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Title:

Expression and purification of the primate foamy virus transmembrane envelope protein

German title of the Thesis:

Expression und Aufreinigung des transmembranen Hüllproteins des Primatenfoamyvirus

Partner of Cooperation: **Robert-Koch-Institute**
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Retrovirus induced immunsuppression

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Presented until: **23.08.2010**

Declaration

I, Alexander Šach, born on 21.07.1987 in Berlin, hereby certify that this report entitled:

Expression and purification of the primate foamy virus transmembrane envelope protein

is the result of my own work and investigation, without external help or any other sources than acknowledged. Quotations and thoughts of any author are marked, the references are given. I am aware that an incorrect declaration can lead to legal outcome.

Place/Date Signature (student)

Zusammenfassung

Seit dem Nachweis breit neutralisierender Antikörper (bNAb), welche von HIV-infizierten Menschen gebildet werden können, konzentrieren sich viele Ansätze auf die Induktion solcher Antikörper zur Entwicklung eines HIV Impfstoffes. Zwei der am besten charakterisierten Antikörper in diesem Zusammenhang sind 2F5 und 4E10. Sie binden an konservierte Bereiche im transmembranen Hüllprotein (TM Protein) gp41 von HIV und neutralisieren bis zu 95% primärer HIV-Isolate, gängige Immunisierungsstrategien waren hier jedoch bisher nicht erfolgreich. Die Entwicklung alternativer Strategien ist daher notwendig. In dieser Arbeitsgruppe erzielte Ergebnisse zeigen, dass die Immunisierung mit dem TM Protein verschiedener Retroviren wie dem Porcinen Endogenen Retrovirus (PERV), dem Katzen-Leukämie-Virus (FeLV) und dem Koala Retrovirus (KoRV) zur Induktion neutralisierender Antikörper führt. Die gebildeten Antikörper richten sich dabei gegen eine ähnliche Region im TM Protein, wie 2F5 und 4E10. Die daraus entstandene Idee, das TM Protein verschiedener Retroviren als Träger für HIV Epitope zu verwenden, wird derzeit in der Gruppe untersucht. Foamyviren besitzen zahlreiche Eigenschaften, die sie als potentiellen Impfstoffvektor interessant machen. Dazu gehören ihre Apathogenität, ihre hohe Verpackungskapazität und das Fehlen präexistierender Antikörper in infizierten Individuen. Als Vorraussetzung für die Konstruktion rekombinanter Foamyviren mit modifiziertem TM Protein, ist die Charakterisierung der immunogenen Bereiche durch Epitopmapping notwendig. Ziel dieser Arbeit war es daher, das TM Protein des Primatenfoamyvirus (PFV) erfolgreich zu exprimieren und in großen Mengen aufzureinigen, um Immuniserungsstudien zu ermöglichen. In einem ersten Versuch wurde dafür ein Plasmidkonstrukt verwendet, welches die Ektodomäne des PFV-TM Proteins als GST-Fusionsprotein exprimierte. Trotz Expressionsoptimierung konnte das Protein nur in unlöslicher Form gewonnen werden. Der Versuch, das Protein durch Detergenzien wieder in Lösung zu bringen war zwar erfolgreich, eine Aufreinigung des resolubilisierten Proteins war unter den getesteten Bedingungen jedoch nur bedingt möglich. Daher wurden Plasmidkonstrukte mit C-terminal angehängtem His-Tag generiert. Aufgrund der zuvor optimierten Bedingungen führte dieser Ansatz zu einer hervorragenden Expressionsrate und der erfolgreichen Aufreinigung von über 90% reinem Protein, welches für spätere Immunisierungen verwendet werden kann. Des Weiteren ist es gelungen, geringe Mengen Protein unter nicht denaturierenden Bedingungen zu gewinnen. Mögliche immunologische Unterschiede zwischen diesem und Detergenz-aufgereinigtem TM Protein, bedingt durch konformationelle Unterschiede, können so untersucht werden.

Abstract

Since broadly neutralizing antibodies (bNAbs) were found to be produced in HIV-infected individuals, recent strategies towards a HIV-vaccine focus on the induction of such antibodies. 2F5 and 4E10, two of the best characterized bNAbs, neutralize up to 95% of primary HIV-isolates and are directed against the highly conserved membrane proximal external region (MPER) of the transmembrane envelope protein (TM protein) gp41. However, despite intense efforts all immunization strategies to induce these antibodies remained unsuccessful to date. Therefore, alternative strategies have to be investigated. Results of our group showed that the immunization with the TM of different retroviruses, such as the porcine endogenous retrovirus (PERV), the feline leukemia virus (FeLV) and the Koala retrovirus (KoRV) led to the induction of bNAbs. Interestingly, those antibodies reacted similarly as 2F5 and 4E10 with the MPER. Therefore, our project group is investigating the use of the TM proteins of different retroviruses as a carrier for the epitopes of HIV. Foamy viruses have several properties which makes them very suitable as a potential vaccine vector. They are apathogenic, have high packaging capacity for the uptake of foreign DNA and, due to their low prevalence in humans, avoid the risk of pre-existing antibodies interfering with the immunization success. As a prerequisite for using recombinant foamy viruses utilizing modified TM proteins, the characterization of the immunogenic regions is necessary. The objectives of this work were therefore the expression and purification of foamy virus TM protein for the later use in immunization studies. As a first attempt a plasmid construct expressing the ectodomain of the primate foamy virus (PFV) TM protein as a GST-fusion protein was used. Despite intense expression optimization the recombinant protein remained insoluble. Although experiments aimed to resolubilize the protein using detergents were successful, the purification thereof was not satisfying. Thus, plasmid constructs containing a C-terminal His-tag were generated. Using the previously optimized conditions, this approach allowed high expression rates and successful purification of recombinant protein with purity above 90%. Furthermore, it was possible to obtain small amounts of purified TM protein under non-denaturing conditions, which will be useful for determination of variation of the humoral immune response, possibly caused by conformational differences.

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Abbreviations

AA	amino acid
Amp	ampicillin
bNAbs	broadly neutralizing antibodies
bp, kbp	base pairs, kilo base pairs
CDNB	1,2 Chlorodinitrobenzene
FPPR	fusion peptide proximal region
g, mg, µg	gram, milligram (0,001g), microgram (0,001mg)
h	hour
HIV	human immunodeficiency virus
i.e.	id est (Latin)
IPTG	Isopropyl-β-D-thiogalactopyranosid
kDa	kilo Dalton
L, mL, µl	liter, milliliter (0,001L), micro liter (0,001mL)
mABs	monoclonal antibodies
MPER	membrane proximal external region
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline + 0,1% Tween
PCR	polymerase chain reaction
PFV	primate foamy virus
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate Polyacrylamid Gel electrophoresis
SIV	simian immunodeficiency virus
SY	sarkosyl
TEMED	tetramethyl-ethylenediamin
TM protein	transmembrane envelope protein
t-RNA	transfer-ribonucleic acid
WB	Western Blot
VLP	virus like particles

1 Introduction

1.1 HIV and the search for a vaccine

The human immunodeficiency virus (HIV) is one of the most challenging retroviral infections worldwide. Originated from non-human primates, the virus was first found and described in humans 1983 [Gallo et al., 1983; Barre-Sinoussi et al., 1983], while initial transmission occurred by zoonosis of the simian immunodeficiency virus (SIV) from chimpanzees to humans in the early 20th century [Korber et al., 2000]. Since then, HIV has become a major public health problem. An estimated number of 4.1 million people became newly infected in 2005 and about 2.8 million deaths were caused by AIDS related diseases. Transmitted from human to human by body fluids like blood, semen or vaginal fluid containing viral particles or infected immune cells, the virus spreads easily between individuals. Infection of cells of the immune system, such as CD4 receptor or co-receptor CCR-5 and CXCR-4 positive T-cells, macrophages and dendritic cells, is generally executed by the surface protein (SU) gp120 of HIV. After attachment conformational changes in the SU allow the insertion of the fusion peptide of the TM protein gp41 into the cellular membrane followed by hairpin formation and fusion of both membranes (Figure 1). After fusion of cells and the virus particle, the virus uses its reverse transcriptase to transcribe its RNA into DNA, which is then inserted into the cellular genome by another viral enzyme called integrase [Zheng et al., 2005]. Within the genome the HI virus can remain inactive for a very long time or lead to the infection of new cells. Progression to AIDS is associated with a decrease in the number of CD4+ cells.

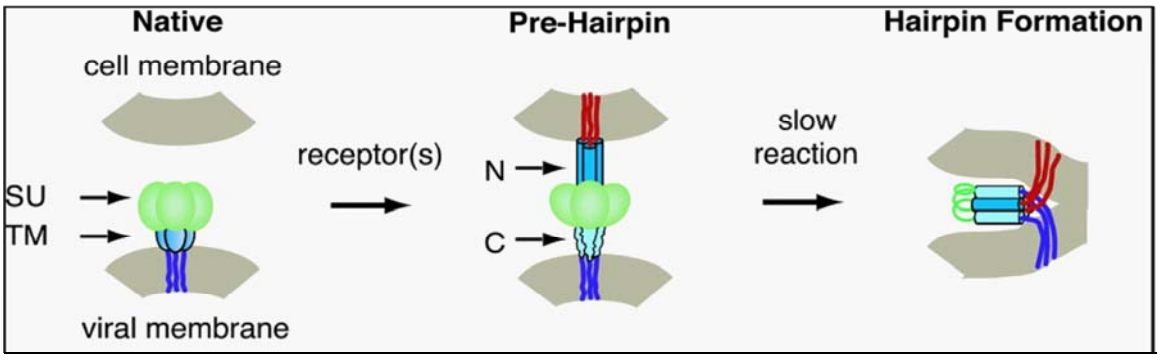


Figure 1: The conformational changes of the trimeric gp120 (green) and gp41 (blue) complex during infection. Upon interaction gp41 undergoes a conformational change, in which the fusion peptide (red) binds to the cell membrane. The hairpin structure results by binding of the N- and C-peptide region. [Chan et al., 1998]

The incubation period varies from patient to patient, ranging from two weeks to beyond 20 years, showing a highly individual course of disease development. It can take years after infection, until the amount of CD4+ cells is below a critical concentration of 200 cells per microliter. Generally, this leads to AIDS associated diseases, where the immune system is highly susceptible for various infections, finally leading to death. HIV is characterized by a high genetic variability caused i.e. by the fast replication cycle and a high error rate of the reverse transcriptase [Robertson et al., 1995]. Furthermore the virus can be divided into several subgroups and clades, complicating vaccine development. Due to frequent superinfections with more than one HIV strain, recombination can form mosaic viruses, some of which can reach epidemiological dominance [Tatt et al., 2001]. Irrespective of the high genetic variability of the virus, one more faced problem seems to be the understanding of several mechanisms during infection and fusion process [Burton et al., 2004]. The fact that the virus remains inactive within the genome, where it can not be found and destroyed by antibodies, represents another major problem in the search for a vaccine.

So far, no preventive vaccine against the infection was found and limited progress was made improving health of infected patients. It has been shown that individuals with a homozygous CCR5 mutation have a longer resistance against the virus [Hütter et al., 2009] but attempts to use this finding by means of stem cell transplantation are still far away from standard clinical application and not feasible for application in highly epidemic areas. Also the treatment with a highly active antiretroviral therapy (HAART), which aims to interrupt the viral replication [Jones, 2002], can only prolong the incubation period. CD8+ T-cells are able to identify some HIV infected cells and to induce their death. One strategy of protection against HIV is to trigger that cellular immunity for the destruction of HIV-infected cells. However, it would be much more comfortable to create a preventive vaccine which protects individuals prior to infection than radically destroying infected cells after infection. Interestingly, some HIV-1 infected individuals produce neutralizing antibodies against HIV, mostly directed against the envelope proteins in the first months after infection. This early response specifically targets the autologous virus and stays ineffective against heterologous viruses [Wei et al., 2003]. Broadly neutralizing antibodies (bNAbs), such as b12, 2G12, 2F5, Z13 and 4E10 only have been detected in later stages of infection [Sather et al., 2009]. Thus, recent studies and research projects investigate several strategies to induce bNAbs prior to infection, aiming the complete prevention of HIV-1. Mostly, these studies are focused on the envelope protein,

which contains the gp120 surface protein and the TM protein gp41. Even though these proteins are well known, the amino acid sequence varies between the different subtypes and shows i.e. within the envelope glycoprotein up to 35% divergence [Girard et al., 2006]. Additionally, the HIV-1 Env trimer is heavily glycosylated, which shields potential neutralization epitopes [Wyatt et al., 1998]. Therefore, highly conserved epitopes need to be found, showing a deep breadth among several different HIV strains. B12, one of the first identified antibodies, was shown to target the CD4 binding site on gp120, but so far immunization strategies thereof have not resulted in bNAbs of such specificity [Burton et al., 2004]. Another immunogenic region is the co-receptor binding site, as well as the membrane proximal external region (MPER) of the envelope protein gp41. The latter contains the binding region for 2F5 and 4E10 bNAbs.

