
Sakari Bochkov

# Site-directed mutagenesis and purification of TmPPase protein for structural analyses

Metropolia University of Applied Sciences

Bachelor of Engineering

Biotechnology and Food Engineering

Bachelor's Thesis

20 November 2024

## Abstract


| | |
|---|---|
| Author: | Sakari Bochkov |
| Title: | Site-directed mutagenesis and purification of TmPPase protein for structural analyses |
| Number of Pages: | 29 pages + 1 appendix |
| Date: | 20 November 2024 |
| Degree: | Bachelor of Engineering |
| Degree Programme: | Biotechnology and Chemical Engineering |
| Professional Major: | Biotechnology and Food Engineering |
| Supervisors: | Adrian Goldman, Professor<br>Keni Vidilaseris, Researcher<br>Orquidea de Castro Ribeiro, Research Technician<br>Hye Jung-Majanen, Senior Lecturer |


---


The project was conducted for the Structural Biology Research Group at the University of Helsinki, led by Professor Adrian Goldman, as part of a bachelor's thesis. The group's research is part of a multinational collaboration involving multiple research teams, aimed at developing potential drugs for diseases such as malaria, toxoplasmosis, and leishmaniasis.

Protozoan parasites from the *Plasmodium* and *Leishmania* genera cause severe diseases. Malaria alone was responsible for 608 000 of deaths in year 2022. These parasites rely on membrane-bound pyrophosphatases (M-PPase) to survive changes in osmotic pressure when entering and leaving a host. These enzymes, however, are not present in humans and, therefore, considered to be potential targets for inhibition.

This project is a continuation of recent research aimed at determining the effects of inhibiting compounds on an analogous M-PPase from *Thermotoga maritima* bacteria (TmPPase) using double electron-electron resonance (DEER) method. This method makes it possible to measure of the distance between cysteine residues, which can be mutated at strategic locations to determine the movement of helices during protein activity.

In this project, the gene encoding TmPPase, which was previously mutated at site 211, was further mutated at site 525 through site-directed mutagenesis, expressed in the yeast membrane, and purified for activity analysis. The activity assay results showed that the double mutation fully inactivates the enzyme, making the mutations unsuitable for DEER studies.



Keywords: M-PPase, DEER, cloning, mutagenesis


---

## Tiivistelmä


| | |
|---|---|
| Tekijä: | Sakari Bochkov |
| Otsikko: | TmPPase-proteiinin mutageneesi ja puhdistus rakenteellisia analyysejä varten |
| Sivumäärä: | 29 sivua + 1 liite |
| Aika: | 20.11.2024 |
| Tutkinto: | Insinööri (AMK) |
| Tutkinto-ohjelma: | Bio- ja kemiantekniikka |
| Ammatillinen pääaine: | Bio- ja elintarviketekniikka |
| Ohjaajat: | Professori Adrian Goldman<br>Tutkija Keni Vidilaseris<br>Tutkimuslaborantti Orquidea de Castro Ribeiro<br>Lehtori Hye Jung-Majanen |



Työ toteutettiin Helsingin yliopiston rakenteellisen biologian tutkimusryhmälle, jota johtaa professori Adrian Goldman. Ryhmän tutkimus on osa monikansallista yhteistyötä, johon kuuluu useita tutkimusryhmiä, ja sen tavoitteena on kehittää potentiaalisia lääkkeitä sairauksiin, kuten malariaan, toksoplasmoosiin ja leishmanioosiin.

*Plasmodium*- ja *Leishmania*-suvun alkueläimet aiheuttavat vakavia sairauksia. Pelkästään malaria oli vastuussa 608 000 kuolemasta vuonna 2022. Nämä loiset käyttävät membraani-pyrofosfataaseja (M-PPaasi) selviytyäkseen osmoottisen paineen muutoksista siirtyessään isäntäeläimeen ja sieltä pois. Näitä entsyymejä ei kuitenkaan esiinny ihmisissä, joten niitä pidetään mahdollisina kohteina inhibitiolle.

Tämä projekti on jatkoa viimeaikaiselle tutkimukselle, jonka tavoitteena on selvittää inhiboivien yhdisteiden vaikutuksia *Thermotoga maritima* -bakteerista peräisin olevaan vastaavaan M-PPaasi-proteiiniin (TmPPaasi) käyttäen kaksoiselektroni-elektroniresonanssi (DEER) -menetelmää. Tämä menetelmä mahdollistaa kysteiiniaminohappotähden välisen etäisyyden määrittämisen, ja aminohappotähtiä voidaan mutatoida kysteiiniksi strategisissa paikoissa proteiinin aktiivisuuden aikana tapahtuviin heliksien liikkeisiin liittyvien tietojen saamiseksi.

Tässä työssä TmPPaasi-geeni, joka oli aiemmin mutatoitu kohdassa 211, mutatoitiin myös kohdassa 525, ilmennettiin hiivan kalvossa ja puhdistettiin aktiivisuusanalyysejä varten. Aktiivisuusmittaustulokset osoittivat, että kaksoismutaatio inaktivoi entsyymin täysin, tehden mutaatioista sopimattomia DEER-tutkimuksiin.



Avainsanat: M-PPaasi, DEER, kloonaus, mutageneesi

## Contents

## List of Abbreviations

M-PPase: Membrane-integral pyrophosphatase

ATC: N-[(2-amino-6-benzothiazolyl)methyl]-1H-indole-2-carboxamide

TmPPase: *Thermotoga maritima* $Na^+$ - membrane-bound pyrophosphatase

DEER: Double electron-electron resonance

FPPS: farnesyl diphosphate synthase

DDM: n-Dodecyl-B-D-maltoside

# 1 Introduction

Membrane-integral pyrophosphatases (M-PPases) are enzymes present in various plant species and unicellular organisms, but they are absent in multicellular animals and fungi. M-PPases play a crucial role in organisms by managing the osmotic pressure and pH of cells by utilising pyrophosphates as energy source and pumping cations. It is hypothesized that M-PPases may have supplied energy to the earliest organisms, given the ability of pyrophosphate to form naturally under geochemical conditions. [1.]

M-PPases help organisms survive under stress conditions [2; 3]. Several human pathogenic protozoan parasites express M-PPases, necessary for osmotic homeostasis, allowing parasites to adapt to new environment when entering a host. These parasites are responsible for serious diseases such as malaria, toxoplasmosis, trypanosomiasis, and leishmaniasis. Since M-PPases are absent in humans, they represent promising targets for the development of structure-based inhibitor drugs. [4; 5.]

Knockout M-PPase mutants of *Toxoplasma gondii* have shown reduced virulence in mice, and bisphosphonate derivatives acting as M-PPase inhibitors also inhibit the growth of multiple protozoan parasites [6; 7; 8]. However, bisphosphonates and other non-hydrolysable pyrophosphate derivatives also inhibit farnesyl pyrophosphate synthase, an enzyme found in humans that is responsible for bone-related cell activities [4; 9]. Understanding the structure and biomechanics of M-PPases could enable the development of bisphosphonate derivatives or other molecules that selectively inhibit M-PPases without affecting human enzymes. [5; 10.]

One such inhibitory compound is N-[(2-amino-6-benzothiazolyl)methyl]-1H-indole-2-carboxamide (ATC), which allosterically inhibits non-parasite *Thermotoga maritima* $Na^+$-pyrophosphatase (TmPPase). This means that ATC binds to a site different from the enzyme active site, causing a structural change in the enzyme. ATC binds to the exit channel of TmPPase but does not show

inhibition signs of parasitic M-PPases due to structural differences between the enzymes. [5; 10.]

To determine the mechanics of the enzyme and identify potential inhibitors, M-PPases must be captured in various states through crystallization and analyzed using different techniques. For this project, TmPPase was intended to be analysed using double electron-electron resonance (DEER) spectroscopy. This method allows us to determine the movement of the active site and exit channel during enzyme inhibition, providing information for a clearer understanding of the protein's function.

DEER works by utilizing exposed cysteine residues, labelling them with an electron spin label molecule, and measuring the changes in electromagnetic frequencies using a specific spectrometer. This process allows to determine the distance between two residues and therefore, the movement of helices during hydrolysis or inhibition. [10; 11.] Residues can be mutated into cysteine residues at strategic locations to track the movement of the desired part of the protein during reactions. However, each mutation site should be selected carefully, as mutations can potentially reduce or inactivate enzyme activity, especially near active sites.

This project aimed to introduce cysteine mutations at sites 211 and 525 to provide a clearer picture of TmPPase inhibition. Since M-PPases are homodimeric proteins, these two mutations would result in two additional cysteine residues per monomer and in a total of four in the protein. Each site was tested individually in single mutants, which showed promising results with DEER. Therefore, it was decided to test both mutations simultaneously. [10.]

The project's goal was to prepare the T211C and S525C double mutant of TmPPase, clone it into a yeast plasmid, express the protein in yeast culture, purify the protein, and measure its activity. Depending on the sample activity, a decision would be made to either continue with the crystallization of the protein or attempt different mutation sites.

# 2 Theoretical Background

## 2.1 M-PPases as target for parasitic drugs

Protozoan parasites from the *Plasmodium* and *Leishmania* genera cause severe diseases such as malaria, toxoplasmosis, and leishmaniasis, affecting millions of lives. In 2022, malaria was responsible for 608 000 deaths [12]. While the primary hosts of these parasites are vertebrate animals, transmission occurs between animals through vectors such as insects, water or food. This process, however, requires the parasites to adapt to new environments. Differences in osmotic pressure between the host and the vector, as well as between intracellular and extracellular environments, requires a quick response from the parasites. [4.]

These parasites manage osmotic stress through an organelle called the acidocalcisome, which functions as an acidic ion-storage compartment [3]. Under osmotic stress, the parasites transfer ions between the acidocalcisome and cytoplasm via ion-pumping enzymes, stabilizing osmotic pressure. For the acidocalcisome to function, it must maintain a low pH, otherwise, the cell will lose its ability to stabilize intracellular pH and will contain significantly lower levels of polyphosphates and calcium. $H^+$-M-PPase, a proton-pumping M-PPase, maintains the acidic pH of the acidocalcisome by hydrolysing pyrophosphates and pumping protons into the organelle. [3; 13.]

