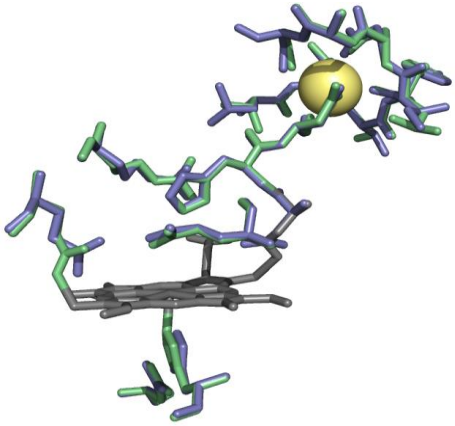
University of Natural Resources and Life Sciences, Vienna

Department of Chemistry, Division of Biochemistry

Metalloprotein Research Group

Cloning, expression and purification of human peroxidasin 1 constructs

Diploma Thesis



submitted by

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ABSTRACT

The peroxidase-cyclooxygenase superfamily is one of two heme peroxidase superfamilies, which are involved in the innate immune system, in thyroid hormone biosynthesis and in modification of extracellular matrix. The human peroxidasin 1 represents the subfamily 2, called peroxidasins, of the peroxidase-cyclooxygenase superfamily and is closely related to mammalian peroxidases. They are glycosylated multidomain oxidoreductases containing a heme peroxidase domain. Furthermore, peroxidasins comprise leucine-rich repeat domains, C-like immunoglobulin domains and a von Willebrand factor C module, which are typical motifs for protein-protein interaction, including cell adhesion and pattern recognition. Peroxidasin was first detected in haemocytes of Drosophila, but was later found in mammalians as well. Recently it was found that Peroxidasin take part in the formation of sulfilimine bonds in the collagen IV network by hypohalous acids and physiological studies showed a role in extracellular matrix formation and consolidation at various developmental stages. The involvement of human peroxidasin 1 in antimicrobial defence has also been suggested.

Biochemical knowledge of this protein family is very poor. Therefore, in this diploma thesis, truncated constructs of human peroxidasin 1 were generated for transient expression, each of them comprising different domains of the full length peroxidasin. In order to probe the role how the individual domains affect protein expression and enzyme activity different biophysical methods like UV-Vis spectroscopy, enhanced chemiluminescence, TMB assay, ABTS assay and halogenation assay were used.

For future research these different variants can be useful for protein-protein interaction studies and protein crystallization to resolve the three dimensional structure of peroxidasin.

ZUSAMMENFASSUNG

Die Peroxidase-Cyclooxygenase Superfamilie ist eine von zwei Häm Peroxidase Superfamilien, welche in der angeborenen Immunabwehr, in der Biosynthese der Schilddrüsenhormone und in der Bildung und Modifizierung der extrazellulären Matrix eine Rolle spielt. Das humane Peroxidasin 1 gehört zur Subfamilie 2, den so genannten Peroxidasinen, der Peroxidase-Cyclooxygenase Superfamilie und ist nah verwandt zu den Säugetier-Peroxidasen. Peroxidasine sind glykosylierte Multidomän-Oxidoreduktasen und besitzen eine Häm Peroxidase Domäne. Weiters bestehen Peroxidasine aus Leucin-reiche Wiederholungssequenzen, Immunglobulin C-ähnliche Domänen und einem von Willebrandfaktor C, welche typische Motive für Protein-Protein Interaktionen, inklusive Zelladhäsion und Mustererkennung, sind. Peroxidasin wurde erstmals in Hämozyten von Drosophila entdeckt, später wurde es auch in Säugetieren gefunden. Kürzlich wurde herausgefunden, dass es eine Rolle in der Bildung von Sulfilimin Bindungen im Kollagen IV Netzwerk durch Hypohalogeniten spielt. Physiologische Studien zeigen eine Rolle in der Bildung der extrazellulären Matrix und Festigung dieser während unterschiedlicher Entwicklungsstufen. Weiters wird die Beteiligung von humanem Peroxidasin 1 in der antimikrobiellen Verteidigung vorgeschlagen.

Aufgrund des bescheidenen biochemischen Wissens über diese Proteinfamilie wurden im Rahmen dieser Diplomarbeit, gekürzte Konstrukte des humanen Peroxidasin 1 für die transiente Expression hergestellt, die aus verschiedenen Domänen des vollständigen Peroxidasins bestanden. Mit den unterschiedlichen biophysikalischen Methoden, wie UV-Vis Spektroskopie, Enhanced Chemiluminescence, TMB Assay, ABTS Assay und Halogenierungsassay, konnte der Einfluss der einzelnen Domänen auf Proteinexpression und Enzymaktivität bestimmt werden.

Diese Konstrukte können für zukünftige Forschung hilfreich sein für Protein-Protein Interaktionsstudien und Proteinkristallisation, um die dreidimensionale Struktur des Peroxidasins aufzuklären.

1. INTRODUCTION

1.1. HEME PEROXIDASE SUPERFAMILIES AND FAMILIES

Heme peroxidases are oxidoreductases (1) and today, there exist two known main peroxidase superfamilies and three smaller families, which all contain heme *b* as prosthetic group (2). One superfamily comprises the peroxidase-cyclooxygenase superfamily and the second one includes the peroxidase-catalase superfamily. The three families consist of di-heme peroxidases, haloperoxidases (mostly Cl^--peroxidases) and dyp-type peroxidases (2).

Characteristic for peroxidase-cyclooxygenase superfamily is their involvement in the innate immune system, which includes peroxidases from mammals as well as from bacteria (2). The peroxidase-cyclooxygenase superfamily can be differentiated into seven subfamilies, including peroxicins, peroxidockerins and primordial peroxidases, mainly from bacteria and cyclooxygenases, peroxinectins, peroxidasins and chordata peroxidases from bacteria, fungi and animals (2). The first subfamily, the chordata peroxidases are represented by myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO), which are well separated from human peroxidasin 1 (hsPxd01) and human peroxidasin 2 (hsPxd02) belonging to the subfamily of peroxidasins (3).

The second heme peroxidase superfamily is the peroxidase-catalase superfamily, which are the most studied superfamily of heme peroxidases. A prominent representative is horseradish peroxidase (HRP). Currently, this superfamily is divided into three classes. Class I contains mainly cytochrome *c* peroxidases, ascorbate peroxidases and bacterial catalase-peroxidases. Class II comprises of fungal lignin and manganese peroxidases and Class III includes secretory plant peroxidases related to HRP (2).

The di-heme peroxidase family is found mainly in various bacteria and in a few archaeal members only. It is unique for containing two heme groups in one protein moiety. A prominent representative is the di-heme cytochrome *c* peroxidase, which is used for the research of intramolecular electron transfer (2).

The dyp-type heme peroxidase family was originally called “dye-decolorizing peroxidases” have the ability to degrade various synthetic dispersive dyes. They are only found among bacteria and fungi (2).

It was discovered that the haloperoxidase family is closely related to plants, although they are mainly found among fungi. Haloperoxidases exist with and without a heme group. Both variants are phylogenetically unrelated and form different gene families (2).

1.2. HUMAN PEROXIDASIN 1

There are human peroxidasins encoded in the human genome, which belong to subfamily 2 of the peroxidase-cyclooxygenase superfamily, namely human peroxidasin 1 (hsPxd01) and human peroxidasin 2 (hsPxd02) (3). Initially, these enzymes were designated as vascular peroxidase 1 (VPO1) and vascular peroxidase 2 (VPO2), because of their expression in the vascular system and heart (4).

Peroxidasin was detected in haemocytes of Drosophila in 1994 for the first time, where it participates in the formation of extracellular matrix (5). In 2000, human peroxidasin, which was then called melanoma gene 50 (*MG50*), was discovered for the first time in human colon cancer and later on in squamous lung carcinoma cells, in which *MG50* showed a 75 % homology to peroxidasin (6).

Recently, Bhave G. *et al.* have described the first demonstrable function of peroxidasin, which is the formation of covalent links in the collagen IV network and thus reinforcing its structure (7). Peroxidasin generates hyphalous acids (HOBr and HOCl) and halogenating intermediates, which catalyze the formation of a sulfilimine bond between a methionine sulphur and hydroxylysine nitrogen (7) (8). Thereby, the sulphur of a specific Met residue of the human collagen IV protomer is oxidized to a halosulfonium cation intermediate, which is then trapped by the hydroxylysine amine and the sulfulimine bond is formed (7). Further functions are extracellular matrix formation and consolidation at various developmental stages (3). The involvement of hsPxd01 in antimicrobial defence has also been suggested (4).

1.2.1. Structure of human Peroxidasin 1

The molecular mass of peroxidasin is about 165 kDa (4). It is a multidomain protein, as shown in Figure 1. The metalloprotein consists of a signal peptide (S) for extracellular secretion (3), five leucine-rich-repeats (LRR) with N-terminal and C-terminal capping motifs (3), four immunoglobulin C-domains (Ig), a heme-binding peroxidase domain (POX) and a von Willebrand factor type C domain (VWC) at the extreme C terminus (3) (4).

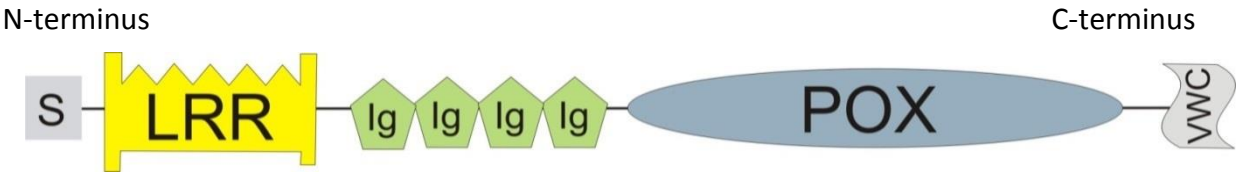


Figure 1: Domain structure of full length human peroxidasin 1

The peroxidase domain and the VWC are located in the C-terminal half of the protein whereas the signal peptide, LRR and immunoglobulin C domains are located in the N-terminal half (4). LRR, immunoglobulin C domains and VWC, the flanking domains of the peroxidase domain, play an important role for protein-protein interaction or cell adhesion (3).

The three dimensional structure of the individual domains (Figure 2-5) were modelled on proteins with high sequence homology and known structures (3). hsPxd01 is a highly N-glycosylated protein with 10 glycosylation sites in total (3).

Table 1: Glycosylation of hsPxd01

hsPxd01 structure	Glycosylation
Signal sequence	1 between POX and VWC
LRR regions	-
IgG regions	1 (N390)
POX	7
VWC	1

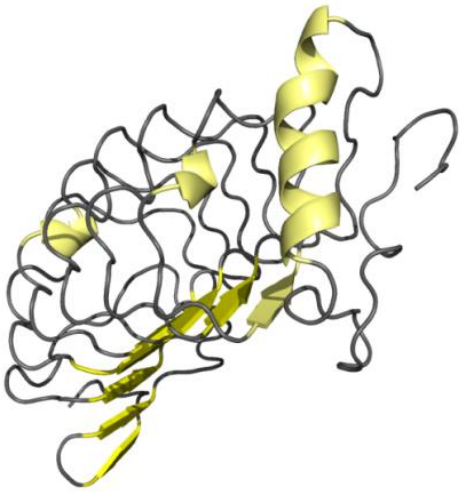


Figure 3: Leucine-rich repeat domains

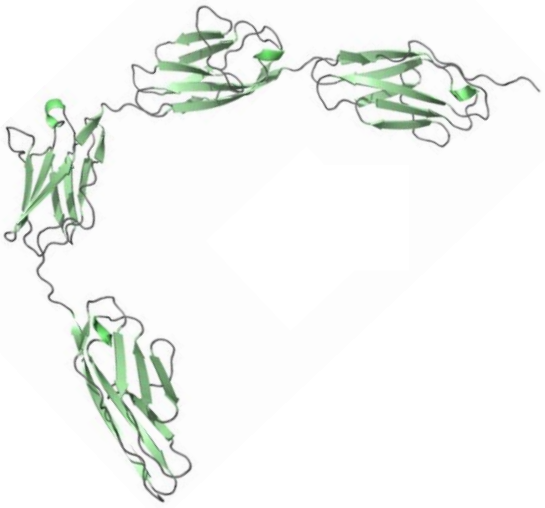


Figure 2: Immunoglobulin C-domains

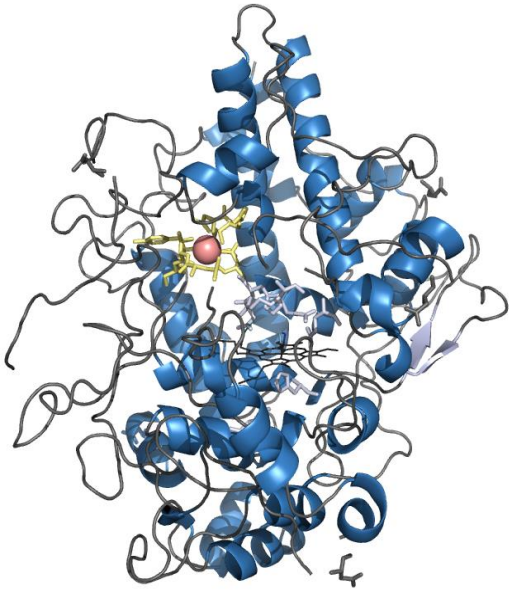


Figure 4: heme-binding peroxidase domain

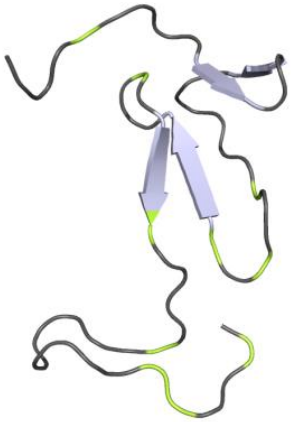


Figure 5: von Willebrand factor type C domain

The peroxidase domain of peroxidasin has high homology to the mammalian peroxidases, in particular to LPO (3) but also to MPO, EPO and TPO. In Figure 6, a multiple sequence alignment (Clustal X 1.81) of hsPxd01 (POX domain, amino acid residues 611-1314 omitting LRR, Ig domains and VWC), human MPO (hMPO) and goat LPO (gLPO) is shown.

The heme binding site and catalytically important amino acid residues are highly conserved. Another common trait of these animal peroxidases is a high-affinity calcium binding site, which is also highly conserved among them (5). This binding site plays an important role in

the stabilization of the distal heme cavity architecture and mediation of the assembly of the mature peroxidases (3). The sequence alignment would suggest that the accessibility of the heme prosthetic group is similar to LPO and other mammalian peroxidases. Our results though indicated that the catalytic site is somehow obstructed as will be discussed later.

Further highly conserved secondary structural features consist of two long helices that form a twisted helical hairpin structure, which forms the backbone of the protein's fold. This spine includes three additional well-conserved helices, which run across the faces of the heme, and forming with adjacent loop regions which contain the distal and proximal histidines and many of the hydrophobic residues that line the heme pocket (5).

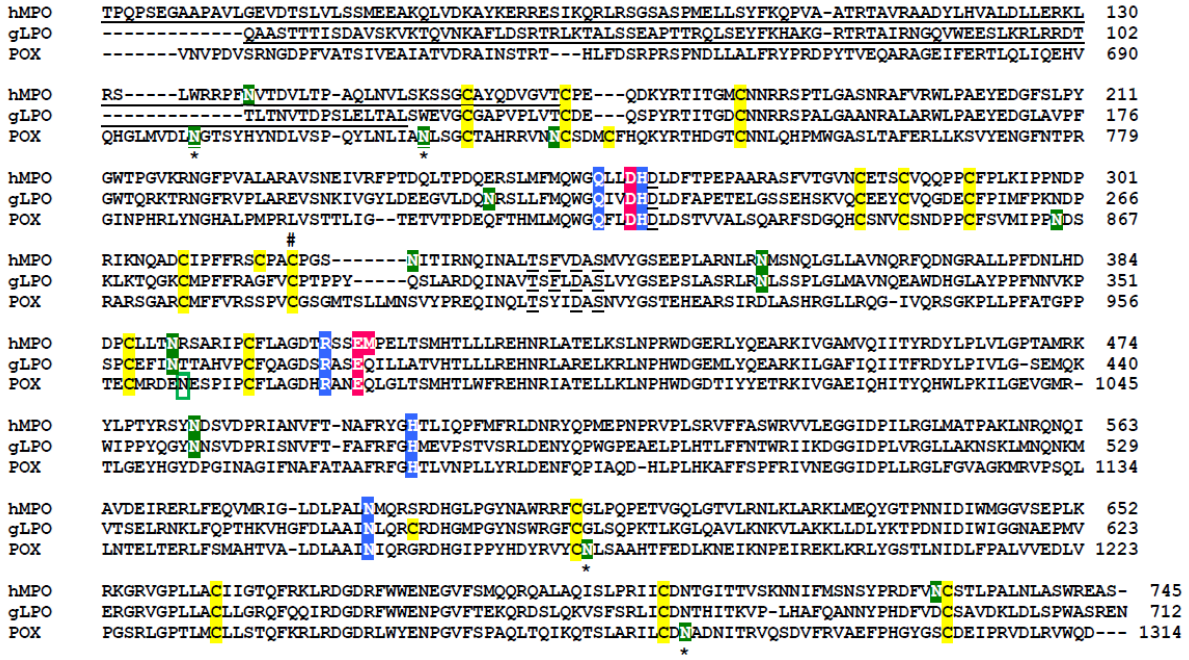


Figure 6: Multiple sequence alignment of human MPO, goat LPO and the peroxidase domain of hsPxd01. The signal peptides of hMPO and gLPO (41 and 26 amino acid residues, respectively) were omitted, the respective propeptides are underlined. Important catalytic residues are highlighted in blue, amino acid residues forming the covalent links with the prosthetic group are depicted in red. Cysteine residues are marked in yellow, the cysteine residue in hMPO responsible for dimer formation is marked by #. Calcium ion binding sites are underlined. Glycosylation sites are highlighted in green, partially glycosylated residues of POX are shown in green and marked with *. One putative not glycosylated site on POX is boxed in green.

1.2.2. The Heme prosthetic Group

The heme is located in the peroxidase domain (3) and as reported by Cheng G. *et al.* it is covalently bound to the protein (4). Mammalian peroxidases, like human peroxidasin I, MPO, LPO, EPO and TPO, contain a ferri-protoporphyrin IX derivative covalently linked via two

ester bonds to the protein. One conserved aspartate residue forms one ester bond and, a conserved glutamate forms the second one in all mammalian peroxidases as shown in Figure 6 (3). The prosthetic group has eight side chains: four methyl groups (positions 1, 3, 4 and 8), two vinyl groups (2 and 4) and two propionate groups (6 and 7). The carbon atoms of the methine bridges are commonly labelled α, β, γ and δ. The α-carbon is located between side-chain positions 2 and 3 (Figure 8). The iron atom is coordinated to the four pyrrole nitrogen atoms of the protoporphyrin as well as to the proximal histidine (9).

Figure 7 shows the heme environment of POX (purple residues) modeled on the structure of goat LPO (green), showing the covalent links to the heme. The heme prosthetic group can be seen in grey and the calcium binding site is shown in yellow.

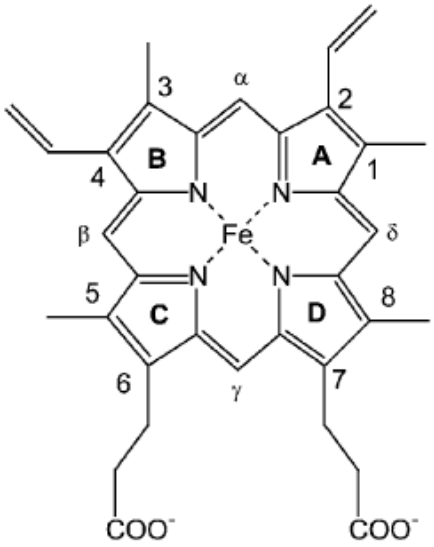


Figure 8: Heme structure

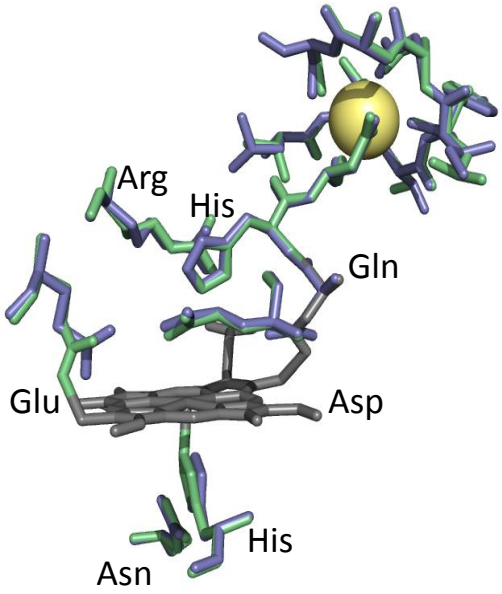


Figure 7: PDB code: 3QF1, 2.6 Å resolution; Active site of hsPxd01 modeled on gLPO (PyMol)

1.2.3. Reaction Mechanism

Human peroxidasin 1 shows peroxidase activity (one-electron oxidation reactions, e.g. tetramethylbenzidine oxidation) as well as halogenation activity (two-electron oxidation of halides). hsPxd01 is able to oxidize tyrosine, which can lead to the formation of dityrosine. The measured activities are very low compared to other mammalian peroxidases (3).

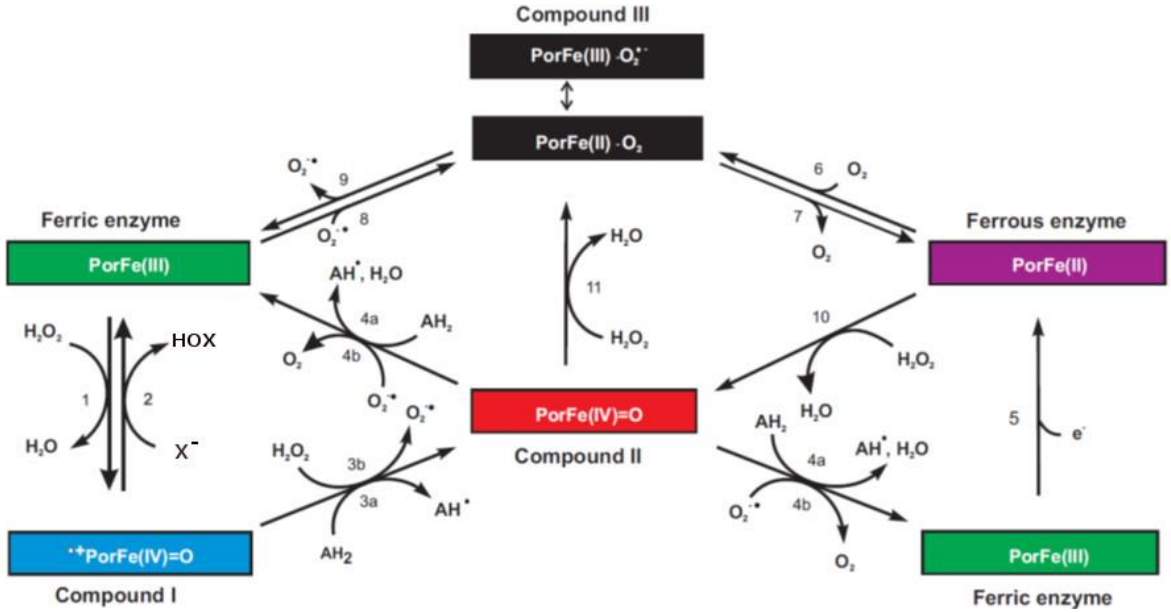


Figure 9: Reaction mechanism of peroxidases (10)

The reaction mechanism of peroxidases will be described below. Due to changes of oxidation- and spin status of heme proteins, spectrophotometrical observations of redox intermediates and their interconversion can be followed using steady-state and stopped-flow spectrophotometry (11).

Both peroxidase and halogenation-cycles start with the reaction of the Fe(III) heme of the native enzyme with hydrogen peroxide (H_2O_2) and the formation of *compound I* (reaction 1) (12) (10).

Reaction 1 $Fe(III) + H_2O_2 \rightarrow H_2O + compound\ I$

Reaction 3a $compound\ I + AH_2 \rightarrow compound\ II + {}^{\bullet}AH$

During heterolytic cleavage (reaction 3a) one oxygen of H_2O_2 is released as water and the second oxygen stays bound to the iron. Due to this, the iron-bound oxygen contains six valence electrons now and becomes therefore, a strong oxidizing agent. An oxoiron(IV) intermediate is formed by removing one electron from the iron. A second electron is removed from the porphyrin, which leads to the formation of a porphyrin π-cationic radical (2).

In conventional peroxidase *compound I* is formed within a few milliseconds and its half-life is about 100 ms only (23). It is converted to compound II (reaction 3a) in presence of an electron donor (2).

Reaction 2 $compound\ I + X \rightarrow Fe(III) + HOX$

Peroxidation cycle

The peroxidation cycle comprises two consecutive one electron reduction steps: *compound I* is reduced to *compound II* (reaction 3a), which is then reduced by a subsequent one electron reduction to the native enzyme creating Fe(III) (reaction 4a) (10).

Reaction 4a $compound\ II + AH_2 \rightarrow Fe(III) + {}^{\bullet}AH + H_2O$

Reaction in absence of exogenous electrondonors

In absence of a suitable electron donor *compound I* converts to *compound II* and a protein radical is formed. Furthermore, c*ompound II* can react with hydrogen peroxide to form *compound III* (reaction 11).

Reaction 11 $compound\ II + H_2O_2 \rightarrow compound\ III + H_2O$

Compound III can be formed in three different ways, namely through ferric enzyme and superoxide (reaction 8), ferrous enzyme and O_2 (reaction 6) and as mentioned before, through *compound II* reacting with H_2O_2 (reaction 11). *Compound III* is unstable and degrades to either ferric enzyme while releasing superoxide (reaction 9) or ferrous enzyme while releasing oxygen (reaction 7) (10).

Halogenation cycle

As mentioned before the halogenation cycle starts also at the ferric native enzyme reacting with H_2O_2 to form *compound I*. Compound *I* mediates the two-electron oxidation of halides and thiocyanate, which results in the reduction of *compound I* back to the native enzyme (reaction 2) (10). In case of peroxidasin the reaction of *compound I* with halides (X^-) to the native enzyme has to be investigated in more detail, since it is not fully known which halides participate in this reaction. In contrast to MPO, which clearly shows chlorination activity (10), human peroxidasin 1 seems to have no chlorination activity and very low bromination activity, which will be discussed in more detail later.

All heme peroxidases are able to oxidize iodide, LPO and EPO are able to oxidize bromide and only MPO is able to oxidize chloride due to its high oxidation capacity (10).

Ligand binding

Due to the tightly bound cyanide to Fe(III) of mammalian peroxidases, they are converted into a low-spin species. Thereby, a deprotonated group, which seems to be the distal histidine, is necessary. The distal histidine is responsible for deprotonation of HCN, which leads to binding of the cyanide to the heme iron and thus, produces an *S=1* low-spin complex. Conformational changes mediated by cyanide binding are negligible (12).

Nitrite is similar to cyanide and converts ferric MPO from high-spin to low-spin. Nitrite binds directly to Fe(III). It prefers a slightly acidic pH in contrast to cyanide binding, which favors a neutral pH (12). Due to these low-spin ligands, a significant red shift in the UV/VIS spectra can be observed (11). In contrast to cyanide and nitrite, halides are high-spin ligands, and thus only small spectral changes can be observed (12).