There are completely different approaches to elicit an immune response including bNAbs, i.e. live attenuated vaccines like nef deleted mutants of simian immunodeficiency virus (SIV) in rhesus macaques, which establishes a low grade viral infection, but does not protect against superinfection and AIDS [Hofmann-Lehmann, 2003]. Inactivated vaccines, whose production without loose of antigenicity remains challenging, virus like particles (VLP) containing just envelope and core proteins, as well as envelope-based subunit vaccines are only a few strategies towards an HIV vaccine, so far resulting in small success, rather in understanding some of the faced difficulties than in inducing neutralizing antibodies [Burton et al., 2004]. The development towards an HIV vaccine might be slow but different immunization strategies established against several various viral infections like measles, mumps or rubella indicate that the protection against enveloped viruses is already possible, leading to a promising perspective.

1.2 The use of foamy viruses as a vector and its advantages

Recently obtained data, showing that the immunization with the transmembrane protein p15E of different gammaretroviruses like porcine endogenous retrovirus (PERV), feline leukemia virus (FeLV) and Koala retrovirus (KoRV) induce antibodies recognizing two epitope domains, one of them located in the MPER (Figure 2), similar to 2F5/4E10 epitopes, led to the idea of using TM proteins as backbone carrier for HIV epitopes.

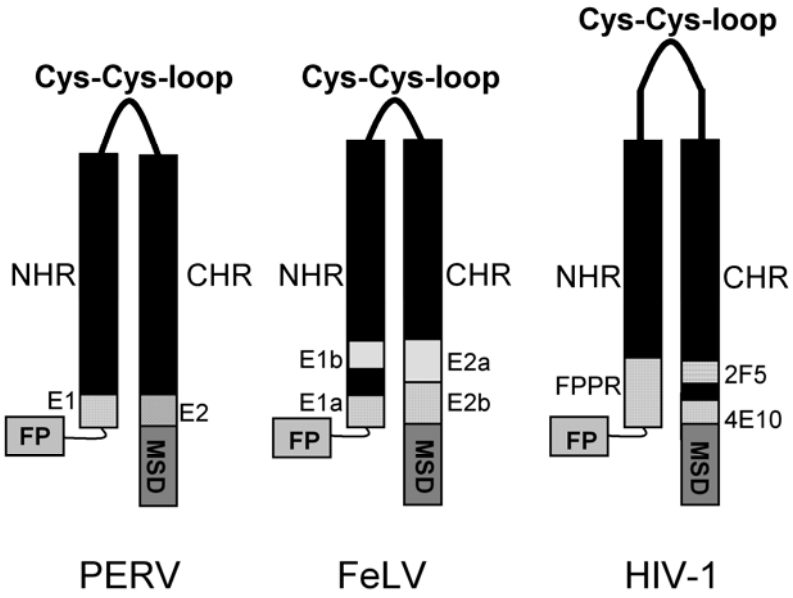


Figure 2: Schematic presentation of the localization of the two Epitope (E1 and E2) domains recognized by bNAbs within the TM protein either p15E from PERV and FeLV or gp41 from HIV-1. Epitopes were found near the membrane spanning domain (MSD) in the membrane proximal external region (MPER) and near the fusion peptide (FP) in the fusion peptide proximal region (FPPR). [Fiebig et al., 2009]

Even though sequence homology of the epitope in the MPER of gammaretroviruses and HIV was found, 2F5 and 4E10 were not able to neutralize gammaretroviruses and specific bNAbs for p15E of PERV, FeLV and KoRV did not cross-neutralize HIV-1. Considering, that structure and function of TM proteins of retroviruses are very similar and contain several highly conserved domains, a new approach of inducing bNAbs against HIV by using different retroviruses as a backbone and exchange the epitope regions, was investigated. At present this approach is tested with the TM protein of PERV and foamy virus (FV) as a vaccine vector, modified with epitopes of gp41 to elicit a humoral immune response. While the pathogenicity of PERV is still discussed, the foamy virus has only been shown to be pathogenic within cell culture, but not in the human host. This might be related to the estimated 60 million years of coevolution of FV and their primate hosts [Murray et al., 2006]. The primate foamy virus

(PFV) was first isolated from a human cancer-derived cell line [Achong et al., 1971], but was found to have originated in chimpanzees [Herchenroder et al., 1995]. As a spumaretrovirus it contains all the typical retroviral genes for gag, pol and env, but different to most orthoretroviruses it contains full-length double stranded DNA as well as RNA packed in the viral particles. Before the virus infects new cells, the reverse transcriptase completely transcribes the RNA into DNA. Extracted DNA from the virions is already able to produce the virus after transfection [Yu et al., 1998]. Several advantages making the foamy virus suitable for the use within this new approach, for instance the virus has been shown to be apathogenic, offering the potential to use replication competent vectors for constant antigen delivery resembling the situation found in HIV infected patients. By the use of PFV as shuttle vector for HIV-1 epitopes, a risk of unintentional HIV infection is not present. The virus is capable to take up additional DNA sequences like transgenes up to 8 kbp and is, in contrast to HIV, genetically stable, suggesting that the integrity of transgenes is maintained. The wide tissue tropism of foamy viruses, which is able to infect almost every cell line including hematopoietic stem cells, would lead to an infection of all cells and therefore a higher immune response.

Although HIV is well examined to date, several important mechanisms during infection remain unclear. To begin with, the conformation changing mechanism of the envelope protein gp41 during the fusion of the virus and the cell membrane is speculative. Therefore it is not known, if epitopes for antibodies like 2F5 are only present during the membrane fusion process, but shielded afterwards within the new conformation [Frey et al., 2008]. In general, target sites for bNAbs are often buried under the heavily glycosylated conformation [Zhou et al., 2007]. Additionally, it has been shown, that the virus can rapidly mutate glycans on the Env surface, in order to escape the bNAbs of the host [Wei et al., 2003]. This theory is encouraged by the realization, that antibodies to gp41 epitopes can efficiently impede Env-mediated fusion, if the fusion process is slowed by cooling the reaction to 32°C [Burton et al., 2004]. Another feasible reason for the ineffective neutralization of primary viruses or even cell-culture adapted viruses by several antibodies, is thought to be the limited space for large antibodies to gain access to gp41 epitopes, which are exposed transiently [Burton et al., 2004]. All these problems, only concentrating on strategies based on the TM protein, point towards the need to understand the mechanisms in the native virus to elicit bNAbs. Therefore a part of this work concentrated on the purification under non-denaturing conditions of the TM envelope protein of the PFV, which is planned to be used as a vaccine vector, containing HIV epitopes. An-

other problem in recent vaccine studies is the lack of animal models. Even though strategies, which have been shown to work in cell culture, can be tested in non-human primates, experiments still can have a completely different outcome as probably expected. The strategy followed in this project can circumvent that problem, by using the foamy virus as a backbone. By exchanging the epitopes in later experiments, the foamy virus vector could be directly used in humans to induce neutralizing antibodies.

Several different studies using different vector models have been unequal successful so far. While a canarypox virus vector (ALVAC) was developed and evaluated through multiple Phase I/II trails [Belshe et al., 2001], the fowlpox virus has been tested as a vector in Phase I trails in Australia [Kent et al., 1998], expressing a variety of HIV antigens, such as gag, pol, env and nef. However, poxvirus-based HIV vaccines showed weak immunogenicity so far, less than 35% of the vaccines scoring positive for T-cell responses [Girard et al., 2006]. The most promising approaches of live viral vectors for HIV vaccines so far were replication-defective adenovirus type 5 (Ad5) models [Shiver et al., 2004]. More than 50% of the volunteers showed significant, long-lasting HIV-1-specific CD8+ T-cell responses to HIV-1 peptides [Girard et al., 2006]. Since then, several studies using adenovirus-based vectors were invented, leaving almost no doubt, that the best results so far have been obtained with these recombinant vaccines [Santra et al., 2005]. However, these vaccines contain the problem of pre-existing antibodies against the vector Ad5 in the human population, interfering with the immune response [Barouch et al., 2004].

Because foamy viruses have no prevalence in humans, there is also a low prevalence of pre-existing antibodies against it [Murray et al., 2006]. That, among other advantages, makes it very suitable as a vector in further vaccination strategies.

All these advantages lead to the conclusion, that the TM protein of the primate foamy virus would be an optimal candidate for the exchange of its epitopes against the epitopes of HIV-1, for further immunization studies, leading to the induction of broadly neutralizing antibodies like 2F5 and 4E10 prior an infection of HIV and hereby representing a natural induced immune resistance.

1.3 Objectives

To combine both ideas of using the TM protein of retroviruses for the induction of neutralizing antibodies and the use of foamy viruses with its described advantages as a vaccine vector, it is necessary to characterize the PFV and to determine a homology between the epitopes of the TM protein of PFV and HIV. Therefore, rats should be immunized with the TM protein of the primate foamy virus, to induce antibodies, which should be tested in neutralization assays. In addition their epitopes can be mapped. Thus, the objectives of this thesis were the expression and purification of primate foamy virus TM protein in milligram amounts. The detailed project overview is visible on the flowchart below (Figure 3).

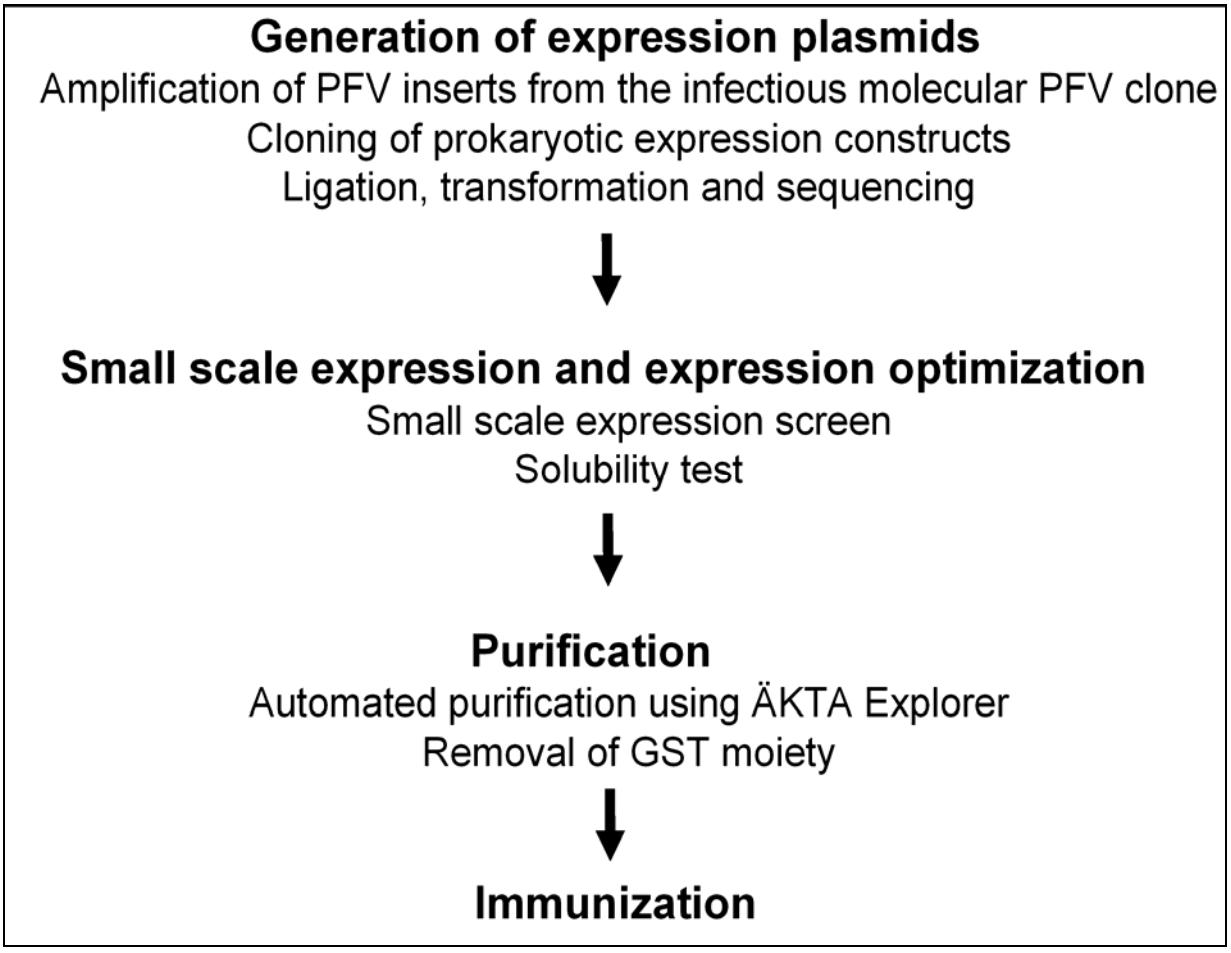


Figure 3: General project overview flowchart

2 Material and Methods

2.1 Material

2.1.1 Antibiotics

Name	Vendor	Catalog Number
Ampicillin Sodium Salt	Roth	K029.1
Kanamycin	Roth	K012.1