To see how important $H^+$-M-PPase is for parasite, a knockout mutant of *Toxoplasma gondii* was generated [6]. Compared to the wild-type *T. gondii*, the mutant had troubles growing in controlled environments and within mice. The mutant also showed defects in attachment and invasion, which significantly reduced the fatal outcomes in infected mice. [6.]

The effects of inhibiting $H^+$-M-PPase were also tested using existing bisphosphonate compounds [14]. The compounds were tested on five different parasites, and all demonstrated the ability to inhibit parasite proliferation rate by 50% at nanomolar or low-micromolar concentrations. However, the same

bisphosphonates also inhibit farnesyl pyrophosphate synthase (FPPS), an enzyme responsible for producing farnesyl pyrophosphate in humans, which can lead to bone resorption and reduced cell motility. [7; 14.]

## 2.2 Determining mechanics of M-PPase and drug design

To identify molecules that can specifically inhibit protozoan M-PPases, the structure of M-PPase proteins must be studied. So far, M-PPase crystals from only three organisms have been obtained and resolved: *Vigna radiata*, *Thermotoga maritima*, and, most recently, *Pyrobaculum aerophilum*. On the basis of these solved structures, common structural features of M-PPases can be determined. [10.]

### 2.2.1 Common structural features of M-PPases

M-PPases are homodimeric enzymes, meaning the protein consist of two identical monomers. Each monomer consists of 15-17 helices, forming an inner and an outer ring. The active site is located within the helices of the inner ring, while the outer helices connect to the other monomer and the membrane. [4.]

Further research led to the categorization of four distinct regions within the active site: the hydrolytic center, coupling funnel, ion gate, and exit channel [4]. The residues responsible for enzyme activity were identified by mutating individual residues in the active site and observing any resulting decrease in activity. These residues are largely conserved across the M-PPase family, highlighting which parts of M-PPases are crucial and may be suitable targets for inhibition. The exit channel is the only region not conserved among M-PPases, suggesting it has minimal function in the catalytic reaction. [4; 15.]

Research is often done on TmPPase due to the relative ease of purifying the protein. TmPPase is derived from *Thermotoga maritima*, a thermophilic anaerobic bacteria, which makes TmPPase thermostable. This stability enables easier solubilization and purification using the hot-solve method. In this process,

the extracted membrane is incubated with detergent at 75 ºC for 1.5–2 hours. Denatured proteins are then removed by centrifugation, and the remaining proteins are purified using His-tag affinity chromatography. [16.]

Since the key residues of the active site, coupling funnel, and ion gate remain largely conserved across all M-PPases based on protein sequence comparisons, experiments can be conducted using the more easily purified TmPPase [17]. Since the goal is to target the active site with inhibitors, using TmPPase makes the research process significantly more cost-effective and efficient.

### 2.2.2 Structure-based drug design

Solving protein structure is essential for modern computational screening to identify suitable inhibitors. Screening and matching tools use compound libraries to identify potential inhibitors for the solved structure. First, synthesized compounds with the best matches are experimentally tested. On the basis of the inhibitory results, protein structures are resolved with these compounds to determine the structural changes induced. The newly solved structures are then fed back into computational tools, which can help identify more optimized inhibitors. This creates a feedback loop that can ultimately lead to a unique compound capable of specifically inhibiting the target protein. [4.]

ATC was the first non-substrate analogue inhibitor synthesized based on structural analysis of TmPPase [5]. As an allosteric inhibitor, it binds to the exit channel of TmPPase, the least conserved region of the protein, rendering ATC ineffective against parasitic M-PPases. However, solving the structure of TmPPase with ATC was the first structural proof that binding and catalysis might occur asymmetrically between the two monomers. Solved structures with substrate-analogue imidodiphosphate and phosphate-analogue $WO_4$ inhibitors would always have both monomers’ active sites open, which did not accurately reflect the protein's natural function. [5.]

### 2.2.3 Proving the asymmetric catalysis using DEER method

To determine if the catalytic reaction of M-PPases occurs asymmetrically in solution, the DEER method was employed. This technique involves introducing a molecule containing an unpaired electron to cysteine residues. In homodimeric M-PPases, the cysteine residues are mirrored, resulting in two electron-spin-labeled residues on each monomer. The distance between these electron spins can be measured up to 80 Å. [18.]

The goal of using DEER is to determine the movement of TmPPase helices during inhibition or hydrolysis. To achieve this, the cysteine residues must be located on helices near the active site and exit channel, without interfering with the protein's function. Additionally, all other exposed cysteine residues must be mutated into other amino acids to ensure that the label molecule attaches only to the desired location. The measured distances between electron spins allow prediction of helix movements, further confirming the asymmetry in TmPPase function. [10.]

For this thesis project, sites 211 and 525 were selected for mutation to cysteines. Site 211 is located near the active site, while site 525 is near the exit channel. Although both sites were tested separately, testing them simultaneously could provide clearer evidence to support the theory of hydrolysis happening asymmetrically. [10.]

# 3 Materials

## 3.1 Components for cloning

For this project, all necessary components were provided by the research group. Reagents for cloning, including polymerases, restriction enzymes, buffers, and loading dyes, were from Thermo Fisher Scientific.

From previous DEER experiments, the cysteineless TmPPase gene was modified to include a single cysteine mutation at site 211. All external cysteine

residues had been removed by mutating each cysteine residue to some other, while the residue at site 211 was mutated to cysteine. The gene was His-tagged for easy metal affinity purification. The gene was inserted into the yeast shuttle vector for yeast expression, serving as the starting point of the experiment. However, for site-directed mutagenesis, the gene needed to be cloned into an *E. coli* vector.

For site-directed mutagenesis, the commercial *E. coli* plasmid pProEX-HTa (Figure 1) was used. It was pre-cut with XbaI and SalI restriction enzymes, treated with calf intestinal phosphatase to prevent re-ligation, and stored at -20 ºC until cloning.

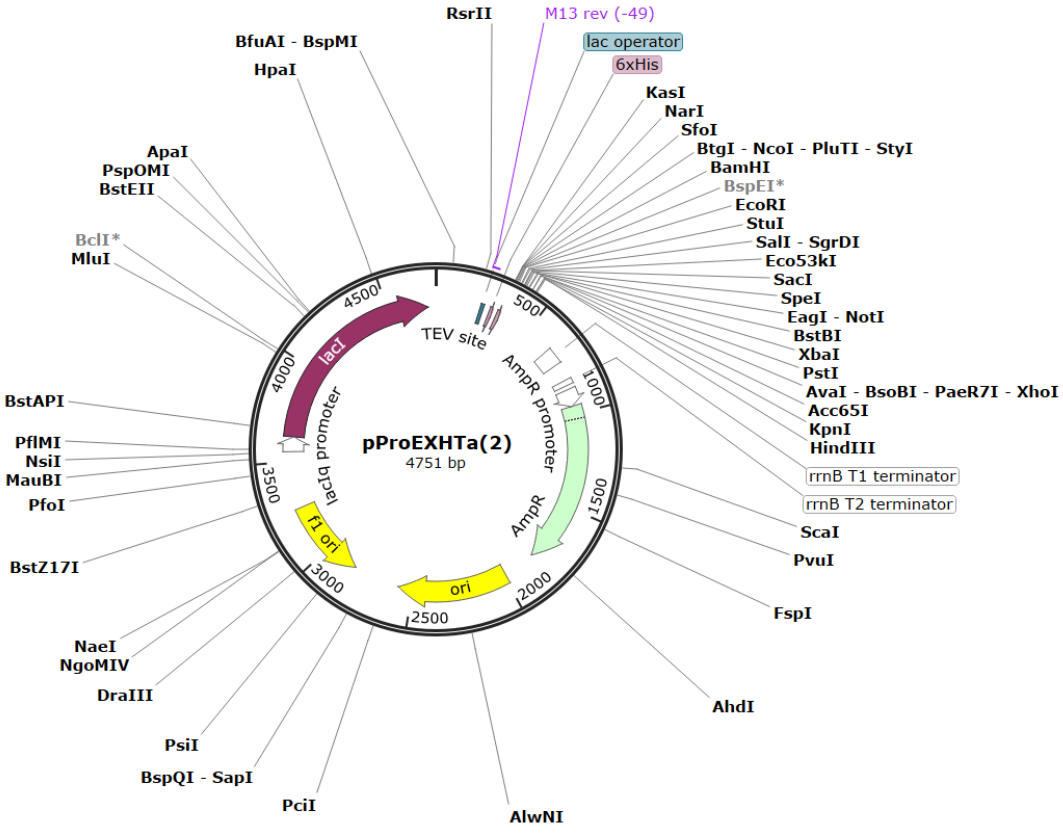


Figure 1. The commercial pProEX-HTa plasmid used for cloning and site-directed mutagenesis. This plasmid is ampicillin-resistant, contains a multiple cloning site with required restriction sites, and is relatively small 4.7 kb, making it easy to work with.

For transformation, Stellar strain competent *E. coli* cells from Takara Bio were used. These cells were provided in kits and stored at -78 ºC until transformation.

For yeast expression, the commercial PMP649 plasmid (Figure 2) was used, which is a shuttle vector compatible with both *E. coli* and yeast. The original PMP649 is approximately 8 kb in size and lacks XbaI and SalI sites. Instead, different restriction sites are used for ligation which are compatible with XbaI and SalI overhangs.