Halides, as bromide, do not bind the heme iron. The X-ray structure of the bromide-MPO complex shows the binding site of the bromide to be at the position of a water molecule, which is hydrogen-bonded to the amide nitrogen of a glutamine (12).

Another important ligand worth mentioning is thiocyanate, a pseudohalide, which also acts as substrate of human peroxidases. Like bromide, no significant protein conformational changes with SCN^- are reported (12).

1.3. TRANSIENT EXPRESSION OF PROTEINS

1.3.1. TRANSIENT EXPRESSION OF RECOMBINANT PROTEINS

Transient transfection is a simple, fast and convenient way to analyse a large number of samples within a short period of time (13) by expressing great amounts of products. Since the transfected DNA is not integrated into the host chromosome, the cells are only able to express the target protein temporarily (14).

Non-viral gene delivery expression becomes of greater interest in transient gene expression in cell cultures and especially in suspension cultures. (15) Advantages are faster processes to express milligram amounts of recombinant proteins (15) and simultaneous transfection of cells to compare different methods with each other (16). Furthermore, recombinant protein (r-protein) production with transient mammalian cell technology is recommended due to its cost-effectiveness and speed in contrast to the establishment of stable cell clones, which is time consuming and laborious (16).

Especially useful non-viral transfer methods include a calcium phosphate-based method called Calfection and a polyethylenimine-based method (PEI) (16). In my work only the latter method was used and thereby is described in more detail. Polyethylenimine, a cationic polymer, can be used as gene-delivery agent in serum-free suspension HEK 293 cells (16). PEI is cost-effective, simple to use, non-cytotoxic and stable (17). PEI can either be branched and highly cationic charged due to the presence of a protonable amino nitrogen at every third atom, which is favourable to clasp the negatively charged DNA easily at any pH (18) or it can be linear (19). The linear 25 kDa PEI was used for PEI/DNA complexes to insert the DNA into the cell via interactions with heparan sulphate proteoglycans, which are expressed on the cell surface. Through endocytosis into acidified endosomal compartments PEI/DNA complexes are able to enter the cell (19).

1.3.2. HEK 293 CELLS

HEK 293 cell line are human embryonic kidney cells, which contain stably transformed sheared fragments of adenovirus type 5 (Ad5) DNA (20). The viral DNA fragment is located in chromosome 19 (19q13.2) of the HEK genome and extends from nucleotide 1 to 4344. The

early region 1 (E1) contains the transforming region of the human adenovirus and consists of E1a and E1b, which are two transcription units. Their products are necessary for mammalian cell transformation by adenoviruses (21).

To express complex r-proteins successfully mammalian cell technology has been established (16). Its big advantage, compared to lower eukaryotes or prokaryotes, lies in their ability to provide posttranslational modifications for r-proteins (22). Thereby, the human cell line HEK 293 has been proved especially useful for transient gene expression because of its high transfection yields, in milligram amounts, and the ability to grow in serum-free suspension to high cell density (22).

1.3.2.1. HEK 293 6E CELLS

The HEK 293-6E cells are a variant of the stably expressed Epstein-Barr virus nuclear antigen-1 (HEK 293-EBNA1 or 293E) (23). They are especially useful for recombinant expression of secreted, membrane and intracellular proteins (24). It is reported that HEK 293 cells containing EBNA1 are advantageous for higher protein expression because of its oriP in the vector backbone (17) (23).

2. Aim of Thesis

2.1. Cloning of different human Peroxidasin 1 and truncated Variants

The expression of the full length human peroxidasin 1 in stably transformed HEK 293 cells is time consuming and resulted in a relatively low protein yield. For the reasons described above transient expression in HEK 293-6E cells was chosen to overcome this problem. In addition to the full length protein several truncated constructs were generated for transient expression, each of them comprising different domains of the full length peroxidasin. The truncated versions of the protein were designed to obtain insights into how the individual domains effect protein expression, protein folding, enzyme activity, and the polimerization state of the protein. The different variants can also be useful for protein-protein interaction studies and protein crystallization to solve the three dimensional structure of peroxidasin.

2.2. Protein Production by transient HEK 293-6E cells and subsequent Characterization

The main goal of this thesis was to obtain sufficient amounts of purified active protein of both the full length protein and the truncated variants, by transient expression of peroxidasin in HEK 293 cells. Twelve constructs were designed, cloned and transfected. Protein expression levels, heme incorporation and activity of the different constructs were analysed. The most promising variants were expressed in large scale to obtain sufficient amounts of protein for further characterization including UV-Vis, TMB, ABTS and halogenation assays and steady-state kinetics.

3. MATERIAL AND METHODS

3.1. CLONING OF THE RECOMBINANT PROTEINS

Since expression of full length human peroxidasin 1 in stably transfected HEK293 cells resulted in a low protein yield, a different expression system and several different truncated variants of hsPxd01 were designed in order to improve the yield. In the end twelve different constructs were designed, each of them comprising different domains of the wild-type peroxidasin and six of them including a C-terminal TEV cleavage site and the Fc domain (IgGκ chain) of human immunoglobulin G, resulting in twelve variants of different molecular mass.

The first six constructs contained a C-terminal strep tag. The different constructs and the resulting protein variants were named Kon1 to Kon6 and are described in more detail below. The endogenous hsPxd01 signal peptide was omitted in all twelve constructs and replaced with the signal peptide of IgG as it is known to mediate efficient secretion of recombinant proteins.

Table 2: Information about peroxidasin structures

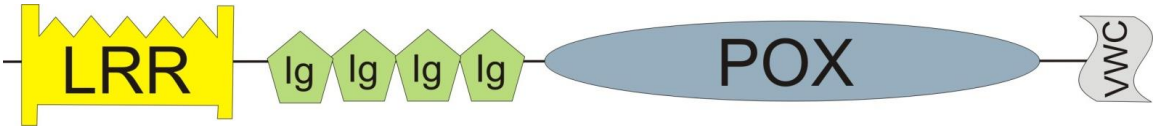
hsPxd01 structure	Amino acid sequence	Amino acids	Cysteines	Glycosylations
Signal sequence	1-26	26	2	1
LRR regions	27-245	219	8 (4 LRRNT, 4 LRRCT)	-
IgG regions	246-619	374	8 (4 Disulphide bonds)	1 (N390)
POX	620-1314	696	15 (7 Disulphide bonds, 1 free Cys)	7
VWC	1413-1471	58	10 (5 Disulphide bonds)	1
	1479		**43**	**10**

Table 3: Detailed information about amino acid sequence of strep constructs

Construct	Amino acid sequence	Amino acids
Kon1	27-1479	1453
Kon2	246-1479	1234
Kon3	27-1314	1288
Kon4	246-1314	1069
Kon5	620-1314	695
Kon6	722-1314	593

- Construct 1 (Kon1): hsPxd01

Construct 1 was the full length human peroxidasin 1 but without its endogenous signal peptide. Kon1 consisted of 1453 amino acids which resulted in a molecular mass of 163 kDa.

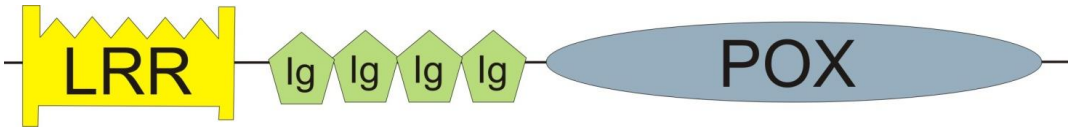


- Construct 2 (Kon2): hsPxd01 without LRR

Construct 2 was missing the signal peptide and the LRR domains, resulting in a 1234 amino acid protein with a molecular mass of 138 kDa.

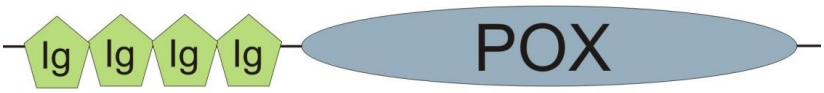
- Construct 3 (Kon3): hsPxd01 without VWC

In construct 3 the signal peptide and VWC were omitted, leading to a protein of 1288 amino acids with a molecular mass of 145 kDa.



- Construct 4 (Kon4): hsPxd01 without LRR and VWC

Construct 4 comprised the Ig domains and the peroxidase domain, omitting the signal peptide, LRR and VWC domains. The obtained protein of 1069 amino acids had a molecular mass of 120 kDa.



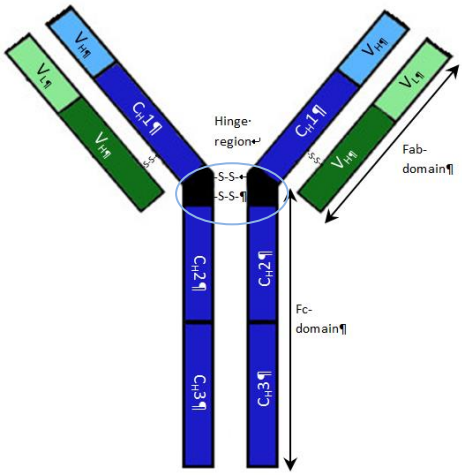
- Construct 5 (Kon5): POX with propeptide

Construct 5 was made up only of the peroxidase domain, which contained 695 amino acid residues and a molecular mass of 79 kDa.

- Construct 6 (Kon6): POX without propeptide

Construct 6 consisted of the peroxidase domain without the N=terminal amino acid region with high homology to the propeptide of LPO, EPO, MPO and TPO. This variant consisted of 593 amino acids, leading to a protein with a molecular mass of 68 kDa.

The further six protein variants were identical to the ones described above but contained an additional N-terminal TEV cleavage site and the Fc domain adding 25 kDa to the molecular mass of the variants. These constructs were created because the expression of recombinant proteins fused with the Fc-domain can improve the protein yield. Furthermore, the Fc-domain was used as means for purifying the fusion proteins using Protein A affinity chromatography.



C	constant region
V	variable region
H	heavy chain
L	light chain (κ or λ)

Figure 10: Immunoglobulin G

The Fc region (**F**ragment **c**rystallizable region) is part of the heavy chain of the immunoglobulin G_1 and consists of the constant regions C_H2 and C_H3 including the hinge region. The cysteine residues in the hinge region form disulphide bonds, leading to dimer formation. The Fc DNA sequence was inserted in Kon1-Kon6 as shown in Figure 11. The Fc domain is able to bind to a Protein A column, which allows purification.

The overall cloning procedure is shown in Figure 11. The double stranded pTT5 DNA plasmid was used for cloning.

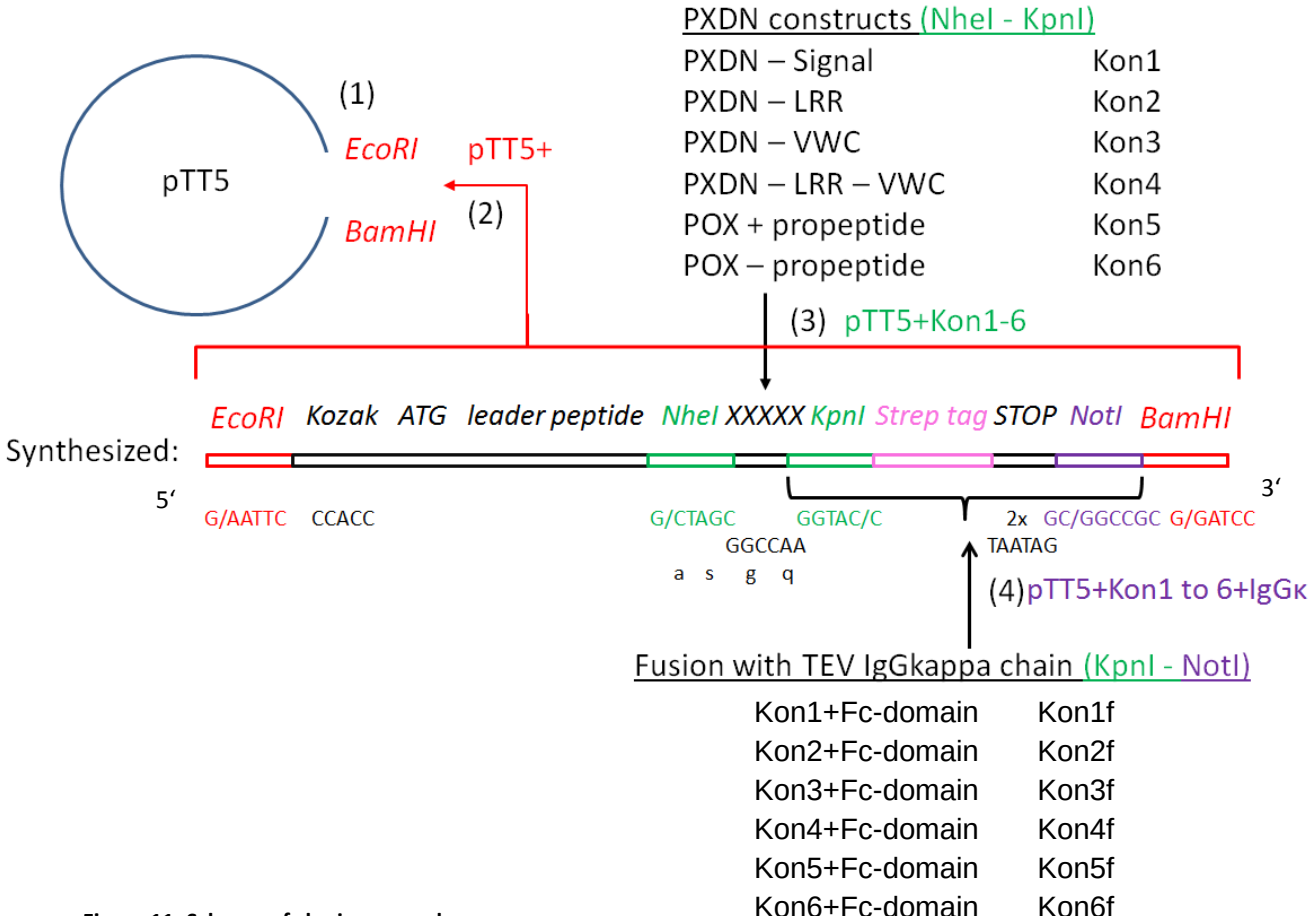


Figure 11: Scheme of cloning procedure

The pTT5 vector was digested using *EcoRI* and *BamHI* on their assigned restriction sites to remove the whole multiple cloning site (MCS). Instead of the MCS a newly synthesized double stranded DNA fragment, consisting of an *EcoRI site, the* Kozak region, the ATG start codon, followed by the DNA sequence encoding for the signal peptide of IgG and furthermore the restriction sites for *NheI* and *KpnI*, the strep tag sequence, two stop codons and finally a *NotI* restriction site and a *BamHI* restriction site (2), was inserted after *EcoRI* and *BamHI* digestion. This plasmid construct was named pTT5+ for easier identification. To generate the individual constructs Kon1 to Kon6, their respective sequences were inserted in the pTT5+ vector after digestion with the restriction enzymes *NheI* and *KpnI* (3). For the fusion proteins Kon1f to Kon6f the additional IgG-κ DNA sequence was inserted using the restriction sites *KpnI* and *NotI* (4).

3.1.1. Plasmid Preparation

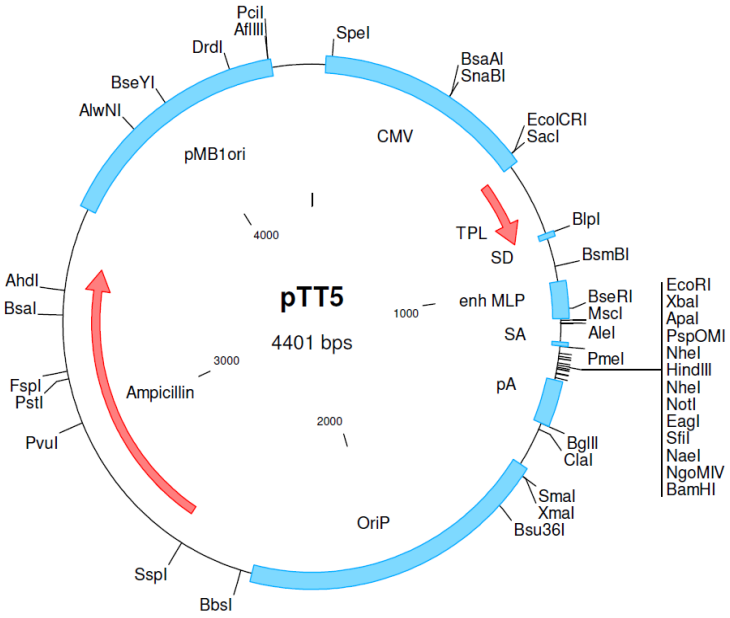


Figure 12: pTT5 vector

The pTT5 plasmid purchased from Invitrogen, was used as vehicle for all constructs. To insert the respective construct sequences, the plasmid had to be prepared by removing its multiple cloning site using its restriction sites *EcoRI* and *BamHI*. The *EcoRI* and *BamHI* digested synthesized double stranded DNA piece was ligated into the CIP treated vector for the insertion of the constructs.

Procedure

The pTT5 plasmid was digested at its *EcoRI* and *BamHI* restriction sites by using the enzymes *EcoRI* and *BamHI*.

Table 4: Digestion of pTT5

5 µL	10x EcoRI buffer
0.5 µL	100x BSA
0.5 µL	*EcoRI*
0.5 µL	*BamHI*
2 µL	pTT5 [2.25 µg/µL]
40,5 µL	HQ-H_2O
1 µL	Alkaline Phosphatase of Calf Intestine (CIP)
50 µL	

Table 5: Restriction site of EcoRI and BamHI

Enzyme	**Sequence**
EcoRI	5' G/AATTC 3'
BamHI	5' G/GATCC 3'

This sample was incubated for 1 hour at 37°C. Then 1 µL CIP was added and the mixture was further incubated for 1 hour at 37°C. CIP was added to prevent re-ligation of the cut vector by dephosphorylation of the digested ends.

The digested vector was purified using agarose gel electrophoresis, described under 3.1.3.1. The sample was applied onto a 1 % agarose gel and the pTT5 band of 4 334 bp was made visible under UV light. The corresponding band was excised and GFX purified, as described in 3.1.1.1. The amount of plasmid was determined at 260 nm using the Nanodrop spectrophotometer and then the plasmid was stored at -20°C until further use.

Material and equipment

pTT5 [2.25 µg/µL]	Invitrogen
10x EcoRI buffer	New England BioLabs
100x BSA	New England BioLabs
EcoRI	New England BioLabs
BamHI	New England BioLabs
CIP	New England BioLabs

Incubator	Memmert BE400
GFX Kit	GE Healthcare
Microcentrifuge	Thermo Sigma 1-15PK
Rotor	Sigma 12024
Nanodrop	1000 Spectrophotometer peqLab

3.1.1.1. GFX PURIFICATION

For purification and concentration of DNA in solution (e.g. restriction enzyme digestions, PCR products, DNA ligations) or from excised DNA bands of agarose gels the GFX Kit by Life Sciences, was used. Thereby, DNA sizes from 50 bp to 10 kbp can be purified. The GFX kit uses a chaotropic agent to extract DNA from the solution or to dissolve agarose and to denature proteins. DNA is bound to the silica membrane, washed with ethanolic buffer to remove salts and other bothering components and eluted with sterile HQ-H_2O.

Procedure

To a sample of 100 µL or less 500 µL Capture buffer type 3 were added. In case of extracting DNA from an agarose gel, the relevant DNA band was excised from the gel. The excised gel band was transferred into a DNase-free 1.5 mL microcentrifuge tube and weighed. For each 10 mg of gel slice 10 µL of Capture buffer type 3 was added (e.g. 300 µL Capture buffer type 3 were added to a 300 mg gel slice). The tube was heated to 65 °C until the gel was dissolved. The Capture buffer type 3/sample mix was mixed thoroughly, loaded onto the GFX MicroSpin column, which was inserted into a Collection tube and centrifuged at 16 000 x g for 30 seconds. The flow through got discarded, 500 µL Wash buffer type 1 was added onto the GFX MicroSpin column and the sample was centrifuged at 16 000 x g for 30 seconds again. The Collection tube was discarded and the column was transferred to a fresh DNase-free 1.5 mL microcentrifuge tube. 20-50 µL sterile HQ-H_2O was added onto the membrane of the column and incubated for 1 minute at room temperature (RT). Afterwards, the sample was centrifuged at 16 000 x g for 1 minute to recover the purified DNA and stored at -20°C.

Material and equipment

illustra GFX PCR DNA and Gel Band Purification Kit	GE Healthcare
Microcentrifuge	Thermo Sigma 1-15PK
Rotor	Sigma 12024

3.1.2. Synthesized Insert Preparation

The synthesized insert was digested with *EcoRI* and *BamHI* and ligated with the previously prepared pTT5 vector (see above).

Procedure

800 ng lyophilised insert was dissolved in 43.5 µL H_2O before digestion for 2 hours at 37°C with the enzymes *EcoRI* and *BamHI*, which can be seen in Table 6.

Table 6: Digesting mix for the insert

43.5 µL	*Synthesized insert*
0.5 µL	*EcoRI*
0.5 µL	*BamHI*
0.5 µL	100x BSA buffer
5 µL	EcoRI buffer
50 µL	

To purify the digested insert the GFX Kit was used, which is described in 3.1.1.1. The amount of DNA was measured using Nanodrop, which is described in3.1.7.6, to quantify the amount of insert for ligation with the prepared pTT5.

Material and equipment

Synthesized insert	Invitrogen
EcoRI	New England BioLabs
BamHI	New England BioLabs
100x BSA buffer	New England BioLabs
10x EcoRI buffer	New England BioLabs

Incubator	Memmert BE400
Nanodrop	1000 Spectrophotometer peqLab

3.1.3. LIGATION OF PTT5 PLASMID AND SYNTHESIZED INSERT

The prepared pTT5 vector was ligated to the digested synthesized insert.

Procedure

The ligation mixture is shown in Table 7. Ligation proceeded for 2 hours at RT. For easier identification the plasmid will be referred to as pTT5+ from now on.

Table 7: Ligation mix for pTT5 and insert

2 µL	T4 Ligation buffer
2 µL	T4 DNA ligase
10 µL	Insert [14.2 ng/ µL]
1 µL	pTT5 [2.25 µg/µL]
6 µL	HQ-H_2O
21 µL	

The ligated pTT5+ vector was transferred into electrocompetent *E. coli Top10* cells by electroporation, which is described in 3.1.6.2. To perform a vector control, the ligation mix was used without insert but HQ-H_2O. This is a quality control of the vector preparation. Ideally the electroporation of the vector control should not produce any colonies because the total amount of vector should be linearized and dephosphorylated.

Afterwards it had to be verified if the ligated plasmid got transferred into the bacterial cells (see electroporation 3.1.6.2) and if the synthesized insert was correctly incorporated into the pTT5 plasmid.

This can be either done by PCR using primers that prime on the pTT5 plasmid or by digesting isolated mini prep DNA of colonies which were picked for screening. Therefore, colonies were picked and cultivated in LB-medium$_{AMP}$ at 37°C overnight, for extraction of plasmid DNA using mini prep (described 3.1.7.1). PCR was done with the same colonies, using respective pTT5 forward and reverse primers, for verification if the plasmid contains the synthesized insert. The respective colonies provided template DNA for the PCR mix. Cells were transferred using a sterile tooth pick or pipette tip and mixed well into the PCR mixture. Before starting the PCR, an initial denaturation step of 10 minutes at 98°C was necessary to break down the cell wall.

Table 8: PCR mix for screening of pTT5+

	Colony picked for screening
2.5 µL	pTT5 forward primer
2.5 µL	pTT5 reverse primer
8 µL	dNTPs [2.5 mM]
20 µL	GC rich phusion buffer 5x
1 µL	Phusion DNA polymerase
66 µL	HQ-H_2O
100 µL	

Table 9: PCR parameters

Initial denaturation	**98°C**	**10 min**	
Denaturation	98°C	30 sec	35 cycles
Annealing	72°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	10 min	
Cooling	4°C	-	

Agarose gel electrophoresis was performed to visualize PCR products. Once a clone was identified positive (either by PCR screening or mini prep DNA digestion) the mini prep DNA was sequenced to verify correct insertion and absence of mutations (Microsynth or LGC Technology in Germany). After identification of a clone with correctly inserted synthesized DNA, pTT5+ vector DNA was prepared for further cloning steps of hsPxd constructs. The picked *E. coli* clone was cultivated in LB-medium$_{AMP}$ at 37°C overnight, for purification of bigger amounts of plasmid DNA via several mini preps (described 3.1.7.1).

Material and equipment

pTT5 vector	Invitrogen
Synthesized insert	Sigma
T4 Ligation buffer	New England BioLabs
T4 DNA ligase	New England BioLabs
pTT5 forward primer	Sigma
pTT5 reverse primer	Sigma
dNTPs	New England BioLabs
5x Phusion GC Reaction Buffer	New England BioLabs
Phusion DNA polymerase	New England BioLabs

Incubator	Memmert BE400
PCR equipment	ESCO.Swift maxi
Electrophoresis chamber	peqLAB
Energy supply	LKB Bromma 2301 Macrodrive 1 Power Supply

3.1.3.1. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is used to resolve a mixed population of DNA fragments by size. Excision of the band (fragment) of the correct size contains the DNA of interest separated from other DNA fragments (e.g. several PCR products, digested vector DNA, etc.).

To verify if the PCR worked and the DNA construct of the correct size was amplified, gel electrophoresis was used. Thereby, an agarose gel was prepared, the PCR mixture were applied onto the gel and the DNA fragments were separated in order of their sizes and shapes in an electrical field. Depending on the amount of used agarose different sizes of pores in the gel can be obtained, where a larger amount of agarose results in smaller pores and vice versa. Smaller DNA fragments are able to move faster through the gel than larger ones. The DNA bands become visible under UV light.

Procedure

Depending on DNA size different amounts of agarose were used. For a preparative gel 150 mL were prepared (twelve slots for a sample volume of 60 µL), whereas for an analytical gel only 100 mL were needed (20 slots for a sample volume of 30 µL). The running conditions for a preparative gel was 70-80 Volt, whereas an analytical gel was run at 100 V, both for approximately 40 minutes, depending on the DNA size and gel.

Table 10: Preparative gel

Agarose	0.5 %	> 5000 bp
	1 %	> 1000 bp
	2 %	< 1000 bp
	2.5 %	<150 bp
TAE (50x)	3 g	
RO-H_2O	Fill up to 150 g	

The mixture was melted in a microwave and cooled down a little bit before adding SYBR-safe (1:10 000). The liquid gel was poured into the prepared gel form, well combs were inserted and the gel was left to solidify before the DNA samples were applied, which had been mixed with 6x loading dye to a 1x final concentration. A DNA ladder with known DNA sizes had to be applied as well to identify the sizes of the DNA bands. Under UV light the bands were detected.

Amplified DNA of the expected size was excised and purified using the GFX gel extraction kit, which is described in 3.1.1.1 and stored at -20°C until further use.

Material and equipment

Agarose	Biozym
SYBR-safe	Invitrogen
6x MassRuler Loading Dye	Fermentas
Gene Ruler 1kb DNA Ladder	Fermentas

Electrophoresis chamber	peqLAB
Power supply	LKB Bromma 2301 Macrodrive 1 Power Supply
GFX kit	GE Healthcare

3.1.4. Preparation of Construct DNA

The prepared pTT5 vector was ligated to the digested synthesized insert.

