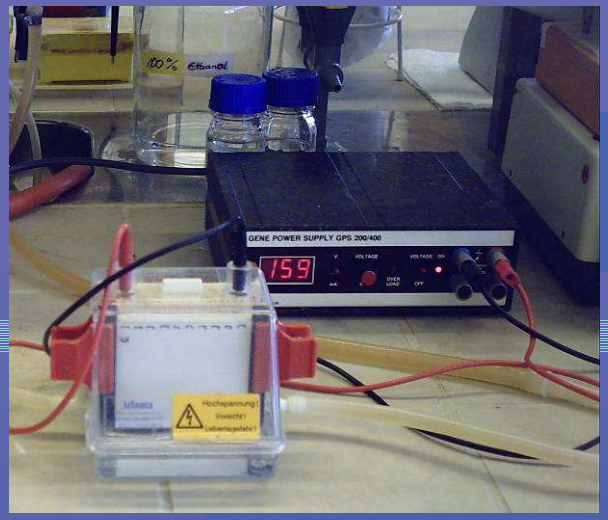


A physiologically refolded enzyme with accessible active site cleft ?

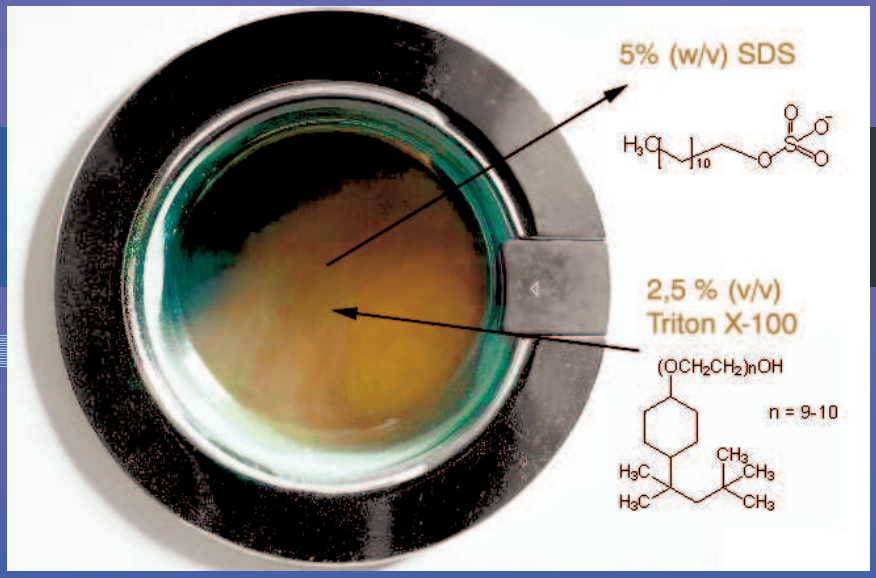
A denaturated inaktive protein knot ?

introducing zymography

- zymography is an extremely sensitive means to show even picogram amounts of gelatinase
- provides the ability to visualize activity and protein size at once
- no blotting on nitrocellulose or agar plates is needed
- however, most zymographic methods in literature base on serine proteases whereas cathepsin B is a cysteine protease



1) 0,5 % (w/v) copolimerized gelatin in 10 % SDS-PAGE



2) washing out SDS

3) renaturing (removing Triton)

4) digesting gelatin @ 37 ℃

introducing zymography

General protocol:

Always work in the cold-room and in darkness.

- **Prepare gels (10 %), add 0,5 % (w/v) gelatin**

- **Combine protein sample and loading buffer.**
- **Incubate 10' @ room temperature. DO NOT HEAT.**
- **Perform SDS-PAGE @ 4 ℃**

- **Incubate gel in washing buffer for 30' @ room temperature**
- **Incubate gel in development buffer for 30 ' @ room temperate**
- **Refresh development buffer. Incubate 4-8 h @ 37 ℃**

- **Stain with Coomassie Brilliant Blue R-250 for 30'.**
- **Destain with MeOH : Acetic acid : Water (50 : 10 : 40)**

Zymogram-SDS-PAGE

Optimized on Trypsin, giving one distinct band without any smear

(thanks to Khalid Muhammed)

normalization on papain

before a new method is useable it has to be

- **optimized to show at which conditions it works best**
- **normalized to a standard so that sensitivity is specified and comparison is made possible**

as we want to visualize cathepsin B, which is a papain-like cysteine protease, we focused on papain as a standard.

pure cathepsin B would give less universal results at much higher costs:

papain	**10 U (papaya latex)**	**1 €**
cathepsin B	**10 U (human liver)**	**90 €**
	10 U (bovine spleen)	**100 €**
	10 U (human placenta)	**163 €**

normalization on papain

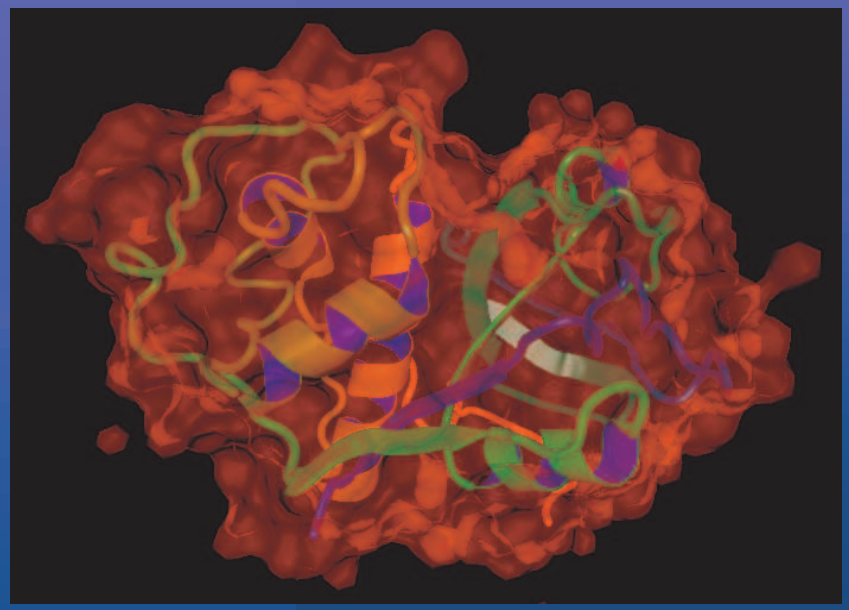
Washing Buffer:

2.5 % (v/v)	**Triton X-100 in water**

Initially used Development Buffer:

50 mM	**Sodium acetate pH 5,0**
10 mM	**Calcium chloride**
100 mM	**Sodium chloride**

normalization on papain



Papain is a monomeric cysteine-protease with 212 residues.

