Expression of membrane associated proteins for structure determination

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ABSTRACT

Membrane-attached cytochrome P450 and reductase enzymes play a key role in biosynthesis of natural compounds and are involved in drug metabolism. Thus, these enzymes are often used as catalysts for industrial applications. Membrane proteins have special properties and are, therefore, more difficult to handle. Cofactor regeneration, activity and stability of the enzymes are limiting factors.

To achieve a better knowledge of protein functions, structural analyses are very important and helpful. In this work, the overall goal is the crystallisation and structural analyses of several membrane-bound and soluble cytochrome P450 and reductase enzymes. With this knowledge, the limiting factors in cytochrome P450 and reductase application like activity, stability and cofactor regeneration can be improved. *Pichia pastoris* was selected as expression system, because of its potential in functionally expressing these enzymes. The proteins were purified using Ni/NTA affinity chromatography for crystallization studies.

ZUSAMMENFASSUNG

Membrangebundene Cytochrom P450 und Reduktase Enzyme spielen eine zentrale Rolle in der Biosynthese von Naturstoffen und sind im Stoffwechsel von Arzneimitteln involviert. Deshalb werden diese Enzyme häufig als Katalysatoren für industrielle Anwendungen verwendet. Membranproteine haben spezielle Eigenschaften und sind deshalb schwierig zu handhaben. Cofaktor-Regenerierung, Aktivität und Stabilität der Enzyme sind limitierende Faktoren bei der Anwendung dieser Proteine.

Um ein besseres Verständnis der Funktionen von Membranproteinen zu erhalten sind Strukturanalysen sehr wichtig und hilfreich. In dieser Arbeit ist das allgemeine Ziel die Kristallisation und Strukturanalyse verschiedener membrangebundener und löslicher Cytochrom P450 und Reduktase Enzyme, um das Wissen über die limitierenden Faktoren, wie Aktivität, Stabilität und Cofaktor Regeneration, zu erweitern. Als Expressionssystem wurde *Pichia pastoris* gewählt, da in dieser Hefe die Enzyme funktionell expremiert werden können. Die Proteine wurden mittels Ni/NTA-Affinitätschromatographie für die anschließende Kristallisation gereinigt.

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1. LIST OF ABBREVIATIONS

Table 1. List of abbreviations

Abbreviation	Description
2D6	*Homo sapiens* cytochrome P450 2D6, CYP2D6
ADH	Alcohol dehydrogenase
Amp	Ampicillin
AOX1	Alcohol oxidase I gene
CMC	Critical micellar concentration
cPCR	Colony polymerase chain reaction
CPR	*Arabidopsis thaliana* NADPH-cytochrome P450-reductase
CV	Column volume
P450	Cytochrome P450 enzyme
P_{AOX1}	AOX1 promoter
ddH2O	Water, double distilled
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
DWP	Deep well plate
ER	Endoplasmic reticulum
EtOAc	Ethyl acetate
EtBr	Ethidium Bromide
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
fw	Forward
GC-FID	Gas chromatography-flame ionization detection
GDH	Glucose 1-dehydrogenase
gDNA	Genomic DNA
hCPR	Human cytochrome P450-reductase
HPO	*Hyoscyamus muticus* premnaspirodiene oxygenase
HIS- tag	6x Histidine tag
Kan	Kanamycin
KP_i	Potassium phosphate
L	Ladder
mp	Microsomal preparation
MS	Mass spectrometry
MW	Molecular weight
ONC	Over night culture
o/n	Over night
p	pellet
PM17	*Mentha piperita* Limonene-3-Hydroxylase
RT	Room temperature
rv	reverse
s	soluble
t2D6	Truncated *Homo sapiens* cytochrome P450 2D6
tCPR	Truncated *Arabidopsis thaliana* NADPH-cytochrome P450-reductase

thCPR	Truncated human cytochrome P450-reductase
tHPO	Truncated *Hyoscyamus muticus* premnaspirodiene oxygenase
TMD	Transmembrane domain
tPM17	Truncated *Mentha piperita* Limonene-3-Hydroxylase
WT	Wild type
Zeo	Zeocin

2. INTRODUCTION

2.1. PROTEINS OF INTEREST

To date the structures of membrane-bound cytochrome P450 proteins and CPRs are not known in detail. To enhance the chances of improving expression level and activity of these proteins, it is important to know the structure and therefore increase knowledge on the function of the proteins. To improve the applicability of membrane-bound cytochrome P450 and CPR enzymes, these proteins will be expressed in *Pichia pastoris*, purified using affinity chromatography and will be crystallized. Crystallisation studies are important for structural determinations and for a better understanding of protein function. Membrane proteins are attached to cellular membranes by one or more transmembrane domains that are highly hydrophobic. Truncation of single transmembrane domains led to soluble cytochrome P450s and, therefore, to high-level expression and activity if expressed in *E. Coli*. [1] Abundant, soluble cytochrome P450 proteins can be easily purified. The purification of the membrane proteins is more complicated, because of their membrane attachment. The membrane bound proteins have to be solubilised, i.e. treated with appropriate detergents, before purification.

2.1.1. Cytochrome P450s

Cytochrome P450`s (CYPs) belong to the superfamily of heme containing enzymes. The active heme site is tethered to the enzyme via a thiolate ligand derived from a cysteine residue. *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO), limonene-3-hydroxylase (PM17), cytochrome P450 2D6 (2D6) and cytochrome P450 reductases (CPR, hCPR) are integral membrane proteins. They are involved in the biosynthesis of important natural compounds, but also contribute to detoxification mechanism in primary and secondary metabolic pathways of microorganisms, plants and animals [3]. CYP enzymes have been identified in all domains of life, i.e., in animals, plants, fungi, protists, bacteria, archaea, and even viruses. Due to their ability to regio- and enantioselectively oxidize a wide variety of compounds, CYPs are attractive and versatile biocatalysts for industrial applications. The substrates of cytochrome P450 and reductase enzyme are lipids and steroidal hormones, xenobiotic substances like drugs and toxins. CYPs and CPRs are involved in drug metabolism and bioactivation [3], [4].

CYPs may convert small and large substrates in enzymatic reactions. To reduce the heme iron one or more electrons are provided by protein partners. Therefore, cytochrome P450 reductase is reduced by NADPH and the electron is transferred to the CYP. They often form multi-component electron transfer chains, called P450-containing systems (Figure 1) [4]. The substrate (RH) is bound to the heme group for conversion and a change in the conformation of the active site is induced. This confirmation change triggers cytochrome P450 reductase to

transfer an electron from the required cofactor NADPH. Due to the electron transfer, the ferric heme iron is reduced to the ferrous state. Then, molecular oxygen binds covalently to the heme center. A second oxygen is transferred via the electron transport system from the corresponding reductase, reducing the dioxygen adduct to a negatively charged peroxo group. This peroxo group is rapidly protonated from surrounding amino acid side chains, releasing a water molecule and forming a highly reactive iron(V)-oxo species. This activated compound causes finally the oxidation of the vicinal bound substrate (ROH), which is released in the next step [5].

Class II cytochrome P450s are the most common class in eukaryotes and are responsible for oxidative metabolism of endogenous compounds and are involved in detoxification processes. In its simplest form, the monooxygenase system of class II enzymes is located in the endoplasmic reticulum (ER) of eukaryotes. The cytochrome 450 enzyme and the NADPH dependent cytochrome P450 reductase (CPR) contain a flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) subunit, which transfer the required redox equivalents from NADPH to the cytochrome P450 enzyme. Cytochrome P450 and CPR are membrane proteins with a single N-terminal transmembrane domain leaving the main part of the protein including the active site facing the cytosol [4] [4] [6].

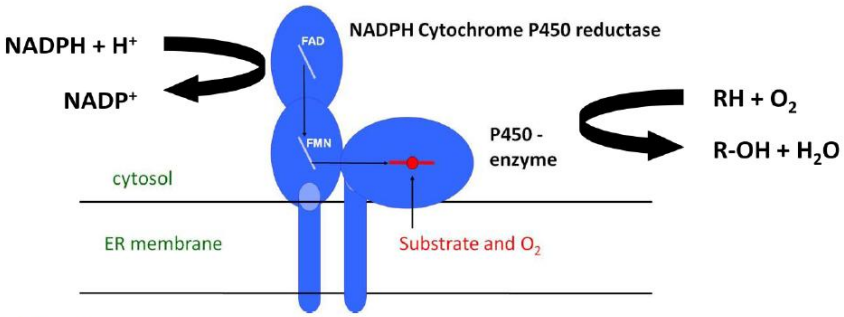


Figure 1. Scheme of a class II P450 enzyme complex

2.1.1.1. HPO, *Hyoscyamus muticus* premnaspirodiene oxygenase

The aroma of grapefruits is mainly based on the terpenoid compound nootkatone, which is highly interesting for industrial research [7]. Its characteristic grapefruit smell and taste turn it into a highly demanded product for fragrance, food, cosmetics and pharmaceutical industries. Unfortunately, nootkatone is an expensive flavor and only trace amounts of nootkatone can be found in citrus fruits [8]. Valencene in contrast, is a cheap and easily available fragrance of Valencia oranges. HPO as a biocatalyst can be applied for the production of nootkatone by oxidation of valencene at the C-2 atom yielding trans-nootkatol [1]. Trans-nootkatol is further oxidised to nootkatone.

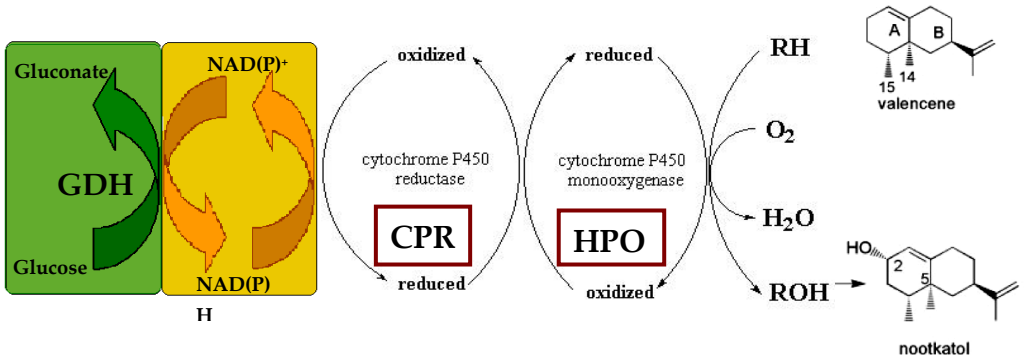


Figure 2. Reactions which take place at the Valencene in vitro activity assay

HPO is produced in the plant *Hyoscyamus muticus* (Figure 3) catalysing the hydroxylation of premnaspirodiene and solavetivol. During the reaction, carbon 2 (C-2) of the spirane substrate is successively hydroxylated (Figure 4) [2].

Figure 3. *Hyoscyamus muticus* (Egyptian henbane)

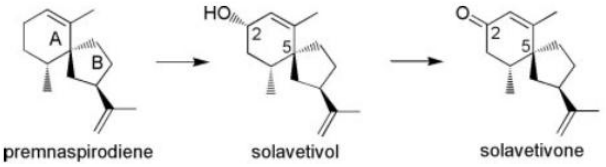


Figure 4. Natural substrate and reaction catalyzed by HPO from premnaspirodiene [2]

2.1.1.2. PM17, (-)-(4S)-Limonene-3-hydroxylase

Limonene-3-Hydroxylase is naturally produced by *Mentha piperita* (Figure 5). In plants, oxygenases play an essential role in the biosynthesis of terpenoid, alkanoid and phenylpropanoid natural products and in the catabolism of herbicides and other xenobiotics [9].

Figure 5. *Mentha piperita*

The N-terminal transmembrane domain anchors PM17 to the endoplasmic reticulum. The protein catalyses the hydroxylation of (-)-(4S)-limonene to (-)-trans-isopiperitenol, a precursor of (-)-menthol, responsible for the cooling sensation of peppermint [10].

2.1.1.3. CYP2D6, Cytochrome P450 2D6

Cytochrome P450 2D6 is a mammalian enzyme, responsible for the oxidation and, therefore, for the metabolism of many drugs and environmental chemicals. Substrates of CYP2D6 contain basic nitrogen and a planar aromatic ring [4].

2.1.2. CPR, NADPH – cytochrome P450 reductase and hCPR, human NADPH – cytochrome P450 reductase

NADPH-cytochrome P450 oxidoreductase serves as the electron donor for almost all eukaryotic cytochrome P450s. It belongs to a small family of diflavin proteins and harbours FAD and FMN cofactor binding domains with high structural homology to bacterial flavodoxins and to ferredoxin-NADP (+) oxidoreductases. CPR shuttles electrons from NADPH through the FAD and FMN cofactors into the central heme -group of the cytochrome P450s. Mobile domains in CPR are essential for electron transfer between FAD and FMN and for cytochrome P450 interaction [11].

2.2. *PICHIA PASTORIS* AS EXPRESSION HOST

The methylotrophic yeast *Pichia pastoris* is one of the most important hosts for heterologous protein expression and the most used yeast species for protein production [12]. Tightly regulated and efficient promoters and a strong tendency for respiratory growth are the greatest advantages using *P. pastoris*. Moreover, the ability of manipulating *P. pastoris* at the molecular level with simple DNA transformation systems has made the yeast a model system for basic and applied studies [13]. The most successful system for high-level expression of foreign proteins in *Pichia pastoris* is to make use of the alcohol oxidase I promoter (P_{AOX1}). P_{AOX1} is tightly regulated by a carbon source dependent repression/ induction mechanism [14]. During growth of *P. pastoris* on glucose or glycerol the expression from P_{AOX1} is repressed and it is maximally induced by methanol (MeOH) as sole C-source [15]. In this work, the *Pichia pastoris* wild type strain CBS7435-his (CBS, Centraalbureau voor Schimmelcultures) was used as starting point for all experiments. *Pp*CBS7435 has also been classified as *Komagataella phaffii* and is the parental strain of the two most frequently used protein expression host strains GS115 and the prototrophic strain X-33 [16], [17]. *HIS4* has been knocked out resulting in a histidine auxotrophic strain, which expanded the possibilities of strain manipulation, as only a very limited number of selectable markers are available for molecular genetic manipulation of *Pichia pastoris* [13].

2.3. PROTEIN PURIFICATION

For crystallization studies, protein purity should be at least 95 %. Therefore, in this work the proteins were purified using the Ni-NTA purification system based on affinity chromatography of 6xHis-tagged recombinant proteins expressed in bacteria, insect or mammalian cells [18]. The Ni-NTA Protein Purification System is based on the remarkable selectivity of Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues, the 6xHis tag. This technology allows one-step purification of almost any His-tagged protein from any expression system under native or denaturing conditions. NTA, which has four chelation sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that only have three sites available for interaction with metal ions. The extra chelation site prevents nickel ion leaching and results in a greater binding capacity and protein preparations with higher than those obtained using other metal-chelating purification systems. The purification of 6xHis-tagged proteins consists of 4 steps, the cell lyses, binding, washing, and elution. Strong denaturants and detergents can be used for efficient solubilization and purification of receptors, membrane proteins, and proteins that form inclusion bodies. Purified proteins are eluted under mild conditions by adding 100–500 mM imidazole as competitor [19].

To purify membrane bound proteins they have to be solubilized with detergents. Different detergents may have different influences on the proteins, some can solubilize them well and some can denaturate or inactivate them. These influences are tested by detergent screenings. Therefore, membrane samples will be mixed with detergent solution. The detergent concentration always has to be above the critical micellar concentration (CMC) in order to form mixed micelles containing the solubilized proteins.

2.4. CRYSTALLIZATION

Protein crystallization is inherently difficult because of the fragile nature of protein crystals. Proteins have irregularly shaped surfaces, which results in the formation of large channels within any protein crystal. Therefore, the noncovalent bonds that hold together the lattice must often be formed through several layers of solvent molecules [20]. Soluble proteins crystallize from solution and membrane proteins are crystallized after being solubilized in detergent solutions [20]. Before crystallization proteins have to be purified. Most protein structures currently available are from proteins that have been crystallized from aqueous solutions. When forming a crystal, individual protein molecules align themselves in a repeating series of unit cells by adopting a consistent orientation [20]. The importance of protein crystallization is that it serves as the basis for X-ray crystallography, wherein a crystallized protein is used to determine the protein's three-dimensional structure via X-ray diffraction. The X-ray diffraction pattern can be analyzed to discern the protein's tertiary structure. Several factors decide

whether a protein sample will crystallize or not, e.g. protein purity, pH, concentration of protein, temperature, precipitants and additives. The more homogeneous a protein in solution, the better the chances are for it to form a crystal. Protein purity has to be at least 95% for crystallization. The pH conditions are very important due to the fact that different pH values can result in different packing orientations. Buffers, such as Tris-HCl, are often applied for the maintenance of a particular pH [22]. In this work, the proteins were crystallized using vapor diffusion method and crystals were prepared by hanging drop or sitting drop method. Both entail a droplet containing purified protein, buffer, and precipitant being allowed to equilibrate with a larger reservoir containing similar buffers and precipitants in higher concentrations. Initially, the droplet of protein solution contains an insufficient concentration of precipitant for crystallization, but as water vaporizes from the drop and transfers to the reservoir, the precipitant concentration increases to a level optimal for crystallization. Since the system is in equilibrium, these optimum conditions are maintained until the crystallization is complete. The hanging drop method differs from the sitting drop method in the vertical orientation of the protein solution drop within the system [20],[23].

2.5. *IN VITRO* ACTIVITY ASSAYS

Membrane proteins can be inactivated when they are outside their natural environment and when they are truncated or solubilized using detergents. To use the crystal structure for structure determinations and furthermore for the improvement of different protein properties like stability and activity, the proteins have to be in their active form for crystallisation. Therefore the activity of the expressed proteins will be controlled via *in vitro* activity assays.

2.5.1. Limonene *in vitro* activity assay

In this assay, limonene is oxidized to form trans-isopiperitenol, which can be further converted to menthol (Figure 7). In this work, full-length (PM17) and truncated limonene hydroxylase (tPM17) are tested for their ability to catalyze this reaction. A NADPH dependent cofactor regeneration system was used. *Glucose 1-dehydrogenases (GDH)* catalyze the oxidation of glucose to gluconolactone, under concomitant reduction of NAD^+ and/or $NADP^+$ (Figure 7) [24]].

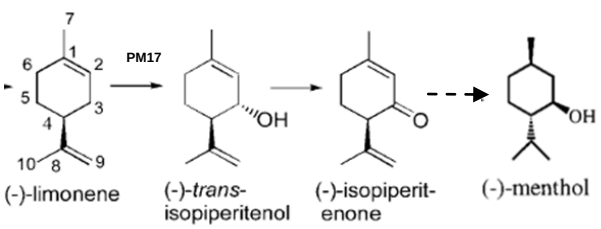


Figure 6. Reaction products generated of PM17 from (-)-limonene [9]

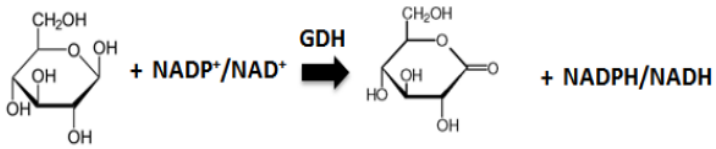


Figure 7. Nicotinamide cofactor regeneration system

2.5.2. Valencene *in vitro* activity assay

The sesquiterpene valencene is oxidized to intermediately form trans-nootkatol, which can further be converted to the final product nootkatone by a second oxidation event at the C-2 atom. The activity of full-length (HPO) and truncated (tHPO) *Hyoscyamus muticus* premnaspirodiene oxygenase are tested for their ability to convert valencene to trans-nootkatol. Triton X-100 was added to support enzyme-substrate-complex formation by facilitating phase transfer of substrate, which is considered to be the rate limiting step. The creation of emulsions on the one hand facilitates substrate solubility, but also stabilizes hydrophobic membrane proteins in cell homogenates [25]. A NADPH dependent cofactor regeneration system was used as described above.

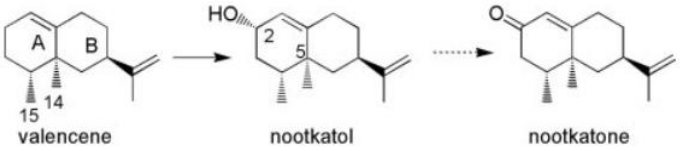


Figure 8. Industrially desired transformation of valencene by HPO [2]

2.5.3. Cytochrome c *in vitro* activity assay

The activity of the cytochrome P450 reductases is very important for the crystallization studies. This assay measures the reduction of cytochrome c by NADPH-cytochrome c reductase in the presence of NADPH. The absorption spectrum of cytochrome c changes with its oxidation/reduction state. Upon reduction, an absorption peak is observed at 550 nm. The reduction of cytochrome c is monitored by increase of absorbance at 550 nm [26].