Procedure

To generate the DNA sequences of constructs Kon1 to Kon6, the full length human peroxidasin 1 encoded on a pcDNA™3.1/V5-His TOPO as template and different primers were used. The vector was kindly bestowed by Miklós Geiszt (Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary).

Each construct DNA sequence was amplified by Polymerase chain reaction (PCR) using a forward primer introducing an *NheI* restriction enzyme site (5'GCTAGC3') and a reverse primer, which introduced the *KpnI* site (5'GGTACC3'). 20-50 bases of the two primers were complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target to amplify the gene.

PCR is a method to amplify DNA *in vitro*. Thereby, DNA itself functions as template, which leads to an exponential amplification during each cycle. To perform PCR template DNA, a DNA-polymerase, forward and reverse primers, desoxyribonucleotidephosphates (dNTPs) and buffer have to be mixed together. The process includes one initial denaturation step, then three cyclic steps of denaturation of DNA, followed by primer annealing and elongation, which are repeated 20-45 times.

The final step is a 10 minutes elongation step to ensure that all PCR products are entirely elongated.

Table 11: Primer for constructs

Construct	Primer	Sequence (5'-3')
Kon1	Kon_1forward	ATGCGCTAGC GTGGTGGCCCAGAAGCCGGGCGCAGGGTGTCCG
	Kon_1reverse	ATGCGGTACCGGGCTTTTCCTCCGCCCTCTTCTGTAAGCAGACTGG
Kon2	Kon_2forward	ATGCGCTAGC CCCCGGATCACCTCCGAGCCCCAGGACGCAGATGTGACC
	Kon_2reverse	=Kon_1reverse
Kon3	Kon_3forward	=Kon_1forward
	Kon_3reverse	ATGCGGTACC GTCCTGCCACACCCGGAGGTCCACCCTGGGG
Kon4	Kon_4forward	=Kon_2foward
	Kon_4reverse	=Kon_3reverse
Kon5	Kon_5forward	ATGCGCTAGC GGAGATCCGTTTGTAGCTACCTCCATCGTGG
	Kon_5reverse	=Kon_3reverse
Kon6	Kon_6forward	ATGCGCTAGC GGCTGTACCGCCCACCGGCGCGTGAACAACTGC
	Kon_6reverse	=Kon_3reverse

Procedure

Each construct DNA was amplified using polymerase chain reaction (PCR) by adding the following components:

Table 12: PCR mix for construct amplification

Amounts	Final concentration	component
20 µL	1x	5x Phusion GC Reaction Buffer
8 µL	0.2 mM	dNTP mix [2,5 mM]
2.5 µL	2.5 µM	Respective forward primer [100 µM]
2.5 µL	2.5 µM	Respective reverse primer [100 µM]
1 µL	1.0 units/50 µL PCR	Phusion HF DNA Polymerase
1 µL	83 ng	pcDNA 3.1 template (83 ng/µL)
65 µL		HQ-H_2O
100 µL		

PCR was then started by using the following parameters:

Table 13: PCR parameters

Initial denaturation	98°C	30 sec	
Denaturation	98°C	10 sec	35 cycles
Annealing	72°C	30 sec	
Extension	72°C	1:20 min for Kon5 and Kon6	
		2:30 min for Kon1, Kon2, Kon3 and Kon4	
Final extension	72°C	10 min	
Cooling	4°C	-	

Depending on DNA sequence length and DNA polymerase, different extension times were used. The Phusion HF DNA polymerase is able to extend 1000 bp in 30 sec.

Table 14: Information on basepairs of each construct

Kon1	4 359 bp
Kon2	3 702 bp
Kon3	3 864 bp
Kon4	3 207 bp
Kon5	2 085 bp
Kon6	1 779 bp

Amplified DNA was screened using agarose gel electrophoresis to verify if the PCR resulted in a PCR product of the correct size. Afterwards, the amplified DNA was purified using the GFX kit, as described under 3.1.1.1.

Material and equipment

pcDNA™3.1/V5-His TOPO	Miklós Geiszt (Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary)
5x Phusion GC Reaction Buffer	New England BioLabs
dnTP	New England BioLabs
All primers	Sigma
Phusion HF DNA Polymerase	New England BioLabs

PCR equipment	ESCO Swift maxi
Incubator	Memmert BE400

Centrifuge	Thermo Sorvall 1-15PK
Rotor	Sigma 12024
GFX Kit	GE Healthcare

3.1.4.1. DIGESTION OF CONSTRUCT DNA

The last step of construct DNA preparation was digesting each DNA construct using the enzymes *NheI* and *KpnI* before ligation with the prepared, *NheI* and *KpnI* digested, CIP treated pTT5+ vector.

Procedure

Table 15: Digesting mix of PCR products

40 µL	PCR construct DNA
5 µL	10x NEB buffer 1
0.5 µL	*KpnI*
0.5 µL	*NheI*
4 µL	HQ-H_2O
50 µL	

The samples were incubated for 2 hours at 37°C. Then the DNA got purified using the GFX kit again, the amount of DNA was measured and stored at -20°C until further use.

Material and equipment

10x NEB buffer 1	New England BioLabs
KpnI	New England BioLabs
NheI	New England BioLabs

Incubator	Memmert BE400
Centrifuge	Thermo Sorvall 1-15PK
Rotor	Sigma 12024
GFX Kit	GE Healthcare
Nanodrop	1000 Spectrophotometer peqLab

3.1.5. Insertion of Construct DNA into pTT5+

The final step to complete the cloning part of the constructs before transforming them into bacterial cells, is the insertion of prepared construct DNA into prepared pTT5+ vector.

Procedure

The amounts of prepared construct DNA and pTT5+ plasmid were measured using Nanodrop, as described under3.1.7.6, to use about 500 ng of construct DNA and 100 ng of plasmid DNA for the ligation. In Table 16 the composition of the ligation mixture is shown:

Table 16: Ligation mixture of construct DNA and pTT5+

500 ng	Respective construct DNA
100 ng	pTT5+ plasmid
2 µL	T4 DNA ligase
2.5 µL	T4 Ligation buffer
Filled up to 25 µL with sterile HQ-H_2O	

Ligation was performed at RT for 2 hours and each ligation was purified using the GFX kit, which is described 3.1.1.1. A positive control with plasmid DNA as well as a negative control with sterile HQ-H_2O was done to confirm the effectiveness of the electroporation. The DNA was eluated in 20 µL sterile HQ-H_2O and was stored at -20°C until further use.

Material and Equipment

pTT5+ plasmid	As prepared above
Respective construct DNA	
T4 ligase	New England BioLabs
T4 Ligation buffer	New England BioLabs

GFX Kit	GE Healthcare
Nanodrop	1000 Spectrophotometer peqLab

3.1.6. TRANSFORMATION OF BACTERIA

The pTT5+ construct ligation mixture was transformed into electrocompetent *E. coli Top10* cells using electroporation. After electroporation the cells were plated and the colonies formed were checked for the presence of the inserted gene of interest (either by using PCR screening or by mini prep DNA digestion). Positive clones were sequenced to verify the correct insertion of the construct without mutations and subsequently cryocultures were made.

3.1.6.1. PREPARATION OF ELECTROCOMPETENT *E. COLI TOP10*

For electroporation electrocompetent *E. coli* cells in a medium of low conductivity were prepared.

Procedure

An overnight *E. coli* 10 mL LB-medium culture was incubated at 37°C and 180 rpm, which was used to inoculate 500 mL LB-medium on the next day. This culture was incubated at 37°C and 180 rpm until the OD_{600} reached 0.6. The culture was incubated on ice for 30 min and then the cells were harvested by centrifugation at 4 000 rpm at 4°C for 8 minutes. The supernatant was discarded, the pellet resuspended in 250 mL chilled 1 mM Hepes and the cells got harvested by centrifugation at 4 000 rpm at 4°C for 10 minutes again. As before, the supernatant was discarded, the pellet resuspended in 125 mL chilled 1 mM Hepes and harvested again under the same conditions. The cells were resuspended in 50 mL chilled 1 mM Hepes, centrifuged once again and the pellet was dissolved in 15 mL cooled 10 % glycerol. The suspension was centrifuged at 5 000 rpm at 4°C for 10 minutes and the pellet was finally resupended in 2 mL cooled 10 % glycerol. Aliquotes of 100 µL were stored at -80°C.

Material and Equipment

1 mM HEPES

238.3 mg Hepes

Added to 1 000 mL with HQ-H_2O

10 % (v/v) Glycerol

5.05 mL 99% Glycerin

Added to a final volume of 50 mL with HQ-H_2O

LB-medium	Self prepared
E. coli Top10	
Hepes	Sigma-Aldrich
Glycerol	Sigma
Incubator	INFORS HT Ecotron
Spectrophotometer	Agilent-8453 UV-Visible System
Centrifuge	Thermo Sorvall RC6 Plus
Rotor	Sorvall SLA 1500

3.1.6.2. ELECTROPORATION

Electroporation is a method that is used to transform plasmid DNA into bacterial cells. This is done in an electrical field with an electric pulse, where the bacterial cell membrane becoms permeable for the plasmid temporarily.

Procedure

Electrocompetent cells were carefully thawed and then added to the plasmid. Cells and plasmid had to be well mixed and put into an electroporation cuvette. The sample was electroporated at 1000 Ohm, 25 µF and 2.5 kV. Afterwards, 1 mL pre-warmed SOC medium was added to the electroporated bacteria to provide an optimal surrounding quickly. Then the solution was pipetted into a 1.5 mL microtube, incubated at 37°C for 30 minutes and plated onto selective LB agar. The plates were incubated at 37°C overnight.

Table 17: Samples for electroporation

Sample	10 µL ligation sample (Kon1-6)
Vector control	10 µL ligation mix without insert
Negative control	10 µL HQ-H_2O
Positive control	1 µL plasmid DNA

Material and equipment

SOC medium

0.5 %	Yeast Extract	AppliChem
2 %	Tryptone	Merck
10 mM	NaCL	Sigma-Aldrich
2.5 mM	Potassium chloride	Sigma-Aldrich
10 mM	Magnesium chloride	Sigma
10 mM	Magnesium sulfate	Merck
20 mM	Glucose	Sigma

Every component, besides glucose, was put together, dissolved in RO-H_2O and then the medium was autoclaved. After cooling , glucose was added after the glucose solution was sterile filtrated through a 0.2 µm filter.

LB – medium

10 g	Peptone	Sigma Aldrich
5 g	Yeast extract	AppliChem
10 g	NaCl	Sigma-Aldrich

Added to 1000 mL with RO-H_2O
and autoclaved

Ampicillin stock solution

100 mg/mL Sodium ampicillin in RO water
Sterile filtrated and stored at -20°C

LB_{AMP} –plates

10 g	Peptone	Sigma Aldrich
5 g	Yeast extract	AppliChem
10 g	NaCl	Sigma-Aldrich
15 g	Agar Agar	Roth

Added to 1000 mL with RO-H_2O

After autoclaving at 122°C for 30 minutes the LB-agar was cooled down to 55°C and 1mL ampicillin solution was added to a final concentration (f.c.) of 100 µg/mL. Then the LB_{AMP}-agar was poured into Petri dishes.

LB_{AMP}- medium

1 mL ampicillin solution (f.c. 100 µg/mL) was used for 1 L of LB- $medium_{AMP}$

Electroporation equipment	BioRad
Electroporation cuvette	VWR
Gene pulser	BioRad
LB_{AMP}-agar or LB_{KAN}-agar	LB-agar+ 1:1000 antibiotic
Shaker	IKA VIBRAX VXR

3.1.7. Screening of transformed bacterial Cells

3.1.7.1. Mini Prep

To maintain small amounts of highly purified plasmid DNA quickly, the mini prep kit illustra™ plasmidPrep Mini Spin Kit by GE Healthcare was used. Thereby, bacteria were grown, harvested, lysated and purified to get the plasmid DNA.

This procedure is based on alkaline lysis of bacterial cells, in which the cells are exposed to chaotropic salts to denature protein components. Chromosomal and plasmid DNA can be separated because plasmid DNA is smaller and more super coiled than chromosomal DNA. Furthermore, plasmid DNA is able to renature, whereas chromosomal DNA precipitates and can thereby be separated from plasmid DNA. In the last step, plasmid DNA is bound onto a silica-based membrane and precipitated by ethanol.

Procedure

This procedure refers to the illustra™ plasmidPrep Mini Spin Kit by GE Healthcare .

The day before the mini prep procedure was started a 10 mL LB_{AMP}-overnight culture with the respective bacterial culture was incubated.

1.5 mL of bacterial culture was centrifuged at 16000 x g for 30 seconds and the supernatant was discarded. This step was repeated once. Afterwards, the pellet was resuspended in 175 µL Lysis buffer type 7. 175 µL Lysis buffer type 8 was added and the microtube was inverted gently. Then 350 µL Lysis buffer type 9 was added and the tube was inverted gently again. The sample was centrifuged at 16000 x g for 4 minutes.

A plasmid mini column inside a Collection tube was prepared, the supernatant was transferred into it and the sample was centrifuged at 16000 x g for 30 seconds. The flowthrough was discarded, the column was washed with 400 µL Lysis buffer type 9 and was centrifuged at 16000 x g for 30 seconds again. The flowthrough was discarded once again, 400 µL Wash buffer type 1 was added and the sample was centrifuged at 16000 x g for 1 minute to let the membrane dry. The flowthrough and Collection tube were discarded and the plasmid mini column was transferred into a fresh Microtube. 100 µL HQ-H_2O was added, the sample was incubated for 30 seconds at RT and centrifuged at 16000 x g for 30 seconds.

Afterwards the amount of DNA in the eluent was measured as described in 3.1.7.6 and stored at -20°C.

Material and equipment

Mini Prep Kit	GE Healthcare
Microcentrifuge	Thermo Sigma 1-15PK
Rotor	Sigma 12024

3.1.7.2. Polymerase Chain Reaction (PCR) Colony Screening

PCR can also be used to determine if the transformed DNA produced by bacteria contains the DNA of interest. By using specific primers, which can either prime on the vector on the flanking regions or directly on the DNA of interest. The PCR product can then be identified by gel electrophoresis.

Procedure

The following table shows the mixture of the PCR solution:

Table 18: Mixture of PCR solution

4 µL	GC-rich buffer
1.6 µL	dNTPs [2.5 mM]
0.5 µL	pTT5 forward primer
0.5 µL	pTT5 reverse primer
0.2 µL	Phusion DNA polymerase
13.2 µL	HQ-H_2O
20 µL	

Then a transformed colony of the overnight LB_{AMP}-plates was picked and put into the PCR mixture to serve as template DNA. With the same colony a master cell plate and an overnight culture was created.

The PCR was then started by using the following parameters:

Table 19: PCR program

Initial denaturation	98°C	10 min	
Denaturation	98°C	30 sec	35 cycles
Annealing	72°C	30 sec	
Extension	72°C	1:30 min: Kon5, Kon6	
		2:30 min: Kon1-Kon4	
Final extension	72°C	10 min	
Cooling	4°C	-	

The initial 10 minutes denaturation step destroyed the cell wall to free plasmid DNA for amplification. Depending on the length of the DNA different elongation times were used. The Phusion HF DNA Polymerase elongates approximately 1 000 bp per 30 seconds.

Material and equipment

LB_{AMP} agar	Preparation see 3.1.6.2
dNTPs	New England BioLabs
Phusion HF DNA Polymerase	New England BioLabs
5x Phusion GC Reaction buffer	New England BioLabs
pTT5 forward primer	Sigma
pTT5 reverse primer	Sigma
PCR equipment	ESCO Swift maxi
PCR tubes	Biozym

3.1.7.3. DNA SEQUENCING

To check if the construct DNA was correctly amplified and inserted into the plasmid, DNA sequencing was performed. A specific amount of DNA was mixed together with one specific primer. Approximately 1 000 bp can be covered with one primer, therefore a series of different primers which anneal less than 1000 bp apart were used to cover the whole

sequence of the construct DNA. The sequencing results were compared with the expected sequences.

Procedure

A final volume of 14 µL was acquired in which 400 nM (f.c.) DNA and 2 µM (f.c.) specific primer were added. Different primers were used:

Table 20: Information on different primers

Primer	Sequence (5'->3')	Binding site
pTT5 forward	AAAAACGAGGAGGATTTGATATTCACCTGG	1057-1086
606 forward	CCTGTGGTTGGCGGATTTG	600-618
2038 forward	ATCTTTGAACGGACATTGC	2032-2050
3034 forward	GACGGCGACACCATCTAC	3028-3045
4030 forward	TACCAGGAGGACAAGCCGAC	4024-4043
Seq 2 forward	CATCGTGCAGCGGTCC	2817-2832
Seq 3 forward	GGCTCTTCCTCAGTTCACT	1290-1308
1547 reverse	ACGACCTTCTGGGAGCCG	1541-1524
2111 reverse	CTTGTTCCGTTGAGGTCG	2105-2088
3119 reverse	ATCTTCGGGAGCCAGTGC	3113-3096
4130 reverse	GTGCTGAAGGCTGAGGTGC	4124-4106
pTT5 reverse	CCGAGGGATCTCGACCAAATGATTTGC	1440-1414

The samples were sent to LGC Genomics in Germany to get analysed.

Material and equipment

Primers as shown in table 6 Designed according to the DNA sequence of the constructs in pTT5+ plasmid

3.1.7.4. CRYOCULTURES

Cryocultures were produced of positively screened clones to gain a stock of the inserted plasmid containing the construct for future work. These are stored at -80 °C and used when needed.

Procedure:

10 mL LB_{AMP} overnight culture was incubated and 0.5 mL of culture was mixed with 0.5 mL 30 % glycerol. The sample was stored at -80 °C.

Material and equipment

30 % (v/v) glycerol

30.3 mL Glycerol (99%) Sigma

Added to a final volume of 100 mL with HQ-H_2O

LB_{AMP} Preparation see 3.1.2.2

Shaker IKA VIBRAX VXR

3.1.7.5. MIDI PREP

The midi prep is similar to the mini prep but is used to get larger amounts of DNA. In my thesis the midi prep kit NucleoBond Xtra Midi by Healthcare was used and therefore the general information and procedure refers to this kit.

The procedure is based on alkaline lysis, followed by clarification of lysate and binding of plasmid DNA to an anion exchange resin under low salt and pH conditions. Due to insertion of the filter into the column, parallel clarification of bacterial lysate and loading DNA onto the column can be attained. Undesired components like RNA, proteins, dyes and low-molecular mass impurities are removed by a medium-salt wash. Plasmid DNA is eluted by a high-salt buffer, precipitated, desalted and concentrated by isopropanol and centrifuged to obtain the DNA.

Procedure

150 mL of overnight LB_{AMP} culture were grown at 37 °C and centrifuged at 6 000 x g for 15 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 8 mL chilled resuspension buffer RES (with RNase A end concentration of 100 µg/mL). 8 mL of lysis buffer LYS was added, which turned the cell suspension blue and the sample was inverted 4-6 times and incubated at RT for 5 minutes. Meanwhile the column/filter construct was equilibrated with 12 mL of equilibration buffer EQU and emptied by gravity. 8 mL neutralization buffer NEU was added to the sample and inverted 10-15 times until the lysate

turned colourless. The lysate was poured into the filter and let be emptied by gravity. The filter was washed with 5 mL equilibration buffer and was then removed.

The column was washed with 8 mL washing buffer (WASH). Afterwards the DNA got eluted by gravity into a clean centrifugation tube with 5 mL elution buffer ELU. The DNA was precipitated by adding 3.5 mL RT isopropanol and by vortexing thoroughly. The eluation was then centrifuged at 15 000 x g for 30 minutes at 4°C. The supernatant was removed and the DNA pellet was washed with 2 mL ethanol. The sample was centrifuged at 15 000 x g for 5 minutes at RT and the supernatant was removed again. The pellet got dried for 10 minutes before it was dissolved in 1 mL sterile HQ-H2O.

Afterwards the amount of DNA in the eluent was measured as described in 3.1.7.6 and stored at -20°C.

Material and equipment

LB-medium$_{AMP}$	Self prepared
Shaker	INFORS HT Ecotron

Midi Prep Kit	Macherey - Nagel
Centrifuge	Thermo Sorvall RC 6 Plus
Rotor	SLA-1500

3.1.7.6. Quantification of DNA Amount

To measure the amount of DNA in a sample the Nanodrop spectrophotometry can be used. Only 2 µL of sample have to be pipetted onto the fiber optic cable. A second fiber optic cable is put onto the first and the drop fills the gap between the two optic ends. The light is provided by a pulsed xenon flash lamp, which works in combination with a spectrometer utilizing a linear charge-coupled device (CCD) array.

DNA has its maximum absorption at 260 nm and proteins at 280 nm. The ratio between these maxima provides the purity of the DNA sample and should be around 1.8.

Procedure

2 µL DNA sample was pipetted on the lower measurement pedestal, the sampling arm was closed and the sample was measured.

Material and Equipment

Nanodrop 1000 Spectrophotometer peqLab

3.1.8. Preparation of the Fusion-protein Constructs

3.1.8.1. PCR Amplification of the Fc-Domain Sequence

In order to obtain the fusion-proteins, the construct DNA of each construct Kon1-6 was fused C-terminally with the Fc-domain. The Fc-domain of hIgG consists of the C_H3, C_H2 and Hinge region of the immunoglobulin G and is able to bind to Protein A, which was used for purification later.

The vector pYD11 carrying the Fc-gene was used as template to provide the DNA sequence, which was PCR amplified.

Table 21: Primer for Fc-domain

Construct	Primer	Sequence (5'-3')
Fc-domain	TEV IgG forward	ATGCGGTACCGAAAACCTGTATTTTCAGGGCACTCACACATGC
	TEV IgG reverse	ATGCGCGGCCGCTTATCATTTCCCGGGAGACAGGGAGAGGC

Procedure

The Fc-domain DNA of 716 bp was amplified using polymerase chain reaction (PCR) by adding the following components:

Table 22: PCR mix for constructs

Amounts	Final concentration	component
20 µL	1x	5x Phusion GC Reaction Buffer
8 µL	0.2 mM	dNTP mix [2,5 mM]
2.5 µL	2.5 µM	Respective forward primer [100 µM]

2.5 µL	2.5 µM	Respective reverse primer [100 µM]
1 µL	1.0 units/50 µL PCR	Phusion HF DNA Polymerase
1 µL	36 ng	pYD11 vector (36 ng/µL)
65 µL		HQ-H_2O
100 µL		

PCR was then started by using the following parameters:

Table 23: PCR parameters

Initial denaturation	**98°C**	**30 sec**	
Denaturation	98°C	10 sec	35 cycles
Annealing	72°C	30 sec	
Extension	72°C	60 sec	
Final extension	72°C	10 min	
Cooling	4°C	-	

Amplified DNA was screened using agarose gel electrophoresis to verify if the PCR resulted in a PCR product of the correct size. Afterwards, the amplified DNA was purified using the GFX kit, as described under 3.1.1.1 and stored at -20°C until further use.

Material and equipment

pYD11	
5x Phusion GC Reaction Buffer	New England BioLabs
dNTP	New England BioLabs
All primers	Sigma
Phusion HF DNA Polymerase	New England BioLabs

PCR equipment	ESCO Swift maxi
Incubator	Memmert BE400
Centrifuge	Thermo Sorvall 1-15PK
Rotor	Sigma 12024
GFX Kit	GE Healthcare

3.1.8.2. DIGESTION OF FC-DOMAIN

To prepare the Fc-domain DNA for ligation with the construct DNA, it was digested using *KpnI* and *NotI*.

Procedure

First, the Fc-domain DNA was *KpnI* digested using the following mix:

Table 24: Digesting mix of Fc-domain DNA

40 µL	PCR Fc-domain DNA
5 µL	10x NEB buffer 1
0.5 µL	*KpnI*
4.5 µL	HQ-H_2O
50 µL	

The sample was incubated for 2 hours at 37°C. Then the DNA got purified using the GFX kit and the sample was digested with *NotI* using the following mix:

Table 25: Digesting mix of Fc-domain DNA

40 µL	*KpnI* digested Fc-domain DNA
5 µL	10x NEB buffer 3
0.5 µL	*NotI*
4.5 µL	HQ-H_2O
50 µL	

The sample was incubated for 2 hours at 37°C again. Then the DNA got purified using the GFX kit, the DNA amount was measured using the Nanodrop and the DNA was stored at -20°C until further use.

Material and equipment

10x NEB buffer 1	New England BioLabs
10x NEB buffer 3	New England BioLabs
KpnI	Fermentas
NotI	New England BioLabs

Incubator	Memmert BE400
Centrifuge	Thermo Sorvall 1-15PK
Rotor	Sigma 12024
GFX Kit	GE Healthcare
Nanodrop	1000 Spectrophotometer peqLab

3.1.8.3. Preparation of the Constructs Kon1-6 for Fc-DNA Sequence Insertion

In order to insert the Fc-domain sequence into the pTT5+ plasmids containing the constructs Kon1-6, they were digested using the enzymes *KpnI* and *NotI* in two digestion steps.

Procedure

First, each construct of Kon1-6 was *KpnI* digested using the following mix:

Table 26: *KpnI* digestion mix of pTT5+ Kon1-6 plasmid DNA

3 µg	Mini Kon1-6
5 µL	10x NEB buffer 1
0.5 µL	*KpnI*
4.5 µL	HQ-H_2O
Fill up to 50 µL with sterile HQ-H_2O	

The samples were incubated for 2 hours at 37°C. Then the DNA got purified using the GFX kit and the samples were digested with *NotI* using the following mix:

Table 27: *NotI* digestion mix of PCR products

40 µL	*KpnI* digested Kon1-6
5 µL	10x NEB buffer 3
0.5 µL	*NotI*
1 µL	CIP
3.5 µL	HQ-H_2O
50 µL	

The sample was incubated for 1 hours at 37°C, 1 µL CIP was added and then the digestion was incubated for 1 hour at 37°C again. Afterwards the DNA got purified using a GFX kit, the

DNA amounts were measured using the Nanodrop and the DNA was stored at -20°C until further use.

Material and equipment

10x NEB buffer 1	New England BioLabs
10x NEB buffer 3	New England BioLabs
KpnI	Fermentas
NotI	New England BioLabs
CIP	New England BioLabs

Incubator	Memmert BE400
Centrifuge	Thermo Sorvall 1-15PK
Rotor	Sigma 12024
GFX Kit	GE Healthcare
Nanodrop	1000 Spectrophotometer peqLab

3.1.9. Ligation of the digested pTT5+ KON1-6 Plasmids with the Fc-Domain

After Fc-DNA and the pTT5+ Kon1-6 construct preparation, the final step of ligation of both components was performed.

Procedure

The amounts of prepared pTT5+ Kon1-6 plasmid DNA and Fc-domain DNA was measured using Nanodrop, described 3.1.7.6 , to use about 120 ng pTT5+ Kon1-6 DNA and 70 ng Fc-DNA. The following ligation mixture was used:

Table 28: Ligation mixture to obtain the fusion-proteins

100 ng	*KpnI* and *NotI* digested pTT5+ Kon1-6
70 ng	*KpnI* and *NotI* digested Fc-DNA
2 µL	T4 Ligation Buffer
2 µL	Ligase T4
4 µL	HQ-H_2O
Fill up to 20 µL with sterile HQ-H_2O	

Ligation was performed at RT for 2 hours and each ligation was purified using the GFX kit, which is described under 3.1.1.1. The DNA was eluated in 20 µL sterile HQ-H_2O and was stored at -20°C until further use.

The ligated Kon1f-Kon6f constructs were electroporated into *E. coli Top10 cells* to obtain the clones for protein expression, as described under 3.1.6.2.