2.1.2 Antibodies

Name / Source	Vendor	Catalog Number
Anti Goat / Rabbit	Dako	P 0449
Anti Mouse / Rabbit	Dako	P 0161
Anti GST / Goat	GE Healthcare	27-4577-50
Penta His Antibody / Mouse	Qiagen	34660

2.1.3 Detergents

Name	Vendor	Catalog Number
Triton X-100	Roth	305
Tween 20	Roth	9127.1
Sodium Dodecyl Sulfate (SDS)	Roth	2326.2
N-lauroyl-sarcosine (Sarkosyl)	Sigma	L 9150

2.1.4 Buffer

Name	**Vendor**	**Catalog Number**
PBS	Biolabs	B0202S
PBS-T	Invitrogen	52806
Tris	Roth	4855.2
Tris-HCl	Roth	9090.1
Name	**Recipe**	
Anode Buffer (10x)	2M Tris, pH 8,9	
Cathode Buffer (10x)	1M Tris, 1M Tricin, 0,03M SDS	
Gel Buffer	1,5M Tris, 0,3% SDS, pH 8,4	
GST Elution Buffer	0,1% Sarkosyl, 20mM Imidazole, 10mM Glutathione, pH 7,4	
His Elution buffer	0,1% Sarkosyl, 500mM Imidazole, pH 7,4	
Lysis Buffer	PBS, 1mg/ml Lysozyme, 1 pill Protease inhibitor / 10mL, 10u/mL Benzonase	
Potassium phosphate buffer	100mM Potassium phosphate buffer, 1mM EDTA, pH 6,5	
TAE-Buffer (50x)	2M Tris, 1,1M Glacial acetic acid, 0,4M EDTA	
TFB Buffer 1	100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% Glycerol, pH 5,8, Sterile-filtered	
TFB Buffer 2	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% Glycerol, pH 8,0, Autoclaved	
Transfer Buffer	48mM Tris, 39mM Glycine, 20% Methanol, 0,03% SDS	
2x SDS Sample Buffer	50mM Tris-HCl, 12% Glycerol, 4% SDS, 5% 2-Mercaptoethanol, 0,01% Coomassie Brilliant Blue G-250	
Washing Buffer	0,1% Sarkosyl, 20mM Imidazole, pH 7,4	

2.1.5 Primer

PFV-ED fwd	5’ agatattaatgatgaaaactta 3’
PFV-ED_Xa	5’ aggatctggctctggatctggtatcgagggaagggatattaatgatgaaaactta 3’
PFV-ED rev	5’ atactcgagttagtgatggtgatggtgatggtggtgaggctttaagtatcccaagag 3’

2.1.6 Software

Name	**Used for**
GelDoc 2000	Biorad gel documentation system
Lasergene 8	Generation of plasmid cards
NanoDrop 1000	Peqlab Spectrophotometer
Tecan Classic	Tecan plate reader

2.1.7 Devices

Name	**Vendor**	**Catalog Number**
Avanti J-20 XP Ultracentrifuge	Beckman Coulter	
Semi Dry Transfer Cell	Biorad	170-3940
UV transilluminator system	Biorad	170-8100
Sonifier 250	Branson	UIP2000
SDS-Page System	CBS Scientific	C-DASG-400-50
Centrifuge 5804R	Eppendorf	5804.000.013
PCR Mastercycler	Eppendorf	5331 000.010
1,5ml Tube Centrifuge	Eppendorf	5415D
Äkta Explorer 10S	GE Healthcare	18-1112-41
Thermocycler	MJ-Research	PTC-200
Nanodrop spectrophotometer	Peqlab	ND-1000
Plate Reader	Tecan	7177

2.1.8 Enzymes

Name	Vendor	Catalog Number
T4 Ligase	Biolabs	MO202L
Fast digest *SmaI*	Fermentas	FD 0663
Fast digest *XhoI*	Fermentas	FD 0694
Pfx Platinum Polymerase	Invitrogen	11708-021
Benzonase	Novagen	70664-3
Lysozym	Sigma Aldrich	L6876-5G
Fire-taq DNA polymerase	Steinbrenner	AB-0241

2.1.9 Media

Name	Recipe
LB-Medium	10g/L Tryptone, 5g/L Yeast extract, 100mM NaCl, pH 7,0
TB-Medium	12g/L Tryptone, 24g/L Yeast extract, 4ml/L Glycerol, add 100mL 170M Potassium dihydrogene phosphat after autoclaving
2YT-Medium	16g/L Tryptone, 10g/L Yeast extract, 100mM NaCl, pH 7,0

2.1.10 Additional solutions

Name	Recipe
Coomassie staining solution	1g/L Coomassie Brilliant Blue G-250, 40% Methanol, 10% Glacial acetic acid
Decolorizing solution	10% Glacial acetic acid, 45% Methanol, 45% Aqua dest.

2.1.11 Online Databases

Name	Function (Address)
Blastn	nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
Blastx	protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
NEBcutter	DNA tool to find restriction sites (http://tools.neb.com/NEBcutter2/index.php)
pubMed	publication research (http://www.ncbi.nlm.nih.gov/pubmed)
Uniprot	protein information data (http://www.uniprot.org/)

2.1.12 Others

Name	Vendor	Catalog Number
Big Dye 3.1 Terminator	Applied Biosystems	4337456
O´gene ruler (DNA marker)	Fermentas	SM 1173
Page ruler (protein marker)	Fermentas	SM 0671
DNA Extraction Kit	Invitek	10201103
Plasmid preparation kit	Invitek	AG 070054
PVDF Membrane (Pore size 0,45µm)	Millipore	IPVH00010
Nuclease Free Water	Promega	P119C
pUC 18 plasmid	Promega	46-O124
ECL Western Blot substrate	Pierce	32106
BCA Reagent A	Pierce	23223
BCA Reagent B	Thermo Scientific	23224
Coomassie Brilliant Blue G-250	Thermo Scientific	20279
ECL X-posure Film	Thermo Scientific	34090
Protease Inhibitor	Roche	04693132001
Agarose	Roth	2267.4

Glacial acetic acid	Roth	3738.5
Glycerin	Roth	3783.1
HCl (25%)	Roth	X897.2
Imidazole	Roth	X988.4
IPTG	Roth	CN08.1
Methanol	Roth	8388.6
2-Mercaptoethanol	Roth	4227.1
Orange G (DNA Sample Buffer)	Roth	0318.1
Rotiphorese Gel 30	Roth	3029.1
Tricine	Roth	6977.3
EDTA	Serva	11280
1,2 Chlorodinitrobenzene	Sigma Aldrich	237329
Ethidium bromide	Sigma Aldrich	E1510
Glycerol	Sigma Aldrich	G-8898
L-Glutathione	Sigma Aldrich	G470-5
Soc-medium	Sigma Aldrich	S1797
TEMED	USB	76320 100GM

2.2 Methods

2.2.1 Selection of primer sequences

To start this project, primer for the amplification of the primate foamy virus TM protein (Uniprot accession number: P14351) needed to be designed. This was realized by analyzing the respective sequence of the PFV genome (accession number P14350) by an online restriction digestion program (NEBcutter 2.0) to avoid interference with natural occurring internal restriction sites. The respective sequences plus additional nucleotides containing restriction sites for

cloning, tag-sequences and three additional nucleotides for improvement of restriction digestion were delivered to “Eurofins MWG” for synthesis.

2.2.2 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for the amplification of the future inserts within the infectious molecular clone of PFV (kindly provided by Prof. M. Löchelt, DKFZ, Heidelberg). A standard PCR reaction using hot-start proof reading polymerase to avoid PCR mediated mutations was set up as follows:

Pfx-PCR Mix

Component	Volume (final conc.)
Pfx-buffer (10x)	5µl (1x)
$MgSO_4$ (50mM)	1µl (1mM)
dNTPs (10mM)	1µl (0,2mM)
5`-primer (10µM)	1,5 µl (0,3µM)
3`-primer (10µM)	1,5 µl (0,3µM)
Pfx-Platinum (2,5U/µl)	0,4µl (1 U)
DNA-template	1µl
H_2O	Filled up to 50µl

Cycler conditions

Step	Temperature
Denaturation	94°C
Annealing	45°C
Elongation	68°C
Number of cycles	25

For initial screening of transformants colony-PCR was used with bacteria directly taken from agar plates as template material. A standard colony-PCR reaction was set up as follows:

Colony-PCR Template

Component	Volume (final conc.)
Fire-taq buffer (10x)	2µl (1x)
MgSO4 (25mM)	0,4µl (0,2mM)
dNTPs (10mM)	0,8µl (0,16mM)
5`-primer (10 µM)	0,4µl (0,08µM)
3`-primer (10µM)	0,4µl (0,08µM)
Fire-Taq (1U/µl)	0,4µl (0,4U)
DNA-template	1µl
H_2O	filled up to 20µl

Cycler conditions

Step	Temperature
Denaturation	96°C
Annealing	42°C
Elongation	72°C
Number of cycles	35

2.2.3 Agarose gel electrophoresis

Agarose gels were made by dissolving 1% of agarose in 1x TAE-buffer and boiling it until a homogenous solution developed. After adding 0,001% Ethidium bromide to visualize the DNA fragments in the later use, the entire solution was poured in a horizontal gel chamber and cooled to room temperature.. After the gel-solution was solidified, the samples, diluted with 10x DNA sample buffer, were loaded and separated under an applied voltage of 120V. The gels were documented with the UV transilluminator system and software.

2.2.4 Purification of PCR fragments from agarose gels

The targeted band was cut out of the gel with a scalpel and purified with a gel purification kit (Invitek) as recommended by the manufacturer. Briefly, gels were solubilized by warming in solubilization reagent and loaded on a DNA binding column. After washing, purified DNA was eluted with nuclease free water (Promega).

2.2.5 Restriction digestion

Before amplified sequences could be introduced into the target expression vector, inserts and empty vector were digested using *SmaI* and *XhoI* restriction enzymes (Fast Digest, Fermentas). This step was performed within a 20µl reaction mixture, containing the plasmid or purified PCR inserts, diluted in 10x restriction enzyme buffer and nuclease free water, for at least 20 minutes to 1,5 h. The compatibility of the fast digestion enzymes (FD) allowed the performance of a double digestion.

2.2.6 Ligation

Digested PCR insert and empty vector were incubated together with 1-2 units of T4-Ligase, Ligase-buffer and nuclease free water in a total reaction volume of 20µl. Typically the ligation-efficiency was improved with an excessive amount of insert. The entire mixture was incubated for at least 1,5h in a thermocycler, switching from 16°C to 4°C every 30 minutes.

2.2.7 Preparation of competent bacteria

For the later use of transformation, chemical competent E. coli were necessary. The host strain was plated on agar plates and a single colony used for inoculation of LB-Medium containing appropriate antibiotics and incubated overnight. Afterwards 1mL of the overnight culture was added to 100mL LB medium and bacteria grown up to an optical density about OD_{600}=0,5. Subsequently the cells were centrifuged and resuspended in TFB Buffer 1 and 2, leading to a partly perforated cell membrane, which allows the uptake of plasmid DNA. Aliquots were made and stored at -80°C until use. To determine the competence of the created bacteria, pUC-18 control plasmids were transformed in produced E.coli, spread out on agar plates and counted after 12-14h incubation.

2.2.8 Transformation in competent bacteria

Chemical competent bacteria were transformed by incubation of plasmid DNA with respective cloning or expression strains of E. coli on ice. After 30 minutes cells were heat-shocked for thirty seconds at 42°C and subsequently put on ice again for additional 2 minutes. To allow expression of the respective antibiotic resistance gene cells were grown for one hour at 37°C in an orbital shaker incubated with SOC-medium and then spread on agar plates containing an appropriate antibiotic. For an optimal colony growth the agar plates were incubated at 37°C overnight.