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Instruments and devices

Table 2. Instruments and devices

Task	Instrument/Device	Manufacturer
Absorption measurement	Micro plate, 96 well, PS, U-bottom, MICROLON® Bio Photometer Plus DU® 800 Spectrophotometer	Greiner bio-one GmbH, Germany Eppendorf, Germany Beckman Coulter™, USA
Agarose gel electrophoresis	PowerPac™ Basic + Sub-Cell GT	Bio-Rad, USA
Clarification (detergent screening)	Optima ™ TLX Ultracentrifuge 120 000 rpm	Beckman Coulter™, USA
Cell harvest	Tabletop centrifuge 5810R Avanti™ Centrifuge, JA-10 and JA-25.50 rotors	Eppendorf, Germany Beckman Coulter™, USA
Cell homogenizer	MSK homogenizer	Braun, Germany
Cell cultivation	HT MultitronII, HT	Infors AG, Switzerland
Electrophoresis	PowerPac™ Basic, Sub-Cell GT NuPAGE® Novex® Bis-Tris Mini Gels	BIO-RAD, USA Invitrogen, USA
Electrotransformation	Micro Pulser™ Electroporation Cuvettes (2 mm gap)	BIO-RAD, USA Molecular BioProducts Inc., USA
Extraction (activity assay)	Vibrax VXR basic	IKA, Germany
GC-FID	Hewlett Packard 6890 Series with a FID detector Agilent 19091J-141 (Capillary 10.0 m x 0.1 mm x 0.1 µm film)	Agilent Technologies, Austria
GC -MS	Hewlett Packard 5890 Series II with a MS detector HP 5 MS column (Capillary 28.8 m x 0.25 mm x 0.25 µm film)	Agilent Technologies, Austria
Imaging	Syngene G:Box	Syngene, Great Britian
Incubator (28°C, 37°C)	BINDER Kühlbrutschränke	Binder GmbH, Germany
Laminar flow	UNIFLOW KR130 biowizard	UNIEQUIP, USA
Resuspension of cell pellets	Vortex – Genie 2	Scientific industries Inc., USA
Micro centrifuge	Centrifuge 5415R	Eppendorf, Germany
Mixing (small volumes)	Vortex – Genie 2	Scientific industries Inc., USA
Membrane harvest	Avanti™ Centrifuge, JA-10 and JA-25.50 rotors Optima TLX ultracentrifuge 100 000 xg	Beckman CoulterTM, USA Beckman CoulterTM, USA
OD measurement	Bio Photometer Plus Cuvettes (10 x 4 x 45 mm)	Eppendorf, Germany Sarstedt, Germany
PCR	GeneAmp® PCR System 2700	Applied Biosystems, USA
Protein purification	Amicon® Ultra-15 Centrifugal filter devices for volumes up to 15 ml (10 K, 30K)	Merck Millipore, Ireland

Shaker (small volumes)	Thermomixer comfort	Eppendorf, Germany
Shaker (cell cultures)	Certomat® BS-1	Sartorius, Germany
Sterile work	Uniflow KR130 biowizard	UniEquip GmbH, Germany
Sonication	Sonifier 250	Branson, USA
SDS-PAGE and Western blot	Power Ease 500 Power Supply XCell SureLock™ Mini-Cell Nitrocellulose membrane, Hybond-ECL™ NuPAGE® Novex 4-12% Bis-Tris Gels 1.0 mm, 17 well	Invitrogen, USA Invitrogen, USA Amersham Bioscience, Sweden Invitrogen, USA
Thermomixer	Thermomixer comfort	Eppendorf, Germany
Weighing	Lab scale: TE 1502S Precision scale: Explorer	Sartorius, Germany OHaus, Germany

3.1.2. Employed kits

Table 3. Employed kits

Name	Supplier
Gene Jet™ Plasmid Miniprep Kit	Fermentas GmbH, Germany
Maxima® Hot Start Green PCR Master Mix	Fermentas GmbH, Germany
SuperSignal West Pico Chemiluminescent Kit	Pierce, USA
Wizard® SV Gel and PCR Clean Up System	Promega Corporation, USA

3.1.3. Reagents

Table 4. Reagents and supplier

Reagent	Supplier
Acetic acid (CH_3COOH)	Roth GmbH, Germany
Agar	Roth GmbH, Germany
Agarose LE	Biozyme, Germany
5-Aminolevulinic acid hydrochloride (δ-ALA)	Sigma – Aldrich, Germany
Ampicillin (Amp)	Sigma - Aldrich, Germany
Aqua bidest.	Fresenius Kabi GmbH, Austria
Bacto™ Agar	Becton, Dickinson and company, USA
Bicine	Roth GmbH, Germany
Biotin	Roth GmbH, Germany
Bovine Serum Albumin (BSA)	Roth GmbH, Germany
Bradford- BIO RAD reagent	BIO-RAD, USA
Bacto™ Yeast Extract	Becton, Dickinson and company, USA
Cis-, trans-Nootkatol	DSM Pharma Chemicals, Netherlands
Dimethylsulfoxyde (DMSO)	Roth GmbH, Germany
Dithiothreitol (DTT)	Roth GmbH, Germany
Dream Taq Polymerase [5 U/µL]	Fermentas GmbH, Germany
Dry milk powder	Low fat milk powder, from a local supermarket
Difco™ Yeast Nitrogen Base w/o Amino Acids	Becton, Dickinson and company, USA
Ethidium bromide (EtBr)	Roth GmbH, Germany
Ethyl acetate (EtOAc)	Roth GmbH, Germany

Ethylen glycol	Roth GmbH, Germany
Ethylenediamine tetraacetic acid (EDTA)	Roth GmbH, Germany
Enzymes and adequate buffers, various	Fermentas GmbH, Germany
$FeCL_3$	
FAD (Flavin adenine dinucleotide disodium salt hydrate)	Sigma - Aldrich, Germany
FMN (Riboflavin 5`monophosphate sodium salt hydrate)	Sigma - Aldrich, Germany
GDH (glucose dehydrogenase, NADP 12 U/mg)	DSM Pharma Chemicals, Netherlands
G6P (D-Glucose 6-phosphate sodium salt)	Sigma - Aldrich, Germany
Gene Jet™ Plasmid Miniprep Kit	Fermentas GmbH, Germany
D-Glucose	Roth GmbH, Germany
Glycin	Roth GmbH, Germany
Glycerol	Roth GmbH, Germany
Hydrochloric acid	Roth GmbH, Germany
Imidazol	Roth GmbH, Germany
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Peqlab Biotechnologie GmbH, Germany
Kanamycin (Kan)	Sigma- Aldrich, Germany
Lactose	Sigma- Aldrich, Germany
LB (Luria Bertani) Lennox	Roth GmbH, Germany
Loading Dye (10x)	Fermentas GmbH, Germany
Ni-Sepharose™ 6 Fast Flow	GE-Healthcare, Sweden
Methanol (MeOH)	Roth GmbH, Germany
Magnesiumchloride heptahydrate (MgCl2*7 H2O)	Roth GmbH, Germany
MOPS SDS Running buffer	Invitrogen, USA
Nootkatone	DSM Pharma Chemicals, Netherlands
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, Germany
Peptone	Roth GmbH, Germany
ProteoPrep® Detergent Sample Kit	Sigma - Aldrich, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma - Aldrich, Germany
Ponceau S ($C_{22}H_{16}N_4O_{13}S_4$)	Sigma - Aldrich, USA
Potassium chloride (KCl)	Roth GmbH, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Roth GmbH, Germany
Potassium hydrogen phosphate (K_2HPO_4)	Roth GmbH, Germany
SuperSignal West Pico Chemiluminescent Kit	Pierce, USA
Sorbitol	Roth GmbH, Germany
Sodium hydroxide (NaOH)	Roth GmbH, Germany
Sodium Chloride (NaCl)	Roth GmbH, Germany
Trichloroacetic acid	Roth GmbH, Germany
Tris	Roth GmbH, Germany
Triton X-100	Sigma - Aldrich, Germany
T4 DNA Ligase [10 U/µl]	Fermentas GmbH, Germany
Tryptone	LAB M, UK
Tween 20	Roth GmbH, Germany
Valencene	DSM Pharma Chemicals, Netherlands
Zeocin™	Invitrogen, USA
Zwittergent® Test Kit	Calbiochem, Germany

3.1.4. Media, buffers and solutions

Table 5. Media, buffers and solutions

Media / Buffer	Composition
Ampicillin Stock (Amp), 100 µg/ml	100 mg dissolved in 1 ml ddH_2O
1 M δ-ALA	1.68 g diluted in 10 ml ddH_2O
BEDS medium	1 ml of 0.1 M Bicine NaOH, 300 µl ethylene glycol, 500 µl DMSO, 2 ml of 5 M Sorbit dissolved in 6.2 ml ddH_2O
BMGY	10 g yeast extract and 20 g peptone dissolved in 700 ml of H_2O. After seperatly autoclaving add 100 ml 1M KPi buffer (pH 6.0), 100 ml 10x YNB, 2 ml 500x Biotin, 100 ml 10x Glycerol
BMMY	10 g yeast extract and 20 g peptone dissolved in 700 ml water. After separately autoclaving,100 ml 1 M KP_i (pH6.0),100 ml 10x YNB, 2 ml 500x Biotin and 10 ml MeOH are added.
Biotin (500x)	10 mg Biotin dissolved in 50 ml ddH_2O and filter sterilized
Binding buffer (Purification),	0.5 M NaCl, 10 mM imidazol dissolved in 20 mM KP_i (pH 7.4)
Breaking buffer	6 g sodium phosphate, 1 mM PMSF, 372 mg EDTA and 50 ml 5% glycerol are separately autoclaved, dissolved in 900 ml ddH_2O and adjusted to pH 7.4
DTT (1 M)	1.54 g DTT dissolved in 10 ml ddH_2O and filter sterilized
1 M Di-potassium-hydrogen phosphate stock	174,18 g dissolved in 1 l ddH2O
10 mM $FeCL_3$	27 mg dissolved in 10 ml ddH_2O
Elution/wash buffer (purification), pH 7.4	20 mM KP_i, 0.5 M NaCl, 20 mM – 500 mM imidazol
Glycerol (10x)	100 ml glycerol dissolved with 900 ml ddH2O
IPTG stock (100 mM)	1.19 g IPTG dissolved in 50 ml ddH_2O
Kanamycin Stock (Kan)	40 µg dissolved in 1 ml ddH_2O
LB - Media	10 g tryptone, 5 g yeast extract, 10 g/l NaCl dissolved in 1 l ddH_2O
LB - Agar	15 g agar dissolved in 1 l LB – Media
20x NPS media	66 g $(NH_4)_2SO_4$, 136 g KH_2PO_4, 142 g Na_2HPO_4 dissolved to 1 l of ddH_2O
50x5052 media	250 g Glycerol, 25 g Glucose, 100 g lactose, dissolved in 730 ml ddH_2O
MOPS-running buffer (1x)	100 ml NuPage®-MOPS (10x) dissolved with 900 ml with ddH_2O
Ponceau S solution	Ponceau S dissolved in 5 % acetic acid
1 M Potassium-dihydrogen phosphate stock	136.09 g dissolved in 1 l ddH_2O
Potassium phosphate buffer (1 M KP_i buffer, pH 6.0)	132 ml 1 M K_2HPO_4 and 868 ml 1 M KH_2PO_4 dissolved in 1 l ddH_2O
SOC-Media	20 g bacto tryptone, 0.58 g NaCl, 5 g bacto yeast extract, 2 g $MgCl_2$, 0.16 g KCl, 2.46 g $MgSO_4$, 3.46 g dextrose dissolved to 1 l with ddH_2O
10x SDS-Running buffer	28 g Tris, 144 g Glycin, 10 g SDS dissolved to 1 l with ddH_2O
SDS – sample buffer	780 µl dissociation buffer (20 mM KH_2PO_4, 6 mM EDTA, 6% SDS, 10% glycin, 0.05% bromophenol blue) and 200 µl Tris-HCl buffer (1 M, pH 8.8) mixed with 20 µl β-mercaptoethanol

TCA (50 % w/v)	50 g TCA dissolved in 100 ml ddH_2O
Tris-HCl buffer (1 M, pH 8.8)	122 g dissolved in 1 l ddH_2O
20x Transfe rbuffer	14.5 g Tris, 72 g Glycin diluted to 500 ml with ddH_2O
1x Transfer buffer (Western blot)	75 ml 20x Transferbuffer , 150 ml Methanol dissolved to 1.5 l with ddH_2O
10x TBS buffer (pH 7.5)	30.3 g Tris and 87.6 g NaCl dissolved to 1 l with ddH_2O
1x TBST buffer	100 ml 10x TBS buffer, 300 µl Tween-20 dissolved to 1 l with ddH_2O
TBST - milk	5 g dry milk powder dissolved in 100 ml 1x TBST buffer
10x YNB	134 g dissolved in 1 l ddH_2O
YPD - Media	10 g yeast extract and 20 g peptone dissolved in 900 ml H_2O. After separate autoclaving 100 ml 10x Dextrose is added.
YPD - Agar	YPD media + 20 g/l agar
ZY-media	10 g N-Z-amine AS (e.g. tryptone) and 5 g yeast extract dissolved in 925 ml ddH_2O
ZYP-5052 media	928 ml ZY-media, 1 ml of 1 M MgSO4, 20 ml of 50x 5052, 50 ml of 0x NPS, 4 ml Kanamycin (25 mg/ml) dissolved to 1 l with ddH_2O

3.1.5. Enzymes and antibodies

Table 6. Enzymes and antibodies

Enzyme / Protein	Supplier
FastAP™ alkaline phosphatase	Fermentas GmbH, Germany
FastDigest™ restriction enzymes	Fermentas GmbH, Germany
Goat anti-rabbit IgG - HRP	Invitrogen, USA
Maxima® Hot Start Taq DNA Polymerase	Invitrogen, USA
Phusion® DNA Polymerase	Sigma-Aldrich, USA
Rabbit anti-6xHIS polyclonal antibody	Rockland, USA
Restriction enzymes	Finnzymes, Finnland
T4 DNA Ligase	Fermentas GmbH, Germany

3.1.6. Strains and plasmids

Table 7. *E. coli* and *Pichia pastoris* strains

Cell	Strain	Genotype	Source
E. coli	Top 10 F`	F'{*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80 *lac*ZΔM 15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U galK *rps*L *end*A1 *nup*G	Invitrogen
E. coli	BL21 Star™ (DE3)	F- *ompT hsdS*B (rB-mB-) *gal dcm rne131*(DE3)	Invitrogen
P. pastoris	Wt CBS 7437-his	*Δku70Δhis4*	T. Wriessnegger

Table 8. Expression plasmids

Plasmid	Features	Source
pPpB1	Vector containing AOX 1 promoter and Zeocin resistance gene	T. Wriessnegger
pET-26b (+)	Expression vector with T7 promoter and Kanamycin resistance gene, pelB signal sequence for potential periplasmic localization	T. Wriessnegger

3.1.7. Genes

All genes were provided by DSM and T. Wriessnegger. Codon optimizations of the wild type sequences were done by T. Wriessnegger and DSM.

Table 9. Cloned genes

Gene	Features	Protein	Organismus	Protein size
HPO	HPO sequence with TMD, codon optimized for *P. pastoris* by DSM	Premnaspirodiene oxygenase	*Hyoscyamus muticus*	58.7 kDa
tHPO	Truncated HPO sequence, codon optimized for *P. pastoris* by DSM	Premnaspirodiene oxygenase	*Hyoscyamus muticus*	55.8 kDa
PM17	PM17 sequence with TMD, codon optimized for *P. pastoris* by T. Wriessnegger	Limonene-3-Hydroxylase	*Mentha piperita*	58.4 kDa
tPM17	Truncated PM17 sequence, codon optimized for *P. pastoris* by T. Wriessnegger	Limonene-3-Hydroxylase	*Mentha piperita*	57.1 kDa
CPR	CPR sequence with TMD, codon optimized for *P. pastoris* by DSM	NADPH-cytochrome P450-reductase	*Arabidopsis thaliana*	78.6 kDa
tCPR	Truncated CPR sequence, codon optimized for *P. pastoris* by DSM	NADPH-cytochrome P450-reductase	*Arabidopsis thaliana*	74.4 kDa
hCPR	hCPR sequence with TMD, codon optimized for *P. pastoris* by T. Wriessnegger	Human cytochrome P450-reductase	*Homo sapiens (Human)*	80.1 kDa
thCPR	Truncated hCPR sequence, codon optimized for *P. pastoris* by T. Wriessnegger	Human cytochrome P450-reductase	*Homo sapiens (Human)*	75.3 kDa
2D6	CYP2D6 sequence with TMD, codon optimized for *P. pastoris* by T. Wriessnegger	Cytochrome P450 CYP2D6	*Homo sapiens (Human)*	58.6 kDa
t2D6	Truncated CYP2D6 sequence, codon optimized for *P. pastoris* by T. Wriessnegger	Cytochrome P450 CYP2D6	*Homo sapiens (Human)*	56.2 kDa

3.1.8. Primers

3.1.8.1. Primers used for cloning in *P. pastoris*

Table 10. List of primers used for constructs coding for membrane proteins. *Eco*RI (highlighted in yellow), *Not*I (highlighted in green), Kozak sequence (red, bold), His-Tag (blue, bold)

Primer	Sequence 5`- 3`
FwHPO	CGGAATTC**CGAAACG**ATGCAATTCTTCTCCTTGGTC
RvHPO-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**TTCTCTGGATGGTTGGTATG
FwCPR	CGGAATTC**CGAAACG**ATGACTTCTGCTTTGTACGCTTC
RvCPR-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CCAGACATCTCTCAAGTATCTAC C
Fw2D6	CGGAATTC**CGAAACG**ATGGGTTTGGAAGCCTTGGTC
Rv2D6-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CTTATCGTCGTCATCCTTGTAATC
Fw_hCPR	CGGAATTC**CGAAACG**ATGATTAACATGGGTGACTCC
Rv_hCPR-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CAAATCCTCTTCAGAAATCAATTT TTG
FwPM17	CGGAATTC**CGAAACG**ATGGAATTGCAAATCTCTTCTG
RvPM17-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CTTATCGTCGTCATCCTTGTAATC

Table 11. List of primers used for constructs coding for truncated proteins. *Eco*RI (highlighted in yellow), *Not*I (highlighted in green), Kozak sequence (red, bold), His-Tag (blue,bold)

Primer	Sequence 5`- 3`
FwHPOnoTMD	CGGAATTC**CGAAACG**ATGTCCAACTCTCAATCCAAGAAATTG
RvHPOnoTMD-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**TTCTCTGGATGGTTGGT ATG
FwCPRnoTMD	CGGAATTC**CGAAACG**ATGGTTGTCTTATTGTGGAAGAAGAC
RvCPRnoTMD-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CCAGACATCTCTCAAGT ATCTACC
Fw2D6noTMD	CGGAATTC**CGAAACG**ATGAGAAGACAAAGATGGGCTGC
Rv2D6noTMD-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CTTATCGTCGTCATCCT TGTAATC
Fw_hCPRnoTMD	CGGAATTC**CGAAACG**ATG TTGTTTAGAAAGAAGAAGGAAGAAG
Rv_hCPRnoTMD-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CAAATCCTCTTCAGAAA TCAATTTTTG
FwPM17noTMD	CGGAATTC**CGAAACG**ATGAAGCAATGGAGAAAACCAAAG
RvPM17noTMD-His	ATAGTTTAGCGGCCGCTTATTA**GTGGTGATGGTGATGATG**CTTATCGTCGTCATCCT TGTAATC

3.1.8.2. Primers used for cloning in *E. coli*

Table 12. List of primer used for constructs without leader sequence (pelB), which are coding for membrane proteins. *Hind*III (highlighted in yellow), *Nde*I (red, bold), His-Tag (blue, bold)

Primer name	Sequence 5`- 3`
FwHPOnoTMD_E2	GGAATTC**CATATG**TCCAACTCTCAATCCAAGA
RvHPO_His_E	ATAGTTTAAAGCTTTTATTA**GTGGTGATGGTGATGATG**TTCTCTGGATGGTTGGTATGGA
FwCPRnoTMD_E2	GGAATTC**CATATG**CGTTGTCTTATTGTGGAAG
RvCPR_His_E	ATAGTTTAAAGCTTTTATTA**GTGGTGATGGTGATGATG**CCAGACATCTCTCAAGTATCTA CC
Fw2D6noTMD_E2	GGAATTC**CATATG**AGAAGACAAAGATGGGCTG
Rv2D6_His_E	ATAGTTTAAAGCTTTTATTA**GTGGTGATGGTGATGATG**CTTATCGTCGTCATCCTTGTAA TC
Fw_hCPRnoTMD_E2	GGAATTC**CATATG**TGTTGTTTAGAAAGAAGAAGGAA
Rv_hCPR-His_E	ATAGTTTAAAGCTTTTATTA**GTGGTGATGGTGATGATG**CAAATCCTCTTCAGAAATCAAT TTTTG
FwPM17noTMD_E2	GGAATTC**CATATG**AAGCAATGGAGAAAACCAAAGCCTC
RvPM17_His_E	ATAGTTTAAAGCTTTTATTA**GTGGTGATGGTGATGATG**CTTATCGTCGTCATCCTTGTAA TC

3.2. METHODS

3.2.1. General methods

Plasmid isolation and purification from *E. coli*

Isolation and purification of plasmids was done using the Gene Jet™ Plasmid Miniprep Kit based on the method of silica-gel membrane adsorption. DNA was finally eluted with 30 µl ddH_2O.

Determination of DNA concentration

One µl of purified DNA fragments were supplied with 8 µl ddH_2O and 1 µl 10x loading dye. The whole volume was loaded onto an 1% agarose gel and separated at 120 V. Concentrations were estimated by comparing band intensities to those of a DNA ladder standard. Five µl of DNA Ladder were loaded on the argarose gel.

PCR reaction (polymerase chain reaction)

PCRs for insert generation were done with Phusion® DNA polymerase according to the Finnzymes Phusion® High-Fidelity DNA Polymerase protocol. Primers, amounts of different reagents used and cycling conditions are stated in the results section. The templates for PCR reactions were provided by DSM and T. Wriessnegger. In general, 0.5 µl of template DNA were used for PCR reactions. After PCR, the whole volume, in general 50 µl, was loaded onto a preparative 1% agarose gel and the bands representing the amplified gene were cut out and cleaned up for further processing.

Gel electrophoresis

Gel electrophoresis was done using 1% agarose gels containing EtBr in TAE-buffer. Analytical gels were run at 120-130 V for approximately 45 min, preparative gels were run at 90 V for approximately 60 min. The sizes and concentrations of DNA fragments were assessed by comparison to DNA standards. For all samples, GeneRuler™ 1 kb DNA Ladder was used.

DNA gel purification

All DNA fragments were separated with standard 1% agarose gels at 90 V and purified according to the manual of the Wizard® SV Gel and PCR Clean Up System. 30 µl of ddH_2O were used for final DNA elution.

DNA restriction

For preparative restriction cuts, the full volume of a single plasmid purification event and the full volume of the PCR products were mixed with the restriction enzymes and appropriate

buffer at the recommended concentration. The mixtures were incubated for at least 2 h at 37°C. Exact volumes are recommended in the result section.

Control cuts

For control restriction cuts, 1 µl of the purified plasmid was mixed with 1 µl of restriction enzyme, 1 µl of recommended buffer (10x) and 6 µl of ddH_2O. The reaction was incubated for 30-60 min at 37°C.

Dephosphorylation

Dephosphorylation of the vector backbone was done using Fermentas® FastAP™ alkaline phosphatase. 30 µL of vector DNA was mixed with 1 µL FastAP™ alkaline phosphatase and 3 µL recommended buffer (10x). The reaction was incubated for 10 min at 37°C and again 1 µL of FastAP™ alkaline phosphatase was added before incubating for 10 min at 37°C. Then, the DNA was purified according to the Wizard® SV Gel and PCR Clean Up System.