Material and Equipment

Fc-DNA	
Respective pTT5+ construct DNA	
T4 ligase	New England BioLabs
T4 Ligation Buffer	New England BioLabs

GFX Kit	GE Healthcare
Nanodrop	1000 Spectrophotometer peqLab

3.2. PROTEIN PRODUCTION

3.2.1. STERILE TECHNIQUE

Working with human cell lines, cell culture media and all materials which are needed to handle cells demand sterile techniques. All work was done in the laminar flow hood, which was always cleaned with 70% ethanol before and after use. Solutions and media were bought sterile or they were autoclaved or sterile filtrated. Every vessel and other material was disinfected with 70% ethanol before entering into the laminar. One way material was autoclaved before it got disposed of and reusable material was autoclaved and then cleaned before the next use.

Material and Equipment

Laminar flow hood	Holten LaminAir HBB 2472
Ethanol	VWR
CO_2 incubator	Infors HT Multitron Cell

3.2.2. Cell Culture

HEK 293-6E cells were used to produce the peroxidasin constructs. This special type of HEK 293 cells is especially suitable for cultivation in suspension. Preparation of HEK 293-6E cells, transfection with DNA, addition of hematin for improved heme incorporation, feeding with peptone and harvesting the cell supernatant containing the produced protein was performed in one week cycles.

3.2.2.1. Preparation of FreeStyle™ Expression Medium

This special medium is used to cultivate and grow the HEK 293-6E cells. It is a chemically defined and serum-free complete medium, which contains no proteins or hydrolysates and is especially designed for suspension cultures.

Procedure

Before use 20 mL of 200 mM L-glutamine (f.c. 4 mM) and 10 mL Pluronic F68 (f.c. 0.1 %) were added to the purchased medium. The antibiotic Geneticin (G418) was added from a stock solution of 50 mg/mL to a final concentration of 50 µg/mL. For all works the complimented F17 medium was used. It is explicitly mentioned if F17 medium without G418 was used for thawing of cryo cultures, which is described under 3.2.2.4.

Materials and equipment

Preparation of 50 mg/mL geneticin

5 g geneticin were dissolved in 100 mL HQ-H_2O and sterile filtrated using a 22 µm filter.

Geneticin	Gibco
22 µm PVDF Rotilabo ® syringe filter, sterile	Roth

FreeStyle™ F17 Expression Medium	Gibco
100x Pluronic F-68, non-ionic surfactant	Gibco
200 mM L-glutamine	Gibco
50 mg/mL Geneticin	Gibco

3.2.2.2. Preparation of HEK 293-6E Master Seed Lot

A master seed lot was prepared to contain a stock of HEK 293-6E cells. They were stored at -196°C in liquid nitrogen and were thawed when needed.

Procedure

The HEK 293-6E cells were cultivated in F17 medium until they reached a cell density between $0.8*10^6$ and $1.2*10^6$ cells/mL. The cell culture was centrifuged at 250 x g for 5 minutes. The supernatant was discarded and the pellets were dissociated by gently tapping the tube.

Freezing mixture was added until the desired cell density of $5\text{-}10*10^6$ cells/mL was obtained. The suspension was homogenized without stressing the cells too much and aliquoted in cryoculture vials. The cells were immediately put into a -80°C freezer, using a container filled with isopropanol which resulted in the cell suspension freezing slowly at 1°C/min.

The cryocultures were transferred to liquid nitrogen for long term storage on the following day.

Material and equipment

FreeStyle™ F17 Expression Medium Preparation see 3.2.2.1

Centrifuge Beckman CS-6KR Centrifuge

3.2.2.3. Thawing of HEK 293-6E Cells

Thawing HEK 293-6E cells was necessary after about every 2 months because the cultivated cells age and do not express protein sufficiently anymore. One 1 mL vial of master seed lot, containing $5\text{-}10*10^6$ cell/mL was thawed.

Procedure

First 16 mL fresh F17 medium without antibiotics was warmed in a 125 mL shaker flask under agitation at 120 rpm in humidified 37°C and 5 % CO_2.

The frozen cells were thawed at 37 °C and immediately added into the prepared shaker flask. The starting density was $0.3*10^6$ cells/mL. The cells were not diluted until they reached a density of $1*10^6$ cells/mL, which should be after two to three days.

As soon as a cell doubling time of about 24 hours was established, the cells were cultivated in F17 medium with a final concentration of 50 µg/mL G418.

Material and equipment

FreeStyle™ F17 Expression Medium	Preparation see 3.2.2.1
Geneticin	Gibco

125 mL flask	Corning Incorporated Corning®
CO_2 incubator	Infors HT Multitron Cell
Haemocytometer	Neubauer improved Marienfeld, Germany
Microscope	Olympus CK2

3.2.2.4. THE SEED FLASK

A seed flask was maintained to grow cells for transfections. It was obtained of a vial of thawed master seed lot and was kept for about two months until the cells did not produce protein sufficiently any more. The seed flask was passaged regularly to keep the conditions perfect for the HEK 293-6E cells for growing.

Procedure

15-25 mL of cell culture were kept in a 125 mL shaking flask. The cells were passaged to keep the cell density below $1.8*10^6$ cells/mL and to provide them with fresh nutrition. Cells, which were passaged next in 48 hours, were diluted to a cell density of $0.45*10^6$ cells/mL (Mondays, Wednesdays). In case of cells, which were passaged next in 72 hours, were diluted to a cell density of $0.25*10^6$ to account for the 72 hour growth period.

Material and equipment

FreeStyle™ F17 Expression Medium	Preparation see 3.2.2.1

125 mL flask	Corning Incorporated Corning®
CO_2 incubator	Infors HT Multitron Cell

Haemocytometer	Neubauer improved Marienfeld, Germany
Microscope	Olympus CK2

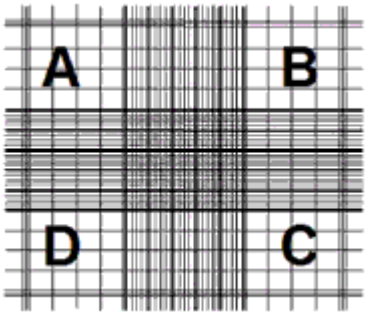
3.2.2.5. DETERMINATION OF CELL NUMBER

The cell number had to be determined in order to keep the cells within their optimal cell density between 0.2 and $2*10^6$ cells/mL. After counting the cells, the dilution factor was calculated.

Procedure

To determine the cell number a 100 µL aliquot was taken from the cell suspension. This aliquot was added to 400 µL of PBS and 100 µL Trypan Blue solution.

Then this mixture was applied to a haemocytometer and the cell number was determined:



The cells in squares A, B, C and D (10 000 mm², 100 nl) were counted using a counting watch. This number was divided by 4, because of the 4 squares counted, and multiplied by $6*10^4$ to calculate the cells per millilitres.

Figure 13: Hemocytometer

An example is given:

97 cells were counted in the four squares:

$$\frac{97}{4} * 6 * 10^4 = 1{,}45 * 10^6 \frac{cells}{ml}$$

The diluted density should be about $0.45*10^6$ cells/mL:

$$\frac{1{,}45 * 10^6 cells/ml}{0{,}45 * 10^6 cells/ml} = 3{,}2$$

The dilution factor was calculated to be **1:3** and therefore 8 mL of cell culture would be diluted with 16 mL fresh medium, which gives a total volume of 24 mL.

Material and equipment

FreeStyle™ F17 Expression Medium Preparation see 3.2.2.1

Flasks	Corning Incorporated Corning®
CO_2 incubator	Infors HT Multitron Cell
Hemocytometer	Neubauer improved Marienfeld, Germany
Microscope	Olympus CK2

3.2.2.6. TRANSFECTION OF HEK 293-6E CELLS

Transfection is introducing nucleic acids into eukaryotic cells, such as HEK cells. There are two types of transfection: stable transfection, where the foreign DNA is permanently incorporated into the eukaryotic genome, and transient transfection, where the eukaryotic cell holds the plasmid with the gene of interest only for a short amount of time until it gets degraded. The great advantage of transient transfection is the short time between transfection and protein production. Protein can be harvested already 24-96 hours after transfection. Picking stably transfected clones is work and time consuming, therefore stable transfection is preferably used for long term studies, large scale protein production or gene therapy, whereas transient expression is also ideal for protein mutant studies.

For peroxidasin constructs expression transient transfection was used. Midi prep DNA of the different constructs was prepared beforehand as described in 3.1.7.5. The DNA was then transferred into HEK 293-6E cells using Polyethyleneimine (PEI). PEI is an organic polymer, which contains a great amount of amino groups that can be protonated. It can bind very effectively to DNA and carry it through the cell membrane of eukaryotic cells (described under 1.3.1). A 25 kDa linear PEI variant was used for transient transfection.

Procedure

First the cell density was determined. The ideal cell density for transfection is approximately 1.7 x 10^6 cells/mL.

Small scale

For a 25 mL cell suspension transfection, a 12-well plate was used. For one transfection two wells are filled with 1.25 mL fresh medium. In one well 25 µg midi DNA in H_2O was added

and in the second well 50 µL PEI solution was added. Both was mixed thoroughly and the PEI/medium solution was added to DNA solution and the mixture was incubated for 10 minutes at RT. Afterwards the mixture was added to the flasks containing the cells, mixed carefully and returned to the incubator.

Approximately 20 hours later hematin was added to a final concentration of 5 µg/mL, which enhanced heme insertion in the peroxidasin variants. Since the solution is alkaline it was added dropwise under swirling. 48 hours later the cells were fed with 20 % tryptone N1 to a final concentration of 0.5 %.

Large scale

For a 2 l cell suspension transfection, a sterile 500 mL flask was filled with 100 mL fresh medium and 2 mg midi DNA in H_2O was added. In another sterile 250 mL flask 100 mL fresh medium was pipetted and 4 mL PEI solution was added. Both was mixed thoroughly and the PEI/medium solution was added to the DNA solution and the mixture was incubated for 10 minutes at RT. Afterwards the mixture was added to the flasks containing the cells, mixed carefully and returned to the incubator.

Approximately 20 hours later hematin was added to a final concentration of 5 µg/mL, which enhanced heme insertion in the peroxidasin variants. Since the solution is alkaline it was added dropwise under swirling. 48 hours later the cells were fed with 20 % tryptone N1 to a final concentration of 0.5 %.

Material and equipment

Preparation of 1 mg/mL Polyethyleneimine

It is important to work endotoxine free, so there had to be used endotoxine free materials. First, 0.5 g PEI was diluted in 450 mL cell culture water. PEI dissolved only at very low pH, so the pH has to be <2.0 which was achieved by using HCl. PEI dissolved in 2-3 hours, in which the pH had to be checked regularly. Once PEI was fully dissolved the pH had to be adjusted to 7.0 using NaOH and the solution was filled up to 500 mL with cell culture water. The last step was filtrating the PEI solution using a 22 µm filter and splitting up the solution in aliquots. The 25 kDa, linear PEI solutions was stored at 4°C for constant use and at -20°C for long term storage.

Polyethyleneimine	Sigma-Aldrich
Cell culture water	PAA
22 µm PVDF Rotilabo ® syringe filter, sterile	Roth

Preparation of 2 mg/mL hematin

Hematin solution was always freshly prepared before use. The necessary amount of hematin was dissolved in 0.1 M NaOH and filtered using a 22 µm filter.

Hematin	Sigma
NaOH	Sigma-Aldrich
22 µm PVDF Rotilabo ® syringe filter, sterile	Roth

Preparation of 20 % (w/v) tryptone N1

100 g tryptone N1 was dissolved in F17 medium containing 20 mL 200 mM L-glutamine and 10 mL Pluronic F68, but without antibiotics. It was stirred for about 2 hours until it was fully dissolved, filled up to 500 mL with F17 medium and then was filtered using a 22 µm filter. Afterwards the solution was split up in sterile 50 mL tubes and stored at 4°C.

20 % (w/v) tryptone N1	TekniScience
FreeStyle™ F17 Expression Medium	Gibco
100x Pluronic F-68, non-ionic surfactant	Gibco
200 mM L-glutamine	Gibco
22 µm PVDF Rotilabo ® syringe filter, sterile	Roth

FreeStyle™ F17 Expression Medium	Self prepared
Midi DNA	Self prepared
1 mg/mL PEI	Self prepared
2 mg/mL Hematin	Self prepared
20 % (w/v) Tryptone N1	Self prepared

3.2.2.7. COLLECTION OF CELL SUPERNATANT

The cells were harvested on the fifth day after transfection.

Procedure

The cell suspension was poured into centrifugal tubes and centrifuged at 6500 x g for 20 minutes. The cell pellets were discarded and the supernatant was filtered by using a 0.45 µm filter. To speed up the filtration vacuum was used.

Material and equipment

0.45 µm filter	Durapore® Membrane Filters, Millipore
Rotor	Thermo Sorvall SLA 1500
Centrifuge	Thermo Sorvall RC 6 Plus
Vacuum pump	Vacuubrand CVC2
pH meter	PHM92 LAB pH METER, Radiometer Copenhagen

3.2.2.8. PRODUCTION OF N-HIS6-TAGGED TEV PROTEASE

Tobacco Etch Virus (TEV) protease is a highly site-specific, highly active and stable protease, which recognizes a seven amino acid sequence and thus making it useful for removing affinity tags from fusion proteins.

Procedure

The pET-24d(+) vector containing the TEV protease gene and kanamycin resistance was transformed into the *E. coli* BL21 (as described in 3.1.6.2). Afterwards the bacteria was plated on LB_{KAN}-agar and incubated overnight at 37 °C. Two colonies were picked and inoculated in 2 x 4 mL LB_{KAN} medium and incubated overnight at 37 °C and agitation at 180 rpm. Two 2 L flasks were prepared with 2 x 0.5 L of LB_{KAN} medium and incubated until an OD_{600} of approx. 0.6 was reached. Then the cultures were cooled down on ice to 20 °C and induced by addition of 100 µL of 1 M IPTG to a final concentration of 0.2 mM. Afterwards, the cultures were incubated overnight at 20 °C and agitation at 180 rpm. The cells were harvested and stored at -80 °C until further use.

Material and equipment

1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG)

238 mg/mL IPTG in RO-H_2O

Sterile filtrated and stored at -20°C

pET-24d(+)

E. coli BL21	Invitrogen
LB_{KAN}-agar	Procedure see 3.1.6.2
Incubator	Memmert BE400
LB_{KAN}-medium	Procedure see 3.1.6.2
Shaker	IKA VIBRAX VXR
Spectrophotometer	Agilent-8453 UV-Visible System
IPTG	Sigma Aldrich

3.3. PROTEIN PURIFICATION

3.3.1. FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC)

Fast protein liquid chromatography (FPLC) works with a column filled with a matrix, the stationary phase, and a moving fluid, the mobile phase. FPLC has a broad range of different chromatography modes, such as ion exchange and gel filtration, which are the most popular ones, but also hydrophobic interaction and reverse phase. Additional to the different usage it can also deal with a wide range of aqueous, biocompatible buffer systems (25).

The cell supernatant containing the respective expressed protein variant was applied onto the column and the protein bound to the column, due to its affinity to the matrix. For Kon1-6 a StrepTrap HP column for the fusion-proteins Kon1f-Kon6f a HiTrap Protein A column was used and for the TEV protease a His Trap FF column was used.

The 5 mL StrepTrap HP column was prepacked with Strep-Tactin Sepharose, which can be used to purify proteins containing a strep tag. Its binding capacity is about 5 mg StrepTactin/mL matrix.

The 5 mL HiTrap Protein A FF column was a prepacked Protein A Sepharose Fast Flow column with a binding capacity of 15 mg human IgG/mL matrix. The Fc region of the IgG

binds to the protein A of the column and can thereby be separated from other proteins in supernatant.

The flow rate of the buffers and sample were controlled by the setting of the pumps and was chosen as low as possible to improve binding of the protein to the matrix.

Procedure

Depending on the purification, the corresponding column was chosen and installed in the FPLC. A flow rate up to 1 mL/min for a 1 mL column or 5 mL/min for a 5 mL column was chosen depending on the solution that was applied onto the column. For the protein supernatant a flow rate as low as possible was chosen. The high pressure had to be under 0.3 MPa and absorbances at 280 nm and 410 nm were selected for the hsPxd01 variants and 280 nm only for the TEV protease.

3.3.1.1. Purification of the Strep-Tag Proteins Kon1-Kon6

Procedure

The purification was performed by using a 5 mL StrepTrap HP column. The column was stored in 20 % EtOH, which was removed by filtrated RO-H_2O (5-10 column volumes). Afterwards the column was equilibrated using 100 mM phosphate buffer, pH 7.4 (5-10 column volumes). Then the filtrated cell supernatant was applied as slowly as possible, while the sample was cooled on ice. The column was washed with 100 mM phosphate buffer, pH 7.4 (5-10 column volumes) once again and the protein was eluated using 1 mM desthiobiotin in 100 mM phosphate buffer, pH 7.4. 1 mL fractions were collected and the column regenerated by washing with H_2O, followed by 0.5 M NaOH (2.5-5 column volumes) and finally with H_2O once again. The column was stored in 20 % EtOH at 4°C.

Material and Equipment

1 mM desthiobiotin in 100 mM phosphate buffer

0,214 g	Desthiobiotin	Sigma Aldrich
Filled up to 1 L with 100 mM phosphate buffer, pH 7.4		

RO-H_2O	
100 mM phosphate buffer, pH 7.4	Self prepared
1 mM desthiobiotin in 100 mM phosphate buffer, pH 7.4	Self prepared
0.5 M NaOH	Self prepared

FPLC	Amersham Pharmacia Biotech
1 mL StrepTrap HP column	GE Healthcare

3.3.1.2. PURIFICATION OF THE FUSION-PROTEINS KON1F-KON6F

Procedure

The purification was performed by using the HiTrap protein A FF column. The column was stored in 20 % EtOH, which had to be removed by filtrated RO-H_2O (5-10 column volumes). Afterwards the column was equilibrated using 100 mM phosphate buffer, pH 7.4 (5-10 column volumes). Before application onto the column the cell supernatant was filtrated and added with 10 % (v/v) 10 x PBS and the pH was set at 7.2 with 200 mM phosphate buffer, pH 8.0 or 8 M NaOH. Then the sample was applied onto the column as slowly as possible. Depending on the volume the application was also done over night. The sample was cooled during the whole application. This step was followed by washing the column with 100 mM phosphate buffer, pH 7.4 (5-10 column volumes). Then the protein was eluated using 0.1 M glycine, pH 3.0 as elution buffer. 1 mL fractions were collected and mixed with 8 µL 2 M Tris buffer, pH 12 immediately, to neutralize the acidic fractions to prevent the protein's denaturation. After eluation the column had to be regenerated by using 0.1 M glycine, pH 2.0 (about 2 column volumes), followed by neutralizing with 100 mM phosphate buffer, pH 7.4 (5-10 column volumes). Then the column was washed with RO-H_2O and stored in 20 % EtOH at 4°C.

Material and Equipment

0.1 M Glycine

7.5 g	Glycine	Sigma Aldrich
Filled up to 1 L with RO-H_2O		
pH 2 and 3respectively; pH adjusted with HCL		

2 M Tris, pH 12

24,2 g	Tris	Roth
Filled up to 100 mL with RO-H_2O		
pH 12 was adjusted with NaOH		

RO-H_2O	
100 mM phosphate buffer, pH 7.4	Self prepared
0.1 M glycine, pH 3.0	Self prepared
0.1 M glycine, pH 2.0	Self prepared
2 M Tris, pH 12.0	Self prepared
10x PBS without Ca and Mg	PAA The Cell Culture Company
200 mM phosphate buffer, pH 8.0	Self prepared
FPLC	Amersham Pharmacia Biotech
5 mL HiTrap Protein A FF column	GE Healthcare

3.3.1.3. PURIFICATION OF TEV PROTEASE

For purification a 1 mL His Trap FF column was used. The column was stored in 20 % EtOH, which was removed by filtrated RO-H_2O (5-10 column volumes). Then the column was equilibrated using binding buffer (5-10 column volumes) charged with 100 mM $NiSO_4$.

Meanwhile, the pellet stored in -80°C (preparation described under 3.2.2.8) was thawed and resuspended in 30 mL lysis buffer. Then the cells were broken using a sonicator and the lysate was centrifuged at 18 000 rpm for 10 min. Imidazole was added to a final concentration of 20 mM from a 2 M stock solution to the supernatant. The sample was applied to the equilibrated column. Then the column was washed with binding buffer (10-15 column volumes) until no protein elutes anymore, which was monitored by the absorbance at 280 nm. Then binding buffer containing 50 mM imidazole + 10 % elution buffer was used to wash the column a second time (10-15 column volumes). The protein was eluted with elution buffer and the eluate was collected in 50 mL tubes, with 20 mL of dialysis buffer. The eluate was dialyzed overnight against 1 L of storage buffer. The solution was stored in 0.5 mL aliquots at -80 °C.

The column was stripped with 50 mM EDTA (5-10 column volumes) to remove all Ni^{2+} ions , was washed with RO-H_2O (5-10 column volumes) and loaded with 0.1 M $NiCl_2$. Once again, the column was washed with RO-H_2O (5-10 column volumes) and it was stored in 20 % EtOH at 4°C.

Material and equipment

1 M Tris-HCl

121.14 g	Tris	Roth
Fill up 1 L	RO-H_2O	

HCl (AppliChem) was used to adjust to pH 8

Lysis Buffer

50 mM	Tris-HCl, pH 8.0	
300 mM	NaCl	Sigma-Aldrich
0.2 % (v/v)	Igepal CA-630	Sigma-Aldrich
1.5 mM	β-mercaptoethanol	Sigma

Adjusted to pH 8

Binding Buffer

50 mM	Tris-HCl, pH 8.0	
300 mM	NaCl	Sigma-Aldrich
20 mM	Imidazole	Sigma-Aldrich
0.01 % (v/v)	1-thioglycerol	Sigma-Aldrich
20 % (v/v)	Glycerol	Sigma

Adjusted to pH 8

Elution Buffer

50 mM	Tris-HCl, pH 8.0	
300 mM	NaCl	Sigma-Aldrich
300 mM	Imidazole	Sigma-Aldrich
0.01 % (v/v)	1-thioglycerol	Sigma-Aldrich
20 % (v/v)	Glycerol	Sigma

Adjusted to pH 8

Dialysis/ Storage Buffer

50 mM	Tris-HCl, pH 8.0	
150 mM	NaCl	Sigma-Aldrich
0.01 % (v/v)	1-thioglycerol	Sigma-Aldrich
50 % (v/v)	Glycerol	Sigma

Adjusted to pH 8

Centrifuge	Thermo Sorvall RC 6 Plus
Rotor	Sorvall SS-34
5 mL His Trap FF Column	GE Healthcare

3.3.2. Concentration of Protein Solutions

To minimize the volume of the purified protein solution and therefore, to concentrate the protein, different kinds of methods can be used, like concentration using centrifugal filters or polyethylene glycol. If centrifugal filters are used, the salt concentration can be lowered and the buffer can be changed as well by diluting the solution again and again with the respective buffer. Polyethylene glycol dehydrates the solution only by osmosis.

3.3.2.1. Centrifugal Filters

Centrifugal filters by Millipore called Centripreps were used. Depending on the size of the protein, Centripreps with different molecular mass cut off membranes (MWCO) were used

(30 kDa, 50 kDa or 100 kDa). The protein solution was applied onto the sample container and due to centrifugation substances with a lower molecular mass than the MWCO of the used Centriprep were removed through the membrane. Substances with a higher molecular mass were kept in the sample container.

Procedure

Fractions of eluated protein samples were pooled and applied onto the sample container of the Centriprep. Then, the sample was centrifuged at 4 500 rpm for 10-15 minutes at 4°C, depending on the concentration of the protein solution. The flowthrough was discarded and this step was repeated until the desired volume for the protein solution was reached.

Material and Equipment

Centrifugal filters	Millipore, Amicon® 4 and 15 Ultra Centrifugal Filters 30K, 50K, 100K
Rotor	Thermo Sorvall SLA 1500
Centrifuge	Thermo Sorvall RC 6 Plus

3.3.2.2. CONCENTRATION USING POLYETHYLENE GLYCOL

Polyethylene glycols (PEGs) are synthetic, water-soluble polymers, with a variety of different molecular weights. PEGs with molecular weights of 200-600 g/mol are clear, viscous liquids, while PEGs with molecular weights of 1 500-4 000 g/mol are white powders and PEGs above 4 000 g/mol are available as creamy white flakes (26). For concentration of protein solutions PEG of 20 000 g/mol was used. PEGs show characteristics as complete solubility in water, good stability, low toxicity levels and wide compatibility with other substances (26). Another important trait is its high hygroscopicity (26), which can be used to concentrate solutions.

Procedure

The pooled fractions were filled into a dialysis tube and placed into a vessel, and the tube was completely surrounded by polyethylene glycol. After 1-2 hours, depending on the degree of the desired concentration, the dialysis tube with the concentrated protein solution was wasted thoroughly with RO-H_2O to remove all remaining polyethylene glycol.

Material and Equipment

SnakeSkin Dialysis Tubing, 3.5K MWCO	Thermo Scientific
Polyethylene glycol, 20 000	Roth

3.3.3. Dialysis

Dialysis works on the basis of diffusion of solutes through a semi-permeable membrane with defined sizes of pores. Thereby, substances have the urgency to move from higher concentrations to lower concentrations. Due to this, solutions can be desalted easily and only substances smaller than the pores can pass the semi-permeable membrane.

Procedure

After concentration of the protein solution it was dialysed to desalt the sample and to exchange the buffer. Thereby, the sample was always dialysed 1:100 in the respective dialysis buffer. The dialysis tube, which was filled with the protein solution for concentration using PEG 20 000, as described above, was put into the dialysis buffer. After 2-4 hours the dialysis buffer was renewed and dialysis was performed overnight.

Material and Equipment

100 mM phosphate buffer, pH 7.4	Self prepared
SnakeSkin Dialysis Tubing, 3.5K MWCO	Thermo Scientific

3.3.4. Lyophilisation

Lyophilisation is used for dehydration of samples. Thereby, aqueous or organic solvents evaporate in vacuum in combination with centrifugal force. Evaporation can be accelerated when heat is used.

Procedure

The protein solution was pipetted into lyophilisation tubes and put into the centrifugal evaporator without caps. The apparatus was started including heating and lyophilisation was performed for 4-6 hours until the protein sample was dry. The samples were stored at -20°C until further use.

Material and Equipment

Lyophilisation tubes

SpeedVac Concentrator SVC 100H Savant

3.3.5. TEV Digestion

To remove the F-domain from the fusion-proteins, TEV protease was used. It recognizes a seven amino acid sequence and thus is useful for removing affinity tags from fusion proteins.

Procedure

Digestion took place in a glutathione buffer, called TEV buffer. Hence, the following reagents were mixed together:

Table 29: Reagents for TEV buffer

50 mM	Tris-HCl, pH 8.0
0.5 mM	EDTA
3 mM	Reduced Glutathione
0.3 mM	Oxidized Glutathione

723.8 µg Kon3f was dissolved in 700 µL TEV buffer, centrifuged to remove insoluble protein and the concentration of the protein was calculated spectrophotometrically.

Table 30: Information on Kon3f

V [µL]	700
Concentration [µM]	7.13
Amount [µg]	723.8
Purity number	0.66

Different TEV to protein ratios were chosen (1:10, 1:20 and 1:50) and the respective amounts of protein and TEV were mixed and incubated at RT for 3 hours.