21 kDa

It contains three intramolecular disulfide bonds.

Its pH optimum range is 6.0-7.0

Sigma-Aldrich #76218, faintly beige hygroscopic lyophylisate soluble up to 5 mg/ml, giving brown solution of clear turbidity Specific activity 11.4 U / mg

experimental part

First some thoughts before starting:

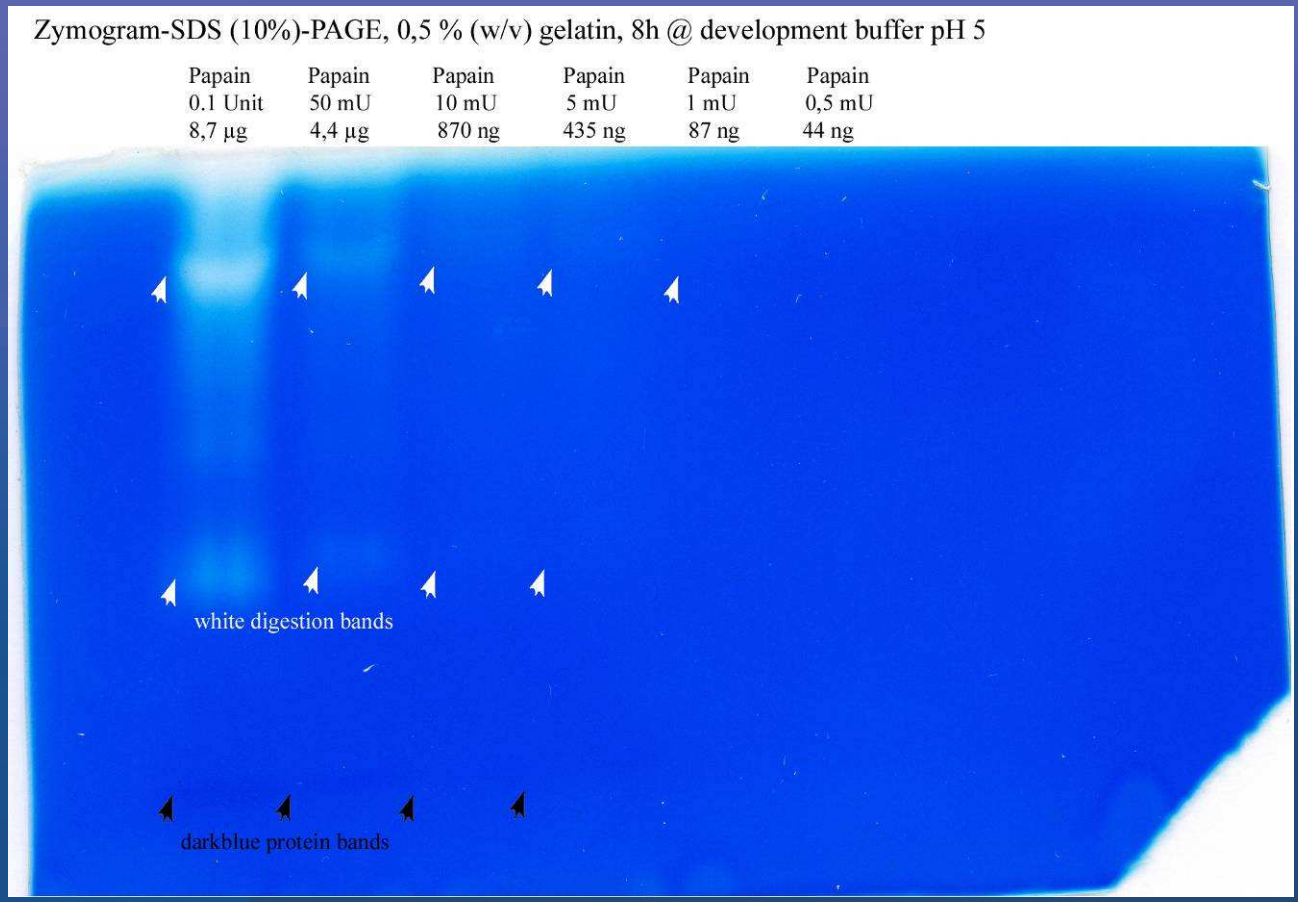
- **Papain-like cathepsins are rather non-specific enzymes with no clear substrate-recognition site, so that gelatin might work as substrate ☺**
  - **Gelatin is denaturated form of fibrilic collagen I/II/III**
  - **Gelatin is build up of repetitive Pro/Pro-OH/Gly motifs**
  - **Unusually winds to a left-handed helix**
- **Cathepsins B, C, H and X are exopeptidases ☹**
  - **but: cathepsin Bs 20 residues occluding loop is flexible and can move aside to allow access to active site cleft → endopeptidase activity**

experimental part

- Cathepsin B is member of papain-like enzymes. It is localized in lysosomes, so that we decided developing at pH 5, although papain prefers higher values.
- Although that could lower the activity of our papain standard, we hoped to generate better reaction conditions for the extracted cathepsin B

experimental part

10 % SDS-PAGE
0,5 % gelatin
self-made non-red load. b.
EDTA-free washing b.
8 h developing b. pH 5
overnight coomassie