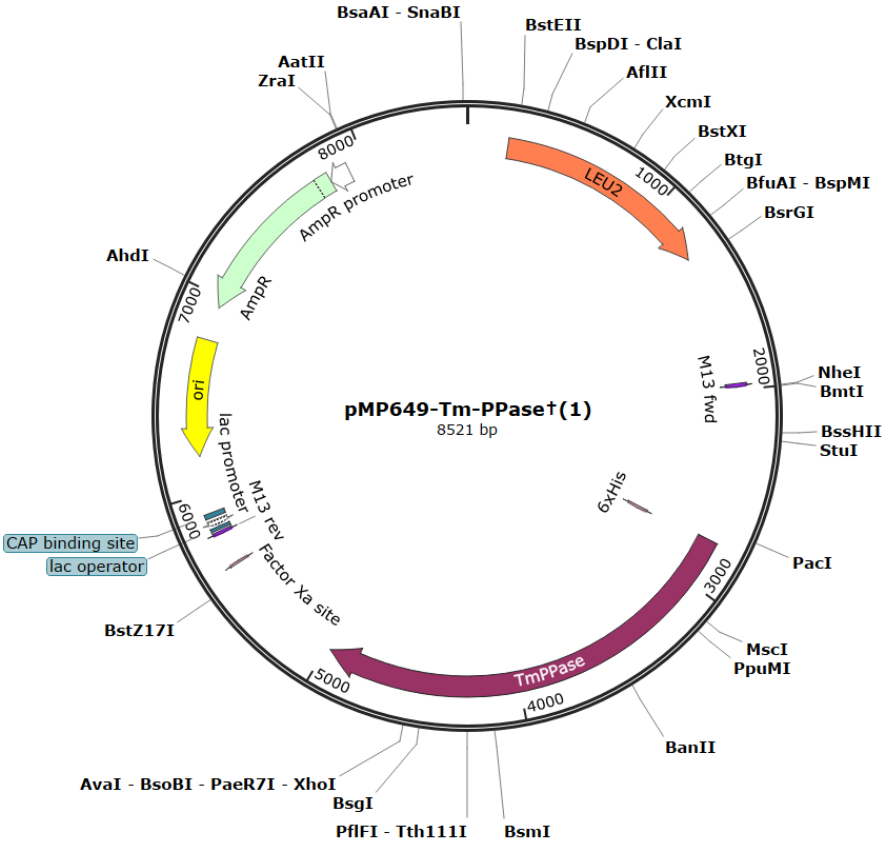


Figure 2. Shuttle vector used for protein expression in yeast with TmPPase. The vector includes ampicillin resistance and a bacterial origin of replication for *E. coli* cultivation. For yeast, it features LEU2 as a selectable marker, enabling the host to survive on leucine-deprived media and has yeast origin of replication.

The primers used in this project were stored at -20 ºC until needed. The primers utilized in the work are listed in Table 1. For gene amplification and the introduction of restriction sites, SalI forward and XbaI reverse primers were

used. Site-directed mutagenesis was performed using the 525 forward and 525 reverse primers. For sequencing, the 503 forward, 525 forward, and SalI forward primers were used.

Table 1. Primers used in this work. The regions in bold for the SalI and XbaI primers represent the restriction sites introduced at both ends of the TmPPase gene. The two bases in bold for the 525 primers differ from the gene sequence and introduce the cysteine mutation.

| | |
|---|---|
| SalI forward | 5'-TTTTTT**GTCGAC**ATGCGAGGATCACATCA CCATCACCATCACTACGTCGCTGCTCTTTTC-3' |
| XbaI reverse | 5'-TTTTTT**TCTAGA**TCAGAACAGGTGAACGTG-3' |
| 525 forward | 5'-CAGTCCTT**G**TGATATTGGGAAGCCTCCTTC-3' |
| 525 reverse | 5'-ATATCA**C**AAGGACTGATCTGGGAGAACA-3' |
| 503 forward | 5'-CTTTGTTCCTTTTGCGATGACC-3' |

The mutation principle is showcased in Figure 3. Primers replicate the gene sequence around site 525 with a single base pair change from cytosine to guanine, converting a serine residue to cysteine.


Figure 3. Primers used for site-directed mutagenesis with the part of TmPPase gene sequence around site 525. The primers were designed to amplify the entire plasmid while changing cytosine to guanine and converting serine to cysteine.

Self-prepared 1% agarose gels were used for DNA fragment isolation, run on a Bio-Rad horizontal electrophoresis system in Tris-acetate-EDTA buffer. Macherey-Nagel kits were utilized for DNA fragment extraction from gels and plasmid purification.

## 3.2 Components for expression and purification

The BJ1991 strain of *Saccharomyces cerevisiae* was used for TmPPase expression. This yeast strain has proven to be a reliable and cost-effective method for expressing various types of M-PPases. The BJ1991 strain lacks the leucine-synthesizing enzyme, enabling selective growth after transformation. [19.]

The yeast growth media contained yeast extract, peptone, glucose as well as ampicillin. For membrane extraction, the buffers contained MES or Tris to maintain a pH of around 6.5 or 7.5, glycerol to stabilize the protein, protease

inhibitors to prevent degradation by the yeast's natural enzymes, and detergent for solubilization.

The detergent used was n-Dodecyl-β-D-maltoside (DDM). This detergent can efficiently solubilize the protein and has been experimentally proven to keep TmPPase stable and active after solubilizing the membrane [20]. Detergents, in general, are used to dissolve hydrophobic membrane molecules and are synthesized in specific ways to avoid damaging the protein of interest, therefore, detergents can be very expensive [21].

SDS-PAGE gels used for protein analysis were self-made 12% of acrylamide. The gels were stored on wet towels in a +4 ºC and were made one week prior to the experiments. The equipment used for the purification is listed in Appendix 1.

# 4 Experiments and results

## 4.1 TmPPase cloning and site directed mutagenesis

### 4.1.1 Cloning into pProEX plasmid

The TmPPase gene was previously inserted into the yeast plasmid PMP649 for expression, which made site-directed mutagenesis difficult due to the plasmid’s instability. Since the selected mutagenesis method amplifies the entire plasmid via PCR, this further compromised its stability. Therefore, the gene had to be cloned into a more stable plasmid intended only for *E. coli*.

To extract the TmPPase gene from the yeast plasmid, the gene was amplified by PCR using high-fidelity polymerase and SalI and XbaI primers. High-fidelity polymerase was used for amplification to reduce the chance of unwanted mutations. Usually, PCR is not the optimal way to extract the gene of interest from a plasmid; a more preferable method is digestion using restriction enzymes. However, due to the cloning methods selected for this and the

previous project, the restriction sites were lost after cloning the gene into the yeast plasmid.

After PCR, the gene was purified using an agarose gel and a DNA purification kit from Macherey-Nagel. The gel was run at 80 V for 50 minutes. The gene was purified according to the kit's protocol. The gene was then digested using SalI and XbaI fast digestion restriction enzymes for one hour at 37 ºC and purified from the gel in the same way as before.

The gene and digested pProEX plasmid were ligated at a 3:1 ratio of insert to vector using DNA ligase for one hour at room temperature. Chemically competent cells were brought in ice from -78 ºC freezer and transformed with the ligation mixture using the heat-shock method. Cells were kept on ice for 30 minutes with the ligation mixture, heat-shocked for 30 seconds at 42 ºC, and then incubated at 37 ºC with S.O.C. media (without antibiotics) for one hour. After the heat shock, cells were concentrated by centrifugation and plated onto LB-Lennox agar plates with 100 µg/ml of ampicillin, then incubated overnight.

On the next day, about 30 colonies had grown on the plate. However, due to the possibility of digested plasmids religating without the insert, the colonies had to be screened for the presence of TmPPase. For this reason, colony PCR was performed on individual colonies.

The cells from the colonies were transferred to another plate and into PCR tubes using sterile pipette tips. To the tubes PCR mixture was added, and PCR was performed with the same settings as DNA amplification. During the denaturation step of PCR, the bacterial cells denature and release DNA into the mixture, allowing the primers to amplify the gene of interest. This method is tricky to perform, as it requires a very small amount of *E. coli* cells, but enough to start the reaction. Positive and negative controls were also included in the PCR. Purified plasmid with TmPPase was used as the positive control and PCR mixture without any cells was used as negative control.

Bands from two colonies in the expected range appeared on the second attempt of colony PCR (Figure 4). The colony PCR method was slightly modified so that cells were mostly removed from the tips by streaking against agar until no visible cell traces remained on the tips. The tips were then washed in the PCR mixture instead of scraping the tip against the bottom of a PCR tube and pipetting the mixture into a tube. This ensured that some cells would consistently enter the PCR mix without the risk of scraping away too many cells or too little cells.

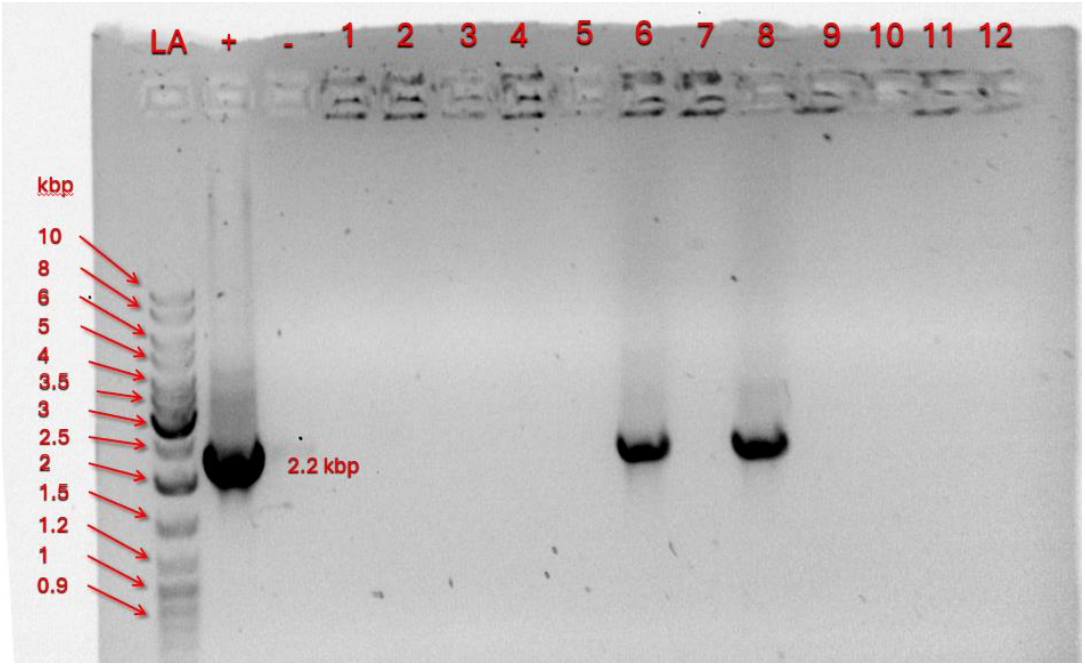


Figure 4. Second attempt at colony PCR. The size of the TmPPase gene is around 2.2 kbp. The original construct of the yeast plasmid with TmPPase was used as a positive control. XbaI and SalI primers were used for amplification. Only the sixth and eighth colonies produced bands, but this does not mean the other colonies lacked the insert and might have been false negatives.