DNA ligation

Vector backbone and insert were used at 1:3 molar ratio. One µl T4 DNA ligase [10 U/µl] and 2 µl T4 DNA ligase buffer (10x) were added to the linear vector and insert and the volume was adjusted to 20 µl with ddH_2O. A useful tool for calculation of size-dependent insert amounts was the online ligation calculator offered by insilico-Düsseldorf [27]. Reactions were performed at room temperature for two h. Afterwards, mixtures were heated to 65°C for 10 min to inactivate the enzyme, followed by desalting for 30 min on a 0.025 µm Millipore filter floating on ddH_2O. Solutions were transferred to sterile Eppendorf tubes and immediately used for electrotransformation or stored at -22°C.

E. coli electrocompetent cells

Single colonies of *E. coli* cells were inoculated into 30 ml of LB media for overnight cultures at 37°C and 220 rpm. Then, 500 ml of LB media in 2 l baffled flasks were inoculated with 5 ml of the overnight culture and incubated at 37°C and 170 rpm until an OD_{600} between 0.7 and 0.9 was reached. Before harvesting, cells were chilled on ice for 30 min. Cells were centrifuged in sterile, pre-chilled 500 ml centrifuge bottles at 2,000xg and 4°C for 15 min. After discarding the supernatants, the pellets were resuspended in 500 ml pre-chilled ddH_2O, and centrifuged as above. This step was repeated. Then, the cells were resuspended in 35 ml of pre-chilled and sterile 10% glycerol and centrifuged at 4,000xg and 4°C for 15 min. Supernatants were discarded and the pellets were finally resuspended in 1 ml of ice cold, sterile 10% glycerol. 80 µl aliquots of electrocompetent cells in Eppendorf tubes were either stored on ice for instant use or frozen in liquid N_2 for storage at -80°C.

Transformation into *E. coli* cells (Electroporation)

For electrotransformation, 80 µl of electrocompetent cells were mixed with 10 µl plasmid DNA in pre-chilled electroporation cuvettes and pulsed with the EC2 program for 5-6 ms at 2.5 kV. Immediately after transformation, 1 ml SOC-medium was added and the whole broth was incubated on a thermomixer at 650 rpm and 37°C for 1 h. Defined amounts of the cells were plated on LB-Antibiotic (Zeocin or Kanamycin) plates and incubated overnight at 37°C.

Plasmid isolation and control cuts

After successful transformation, which was determined by comparing the colony numbers of the plates with the corresponding negative controls, several transformants were streaked onto quarters of adequate plates and grown o/n at 37°C. A quarter of *E. coli* cells were abraded and the plasmids were isolated using GeneJet Mini Prep Kit. In order to ensure that ligation was successful and the right plasmid was generated, restriction control cuts using Fast Digest enzymes were performed. After confirming correct cloning, the plasmids were stored at -20°C until further use.

Linearization of a plasmid

To linearize the isolated plasmid, for transformation into *P. pastoris,* 50 µl of plasmid DNA was mixed with 6 µl of the recommended buffer (10x), 4 µl of recommended restriction enzyme and adjusted to 60 µl with ddH_2O. The mixture was incubated for 3 h at 37°C and a control gel was performed to control correct linearization.

P. pastoris electrocompetent cells

Generation of electrocompetent *P. pastoris* cells and transformation were based on the "Condensed protocol for competent cell preparation and transformation of *Pichia pastoris*" [28]. A single *P. pastoris* colony was inoculated in 5 ml YPD medium and was grown o/n at 28°C. 50 mL of YPD medium were inoculated to an A_{600} of 0.2 with the ONC and grown to an A_{600} of 0.8-1.0 at 28°C. Cells were harvested by centrifugation in Grainer tubes for 5 min at 500 xg and room temperature, and the pellet was resuspended in 9 ml of freshly prepared BEDS medium before 1 ml DTT medium was added. The solution was centrifuged again for 5 min at 500 x g and resuspended in 1 ml BEDS medium. The cells were used immediately for transformation. Storing the cells at -20°C or -80°C significantly decreased their electrocompetence. For every transformation, the cells were freshly prepared.

Transformation into *P. pastoris* cells (Electroporation)

After linearization of the respective plasmid carrying the insert, the linear DNA was transformed into *P. pastoris* electrocompetent cells. Transformation was based on the

"Condensed protocol for competent cell preparation and transformation of *Pichia pastoris*" [28]. For transformation, at least 50-100 ng of a linearized plasmid DNA was mixed with 80 µl of competent cells and incubated for 2 min on ice. Electroporation was carried out at 1500 V, 200 Ω and 25 µF and the program Fungi/Pic was used. Immediately after the electropulse, 0.5 ml YPD and 0.5 ml of 1 M sorbitol were added and the cells were regenerated for 2-3 h at 28°C without shaking. 50 µl of the cell suspension and the transformation rest were centrifuged, resuspended and plated on selective media before incubation for 2-3 days at 30°C.

Colony PCR (polymerase chain reaction)

Colony PCR was performed to determine integration of different genes into the after transformation. Colony PCR was done using Phusion DNA polymerase, dNTPs and the recommended buffer according to the recommended protocol or using Maxima® Hot Start Green PCR Master Mix (2x) kit according to the recommended protocol. The template was prepared in the same manner as described above. After PCR, 5 µl of the PCR-mix was mixed with 5 µl 6 x loading dye and loaded onto an analytical 1% agarose gel.

3.2.2. *E. coli* cultivation

Autoinduction

To prepare the pre-culture, a single colony was inoculated in 100 ml ZY media (growth medium) and incubated over night at 120 rpm and 37°C. 500 ml ZYP-5052 media (induction medium), containing 40 µg/ml kanamycin, were inoculated the next day to an A_{600} of 0.1 and autoinduced at 120 rpm and 20°C for 16-20 h. Every two hours samples were taken for protein analysis. Cells were harvested by centrifugation at 5000 rpm ((Avanti^{TM} Centrifuge, JA 10) for 10 min.

IPTG-induction

To prepare the pre-culture, 100 ml of LB-medium (growth media), containing 40 µg/ml kanamycin, were inoculated with a single colony and incubated over night at 20°C and 250 rpm. For the main culture, 500 ml of LB medium (induction medium) were inoculated with the pre-culture to an A_{600} of 0.1 and incubated at 20°C and 250 rpm for approximately 24 hours. At an A_{600} of 0.3, after around 4 hours, the cells were induced with 0.05 - 0.5 mM IPTG.

3.2.3. Protein preparation from *E. coli* cells

Sample preparation

Cell pellets were resuspended in 20 mM KP_i, pH 8.0, and cells were disrupted using B-Per reagent for samples up to 1 ml and using sonication for higher volumes.

B-Per cell disruption

Cells were disrupted following the “Instruction manual for B-PER® Bacterial Protein Extraction Reagent” from Thermo Scientific. 200 µl B-PER reagent were added to 1 ml cell suspension and incubated for 10-15 min at room temperature. The lysate was centrifuged at 15 000 x g for 5 min to separate the soluble fraction from the insoluble fraction. The fractions were stored at -20°C.

Ultrasound cell disruption

Cells were sonicated for 5 min during cooling with ice water. The adjustments of the sonifier were 80% duty cycle and 8 output control. After sonication, the cell extract was centrifuged for 1 h at 75 600 x g and 10°C. The solutions were stored at 4°C.

3.2.4. *P. pastoris* cultivation

Pichia pastoris colonies were cultivated in deep well plates (DWP) and 2 l baffled shake flasks according to the requirements of the experiments.

Micro-scale cultivation in DWPs

To screen for positive clones and to prepare strains for Western blot analysis, *P. pastoris* transformants were grown in 96 DWPs. One well per row was filled with medium only to serve as a sterile control and a wild type *P. pastoris (*WT *CBS7435)* colony was inoculated as negative control. Single *P. pastoris* colonies were inoculated into 250 µl BMGY media and grown at 28°C, 320 rpm and 80% humidity for approximately 48 h to reach the stationary growth phase and deplete glycerol. Then, the cells were pinned onto YPD-zeocin plates, containing different antibiotic concentrations (100 µg/ml, 1 mg/ml, 2 mg/ml) for selection. The culture was induced by addition of 250 µl of BMMY2 (1% MeOH) resulting in a final concentration of 0.5% MeOH. 12 h, 24 h and 36 h after the induction start, 50 µl of BMMY10 (5% MeOH) were added to compensate for MeOH loss due to evaporation and consumption. The cultivation was stopped 12 h after the last MeOH addition, resulting in an overall induction period of 48 h.

Shake-flask cultivation

Two l baffled shake-flasks were filled with 100-125 ml BMGY medium and inoculated with single colonies of *P. pastoris* strains. The cells were grown at 28°C and 110 rpm for 48- 60 h to reach the stationary growth phase and deplete glycerol. 100-125 ml of BMMY2 (1% MeOH) were added, resulting in a final concentration of 0.5% methanol. At 12 h, 24 h, 36 h, 48 h and 60 h after the induction start, 1 - 1.25 ml of sterile filtered MeOH was added, resulting in a final MeOH concentration of 0.5%, to adjust for MeOH loss due to evaporation and consumption. The cultivation was stopped 12 h after the last addition of MeOH, resulting in an overall

induction period of 72 h. Every 12 h, 1 ml of cell culture was withdrawn, centrifuged at 5000 rpm (AvantiTM Centrifuge, JA10) for 1 min and the cell pellet was stored at -20°C for expression level control.

3.2.5. Protein preparation from *P. pastoris* cells

Sample preparation

Strains tested for protein expression by Western blot analysis were grown and induced in DWPs. Cells taken every 12 h from strains grown in 2 l baffled flasks, were used for SDS-PAGE. Cells from 2 l baffled flask cultivation were used for protein isolation and the protein was used for further experiments.

Cell harvest and glass bead disruption

Glass bead disruption was performed according to the "Pichia Expression Kit manual" [27]. The cell cultures from the DWPs were transferred into an Eppendorf tube and centrifuged at 13 200 rpm and 4°C for 10 min. The cells were resuspended in 200 µl of ice-cold breaking buffer, containing 1 mM PMSF. An equivalent volume of glass beads was added to the solution and the cells were disrupted in 8 alternating 30 s cooling / vortexing cycles. The mixture was centrifuged again at 5 000 rpm and 4°C for 5 min and the supernatant, i.e. cell free protein extract, and was transferred into Eppendorf tubes or microtiter plates. The extracts were used immediately or stored at -20°C. To determine the concentrations of the cell lysate a Bio-Rad protein assay was performed.

Cells from 2 l baffled flask cultures were also disrupted using glass beads. Therefore, cells were harvested by centrifugation at 5000 rpm and 4°C for 5 min. Cells were resuspended in 20 mM KP_i, pH 7.4, followed by addition of PMSF to 1 mM. One third of the disruption vessel was filled with the cell suspension and one third of the vessel volume was filled with glass beads. The cells were disrupted in the cell Homogenizer MSK for 30 min with CO_2 cooling every 30 s. After disruption the suspension was centrifuged for 20 min at 20 000 rpm (Avanti^{TM} Centrifuge, JA10) to get rid of glass beads and undisrupted cells.

3.2.6. Protein analysis

Determination of protein concentration (Bradford assay)

Protein concentrations were determined based on the method of BioRad according to a 96 well plate protocol established by A. El-Heliebi. Therefore, cell lysates, cell fractions or protein solutions were added undiluted or diluted at a ratio of 1:10 and 1:100 with ddH_2O or buffer and 10 µl were transferred to each well. As blank, 10 µl ddH_2O or buffer were transferred to a well. 200 µl of 1:5 diluted BioRad reagent were added to each well of a standard microtiter plate.

Absorptions were recorded after incubation for at least 5 min, but less than 60 min at 595 nm in a microplate reader. Bovine serum albumin (BSA) was used for calibration at concentrations of 0.05 to 1 mg/ml (Figure 9).

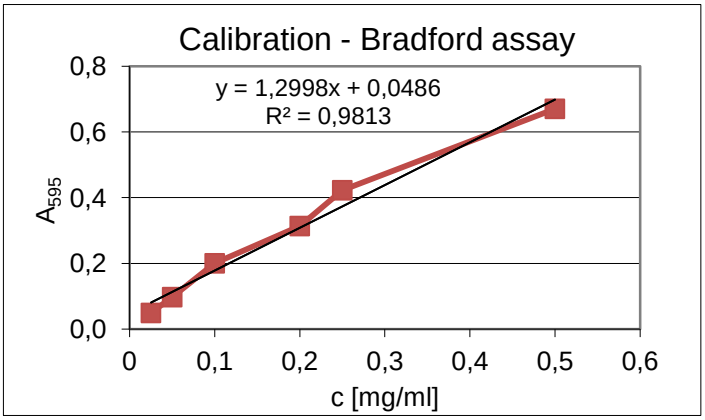


Figure 9. Calibration with BSA for the Bradford assay

TCA precipitation

In order to concentrate and purify the protein extracts, 20 µg of protein were precipitated using TCA. The volume of protein extract, equivalent to 20 µg, was added to 400 µl of ice-cold ddH_2O. 100 µl of ice-cold TCA (50%, w/v) were added and the protein was precipitated for at least 1 h on ice. The protein was pelleted by centrifugation for 10 min at 4°C and 13 200 rpm in a table top centrifuge and washed with ice–cold ddH_2O or ice–cold acetone. Supernatants were removed using the water jet pump. For SDS-PAGE, 20 µg of precipitated protein was dissolved in 10 µl of SDS sample buffer.

SDS – PAGE

SDS-PAGE was performed according to the manual of NuPage®, Invitrogen. 20 µg of precipitated protein were mixed with 10 µl of SDS sample buffer. The protein was denatured at 70 / 95 °C for 10 / 5 min, depending on the different samples. SDS-PAGE samples were used immediately or stored at -20°C. 10 µl of protein ladder (Fermentas® PageRuler™ prestained protein ladder) were loaded on the gel. Self-made Tris-Glycine gels, NuPage® gels and Native gels were used. All gels were further used for Western blot analysis or stained with Coomassie Blue over night under gentle shaking and destained using 10% acetic acid solution.

NuPage® gels

Running conditions for NuPage ready-to-use gels were maximum voltage, maximum power and 110 mA for 50 min.

Self-made Tris-Glycine gel

For the separating gel (12.5%), 9.43 ml ddH_2O, 7.03 ml Tris-HCl buffer (pH 8.8, 1.5 M), 11.25 ml Acrylamid (30%), 281.25 µl SDS (10% w/v), 140.63 µl APS (Ammonium persulfate, 10%

w/v) and 28.13 µl TEMED were mixed and filled into the pouring chamber for five gels. The gel was polymerized for about one h. For the stacking gel 11.45 ml ddH_2O, 4.7 ml Tris-HCl buffer (pH 8.8, 1.5 M), 2.45 ml Acrylamid (30%), 187.5 µl SDS (10% w/v), 93.75 µl APS (Ammonium persulfate, 10% w/v) and 18.75 µl TEMED were mixed and polymerized for about one h. The running conditions for the Tris-Glycine gel were maximum voltage, maximum power and 25 mA gel for 10 min and 40 mA for a duration time of 40 min.

SDS-PAGE gels were placed in a well sized plastic box, for Coomassie blue staining or gels were immediatly used for Western blot analysis. Coomassie staining was carried out overnight under moderate shaking at room temperature. Gels were destained with 10% acetic acid, which was exchanged until protein bands were clearly seen.

Basic-NATIVE gel

For the running buffer, 1.21 g (0.05 M) of Tris and 5.32 g (0.38 M) of Glycine were mixed, filled up to 200 ml with distilled water and the pH was adjusted to 8.9 with HCl. To dissolve the protein sample a 5x dissolving buffer was prepared, containing 5 ml Glycerol, 2.7 ml ddH_2O, 2.13 ml Tris-HCl (0.5 M, pH 6.8) and traces of bromophenol blue. Aliquots of 1 ml were kept at -20° C. The gel was prepared according to the conditions listed in Table 13 and 14. . TEMED and APS were added at the end. The solution was pipetted to a level of 4 cm from the top of the pouring chamber. 500 µl of n-buthanol was added. The gels were polymerized for at least 60 min. The surface of the gel was rinsed with H_2O before pouring the stacking gel. The sandwich was filled with stacking gel solution. The gel was fully polymerized after 1 h was stored at 4°C for no more than 2 days. Running conditions were 30 mA / 250 V max.

Table 13. Composition of the separating gel for native PAGE

% Acrylamide	12%	12%
Number of Minigels	5	1
1.5 M Tris-HCl, pH 8.8	7.0 ml	1.4 ml
30% Acrylamide, 0.8% Methylene bis Acrylamide	11.3 ml	2.3 ml
H_2O	9.3 ml	1.9 ml
10% APS	100 µl	20 µl
TEMED	23 µl	4.6 µl

Table 14. Composition of the stacking gel for native PAGE

Number of Minigels	1	5
0.5 M TrisHCl, pH 6.8	1.25 ml	4.0 ml
30% Acrylamide, 0.8% Methylene bis Acrylamide	0.5 ml	1.5 ml
H_2O	3.2 ml	9.6 ml
10% APS	50 µl	150 µl
TEMED	5 µl	15 µl

Western blot

Western blot was performed according to the manual of NuPage®, Invitrogen. Blotting pads, filter paper and nitrocellulose membrane were soaked in 700 mL 1x transfer buffer to remove air bubbles. The blotting sandwich was built as described in Figure 10 using soaked pads, gel(s) from SDS-PAGE, and filters. Air bubble formation was thoroughly avoided.

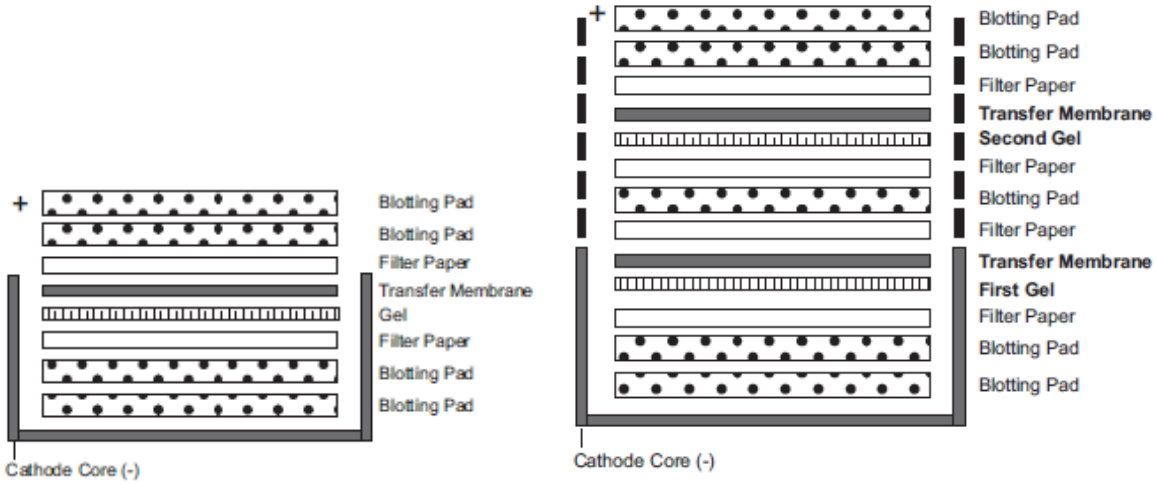


Figure 10. Left scheme: Blotting sandwich used for blotting one gel; Right scheme: Blotting sandwich used for blotting two gels (Manual NuPAGE®, Invitrogen)

The blotting sandwich was fixed with a Gel Tension Wedge in the XCell II unit and blotting was carried out according to the manual, exposing the sandwich to 250 mA, maximum voltage and maximum power for 1 h. After blotting, the membranes were stained with PonceauS solution to detect transfer efficiency of the proteins. After washing away excessive PonceauS with ddH_2O, the membranes were blocked with 50 ml of TBST-milk at RT for 1 h or o/n. Afterwards TBST-milk was discarded and the membranes were incubated with the primary antibody by moderately shaking at RT. This step was followed by washing three times with TBST for 5 min each. The membranes were treated with the secondary antibody at RT and moderate shaking, which was followed by three washing steps with TBST for 5 min each. Detection was carried out by covering the membrane with 3 ml of SuperSignal West Pico Chemoluminescent Substrate mixture. Chemoluminescence detection was done in a G:Box (Syngene) after 3-5 min incubation. Used antibodies, their dilution rates and incubation times are stated in the Results section. For all Western blots the proteins were detected with rabbit anti-His primary antibody, 1:7500 diluted with TBST-milk. Secondary antibody used was goat anti-rabbit IgG-HRP, 1:10 000 diluted with TBST-milk.

3.2.7. Purification

Detergent screening for membrane-bound proteins

For the following purification step of the proteins, membrane proteins were solubilized using "GE Healthcare Challenging proteins protocol" [29].

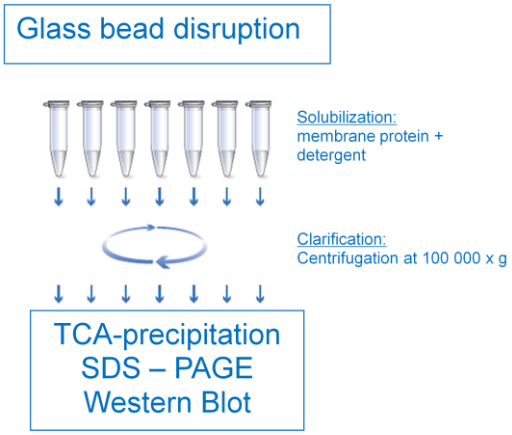


Figure 11. Scheme of the detergent screening for of membrane protein solubilisation

After glass bead disruption of the cells, the cell extract was used for the detergent screening. Different detergents were tested as listed in the Results section. The membrane protein fractions were solubilized by mixing 5 mg of total protein, 10 mM KP_i, pH 7.4, containing 100 mM NaCl and 1 mM PMSF, and 100 / 200 µl of 10% detergent solution to an overall volume of 1 ml (Table 15). For detergents with a higher CMC, 2 % of detergent was used. The solution was incubated at 25°C for 3 h and then clarified by ultracentrifugation at 100,000 x g for 1 h. The supernatants (soluble fraction) and the pellets (membrane fraction) were used for TCA precipitations, SDS-PAGE and Western blot analysis.