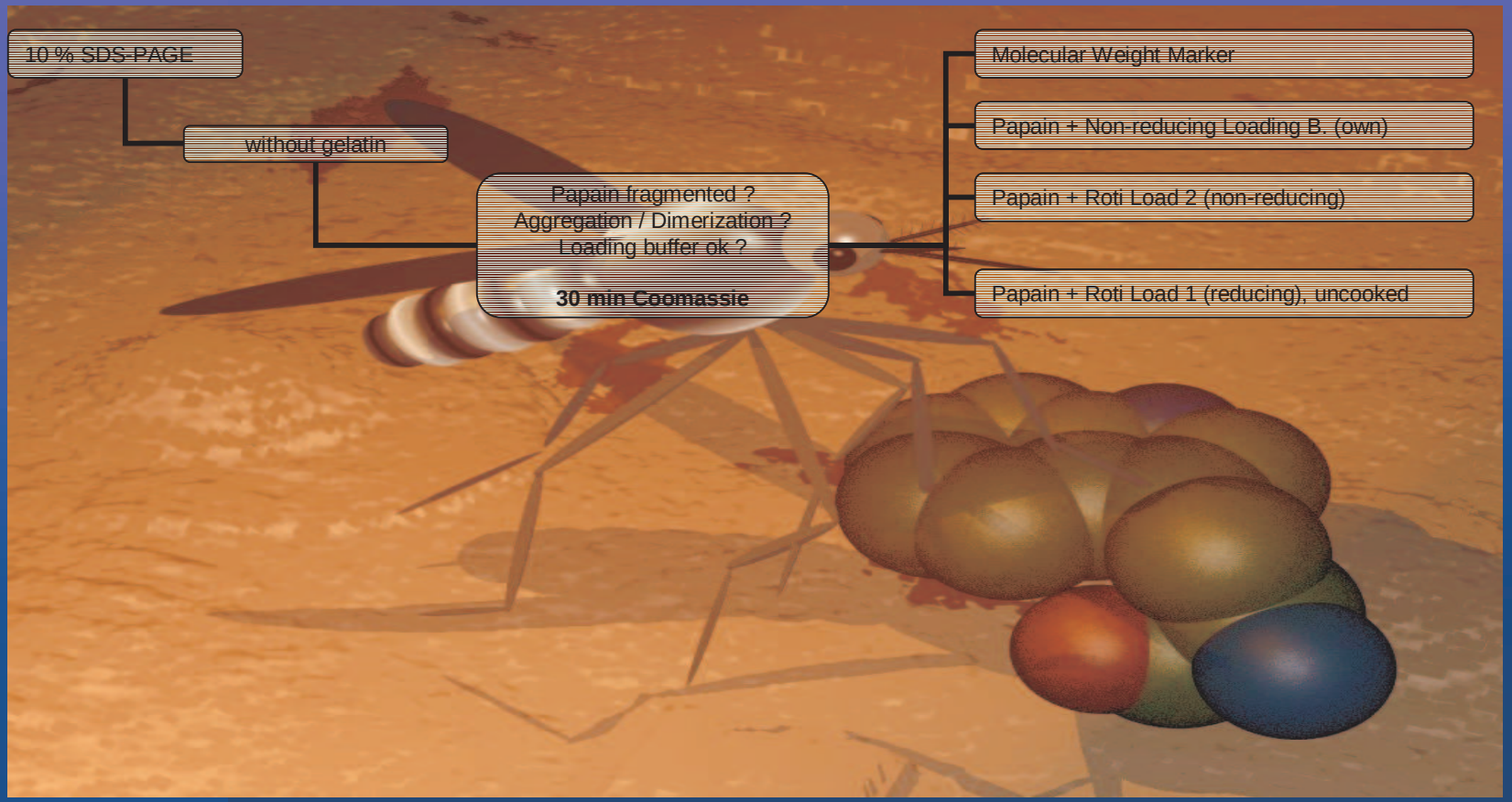
- **Best results with 0.1 U of papain**
- **two digestive bands (why?)**
- **one darkblue protein band**

experimental part

- 100 mU (8,7 µg papain) showed to be the best choice for carrying out zymography at the applied conditions
- however, results were very confusing:
  - both digestive bands occur at high molecular weights and could therefor not be papain
  - a dark blue protein band seemed to be at a size of 21 kDa but were not active
- so what could have caused such effects ?

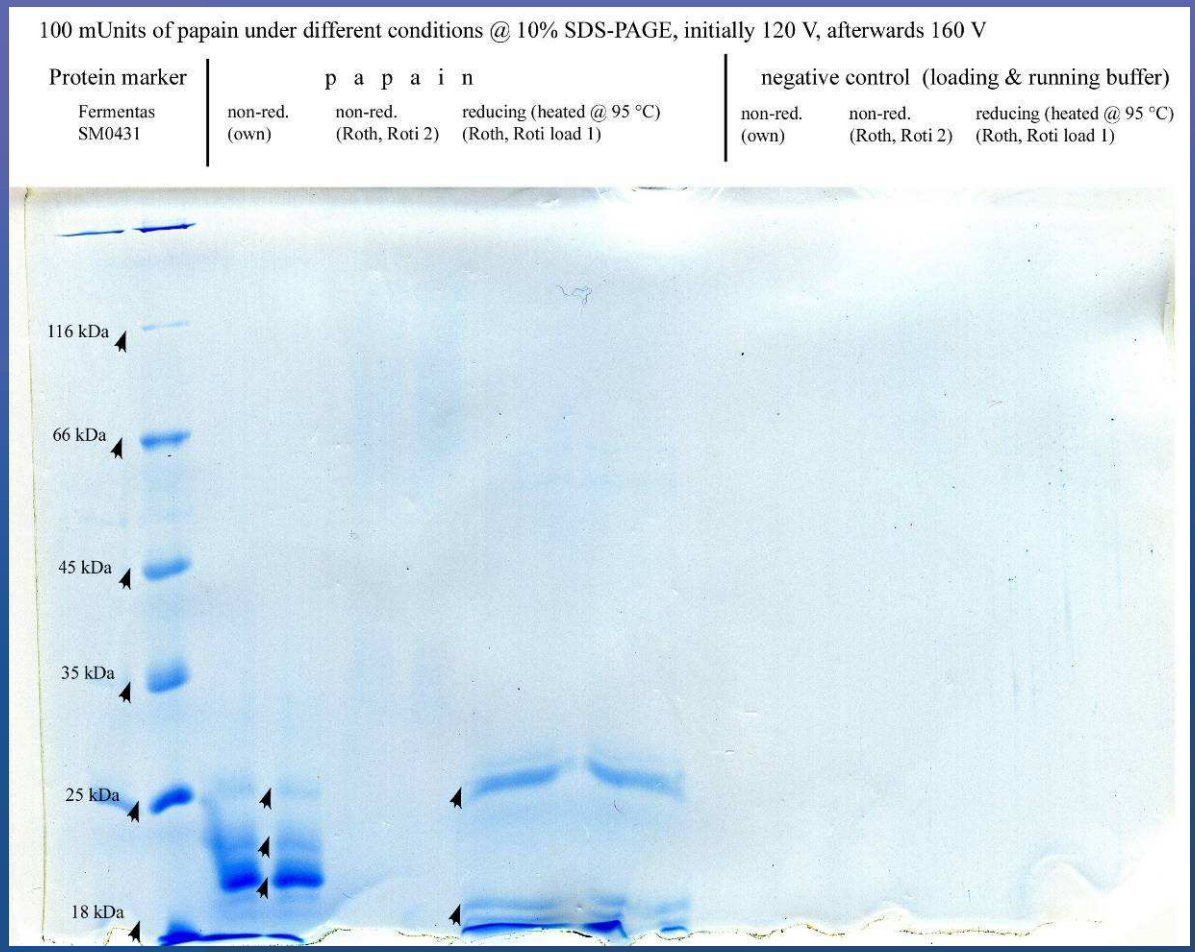
  as there are a number of possibilities we prepared a lot of test systems