DNA were extracted from the colonies using miniprep kit from Macherey-nagel and insert was confirmed to be in the plasmid with PCR. The plasmids from colonies 6 and 8 were then used for the mutagenesis.

### 4.1.2 Site-directed mutagenesis

Mutagenesis was performed using PCR with high-fidelity polymerase, 525 forward and reverse primers, and cells from colonies 6 and 8. There was no requisite to separate the parent plasmids from the PCR product because only 1 ng/µl of the initial plasmid concentration was used. After PCR, the solution was directly mixed with competent cells and transformed as previously described. Since plasmids from two different colonies were used, one tube of 50 µl of competent cells was divided into two and transformed with mixtures containing mutated plasmids from colony 6 and colony 8, then plated onto separate plates. After one week of attempting mutagenesis, approximately 10 to 20 colonies grew on both plates.

Plasmids from two colonies from each plate were extracted with miniprep and sent for sequencing to Eurofins Genomics. For the sequencing, 2 µl of 10 pM of primer 503 forward was mixed with 15 µl of 100 ng/µl of plasmid. Results showed that both mutations at sites 211 and 525 were present (Figure 5).

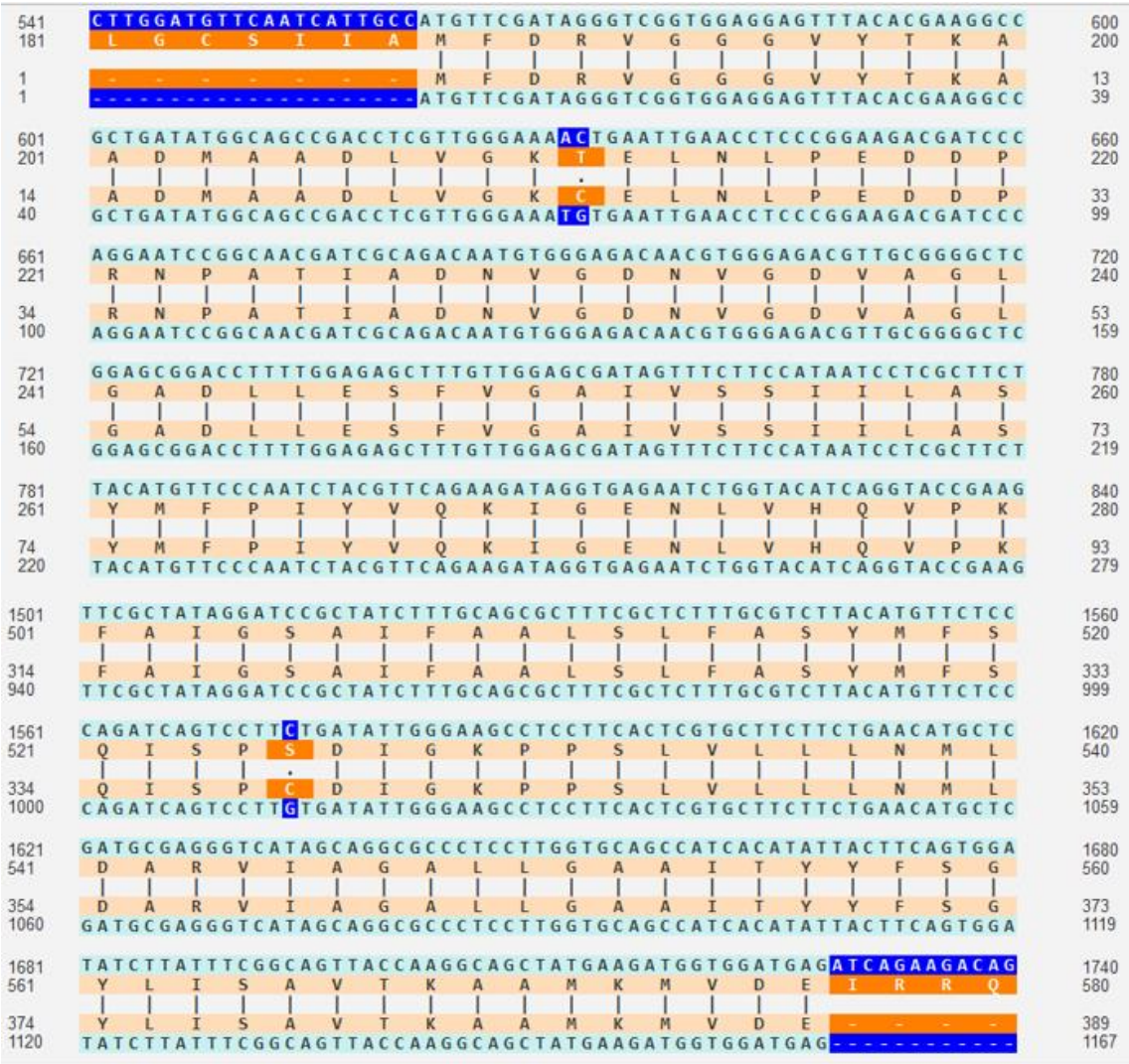

Figure 5. Sequence comparison of the double mutant TmPPase (Bottom) to cysteinless TmPPase (Top). Both cysteine mutations at sites 211 and 525 are present.

The gene was sequenced using the Sanger sequencing method. This method can sequence around 1000 base pairs per reaction. This leaves parts of the gene unchecked, requiring additional sequencing to determine if there are any unwanted mutations.

### 4.1.3 Cloning into the PMP649 plasmid

With the TmPPase gene containing both mutations, it had to be cloned back into the yeast plasmid for expression. The gene with both mutations was

amplified using the same XbaI and SalI primers and digested with XbaI and SalI restriction enzymes, following the same procedure as for the first cloning. This was not the preferred method for extracting the gene, but for an unknown reason, the XbaI restriction enzyme could not digest the pProEX plasmid with TmPPase, as if the restriction site was not present. Different XbaI enzymes from various sources were used without success, leaving PCR amplification as the only remaining option.

This time, the PMP649 plasmid had to be digested as well, but with XhoI and BcuI restriction enzymes. The restriction sites of the gene and the plasmid produce matching overhangs after digestion, allowing for ligation between TmPPase and PMP649 (Figure 6). Unique sites are formed after ligation, meaning the gene can no longer be extracted via digestion but can only be amplified using primers and PCR. This was the reason why the gene had to be extracted via PCR the first time.

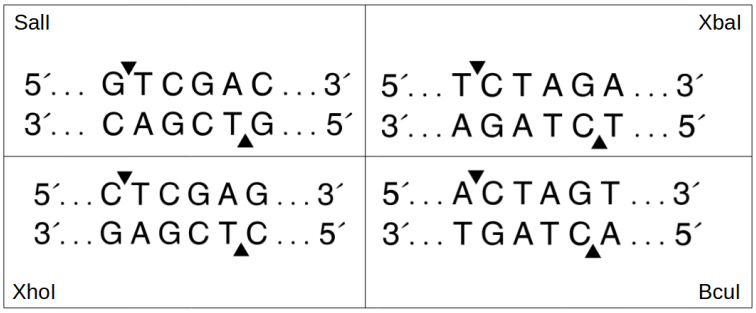


Figure 6. Restriction sites used for TmPPase cloning with PMP649. The insert's sites are on top, while the vector's sites are on the bottom. The sites produce matching overhangs without retaining the restriction sites.

While digesting PMP649 with XhoI and BcuI enzymes, another problem was differentiating the cut plasmid from the supercoiled plasmid. In Figure 7, the gel with undigested, single digestion, and double-digested plasmid is presented. While the recommended digestion time by the enzyme manufacturer is about 15 minutes, in this particular case, the digestion time had to be at least one hour.

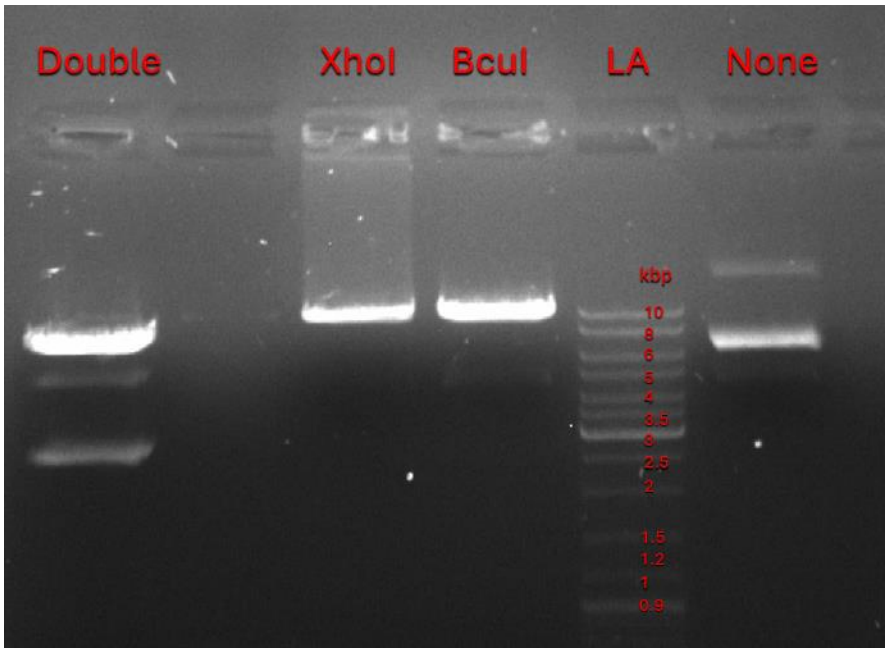


Figure 7. Gel image of PMP649 digestion. The plasmid was single-digested (XhoI and BcuI) and double-digested (Double) and compared to the undigested plasmid (None). The double-digested plasmid moves at the same speed as the supercoiled undigested plasmid, making it difficult to separate the undigested from the digested plasmids. GeneRuler DNA Ladder Mix was used as ladder (LA).