Table 15. Conditions for the detergent screening

Membrane protein concentration [mg/ml]	Detergent concentration [%]	NaCl [mM]	PMSF [mM]	Temp.[°C]	Time [h]
5	1 (above CMC)	100	1	25	2
5	2 (for detergents 5, 6, 9)	100	1	25	2

Isolation of total microsomes

The cells were harvested by centrifugation at 5000 rpm (JA 10) at 4°C for 5 min. Cells were resuspended in KP_i buffer, pH 7.4, and were disrupted using glass beads. After centrifugation at 5000 rpm (JA 10) and 4°C for 10 min to remove unbroken cells and glass beads, homogenate was centrifuged at 10 000 rpm (JA 25.5) and 4°C for 15 min to remove the mitochondrial fractions. The supernatants were centrifuged at 13 000 rpm (JA 25.5) and 4°C for 15 min to remove the rest of the mitochondrial fraction. The supernatants were ultra-centrifuged at 45 000 rpm (JA 25.5) and 4°C for 45 min to pellet the microsomal membranes.

The resulting pellets were the total microsomal fractions, which included the ER membranes where the proteins of interest were located. Pellets were resuspended in 1-2 ml 20 mM KP_i buffer, pH 7.4, containing 1 mM PMSF. These fractions were used for solubilisation of the proteins according to the conditions listed above (Table 15).

Protein purification using Ni - sepharose beads

For the purification of the His-tagged proteins, Ni-NTA affinity chromatography was used, according to the "InvitrogenTM Ni-NTA purification system protocol" [30]. The cells were disrupted by glass bead disruption and the cell extract was directly used for purification of truncated, soluble proteins. The membrane proteins were purified after solubilisation. The Ni-Sepharose beads were prepared and used according to the "GE Healthcare Instruction manual (affinity media) for Ni Sepharose™ 6 Fast Flow" [31]. Therefore, the used beads were washed with ddH_2O by shaking for 3 min and resedimented by centrifugation at 4000 rpm in a tabletop centrifuge for 5 min. Supernatants were discarded and the beads were washed again with 20 mM KP_i buffer, pH 7.4. For protein binding, beads and extracts were mixed and gently shaken over night at 4°C. Then, the beads with bound protein were filled into an empty PD10 column. All solutions were prepared with 20 mM KP_i buffer, pH 7.4, containing 0.5 M NaCl. Different buffers were used for the washing and elution steps (Table 16). To optimize protein elution for imidazol concentrations, the columns were washed with buffers containing increasing imidazol concentrations (Table 17).

Table 16. List of buffers used for Ni-NTA affinity chromatography

Buffer	Composition
Binding buffer	20 mM KP_i, pH 7.4, 0.5 M NaCl, 10 mM imidazol
Washing buffer	20 mM KP_i, pH 7.4, 0.5 M NaCl, 15 - 50 mM imidazol
Elution buffer	20 mM KP_i, pH 7.4, 0.5 M NaCl, 100 - 500 mM imidazol

Table 17. List of washing and elution steps used for the purification

Purification step	Column volumes (CV)	Imidazol concentration [mM]
Wash 1	10	10
Wash 2	10	15
Wash 3	10	20
Wash 4	10	30
Wash 5	10	50
Elution 1	5	100
Elution 2	5	200
Elution 3	5	300
Elution 4	10	500

For all further purifications, the column was washed and eluted only with buffer containing one fixed concentration of imidazol, but with higher volumes. The column was washed with buffer containing 15-50 mM imidazol and directly eluted with buffer containing 100-500 mM imidazol. After purification, the eluted proteins were desalted to get rid of imidazol and NaCl using P-10 desalting columns and the protein was concentrated using Millipore Amicon® Ultra- 15 concentrator with 10000-30000 MWCO, depending on the protein size, by centrifugation at 4 000 rpm (tabletop centrifuge) and 4°C for 20-30 min. The protein was stored at 4°C. All buffers were sterile-filtered before use.

Preparation and cleaning of the Ni-sepharose medium

According to the Invitrogen™ Ni-NTA purification system protocol, the slurry containing the used Ni-Sepharose beads was transferred to a centrifuge tube and sediment by centrifugation at 4000 rpm for 5 min. Supernatants were discarded and replaced with 5 ml of distilled water. Beads were washed by shaking for 3 min and resedimented by centrifugation at 4000 rpm (tabletop centrifuge) for 5 min. These steps were repeated one time using binding buffer instead of distilled water. The Ni- Sepharose beads were dispersed in binding buffer to a 50% slurry.

Regenerating of the medium

The residual Ni^{2+} was removed by washing with 5 column volumes of 20 mM KP_i buffer, pH 7.4, containing 0.5 mM NaCl and 50 mM EDTA. The residual EDTA was removed by washing with at least 5 CVs of binding buffer followed by 5 CVs of distilled water before recharging the column. To recharge the column, 0.5 CVs of 0.1 M $NiSO_4$ in distilled water were added. The beads were washed with 5 column volumes of distilled water followed by 5 column volumes of binding buffer before storage in 20% Ethanol.

3.2.8. *In vitro* activity assays

Limonene assay

All measurements were performed in duplicate in an overall volume of 1 ml using 100 mM KP_i, pH 7.4, as buffer. 500 pmol - 1 nmol of PM17 protein (suspended membranes and solubilized protein) were mixed with 500 pmol - 1 nmol CPR (ER- fraction) or tCPR (Homogenate) or 50 - 100 pmol of purified tCPR, 250 mM KCl and 50 mM $MgCl_2$ in Pyrex vials and incubated at room temperature for 30 min. To initiate the reaction, 200 µM (-)-limonene, 2 mM Glucose-6-phosphate, 0.8 U Glucose-6-phosphate dehydrogenase (NADP 12 U/mg), 0.5 mM NADPH, 5 µM FAD and 5 µM FMN were added to the reaction solution and filled up to 1 mL with assay buffer. The reaction was incubated for 2 h at 30°C and gently shaking to convert (-)-limonene to trans-isopiperitenol. The reaction was terminated on ice and extracted with 0.5-1 ml EtOAc on the Vibrax VXR basic for 30 min. The mixture was centrifuged for 15 min at 4°C and 4000

rpm (tabletop centrifuge) to separate the fractions. The upper, organic fraction contained the substrate and the products. The extract was used for GC-FID measurements along with the standards, (-)-limonene (100 µg/ml), D-camphor (100 µg/ml), a standard mix containing (-)-limonene, (-)-menthol, D-campher and L-carvone and EtOAc as blank [9].

Valencene assay

The assay was performed in Pyrex tubes in an overall reaction volume of 1 ml. All reactions were performed with assay buffer, 100 mM KP_i, pH 7.4. Five mg of HPO (ER fraction and homogenate) were mixed with 5 mg homogenate of tCPR, 4% (v/v) DMSO and reaction buffer before incubating for 5 min at 30°C. The reaction was initiated by addition of 2.4 mM NADPH, 200 µM valencene, 0.1% Triton X-100, 5 µM FMN and 5 µM FAD and incubated over night at 30 °C to convert valencene to nootkatol. The reaction was terminated on ice and extracted with 1 ml of EtOAc on the Vibrax VXR basic for 30 min. The mixture was centrifuged for 15 min at 4°C and 4000 rpm in a table top centrifuge to separate the fractions. The organic phase was concentrated under N_2 steam and the residues were dissolved resuspended in 100 µl EtOAc and GC-FID analysis was performed [2].

GC-FID measurement

GC measurements were performed in cooperation with Professor Erich Leitner and the Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology. The methods were established for the efficient separation and analysis of the terpenes and terpenoids of interest by Erich Leitner. For analysis of the results, Instrument #1 Data Analysis was used. GC-FID measurements were carried out using a HP-5 column (cross linked 5% Ph-Me Siloxane; 10 m, 0.10 mm diameter and 0.10 µm film thickness) on a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID).

Quantification of the products with GC-FID

For the quantification of valencene, *cis/trans*-nootkatol, nootkatone, (-)-limonene and trans-isopiperitenol, respective standards were prepared at different concentrations and analyzed by GC-FID. The peak areas were integrated and calibration curves were created by correlating to the areas with known concentrations. Based on this data, Erich Leitner programmed an automatic peak recognition and integration program that directly quantified the substances yielding in ng/µl values.

GC-program for limonene assay

A HP-5 column was used (cross linked 5% Ph-Me Siloxane; 10 m, 0.10 mm diameter and 0.10 µm film thicknes) on a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID). Sample aliquots of 1 µl were injected in split mode at 250°C injector temperature and

320°C detector temperature with N_2 as carrier gas and with a flow rate set to 0.4 ml/min in constant flow mode. The oven temperature program was as follows: 40°C for 1 min, 4°C/min ramp to 90°C and 30°C/min ramp to 280°C (0.5 min).

GC -program for valencene assay

A HP-5 column was used (cross linked 5% Ph-Me Siloxane; 10 m, 0.10 mm diameter and 0.10 µm film thicknes) on a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID). Sample aliquots of 1 µl were injected in split mode at 250°C injector temperature and 320°C detector temperature with N_2 as carrier gas and with a flow rate set to 0.4 ml/min in constant flow mode. The oven temperature program was as follows: 100°C for 1 min, 20°C/min ramp to 250°C, and 45°C/min ramp to 280°C (0.5 min). Total run time was 8 min.

Cytochrome c assay

The activity assay was performed according to an instruction by T. Wriessnegger [32]. The assay was performed in cuvettes measuring the reduction of cytochrome c and therefore, the increase of the extinction at 550 nm over a time range of 2 min. Dilutions of 1:10, 1:50 and 1:100 were prepared for the protein samples. 500 µl of Tris-HCl, pH 7.5, containing 1 mM EDTA was provided in a cuvette and 125 µl of Cytochrome c solution were added. To mask oxidase activities, 50 µl of KCN, pH 7.7, and 25 µl of diluted protein sample were added to the solution. To start the reaction, 50 µl of NADPH solution (0.1 mM) were added. The reactions were blanked against a cytochrome c, buffer and KCN mixture.

Crystallization studies

Crystallization studies were performed by Andrzej Lyskowski from Structural biology group of Molecular Biology of Graz University. The vapor diffusion method was used, crystals were prepared by hanging drop or sitting drop method and structure determination was performed by using X-Ray diffraction systems. 30 µl of protein solution of 5-10 mg/ml was needed for as starting point in a single drop.

4. RESULTS

4.1. AIMS

The overall aim of the project was the expression, purification and crystallization of five different membrane bound and for truncated cytochrome P450 and CPR proteins. Genes optimized for *P. pastoris* and coding for an additional His-tag were cloned into *E. coli* and *P. pastoris*. All proteins were expressed in *P. pastoris* using the P_{AOX1} by induction with MeOH. The truncated proteins were also expressed in *E. coli* using the *T7* promoter by autoinduction and induction with IPTG. The His-tagged proteins were isolated and purified using Ni/NTA affinity chromatography. Therefore, the membrane-bound proteins were solubilized using detergents. The truncated proteins were purified directly from cell extracts. The purified proteins were crystallized by the core facility of structural biology of Graz University using vapor diffusion method.

4.2. EXPRESSION OF MEMBRANE - BOUND AND TRUNCATED, SOLUBLE PROTEINS IN *P. PASTORIS*

4.2.1. Cloning

The Vectors and genes were provided and optimized by T. Wriessnegger and DSM. The genes listed in Table 18 were cloned into a pPpB1 vector as full length and truncated variants lacking the transmembrane domain coding sequence.

Table 18. List of genes cloned into a pPpB1 vector for *P. pastoris*

Protein	TMD	Host	Vector	Promotor	Tag
tHPO	no	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
HPO	yes	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
tPM17	no	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
PM17	yes	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
tCPR	no	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
CPR	yes	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
thCPR	no	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
hCPR	yes	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
t2D6	no	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His
2D6	yes	*P. pastoris* WT CBS 7435-his	pPpB1	AOX1	His

The genes for premnaspirodiene oxygenase, limonene-3-hydroxylase, cytochrome P450 reductase, human cytochrome P450 reductase and CYP2D6 were amplified via PCR, using primers containing the Kozak sequence, restriction sites for cloning into the used vector and a His-Tag for purification. Genes, optimized for *P. pastoris,* which code for an N-terminally linked

trans-membrane domain are indicated by the attachment “TMD” (Table 19). To generate the truncation, the TMD was cut off using different primer pairs as listed in Table 20.

Table 19. N-terminal trans-membrane domains (TMDs) as predicted by the tool TMHMM Server v. 2.0

Gene	N-terminal TMD (AA code)	Amino acid
HPO	QFFSLVSIFLFLSFLFLLRKWK	2-23
CPR	TSALYASDLFKQLKSIMGTDSLSDD	2-26
2D6	RNSETMGLEALVPLAVIVAIFLLLVDL	1-27
hCPR	INMGDSHVDTSSTVSEAVAEEVSLFSMTDMILFSLIVGLLTYWF	1-45
PM17	ELQISSAIIILVVTYTISLLII	2-23

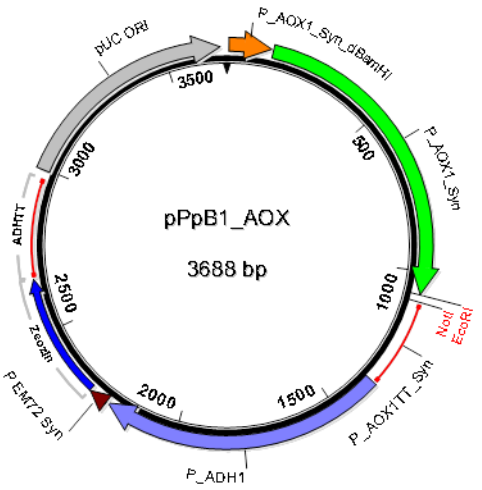
For all reactions, 0.5 µl of template DNA were mixed with 3 µl of forward as well of reverse primers, 2.5 µl dNTPs (2 mM each), 10 µl Phusion® HF Buffer (5x), 0.5 µl Phusion® DNA Polymerase (Finnzymes) (5 U/µl) and 30.5 µl of ddH_2O. Used annealing temperatures and extension times were calculated for the primer length and listed in Table 20.

PCR - cycling program: 98°C/30’’-(98°C/10’’- X°C/10’’ -72°C/Y’’) x30 -72°C/7 min -4°C/∞

Table 20. Templates, primers (forward / reverse) and conditions for tagging the genes with related pair of primers, specific annealing temperature (predicted by Tm calculation tool) and name of product

N°	Template	Fw / Rv primer	Annealing-temp. (X) [°C]	Extension time (Y) [s]	Product
1	HPO	FwHPO/ RvHPO-His	63	30	HPO-His
2	CPR	FwCPR/ RvCPR-His	63	40	CPR-His
3	2D6	Fw2D6/ Rv2D6-His	65	30	2D6-His
4	hCPR	Fw_hCPR/ Rv_hCPR-His	63	40	hCPR-His
5	PM17	FwPM17/ RvPM17-His	65	30	PM17-His
6	tHPO	FwHPOnoTMD/ RvHPOnoTMD-His	63	30	tHPO-His
7	tCPR	FwCPRnoTMD/ RvCPRnoTMD-His	62	40	tCPR-His
8	t2D6	Fw2D6noTMD/ Rv2D6noTMD-His	65	30	t2D6-His
9	thCPR	Fw_hCPRnoTMD/ Rv_hCPRnoTMD-His	63	40	thCPR-His
10	tPM17	FwPM17noTMD/ RvPM17noTMD-His	65	30	tPM17-His

The *E. coli – P. pastoris* shuttle vector pPpB1 (TU Graz strain collection 5709) was isolated from *E. coli* Top10F` cells (Figure 12). The pPpB1 vector provides single or multi-copy chromosomal integration of target genes in *P. pastoris.* Features of the vector are the origin of replication of the *E. coli* plasmid pBR322 [34], the P_{AOX1}, a multiple cloning site with unique restriction sites for *Eco*RI and *Not*I, the *AOX1* transcription termination sequence and an antibiotic resistance cassette consisting of a synthetic bacterial promoter called EM72 in tandem with a truncated version of the *P. pastoris ILV5* (*acetohydroxyacid reductoisomerase*) promoter. A synthetic gene, coding for the amino acid sequence of bleomycin (Zeocin), confers resistance against Zeocin from *Streptoalloteichus hindustanus*. The *ble* gene was codon optimized for function in *E. coli* as well as for *P. pastoris* [34].



Special considerations:

Zeocin™ selection in *Escherichia coli* and *Pichia pastoris*

For intracellular expression of gene of interest

For integration at *AOX1* locus, linearization with *Bgl*II (recommended)

For integration at *AOX1TT*, linearization with *Bam*HI

Recommended Regeneration: 500 µL of 1 M sorbitol +1%EtOH, after 1 h addition of 500 µL YPD

Completely sequenced

Figure 12. pPpB1 vector used for cloning into *P. pastoris* provided by T. Wriessnegger

50 µl of the vector backbone pPpB1 and 50 µl of the PCR products (inserts) were digested using restriction enzymes *Eco*RI and *Not*I. After digestion, the linearized vector backbone was dephosphorylated as described in the methods sector. The products were cleaned up on a preparative agarose gel. The DNA concentrations and the correct sizes of the constructs were determined with an analytical agarose gel (Figure 13) by comparing the bands to the bands of the ladder with known sizes and band intensities. Table 21 gives the determined concentrations of the digested DNAs and Table 22 shows the expected band sizes for the constructs.

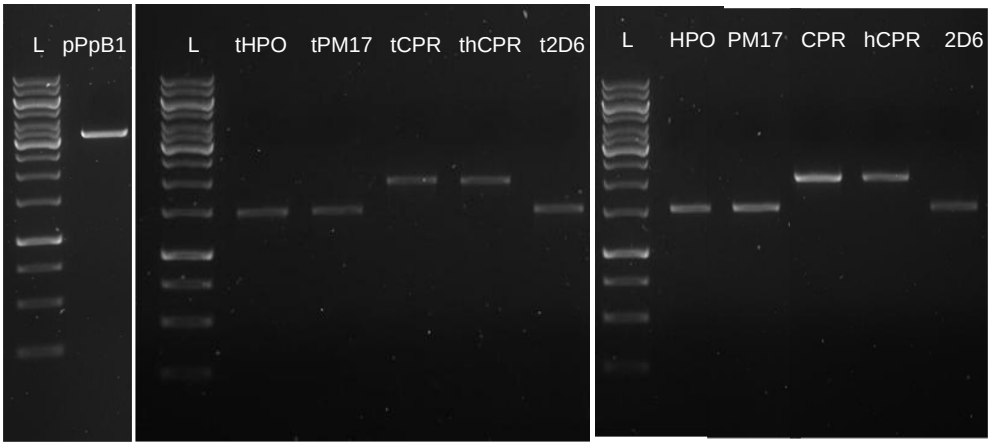


Figure 13. Control gel, double digest *Eco*RI and *Not*I, pPpB1 and insert verification

Table 21. Lane description of Figure 13

Lane	Description	Amount [µL]	Concentration [ng/µL]
L	GeneRuler™ 1kb DNA ladder	5	/
pPpB1	Vector backbone with AOX1 locus for ligation, cut (*Eco*RI and *Not*I) and purified	1	70
tHPO	Truncated HPO insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	15
tPM17	Truncated PM17 insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	20
tCPR	Truncated CPR insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	20
thCPR	Truncated hCPR insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	25
t2D6	Truncated CYP2D6 insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	20
HPO	HPO insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	30
PM17	PM17 insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	30
CPR	CPR insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	60
hCPR	hCPR insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	45
2D6	CYP2D6 insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	20

Table 22. Expected band sizes for inserts and the vector backbone

Construct	Expected band size (bp)
pPpB1	3700
HPO-His	1550
tHPO-His	1500
PM17-His	1600
tPM17-His	1500
CPR-His	2100
tCPR-His	2000
hCPR-His	2100
thCPR-His	2000
2D6-His	1600
t2D6-His	1500

The digested inserts were ligated into 50 ng of digested pPpB1 vector backbone in a molar ratio of 3:1 and the resulting plasmids were desalted for 30 min using microdialysis. The plasmids represented in Figure 14 were electrotransformed into E. coli TOP10F' cells. Transformants were selected for Zeocin resistance on LB-Zeo (100 µg/ml) plates for 1 day at 37°C and several colonies were streaked out for plasmid isolation. After plasmid isolation, positive clones were identified by restriction cut analysis, using Fast digest enzymes EcoRI and NotI. The cut was controlled via gel electrophoreses (Figure 15). The enzymes cut the insert sequence out of the vector and sizes of the fragments are listed in Table 23.