2.2.9 Preparation of plasmid DNA

A column based plasmid preparation kit (Invitek) was used for a small scale isolation of plasmid DNA from bacteria. The method was based on the alkaline lysis method, which breaks up bacteria with an alkaline buffer containing SDS and makes it possible to separate the plasmid DNA from the genomic DNA. The amount of DNA was checked by determining the optical density (OD_{260}) with the NanoDrop 1000. Furthermore the relation between DNA (260nm) and protein (280nm) was calculated to estimate purity. A ratio of 1,9 was considered to be pure plasmid DNA.

2.2.10 Sequencing

All generated expression constructs were used for fluorescence based cycle sequencing using Big Dye 3.1 (Applied Biosystems) fluorescent dNTPs to check integrity of the cloned sequences. Only sequenced constructs were used for all subsequent experiments.

2.2.11 Preparation of glycerol stocks

To assure expression under identical condition in future experiments and create backups of generated expression constructs, plasmids transformed into expression or cloning strains were kept frozen at -80°C. Therefore, glycerol stocks were prepared by mixing equal amounts of grown cultures with 50% glycerol and direct freezing in liquid nitrogen.

2.2.12 Small scale expression optimization screen

For expression optimization PFV-TM plasmids were transformed into different expression strains (C3030H, C43, SCS1, Rosetta 2) and two colonies per strain taken for overnight cultures. On the next morning 100µl of stationary cultures were diluted in 1ml of media in 96 deep well plates and grown for 3h before induction of expression with IPTG. IPTG activates the Lac-promoter of pGEX vector and allows the transcription and translation of the inserted fusion protein. Three hours after induction the cells were centrifuged and the supernatant discarded. Remaining pelleted cells were disrupted by three freeze thaw cycles in liquid nitrogen and then resuspended and incubated for 30 min in lysis-buffer. Afterwards 10µl of cellular lysate were taken for SDS-PAGE for analysis. The setup of an expression optimization screen in a 96 deep well plate allowed the simultaneously investigation of four different bacterial strains, three different IPTG concentrations, three different media and two transformants plus the empty vector (Figure 4).

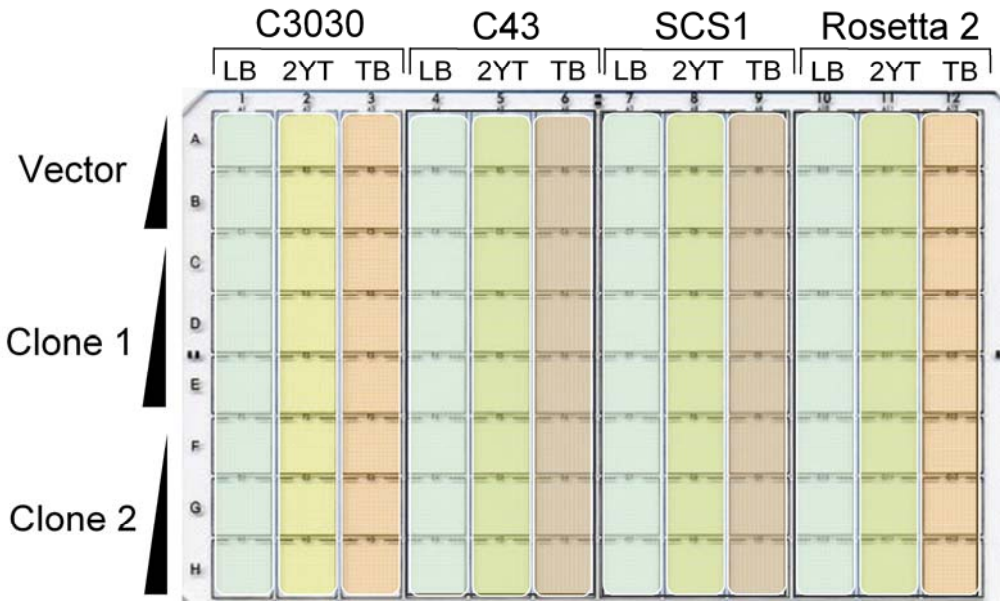


Figure 4: Layout of a 96 deep well plate for the small scale expression optimization screen using three different media (LB, 2Yt and TB). The empty vector was inoculated in two wells of each lane, one induced later with IPTG, the other not. Two clones were picked and inoculated in each lane, one not induced while IPTG was applied on the other two wells in a rising concentration. The four expression strains used were C3030H, C43, SCS1 and Rosetta 2, each of them inoculated in one lane of different media.

2.2.13 Large scale expression

The large scale expression was performed using the conditions found to be suitable in the expression optimization screen to receive high amounts of expressed protein. Usually, 100 ml of an overnight culture were diluted in 1L of TB-media and grown for 3h at 37°C (OD_{600} ~1,0) prior to induction with 1 mM IPTG and growth at 25°C. Another 3h after induction, the cells were pelleted by centrifugation (8000 rpm, 15 minutes) and the supernatant discarded. Until purification the pellets were stored at -20°C.

2.2.14 Protein purification by affinity chromatography

To proceed after the large scale expression a 1L pellet was resuspended in 25 mL lysis-buffer and held on ice for 30 min. Subsequently, the cells were disrupted by using a sonicator three times, 45 seconds each, and were pelleted by high speed centrifugation (25.000 rpm, 1h, 4°C). For non-denaturing purification supernatant was collected for further analysis. For purification using detergents, resulting pellets were incubated in extraction buffer for at least 1h under

gently shaking and resolubilized proteins were separated from insoluble material afterwards by a second high speed centrifugation step (25.000 rpm, 30 min, 4°C). For purification under non-denaturing conditions the collected supernatant was diluted to a final amount of 50 ml in washing buffer without detergents, loaded on a nickel-nitrilotriacetic (NiNTA) affinity column and eluted by applying an elution buffer gradient, which again did not contain detergents. The resolubilized proteins were either applied on a GSH matrix or on a NiNTA affinity column. In both ways prior to the purification on the matrices, the solution was diluted to a final concentration of 0,1% sarkosyl and applied to prepacked affinity columns (His-Trap FF Crude, 1 ml), which were installed in a Äkta Explorer FPLC system. After intense washing, protein was eluted by applying an elution buffer gradient.

2.2.15 Protein estimation with the BCA-Assay

To specify the amount of protein after the purification a bicinchroninic acid (BCA)-assay was performed, comparing a colorimetric signal, caused by the BCA Reagent mixture with known amounts of BSA standard with the unknown amount of the purified protein. More precisely, 196µL of a 50:1 mixture of BCA Reagent A and B were added to 4 µL of the protein. The read out was performed with the Tecan plate reader at 560 nm.

2.2.16 Activity test

To investigate the activity of the purified protein a CDNB enzymatic assay was performed, based on the principle, that active GST is able to conjugate GSH to 1,2 chlorodinitrobenzene (CDNB) resulting in a measurable colorimetric signal, correlating with the activity of the GST-tagged protein. In detail, a defined amount of protein, in this case 10µg, was added to 5µl of 75 mM glutathione solution mixed with 5µl of 30 mM CDNB solution within 215 µl potassium phosphate buffer. The samples were applied as triplicates in a 96 well multititerplate and read out with the Tecan spectrophotometer at 370 nm wavelength.

2.2.17 SDS-PAGE

A SDS-Page for the size dependent separation of proteins was performed, using the protocol described by Schägger and Jagow [Schägger et al., 1987] in a standard SDS-PAGE gel system

(CBS Scientific). Except if labeled different, the SDS-gels were always used with a 10% polyacrylamid concentration. Prior to loading, the samples were incubated in 2x sample buffer and denaturized for 15 min at 95°C. The separation was performed at a maximum voltage of 100 V. The following staining was always performed with a 20 min Coomassie blue bath followed by a stepwise discoloration using decolorizing-solution.

2.2.18 Western Blot analyzes

Whenever a higher sensitivity was necessary to visualize bands, a Western Blot analyzes was performed instead of the Coomassie staining after the SDS-PAGE. Therefore the separated proteins were transferred to an activated PVDF membrane in a semi-dry transfer cell for 30 minutes at 25 V. To prevent unspecific binding of proteins to the membrane it was blocked 1h in 5% non fat dry milk in PBS-T before the primary antibody was applied for at least 1h in an appropriate dilution. After washing the membrane three times in PBS-T for 5 minutes, the secondary antibody was incubated on the membrane, again for at least 1h. One last washing step in PBS-T (3x5 min) was performed before the blots were developed using ECL-Reagent and light sensitive films.

3 Results

3.1 Small scale expression and optimization of expression

In this part of the project, expression plasmids from our cooperation partner at the DKFZ, Heidelberg were used for the expression of PFV TM protein. The used vector pGEX4T3 fuses the TM protein sequence N-terminal to GST allowing efficient purification for soluble proteins. Furthermore those constructs were attached to a C-terminal Simian Virus 40 (SV40)-tag for detection of full length proteins using sera from infected primates. Sequencing revealed that the obtained constructs contained two silent mutations not altering the amino acid sequence.

3.1.1 Expression of PFV TM protein and test of solubility

The plasmid DNA was used for transformation in competent expression strains and spread on agar plates. Transformants were then used for a small scale test expression. The test expression was performed under the standard conditions (37°C, 1 mM IPTG, 3h) in LB-Medium using the Rosetta 2 bacterial strain. Additionally the solubility was checked directly by separating insoluble pellet and supernatant by centrifugation (Figure 5).

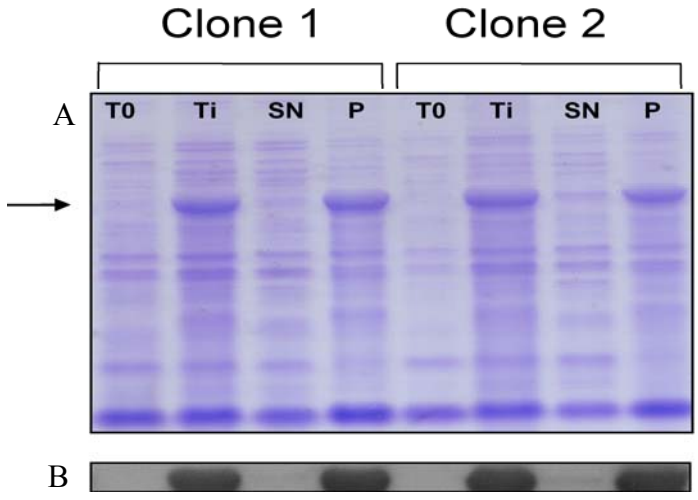
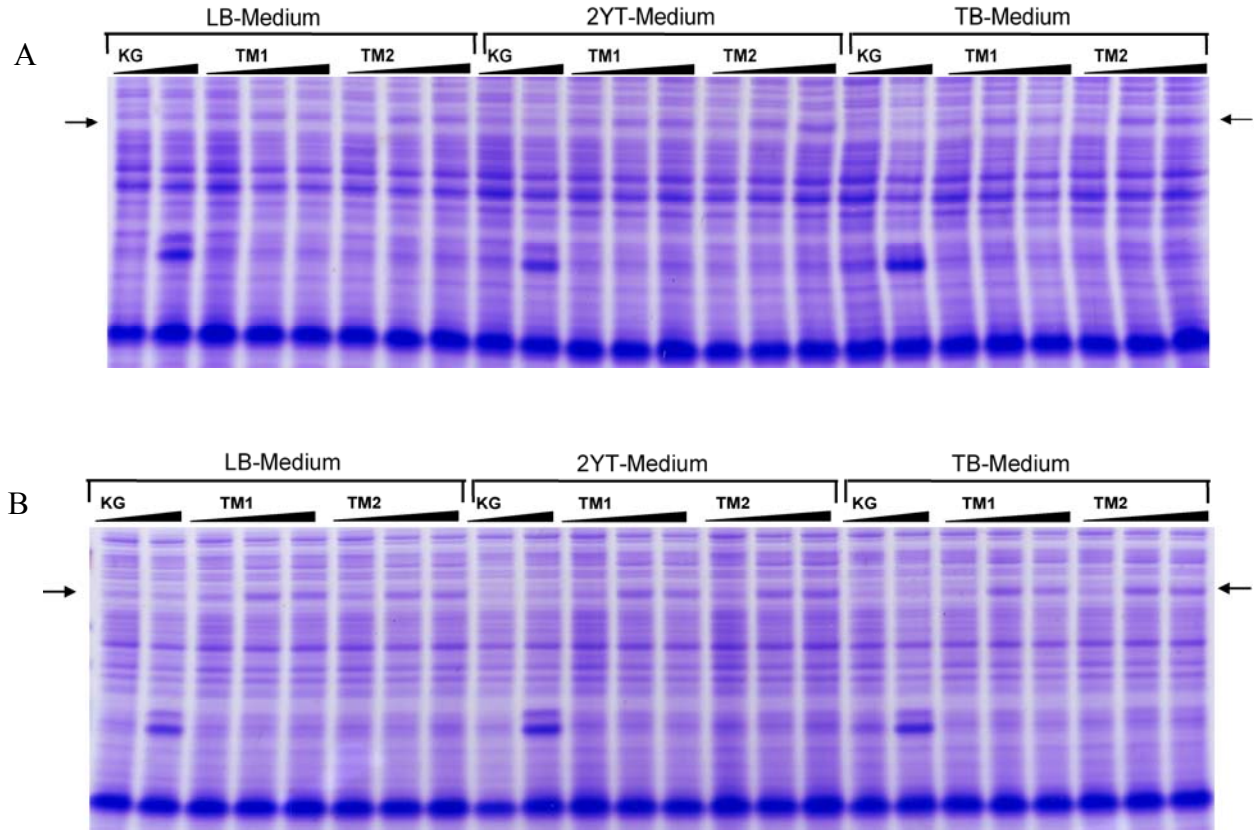


Figure 5: Coomassie stained SDS-gel (A) and corresponding WB (B), showing the expression and solubility of PFV TM protein. Although expression levels were high, visible in the lanes "T0" prior induction and "Ti" after induction of the expression, the whole amount of expressed protein was present as part of the insoluble pellet (P) and no protein remained in the supernatant (SN) under the standard conditions. No differences between the two picked clones "clone 1" and "clone 2" were observed.