Table 31: Amounts of used TEV and fusion-protein

	[12.8 µM] TEV		[3.38 µM] Protein	
1:10	16 µg	17.8 µL	160 µg	155 µL
1:20	8 µg	8.9 µL	160 µg	155 µL
1:50	3.2 µg	3.6 µL	160 µg	155 µL

To analyze which TEV to protein ratio was best to use and how fast the TEV protease worked, aliquots were taken at the beginning and after 1, 2 and 3 hours to perform SDS-PAGE and Western Blot, as described in 3.4.1 and 3.4.3.

Material and Equipment

Tris-HCl, pH 8.0	Self prepared
Kon3f	Self prepared
TEV protease	Self prepared
EDTA	Sigma
Reduced Glutathione	Sigma
Oxidized Glutathione	Sigma

Spectrophotometer	Agilent-8453 UV-Visible System
Quartz cuvette	

3.4. PROTEIN CHARACTERIZATION

3.4.1. SDS-POLYACRYLAMIDE-GEL-ELECTROPHORESIS (SDS-PAGE)

Many biomolecules are charged and are able to move in an electrical field. The ion's ability to move depends on charge, size, shape of the molecule and the medium. These differences are the basics of electrophoresis.

Depending on their net-charge, ions are able to move towards the negative pole, which is called cathode, or the positive pole, the anode. The separation can either take place in solution (carrier-free electrophoresis) or in a large-pored matrix. A matrix is made of an inert and homogenous gel of polyacrylamide. The gel prevents temperature-gradients and heat-transfer, which occurs due to movement of the particles. Furthermore, there is hardly diffusion in a gel which results in good visible bands of the separated proteins after electrophoresis.

A further important component is sodium dodecylsulfate (SDS), which is an anionic detergens. It binds to proteins and charges them highly negative, makes proteins lose their biological activity, makes almost all proteins soluble and prohibits all non-covalent interactions within and between polypeptide chains, which results in complete unfolding of the protein. Dithiothreitol (DTT), a strong reducing agent, is used to reduce inter- and intramolecular disulfide bonds.

After electrophoresis the protein bands can be made visible by using various staining methods, like Coomassie Blue staining or silver staining.

Procedure

The samples were mixed with 4x loading dye and 0.5 mM dithiothreitol (DTT) was added if reduction of the proteins was desired. All samples were heated at 95°C for 5 minutes. Meanwhile, a 10 or 15 well ready-to-use gel was put into the electrophoresis apparatus and running buffer was poured into the chamber. Samples were applied to the wells and the electrophoresis was started. The running conditions were 200 V for about 50 minutes.

Material and Equipment

0.5 mM Dithiothreitol (DTT)

0,078 mg/mL	DTT in RO-H_2O

Running buffer

760 mL	RO-H_2O
40 mL	20x NuPAGE Running Buffer
Total 800 mL	

LDS Sample Buffer (4x)	Novex
4-12 % Bis-Tris Gel	Novex
3-8 % Tris-Acetate Gel	Novex
20x NuPAGE MOPS Running Buffer	Novex
20x NuPAGE Tris-Acetat Running Buffer	Novex
DTT	Sigma-Aldrich
Power Supply	BioRad
Electrophoresis equipment	BioRad
Shaker	IKA VIBRAX VXR

3.4.2. Staining Solutions

3.4.2.1. Coomassie Blue R-250 solution

After SDS-PAGE, the gel was stained in Coomassie Blue R-250 solution for 45 minutes. Afterwards the gel was put into Coomassie destain solution to make the bands visible.

Material and Equipment

Coomassie staining solution

0.1 % (w/v)	Coomassie Blue R-250	
40 % (v/v)	Methanol	Roth
10 % (v/v)	Acetic acid	Roth
50 % (v/v)	RO-H_2O	

Coomassie destain solution

40 % (v/v)	Methanol	Roth
10 % (v/v)	Acetic acid	Roth
50 % (v/v)	RO-H_2O	

Shaker	IKA VIBRAX VXR

3.4.2.2. Silver staining

Silver staining is a more sensitive method (for low ng range) to detect proteins separated by SDS-PAGE than Coomassie Blue staining is. Thereby silver binds on the negatively charged amino acid side chains of proteins and is reduced to free metallic silver, which is visualized as dark brown bands on the gel.

Procedure

After finishing the SDS-PAGE, the gel was put in fixing solution for 30 minutes, washed with RO-H_2O and incubated in incubation solution for 15 minutes. Afterwards, the gel was washed 3 x 5 minutes with RO-H_2O and then the gel was stained with staining solution for 10 minutes. The gel was washed shortly with RO-H_2O and developed in the developing solution until bands were seen. To stop the reaction the gel was put into stopping solution.

Silver staining can also be used after dying the gel with Coomassie Blue staining by skipping the fixation step and starting directly with incubating the gel in incubation solution.

Material and Equipment

Fixing solution

357 mL	Ethanol 70 (v/v)%	VWR
50 mL	Acetic acid (glacial) 100%	Roth

Filled up to 500 mL with AD

Incubation solution

214 mL	Ethanol 70 (v/v)%	VWR
1.57 g	Sodium thiosulfate pentahydrate	Merck
34 g	Sodium acetate	Roth

Filled up to 500 mL with AD

Before using 62.5 µL glutaraldehyde is added to 25 mL incubation solution.

Staining solution

0.5 g Silver nitrate Sigma

Filled up to 500 mL with AD

Before using 5 µL formaldehyde is added to 25 mL staining solution.

Developing solution

12.5 g Sodium carbonate Sigma-Aldrich

Filled up to 500 mL with AD

Before using 5 µL formaldehyde is added to 25 mL developing solution.

Stopping solution

7.89 g Titriplex® III Sigma-Aldrich

Filled up to 500 mL with AD

Shaker IKA VIBRAX VXR

3.4.3. WESTERN BLOT

The Western Blot is a very sensitive method for immunochemical detection of small amounts of protein. After separation of proteins via electrophoresis the proteins are blotted from the gel onto the membrane in an electrical field. To do so filter papers, nitrocellulose membrane, the gel and transfer buffer was needed according to the following scheme:

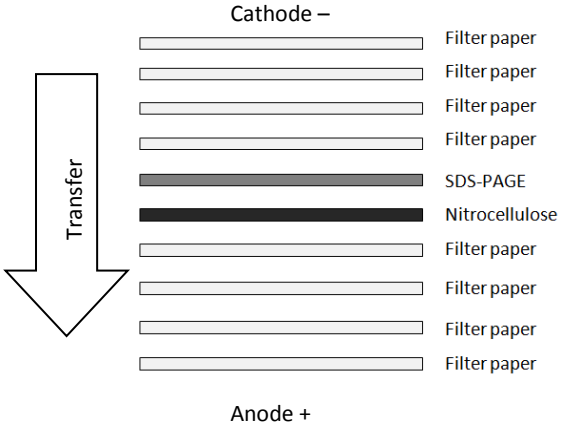


Figure 14: Assembly of a semi-dry Western Blot

Procedure

First, the nitrocellulose membrane and the filter papers were cut to appropriate sizes. The gel was equilibrated in 2x transfer buffer for about 10 minutes. Then four layers of filter paper soaked in 2x blotting buffer, the membrane, the gel and another four pieces of filter papers according to Figure 14 were assembled in the Western Blot apparatus. The staple was soaked in transfer buffer and blotted for 50 minutes at 20 V and RT.

Afterwards the blotted membrane was incubated in 20 mL blocking buffer for 45 minutes. Then the blocking buffer was discarded and the membrane was soaked in binding buffer with the respective antibody to detect the protein of interest. Depending on construct different antibodies where added, which can be seen in the following table:

Table 32: Used primary antibodies for Western Blot

Primary antibody	Binding site	hsPxd01 variants	Secondary antibody	Detection System
Mouse Anti Human IgG CH3 Domain (1:5 000)	Fc-domain	Kon1f-Kon6f	Anti mouse (1:20 000)	Alkaline phosphatase
Anti human PXDN antibody (produced in rabbit) (1:500)	Peroxidasin	All variants	Anti rabbit produced in goat (1:30 000)	Alkaline phosphatase
StrepMAB-classic-HRP mab and Strep-tag HRO conjugate (1:4 000)	Strep tag	Kon1-Kon6	-	Horseradish peroxidase

The membrane was incubated with the respective antibody for 1.5 hours at RT on the shaker. Then the membrane was washed two times with PBST, two times with PBS and one time with RO-H_2O for 10 minutes each. Afterwards the membrane was either developed with the respective developing reagents, which can be seen in Table 33, or the membrane was incubated in binding buffer with the respective second antibody for further 1.5 hours, washed as described before and then developed.

Table 33: Developing systems

Alkaline phosphatase		Horseradish peroxidase	
5 mL	AP Buffer	5 mL	HRP Chromogenic Substrate (TMB)
33 µL	NBT		
16.5 µL	BCIP		

Material and Equipment

2x Transfer Buffer

100 mL	20x Transfer Buffer NuPAGE Tris Acetat	NuPAGE
100 mL	Methanol	Roth
800 mL	RO-H_2O	

PBS Buffer (phosphate buffered saline)

8 g	NaCl	Sigma-Aldrich
0.2 g	KCl	Sigma-Aldrich
2.73 g	Na_2HPO_4*7 H_2O	Sigma-Aldrich
0.24 g	KH_2PO_4	Roth

Filled up to 1 L with AD

pH adjusted to pH 7.4 with HCl or NaOH

PBST Buffer

0.02 % (v/v) Tween 20 (Merck) was added to PBS

Blocking Buffer

2 g	Bovine serum albumin (BSA)	Sigma-Aldrich

Filled up to 100 mL with PBS

Binding Buffer

2 g	Bovine serum albumin (BSA)	Sigma-Aldrich

Filled up to 100 mL with PBST

AP (alkaline phosphatase) buffer

1.21 g	Tris	Roth
5.88 g	NaCl	Sigma-Aldrich
1.02 g	$MgCl_2$	Sigma-Aldrich

Filled up to 1 L with AD

pH adjusted to pH 9.5

Nitrocellulose membrane: BA 45; 0.45 µm	Schleicher & Schuell
Filter paper	Whatman 3MM
Power Supply	BioRad
Western Blot apparatus	Invitrogen
Shaker	IKA VIBRAX VXR
Mouse Anti Human IgG CH3 Domain	BioRad
Anti Mouse	Sigma
Anti human PXDN antibody	
StrepMAB-classic-HRP mab and Strep-tag HRO conjugate	AbD Serotec
AP buffer	Self prepared
Nitro blue tetrazolium (NBT)	BioRad
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	BioRad
Novex® HRP Chromogenic Substrate (TMB)	Invitrogen

3.4.4. ENHANCED CHEMILUMINESCENCE

Covalently linked heme can be easily detected by enhanced chemiluminescence (ECL) (4). Thereby, electrical energy is converted into light, which amount gets measured. The substrate luminol reacts with the iron of the heme prosthetic group and thus, emits light. Only covalently linked heme can be detected because non-covalently bound heme is lost during the SDS-PAGE.

Procedure

After performing an SDS-PAGE, as described in 3.4.1, the proteins were transferred onto a membrane by Western Blot, as described in 3.4.3. The membrane should not dry and thus was kept in 1x PBS until the ECL measurement.

1 mL ECL solution was prepared by mixing Clarity™ Western ECL Substrate Peroxide Solution and Clarity™ Western ECL Substrate Luminol/enhancer solution (BioRad) 1:2. Then the

membrane was placed onto a transparent plastic foil and covered with the prepared ECL solution. Another transparent plastic foil was used to cover the moistened membrane and the membrane was put into the ECL apparatus (ChemiDoc™ MP System by BioRad), where the emitted light was measured.

Material and equipment

Clarity™ Western ECL Substrate Peroxide Solution	BioRad
Clarity™ Western ECL Substrate Luminol/enhancer solution	BioRad
ChemiDoc™ MP System	BioRad

3.4.5. Enzyme Activity

To determine peroxidase activity various peroxidase assays, like the TMB assay or ABTS peroxidase assay were used. Thereby, TMB or ABTS function as substrates. The reaction was started with hydrogen peroxide and if activity exists, the formation of oxidized substrate was followed over time.

3.4.5.1. Calculation of Protein Yield

The protein solution was measured by spectrophotometry from a wavelength of 200-800 nm and then its concentration and purity number (RZ) were calculated using Lambert-Beer's law:

$$A = \varepsilon * c * d$$

A Absorbance

ε molar extinction coefficient [M^{-1} cm^{-1}]

c concentration [mol/L]

d cuvette diameter [cm]

$$RZ = A_{heme}/A_{280}$$

RZ purity number

A_{heme} Absorbance of Soret peak

A_{280} Absorbance of protein peak

Furthermore, the mass of protein can be calculated using the concentration and the molecular mass :

$$m = c * M * V$$

m mass [g]

c concentration [mol/L]

M molecular mass [g/mol]

V Volume [L]

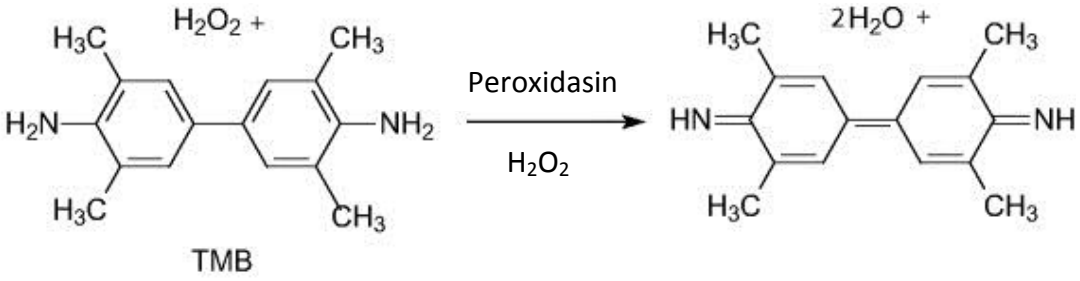
Material and equipment

Spectrophotometer Agilent-8453 UV-Visible System

Quartz cuvette

3.4.5.2. TMB Assay

To determine peroxidase activity by the ability to oxidize tetramethylbenzidine (TMB), the TMB assay was used. Thereby, the absorption at 655 nm at RT was measured either after a certain amount of time or to determine the rate of the reaction.



colourless blue

Figure 15 Oxidation of TMB by hsPxd01

Procedure

The following solutions were mixed together in a plastic cuvette:

Table 34: Reaction mix for TMB assay

Stock solution	Volume	Final concentration
200 mM Sodium acetate buffer pH 5.4	850 µL	170 mM
20 mM TMB in DMF	100 µL	2 mM
Enzyme	Different volumes were used	500 nM-1µM
8 mM H_2O_2	25 µL	0.2 mM
Total	**1000 µL**	

H_2O_2 was added last to start the reaction and the colour was detected within 5 minutes.

Material and equipment

20 mM Tetramethylbenzidine (TMB)

4,8 mg/mL	TMB in DMF

Sodium acetate	Roth
TMB	Sigma-Aldrich
DMF	Sigma-Aldrich
H_2O_2	Sigma-Aldrich

Plastic cuvette	Sarstedt
Spectrophotometer	Zeiss Specord UV VIS S10

3.4.5.3. ABTS Assay

ABTS peroxidase assay is another possibility to determine peroxidase activity. Thereby, hydrogen peroxide is reduced to water while the substrate ABTS is oxidised and forms a green coloured product. This reaction is peroxidase catalyzed and the increase of absorption is measured at 414 nm at RT.

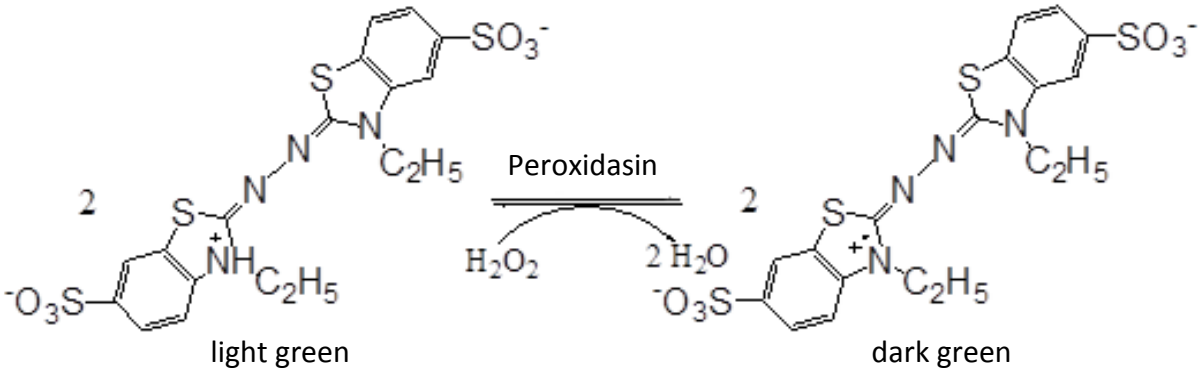


Figure 16: Oxidation of ABTS by hydrogen peroxide

Procedure

A cuvette with a stirring bar was used and all following solutions, except H_2O_2, were mixed together:

Table 35: Reaction mix for ABTS assay

Stock solution	Volume	Final concentration
200 mM phosphate buffer pH 5.0 or pH 7.4	500 µL	100 mM
50 mM ABTS	20 µL	1 mM
Enzyme	Different volumes were used	500 nM-1µM
RO-H_2O	Fill to 1 mL	
10 mM H_2O_2	10 µL	100 µM
Total	**1000 µL**	

The cuvette was put into the spectrophotometer and the reaction was started by adding H_2O_2.

Material and equipment

200 mM phosphate buffer pH 5.0 or pH 7.4	Self prepared
ABTS	Sigma
H_2O_2	Sigma-Aldrich

Quartz cuvette	QA 10,00 mm
Spectrophotometer	Hitachi UV-3900

3.4.5.4. HALOGENATION ASSAY

The halogenation assay is used to verify if an enzyme produces HOCl or HOBr, respectively, oxidizing the corresponding halogen Cl^- or Br^- respectively, which is described in 1.2.3. If so, HOCl/ HOBr reacts with taurine and forms taurinechloramine or taurinebromamine, which oxidises the TMB in the developer solution, which can be seen by blue colouring of the solution. Taurinechloramine needs potassium iodide for this reaction, whereas taurinebromamine does not.

Procedure

The following solutions were mixed together, whereby H_2O_2 was added last to start the reaction:

Table 36: Rection mix for Halogenation assay

Stock solution	Volume	Final concentration
200 mM phosphate buffer, pH 7.4	500 µL	100 mM
1 M NaCl/KBr	100 µL	100 mM
Enzyme	Different volumes were used	1 µM
100 mM taurine	100 µL	5 mM
Total	**Filled up to 980 µL with H_2O**	
10 mM H_2O_2	20 µL	200 µM

The bromination absorption was measured at 289 nm and the chlorination absorption was measured at 252 nm.

Material and equipment

200 mM phosphate buffer, pH 7.4	Self prepared
NaCl	Sigma-Aldrich
Potassium bromide	Merck
Taurine	Sigma
H_2O_2	Roth
Sodium acetate	Roth
TMB	Sigma-Aldrich
Potassium iodide	Merck

Quartz cuvette	QA 10,00 mm
Spectrophotometer	Hitachi UV-3900

4. RESULTS

4.1. CLONING OF THE CONSTRUCTS

4.1.1. PTT5+ VECTOR PREPARATION

The complete pTT5 vector contains 4 401 basepairs (bp). By using the enzymes *EcoRI* and *BamHI* the vector was digested and its MCS of 73 bp was removed. The digested pTT5 vector was CIP treated to prevent its religation by dephosphorylation of the 5' and 3' ends. Thus, the plasmid was prepared for the incorporation of the synthesized insert. To purify the digested vector agarose gel electrophoresis was performed. The first lane shows the digested linearized vector without its MCS of 4 328 bp in length (Figure 17).

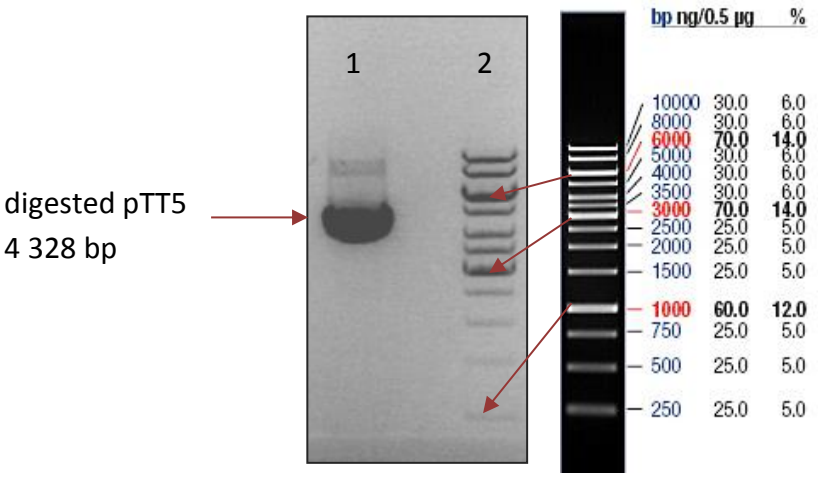


Figure 17: pTT5 digestion Lane 1: digested pTT5 vector; lane 2: 1 kb DNA ladder

In order to obtain the pTT5+ vector, the MCS of the pTT5 vector was replaced by the synthesized insert. To ligate this insert of 131 bp into the vector it was prepared by digestion with *EcoRI* and *BamHI*. The digested insert was GFX purified and then ligated into the prepared pTT5 vector to finally obtain the pTT5+ vector of 4 459 bp.

After ligation the pTT5+ plasmid was transported into *E. coli Top10* by electroporation. Colonies were picked and screened to verify if the cells contained the vector including the insert. Finally, the obtained pTT5+ plasmid was sequenced to verify that the vector and insert were correctly ligated.

4.1.2. Preparation of DNA Constructs Kon1-6

Each construct DNA sequence was PCR amplified using the respective forward and revers primers and the pcDNA™3.1/V5-His TOPO was used as template to provide the DNA sequence of hsPxd01. In order to separate the PCR products of the correct size from non-specific products and the template the PCR mix was subjected to agarose gel electrophoresis (Figure 18-Figure 20).

Table 37: Length of PCR construct DNA

Kon1	4 359 bp	**Kon4**	3 207 bp
Kon2	3 702 bp	**Kon5**	2 085 bp
Kon3	3 864 bp	**Kon6**	1 779 bp

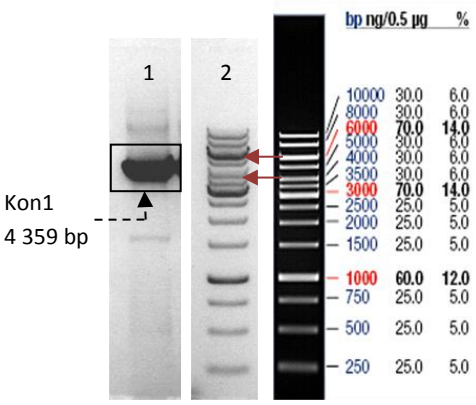


Figure 19: Agarose gel electrophoresis of Kon1 PCR product Lane 1: Kon1; lane 2: 1 kb DNA ladder

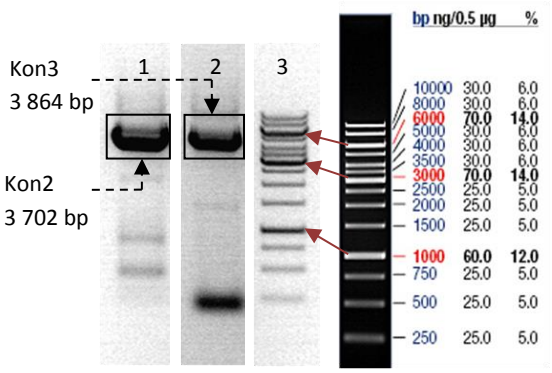


Figure 18: Agarose gel electrophoresis of Kon2 and Kon3 PCR product Lane1: Kon2; lane 2: Kon3; lane 3: 1 kb DNA ladder

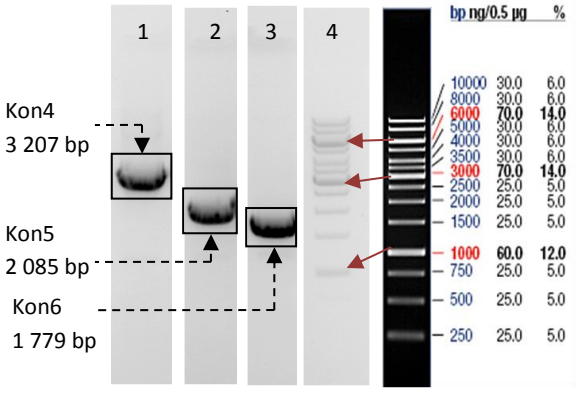


Figure 20: Agarose gel electrophoresis of Kon4, Kon5 and Kon6 PCR product Lane 1: Kon4; lane 2: Kon5; lane 3: Kon6; lane 4: 1 kb DNA ladder

Agarose gel purified, *NheI* and *KpnI* digested and GFX purified PCR products were ligated into the pTT5+ vector, that was also digested with *NheI* and *Kpn*, CIP treated and GFX purified. The ligation mix was electroporated into *E. coli Top10.* Colonies were picked and screened to confirm that the plasmid contained the construct DNA.

In order to confirm that the construct DNA was correctly amplified and inserted into the pTT5+ vector, mini prep DNA of pTT5+ Kon1-Kon6 was sequenced. Plasmid DNA was mixed with one specific primer as shown in Table 20. To cover the whole DNA sequence, a series of different primers had to be used. Afterwards the obtained data was compared with the expected sequences to verify their correctness.

4.1.3. Preparation of Fusion Protein DNA Constructs Kon1f-6f

For the fusion proteins Kon1f-6f the **F**ragment **c**rystallisable region (Fc-domain) was inserted into the already designed pTT5+ Kon1-6. Thereby, the Fc-domain was PCR amplified by using the pYD11 vector containing the Fc-gene as template and the respective forward and reverse primers. The Fc-domain is composed of the C_H3 and C_H2 region including the Hinge region of the immunoglobulin G and if of 696 bp in length. The PCR product was purified by agarose gel electrophoresis and digested with *KpnI* and *NotI* (Figure 21).

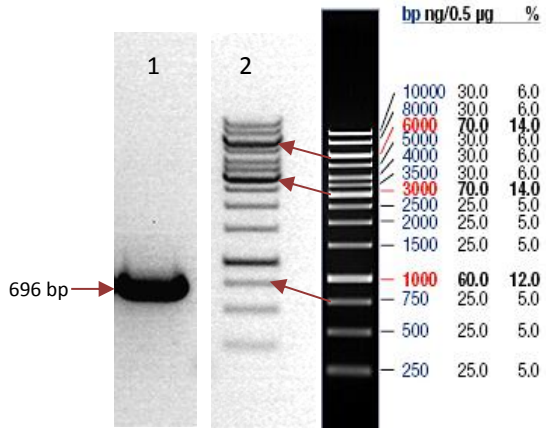


Figure 21: Agarose gel electrophoresis of Fc PCR product; Lane 1: Fc-domain; Lane 2: 1 kb DNA Ladder

Before ligation of the Fc-DNA with the constructs Kon1-6, the respective constructs were digested with *KpnI* and *NotI* as well. To prevent re-ligation of the plasmid, CIP was used. In

Table 38 the length of the fusion-protein DNA construct is shown. After electroporation into *E. coli Top10* cells, the fusion-proteins were labelled Kon1f-Kon6f.