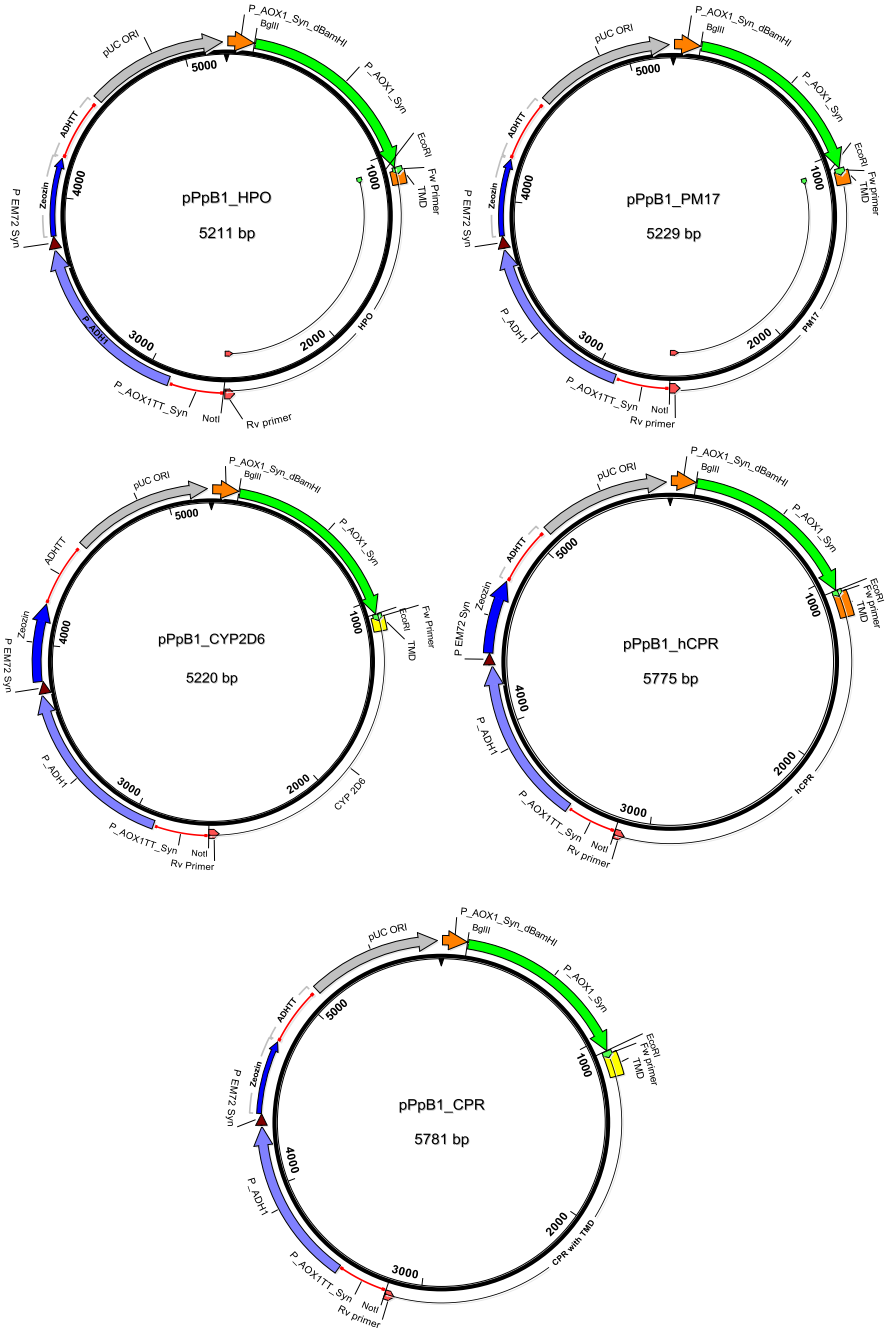


Figure 14. Vector maps of expression vectors pPpB1_HPO, pPpB1_PM17, pPpB1_2D6, pPpB1_hCPR and pPpB1_CPR with marked TMD, which was removed for expression vectors pPpB1_tHPO, pPpB1_tPM17, pPpB1_tCyp2D6, pPpB1_thCPR and pPpB1_tCPR

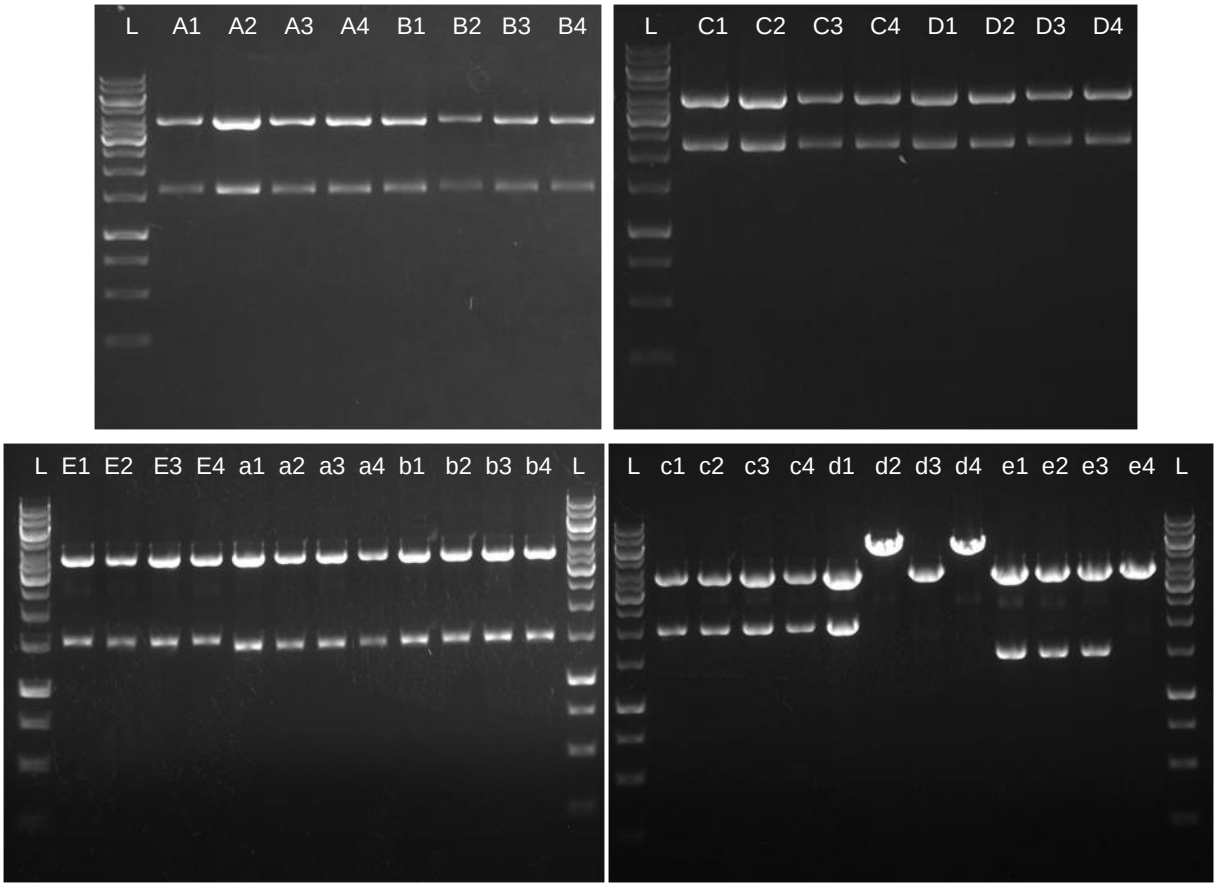


Figure 15. Control cuts of pPpB1_HPO, pPpB1_PM17, pPpB1_CPR, pPpB1_hCPR, pPpB1_2D6, pPpB1_tHPO, pPpB1_tPM17, pPpB1_tCPR, pPpB1_thCPR, pPpB1_t2D6 expression plasmids

Table 23. Lane description of Figure 15 and sizes of fragments

Lane	Description	Visible bands [bp]
L	GeneRuler™ 1kb DNA ladder	/
A1-A4	pPpB1_HPO	3700, 1550
B1-B4	pPpB1_PM17	3700, 1600
C1-C4	pPpB1_CPR	3700, 2100
D1-D4	pPpB1_hCPR	3700, 2100
E1-E4	pPpB1_2D6	3700, 1600
a1-a4	pPpB1_tHPO	3700, 1500
b1-b4	pPpB1_tPM17	3700, 1500
c1-c4	pPpB1_tCPR	3700, 2000
d1	pPpB1_thCPR	3700, 2000
d2, d3, d4	pPpB1_thCPR	Uncut plasmid (5800 bp)
e1-e4	pPpB1_t2D6	3700, 1500

After verification of the pPpB1 plasmid-based expression vectors, the plasmids were linearized using the restriction enzyme *Bgl*II (Figure 16). The linearized plasmids were purified and transformed into electrocompetent wild type *P. pastoris* CBS 7437-his strain, using 50-100 ng of plasmid DNA. Transformants were selected on YPD-Zeocin (100 µg/ml) plates for 3 days at 28°C.

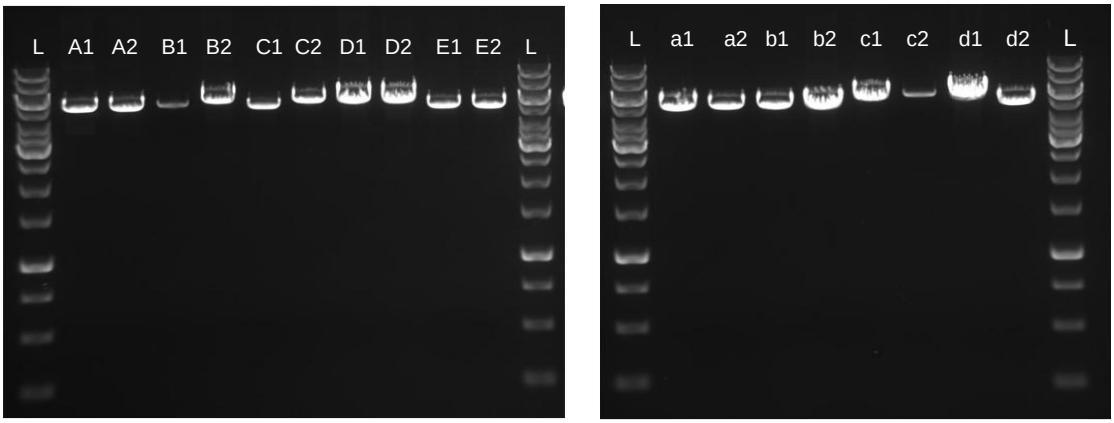


Figure 16. Control cuts of linearized plasmids pPpB1_HPO, pPpB1_PM17, pPpB1_CPR, pPpB1_hCPR, pPpB1_2D6, pPpB1_tHPO, pPpB1_tPM17, pPpB1_tCPR, pPpB1_thCPR, pPpB1_t2D6

Table 24. Lane description of Figure 16

Lane	Description	Visible band [bp]
L	GeneRuler™ 1kb DNA ladder	5211
A1, A2	pPpB1_HPO	5142
B1, B2	pPpB1_PM17	5229
C1, C2	pPpB1_CPR	5163
D1, D2	pPpB1_hCPR	5781
E1, E2	pPpB1_2D6	5659
a1, a2	pPpB1_tHPO	5775
b1, b2	pPpB1_tPM17	5445
c1, c2	pPpB1_tCPR	5220
d1, d2	pPpB1_thCPR	5151
e1, e2	pPpB1_t2D6	5211

30-40 transformants of each construct were tested with multi-copy integration screening (micro-scale cultivation in DWPs) as described in the methods section. A few transformants were tested via colony PCR whether they contain the linearized plasmids (Figure 17).

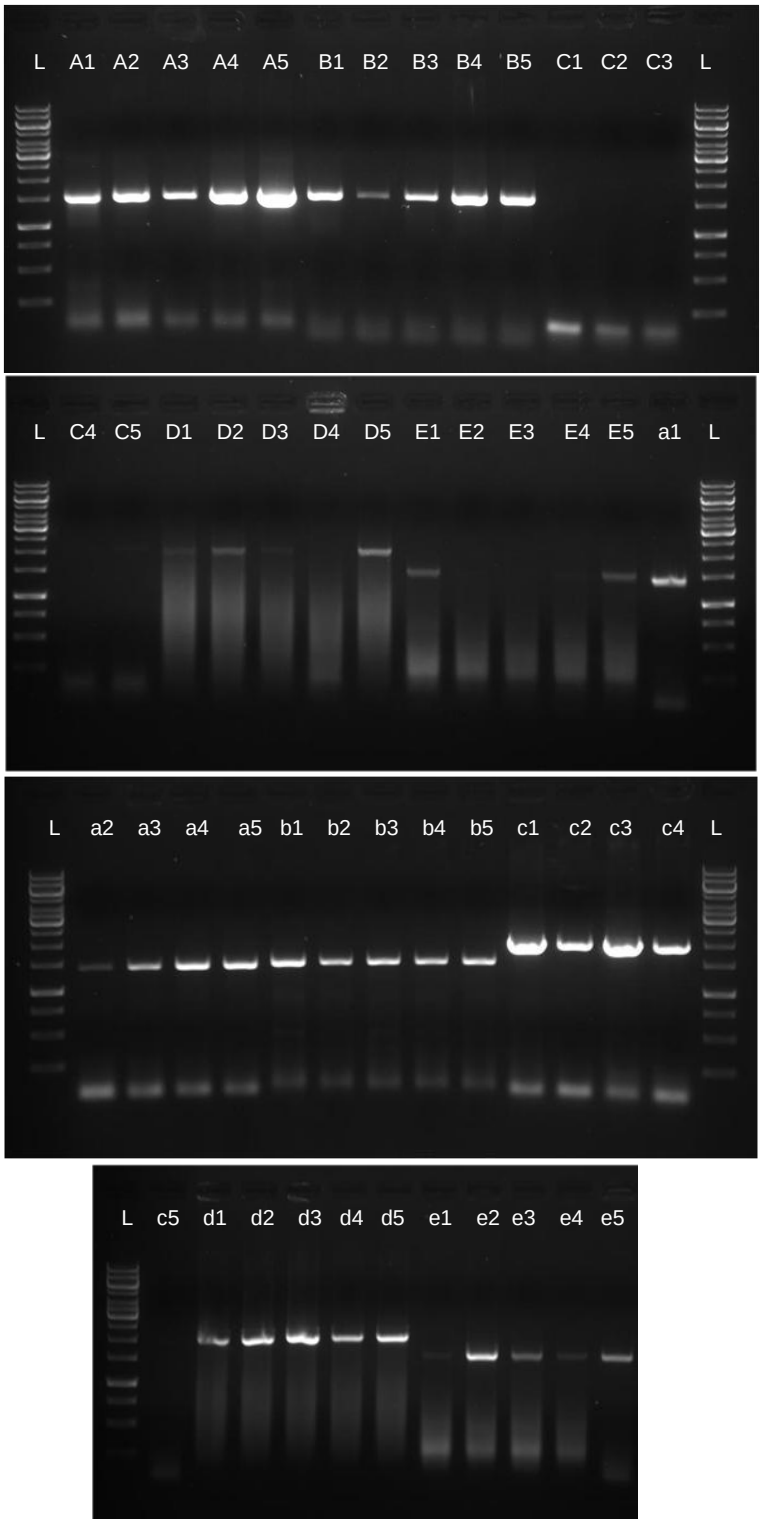


Figure 17. Colony PCR of CBS 7437-his transformants

Table 25. Lane description of Figure 17

Lane	Description
L	GeneRuler™ 1kb DNA ladder
A1- A5	pPpB1_HPO
B1- B5	pPpB1_PM17
C1- C5	pPpB1_CPR
D1- D5	pPpB1_hCPR
E1- E5	pPpB1_2D6
a1- a5	pPpB1_tHPO
b1- b5	pPpB1_tPM17
c1- c5	pPpB1_tCPR
d1- d5	pPpB1_thCPR
e1, e2	pPpB1_t2D6

4.2.2. Expression

Randomly chosen colonies were tested via Western blot according for their ability to express the different membrane proteins (HPO, CPR, hCPR, PM17 and 2D6) and truncated proteins (tHPO, tCPR, thCPR, tPM17 and t2D6). The cells, containing the linearized plasmid, were cultivated in DWPs using BMGY and BMMY media as described in the method section. All cells from DWP cultures were transferred into Eppendorf tubes and disrupted with glass beads. Protein concentrations were determined using Bradford assay and 20 µg of the proteins were precipitated with TCA. After precipitation, the protein pellets were resuspended in SDS-sample buffer and Western blot analysis was performed using anti-His (rabbit) primary antibody and anti-rabbit secondary antibody. The positive clones were chosen to perform further experiments. Western blot results are shown in Figure 18. SDS-PAGE, Western blot and subsequent immuno detection of the proteins were done according to the protocol described in Materials and Methods section.

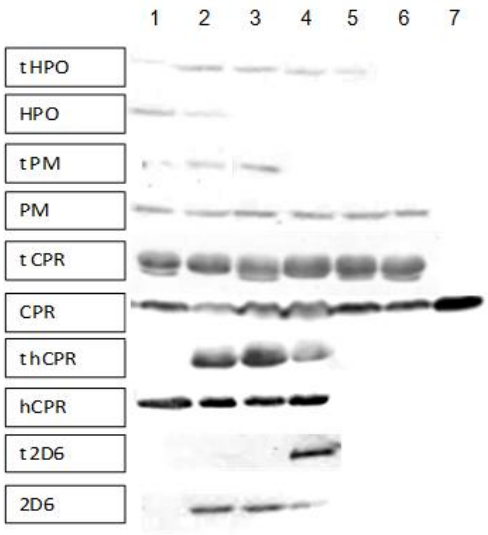


Figure 18. Western blot of positive clones in *P. pastoris* Wt CBS 7437-his strains

Table 26. . Expected sizes of the constructs

Protein	Expected size [kDa]
tHPO	55. 8
HPO	58.7
tPM17	57.1
PM17	58.4
tCPR	74.4
CPR	78.6
thCPR	75.3
hCPR	80.1
t2D6	56.2
2D6	58.6

Highest expression levels were achieved for the proteins CPR, tCPR, hCPR and thCPR. The truncated proteins yielded higher expression levels than the respective membrane-bound proteins. The positive transformants, tested with Western blot, were used for protein production in 2 l shaking flasks as described in the method section. SDS-PAGE was performed with samples taken every 12 h of Induction time to control expression levels. Harvested cells were disrupted using glass beads and precipitated using TCA as described above.

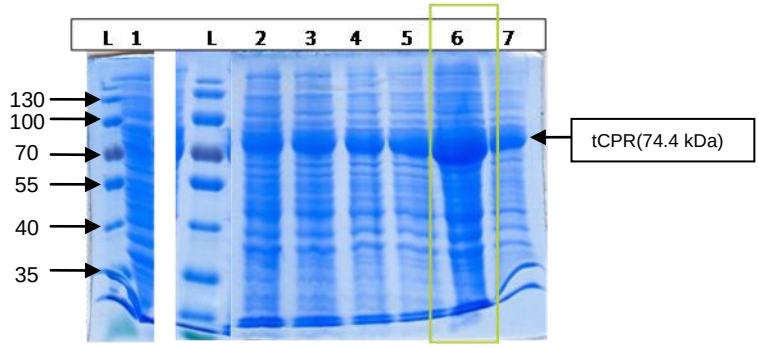


Figure 19. SDS- PAGE of samples of tCPR expression experiments

Table 27. Lane description for Figure 19

Lane	Sample	Induction time [h]
L	PageRuler^{TM} Prestained Protein Ladder	/
1	tCPR	0
2	tCPR	12
3	tCPR	24
4	tCPR	36
5	tCPR	48
6	tCPR	60
7	tCPR	72

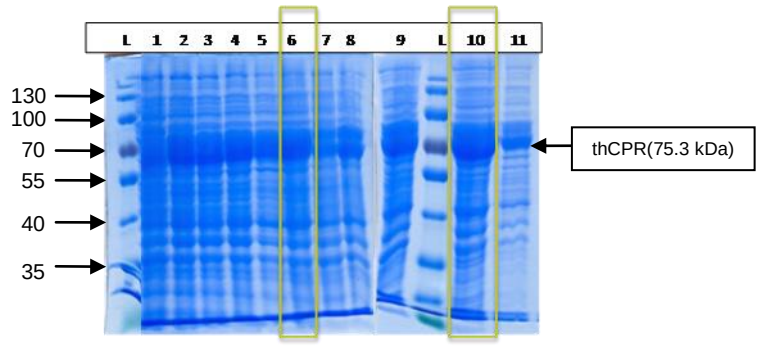


Figure 20. SDS- PAGE of samples of two independent thCPR expression experiments (E1, E2)

Table 28. Lane description of Figure 20

Lane	Sample	Induction time [h]	Lane	Sample	Induction time [h]
L	PageRuler™ Prestained Protein Ladder	/	6	E1, thCPR	60
1	E1, thCPR	0	7	E2, thCPR	12
2	E1, thCPR	12	8	E2, thCPR	36
3	E1, thCPR	24	9	E2, thCPR	48
4	E1, thCPR	36	10	E2, thCPR	60
5	E1, thCPR	48	11	E2, thCPR	72

The highest expression levels were achieved after 60 h of induction with MeOH as shown in the SDS-PAGE in Figure 20. Further expressions of the proteins in *P. pastoris* were performed Highest expression levels were achieved after 60 h of MeOH induction (Figure 20). Further protein expression experiments in *P. pastoris* were performed by growing in BMGY medium for 48 h and induction for 60 h with MeOH, to a final concentration of 0.5%, every 12 h, using the same expression conditions for all other expressed proteins (CPR, hCPR, tPM17, PM17, t2D6, 2D6, tHPO and HPO). Table 29 and 30 list the cell culture values and the final yield of cell extracts with their protein concentrations. Fermentations were done multiple times to generate enough protein material for purification and crystallization studies.

Table 29. Cell cultures used for purification of tCPR and thCPR

Sample	Culture	Cell wet weight [g]	Cell extract volume [ml]	C (protein) [mg/ml]
thCPR	600 ml (3 cultures)	17.00	30.00	22.90
tCPR	600 ml (3 cultures)	15.00	30.00	24.50

Table 30. Cell cultures used for detergent screenings and protein solubilization

Sample	Culture [ml]	Cell wet weight [g]	C(protein) after microsomal preparation [mg/ml]
HPO	600 ml (3 cultures)	20.70	34.14
CPR	600 ml (3 cultures)	22.00	43.73
2D6	600 ml (3 cultures)	17.00	40.41
PM17	600 ml (3 cultures)	18.70	33.24
hCPR	600 ml (3 cultures)	17.00	39.10

4.3. EXPRESSION OF TRUNCATED PROTEINS IN *E. COLI*

The genes (Table 31) were cloned into the pET-26b(+) vector (Figure 21), for expression of the truncated proteins in *E. coli* to achieve higher expression levels of the recombinant proteins than in *P. pastoris*. The vector and the genes were provided by T. Wriessnegger and DSM. For cloning into *E. coli,* the truncated and optimized genes for *P. pastoris* were used.

Table 31. List of genes cloned into the pET-26b (+) vector for cloning into *E. coli*

Protein	TMD	Host	vector	Promotor	Tag
tHPO	no	*E. coli* BL21 star (DE3)	pET-26b(+)	T7	His
tPM17	no	*E. coli* BL21 star (DE3)	pET-26b(+)	T7	His
tCPR	no	*E. coli* BL21 star (DE3)	pET-26b(+)	T7	His
thCPR	no	*E. coli* BL21 star (DE3)	pET-26b(+)	T7	His
t2D6	no	*E. coli* BL21 star (DE3)	pET-26b(+)	T7	His

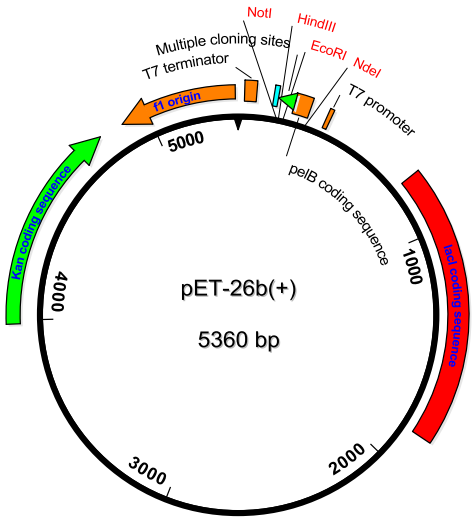


Figure 21. pET-26b(+) vector

All genes were cloned into the vector containing the pelB leader sequence, which is located between the restriction sites *Eco*RI *and Not*I. Genes without pelB leader sequence were cloned using *Nde*I and *Hind*III restriction sites. The pelB leader sequence is responsible for protein transport into the periplasma. Vector constructs containing the inserts are shown in Figure 22.