The test expression indicated that PFV TM can be expressed in large amounts, but not as soluble protein. Therefore an expression optimization screen was investigated to improve the solubility of the TM protein under certain expression conditions.

3.1.2 Expression optimization

An expression optimization screen was performed using the four bacterial strains C3030H, C43, SCS1 and Rosetta 2 each grown in media containing appropriate antibiotics. The media chosen for the optimization were LB-, 2YT- and TB-Medium (Figure 4). The empty vector pGEX KG, acting as control, as well as three IPTG concentrations were used (0 mM; 0,1 mM; 1 mM). The screening was performed in two 96 deep-well-plates, one incubated at 16°C overnight, the other one at 25°C for 3 h after induction (Figure 6).



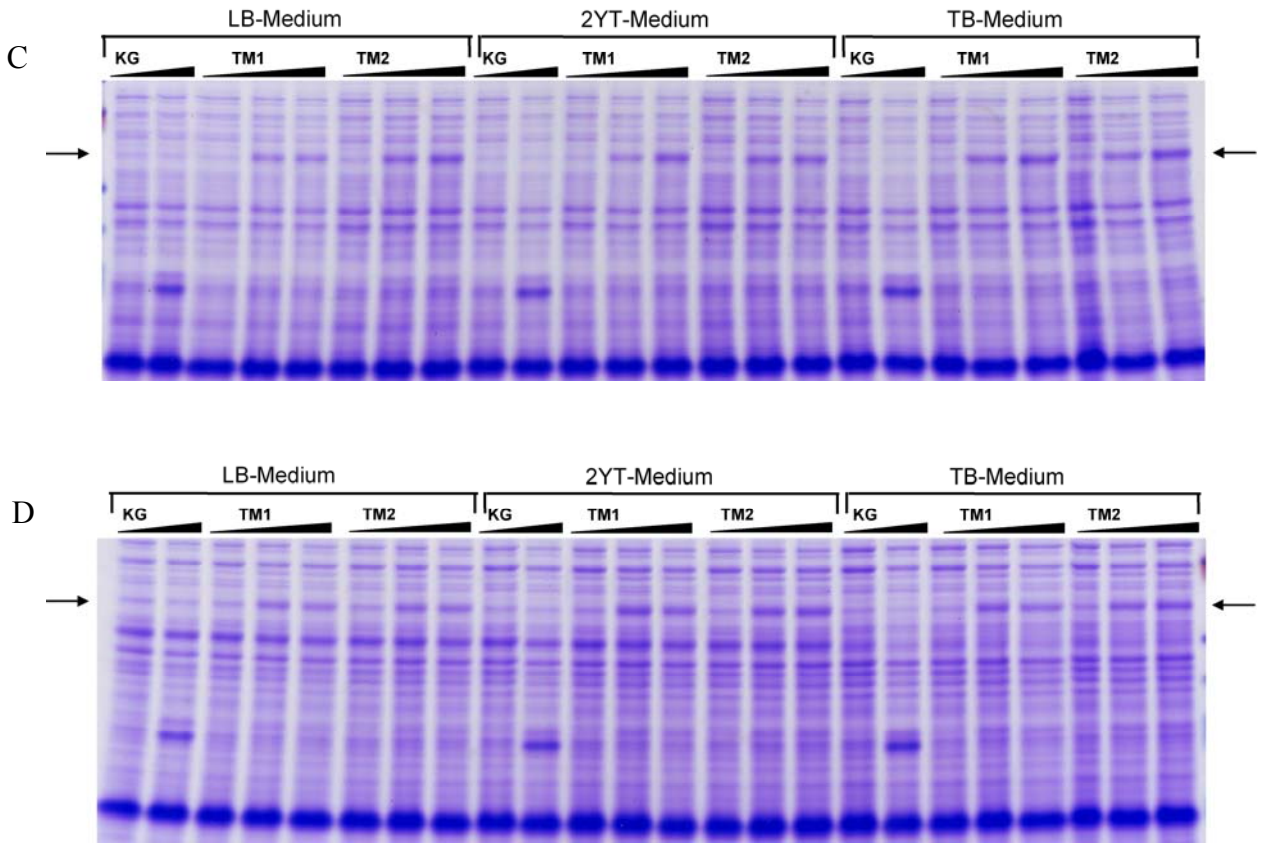


Figure 6: Coomassie stained SDS-gel of the expression optimization screen, showing the expression of the TM protein by the C43 strain (A), the C3030H strain (B), the Rosetta 2 strain (C) and the SCS1 strain (D) at 25°C in the three different media used. The empty vector pGEX KG (lane "KG") clearly expresses GST after induction (first lane KG is non induced, second is induced with 1mM IPTG). The three lanes of each transformant (TM1 and TM2) represents the rising IPTG concentration from 0mM (first lane) over 0,1mM (second lane) to 1mM (third lane). The expressed protein of interest is 68kDa, marked by the arrows.

The expression optimization screen, used to optimize the expression condition for soluble amount of PFV TM protein showed that the empty vector (KG) expresses GST after induction and the two transformants (TM1 and TM2) both express the TM protein at only slightly different levels. Furthermore the expression levels are comparable between the three selected media. Differences between 16°C and 25°C expression temperature were not detected. The C43 strain did not show any expression at all, while the C3030H strain showed a moderate expression. In the case of the Rosetta 2 and SCS1 strain high expression levels were observed, especially when induced with 1 mM IPTG.

3.1.3 Solubility test

Again it was necessary to investigate the solubility of the expressed protein under the optimized conditions. To facilitate subsequent purification, the protein needed to be both, highly expressed and soluble. Thus, only the two best expressing strains, SCS1 and Rosetta 2, each grown in TB-Medium were centrifuged to separate the supernatant and the pellet and both were loaded on a SDS-gel afterwards (Figure 7).

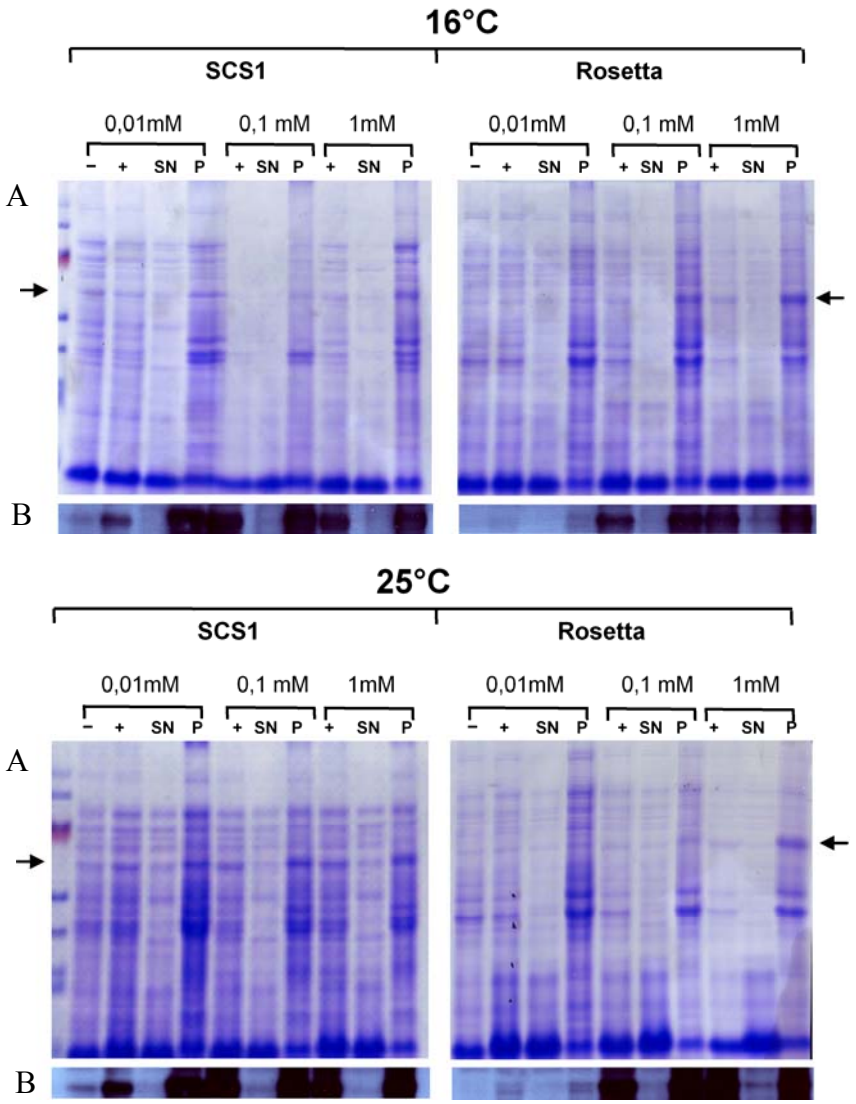


Figure 7: SDS-gel picture (A) and the corresponding WB (B) showing the induction of protein expression at 16°C and 25°C in the two strains with the best expression rates observed, SCS1 and Rosetta 2. While the first lane (-) is not induced, the following are induced (+) with the labeled IPTG concentration above (0,01mM, 0,1mM, 1mM). The supernatant (SN) and the pellet (P) were separated to determine the soluble amounts of protein.

The solubility test, which was performed to determine the solubility of the expressed protein under previously optimized conditions, demonstrates that even under mildest expression conditions the TM protein of PFV TM is predominantly expressed as insoluble protein, independent of the temperature, the bacterial strain or the IPTG concentration used.

3.1.4 Resolubilization and purification

Since almost the whole amount of the expressed protein was in the insoluble fraction, a method for resolubilization should be investigated. For that reason, the insoluble pellet was resuspended and incubated in lysis-buffer containing sarkosyl (SY) detergent in two different concentrations. After 3h in 1% SY and 12h in 0,01% SY the protein was purified, using a GSH bead-solution as batch-process. More precisely, the solution containing the protein was centrifuged after the incubation with SY, the supernatant was diluted to a final concentration of 0,1% SY and 100 µL of the GSH matrix-solution were added, which were able to bind up to approximately 800 µg of recombinant protein. After 1h of incubation the tubes were centrifuged again, the supernatant was stored while the matrix was washed three times with PBS, before a SDS gel was loaded, with the insoluble pellet prior resolubilization (IP), the unbound fraction after the incubation with the GSH affinity-matrix (Flow through, FT) and the resolubilized and purified pellet fraction (E) (Figure 8).

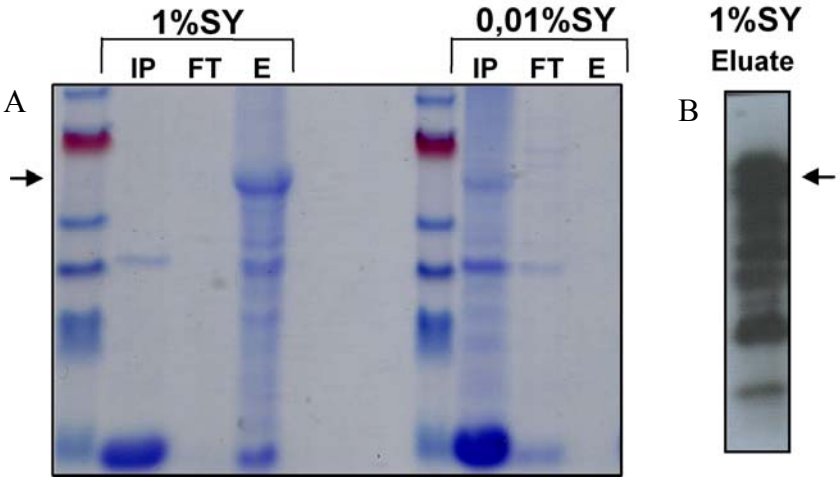


Figure 8: SDS-gel (A) and its corresponding WB (B) of the resolubilization experiment by using sarkosyl. The resolubilization of the expressed PFV TM protein with 1% Sarkosyl from the insoluble fraction (IP) was possible, while 0,01% sarkosyl did not lead to resolubilization of the protein. The corresponding WB on the right indicates that all bands visible in the elution fraction (E) can be detected with a GST antibody.