Table 38: Length of fusion-protein DNAy

Kon1f	5 055 bp	**Kon4f**	3 903 bp
Kon2f	4 398 bp	**Kon5f**	2 781 bp
Kon3f	4 560 bp	**Kon6f**	2 475 bp

To verify that the insertion of the fusion-protein DNA into the pTT5+ Kon1-6 plasmids was correct, clones were picked for a PCR screening using the TEV IgG forward and TEV IgG reverse primers, followed by an agarose gel electrophoresis. In Figure 22 the successful incorporation of the Fc-domain in Kon1-4 is shown.

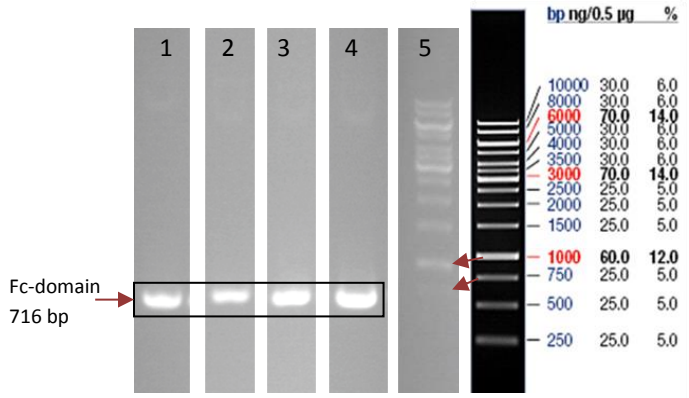


Figure 22: Verification of Fc-domain incorporation by PCR screening; Lane 1: Kon1f, lane 2: Kon2f, lane 3: Kon3f, lane 4: Kon4f, lane 5: 1 kb DNA ladder

Mini prep DNA of positive clones was extracted by performing a mini prep, followed by GFX purification.

Insertion of the Fc-PCR-DNA was also tested by digesting pTT5+ Kon1f-4f with *EcoRI* and *BamH*. The pattern of a theoretical digest of Kon1f-4f is shown in Table 39. Due to the different DNA sequences of the constructs, fragments of different sizes were observed. The obtained bands were compared with the expected ones and so the correctness of the generated constructs was verified.

Table 39: DNA fragments of *EcoRI* and *BamHI* digest of pTT5+ Kon1f-4f

	Kon1f	Kon2f	Kon3f	Kon4f	
pTT5+	4 459 bp	4 459 bp	4 459 bp	4 459 bp	**Length of fragments**
	1 993 bp	-	1 993 bp	-	
	1 502 bp	1 502 bp	1 502 bp	1 502 bp	
	1 339 bp	-	-	-	
	-	1 336 bp	-	1 336 bp	
	-	1 333 bp	-	-	
	-	-	844 bp	844 bp	
	318 bp	-	318 bp	318 bp	
	-	324 bp	-	-	

In Figure 23 *EcoRI* and *BamHI* digestion of pTT5+ Kon1f-4f can be seen on the agarose gel. Only the fragments between 4 459 bp to 1333 bp were visible. The smaller fragments were too small to be resolved on a 1% gel. Since the bands of 1336 bp and 1333 bp of Kon2f are too similar in size, only one band was observed.

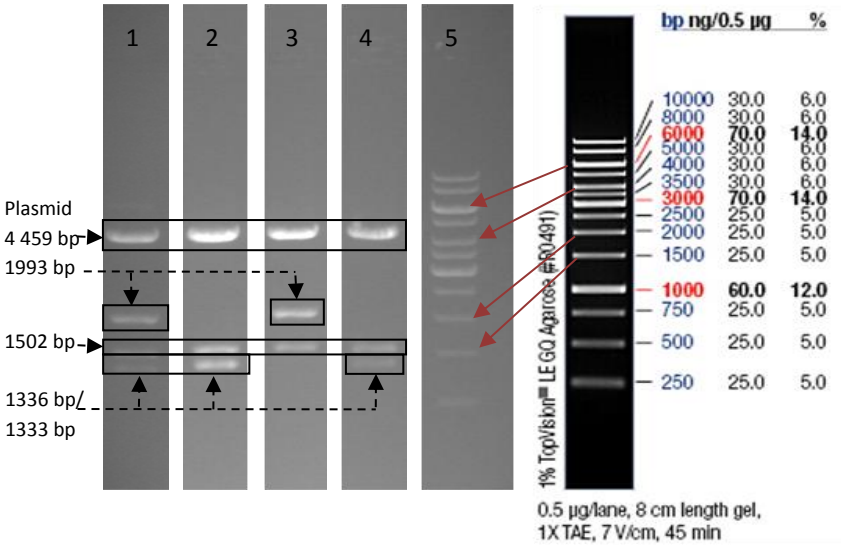


Figure 23: Agarose gel electrophoresis of *EcoRI* and *BamHI* digest of pTT5+ Kon1f-4f. Lane 1: Kon1f; lane 2: Kon2f: lane 3: Kon3f;lane 4: Kon4f; lane 5: 1 kb DNA ladder

As before, in order to confirm if the fusion-proteins were correctly generated without any mutations, DNA sequencing was used. The obtained data was compared with the expected sequences to verify their correctness.

4.2. Optimization of Protein Production and Purification

After generating all 12 constructs, 25 mL small scale transient expression was performed to test if the HEK 296-6E cells produce the respective peroxidasin variants. All constructs differ in molar mass, which is summarized in Table 40. SDS-PAGE and Western Blot were used to detect the variants in the cell supernatant.

Table 40: Molecular weights of all constructs

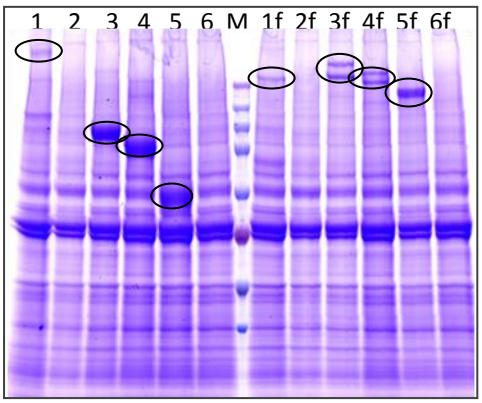
Theoretical size				Observed size		
Kon1	163 kDa	**Kon1f**	188 kDa	**Kon1**	489 kDa	**Trimer**
Kon2	138 kDa	**Kon2f**	163 kDa	**Kon2**	**No protein expression observed**	
Kon3	145 kDa	**Kon3f**	170 kDa	**Kon3**	145 kDa	**monomer**
Kon4	120 kDa	**Kon4f**	145 kDa	**Kon4**	130 kDa	**-**
Kon5	79 kDa	**Kon5f**	104 kDa	**Kon5**	90 kDa	**-**
Kon6	68 kDa	**Kon6f**	93 kDa	**Kon6**	**No protein expression observed**	

As the Hinge region of the Fc-domain contains four cysteine residues, the fusion-protein variants are expected to dimerize due to disulfide bridge formation.

To perform SDS-PAGE, 2 mL cell culture supernatant was 20x concentrated to a final volume of 100 µL using 30 kDa concentration tubes for Kon5, Kon5f, Kon6 and Kon6f, a 50 kDa concentration tube for Kon4 and Kon 4f and a 100 kDa concentration tube for Kon1-Kon3 and Kon1f-Kon3f. 15 µL sample was mixed with 5 µL 4x Loading Dye to perform SDS-PAGE. For reduction of the oligomers to monomers 10x DTT was added as well. All samples were heated at 70°C for 10 minutes and applied onto the gel (Figure 24 and Figure 25).

SDS-PAGE

non reduced



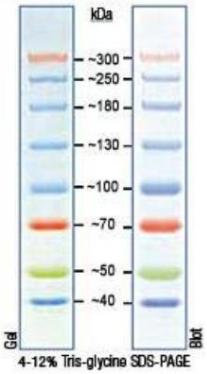


Figure 25: non reduced SDS PAGE of all constructs; lane 1-6: Kon1-Kon6; lane M: peqGOLD VII marker; lane 1f-6f: Kon1f-Kon6f

reduced

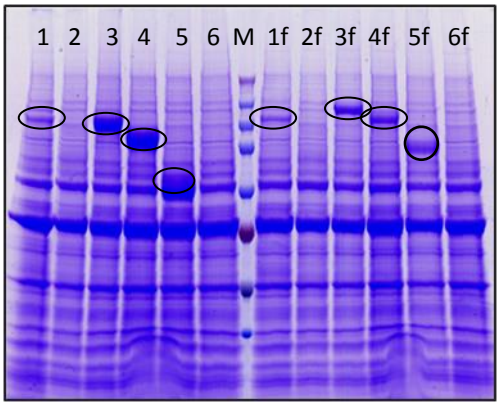


Figure 24: reduced SDS PAGE of all constructs; lane 1-6: Kon1-Kon6; lane M: peqGOLD VII marker; lane 1f-6f: Kon1f-Kon6f

To detect the proteins on the Western Blot different antibodies were used depending on the construct. Kon1-6, which contain a strep tag, were detected by using an anti-strep-tag antibody, which was able to be detected directly as it was conjugated with horseradish peroxidase. Kon1f-Kon6f, which contain the Fc-domain were detected using an anti-CH3 domain primary antibody, which was detected by a secondary anti mouse antibody before developing the bands with alkaline phosphatase reagent (Figure 28-Figure 26).

Western Blot

anti-strep-tag-antibody
non reduced

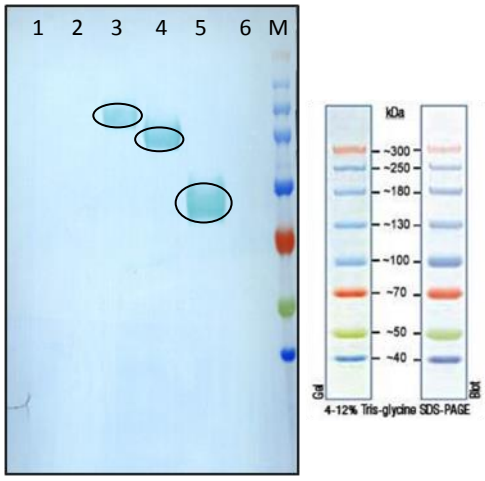


Figure 26: non reduced Western Blot of Kon1-6 lane 1-6: Kon1-Kon6; lane M: peqGOLD VII marker

anti-trep-tag-antibody
reduced

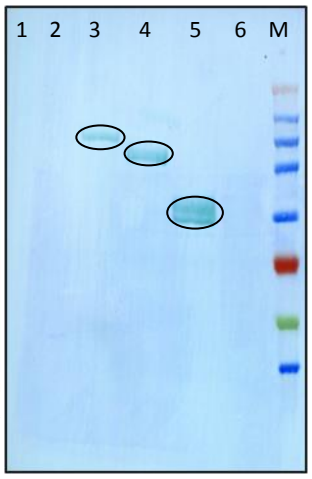


Figure 27: reduced Western Blot of Kon1-6 lane 1-6: Kon1-Kon6; lane M: peqGOLD VII marker

anti-CH3-domain-antibody;
non reduced

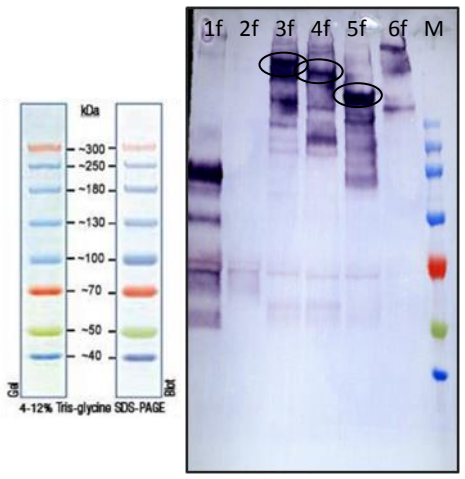


Figure 28: non reduced Western Blot of Kon1f-6f lane 1f-6f: Kon1f-Kon6f; lane M: peqGOLD VII marker

Out of all peroxidasin variants, Kon2, Kon6 and Kon2f were the only ones, which were not produced in detectable amounts and thereby were excluded from future experiments. Furthermore, Kon6f was not produced in higher amounts and the main bands were oligomerized and thus were not further expressed either. Kon1 and Kon1f were both visible on the SDS-PAGE but not on the Western Blot, which indicates that the protein was not recognized by the antibody. Due to this, the protein variants were not further worked with either. In contrast, Kon3, Kon4, Kon5, Kon3f, Kon4f and Kon5f turned out to be promising to produce protein in higher amounts and were suitable for large scale expression and purification.

To verify if the heme was covalently linked to the protein, Enhanced Chemiluminescence (ECL) was performed. Not covalently linked heme was lost during SDS-PAGE and due to this only covalently linked heme can be detected. After blotting the proteins from the gel onto the nitrocellulose membrane, the membrane was soaked in ECL substrate and chemiluminescence was measured in the ChemiDoc. The ECL screening was performed at a later time of this thesis. Due to this, knowledge about the existence of unmodified heme *b* in Kon3f was gained only after several expressions of this construct. If this would have been known earlier, Kon3f would not have been the main point of interest.

ECL

Non-reduced

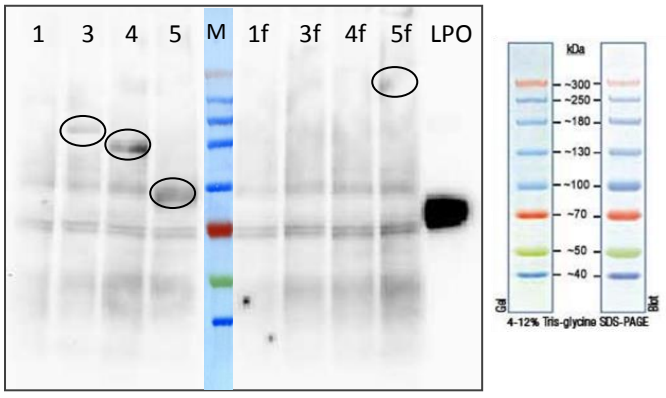


Figure 29: ECL of expressed proteins; lane 1: Kon1; lane 3: Kon3; lane 4: Kon4; lane 5: Kon5; lane M: peqGOLD VII marker; lane 1f: Kon1f; lane 3f: Kon3f; lane 4f: Kon4f; lane 5f: Kon 5f; lane LPO: positive control with LPO

The visualized bands are shown in Figure 29. Thereby, the band of Kon5 is the most prominent one compared to the other constructs, followed by Kon4, Kon3 and Kon5f. There were no bands of Kon1, Kon1f, Kon3f and Kon4f detected, which led to the assumption that the heme group was not covalently linked in these constructs.

After this first overview by using SDS-PAGE, Western Blot and ECL, it was decided to start with the expression of Kon3 in large scale. Purification using a 5 mL Strep trap column turned out to be difficult as no protein bound to the column, although it was known that protein was produced. The binding of the strep tag to the column could have been prevented by the biotin content of the cell culture medium. Still after buffer exchange no protein bound to the column. The purification problems could also be caused by a degraded or not accessible strep tag. Due to this the focus of this work was moved to the purification of fusion-protein variants and the first construct, which was produced in large scale was Kon3f. At a later stage the priorities were shifted to Kon5f, which will be discussed later in more detail.

4.2.1. Supplementation of Media with Heme Precursors

To optimize the protein expression, several trials with heme additives were performed. Protein was produced in reasonable amounts, which was observed on SDS-PAGES but the heme incorporation turned out to be a limiting factor for correctly produced active protein. Due to this, trials with different heme precursors or additives, different time points of heme source addition and different cell densities were performed.

The effect of the addition of heme precursors and of heme in different forms to the Kon3f transfected cells was tested to see if heme incorporation improved. Small scale transfections of Kon3f were performed with six different heme sources which were added at the time of transfection (Table 41).

Table 41: List of heme additives

Final concentration in cell culture	Heme additive stock solution
10 %	Fetal Bovine Serum (FBS)
10 % + [0.5 mM]	FBS + [100 mM] Aminolevulinic acid (ALA)
10 % + [2 mM]	FBS + [100 mM] ALA
10 % + [5 mM]	FBS + [100 mM] ALA
[0.5 mM]	Iron(III)citrate
5 µg/mL	[2 mg/mL] Hematin

Kon3f was purified using Protein A affinity chromatography and spectra of Kon3f were taken.

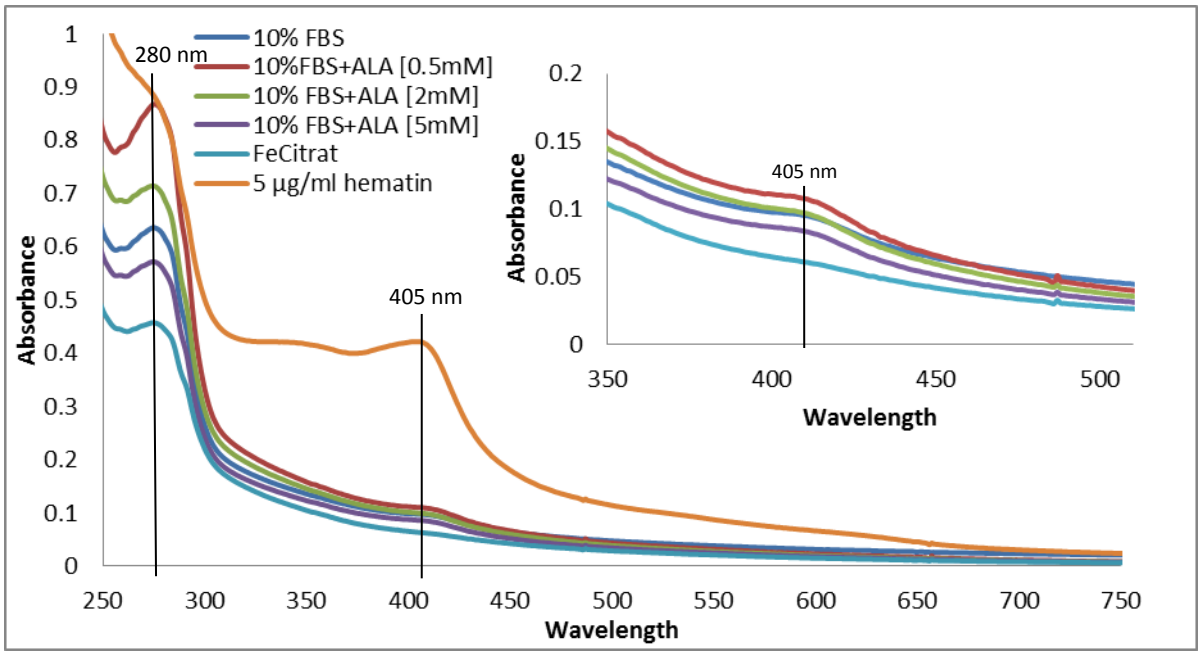


Figure 30: Heme incorporation with various additives added to Kon3f transfected cells

As depicted in Figure 30, hematin addition showed the best results for both, expressed amount of protein and heme incorporation. FBS proved insufficient as heme group provider and it can be seen that with higher ALA concentration, the heme incorporation drops even more. A higher aminolevulinic acid concentration seemed to influence the cells negatively. Due to this, for all further experiments with ALA a final concentration of 1 mM was used.

The worst results were observed by addition of ferric citrate, which shows no heme incorporation at all. A prominent shoulder at about 350 nm can be seen with hematin. Hematin was used for all further expressions. The Soret Peak should be around 405-410 nm, depending on the existence of heme to protein linkages and the heme environment.

Another aspect of interest was the time point of addition of heme precursors. An experiment was done with Kon3f, in which three different heme precursors and additives were added right after transfection and 4 hours later.

Table 42: Time point of addition of heme sources

Label	Final concentration	Heme additive stock solution	Moment of addition
#1	[5 µg/mL]	[2 mg/mL] Hematin	Right after transfection
#2	[5 µg/mL]	[2 mg/mL] Hematin	After 4 hours
#3	[1 mM]+ [0.5 mM]	[1 M] ALA + [0.5 M] Iron(III)citrate	Right after transfection
#4	[1 mM]+ [0.5 mM]	[1 M] ALA + [0.5 M] Iron(III)citrate	After 4 hours
#5	[1 mM]+ [0.5 mM]+ [1 mM]	[1 M] ALA + [0.1 M] Iron(II)sulphate + [0.5 M] Citrate	Right after transfection
#6	[1 mM]+ [0.5 mM]+ [1 mM]	[1 M] ALA + [0.1 M] Iron(II)sulphate + [0.5 M] Citrate	After 4 hours

2 mL of cell culture supernatant was 20x concentrated to 100 µL and subjected to SDS-PAGE and Western Blot.

SDS-PAGE **Western Blot**

non-reduced reduced anti-CH3-domain-antibody; non-reduced

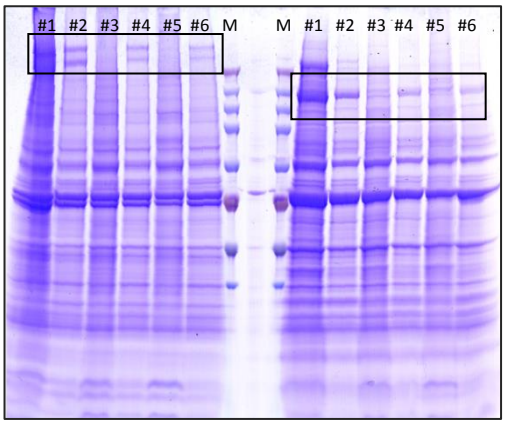
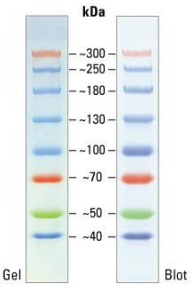


Figure 32: SDS PAGE of addition of heme sources at two different time points; Lane codes are given in **Table** 42; lane M: peqGOLD VII marker



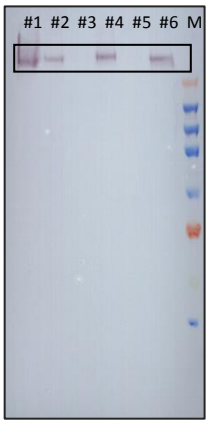


Figure 31: Western Blot of addition of heme sources at two different time points; Lane codes are given in Table 42; lane M: peqGOLD VII marker

Kon3f was transfected and different heme sources were added at two different points in time. Overall it can be said that the addition of a heme additive is to be recommended some hours after transfection and not immediately after transfection. The heme additives seem to prevent effective cell transfection. Transfected cells usually do not show further growth as the cells have switched to protein production. All lanes with heme additives added at the time of transfection show increase in the overall amount of cell material but a decreased expression of Kon3f. Striking are the lanes #1 of Figure 31 and Figure 32, in which the bands are more prominent than as in the other lanes. This would indicate that addition of 5 µg/mL hematin right after transfection was the best choice for optimal protein expression and heme incorporation. This small scale trial should be repeated to reproduce the results for a final conclusion.

Once again hematin was the most effective heme source again. As cells were usually transfected in the afternoon, in future experiments hematin was added in the following day in the morning after approximately 18 hours after transfection.

4.2.2. Influence of Cell Density on Protein Expression

The cell density was another aspect, which was closer analysed to optimize protein expression. Thereby, five different cell densities, namely 0.8, 1, 1.2, 1.4 and $1.8*10^6$ cells/mL

were used to find the optimal one for most effective protein production. For this trial Kon5f was transfected and hemin and hematin were added as heme sources (see Table 43):

Table 43: Cell density trial for Kon5f

Label	Cell density for transfection	Final concentration	Addition after 20 h
#1	1.8	[5 µg/mL]	**[2 mg/mL] Hemin**
#2	1.8	[5 µg/mL]	[2 mg/mL] Hematin
#3	1.4	[5 µg/mL]	**[2 mg/mL] Hemin**
#4	1.4	[5 µg/mL]	[2 mg/mL] Hematin
#5	1.2	[5 µg/mL]	**[2 mg/mL] Hemin**
#6	1.2	[5 µg/mL]	[2 mg/mL] Hematin
#7	1	[5 µg/mL]	**[2 mg/mL] Hemin**
#8	1	[5 µg/mL]	[2 mg/mL] Hematin
#9	0.8	[5 µg/mL]	**[2 mg/mL] Hemin**
#10	0.8	[5 µg/mL]	[2 mg/mL] Hematin

To analyse protein expression cell supernatant was 20x concentrated from 2 mL to 100 µL for SDS-PAGE and Western Blot.

SDS-PAGE

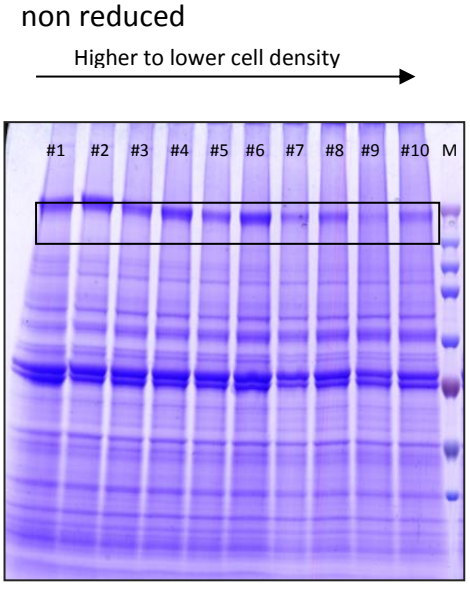


Figure 33: non-reduced SDS-PAGE of Kon5f; Lane codes are given in **Table** 42; lane M: peqGOLD VII marker

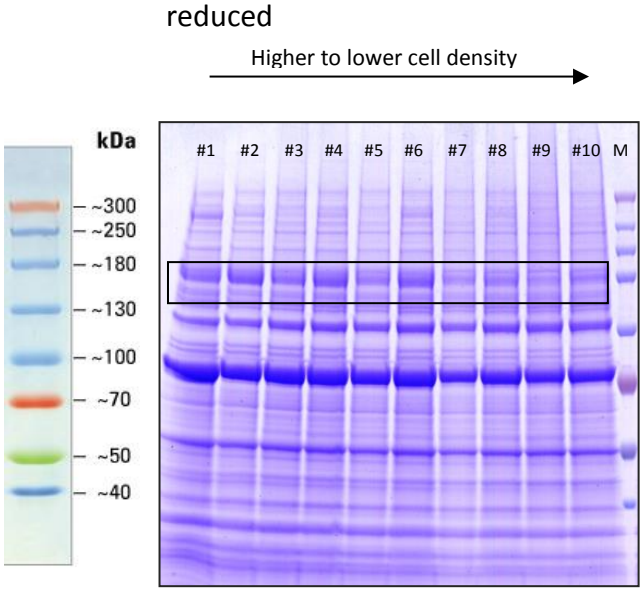


Figure 34: reduced SDS-PAGE of Kon5f; Lane codes are given in Table 43; lane M: peqGOLD VII marker

Western Blot

Anti-CH3-domain-antibody; non reduced

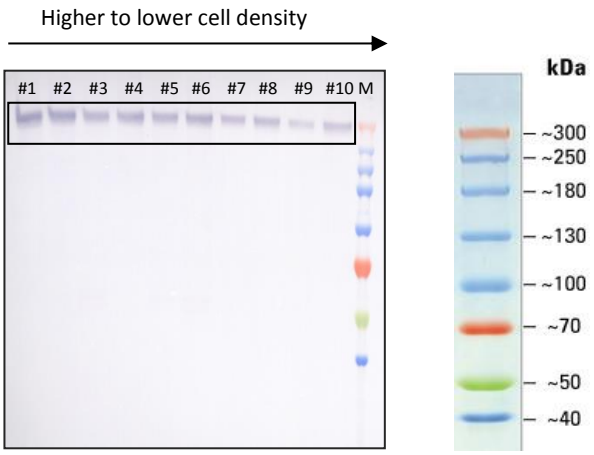


Figure 35: non-reduced Western Blot of Kon5f; Lane codes are given in Table 43; lane M: peqGOLD VII marker

Kon5f has a molecular mass of 104 kDa, and would be expected to form a dimer via the Hinge region of the Fc-domain. On SDS-PAGE Kon5f ran at a higher molecular mass than expected. It can clearly be seen that a higher cell density results in greater amounts of protein expression, whereas the overall amount of cell material was constant. For all further experiments a cell density of 1.4-1.6*10^6 cells/mL was used.