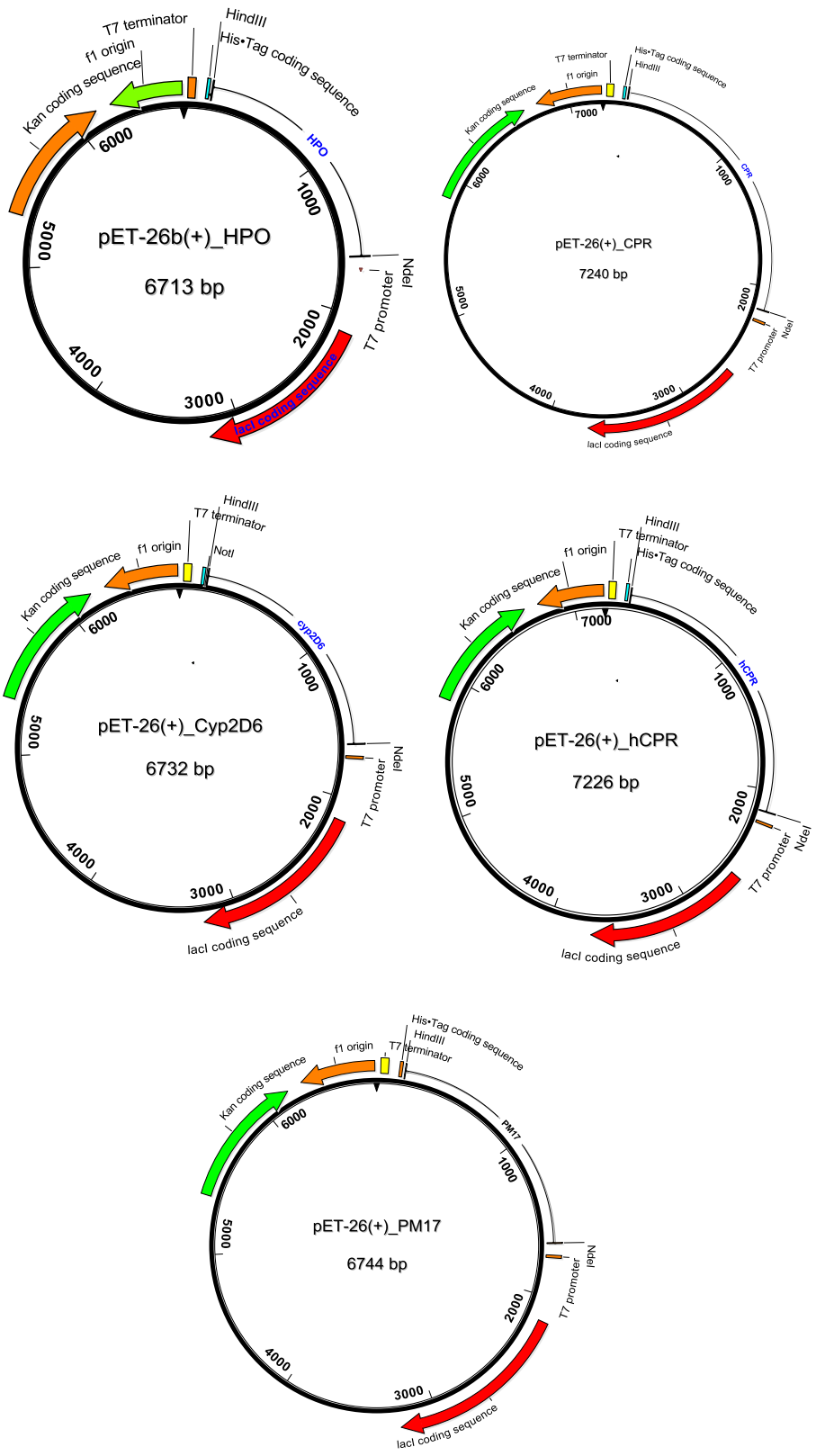


Figure 22. Vector maps of expression vectors pET-26(+)_tHPO, pET-26(+)_tCPR, pET-26(+)_tCyp2D6, pET-26(+)_thCPR and pET-26(+)_tPM17

4.3.1. Expression of truncated cytochrome P450 and CPR proteins in *E. coli,* using *T7* promoter

Five truncated cytochrome P450 and CPR proteins were expressed in *E. coli* to achieve higher expression yields and to get higher protein amounts, because for the crystallisation studies 10 mg/ml of protein concentration were needed. In previous experiments done by Anita Emmerstorfer, expression of cytochrome P450 and CPR proteins in *E. coli* led to inclusion body formation propably due to incorrect, i.e. false or too fast, folding of the proteins. Different strategies were applied to avoid this problem. The proteins were expressed using autoinduction and induction with small amounts of IPTG to achieve a slow and, therefore, correct folding of the proteins and to avoid inclusion body formation. The proteins were expressed without pelB leader sequence, which is the signal sequence for the transport into the periplasm. To achieve a better yield, the proteins were also expressed containing the pelB leader sequence, because in the periplasm the proteolytic activity is reduced and better folding might be achieved.

Cloning

The genes coding for tHPO, tPM17, tCPR, thCPR and t2D6 and optimized for expression in *P. pastoris*, were used for cloning and expression in *E. coli*. The genes were amplified via PCR with primers containing restriction sites for cloning into the used vector and a His-Tag for the purification. For the proteins with pelB sequence the same primers were used as for the expression in *P. pastoris* (Table 32). Primer pairs used to eliminate the pelB leader sequence (Table 33). In *E. coli* only the truncated proteins were expressed, therefore the trans-membrane domains (TMD) were cut off from the optimized genes like described for the expression in *P. pastoris*. For all reactions, 0.5 µl of template DNA were mixed with 3 µl of forward as well of reverse primers, 2.5 µl dNTPs (2 mM each), 10 µl Phusion® HF Buffer (5x), 0.5 µl Phusion® DNA Polymerase (Finnzymes) (5 U/µl) and 30.5 µl ddH_2O.

Cycling program: 98°C/30''-(98°C/10''- X°C/10'' -72°C/Y'') x30 -72°C/7 min -4°C/∞

Table 32. Templates, primers (Forward/reverse) and conditions for His tagging the proteins without pelB leader sequence, T_a…annealing temperature, t_E…extension time

N°	Template	Fw/Rv primer	T_a (X) [°C]	t_E (Y) [sec]	Product
1	tHPO	FwHPOnoTMD_E2/ RvHPO_His_E	63	30	tHPO-His
2	tCPR	FwCPRnoTMD_E2 / RvCPR_His_E	63	30	tCPR-His
3	t2D6	Fw2D6noTMD_E2 / Rv2D6_His_E	63	30	t2D6-His
4	thCPR	Fw_hCPRnoTMD_E2 / Rv_hCPR-His_E	63	30	thCPR-His
5	tPM17	FwPM17noTMD_E2 / RvPM17_His_E	63	30	tPM17-His

Table 33. Templates, primers (forward / reverse) and conditions for His tagging the proteins with pelB leader sequence, T_a…annealing temperature, t_E…extension time

N°	Template	Fw / Rv primer	T_a (X) [°C]	t_E (Y) [sec]	Product
6	tHPO	FwHPOnoTMD/ RvHPOnoTMD-His	63	30	tHPO-His
7	tCPR	FwCPRnoTMD/ RvCPRnoTMD-His	62	40	tCPR-His
8	t2D6	Fw2D6noTMD/ Rv2D6noTMD-His	65	30	t2D6-His
9	thCPR	Fw_hCPRnoTMD/ Rv_hCPRnoTMD-His	63	40	thCPR-His
10	tPM17	FwPM17noTMD/ RvPM17noTMD-His	65	30	tPM17-His

50 µl of the vector backbone pET-26b-(+) and 50 µl of the PCR products (inserts) were digested, using restriction enzymes EcoRI and NotI for proteins containing pelB leader sequence and using NdeI and HindIII for proteins without pelB leader sequence. The pelB leader sequence is located between the NdeI and HindIII cutting sites and eliminated when digested with the enzymes (Figure 23). The linearized vector backbone was dephosphorylated and the products were again cleaned up on a preparative agarose gel. The concentrations and the correct sizes of the constructs were determined with an analytical agarose gel as shown in Figure 25. Table 34 shows the expected band sizes for the constructs.

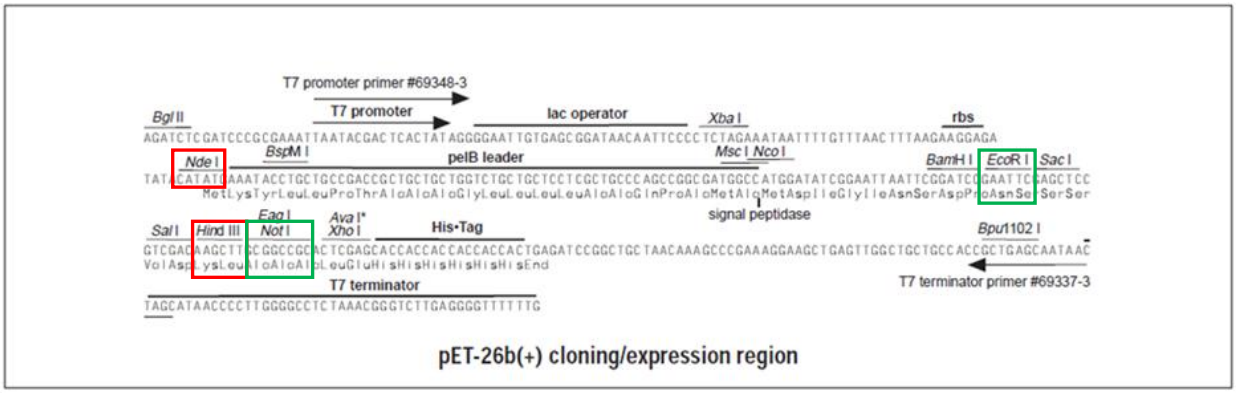


Figure 23.Cloning regions of the pET-26(+) vector with pelB sequence and cutting sites (green for proteins with pelB and red for proteins without pelB)

Table 34. Expected band sizes for inserts and vector backbone

Construct	Expected band size (bp)
pET-26b(+) with pelB	5334
pET-26b(+) without pelB	5245
tHPO-His	1500
tPM17-His	1500
tCPR-His	2000
thCPR-His	2000
t2D6-His	1500

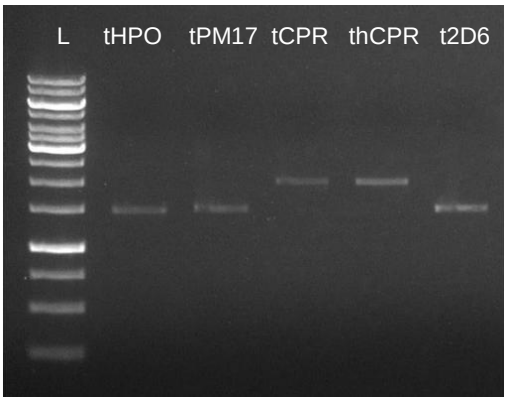


Figure 24. PCR products for cloning in pET-26b (+), L… PageRuler™ Prestained Protein Ladder

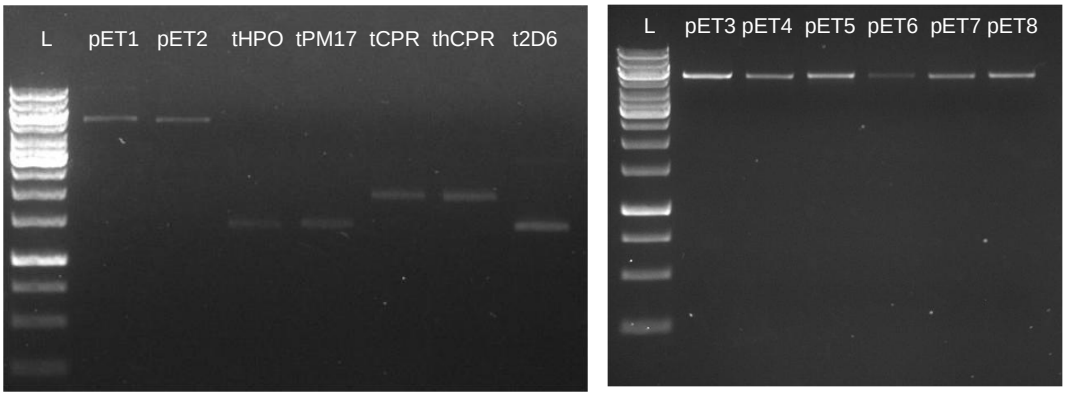


Figure 25. Digested vector (pET1, 3-5: pET-26b (+) cut with *Eco*RI and *Not*I, pET2, 6-8: pET-26b (+) cut with *Hind*III and *Nde*I) and inserts (digested with HindIII and NdeI) , L: PageRuler™ Prestained Protein Ladder

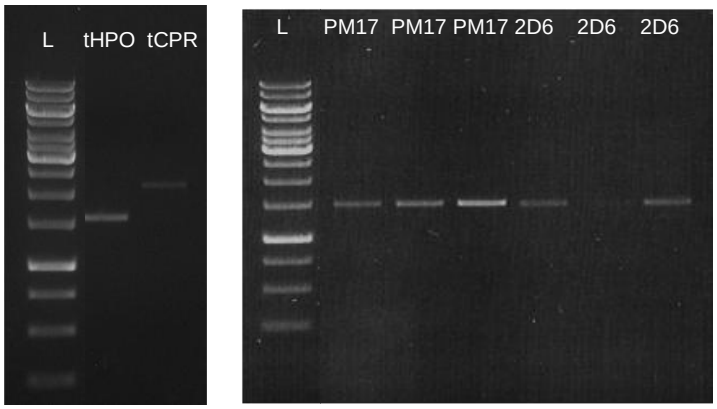


Figure 26. Digested inserts (digested with EcoRI and NotI)

Table 35. Lane description of Figure 24-26 and concentrations determined from Gel electrophoresis

Slot	Description	Amount [µl]	Concentration [ng/µl]
L	GeneRuler™ 1kb DNA ladder	5	/
pET1	pET-26b(+) vector backbone	1	5
pET3	with pelB sequence for periplasma transport ,	1	60
pET4	cut (*Eco*RI and *Not*I) and purified	1	25
pET5		1	30
pET2	pET-26b(+) vector backbone without pelB sequence, cut	1	7
pET6	(*Nde*I and *Hind*III) and purified	1	10
pET7		1	30
pET8		1	40
tHPO	Truncated HPO insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	30
tPM17	Truncated PM17 insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	15
tCPR	Truncated CPR insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	10
thCPR	Truncated hCPR insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	10
t2D6	Truncated CYP2D6 insert for ligation, cut (*Eco*RI and *Not*I) and purified	1	5
tHPO	Truncated HPO insert for ligation, cut (*Nde*I and *Hind*III) and purified	1	15
tPM17	Truncated PM17 insert for ligation, cut (*Nde*I and *Hind*III) and purified	1	20
tCPR	Truncated CPR insert for ligation, cut (*Nde*I and *Hind*III) and purified	1	15
thCPR	Truncated hCPR insert for ligation, cut (*Nde*I and *Hind*III) and purified	1	20
t2D6	Truncated 2D6 insert for ligation, cut (*Nde*I and *Hind*III) and purified	1	25

The concentrations of the inserts and vector backbones were determined from the gel electrophoreses pictures by comparing the bands with the ladder bands (Table 35). The digested inserts were ligated into 50 ng of pET-26b (+) vector backbone in a molar ratio of 3:1 and the resulting plasmids were desalted using microdialysis for 30 min. The plasmids were electrotransformed into electrocompetent *E. coli* TOP10F' cells. Transformants were selected for Kanamycin resistance on LB-Kan (40 µg/ml) plates and several colonies were streaked out for plasmid isolation. Plasmids of four to ten colonies were isolated and the correct ligation was controlled by enzyme restriction analysis, using Fast digest enzymes *Eco*RI and *Not*I for constructs with pelB sequence and using *Nde*I and *Hind*III for constructs without pelB leading sequence to liberate the insert. Some colonies also were controlled with colony PCR as described in the materials and methods sector. The sizes of the fragments from the restriction

analyses are listed in Table 36. The colony PCR was controlled by gel electrophoreses (Figure 27).

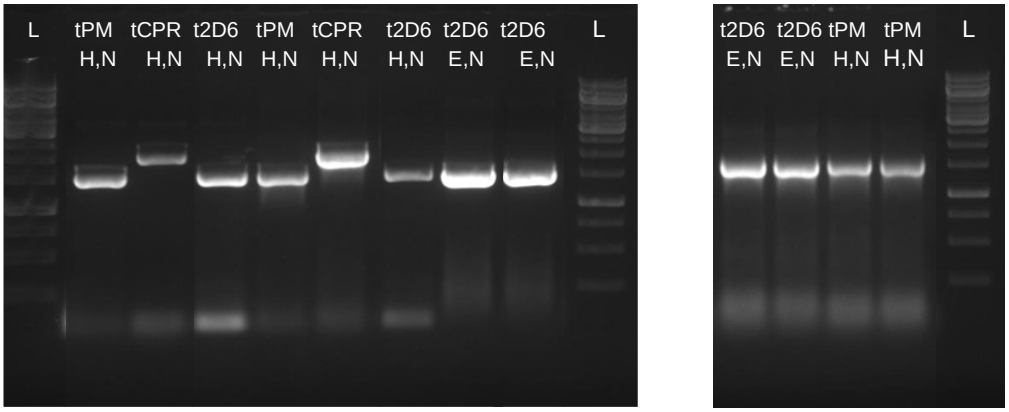


Figure 27. Colony PCR of clones in *E. coli* Top 10F`, H,N: cutting sites *Hind*III and *Nde*I; E,N: cutting sites *Eco*RI and *Not*I

The plasmids, containing insert and vector, were purified and electrotransformed into electrocompetent BL21 star (DE3) cells and correct transformation was controlled with enzyme restriction analysis, using fast digest enzymes *Eco*RI / *Not*I and *Nde*I /*Hind*III and controlled with gel electrophoreses as shown in Figure 28. Transformants grew on LB-Kan (40 µg/ml) plates over night at 37°C. From all five constructs without pelB sequence, except for pET-26(+)_t2D6, positive colonies were derived. For the constructs with pelB leader sequence only for pET-26(+)_t2D6 and pET-26(+)_tPM17 positive clones were found. The quality of the control cut on the gel picture is bad, because plasmid isolation from BL21 is difficult.

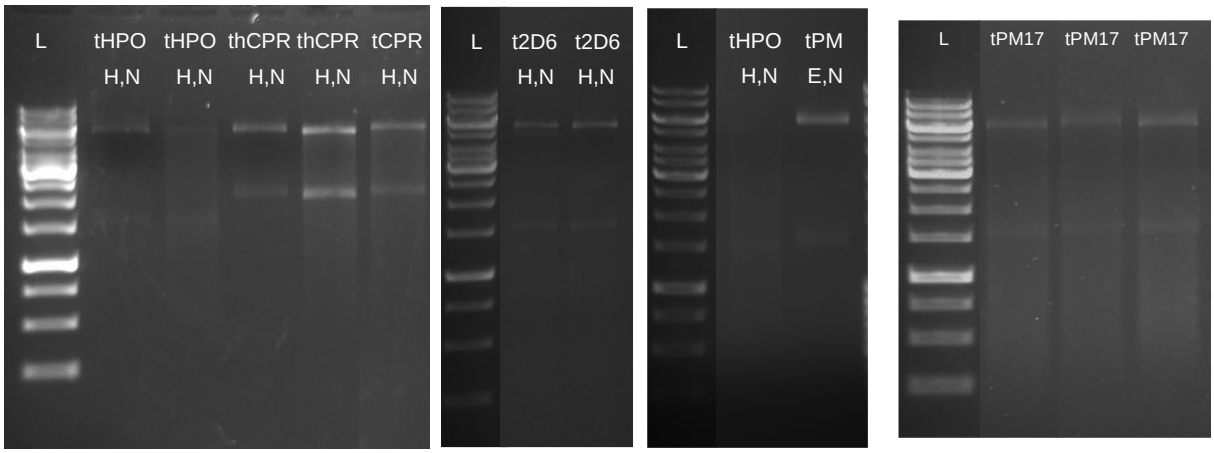


Figure 28. Control cuts using Fast digest enzymes of plasmids from *E. coli* Top 10F` transformants, H,N: cutting sites *Hind*III and *Nde*I; E,N: cutting sites *Eco*RI and *Not*I

Table 36. Sizes of inserts and vector

Linearized plasmid	Plasmid Size	Insert size
pET-26(+)_tHPO	6713 bp	1485 bp
pET-26(+)_tPM17	6744 bp	1506 bp
pET-26(+)_tCPR	7240 bp	2002 bp
pET-26(+)_thCPR	7226 bp	1987 bp
pET-26(+)_tCYP2D6	6732 bp	1494 bp

Expression studies

E. coli colonies were cultivated in 250 ml flasks and in 2 l flasks and protein expression was started by autoinduction or by induction with IPTG according to the materials and methods section. Two µM $FeCl_3$ solution and 0.5 mM δ-ALA were added to the medium to achieve higher expression levels, as described in the Master thesis of A. Emmerstorfer [33]. Cells were harvested by centrifugation at 5000 rpm (JA 10) and 4°C for 10 min and the cells were resuspended in 20 mM KP_i, pH 8.0. For SDS-PAGE studies, cells were disrupted with B-Per reagent and for higher cell numbers disruption was performed by sonication according to the instructions in the materials and methods section. After cell disruption, the cell extract and the cell debris were taken for SDS-PAGE (Figure 29, Figure 30 and 31). SDS-PAGE showed that no or very low amounts of protein were expressed with IPTG and with autoinduction. In the cell debris fractions, more protein was found. Especially upon autoinduction, a stronger band was seen for the theoretical size of tPM17 of ~57 kDa in the cell debris samples, which suggests that inclusion bodies where formed. SDS-PAGE of the proteins was done according to the protocol described in Materials and Methods section. Approximately 20 µg of TCA-precipitated proteins were loaded onto SDS-gels (Self made gels, 12% Bis-Tris).