Using 1% SY within the lysis-buffer, it was possible to redissolve large amounts of expressed protein, whereas lower concentrations of detergent did not show a resolubilization effect. However, although resolubilisation was efficient, several proteins were co-purified with the recombinant protein, which were also detected by the anti-GST antibody in the Western Blot on the right. That led to the conclusion that the purified protein contains C-terminal degradation products probably caused by protein instability or smaller target proteins due to premature termination. For further immunization studies it was preferred to obtain the protein with a higher purity, for clear antibody response. Therefore a new strategy needed to be investigated.

3.2 Expression and purification of PFV using C-terminal His-tags

After there was no possibility to go on with the first strategy, using the PFV TM plasmid constructs from Prof. M. Löchelt, DKFZ, Heidelberg, a second strategy was investigated. Hereby it was necessary to get the sequence of the TM protein out of the PFV plasmid. To solve the problem of C-terminal degradation products, this second strategy involved the introduction of a C-terminal His-tag, with the advantage to purify theoretically only full-length protein and permit the use of denaturing conditions for insoluble proteins.

3.2.1 Cloning strategy

To amplify the targeted sequence from the PFV plasmid, two different primer sets (Figure 9) were designed, both containing a C-terminal His-tag (Figure 10). One of these two sets also coded for a factor Xa cleavage site N-terminal to the TM protein. Both primer sets were used for the amplification of the protein sequence. Afterwards the amplified inserts were fused with the empty vector pGEX-KG [Guan et al., 1991] containing the GST gene, a thrombin cleavage site and a lacIQ repressor coded on the same plasmid (Figure 11). The reverse primer was provided with an 8x His-tag and an *XhoI* cleavage site. To eliminate the GST- and further amino acid residues leading to use only the ectodomain for immunization, either the thrombin site or the factor Xa site could be digested. The factor Xa cleavage site promised an improved cleavage and therefore was introduced additionally to the thrombin site.

```
                    D  I  N  D  E  N  L
HFV-ED fwd:    5′  a  GAT ATT AAT GAT GAA AAC TTA  3′

                 G  S  G  S  G  S  G  I  E  G  R  D  I  N  D  E  N  L
HFV-ED_XA:     5′ a  gga tct ggc tct gga tct ggt atc gag gga agg GAT ATT AAT GAT GAA AAC TTA  3′

                    XhoI    -  H  H  H  H  H  H  H  H  P  K  L  Y  G  L  L
HFV-ED rev:    5′ ATACTCGAG  tta gtg atg gtg atg gtg atg gtg gtg  AGG CTT TAA GTA TCC CAA GAG 3′
```

Figure 9: The starting sequence of the forward primer is indicated (DINDENL), the PFV-ED_XA primer set also provided a Xa cleavage site prior to the starting sequence. The reverse primer provided a C-terminal 8x His-tag and an *XhoI* cleavage site

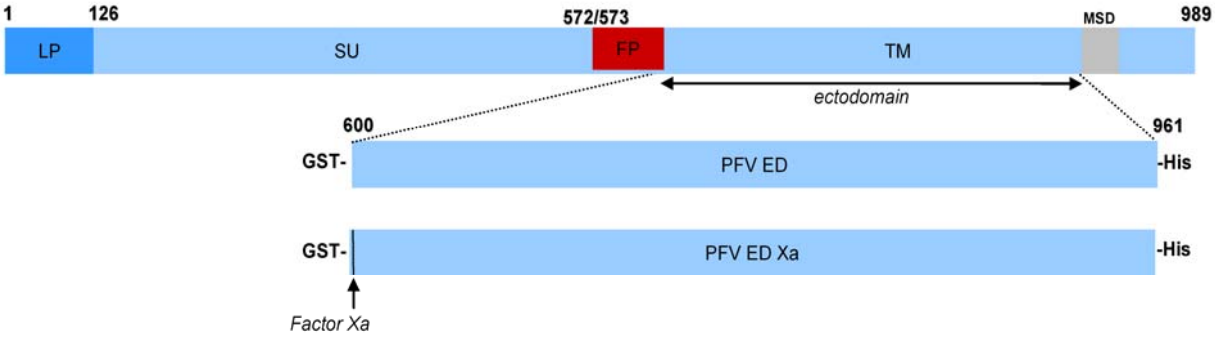


Figure 10: PFV TM envelope protein and two constructs containing the ectodomain. The whole envelope protein contains the leader peptide (LP), the surface protein (SU), the TM protein with the fusion peptide (FP) and the membrane spanning domain (MSD). Only the ectodomain was amplified by the two primer sets.

The inserts PFV ED and PFV ED Xa (Figure 10) were inserted in the *SmaI* /*XhoI* sites of the empty vector pGEX KG (Figure 11).

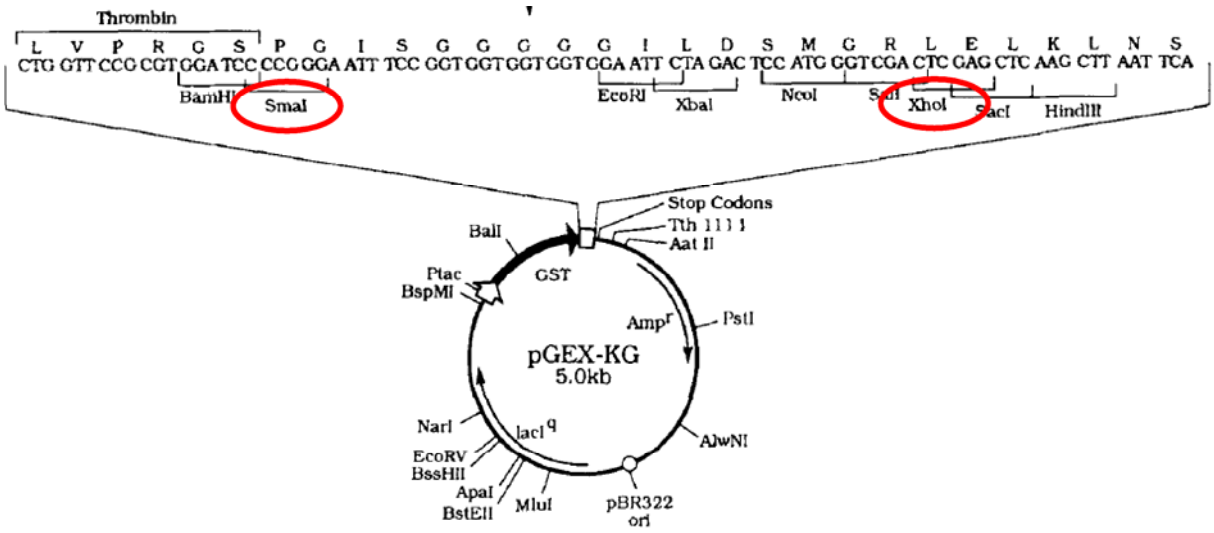


Figure 11: Vector pGEX KG. The restriction sites used for ligation are marked

3.2.2 PCR Optimization

Because of low amplification with initial PCR conditions, it was necessary to optimize the reaction. Therefore the temperature during the annealing step of the PCR and the $MgSO_4$ concentration were adjusted. A temperature gradient was used combined with rising concentration of $MgSO_4$, showing that 5 mM $MgSO_4$ and 42°C annealing temperature (AT) were the optimum for the amplification (Figure 12).

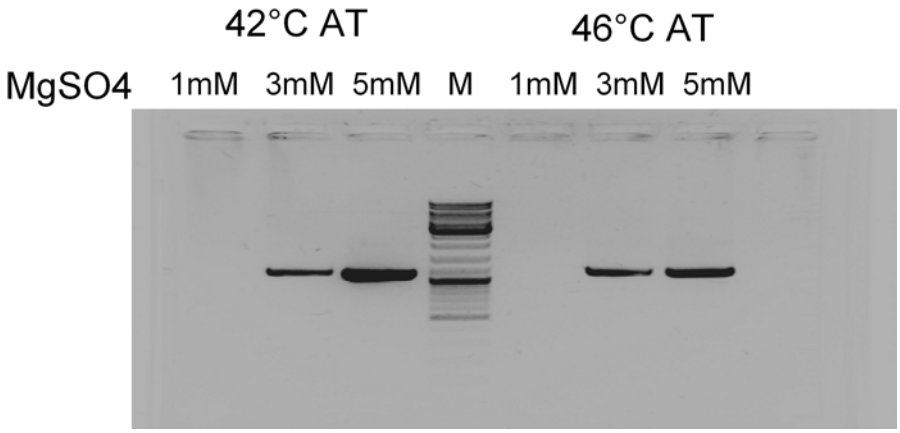


Figure 12: Optimization of PCR using different $MgSO_4$ concentrations within a temperature gradient program showing that 5mM $MgSO_4$ at 42°C annealing temperature (AT) was leading to the best amplification of the sequence of the PFV TM protein inserts.

3.2.3 Investigation of transformants

Due to the optimized PCR amplification, it was possible to get a good amount of PCR amplicons for subsequent steps. As described earlier, the inserts were digested with *XhoI*, purified out of a 1% agarose gel and ligated to the *SmaI* /*XhoI* digested empty vector with T4-ligase. The vector itself was therefore also purified via a gel purification kit prior usage (Figure 13). After the transformation of ligated plasmids into chemical competent bacteria, cells were spread out on agar plates. At least 12 h later a colony-PCR was be performed for all grown transformants, checking the presence of the insert (Figure 14).

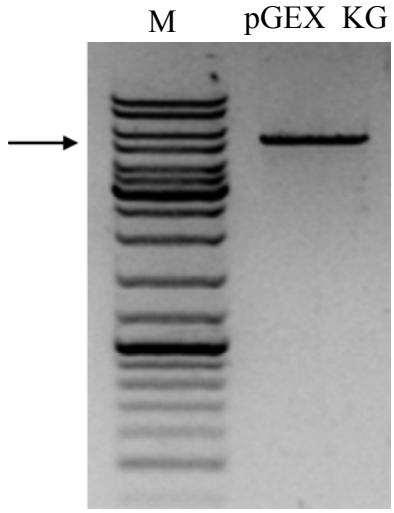


Figure 13: Empty vector (pGEX KG) after digestion with *XhoI* /*SmaI* enzymes and purification using a 1% agarose gel, showing one band at 5000bp (arrow on the left)

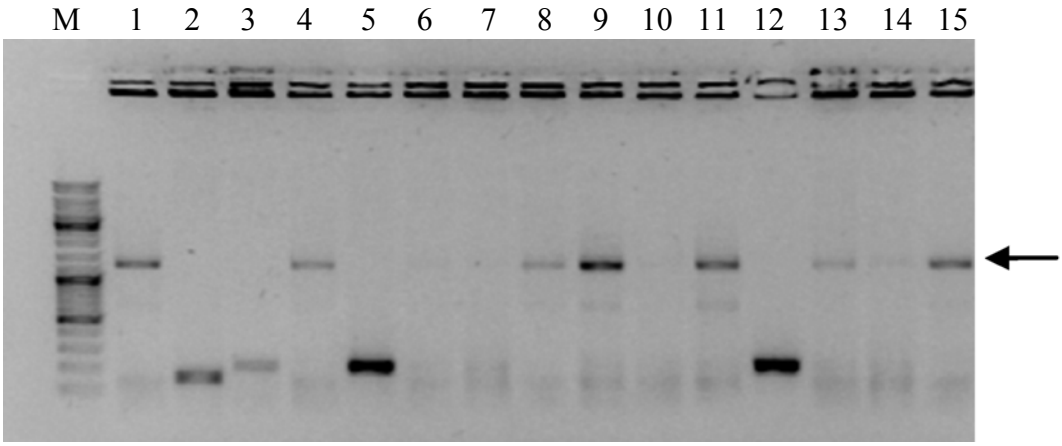


Figure 14: Agarose gel picture of a representative Colony-PCR showing inserts coding for PFV ED at 1200 bp (marked by the arrow on the right)

Positive clones (Nr. 1, 4, 8, 9, 11, 13 and 15) were picked for plasmid preparation and for fluorescence based cycle sequencing. The other transformants were expected to be negative (for instance Nr. 6 and 7) or to have relegated without taking up the insert (for instance Nr. 5 and 12). An alignment of a translated sequence was performed, confirming that it was identical to the published sequence of the PFV ectodomain (Figure 15). Based on these positive results, the plasmid DNA was transformed in expression strains for a large scale expression under the previously optimized conditions for PFV TM.