experimental part



experimental part

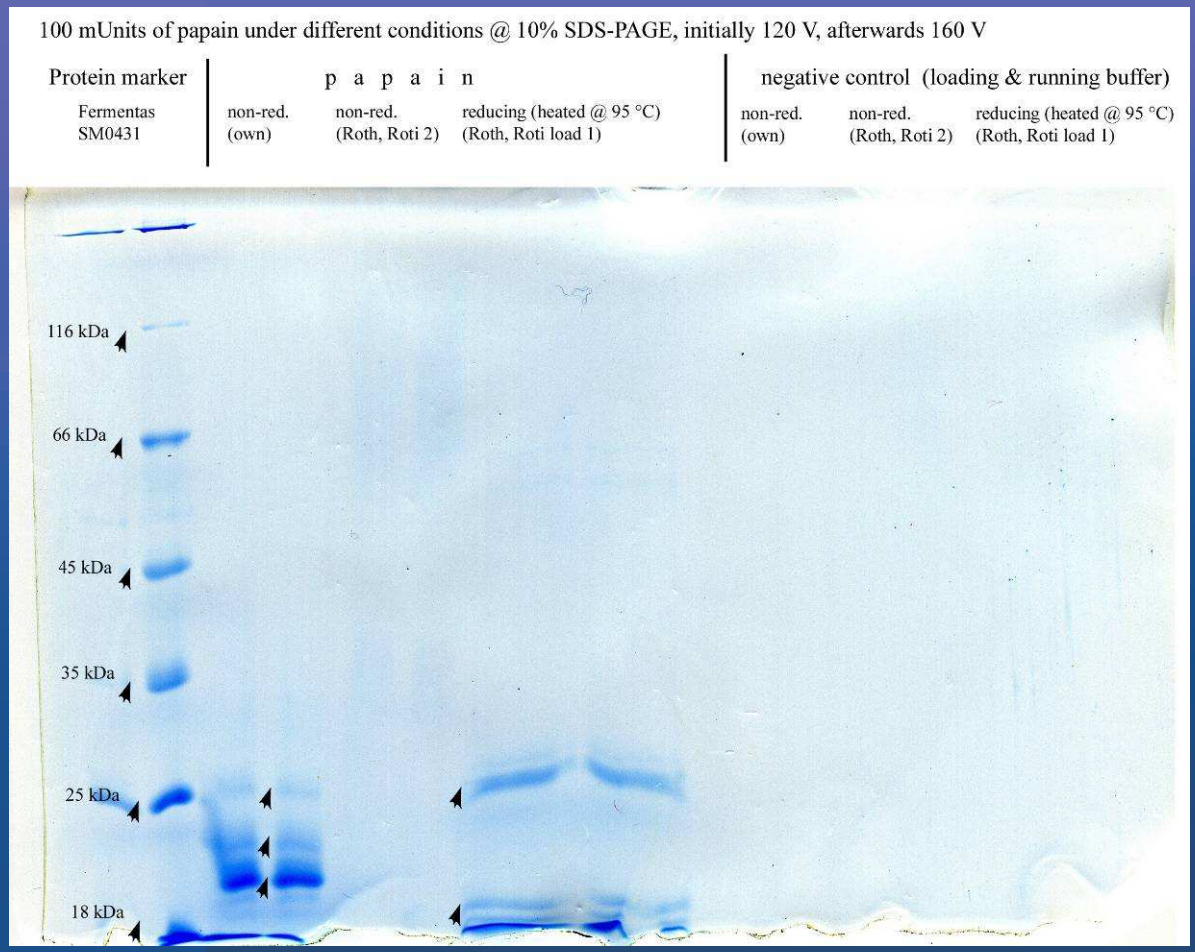
10 % SDS-PAGE
several loading buffers
30 min coomassie



- **self-made non-red. b. fragments papain**
- **Roti Load 2 gives no bands and seems to be not working (repetitively same results)**
- **Roti Load 1 does not fragment papain**

experimental part

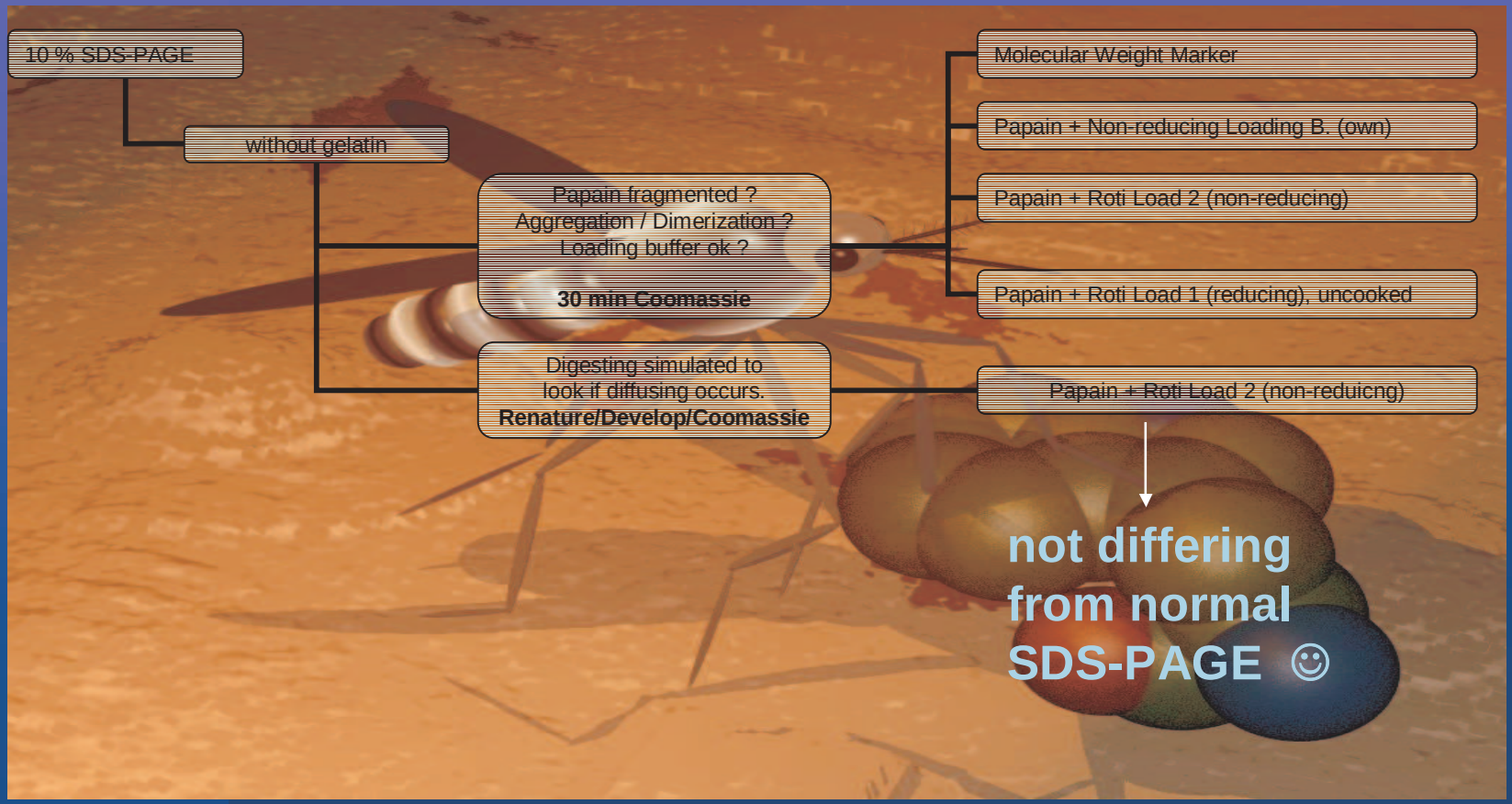
10 % SDS-PAGE
several loading buffers
30 min coomassie