Expression of tPM17 induced with IPTG

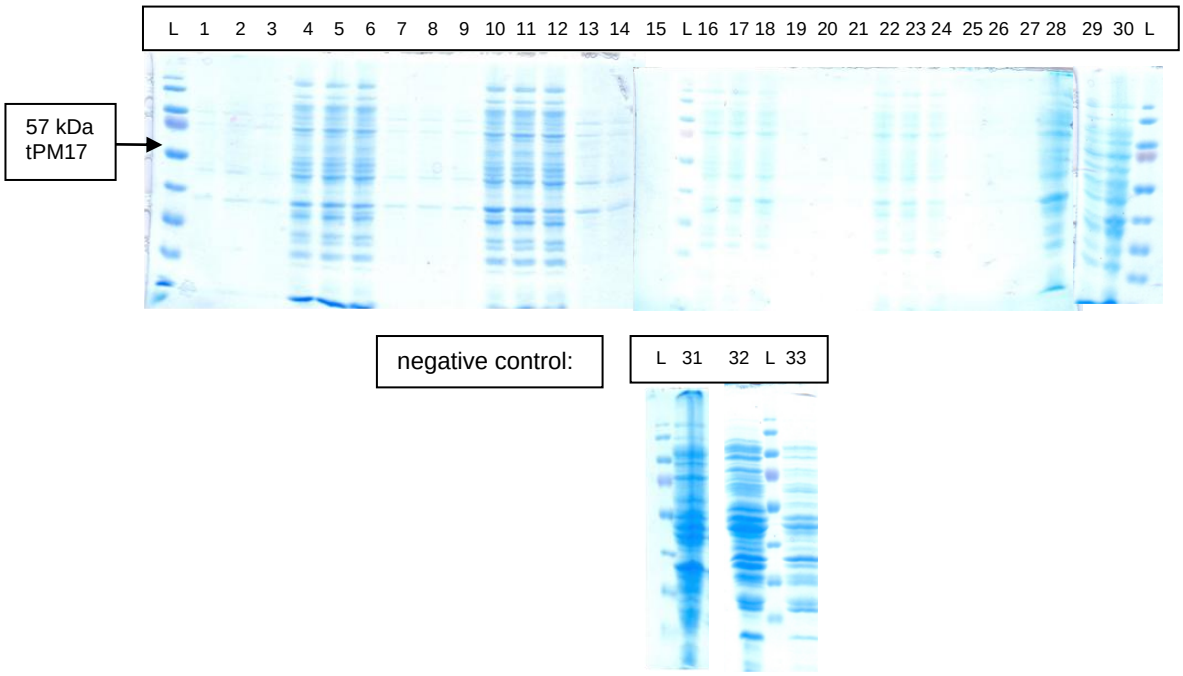


Figure 29. SDS-PAGE of cell extracts upon induction of tPM17expression in *E. coli* at different induction times

Table 37. Lane description to Figure 29, s…supernatant, cd…cell debris, Ladder… PageRuler™ Prestained Protein Ladder

Lane	Sample	C(IPTG) [mM]	Induction time [h]	Fraction	Lane	Sample	C(IPTG) [mM]	Induction time [h]	Fraction
L	Ladder	/	/	/	17	tPM17	0.1	3	cd
1	tPM17	0.05	1	S	18	tPM17	0.25	3	Cd
2	tPM17	0.1	1	S	19	tPM17	0.05	6	S
3	tPM17	0.25	1	S	20	tPM17	0.1	6	S
4	tPM17	0.05	1	Cd	21	tPM17	0.25	6	S
5	tPM17	0.1	1	Cd	22	tPM17	0.05	6	Cd
6	tPM17	0.25	1	Cd	23	tPM17	0.1	6	Cd
7	tPM17	0.05	2	S	24	tPM17	0.25	6	Cd
8	tPM17	0.1	2	S	25	tPM17	0.05	18	S
9	tPM17	0.25	2	S	26	tPM17	0.1	18	S
10	tPM17	0.05	2	Cd	27	tPM17	0.25	18	S
11	tPM17	0.1	2	Cd	28	tPM17	0.05	18	Cd
12	tPM17	0.25	2	Cd	29	tPM17	0.1	18	Cd
13	tPM17	0.05	3	S	30	tPM17	0.25	18	cd
14	tPM17	0.1	3	S	31	pET-26b(+) in BL21 star(DE3)	/	/	Cd
15	tPM17	0.25	3	S	32	BL21 star (DE3)	/	/	S
16	tPM17	0.05	3	Cd	33	pET-26b(+) in BL21 star(DE3)	/	/	S

Expression of tPM17 upon autoinduction

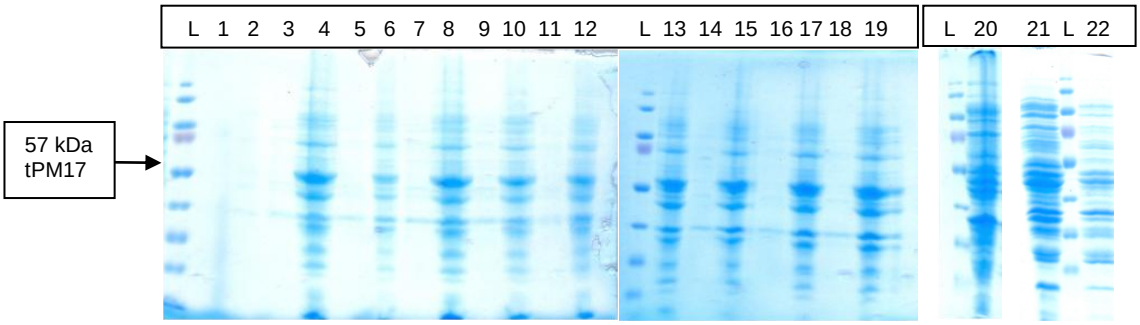


Figure 30. SDS-PAGE of cell extracts upon autoinduction of tPM17 expression in *E. coli* at different induction times

Table 38. Lane description for Figure 30, s…supernatant, cd…cell debris

Lane	Sample	Induction time [h]	Fraction	Lane	Sample	Induction time [h]	Fraction
L	PageRuler™ Prestained Protein Ladder	/	S	12	tPM17	18	s
1	tPM17	0	Cd	13	tPM17	19	Cd
2	tPM17	0	S	14	tPM17	20	S
3	tPM17	14	Cd	15	tPM17	20	Cd
4	tPM17	14	S	16	tPM17	21	S
5	tPM17	15	Cd	17	tPM17	21	Cd
6	tPM17	15	S	18	tPM17	36	S
7	tPM17	16	Cd	19	tPM17	36	cd
8	tPM17	16	S	L	Ladder	/	/
9	tPM17	17	Cd	20	pET-26b(+) in BL21 star(DE3)	/	Cd
10	tPM17	17	S	21	BL21 star (DE3)	/	S
11	tPM17	18	Cd	22	pET-26b(+)in BL21 star(DE3)	/	s

Expression of thCPR in *E. coli* upon autoinduction and induction using IPTG

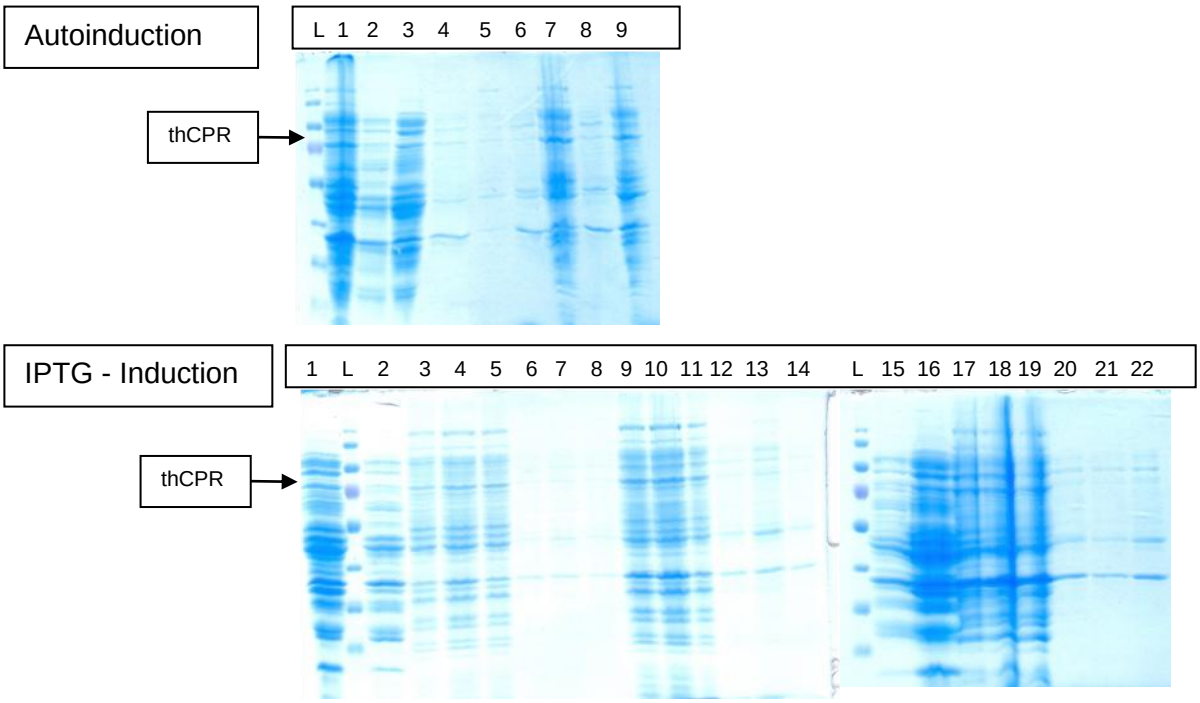


Figure 31. SDS-PAGE of thCPR expression upon autoinduction and induction with IPTG, slot 4 (autoinduction): with thCPR TCA precipitation was done

Table 39. Lane description of Figure 31, autoinduction, S: supernatant, Cd: cell debris

Lane	Sample	Induction time [h]	Fraction
L	PageRuler™ Prestained Protein Ladder	/	/
1	pET-26b(+) in BL21 star(DE3)	/	Cd
2	pET-26b(+)in BL21 star(DE3)	/	S
3	BL21 star (DE3)	/	S
4	thCPR	0	S
5	thCPR	0	Cd
6	thCPR	o.n.	S
7	thCPR	o.n.	Cd
8	thCPR	48	S
9	thCPR	48	S

Table 40. Lane description of Figure 31 , IPTG Induction

Lane	Sample	C(IPTG) [mM]	Induction time [h]	Fraction
1	BL21 star (DE3)	/	/	S
L	PageRuler™ Prestained Protein Ladder	/	/	/
2	pET-26b(+) in BL21 star(DE3)	/	/	S
3	thCPR	0.05	1	Cd
4	thCPR	0.1	1	Cd
5	thCPR	0.25	1	Cd
6	thCPR	0.05	1	S
7	thCPR	0.1	1	S
8	thCPR	0.25	1	S
9	thCPR	0.05	6	Cd
10	thCPR	0.1	6	Cd
11	thCPR	0.25	6	Cd
12	thCPR	0.05	6	S
13	thCPR	0.1	6	S
14	thCPR	0.25	6	S
L	Ladder	Ladder	/	/
15	pET-26b(+) in BL21 star(DE3)	/	/	Cd
16	BL21 star (DE3)	/	/	S
17	thCPR	0.05	o.n.	Cd
18	thCPR	0.1	o.n.	Cd
19	thCPR	0.25	o.n.	Cd
20	thCPR	0.05	o.n.	S
21	thCPR	0.1	o.n.	S
22	thCPR	0.25	o.n.	S

No or very small amounts of thCPR were expressed upon autoinduction and induction with small amounts of IPTG. The specific bands, which were found for the cell extract and for the cell debris samples showed the same patterns as the bands of the wild type samples, pET-26b(+) in BL21 star (DE3) and BL21 star (DE3). SDS-PAGE showed that expression did not work and no inclusion bodies of thCPR were found in cell debris samples. The *T7* promoter was a too strong promoter for the expression of these truncated proteins in *E. coli*. The expression of the truncated proteins in *E. coli* were terminated and for all further experiments and all purifications proteins, expressed in *P. pastoris* WT CBS 7435, were used.

4.4. PURIFICATION OF HIS-TAGGED PROTEINS

4.4.1. Solubilization of membrane proteins

Twelve different detergents were tested for solubilisation of the membrane proteins PM17, 2D6, HPO, CPR and hCPR expressed as C-terminally His-tagged proteins in P. pastoris (Table 41-44). Protein concentrations were determined using the Bradford assay. In the further experiments all detergents will be named with their numbers listed in Table 41. For detergents with higher CMC, a detergent concentration of 2% was used (Table 42).

Table 41. List of tested detergents with different CMC (critic micelle concentration)

No.	Detergent	Description	CMC [mM]	MW	1% solution [mM]
1	CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)	Zwitterionic	6	615	16.3
2	ASB-14 (3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate)	Zwitterionic	8	435	23.0
3	n-Dodecyl-β-D-maltoside	Nonionic	1.8	511	19.6
4	Octyl-β-D-1-thioglucopyranoside (OTG)	Nonionic	9	308	32.5
5	3-(Decyldimethylammonio)-propanesulfonate inner salt (SB3-10)	Zwitterionic	25-40	308	32.5
6	Octyl-β-D-1-Glucopyranoside (OG)	Nonionic	18	292	34.3
7	Polyoxyethylene 10 tridecyl ether	Nonionic	0.125	640	15.6
8	C7BzO (3-(4-Heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate)	Zwitterionic	Not determined	400	25.0
9	n-Octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (3-08)	Zwitterionic	330	229	43.7
10	n-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (3-16)	Zwitterionic	0.01-0.06	391	25.6
11	Tween 20	Nonionic	0.06	1227	8.1
12	SDS (sodium dodecyl sulfate)	Anionic	7-10	288	34.7

Table 42. Detergents with 2% concentration

No.	Detergent	Description	CMC [mM]	MW	2% solution [mM]
5	3-(Decyldimethylammonio)-propanesulfonate inner salt (SB3-10)	Zwitterionic	25-40	308	65.0
6	Octyl-β-D-1-Glucopyranoside (OG)	Nonionic	18	292	68.5
9	n-Octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (3-08)	Zwitterionic	330	229	87.3

Table 43. List of tested detergents and their structure

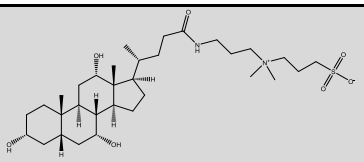
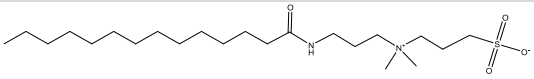
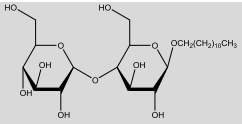
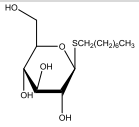
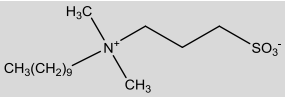
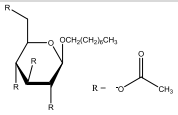
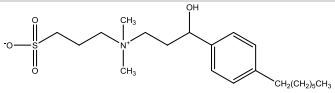
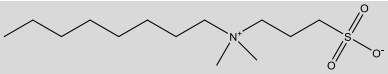
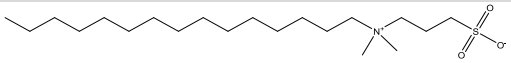
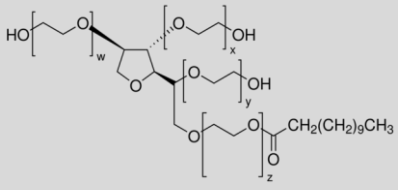
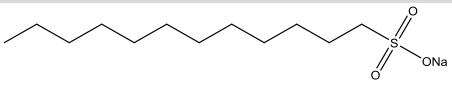
No.	Detergent	Structure
1	CHAPS	
2	ASB-14	
3	n-Dodecyl-β-D-maltoside	
4	OTG	
5	SB3-10	
6	OG	
7	Polyoxyethylene 10 tridecyl ether	$C_{13}H_{27}(OCH_2CH_2)_nOH$
8	C7BzO	
9	3-08	
10	3-16	
11	Tween 20	
12	SDS	

Table 44. Conditions used for the detergent screenings

Membrane protein concentration [mg/ml]	Detergent concentration [%]	NaCl [mM]	PMSF [mM]	Temperature [°C]	Time [h]
5	1 (above CMC)	100	1	25	3
5	2 (for detergent 5, 6, 9)	100	1	25	3

Table 45. Protein concentrations and volumes used for detergent screening

Protein	Concentration [mg/ml]	5 mg protein [µl]
HPO	29.87	200
PM17	26.73	300
CPR	16.15	310
hCPR	30.51	160
2D6	24.95	200

For the whole detergent screening a 10 mM KP_i, pH 7.4, containing 1 mM PMSF and 100 mM NaCl was used for all samples. The cell pellet was resuspended in 10 mM KP_i, pH7.4, and the cells were disrupted by glass bead disruption as described in the Materials and Methods section. 10% detergent solutions were prepared in 10 mM KP_i, pH 7.4. Therefore, 100 mg detergent were solubilized in 900 µl buffer. The homogenate of the protein solution (membrane fraction), containing 5 mg of protein, was mixed with the detergent solution to 1%- 2% final concentration of detergent in a volume of 1 ml with 10 mM KP_i buffer. The membrane fractions were solubilized by incubation with the detergent solution at 25°C for 3 h, and then clarified by ultracentrifugation at 100 000 x g for 1 h. The supernatants (soluble fraction) and the pellets (membrane fraction) were used for SDS-PAGE and Western blot analysis to estimate how much of the protein was solubilized and how much remained in the membrane fraction. Therefore, TCA precipitation was performed. The results of Western blot analysis are shown in Figure 32. Protein concentrations were determined using Bradford assay (Table 46 and Table 47). SDS-PAGE, Western blot and subsequent immunedetection of the proteins were done according to the protocol described in the Materials and Methods section. Approximately, 20 µg of TCA-precipitated proteins were loaded onto SDS-gels (Self made gels, 12% Bis-Tris).

Table 46. Protein concentrations of solubilized membrane proteins (PM17, HPO, hCPR)

Protein fraction	PM17 Supernatant	PM17 Pellet	HPO Supernatant	HPO Pellet	hCPR Supernatant	hCPR Pellet
detergent	**c [mg/ml]**	**c [mg/ml]**	**c [mg/ml]**	**c [mg/ml]**	**c [mg/ml]**	**c [mg/ml]**
1	2.09	0.58	3.81	1.39	1.52	0.21
2	3.80	0.24	7.89	2.75	3.61	0.26
3	2.75	1.13	4.14	2.77	1.78	0.24
4	2.08	0.28	1.91	1.45	1.10	0.11
5	1.83	0.53	2.92	1.52	1.02	0.09
6	1.95	0.69	2.24	1.05	1.02	0.11
7	2.70	0.84	4.15	2.38	1.71	0.20
8	2.92	1.25	3.90	2.39	1.71	0.14
9	1.97	0.89	2.99	1.12	1.19	0.16
10	3.38	0.91	4.53	2.97	3.31	0.18
11	2.68	1.06	4.33	3.86	1.93	0.25
12	2.09	0.18	2.08	0.65	0.81	0.02
no detergent	1.96	1.47	3.68	2.11	1.10	0.24

Table 47. Protein concentrations of solubilized membrane proteins (2D6, CPR)

Protein fraction	2D6 supernatant	2D6 Pellet	CPR Supernatant	CPR Pellet
detergent	c [mg/ml]	c [mg/ml]	c [mg/ml]	c [mg/ml]
1	1.99	0.88	3.48	0.44
2	2.17	0.65	4.79	0.55
3	1.33	0.64	4.43	0.45
4	2.14	1.15	2.51	0.36
5	1.69	1.07	2.06	0.41
6	1.77	1.07	2.15	0.47
7	2.12	0.91	4.04	0.38
8	1.79	0.91	3.68	0.39
9	3.29	0.92	2.00	0.40
10	1.50	0.57	4.45	0.45
11	3.73	0.77	3.75	0.46
12	2.92	0.23	2.20	0.03
no detergent	2.74	0.72	3.50	0.44

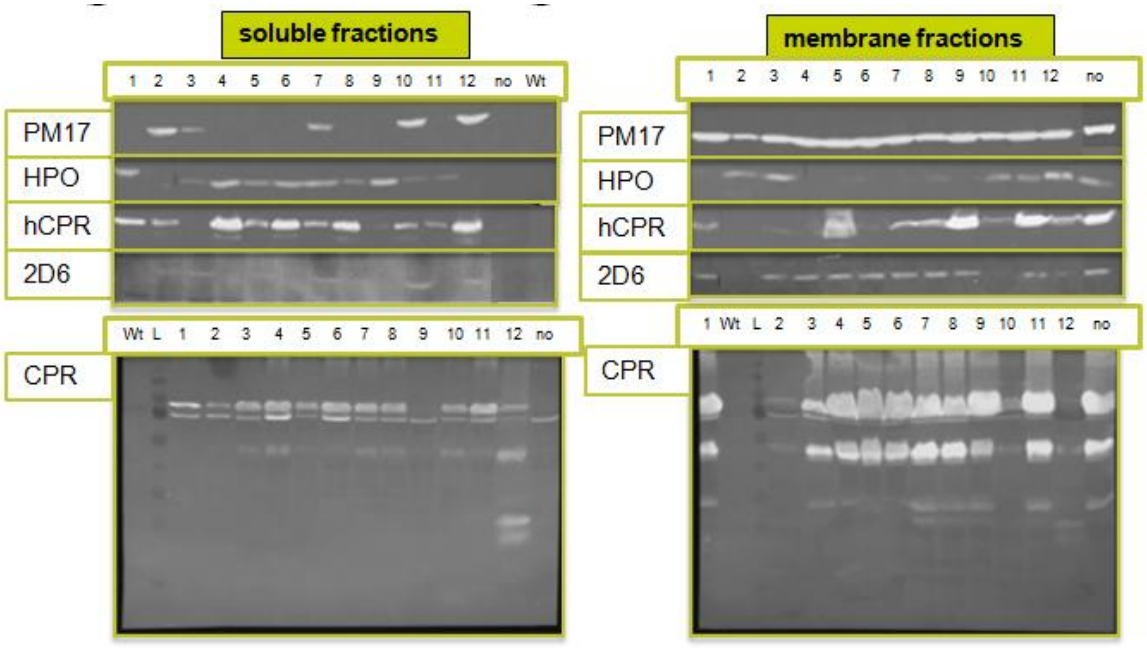


Figure 32. Western blot for His-tag, samples of the detergent screening

Table 48. Lane description of Figure 32

Lane	Detergent	Lane	Detergent
L	PageRuler™ Prestained Protein Ladder	8	C7BzO
1	CHAPS	9	3-08
2	ASB-14	10	3-16
3	n-Dodecyl-β-D-maltoside	11	Tween 20
4	Octyl-β-D-1-thioglucopyranoside	12	SDS
5	3-(Decyldimethylammonio)-propanesulfonate inner salt	no	No detergent
6	Octyl-β-D-1-Glucopyranoside	Wt	WT CBS 7435 – his
7	Polyoxyethylene (10) tridecyl		

Twelve different detergents were tested for solubilisation of the membrane proteins. PM17 was solubilized with the detergents 2, 3, 7, 10 and 12. The highest solubilisation was achieved with the detergents ASB-14, 3-16 and SDS, also in the pellet fraction less protein was detected for the samples with these detergents, which shows that protein was solubilized from the membrane. SDS gave almost complete solubilisation of the membrane protein. HPO was solubilized with nearly every tested detergent, only SDS and ASB-14 did not solubilize the protein. Highest solubilisation was achieved with detergents 1, 4, 6, 7 and 9, because most of the protein was solubilized and also in the pellet fraction no protein was found after solubilisation. For hCPR detergents 4, 6, 8 and 12 worked the best. For CYP2D6 it is hard to see on this Western blot, but detergents 2, 3, 5, 7, 8, 10 and 12 worked and the screening was repeated with these detergents and the ER fractions as shown in Figure 33. More than one band with different sizes for CPR occurred in the Western blot, which shows that the protein was fragmented during the expression of the protein and each fragment still carried the His-Tag. The apparently largest protein band was the CPR, because it is the only band which did not occur in the soluble fraction without detergent (slot 13, Figure 32). The fragmentation was controlled with mass spectrometry (Figure 41). Nearly every detergent solubilised the protein, but the best results were achieved with detergents 3, 4, 6, 7, 11 and 12. For further experiments mainly nonionic or zwitterionic detergents were used, because anionic detergents, like SDS, are very strong detergents and can also inactivate or destabilise the protein.