Alignment 1 against P14351

Score	1894	**E-value**	
Identity	100.0%	**Positives**	
Query length	1091	**Match length**	
Position	**P14351** matches from 600 to 961 (362AA), in the query sequence from 2 to 1087 (1086AA)		
Graphical			

```
2     DINDENLQQGIYLLRDHVITLMEATLHDISVMEGMFAVQHLHTHLNHLKTMLLERRIDWTYMSSTWLQQQ   71 Query
      DINDENLQQGIYLLRDHVITLMEATLHDISVMEGMFAVQHLHTHLNHLKTMLLERRIDWTYMSSTWLQQQ
600   DINDENLQQGIYLLRDHVITLMEATLHDISVMEGMFAVQHLHTHLNHLKTMLLERRIDWTYMSSTWLQQQ  669 P14351

72    LQKSDDEMKVIKRIARSLVYYYVKQTHSSPTATAWEIGLYYELVIPKHIYLNNWNVVNIGHLVKSAGQLTH  141 Query
      LQKSDDEMKVIKRIARSLVYYYVKQTHSSPTATAWEIGLYYELVIPKHIYLNNWNVVNIGHLVKSAGQLTH
670   LQKSDDEMKVIKRIARSLVYYYVKQTHSSPTATAWEIGLYYELVIPKHIYLNNWNVVNIGHLVKSAGQLTH  739 P14351

142   VTIAHPYEIINKECVETIYLHLEDCTRQDYVICDVVKIVQPCGNSSDTSDCPVWAEAVKEPFVQVNPLKN  211 Query
      VTIAHPYEIINKECVETIYLHLEDCTRQDYVICDVVKIVQPCGNSSDTSDCPVWAEAVKEPFVQVNPLKN
740   VTIAHPYEIINKECVETIYLHLEDCTRQDYVICDVVKIVQPCGNSSDTSDCPVWAEAVKEPFVQVNPLKN  809 P14351

212   GSYLVLASSTDCQIPPYVPSIVTVNETTSCFGLDFKRPLVAEERLSFEPRLPNLQLRLPHLVGIIAKIKG  281 Query
      GSYLVLASSTDCQIPPYVPSIVTVNETTSCFGLDFKRPLVAEERLSFEPRLPNLQLRLPHLVGIIAKIKG
810   GSYLVLASSTDCQIPPYVPSIVTVNETTSCFGLDFKRPLVAEERLSFEPRLPNLQLRLPHLVGIIAKIKG  879 P14351

282   IKIEVTSSGESIKEQIERAKAELLRLDIHEGDTPAWIQQLAAATKDVWPAAASALQGIGNFLSGTAQGIF  351 Query
      IKIEVTSSGESIKEQIERAKAELLRLDIHEGDTPAWIQQLAAATKDVWPAAASALQGIGNFLSGTAQGIF
880   IKIEVTSSGESIKEQIERAKAELLRLDIHEGDTPAWIQQLAAATKDVWPAAASALQGIGNFLSGTAQGIF  949 P14351

352   GTAFSLLGYLKP   363 Query
      GTAFSLLGYLKP
950   GTAFSLLGYLKP   961 P14351
```

Figure 15: Alignment of a representative PFV ED-Xa clone (Alignment 1) and the PFV envelope protein (accession number: P14351), showing complete homology in the TM region (alignment was created using blastx online database)

3.2.4 Purification using an ÄKTA FPLC equipment

For purification of the protein, the cells were pelleted and resuspended in lysis-buffer. Because of the previously obtained results that the protein was not soluble, 1% sarkosyl was directly used for extraction of insoluble pellets by incubation with the detergent for at least 1h. Prior to purification, the solution was diluted to a final sarkosyl concentration of 0,1%. Then the purification was performed as described in 2.2.14 by using NiNTA columns for the binding of His-tagged proteins. The estimated flow rate was 12 mg/L. The purity was determined by a SDS-PAGE of the eluted fraction (Figure 16).

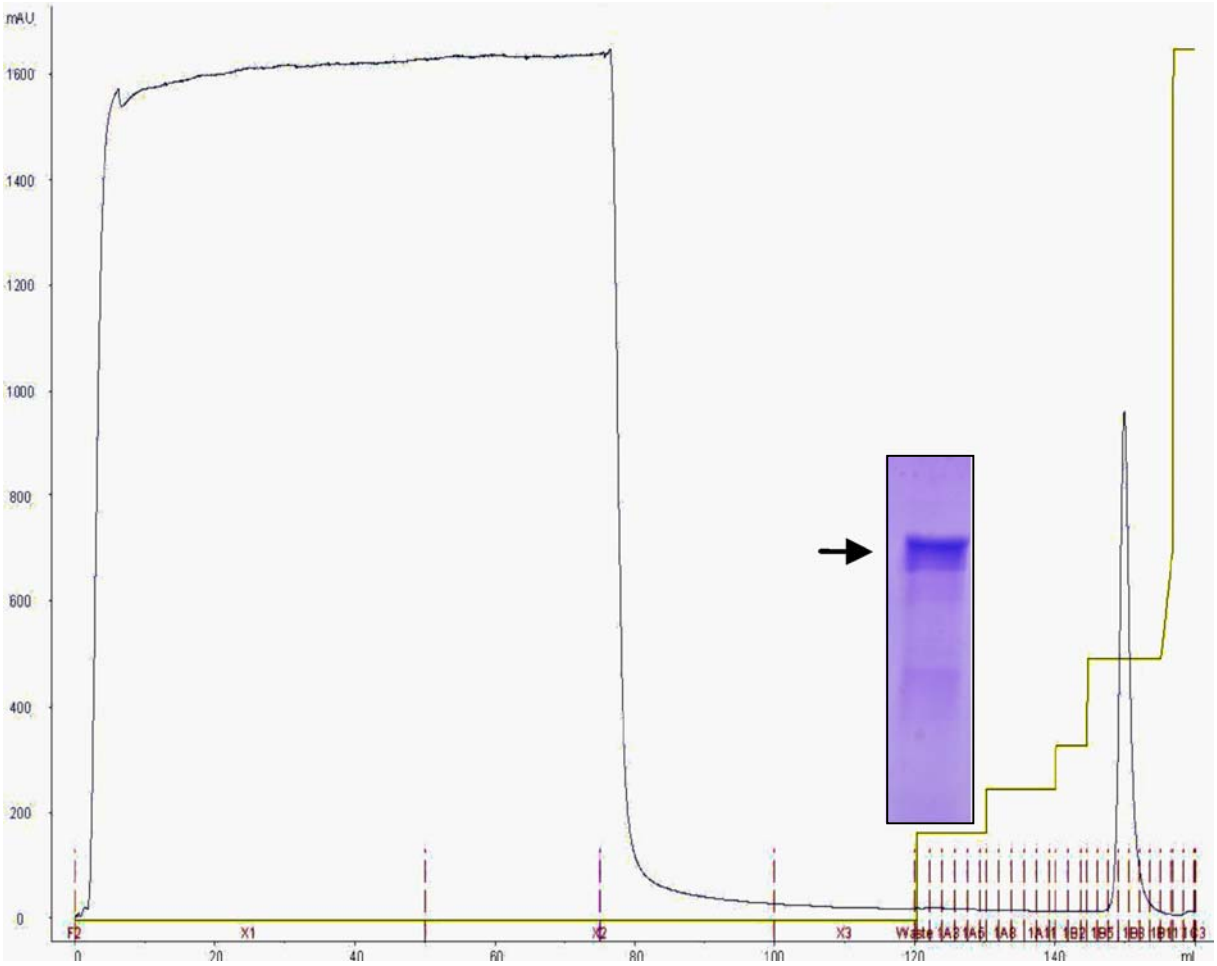


Figure 16: Äkta profile of the protein purification under denaturing conditions, showing the loading of the NiNTA column with the extracted protein solution (x = 0-80), washing (x = 80-120) and elution of the recombinant protein afterwards with an elution buffer containing imidazole (x= 120-160). The visible peak represents the purified protein (~1,2 mg) out of a 100 mL pellet fraction, leading to an estimated expression rate of 12 mg/L. Additionally the SDS-gel picture is inserted, indicating a purity of the protein above 90%, marked by the arrow.

Although it was shown before that there is almost no soluble protein present in the supernatant, a non-denaturing purification was performed using no detergents. As expected, the amount of purified protein was much less compared to the protocol described above (Figure 17). However, yields would be sufficient for immunization of rats, but the corresponding SDS-gel showed a less pure protein, indicating that the purification under non-denaturing conditions was much more complicated and less effective.

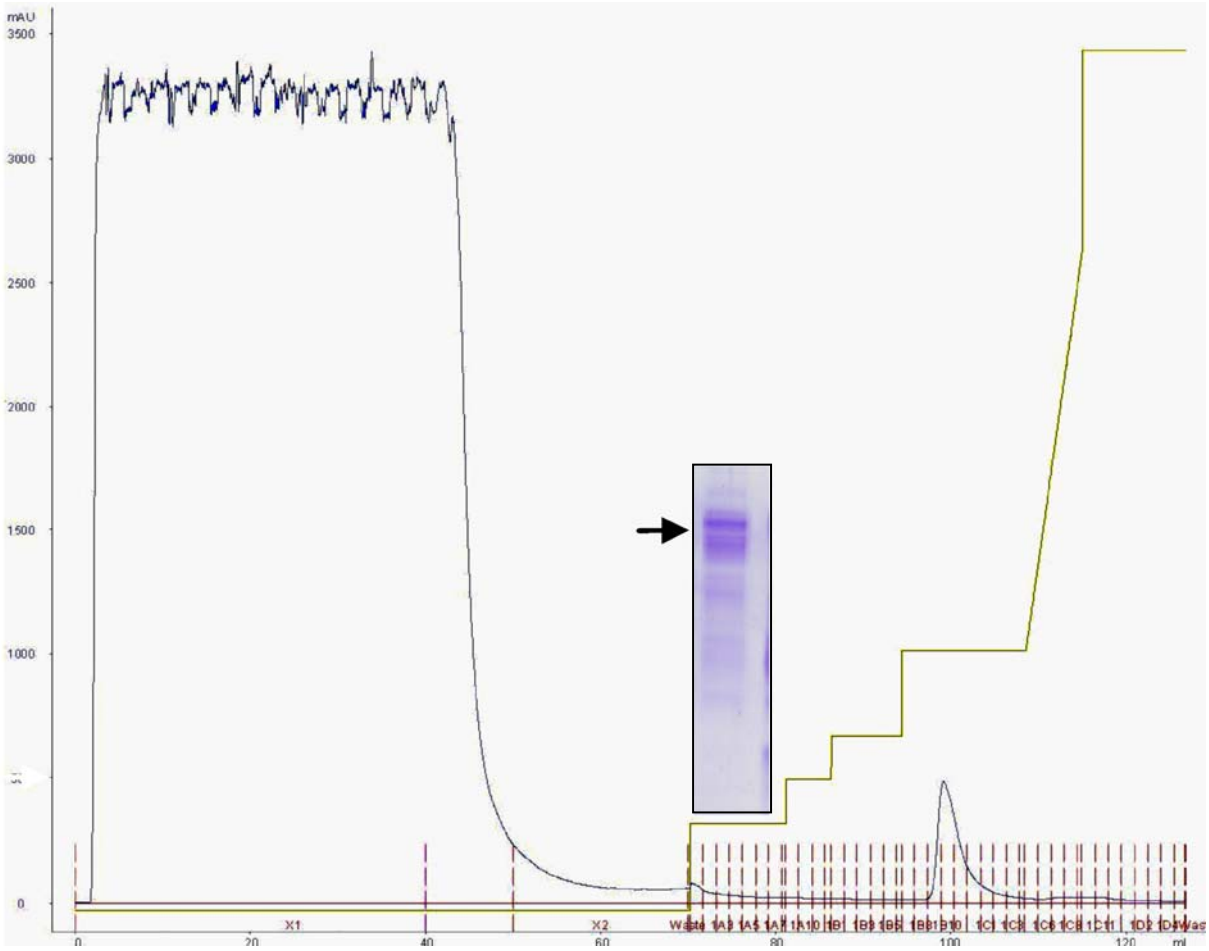


Figure 17: Äkta profile of purified protein under non-denaturing conditions, using no detergents. The elution fraction shows only a small amount of protein (~1mg) eluted from a 1 L pellet, Expression rate was calculated to 1mg/L. The inserted SDS-gel showed many unspecific bands or contamination products besides the targeted protein (marked by the arrow), indicating a low purity.