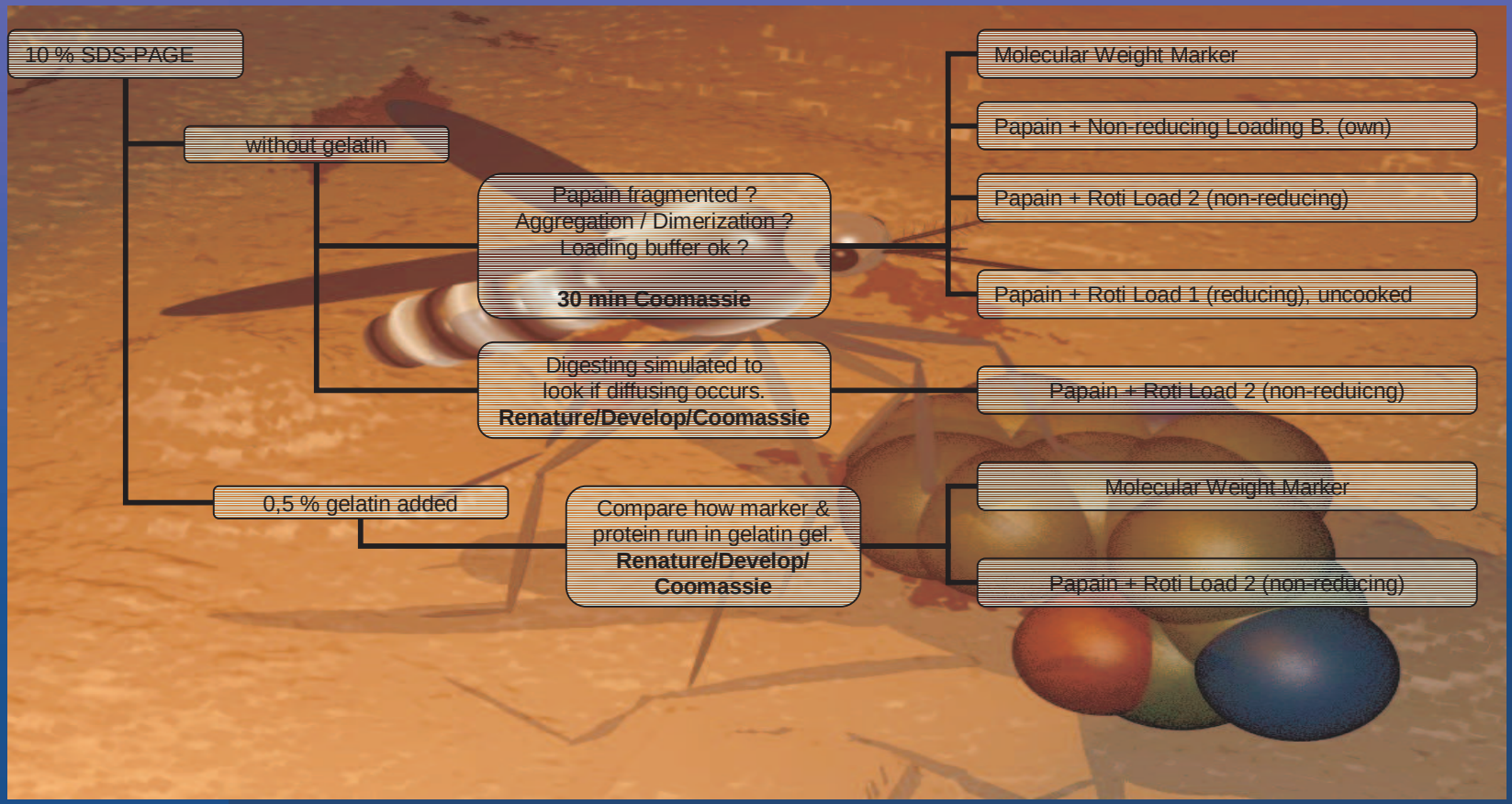
- **no protein contamination/ impureness in any of the applied running buffers**