Usually, during gel extraction, digested samples are separated from undigested ones, but here, some supercoiled plasmids always mixed with the double-digested plasmids if the digestion time was insufficient. Before realizing this, cloning failed multiple times due to undigested plasmids contaminating the transformation. Extracting the plasmid from the gel also had to be done more carefully, as heat generated from running the gel, prolonged exposure to UV, and the buffers from the gel extraction kit can all negatively impact the plasmid quality.

The agarose gel was run at 80 V for 30 minutes to prevent the gel from overheating, which could damage the plasmid. The band was cutout as quickly as possible to minimize UV exposure from transilluminator and reduce potential UV-induced damage. During plasmid extraction from the gel using the Macherey-Nagel NucleoSpin kit, the protocol notes that chaotropic salts in the binding buffer used to dissolve agarose absorb light at 230 nm. Given the low

DNA concentrations after gel extraction, these salts would always interfere with Nanodrop concentration readings. To reduce salt contamination, the column from the kit was washed three times after binding the melted agarose and plasmid, rather than just once.

Ligation was performed in the same way as with pProEX. The transformation procedure had to be adjusted slightly. First, a negative control was introduced, where competent cells were mixed with only the digested plasmid to find if undigested plasmids were present. The second change was to lower the recovery and incubation temperature to 30 ºC instead of 37 ºC. After failing to obtain colonies with inserts, it was decided to decrease the temperature to 30 ºC, as this temperature had worked in previous experiments.

After multiple attempts at cloning, colony PCR resulted in several positive results. Plasmids were extracted from two colonies and sent to sequencing. Both colonies contained the TmPPase gene with the 211 and 525 mutations. The plasmid was now ready for yeast transformation.

## 4.2 TmPPase expression and purification

### 4.2.1 Yeast transformation and TmPPase expression

Yeast cells were transformed using the lithium acetate method, which is a common and effective technique for transforming yeast cells. In this method, yeast were heat-shocked in a mixture with lithium acetate, polyethylene glycol, single-stranded DNA (usually denatured salmon sperm DNA), dimethyl sulfoxide, and the plasmid DNA of interest. While the exact mechanisms of transformation are not fully understood, this method has been optimized and proven to work well compared to other methods [22].

After transformation, cells were spread onto a selective SC-LEU plate, and the plate was incubated at 30 ºC. In three days, colonies became visible on the plate. After six days, the colonies were large enough to be streaked onto a new

plate. Three colonies were selected and transferred onto a fresh SC-LEU plate. The following day, the plate was fully covered with yeast cells.

Half of the plate's worth of cells was inoculated into 250 mL of SC starter culture in a 1-liter Erlenmeyer flask, incubated overnight, and then mixed with 950 mL of YPD media and two drops of antifoam in a 2-liter flask. After exactly 8 hours of incubation, the cells were pelleted by centrifugation and frozen at -20 ºC until membrane extraction.

### 4.2.2 Membrane extraction

TmPPase was expressed in the yeast membrane and had to be extracted from it. When starting membrane purification, cells must be suspended in a buffer containing high concentrations of glycerol to remain active until crystallization. The buffers used for purification always had to contain protease inhibitors to prevent the degradation of TmPPase by natural yeast enzymes.

Cells were lysed with Bead Beater and 0.5 mm glass beads (Appendix 1, Figure 1). This method lyses cells via mechanical force, which generates heat that can heat the sample and reduce the efficiency of lysing. To avoid overheating, the sample container was surrounded by ice water, and lysis was performed in short 1-minute on and 2-minute off cycles for 20 repetitions.

After lysing the cells, the membrane had to be separated from the rest of the cell debris by ultracentrifuging the lysate at 42,500 RPM for 45 minutes (Appendix 1, Figure 2). Due to the incredibly high speeds, the centrifuge tubes had to be balanced with a maximum 0.01-gram difference and filled to the top to prevent the tubes from shattering in the vacuum. The rotor used was a Ti45, allowing for a top speed of 45,000 RPM, and was prechilled in the cold room.

When centrifugation was complete, the supernatant was discarded, and the pellets were homogenized with buffer using a Dounce homogenizer (Appendix 1, Figure 3). The homogenizer is a glass tube and tight-fitting rod used for

careful homogenization of cell samples without disturbing their structure. The sample was flash-frozen after homogenization in liquid nitrogen and stored at -78 ºC until solubilization.

### 4.2.3 Bradford assay and TmPPase solubilisation

Before proceeding with the hot-solve method for solubilization, some of the homogenized membrane was used in Bradford assays to measure the amount of expressed membrane protein. The protein amount in the membrane determines how much detergent should be used for solubilization, which has been experimentally proven to be 3:1 detergent-to-protein ratio [16].

For this, bovine serum albumin was used as a standard protein, and the selected range for the standard curve was 1–20 mg/mL. The sample dilution range was selected to be 1/100–1/2. The sample and standard were pipetted into a 96-well plate with Bradford reagent in triplicates, and absorbance was measured with a Multiskan FC (Appendix 1, Figure 4). The standard curve was calculated and is presented in Figure 8. Based on the standard curve, the approximate concentration of membrane proteins was around 13 mg/ml.

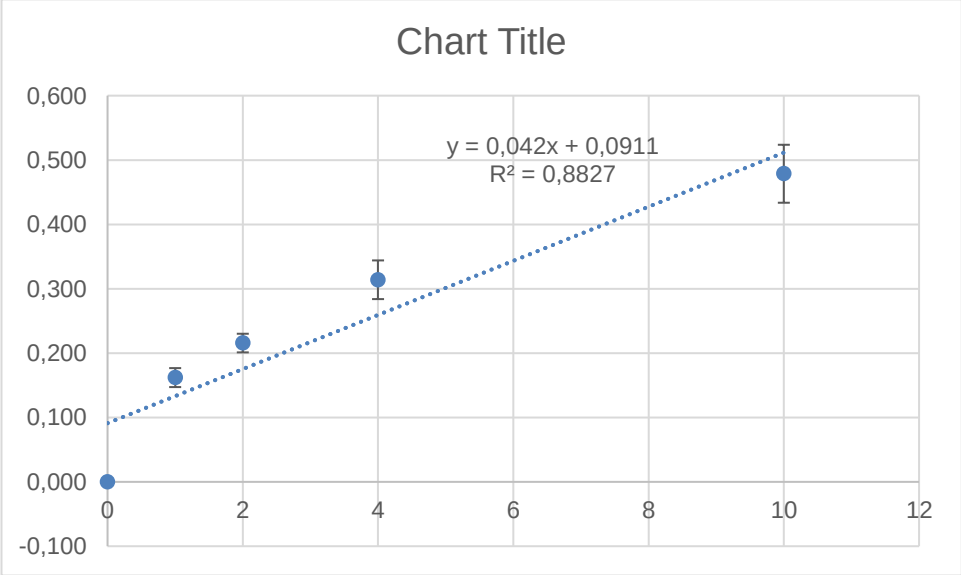


Figure 8. Bradford standard curve with absorbance measured at 595 nm. Bars represent the mean of triplicates.

After determining the approximate amount of protein, the membrane sample was diluted with solubilization buffer in a 15 mL Falcon tube. The buffer contained DDM detergent, glycerol, and protease inhibitors. The sample was solubilized using the hot-solve method, meaning the tube was submerged in a 75 ºC water bath for one and a half hours and mixed every 5 minutes to prevent separation.

When solubilization was complete, the sample was centrifuged twice for 5 minutes at 4500 × g. The supernatant was used in column purification, while the pellet was discarded. Some of the supernatant was stored at -20 ºC for running the gel.

### 4.2.4 Purification of TmPPase with Nickel affinity chromatography

For purification, Ni-NTA beads and a 20 ml gravity chromatography column were used. The beads were stabilized with a buffer containing DDM. From this point on, every buffer contained DDM, in addition to the previously mentioned reagents for protein solubility.

When the beads were stabilized and excess buffer was removed, they were mixed with the sample and incubated for two and a half hours at 40 ºC while shaking at 150 RPM. Wash and elution buffers were prepared with imidazole concentrations of 50 mM and 400 mM, along with the necessary components to keep TmPPase stable.

The sample with beads was pipetted into the column, and the flow-through was collected (Appendix 1, Figure 5). The column was washed twice with wash buffer, using two times the bead volume (800 µl), and the washes were collected in separate tubes. After the washes, the column was eluted three times with elution buffer, and the elution was collected separately in three tubes.

Before proceeding with concentrating the sample, the purified samples were analysed using SDS-PAGE gel to confirm whether TmPPase had been eluted.

The samples were mixed with SDS running buffer and stored in +4 ºC until the next morning. SDS buffer denatures and linearises proteins for gel electrophoresis, and samples can be kept in fridge for at least a day. The following day, the gel was run for 40 minutes at 200 V and stained with Coomassie Blue while shaking. Unfortunately, no bands appeared on the gel for an unknown reason (Figure 9). However, there was some trace of proteins in the elutions.