The purification of PFV TM protein with C-terminal His-tags was successful under denaturing conditions yielding large amounts of protein with a high purity (~90%), as necessary for further immunization studies. The non-denaturing purification did not result in high, pure amounts of protein but was used for further activity tests.

3.2.5 Activity test

Showing that purification without the use of detergents is possible, the question about the activity of the fusion protein was very interesting, since activity would indicate protein integrity. The activity test of both, non-denatured and denatured purified TM fusion protein showed that the deactivated protein after purification under denaturing conditions was completely inactive. In contrast, the non-denatured purified protein showed a weak activity, rising with time elapsing, whereas the activity of pure GST was as high as expected.

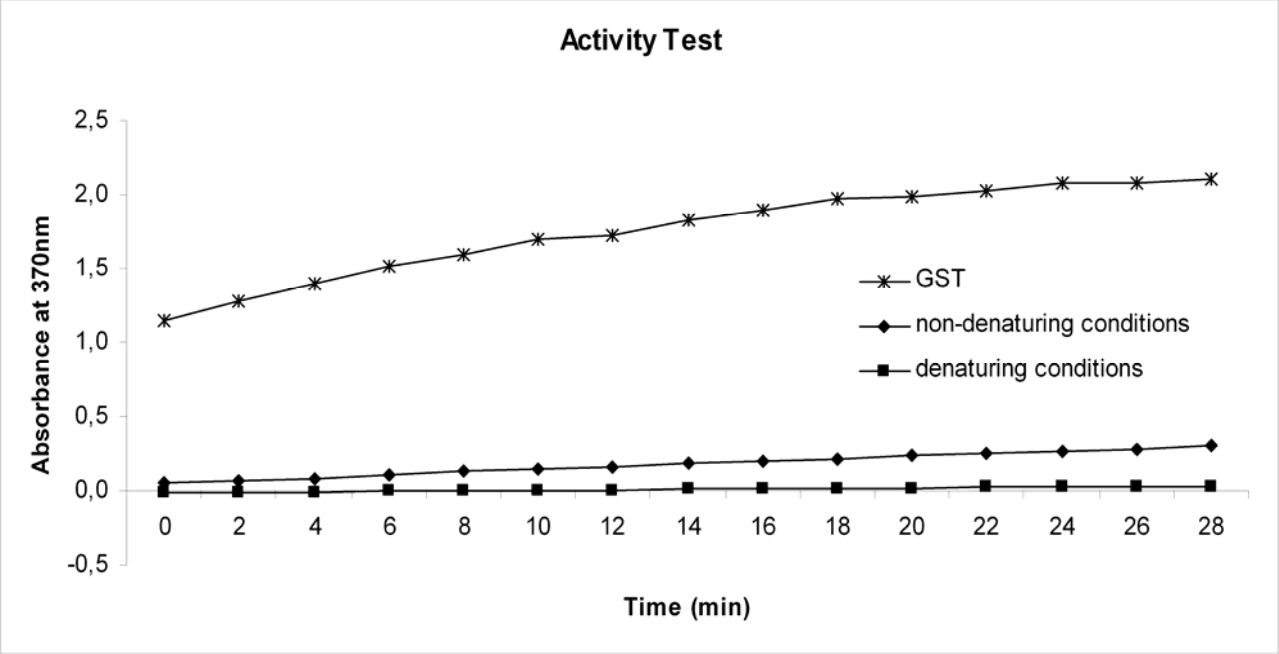


Figure 18: Activity test of purified protein under non-denaturing and denaturing conditions, indicating that non-denaturing purification leads to a slightly higher activity of the fusion protein, while GST shows a high activity.

Even though purification under non-denaturing conditions of the recombinant protein did not result in high amounts of pure protein it was possible to determine a weak activity of those proteins, possibly because of proper folding.

4 Discussion

4.1 Expression and purification of the TM protein of PFV

In the beginning of this project, efforts to purify of the TM protein of PFV were focused on expression constructs obtained from our cooperation partners. It is well known that proteins, especially transmembrane proteins containing several hydrophobic areas such as a fusion peptide (FP) or a membrane spanning domain (MSD) are difficult to express in E. coli and often insoluble. The binding of such proteins to highly soluble proteins such as GST is helpful to increase the chance of obtaining soluble fusion proteins by pulling the insoluble fusion partner "in solution". Due to specific and strong interaction to glutathione, GST additionally allows purification of the expressed protein, making it a common fusion tag for standard applications [Esposito et al., 2006]. As shown in Figure 7 the produced protein was well expressed but insoluble under standard conditions, although an N-terminal GST-tag was present. The insolubility might be due to the large size of the GST fusion protein (68 kDa), since larger proteins have been described to be harder to express for E. coli [Graslund et al., 2008]. Furthermore, the TM protein of PFV contains seven cysteines in its internal loop region which usually build disulfide bonds to assure proper folding and intramolecular stability of the protein. Since bacteria naturally contain a highly reducing environment in their cytoplasm, the natural folding process due to disulfide bonds is impaired, and expression of such proteins is often highly ineffective as well as accompanied with the formation of inclusion bodies. Strong interactions between the hydrophobic domains also promote the aggregation of the protein, while GST monomers itself tend to aggregate as dimers. To circumvent solubility problems, it would have been possible to use different fusion proteins like maltose binding protein (MBP) or calmodulin binding protein (CBP), but subcloning is time-consuming and purification protocols need to be re-established. A more convenient way to improve protein solubility is to decrease the temperature during expression to avoid misfolding of the protein due to high expression rates. This can also be achieved by decreasing amounts of IPTG, to induce the expression of the protein more gently. Additionally, the expression strain and the nutrient condition of media can be varied, leading to varying expression rates and possibly soluble protein.

4.2 Expression optimization

To address the influence of last four points mentioned above, expression was performed under different expression temperatures, IPTG concentrations, different media and expression strains in a small scale expression screen (Figure 6). Each of the selected bacterial strains was chosen because of unique properties advantageous for expression. C3030H is an engineered E. coli strain genetically mutated in two cytoplasmic reductases and overexpressing the disulfide bond isomerase DsbC, allowing the formation of disulfide bonds for an increased natural folding of the recombinant protein [Bessette et al., 1999]. C43, also used in the expression optimization, contains an uncharacterized mutation resulting in improved tolerance and expression of proteins with hydrophobic domains. The SCS1 and Rosetta 2 strain supply one to seven rarely expressed eukaryotic t-RNAs for the expression of eukaryotic genes in E coli to counteract expression problems caused by codon-usage. The SCS1 strain additionally produces the lacIQ repressor to inhibit leaky expression prior induction. According to these properties, the C43 strain was expected to show the best expression rates, however, the expression was not successful, suggesting that the predicted advantages are strongly protein dependent. C3030H showed moderate expression, but the activity of the fusion protein might be higher due to the more natural folding. A test to determine the GST activity thereof has not been performed yet. SCS1 and Rosetta 2 strains were shown to express the recombinant protein at the best levels, leading to the conclusion, that the containing t-RNAs were advantageous. Furthermore, slightly higher expression rates were obtained within 2YT and TB media, indicating that a high amount of nutrients is necessary for an optimal expression. However, although overall expression was high, all of the investigated parameters had no influence on the overall solubility of the produced protein and only minor quantities were detectable in the supernatant on the Western blot level (Figure 7).

4.3 Resolubilization and purification of PFV TM with glutathione affinity matrix

Since expression optimization did not lead to an improvement of overall protein solubility, it was tried to resolubilize the protein from the insoluble fraction by the use of detergents. For that purpose, the anionic detergent sarkosyl has been shown to be very efficient [Frankel et al., 1991]. However, since binding of GST to glutathion requires intact GST, concentrations have to be carefully selected. In literature, concentrations up to 0,3% of sarkosyl have been

used for purification of GST tagged proteins without interference. As can be seen in Figure 8, almost all of the pelleted protein could be resolubilized with 1% sarkosyl and only minor amounts remained in the insoluble fraction (IP) after extraction. In contrast, no effect was observed using only 0,01% sarkosyl. However, in line with what was described by others, in presence of 1% sarkosyl, protein integrity was obviously disrupted and no binding to the matrix occurred (data not shown). Thus, the extracted fraction containing detergent was diluted to a concentration below 0,3% allowing a theoretical refolding of the GST fusion protein due to the low concentration of the detergent. Indeed, under these conditions the recombinant protein bound to the affinity matrix and could be eluted afterwards. As visualized by the corresponding Coomassie stained SDS gel, however, several other proteins were co-purified by this means. Western blot analysis of the eluted fraction using an anti-GST antibody indicated that these breakdown products originate from the recombinant protein, probably caused by protein instability or premature termination. These findings underline that expression of PFV TM protein in E.coli seems to be quite complicated and not well tolerated by this host. Since purification using the N-terminal GST tag would always lead to co-purification of the C-terminal truncation products and the protein was highly insoluble, further efforts were concentrated on the construction of new expression constructs.

4.4 Expression and purification of PFV using C-terminal His-tags

As discussed above, purification under non-denaturing conditions was not feasible for PFV TM protein and the instability remained a major problem. As a result, a C-terminal His-tag was introduced to the amplified sequence of the TM protein, having the advantage of purifying only full length protein under either non-denaturing or denaturing conditions. Since N-terminal fusion partners like GST generally increase protein expression of recombinant proteins [Esposito et al., 2006] the N-terminal part of the protein was not altered. After successful optimization of PCR conditions, cloning and sequencing of PCR positive clones (Figure 12, Figure 14 and Figure 15, respectively) were used to generate constructs which could be used for expression and purification. In view of the fact that the addition of a small tag like an eight histidines His-tag used in this second strategy does generally not influence the overall biochemical properties of recombinant proteins, expression conditions and purification protocols previously optimized for PFV-TM could be used. High amounts of pure protein could be obtained by this way (Figure 16).

Due to the fact that supernatants from disrupted cells from large scale expressions were otherwise discarded, it was tested which amounts of purified protein under non-denaturing conditions could be isolated. As found in the initial experiments comprising PFV TM constructs, only small amounts with moderate purity were detectable (Figure 17). Interestingly, the non-denaturing purified PFV ED constructs showed in contrast to the sarkosyl-treated protein residual GST activity, suggesting an at least partly intact conformation of the GST fusion protein (Figure 18). It is impossible to say if differences in activity compared to purified GST may be caused by the purification process itself or a decreased activity of the GST fusion protein due to the PFV-ED fusion partner. If solubility of the protein can be maintained even after cleavage of the GST residue, it would be interesting to determine potential immunological differences between both proteins in the antibody response induced after immunization. Although bacterial expression lacking the often structure-important eukaryotic glycosylation patterns as well as the lipid environment of the viral membrane is far away from the native situation, it allows at least formation of thermodynamically strongly favored domains and epitopes - especially under non-denaturing conditions.

4.5 Summary and Outlook

Despite huge efforts in recent research to develop a vaccine against the human immunodeficiency virus (HIV-1), no convincing solution has been found. As outlined in the introduction, several attempts aim to induce broadly neutralizing antibodies against the envelope proteins. This work was intended to provide a recombinant protein for a vaccine strategy based on foamy viral vectors. To investigate the immunogenic properties of the primate foamy virus TM protein and subsequent replacement of recognized epitopes, milligram amounts of purified protein needed to be produced for immunization studies. After the systematic optimization of the expression conditions and the establishment of a purification strategy for insoluble GST-tagged proteins, only partly purified PFV TM protein could be obtained. Thus, new expression constructs containing a C-terminal His tag were generated. This strategy proved to be successful to express and purify the TM protein of the primate foamy virus in milligram amounts and a purity of above 90%. Additionally, first successful attempts of purification under non-denaturing conditions allowing the maintenance of the protein integrity were performed, opening an additional alternative to continue this project. As further steps, the purified protein will be digested on the thrombin or factor Xa site to eliminate the GST residue prior to immunization. Furthermore, the conditions for non-denaturing purification need to be optimized, to clarify whether it would be possible to purify larger amounts of active recombinant protein and even more exiting, if immunizations with differently purified proteins induce changes in the immunological response and particularly in the epitopes recognized. A long term goal will be the characterization of the antisera collected from immunized rats and to determine the epitopes recognized by the antibodies. If neutralizing antibodies can be induced by this means as seen for other viruses, the final goal of this project would be the exchange of PFV epitopes against HIV-1 domains for further immunization studies.

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