  (especially of interest with the self-made one)

experimental part

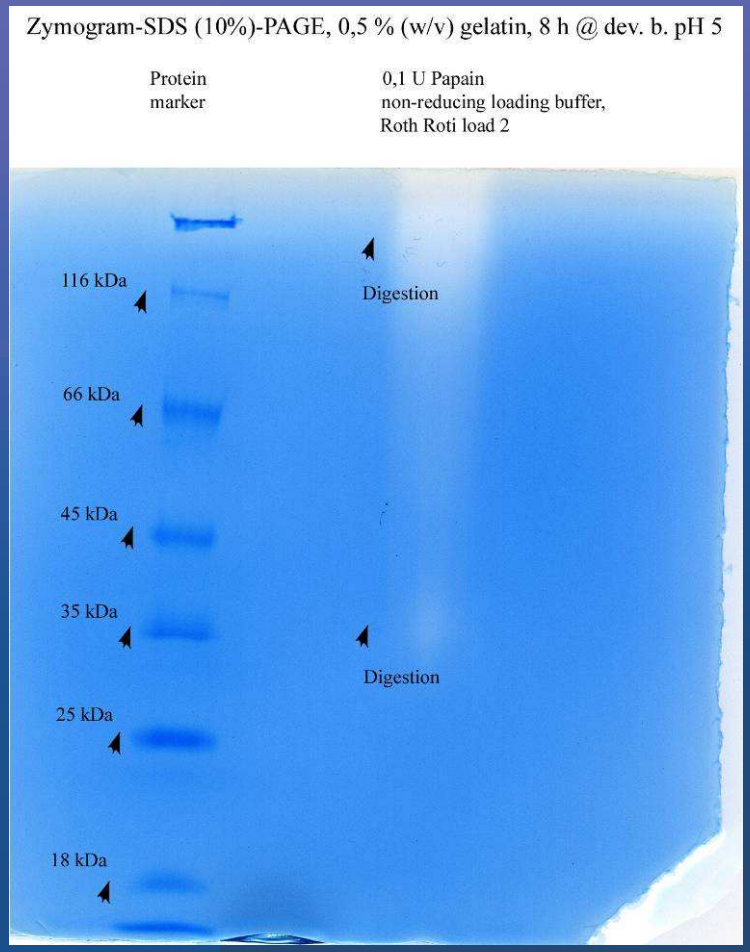


experimental part



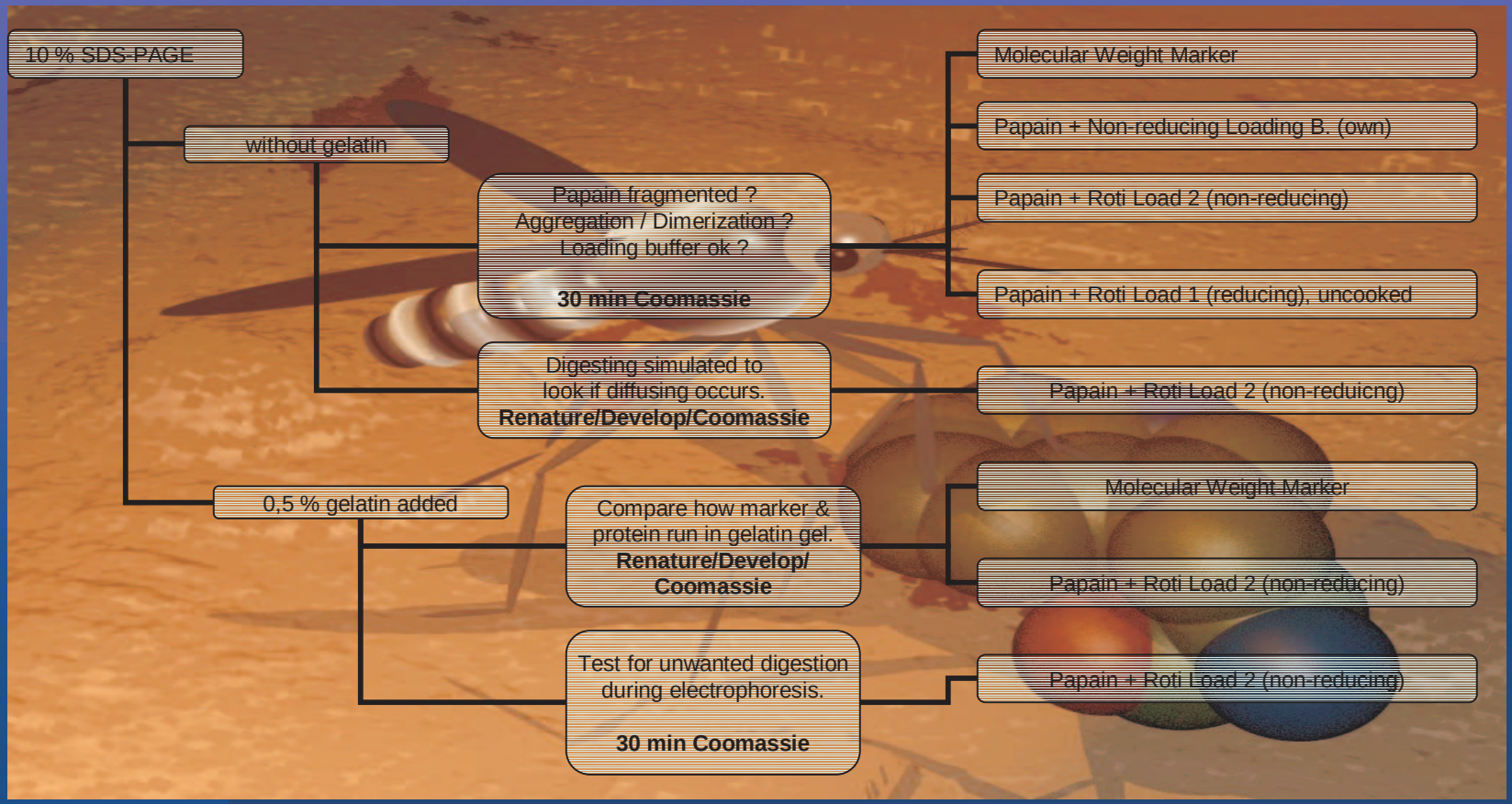
experimental part

10 % SDS-PAGE
0,5 % gelatin
Roti Load 2
EDTA-free washing b.
8 h developing b. pH 5
30 min coomassie



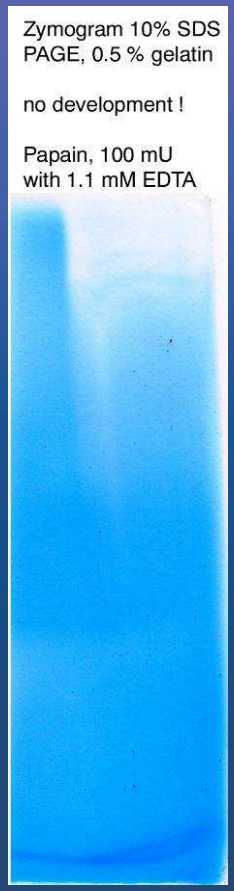
- Protein marker runs normally
- That means gel properties (i.e. pore sizes) do not differ from SDS-PAGE
- Digestive bands are for the first time localized to > 120 kDa and 35 kDa.

experimental part



experimental part

10 % SDS-PAGE
0,5 % gelatin
Roti Load 2 / Roti Load 1
no washing
no developing
30 min coomassie



- 0.1 U Papain with Roti Load 2 does not digest during electrophoresis
- 0.1 U Papain with stabilizing EDTA and Roti Load 1 digests at the top of the gel

experimental part

- Extended investigations led to a technical manual telling that papain should be fully activated before use in a solution of

  1.1 mM EDTA, 0.067 mM HO-Et-SH, 5.5 mM cysteine, pH 6.5

- As stabilisizing agent EDTA was added from the very beginning to the papain solution.