Hematin and hemin as heme additives were compared and no difference was observed. Based on these results for all further protein expressions hematin was used.

4.2.3. Purification of Kon3f

Kon3f was the first construct to be produced in large scale because of the great amounts, which were expressed by HEK 293 cells.

A typical SDS-PAGE of a Kon3f purification procedure is shown in Figure 36 and Figure 37. Protein was detectable in the cell culture supernatant. Under non reducing conditions Kon3f was detected at 340 kDa (dimer formation) and on the reduced gel Kon3f ran at 170 kDa for the monomer. Since Kon3f can also be seen in the flow through, it can be assumed that the flow rate was too high during sample application onto the column and/or the capacity of the column was exceeded. This led to the consideration that the flow rate should be kept as low as possible and a larger column should be used. Prominent protein bands at 340 kDa on the

non-reduced gel and 170 kDa on the reduced gel can be seen in the eluated fractions and in the pooled samples.

SDS-PAGE

Non-reduced

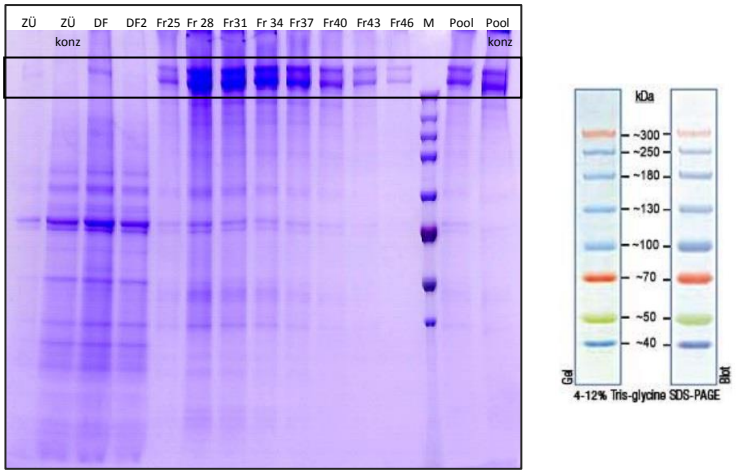


Figure 36: non-reduced SDS-PAGE of Kon3f; lane ZÜ: cell culture supernatant; lane ZÜ konz: 20x concentrated cell culture supernatant; lane DF: flow through of sample application; lane DF2: flow through of washing; lane Fr25-Fr46: fractions; lane M: peqGold VII marker; lane Pool: pooled sample; lane Pool konz: 20x concentrated pooled sample

reduced

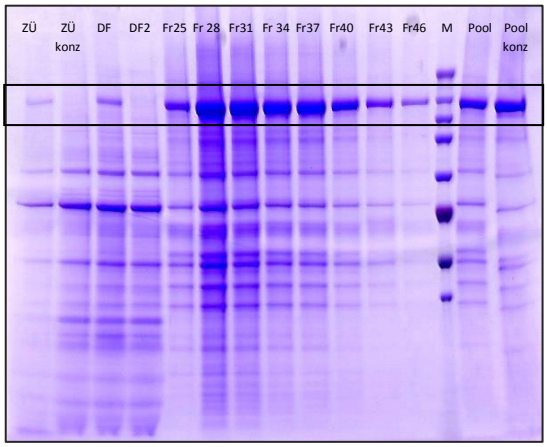


Figure 37: reduced SDS-PAGE of Kon3f; lane ZÜ: cell culture supernatant; lane ZÜ konz: 20x concentrated cell culture supernatant; lane DF: flow through of sample application; lane DF2: flow through of washing; lane Fr25-Fr46: fractions; lane M: peqGold VII marker; lane Pool: pooled sample; lane Pool konz: 20x concentrated pooled sample

A non-reducing gel was blotted onto a nitrocellulose membrane and Kon3f was detected using the ant-CH3-domain antibody and prominent protein bands at 340 kDa can be seen clearly.

Western Blot

anti-CH3-domain antibody; non-reduced

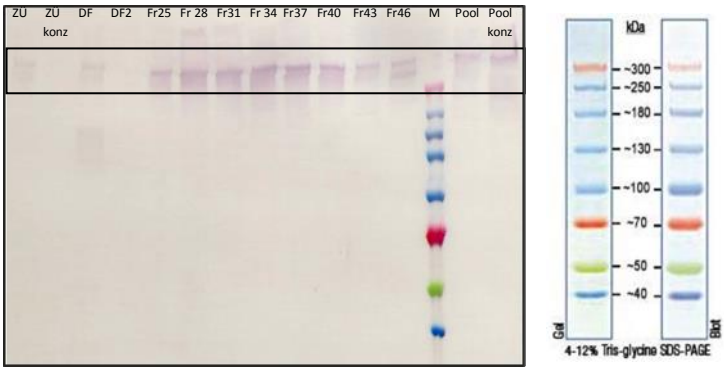


Figure 38: Western Blot of Kon3f; lane ZÜ: cell culture supernatant; lane ZÜ konz: 20x concentrated cell culture supernatant; lane DF: flow through of sample application; lane DF2: flow through of washing; lane Fr25-Fr46: fractions; lane M: peqGold VII marker; lane Pool: pooled sample; lane Pool konz: 20x concentrated pooled sample

Approximately 15-20 mg Kon3f per 1 L cell suspension was produced. After purification of Kon3f using a Protein A column, the fractions were pooled and dialysed. After dialysis the protein solution was concentrated using Centripreps and the amount decreased drastically during the concentration step.

Kon3f was not very stable. During concentration using Centriprep but also when PEG 20 000 was used, most of the protein precipitated, probably due to incorrect folding. Due to this protein amounts of 1-1.5 mg were obtained. Further, after discovery that the prosthetic heme group is not covalently linked, the expression of Kon3f was stopped.

In Figure 39 different fractions of the purification using Protein A column and the concentrated and dialysed Kon3f are shown. It is striking that after concentration and dialysis the amount of protein decreased significantly. Furthermore, after purification the Soret peak shifted from 400 nm to 407 nm.

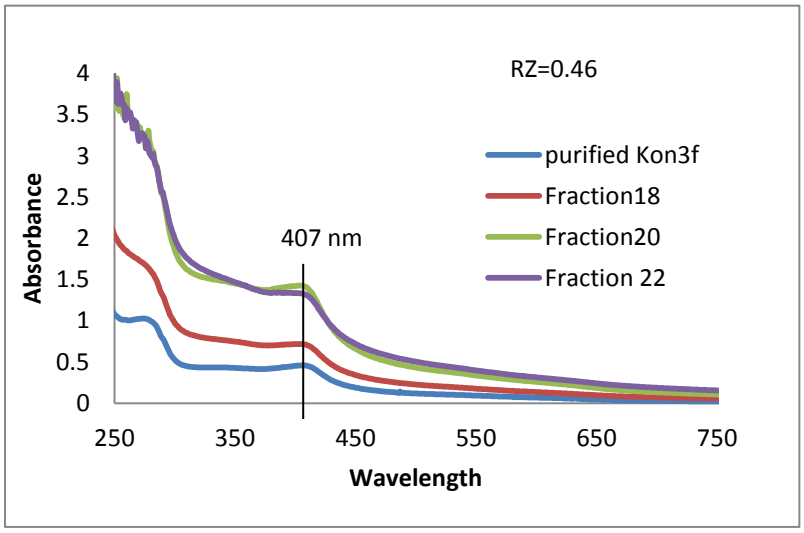


Figure 39: Spectra of fractions of Kon3f and purified Kon3f

After performing ECL, it was observed that the heme group of Kon3f was not covalently linked to the protein, which is shown in Figure 29. Due to this the expression of Kon3f was stopped.

4.2.4. PURIFICATION OF KON5F

Kon5f became of great interest after the discovery that the heme group of Kon3f is not covalently linked to the protein and thereby not worthwhile for further investigation.

The bands of the non-reduced oligomer of Kon5f at 280 kDa were observed and of the reduced monomer at 120 kDa. In Figure 39-41 the purification of Kon5f is shown on SDS-PAGE and Western Blot. Before purification aliquots of the supernatant of Kon5f were taken and applied onto the gel one time without concentration and one time 20 times concentrated. Aliquots after concentration (conc) using Polyethylene glycol and after dialysis (Dia) were taken.

SDS-PAGE **Western Blot**

Non-reduced reduced non-reduced; anti-CH3-domain antibody

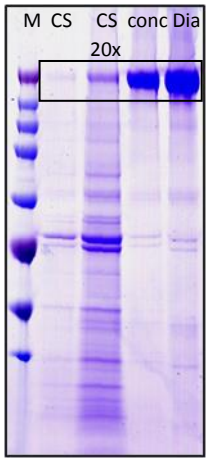


Figure 41: non reduced SDS-PAGE of Kon5f; lane M: peqGOLD VII marker; lane CS: cell supernatant; lane CS 20x: 20x concentrated cell supernatant; lane con: pooled fractions;lane: Dia: pool after dialysis

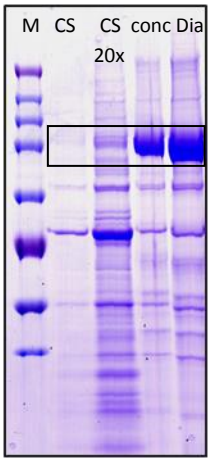
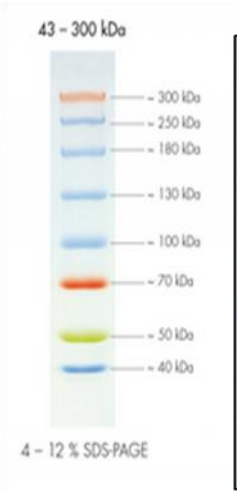


Figure 40: reduced SDS-PAGE of Kon5f; lane M: peqGOLD VII marker; lane CS: cell supernatant; lane CS 20x: 20x concentrated cell supernatant; lane con: pooled fractions;lane: Dia: pool after dialysis



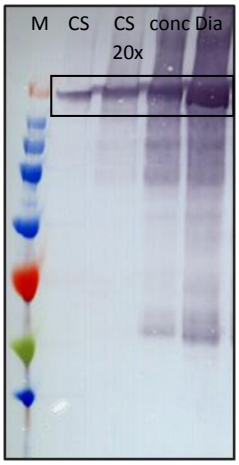


Figure 42: non reduced Western Blot of Kon5f; lane M: peqGOLD VII marker; lane CS: cell supernatant; lane CS 20x: 20x concentrated cell supernatant; lane con: pooled fractions;lane: Dia: pool after dialysis

ECL was performed as well to verify if the heme was covalently linked to the protein. To compare the results one gel was Coomassie stained and a second one was blotted onto a nitrocellulose membrane to perform ECL and Western Blot. 15 µL of 5 µM Kon5f was loaded.

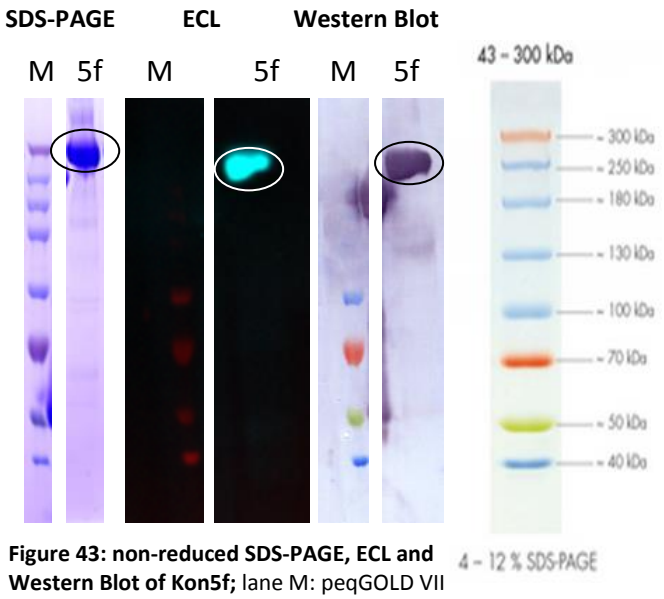


Figure 43: non-reduced SDS-PAGE, ECL and Western Blot of Kon5f; lane M: peqGOLD VII marker; lane 5f: Kon5f; for Western Blot the anti-CH3-domain antibody was used

In Figure 43 SDS-PAGE, ECL and Western Blot of purified Kon5f are shown. A high concentration of Kon5f was loaded to check its purity. Prominent bands of Kon5f were visualized by SDS-PAGE, ECL and Western Blot at 280 kDa. It can be seen on the gel as well as on the Western Blot that Kon5f is very pure. Furthermore, a prominent band was visualized on the ECL blot, which led to the assumption that the heme is covalently linked to the protein.

Kon5f was far more stable than Kon3f, which became very obvious during the concentration of the protein. It was possible to concentrate Kon5f much more than Kon3f, which easily precipitated. In contrast to Kon3f, Kon5f was expressed in far lower amounts than Kon3f. Out of 1 L cell culture 2-2.5 mg were usually purified with a purity number of approximately 0.5. Its theoretical purity number is 1.37

In Figure 44 a spectrum of the pooled fractions after purification of 1 300 mL cell supernatant using a Protein A column and a spectrum after concentration and dialysis of Kon5f is shown. 2.7 mg of protein was purified with a purity number of 0.56. The Soret peak was at 406 nm.

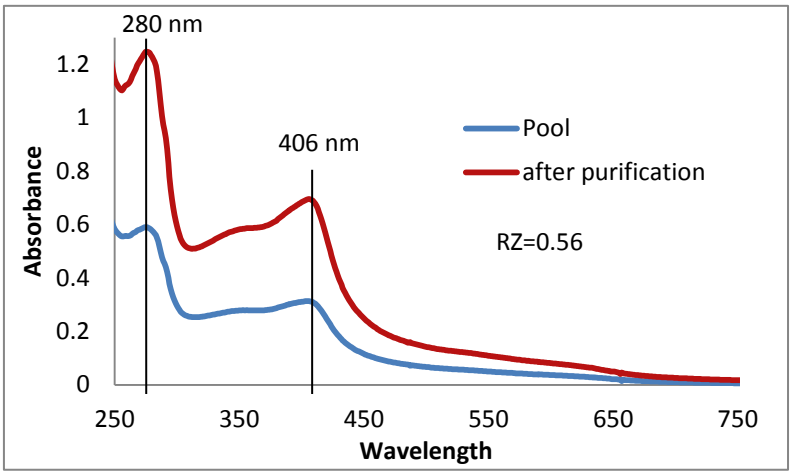


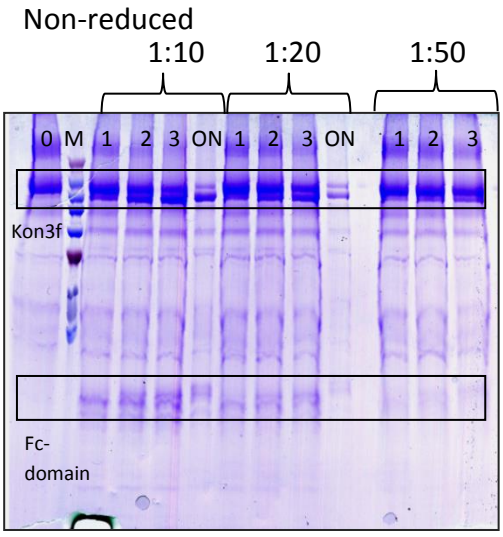
Figure 44: Spectra of Kon5f

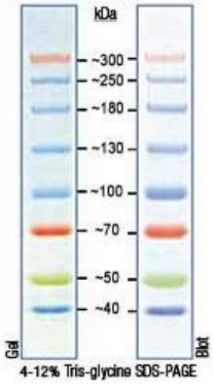
4.2.5. TEV Protease Digestions

4.2.5.1. Digestion of Kon3f with TEV Protease

To remove the Fc-domain of 25 kDa from Kon3f, TEV protease was used. The Fc-domain was used for purification and enhanced protein expression but it could interfere with the subsequent activity measurements, thus was removed. Thereby, different TEV protease to protein ratios were used: 1:10, 1:20 and 1:50. The Kon3f samples were incubated for 3 hours at RT and overnight at 4°C. After 0, 1, 2 and 3 hours and on the next day aliquots for SDS-PAGE (Figure 45and Figure 46) were taken. A Western Blot could not be performed because reduced glutathione was present in the digest acting as a reducing agent. As mentioned earlier the anti-CH3-domain antibody only recognizes the dimerized non-reduced Fc-domain.

SDS-PAGE





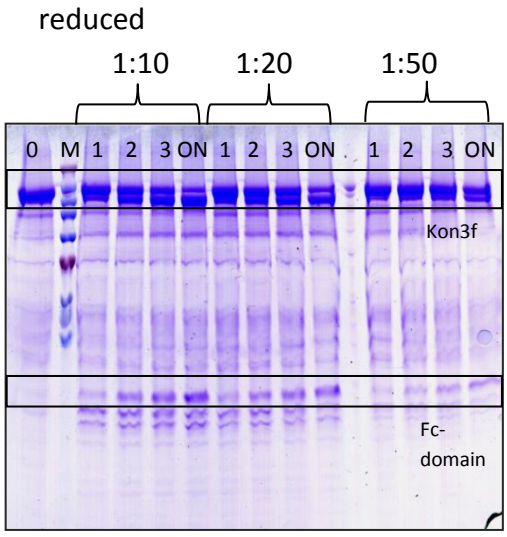


Figure 46: non reduced SDS-PAGE of Kon3f TEV digestion. Lane 0: Kon3f before TEV digestion; lane M: peqGOLD VII marker; lanes 1, 2 and 3: Kon3f after 1h, 2h and 3 h respectively; lane ON: overnight aliquote

Figure 45: reduced SDS-PAGE of Kon3f TEV digestion. Lane 0: Kon3f before TEV digestion; lane M: peqGOLD VII marker; lanes 1, 2 and 3: Kon3f after 1h, 2h and 3 h respectively; lane ON: overnight aliquote

The ratio of TEV protease to protein as well as the amount of time influenced the degree of digestion. 1:10 TEV protease to protein ratio had higher digestion rates than 1:50. Furthermore, after 3 hours the results were better than after 1 hour. Overnight digestion showed slightly more digestion than after 3 hours, which can be seen in Figure 45. This led to the assumption that more TEV protease and longer digestion time improves elimination of the Fc-domain, although overall the digestion is relatively low.

4.2.5.2. DIGESTION OF KON5F WITH TEV PROTEASE

TEV protease digestion was repeated with Kon5f using a 1:10 TEV protease to protein ratio. The sample was incubated for 4 hours at RT. Afterwards, another 1:10 TEV protease to protein ratio was added and the sample was incubated at 10°C on the shaker overnight. Aliquots after 4 hours, overnight and subsequent dialysis were taken and SDS-PAGE (Figure 48) was performed.

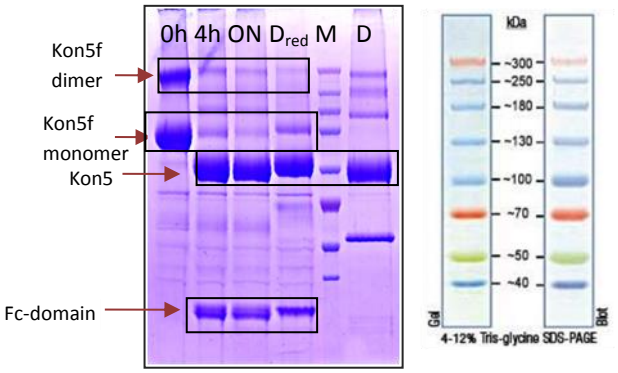


Figure 48: SDS-PAGE of Kon5f's TEV digestion. Lane 0h: undigested Kon5f; lane 4h: after 4 hour; lane ON: overnight aliquote; lane. D_{red} reduced sample of dialysed digested Kon5f; lane M: peqGOLD VII marker; lane D: dialysed digested Kon5f

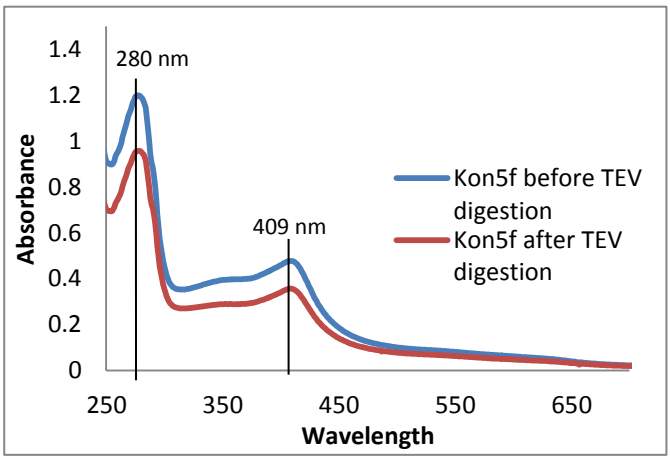


Figure 47: Spectra of Kon5f and TEV digested Kon5f

Table 44: Information on Kon5f TEV protease digestion

	Resuspended	After digestion
Purity number	0,4	0,37
Concentration [µM]	4,8	3,57
Amount [mg]	0,46	0,34
Percentage	**100 %**	**73,9 %**

In Figure 48 the digestion procedure is shown, which worked rather well and about 74% of the protein was digested successfully. Before digestion started an aliquot of Kon5f was taken. Thereby, a prominent band at 130 kDa is visualized, which is partly digested Kon5f. As mentioned before the lyophilised Kon5f was resusupended in TEV buffer, which contained reduced gluthatione. Reduced gluthatione was responsible for the partly digested Kon5f. The Fc-domain band can be seen at 25 kDa and the undigested Kon5f oligomer can be seen at 280 kDa. Without the Fc-domain, Kon5f turns into Kon5 with a molecular mass of 79 kDa as monomer and 237 kDa as trimer. The additional overnight digestion resulted in a minor further cleavage of the Fc-domain.

After digestion a spectrum of the protein was taken, as shown in Figure 47, and it can be seen that the Soret peak did not change, but the purity number decreased slightly, which is summarized in Table 44.

To separate Kon5 from the Fc-domain and the remaining Kon5f the Protein A column was used and to remove the TEV protease the His trap column was used subsequently. Thereby, a significant amount of Kon5 was lost. Due to this, this purification step should be optimized in future.

The digestion was performed because of the possibility that the Fc-domain might interfere with the protein's activity and heme accessibility. Activity measurements with constructs without Fc-domain were not performed though. This should be done in future to analyse its effect on the protein.

4.3. PROTEIN CHARACTERIZATION

4.3.1. SPECTRAL PROPERTIES

UV-Vis absorbance spectra were usually determined in 100 mM phosphate buffer at pH 7.4. This buffer provided the optimal environment for the stability of the protein while maintaining simplicity of composition.

The protein peak is always found at 280 nm and the heme peak-the Soret peak-varied between 406 and 410 nm, depending on protein variant and purification steps. A typical trait, which was always found in the spectra, is the shoulder of free heme at about 350 nm. Due to measuring the spectral properties, the purity number (RZ) is obtained as well. The purity number of a heme protein is the ratio of the absorbance at the heme Soret peak and the protein peak at 280 nm. The calculated purity number of Kon3 is 0.84, of Kon5 is 0.94, of Kon3f is 0.93 and of Kon5f is 1.06.

Figure 49 shows a typical UV-Vis spectrum of Kon5f. After 5f protein was purified from 1 300 mL supernatant, the fractions were pooled and a spectrum of the protein solution was taken to get a first overview of the characteristics of the protein. The characteristic protein peak can be seen at 280 nm and the Soret peak at 405 nm. A small shoulder at around 350 nm is present as well. The pool was concentrated using PEG 20 000 and dialysed against 100 mM phosphate buffer, pH 7.4 and then another spectrum was measured. The Soret peak changed to 406 nm. The shoulder at around 350 nm intensified as well. A purity number of

about 0.5 for Kon5f was usually obtained, which corresponds to 47% of the theoretical purity number of 1.06.

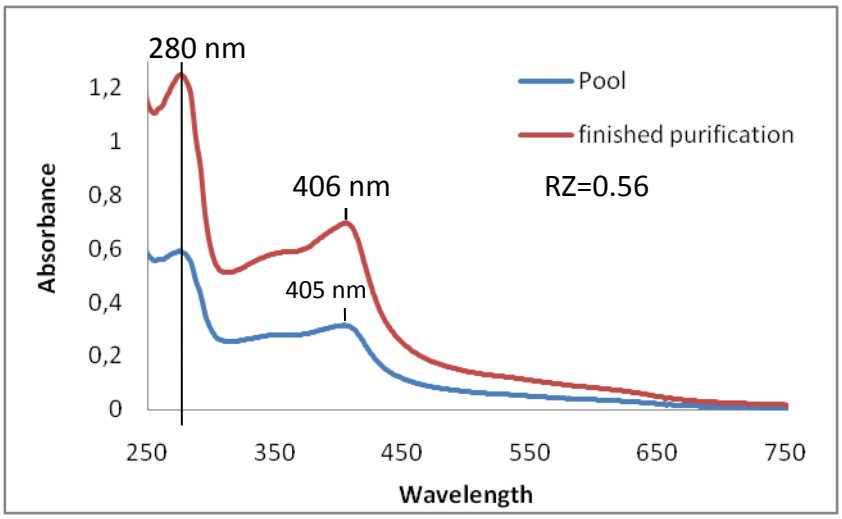


Figure 49: UV-Vis spectrum of Kon5f

From this spectrum the concentration and amount of purified protein can be calculated, as described in 3.3.3. For this purification 2.7mg protein Kon5f was purified from 1 300 mL supernatant with a purity number of 0.56. This purification went exceptionally well. The amount of protein produced is influenced by the age, condition and density of the cells, amount of DNA added, time point of hematin addition and harvest, column constitution and level of sample concentration. Consequently, even small changes can affect the amount of protein greatly.

4.3.2. Enzyme Activity

Different assays were used to measure the enzyme activity. The TMB assay was used to get a first overview of peroxidase activity, while the ABTS assay was performed to get more detailed information on the peroxidase activity of Kon3f and Kon5f.

4.3.2.1. TMB Activity Measurement

After analyzing protein production by SDS-PAGE and Western Blot, the TMB assay was used to determine if there was any peroxidase activity at all. Thereby the constructs Kon3-5 and

Kon4f-6f were analyzed (Figure 51 and Figure 50). TMB is a colourless peroxidase substrate which turns blue when oxidized.

Usually, 500 nM of the different constructs were used in combination with 2 mM TMB as substrate in a total volume of 1 mL of 170 mM sodium acetate buffer at pH 5.4. The reaction was started by adding 0.2 mM H_2O_2.

As positive control 50 nM LPO was used, which instantly oxidized TMB to a dark blue solution (Figure 51). Kon5 (Figure 50) and Kon5f (Figure 51) showed the highest activity of all constructs followed by Kon3 and Kon4. Kon4f showed a slightly blue colouring, whileKon6f was not active at all and therefore the solution remained colourless. After this first activity measurement Kon5 seemed to be the most promising construct. However, purification was impossible because the strep-tag did not bind to the affinity column. Kon5f was very active as well and was able to be purified. Therefore, most of the further characterisation was performed with Kon5f.