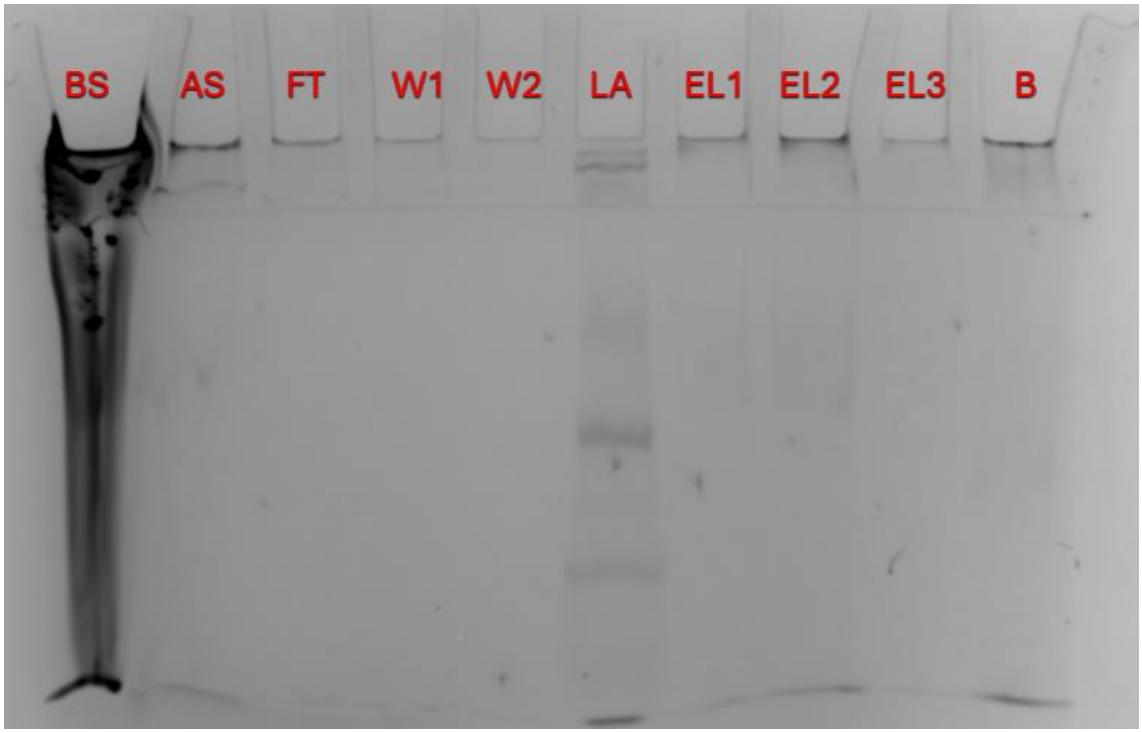


Figure 9. Self-made SDS-PAGE gel with purification samples of the double mutant. The samples from left to right are as follows: before solubilization (BS), after solubilization (AS), flow-through (FT), first wash (W1), second wash (W2), ladder (LA), first elution (EL1), second elution (EL2), third elution (EL3), and beads (B). Since bands did not form properly in the ladder, this suggests that the problem was with the gel itself.

Nevertheless, since there was something visible in the elutions, it was decided to proceed with the concentration and buffer exchange and run the gel afterward.

### 4.2.5 Sample concentration and buffer exchange

For concentrating the sample, a Vivaspin 50 kDa concentration column and centrifuge were used (Appendix 1, Figure 6). The sample was applied to the column and spun at 4000 g for 5 minutes. The column was lightly washed with elution buffer to ensure no protein remained on the walls of the column. The column was then spun again until only 50 µl of sample remained. The flow-through was discarded.

For the buffer exchange, a Bio-Rad Micro Bio-Spin chromatography column was used. The exchange buffer contained glycerol, DDM, and protease inhibitors. The column was stabilized with exchange buffer, and the elution samples were used for the buffer exchange. After concentration, samples were run on an SDS-PAGE gel again (Figure 10). This time, after staining the gel, bands were visible. The concentrated sample showed a clear, strong band at the expected size of 70 kDa. However, it appears that the remaining elution sample was mixed with other samples, and does not resemble the purified protein.

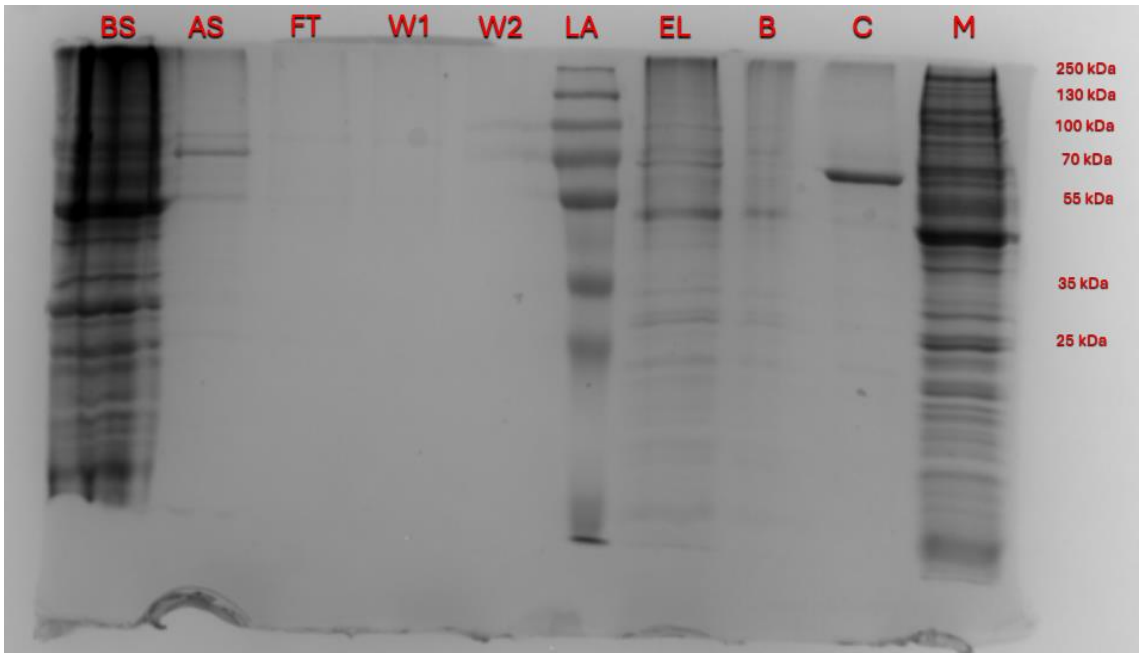


Figure 10. Purification samples with concentrated and buffer exchanged TmPPase. Samples from left to right are as follows: before solubilization (BS),

after solubilization (AS), flow-through (FT), first wash (W1), second wash (W2), ladder (LA), elution leftovers (EL), beads (B), concentrated (C), and membrane (M).

After buffer exchange, the sample concentration was measured using a Nanodrop spectrophotometer at A280. The total concentration was approximately 0.2 mg/ml. Finally, the sample was divided into eight 10 µl aliquots, flash-frozen in liquid nitrogen, and stored at -80 ºC.

## 4.3 Activity assay

For measuring enzyme activity, the colorimetric method is often used. This method relies on the reaction between orthophosphate and molybdate, forming phosphomolybdenum blue after reduction under acidic conditions. The colour intensity increases with the concentration of orthophosphate in the solution. Since M-PPases hydrolyse pyrophosphates to produce orthophosphates, this method can be used to measure the number of reactions catalysed by the enzyme within a specific time frame. [23.]

Activity assays are typically conducted with both the purified wild-type M-PPase and the mutant, with the mutant's activity compared to that of the wild-type. A mutant is considered active if its activity is at least 30% of the wild-type's activity. For TmPPase, the assay is carried out at 71 ºC, the temperature at which the protein is most active. In this project, activity assays were performed with the cysteineless 211 and 525 mutant, the wild-type TmPPase, and the cysteineless 525 mutant.

The concentrated TmPPase samples were reactivated with liposomes, DDM, and reactivation buffer. During this process, the samples were diluted so that the double mutant in this project was four times, two times, and one time more concentrated than the other enzymes. Disodium phosphate was used as the standard, and four standard solutions were prepared. Each standard solution included all the components of the activation buffer to minimize absorbance

variation. The standard solution without disodium phosphate was used as a blank.

Samples and standards were pipetted in triplicates into a 96-well plate and sealed with adhesive tape to prevent evaporation during the reaction. A multichannel pipette was required to initiate reactions simultaneously. The plate was then incubated for 5 minutes at 71 ºC, after which sodium pyrophosphate dibasic (as the pyrophosphate source) was added. To do this, the adhesive tape was briefly lifted, and the solution was pipetted into each strip at 30-second intervals to account for the time needed to open the tape. After adding pyrophosphate, the plate was incubated for another 5 minutes. Following the reaction, samples were cooled on ice for 10 minutes, centrifuged to collect any condensed liquid from the tape, and the tape was removed. Molybdate and ascorbic acid were mixed together on ice and pipetted into the tubes, followed by a 10-minute incubation.

During this incubation, the molybdate reacts with the hydrolysed phosphates to form a blue colour, which can be measured with a spectrophotometer. The reaction is stopped with an arsenite-citrate solution and incubated for another 30 minutes to stabilize the color. The samples are then pipetted into a 96-well plate and measured for absorbance at 860 nm using the Multiskan FC.

The phosphate standard produced a clean trendline (Figure 11) with minimal deviation. Based on the amount of disodium phosphate, the specific activity of the enzymes was calculated by dividing the amount of phosphate by the reaction time and protein mass.