- Whenever possible we worked on ice and in the dark due to papain‘s photosensitivity. Working under argon was not possible, but we tried to prevent oxidation by the use of reducing agents, as is mercaptoethanol.

- As papain is a monomer it would not split into subunits. Disulfide bonds can be refolded by catalytical amounts of HO-ET-SH.

normalization on papain

Washing Buffer:

2.5 % (v/v)	Triton X-100 in water
1.1 mM	EDTA (optimized)

Initially used Development Buffer:

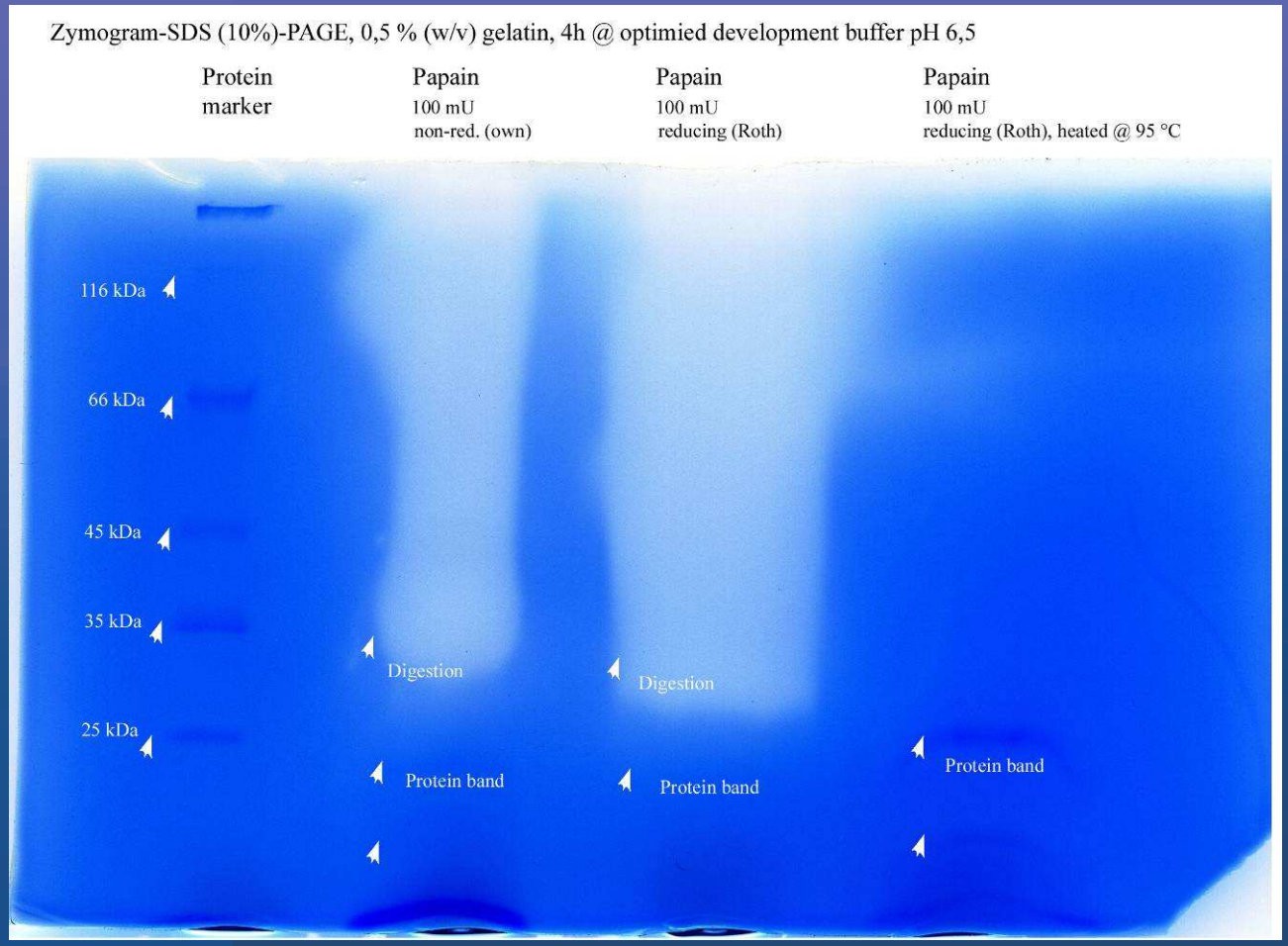
50 mM	Sodium acetate pH 5.0
10 mM	Calcium chloride
100 mM	Sodium chloride

Optimized Development Buffer:

50 mM	Phosphate buffer pH 6.5
1.1 mM	EDTA
0.067 mM	mercaptoethanol
5.5 mM	cysteine