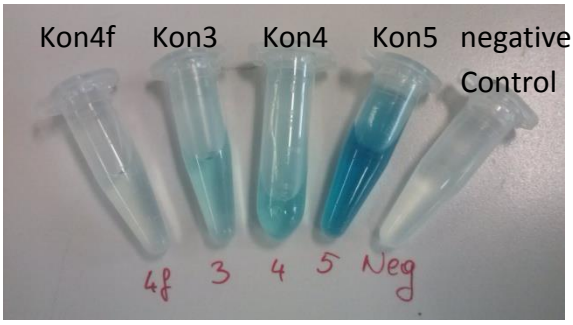


Figure 50: TMB assay of 500 nM Kon 4f and Kon3-5 mixed with 2 mM TMB in 170 mM sodium acetate buffer, pH 5.4. The reaction was started by adding 8 mM H_2O_2

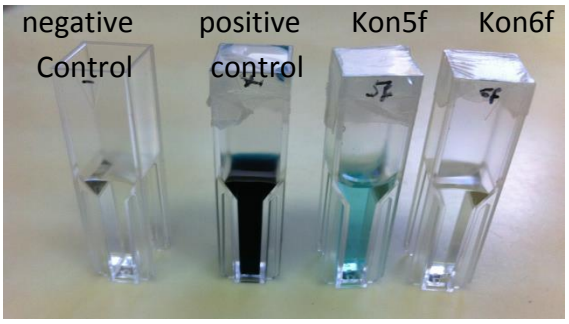


Figure 51: TMB assay of 500 nM Kon5f and Kon6f mixed with 2 mM TMB in 170 mM sodium acetate buffer, pH 5.4. The reaction was started by adding 8 mM H_2O_2

4.3.3. ABTS Assay

To investigate the peroxidase activity in detail the ABTS assay was used. Thereby, 500 nM enzyme were used in combination with 1 mM ABTS as substrate and 100 µM H_2O_2 to start the reaction in 100 mM phosphate buffer, pH 5 and 7.4, respectively. The extinction coefficient of $ABTS^{-\bullet}$ is 36 000 $M^{-1}cm^{-1}$ at 414 nm.

The specific activity was calculated in unit mg^{-1}, where one unit of peroxidase activity is defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute at pH 5.0 and pH

7.4, respectively, and 25°C. An example is given using one data point of pH measurement of Kon5f at pH 5.

Specific activity [A] = [Units mg^{-1}] = [µmol ABTS min^{-1} mg^{-1} protein]

ε_{414nm} 36 000 M^{-1} cm^{-1}

Average absorbance value: $0.004168\ A/sec$

$$0.004168\ A/sec * 60 = 0.2501\ A/min$$

$$A/sec/\varepsilon = \frac{0.2501}{36000} = 6.95 * 10^{-6}\ M/min = 0.00695\ \mu M/min$$

$$\frac{0.00695\ \mu M/min}{0.052mg\ (used\ protein\ amount)} = \mathbf{0.134\ U/mg\ (Specific\ activity)}$$

The assay was used to compare the influence of lyophilisation of protein samples followed by storage at -20°C and freezing of protein samples in buffer solution at -20°C. Furthermore, the ABTS oxidation at pH 5 and pH 7.4 was measured.

4.3.3.1. Influence of Lyophilisation and Freezing on Protein Activity

Protein solutions need to be stored until further use, since storage at 4°C long-term results in decreased activity. To find the most optimal way to store the protein samples, the peroxidase activity of freshly purified, lyophilised and frozen Kon3f, respectively, was measured using the ABTS assay. Thereby, lyophilisation is the more gentle method compared to freezing. Freezing can harm the protein, which results in loss in activity. In contrast, lyophilised protein may not dissolve fully again, which leads to a loss of protein.

Due to this, the influence of lyophilisation and freezing on protein activity was investigated. Thereby, freshly purified, lyophilised and frozen Kon3f, respectively, of the same charge was used.

The purified protein was kept in 100 mM phosphate buffer at pH 7.4. The same charge was portioned evenly and one part was stored at 4°C, another part was frozen at -20°C and the last part was lyophilised, followed by storage at -20°C for several days. The lyophilised

sample was resuspended in water and UV-Vis spectra of all samples were measured (Figure 52).

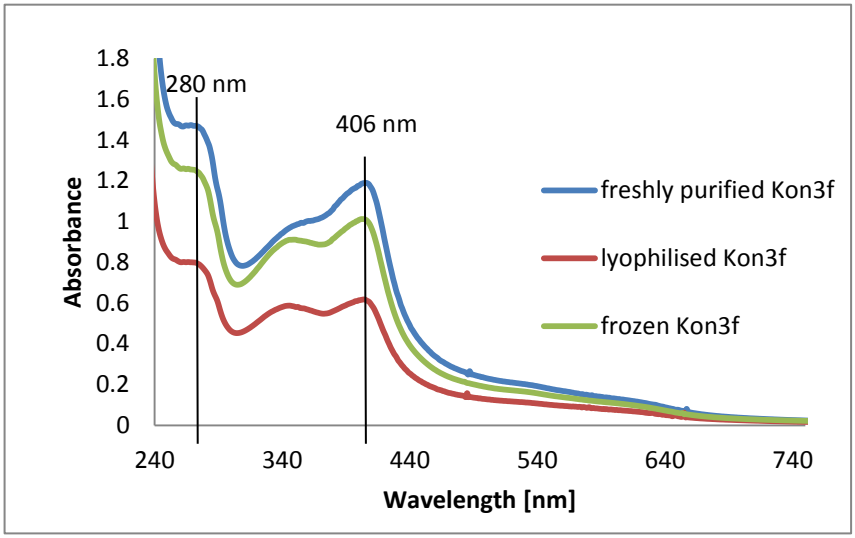


Figure 52: UV-Vis spectrum of freshly purified, lyophilized and frozen Kon3f

Table 45: Information about Kon3f variant

	Purity number	Concentration [µM]	Protein amount [mg/mL]	Percentage based on protein amount
Freshly purified Kon3f	0.84	11.9	2.02	**100 %**
Lyophilised Kon3f	0.8	6.18	0.9	**44.6 %**
Frozen Kon3f	0.84	10.13	1.47	**72.8 %**

After obtaining the UV-Vis spectra of freshly purified, lyophilised and frozen Kon3f (Figure 53), it was observed that lyophilisation led to a significant loss in protein. Only about 45% of protein could be resuspended (Table 45). Freezing decreased the protein amount as well, leading to a loss of about 38% of protein based on the freshly purified Kon3f.

Lyophilisation had an impact on the purity number, which slightly worsened from 0.84 to 0.8, while the purity number of the frozen Kon3f remained at 0.84.

Furthermore, the shoulder around 350 nm of lyophilised and frozen Kon3f was more pronounced compared to the one of the freshly purified protein. Overall, the spectral properties worsened after freezing but especially after lyophilisation.

To analyze the peroxidase activity 500 nM enzyme were used to oxidize 1 mM ABTS in 100 mM phosphate buffer at pH 7.4. The reaction was started by adding 100 µM H_2O_2. The extinction coefficient of $ABTS^{-\bullet}$ is 36 000 M^{-1} cm^{-1} at 414 nm. 50 nM MPO was used as positive control.

Table 46: Specific activity with ABTS of lyophilized and frozen Kon3f

ABTS assay	Freshly purified Kon3f	Lyophilised Kon3f	Frozen Kon3f
conc. [g/L]	0.000085	0.000085	0.000085
slope/min	0.01109	0.1339	0.02258
slope/min	0.009376	0.1765	0.01659
Average [min^{-1}]	0,010233	0.1552	0.019585
U=µmol*min^{-1}*mg^{-1}	**0.003**	**0.051**	**0.006**

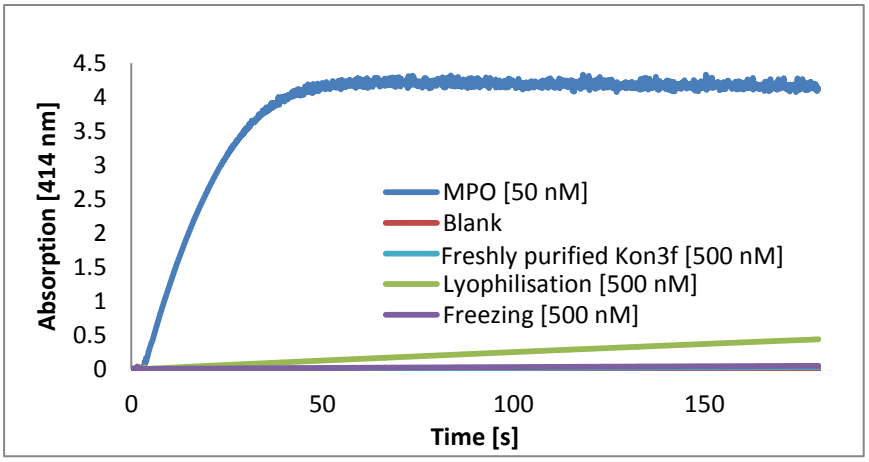


Figure 53: Peroxidase activity of 500 nM freshly purified, lyophilized and frozen Kon3f, respectively, at pH 7.4. 50 nM MPO was used as positive control.

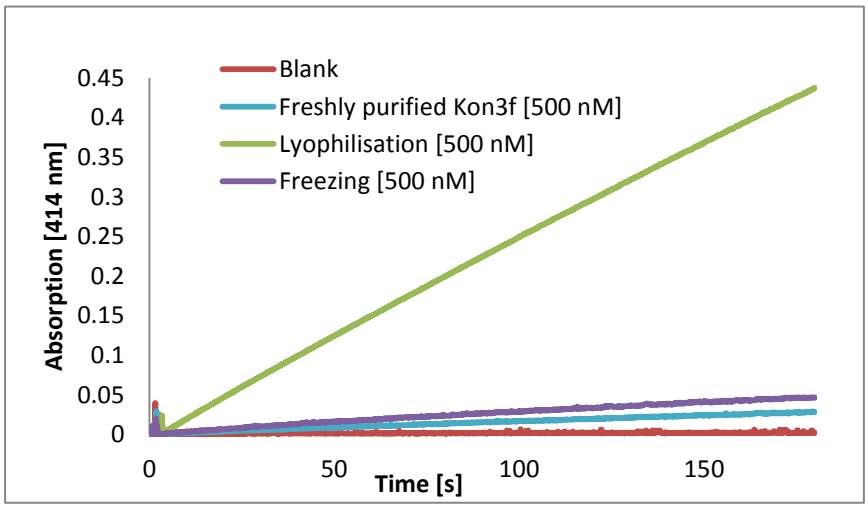


Figure 54: initial part of the reaction without MPO enlarged from Figure 53

This peroxidase activity measurement was done only twice, due to the low amount of available protein. This leads to inaccuracy and does not ensure reproducibility. Due to this, it is recommended to verify these results by another series of measurements and thus gave only a rough overview concerning the peroxidase activity. Especially the peroxidase activity of lyophilised protein was unusual high, which is visualized in Figure 54, and has to be verified necessarily. Lyophilised protein had the highest peroxidase activity, followed by frozen and freshly purified protein.

50 nM MPO was used as positive control. A typical time trace at 414 nm is shown in Figure 53. Due to the high peroxidase activity there was a steep increase in absorbance at the beginning of the reaction followed by reaching a plateau when all hydrogen peroxide was consumed. Since peroxidasin is related to MPO, it is suggested that the behaviour should be similar. In contrast to MPO, Kon3f seemed to be a rather slow reacting enzyme. It stayed reactive over a larger period of time and a plateau could not be observed. To find out the reason for the low peroxidase activity of the peroxidasin constructs clearly more measurements have to be done.

4.3.3.2. ABTS Oxidation at pH 5 and pH 7.4

ABTS reactivity of Kon5f in phosphate buffer, pH 5 and pH 7.4, respectively, was measured to analyse the peroxidase activity in an acidic and neutral environment. Thereby, 500 nM enzyme were used to oxidize 1 mM ABTS in 100 mM phosphate buffer at pH 5 and pH 7.4, respectively. The reaction was started by adding 100 µM H_2O_2. The extinction coefficient of $ABTS^{-\bullet}$ is 36 000 M^{-1} cm^{-1} at 414 nm. 100 nM LPO was used as positive control.

Table 47: Specific activity of Kon5f with ABTS at pH 5 and pH 7.4, respectively

ABTS assay	pH 5	pH 7.4
conc. [g/L]	0.000052	0.000052
slope/min	0.2443	0.01899
slope/min	0.2559	0.0194
Average [min^{-1}]	0.2501	0.0192
U=µmol*min^{-1}*mg^{-1}	**0.134**	**0.01**

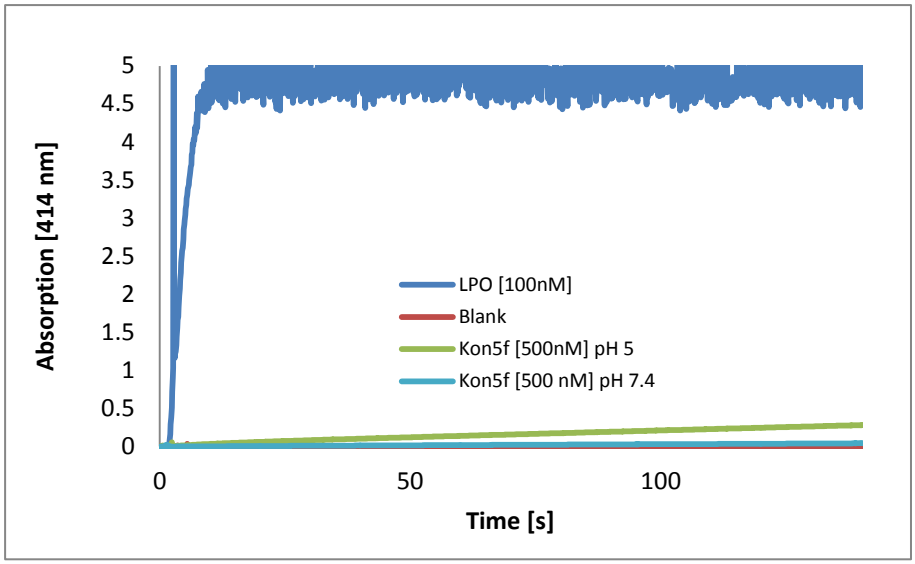


Figure 55: Peroxidase activity of 500 nM Kon5f at pH 5 and pH 7.4, respectively. 100 nM LPO was used as positive control.

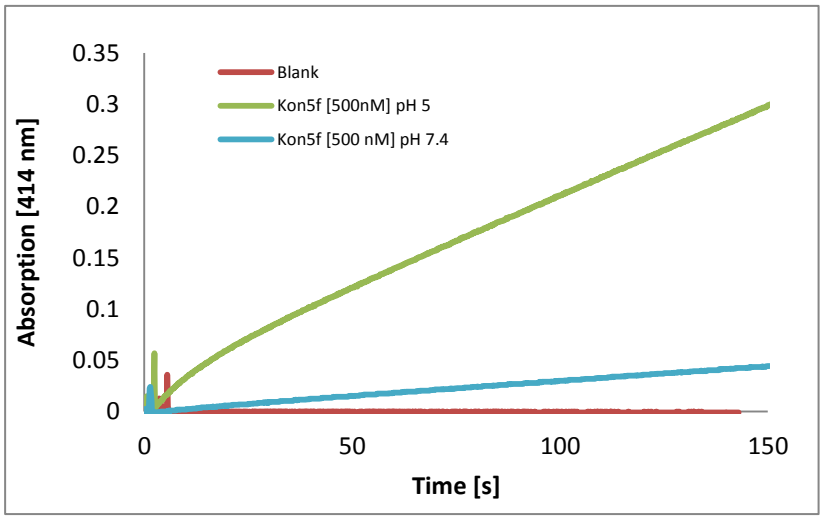


Figure 56: initial part of the reaction without LPO enlarged from Figure 55

These measurements have to be repeated, since this assay was done only twice, due to lack of protein amount. This leads to inaccuracy and does not ensure reproducibility.

The peroxidase activity of Kon5f was higher at pH5, compared to pH 7.4, which led to the assumption that ABTS oxidation was favoured at pH5. LPO was used as positive control, since peroxidasin is LPO-like, suggesting a similar behaviour concerning peroxidase activity. Similar to MPO the peroxidase activity of LPO started with a steep rise in absorbance at 414 nm and reached a plateau after all hydrogen peroxide was consumed. Nonetheless, the peroxidase activity of Kon5f rose slowly and a plateau could not be measured but the activity was 13 times higher at pH 5 compared to the activity at pH 7.4 (see Table 47).

4.3.4. HALOGENATION ASSAY

The oxidation of halide ions like chloride and bromide was measured using the halogenation assay. In this assay generated HOCl or HOBr are trapped as taurine chloramine or taurine bromamine.

The activity measurement was performed with 1 µM Kon3f and Kon5f, respectively, 10 mM taurine and 100 mM NaCl or KBr in 100 mM phosphate buffer, pH 7.4. The reaction was started by adding 200 µM H_2O_2. 50 nM MPO was used as positive control.

Only data about the bromination activity is provided because no chlorination activity could be detected. The extinction coefficient of bromamine is 415 M^{-1} cm^{-1} at a wavelength of 289 nm. Thereby, the measurement was repeated three times to reduce inaccuracy and to ensure reproducibility.

Table 48 Specific activity of Kon3f and on5f

Halogenation Assay	MPO	Kon3f	Kon5f
conc. [g/L]	0.0000085	0.000145	0.000079
Average [min^{-1}]	0.22853333	0.011925	0.0056755
U=µmol*min^{-1}*mg^{-1}	**73.4**	**0.169**	**0.131**

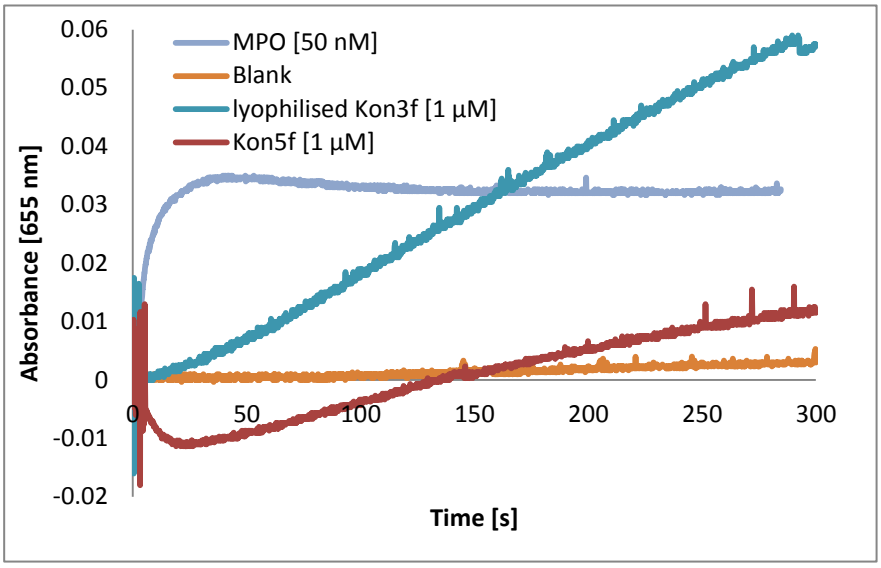


Figure 57: Bromination activity of 1 µM Kon3f and Kon5f, respectively at pH 7.4. 50 nM MPO was used as positive control

Bromination activity of Kon3f as well as of Kon5f is very low. There were no significant differences between Kon3f and Kon5f seen (Figure 57 and Table 48). MPO was used as a positive control, due to its homology to peroxidasin. Its characteristic steep rise at the beginning of the reaction is shown in Figure 57, reaching its plateau after all hydrogen peroxide was consumed. In comparison, the peroxidasin constructs showed a much smaller bromination activity. To clarify the low bromination activity of the peroxidasin constructs pre-steady-state measurements with the individual reaction intermediates should be performed.

5. DISCUSSION

5.1. CLONING OF THE RECOMBINANT PROTEINS

The expression of the full length human peroxidasin 1 turned out to be difficult to accomplish, which led to the generation of six different truncated variants of this metalloprotein. Each construct was designed with different domains to analyse their influences on protein expression and activity.

The pTT5 vector was used as means for insertion of each truncated human peroxidasin 1 construct into the HEK 293-6E cells by transient transfection. Its multiple cloning site was removed and replaced by the synthesized insert, forming the pTT5+ vector.

The pcDNA3.1 vector, which contains the full length human peroxidasin 1, was used as template to design all constructs in combination with the respective forward and reverse primers. Finally, each strep-tag construct was generated by insertion of the construct DNA into the prepared pTT5+ vector.

The strep-tag seemed to be a promising way for purification later, thus designing all constructs with this tag. Due to unknown reason purification was not successful, leading to consideration to generate the fusion-proteins Kon1f-6f. The six different variants Kon1-6 were fused with the **F**ragment **c**rystallizable region (Fc-domain), which is part of the heavy chain C_H3 and C_H2, including the Hinge region, of the antibody immunoglobulin G, was used.

Both variants, the strep-tag constructs as well as the fusion-proteins, were difficult to work with. The strep-tag proteins could not be purified and Kon5f was the only construct of the fusion-proteins, which was expressed, contained a covalently linked heme group and had peroxidase activity but was produced in small amounts of only 1.5-2 mg of 1 litre cell culture. Due to this, the generation of further constructs could be considered. Different tags, as the His-tag, could be used instead.

5.2. PROTEIN PRODUCTION

To produce the twelve different human peroxidasin 1 variants, transient expression using HEK 293-6E cells were used. A one week cycle of growing, diluting, transfecting, heme addition, peptone feeding and finally harvesting was needed to start the protein purification. Transient transfection was performed using PEI, which is bound by DNA and transferred into the HEK cells. Due to poor protein expression of Kon5f, optimization of protein production became a key objective.

To optimize the protein production, several experiments concerning kinds of heme precursor additives, time points of heme precursor addition and cell density were performed. All together seven different heme sources were tested but hematin was clearly the best heme source and led to the best heme insertion.

The time point of hematin addition was of great interest as well, since the timing played a big role in protein yield. Due to this, hematin addition right after transfection and after 4 hours was tested. 4 hours after transfection the heme incorporation showed by far the best results. This led to the assumption that hematin was interfering with the incorporation of the construct DNA. Instead of producing correctly folded, heme inserted protein, the cells kept growing and produced protein without heme incorporated. Due to this, hematin was added 18 hours after transfection.

Analysis of the cell density at the time of transient transfection turned out to be important as well. Hence, different cell densities of 0.8-1.8*10^6 cells/mL were tested. The results were analysed on SDS-PAGE and Western Blot. Thereby, a greater cell density produced more protein, whereas the overall amount of cell material was constant. Due to this, a cell density of 1.4*10^6 cells/mL was used for all further transient transfections.

5.1. PROTEIN PURIFICATION

Purification of the strep-tag proteins Kon1 to Kon6 turned out to be not successful. Reasons for this are unknown, but it is considered that the strep-tag was damaged or not accessible and thus, cannot bind to the matrix of the StrepTactin Sepharose column.

Due to this, the fusion-proteins were generated which can bind to Protein A Sepharose through affinity chromatography. This turned out to be much more promising and the fusion-proteins could be purified. Since huge volumes of 1.5 to 2 litres cell culture were purified it is recommended to apply the cell culture in smaller volumes and use a flow rate as low as possible.

Furthermore, it was noticed that after purification via column, concentration using PEG 20 000, followed by dialysis to remove the glycine was a better choice than the other way around by starting with dialysis, followed by concentration. Purified protein was stored after lyophilisation at -20°C.

A series of SDS-PAGEs, Western Blots and ECLs were done, to verify the expression and purification of the protein variants and its successful heme incorporation. Thereby, it was observed that the heme group of Kon3f was not covalently linked to the protein, which directed the focus of attention to the expression of Kon5f.

It was known that strep-tag proteins were produced in significantly higher amounts than the fusion-proteins but could not be purified. Instead of using the StrepTactin Sepharose column other methods for purification could be tried. Another possibility could be diafiltration, which involves removal of components of a specific molecular size, and thereby purifies the solution.

5.1.1. TEV Digestion

The Fc-domain was only used for protein purification and might not be necessary afterwards. Furthermore, it could even interfere with the protein's activity and thus was removed using the TEV protease. Several trials were performed to find the necessary amount of protease and time to correctly remove the Fc-domain. Naturally, using more time and TEV protease resulted in better and faster digestion of the fusion-proteins. About 74 % of protein was digested successfully. To remove the remaining fusion-protein, Fc-domain and TEV-protease purification using a His-trap column, directly followed by a Protein A column was performed.

Although digestion was done because of the idea it might interfere with the protein's activity, a series of peroxidase activity measurements were performed with the fusion-proteins and it was observed that the Fc-domain did not interfere with the protein's activity in these tests. For better understanding on the influences of the Fc-domain, more comparable assays of strep-tag constructs and fusion-proteins should be done in future.

5.2. PROTEIN CHARACTERIZATION

5.2.1. SPECTRAL CHARACTERIZATION

The two constructs Kon3f and Kon5f, which were analysed in most detail, had their Soret peak at 406-410 nm, which depended on the purification step, whereas the full length human peroxidasin 1 had its Soret peak at 410 nm. At about 350 nm a prominent shoulder was seen, which was slightly more pronounced in Kon3f. The origin of this shoulder was not known and cannot be reduced or removed by purification or desalting.

The purity number, which is the ratio of the absorbance of the heme Soret peak and the protein peak at 280 nm, of Kon3, Kon3f, Kon5 and Kon5f, respectively, was 0.84, 0.94, 0.93 and 1.06, respectively. This purity number was used to get an overview about the effectiveness of the purification.

5.2.2. ACTIVITY MEASUREMENTS

The TMB assay was used to verify if the construct variants had any peroxidase activity at all. Only Kon5 and Kon5f showed sufficient peroxidase activity. Since purification of Kon5 was not successful, the main attention was paid to Kon5f.

ABTS assay was performed to measure the specific peroxidase activity. Thereby, measuring the influence of lyophilisation and freezing and the ABTS reactivity at pH 5 and pH 7.4 of Kon3f and Kon5f were given priority. In general, no significant differences between frozen and freshly purified Kon3f concerning peroxidase activity was observed. In contrast, the peroxidase activity of lyophilized Kon3f was significantly higher, but lyophilisation led to about 50% loss of active protein which is also reflected by a lower purity number.

After performing pH measurements it was observed that the peroxidase activity of Kon5f is higher at pH 5 than at pH 7.4, leading to the assumption that ABTS oxidation was favoured at pH 5.

Compared to LPO and MPO, which have very similar active side architecture to peroxidase, Kon3f and Kon5f have a very low peroxidase activity but showed a moderate bromination activity. Both variants showed no chlorination activity.

Characterization of the protein variants Kon3f and Kon5f should be done in more detail in the future. Some first characterization, like UV-Vis spectroscopy, TMB, ABTS and halogenation assay, were performed but this is only a small step to get an insight of this protein. Especially, biochemical and physical analysis including electronic circular dichroism, stopped-flow spectroscopy, electron paramagnetic resonance (EPR), mass-spectroscopy and X-ray crystallography to resolve the three dimensional structure, should be done in future. This could lead to a better understanding of protein structure, domain interaction, architecture of substrate access channel(s) and heme cavity, spin state of the prosthetic group and substrate pattern.

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