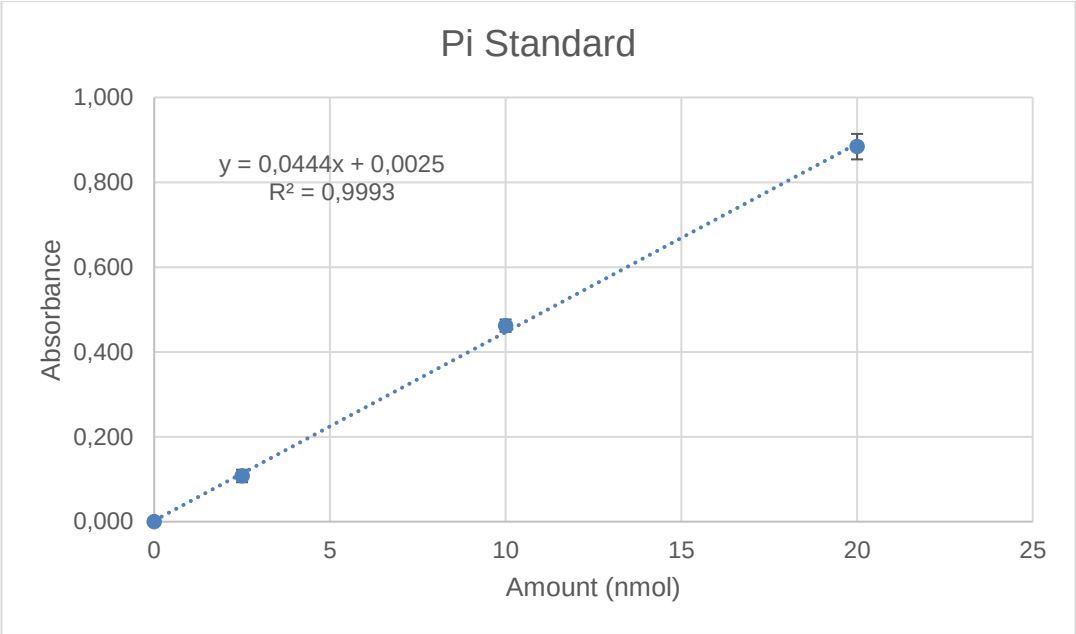


Figure 11. Standard obtained from the TmPPase activity assay.

Specific activity was calculated for each enzyme and compared to wild-type TmPPase (Figure 12). The double mutant did not produce any blue colour after adding molybdate, indicating that no orthophosphate was present in the solution.

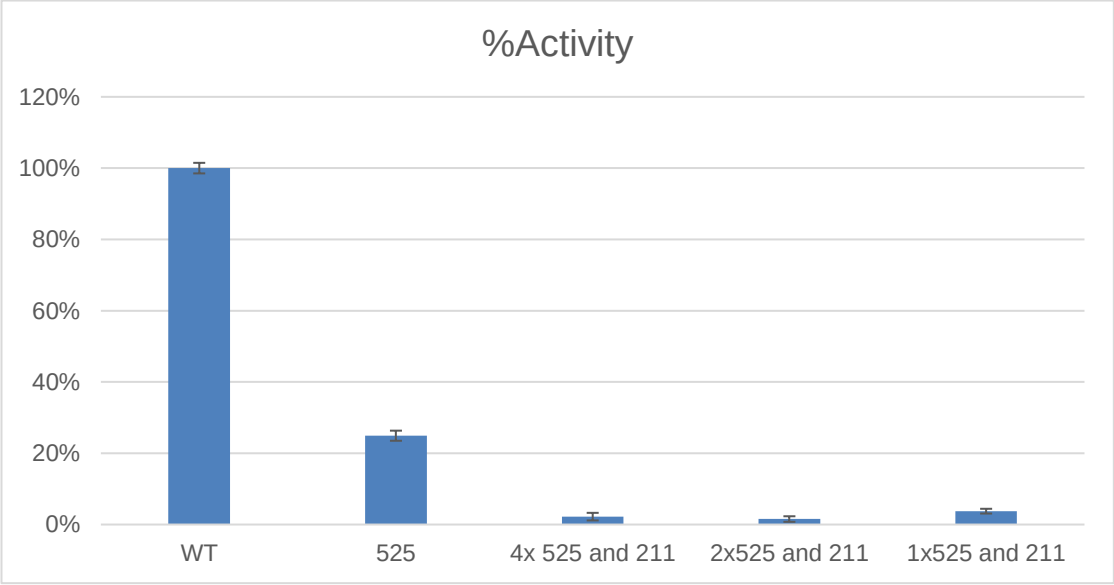


Figure 12. Activity assay results. The specific activity of the mutants is compared to the wild type, which is set to 100%. The 525 mutant exhibited only 25% of the wild-type activity, while the double mutant showed no activity.

Since wild-type and 525 mutant TmPPase served as positive controls for the assay, the activity assay was carried out properly. On the basis of these results, it was decided not to proceed with crystallization of the double mutant TmPPase.

# 5 Discussion

Before concluding that the double mutant was inactive, the gene had to be fully sequenced to determine if there were any unwanted mutations. The gene was sequenced from both ends, and no unwanted mutations were found, suggesting that the gene was coding for the intended protein correctly.

The concentrated sample appeared clean on the gel, with no visible smearing or abnormalities, and it had the correct size. This suggests that the purification was performed correctly without damaging the protein. Unfortunately, the elutions were nearly fully used for concentration, and the gel does not appear to accurately reflect the purification results. The most likely explanation is that the remaining elutions were accidentally mixed with other samples due to a pipetting error. This issue could have been avoided by preserving and correctly storing a sufficient amount of the elution samples.

Another improvement to the purification process could be the measurement of protein loss at each step. Typically, protein samples are retained from each purification stage to measure protein concentration. At the end of purification, protein concentrations from each sample are determined, for example using the Bradford assay. This helps identify where in the process issues may have occurred if no protein was purified or if the yield of purified protein is too low. In the experiment of this thesis, the protein was, nevertheless, successfully purified, and the final concentration was sufficient for the activity assay. Measuring protein loss also allows for optimization of the purification protocol.

The activity assay was conducted correctly, as both the wild type and the single mutant were active. However, the activity of the single mutant was significantly

lower compared to the wild type. Mutations near the active site can significantly reduce the activity of TmPPase, or even render it inactive. Since TmPPase was stripped of its outer cysteines and had one mutation near the active site (211) and another near the exit channel (525), it is not surprising that the double mutant shows no activity.

Ideally, each individual mutant should have been tested in the same activity assay attempt. This would mean testing both single mutants, 525 and 211, as well as the cysteineless mutant alongside the wild-type TmPPase. This would have provided a clearer picture of which mutation impacts the protein's activity the most. However, no other purified mutants were available at that time, and additional purifications would have been costly and with minimal benefit, as there was no longer a requisite for other mutants.

It was concluded that the double mutation made the protein inactive. Therefore, there was no need to continue with the crystallisation and with DEER. Nonetheless, it was worth trying both mutations at the same time. To continue with the project, new mutation sites might be attempted.

# 6 Conclusion

The goal of this thesis was to introduce a cysteine residue at site 525 through site-directed mutagenesis to the TmPPase cysteineless mutant with an existing cysteine mutation at site 211, to express and purify the double mutant TmPPase protein, and to measure the protein's activity. If the double mutant retained at least 30% of the wild-type TmPPase activity, it would have been crystallized and sent for DEER analysis to measure the distance between cysteine residues, providing a clearer understanding of monomer helix movement during inhibition or hydrolysis.

Mutation, expression, and purification of the TmPPase were performed successfully. The protein sequence did not contain any unintended mutations, and the concentrated double mutant was pure, with a concentration of 0.2

mg/ml. However, the activity assay demonstrated that both mutations completely inactivated the protein. The activity assay was performed with the wild-type and the cysteineless 525 mutant TmPPases. When compared to the wild type, the 525 single mutant retained 25% activity, while the 211 and 525 double mutant showed no activity. This result concluded that both mutations together are not suitable for DEER analysis.

Nevertheless, the work was still useful. There is no way of knowing whether a mutant will be active or not. After potential mutation sites are identified, it ultimately has to be experimentally determined if the mutation is suitable for analysis. This work contributed to the tedious part of research that usually goes unrecognized due to not producing presentable results. Most research documentation shows only the tip of the iceberg of the effort involved, as failed attempts are typically not considered suitable for publication.

The overall goal of the research is to aid in developing inhibitors for parasitic M-PPases by analysing protein function and the effects of inhibitors on TmPPase. Since diseases like malaria primarily affect low-income regions, private companies often find it challenging to justify research into widely accessible drugs. This shifts the responsibility of such drug development to a few research groups with limited funding, which slows down progress. [4.]

By experimentally demonstrating that these two mutations cannot function together, further research can explore alternative sites. New sites may not compromise enzyme activity and could be potentially ideal for DEER analysis. This will lead to better understanding of M-PPase function and support the discovery of unique inhibitors, potentially leading to the development of new drugs for diseases such as malaria.

## References


1 Baykov, Alexander A.; Malinen, Anssi M.; Luoto, Heidi H. & Lahti, Reijo. 2013. Pyrophosphate-Fueled Na+ and H+ Transport in Prokaryotes. Online material. ASM Journals. <https://journals.asm.org/doi/10.1128/mmbr.00003-13>. Accessed 15.10.2024.

2 García-Contreras, Rodolfo; Celis, Heliodoro & Romero, Irma. 2004. Importance of Rhodospirillum rubrum H+-Pyrophosphatase under Low-Energy Conditions. Online material. ASM Journals. <https://journals.asm.org/doi/full/10.1128/jb.186.19.6651-6655.2004>. Accessed 15.10.2024.

3 Docampo, Roberto; Souza, Wanderley; Miranda, Kildare; Rohloff, Peter & Moreno, Silvia N. J. 2005. Acidocalcisomes? conserved from bacteria to man. Online material. Nature Reviews Microbiology. <https://www.nature.com/articles/nrmicro1097>. Accessed 15.10.2024.