experimental part

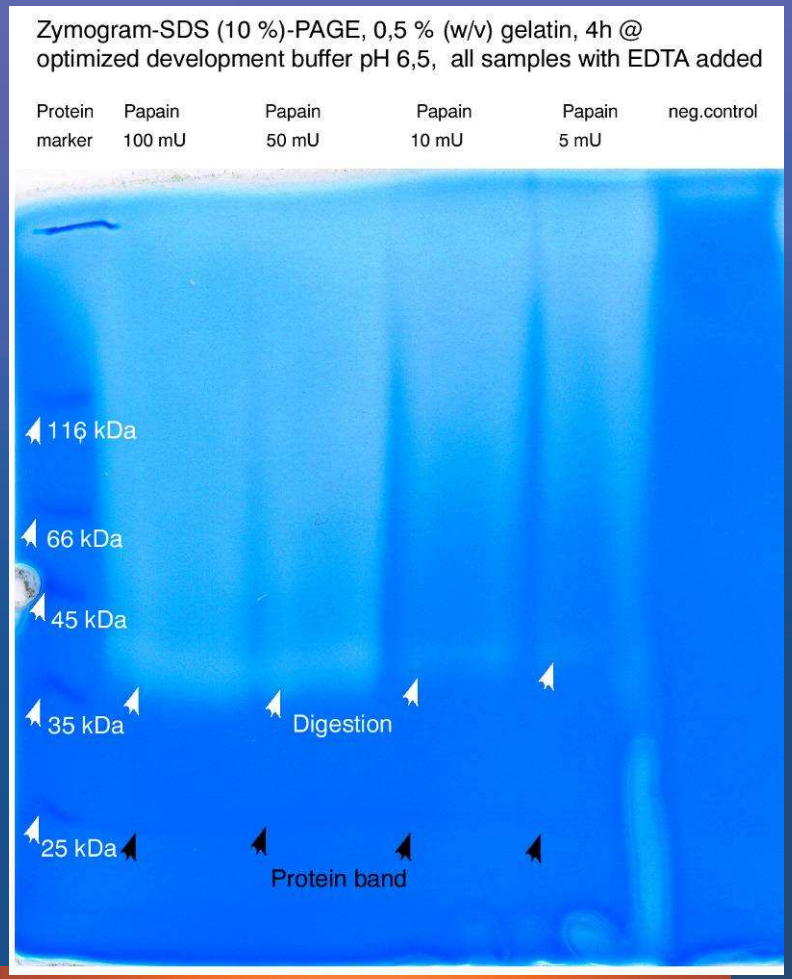
10 % SDS-PAGE
0,5 % gelatin
several loading b.
EDTA-added washing b.
4 h developing b. pH 6.5
30 min coomassie



- **new conditions produce enormous increase of activity**
- **again wrong sized digestive bands & 21 kDa protein band**

experimental part

10 % SDS-PAGE
0,5 % gelatin
Roti Load 1 (not heated)
EDTA-added washing b.
4 h developing b. pH 6.5
30 min coomassie



- 10 mU (870 ng) are the best choice to use in further experiments
- Negative control (EDTA, aqua dest, Roti Load 1 does not digest)
- Size problems still persist

summary and outlook

What has been done ?

- **cathepsin B has been successfully cloned and expressed in *E. Coli***
- **inclusion bodies could be resolved with 6 M guanidinee and refolded by dialysis against urea**
- **affinity column purification of the intein-tagged molecule is still being optimized**

- **in parallel, a protocol for zymography of cysteine proteases has been worked out**
- **normalization was performed with papain as a standard**

summary and outlook

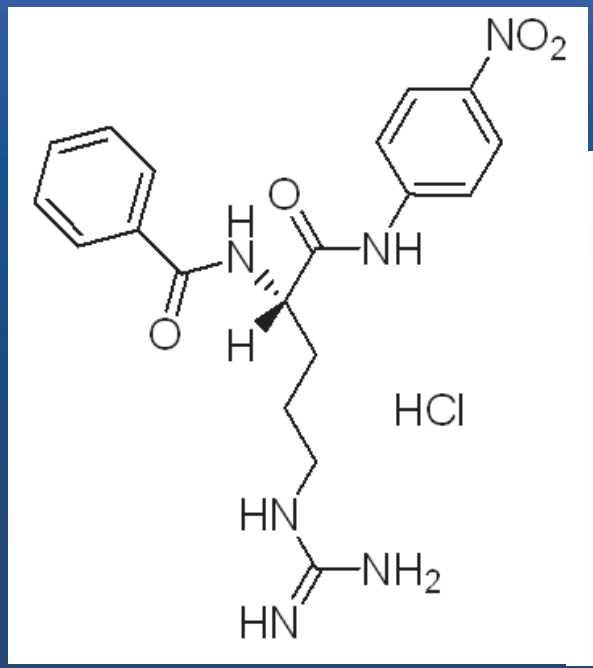
How to continue with purified and active cathepsin B ?

- **optimization of purification**
- **screening for inhibitors without toxic side-effects**

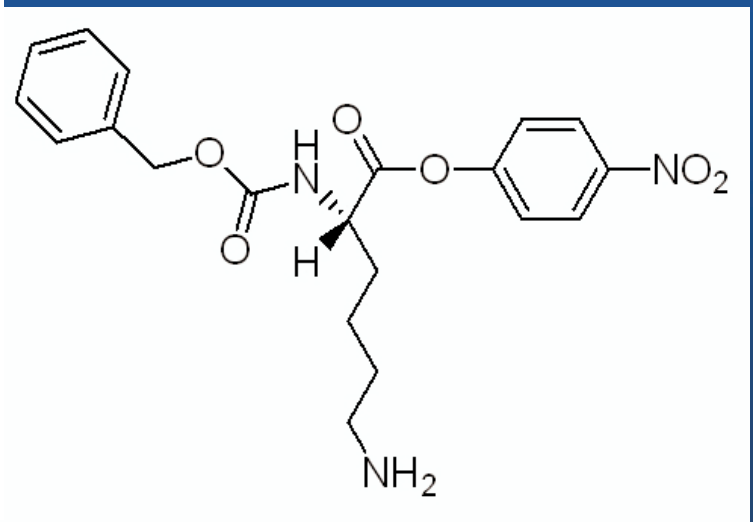
- **at this time a quantitative assay will be needed to analyse kinetics**
- **this could be realized spectrometrically with standard chromogene substrates**

summary and outlook

- **therefor we would recommend BAPNA or Z-K-ONp, as these are commonly used to define activity**



E (405 nm)



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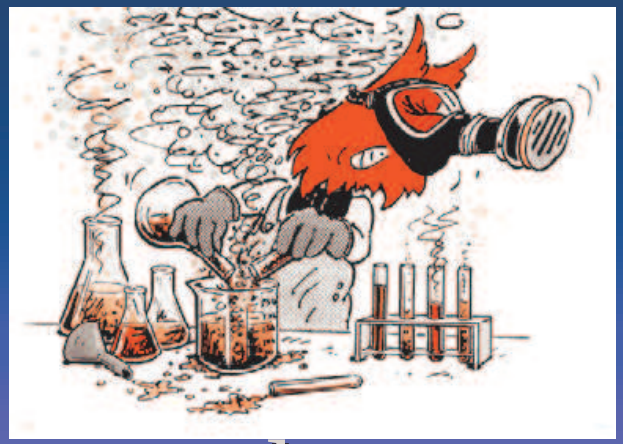

Cathepsin B – a highly potent drug target in *T. brucei*

thanks for your attention