4 Shah, Nita R.; Vidilaseris, Keni; Xhaard, Henri & Goldman, Adrian. 2016. Integral membrane pyrophosphatases: a novel drug target for human pathogens?. Online material. AIMS Biophysics. <http://www.aimspress.com/article/10.3934/biophy.2016.1.171>. Accessed 15.10.2024.

5 Vidilaseris, Keni; Kiriazis, Alexandros; Turku, Ainoleena; Khattab, Ayman; Johansson, Niklas G.; Leino, Teppo O.; Kiuru, Paula S.; Gennäs, Gustav Boije; Meri, Seppo; Yli-Kauhaluoma, Jari; Xhaard, Henri & Goldman, Adrian. 2019. Asymmetry in catalysis by Thermotoga maritima membrane-bound pyrophosphatase demonstrated by a nonphosphorus allosteric inhibitor. Online material. Science Advances. <https://www.science.org/doi/10.1126/sciadv.aav7574>. Accessed 15.10.2024.

6 Liu, Jing; Pace, Douglas; Dou, Zhicheng; King, Thayer P.; Guidot, Daniel; Li, Zhu-Hong; Carruthers, Vern B. & Moreno, Silvia N. J.. 2014. A vacuolar-H + -pyrophosphatase (TgVP1) is required for microneme secretion, host cell invasion, and extracellular survival of Toxoplasma gondii. Online material. Molecular Microbiology: Volume 93, Issue 4. <https://onlinelibrary.wiley.com/doi/epdf/10.1111/mmi.12685?src=getftr>. Accessed 15.10.2024.

7 Martin, Michael B.; Sanders, John M.; Kendrick, Howard; de Luca-Fradley, Kate; Lewis, Jared C.; Grimley, Joshua S.; Van Brussel, Erin M.; Olsen, Jeffrey R.; Meints, Gary A.; Burzynska, Agnieszka; Kafarski, Pawel; Croft, Simon L. & Oldfield, Eric. 2002. Activity of Bisphosphonates against Trypanosoma bruceirhodesiense. Online material. Journal of Medicinal Chemistry, Volume 45, Issue 14. <https://pubs.acs.org/doi/10.1021/jm0102809>. Accessed 15.10.2024.

8 Rodrigues, Claudia O.; Scott, David A.; Bailey, Brian N.; de Souza, Wanderley; Benchimol, Marlene; Moreno, Ben; Urbina, Julio A.; Oldfield, Eric & Moreno, Silvia N. J. 2000. Vacuolar proton pyrophosphatase activity and pyrophosphate (PPi ) in Toxoplasma gondii as possible chemotherapeutic targets. Online material. Great Britain. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1221200/pdf/10903134.pdf>. Accessed 15.10.2024.

9 Tsoumpra, Maria K.; Muniz, Joao R.; Barnett, Bobby L.; Kwaasi, Aaron A.; Pilka, Ewa S.; Kavanagh, Kathryn L.; Evdokimov, Artem; Walter, Richard L.; Von Delft, Frank; Ebetino, Frank H.; Oppermann, Udo; Russell, R. Graham G. & Dunford, James E. 2015. The inhibition of human farnesyl pyrophosphate synthase by nitrogen-containing bisphosphonates. Elucidating the role of active site threonine 201 and tyrosine 204 residues using enzyme mutants. Online material. National Library of Medicine. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4652608/>. Accessed 15.10.2024.

10 Liu, Jianing; Shah, Anokhi; Ma, Yue; Hardman, Katie; Johansson, Niklas G.; Ribeiro, Orquidea; Brookfield, Adam; Bowen, Alice; Yli-Kauhaluoma, Jari; Xhaard, Henri; Jeuken, Lars J.C.; Goldman, Adrian; Pliotas, Christos & Vidilaseris, Keni. 2024. Conformational dynamics and asymmetry in multimodal inhibition of membrane-bound pyrophosphatases. Online material. bioRxiv. <https://www.biorxiv.org/content/10.1101/2024.07.26.605302v2.full>. Accessed 15.10.2024.

11 Jeschke, G.; Bender, A.; Paulsen, H.; Zimmermann, H. & Godt, A. 2004. Online material. Journal of Magnetic Resonance, Volume 169, Issue 1. <https://www.sciencedirect.com/science/article/pii/S1090780704000898?via%3Dihub>. Accessed 15.10.2024.

12 Malaria's Impact Worldwide. 2024. Online material. CDC. <https://www.cdc.gov/malaria/php/impact/index.html>. Accessed 12.11.2024.

13 Lemercier, Guillaume; Dutoya, Sandrine; Luo, Shuhong; Ruiz, Felix A.; Rodrigues, Claudia O.; Baltz, Théo; Docampo, Roberto & Bakalara, Norbert. 2002. A Vacuolar-type H+-Pyrophosphatase Governs Maintenance of Functional Acidocalcisomes and Growth of the Insect and Mammalian Forms of Trypanosoma brucei. Online material. Journal of Biological Chemistry. <https://www.sciencedirect.com/science/article/pii/S002192581836455X?via%3Dihub>. Accessed 12.11.2024.

14 Martin, Michael B.; Grimley, Joshua S.; Lewis, Jared C.; Heath, Huel T.; Bailey, Brian N.; Kendrick, Howard; Yardley, Vanessa; Caldera, Aura; Lira, Renee; Urbina, Julio A.; Moreno, Silvia N. J.; Docampo, Roberto; Croft, Simon L. & Oldfield, Eric. 2001. Bisphosphonates Inhibit the Growth of Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani,

Toxoplasma gondii, and Plasmodium falciparum: A Potential Route to Chemotherapy. Online material. ACS Publications. <https://pubs.acs.org/doi/10.1021/jm0002578>. Accessed 12.11.2024.

15 Asaoka, Mariko; Segami, Shoji & Maeshima, Masayoshi. 2014. Identification of the critical residues for the function of vacuolar H+-pyrophosphatase by mutational analysis based on the 3D structure. Online material. The Journal of Biochemistry Volume 156, Issue 6. <https://academic.oup.com/jb/article/156/6/333/2726268>. Accessed 12.11.2024.

16 López-Marqués, Rosa L.; Pérez-Castiñeira, José R.; Buch-Pedersen, Morten J.; Marco, Sergio; Rigaud, Jean-Louis; Palmgren, Michael G. & Serrano, Aurelio. 2005. Large-scale purification of the proton pumping pyrophosphatase from Thermotoga maritima: A “Hot-Solve” method for isolation of recombinant thermophilic membrane proteins. Online material. Biochimica et Biophysica Acta – Biomembranes Volume 1716, Issue 1. <https://www.sciencedirect.com/science/article/pii/S0005273605002567>. Accessed 12.11.2024.

17 Strauss, Jannik; Wilkinson, Craig; Vidilaseris, Keni; de Castro Ribeiro, Orquidea M.; Liu, Jianing; Hillier, James; Wichert, Maximilian; Malinen, Anssi M.; Gehl, Bernadette; Jeuken, Lars JC; Pearson, Arwen R. & Goldman, Adrian. 2024. Functional and structural asymmetry suggest a unifying principle for catalysis in membrane-bound pyrophosphatases. Online material. EMBO reports. <https://www.embopress.org/doi/full/10.1038/s44319-023-00037-x>. Accessed 12.11.2024.

18 Schiemann, Olav & Prisner, Thomas F. 2007. Long-range distance determinations in biomacromolecules by EPR spectroscopy. Online Material. Quarterly Reviews of Biophysics. <https://www.cambridge.org/core/journals/quarterly-reviews-of-biophysics/article/longrange-distance-determinations-in-biomacromolecules-by-epr-spectroscopy/522C833F4391B6AE50892B8E59CDD1AC>. Accessed 12.11.2024.

19 Kim, Eugene J.; Zhen, Rui-Guang; Rea & Philip A. 1993. Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. Online material. PNAS. <https://www.pnas.org/doi/epdf/10.1073/pnas.91.13.6128>. Accessed 12.11.2024.

20 Kellosalo, J.; Kajander, T.; Palmgren, M.G.; Lopéz-Marqués, R.L. & Goldman, A. 2011. Heterologous expression and purification of membrane-bound pyrophosphatases. Online material. Protein Expression and Purification Volume 79, Issue 1.

<https://www.sciencedirect.com/science/article/pii/S1046592811001343?via%3Dihub>. Accessed 12.11.2024.

21 Membrane Protein Solubilization. Online material. Merck. <https://www.sigmaaldrich.com/FI/en/technical-documents/technical-article/protein-biology/protein-purification/detergent-solubilization-of-membrane-proteins?srsltid=AfmBOopPZL33EPrWxyGmYOch4LTLofhNsS4IBBgpQ11x1TMn0PiWUq72>. Accessed 12.11.2024.

22 Kawai, Shigeyuki; Hashimoto, Wataru & Murata, Kousaku. 2010. Transformation of Saccharomyces cerevisiae and other fungi. Online material. Bioengineered Bugs. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3056089/>. Accessed 12.11.2024.

23 Vidilaseris, Keni; Kellosalo, Juho & Goldman, Adrian. 2018. A high-throughput method for orthophosphate determination of thermostable membrane-bound pyrophosphatase activity. Online material. Analytical Methods. <https://pubs.rsc.org/en/content/articlelanding/2018/ay/c7ay02558k>. Accessed 12.11.2024.

## Equipment used for double mutant TmPPase purification

Figure 1. BeadBeater. The sample was located inside the chamber in the middle and outside is surrounded by ice water.


Figure 2. Closed ultracentrifuge with one of the polycarbonate bottles with metal cap.

Figure 3. Dounce homogeniser that was used for homogenisation of membrane sample with buffer.

Figure 4. Multiskan FC from ThermoScientific used for Bradford and activity assay.

Figure 5. Gravity column with volume of 20 ml that was used for purification. Sample with beads is applied to the column, washed and eluted using a pipette.

Figure 6. Concentration column. The sample is applied to the mash and column is centrifuged, until desired volume.