Table 49. Summary of the results from the detergent screening, +++ …detergent solubilised the most protein, ++…detergent solubilised less protein, +… detergent solubilised fewest protein, -...no protein was solubilised

No.	Detergent	Description	HPO	PM17	CPR	hCPR	2D6
1	CHAPS	Zwitterionic	+++	-	++	++	-
2	ASB-14	Zwitterionic	-	+++	+	+	++
3	n-Dodecyl-β-D-maltoside	Nonionic	+	+	+++	-	+++
4	Octyl-β-D-1-thioglucopyranoside (OTG)	Nonionic	++	-	+++	+++	-
5	3-(Decyldimethylammonio)-propanesulfonate inner salt (SB3-10)	Zwitterionic	+	-	++	+	+
6	Octyl-β-D-1-Glucopyranoside (OG)	Nonionic	++	-	+++	+++	-
7	Polyoxyethylene 10 tridecyl ether	Nonionic	++	++	+++	+	+++
8	C7BzO	Zwitterionic	+	-	++	+++	-
9	n-Octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (3-08)	Zwitterionic	++	-	-	-	-
10	n-Hexadecyl-N,N-dimethyl-3-ammonio-1-propaneulfonate (3-16)	Zwitterionic	+	+++	++	+	+++
11	Tween 20	Nonionic	+	-	+++	+	-
12	SDS	Anionic	-	+++	+++	+++	+++

The detergents with the highest solubilization rates were used for another detergent screening using the ER fractions of the cells, where the proteins are located. Table 49 shows that detergent 7 (Polyoxyethylene 10 tridecyl ether) and detergent 10 (n-Hexadecyl-N, N-dimethyl-3-ammonio-1-propaneulfonate) worked for each of the five membrane proteins. Interestingly the detergent 9 (n-Octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) worked for none of the proteins except for HPO and SDS worked for PM17, CPR, hCPR and 2D6 very well, but it did not work for HPO.

Isolation of total microsomes:

ER membranes were isolated to obtain membrane protein at higher concentration. The ER fractions were used to solubilise the proteins using the best detergents chosen from the detergent screening above. Cells were harvested by centrifugation at 5000 rpm (JA 10 rotor) and 4 °C for 5 min and the cells were disrupted using glass bead disruption. Total microsomes were isolated according to the materials and methods section. The results of the Western blot analysis after solubilisation of the ER membranes (Figure 33). The culture volumes and protein concentrations after microsomal preparations which were used for the detergent screening are listed in Table 50. SDS-PAGE, Western blot and subsequent immunodetection of the proteins were done according to the protocol described in Materials and Methods. Approximately, 20 µg of TCA-precipitated proteins were loaded onto SDS-gels (Self made gels, 12% Bis-Tris).

Table 50. Cell cultures used for detergent screenings and protein solubilisations, c (protein)-mp…protein concentration after microsomal preparation

Sample	Culture [ml]	Cell mass [g]	C(protein) – mp [mg/ml]
HPO	600 ml (3 cultures)	20.7	34.14
CPR	600 ml (3 cultures)	22	43.73
2D6	600 ml (3 cultures)	17	40.41
PM17	600 ml (3 cultures)	18.7	33.24
hCPR	600 ml (3 cultures)	17	39.10

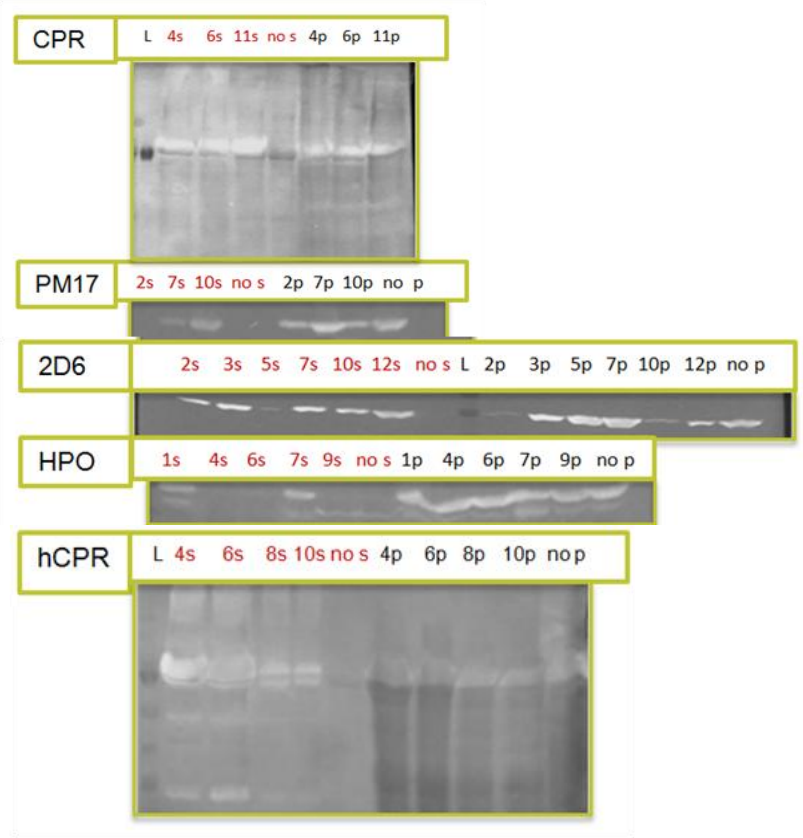


Figure 33. Detergent screening of ER fractions from membrane protein solubilization, red…supernatant, black… pellet

Table 51. Lane description of Figure 33

Lane	Detergent	Lane	Detergent
1	CHAPS	8	C7BzO
2	ASB-14	9	3-08
3	n-Dodecyl-β-D-maltoside	10	3-16
4	Octyl-β-D-1-thioglucopyranoside	11	Tween 20
5	3-(Decyldimethylammonio)-propanesulfonate inner salt	12	SDS
6	Octyl-β-D-1-Glucopyranoside	no	No detergent
7	Polyoxyethylene (10) tridecyl	L	PageRuler™ Prestained Protein Ladder

Best solubilisation results for the ER fractions were achieved with detergents listed in Table 52. These samples were used for further solubilizations of the membrane proteins for purification and *in vitro* activity assays.

Table 52. Detergents achieving best solubilisation results for the ER fractions

Protein	detergents
PM17	Polyoxyethylene (10) tridecyl , 3-16
CPR	Octyl-β-D-1-Glucopyranoside, Tween 20
2D6	n-Dodecyl-β-D-maltoside, 3-16
hCPR	Octyl-β-D-1-thioglucopyranoside , Octyl-β-D-1-Glucopyranoside
HPO	Polyoxyethylene (10) tridecyl, CHAPS

4.4.2. Purification of His-tagged proteins

The truncated proteins tHPO, tCPR, thCPR, tPM17 and t2D6 were purified using affinity chromatography. The protein was bound to the beads by incubating the protein and the Ni-Sepharose beads with binding buffer over night at 4°C by moderate shaking. Then, the column was washed with different imidazol concentrations before eluting with increasing imidazol concentrations to test the best conditions for purification with Ni-NTA affinity chromatography (Table 53 and 54). Purified proteins precipitate when stored at -20°C. To inhibit precipitation the proteins were stored at 4°C for some days or with 5% Glycerin added at -20°C for longer time.

Table 53.List of buffers used for Ni-NTA affinity chromatography

Buffer	Composition
Binding buffer	20 mM KP_i, pH 7.4, 0.5 M NaCl, 10 mM imidazol
Washing buffer	20 mM KP_i, pH 7.4, 0.5 M NaCl, 15 - 50 mM imidazol
Elution buffer	20 mM KP_i, pH 7.4, 0.5 M NaCl, 100 - 500 mM imidazol

Table 54. List of washing and elution steps used for the purification

Purification step	Column volume (CV)	Buffer	Imidazol concentration [mM]
Wash 1	10	Binding buffer	10
Wash 2	10	Washing buffer	15
Wash 3	10	Washing buffer	20
Wash 4	10	Washing buffer	30
Wash 5	10	Washing buffer	50
Elution 1	5	Elution buffer	100
Elution 2	5	Elution buffer	200
Elution 3	5	Elution buffer	300
Elution 4	10	Elution buffer	500

For the first purification, buffers with an imidazol concentration of 100 - 500 mM were used and samples were taken in one ml fractions to test which imidazol concentration will be the best choice for washing the column and eluting the protein. An SDS – PAGE was performed using a Coomassie blue staining to control the purification level. Approximately, 20 µg of TCA-precipitated proteins were loaded onto SDS-gels (Self made gels, 12% Bis-Tris).

Purification of thCPR:

First purification was performed as described above (Figure 34). The protein was incubated with the Ni Sepharose beads for 1 h at 4°C.

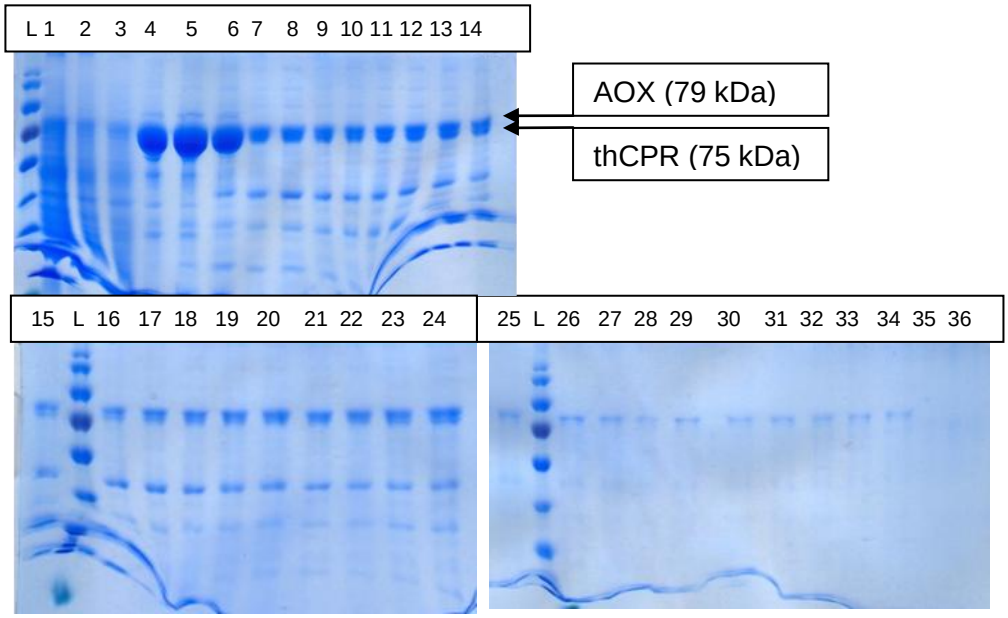


Figure 34. Purification of thCPR with binding at 4°C for 1 h

Table 55. Lane description of Figure 34

Lane	Sample	C(imidazol) [mM]	Lane	Sample	C(imidazol) [mM]
L	PageRuler™ Prestained Protein Ladder	/	19	Elution 3 - fraction 7	300
1	Cell extract	/	20	Elution 3 - fraction 8	300
2	Flow through	/	21	Elution 3 - fraction 9	300
3	Wash 1	10	22	Elution 3 - fraction 10	300
4	Wash 4	30	23	Elution 3 - fraction 11	300
5	Elution 1 - fraction1	100	24	Elution 3 - fraction 12	300
6	Elution 2 - fraction 1	200	25	Elution 4 - fraction 1	500
7	Elution 2 - fraction 2	200	26	Elution 4 - fraction 2	500
8	Elution 2 - fraction 3	200	27	Elution 4 - fraction 3	500
9	Elution 2 - fraction 4	200	28	Elution 4 - fraction 4	500
10	Elution 2 - fraction 5	200	29	Elution 4 - fraction 5	500
11	Elution 2 - fraction 6	200	30	Elution 4 - fraction 6	500
12	Elution 2 - fraction 7	200	31	Elution 4 - fraction 7	500
13	Elution 3 - fraction 1	300	32	Elution 4 - fraction 8	500
14	Elution 3 - fraction 2	300	33	Elution 4 - fraction 9	500
15	Elution 3 - fraction 3	300	34	Elution 4 - fraction 10	500
16	Elution 3 - fraction 4	300	35	Elution 4 - fraction 11	500
17	Elution 3 - fraction 5	300	36	Elution 4 - fraction 12	500
18	Elution 3 - fraction 6	300			

When the protein was incubated with the Ni-Sepharose beads for one hour at 4°C the protein did not bind to the beads and was washed out with the first washing steps and only small amounts of the protein were bound to the Ni-Sepharose beads and were eluted with higher imidazol concentrations as shown for the thCPR purification in Figure 34. The protein was already eluted with imidazol concentrations of 10 mM. The same results were obtained for the purification of tCPR under the same binding conditions. For further purifications, binding conditions were changed to binding over night at 4°C. This strategy worked very well as shown in the SDS- PAGE in Figure 35. The protein bound to the column and was eluted at higher imidazol concentrations. For the purification of thCPR the column was washed with 50 ml binding buffer containing 10 mM imidazol and the protein was eluted with 50 ml elution buffer, containing 200 mM imidazol. The purified proteins were desalted using P-10 desalting columns to get rid of salts and imidazol in the solution. The proteins were concentrated to achieve higher protein concentrations per ml sample using Millipore concentrators.

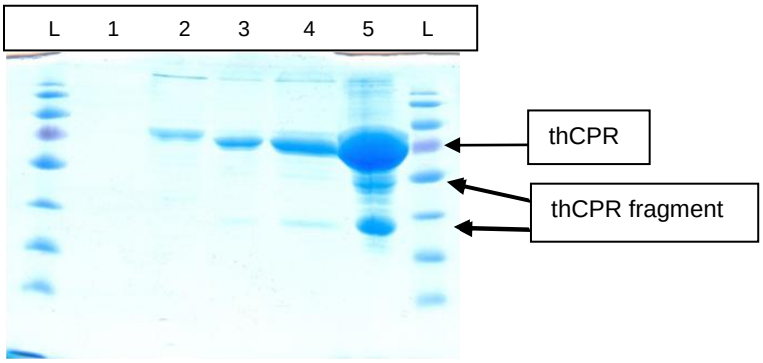


Figure 35. SDS-PAGE of purified and concentrated thCPR

Table 56. Lane description of Figure 35, proteins concentrated using Millipore concentrator

Lane	Sample	C(Imidazol) [mM]	Volume loaded [µl]
L	Ladder	/	5
1	Flow through	/	1
2	Wash 1	10	5
3	Elution 2	200	5
4	Elution 2, concentrated	200	2
5	Elution 2, concentrated	200	5

After purification of thCPR, protein bands with smaller sizes occurred in the SDS-PAGE which were fragments of the thCPR like in the case of CPR as shown above in the detergent screening. The fragments bound to the Ni-Sepharose beads and were eluted with 200 mM imidazol like the full-length protein at 75 kDa. After concentration using Millipore concentrators (30 000 MWKO), protein concentrations of 4.5 mg/ml were obtained.

Purification of tCPR:

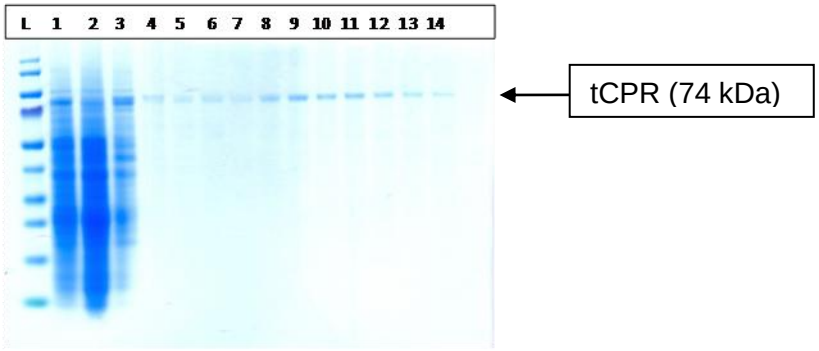


Figure 36. SDS-PAGE of tCPR purification

Table 57. Lane description of Figure 36

Lane	Sample	C(imidazol) [mM]	Lane	Sample	C(imidazol) [mM]
L	Ladder	/	8	Elution buffer 1	100
1	Cell extract	/	9	Elution buffer 1	100
2	Flow through	/	10	Elution buffer 2	200
3	Wash 1	10	11	Elution buffer 2	200
4	Wash 2	15	12	Elution buffer 3	300
5	Wash 3	20	13	Elution buffer 3	300
6	Wash 4	30	14	Elution buffer 4	500
7	Wash 5	50			

SDS-PAGE showed that most of the proteins, except for tCPR, were washed out with the first washing step with buffer containing 10 mM imidazol (Figure 36). The fractions eluted with an imidazol concentration of 200 mM were considered pure and were pooled and used for crystallisation studies. For further purifications of tCPR, the washing step was performed by using buffer containing 20 mM imidazol and the protein was eluted using elution buffer containing 200 mM imidazol. The purified protein was desalted with P-10 desalting columns and concentrated using Millipore concentrators (30 000 MWKO), as described in the Materials and Methods section. This purification was controlled with SDS-PAGE (Figure 37). The protein concentrations were determined with Bradford assay and are listed in Table 59. The sample used for purification of tCPR contained 24 mg/ml of protein, the purified tCPR had a concentration of 0.37-0.60 mg/ml and after concentration a protein content of 2.6 - 5.5 mg/ml was obtained.

Purification of 40 ml tCPR with a protein concentration of 25 mg/ml:

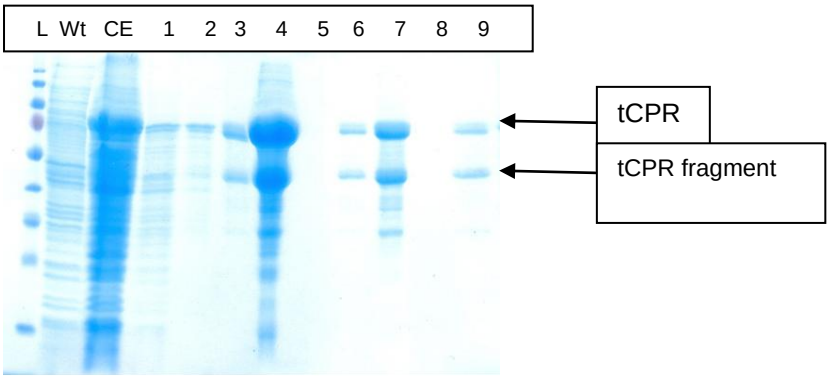


Figure 37.SDS-PAGE of purified and concentrated tCPR

Table 58. Lane description of Figure 37

Lane	Sample	C(Imidazol) [mM]	Loaded volume [µl]
L	PageRuler™ Prestained Protein Ladder	/	5
Wt	Wild type	/	1
CE	Cell extract	/	1
1	Flow trough	/	1
2	Washing buffer 3	20	5
3	Elution buffer 2-1	200	5
4	Elution buffer 2-1, concentrated	200	5
5	Elution buffer 2-1, Filtrate	200	5
6	Elution buffer 2-2	200	5
7	Elution buffer 2-2, concentrated	200	5
8	Elution buffer 2-2, Filtrate	200	5
9	Elution buffer 4	500	5

Table 59. Protein concentration of the purification of tCPR

Sample	Protein concentration [mg/ml]
Cell extract	24.51
Flow through	4.16
Wash 3	0.48
Elution 2 - fraction 1	0.63
Elution 2 - fraction 1, concentrated	5.57
Elution 2 - fraction 1, Filtrate	0.00
Elution 2 - fraction 2	0.37
Elution 2 - fraction 2, concentrated	2.57
Elution 2 - fraction 2, Filtrate	0.00
Elution 4	0.00

Second purification of tCPR:

The same conditions as above were used. The protein was bound to the Ni-Sepharose beads at 4°C over night, washed with washing buffer 3 (20 mM imidazol), eluted with elution buffer 2 (200 mM imidazol), desalted and concentrated (Figure 38).

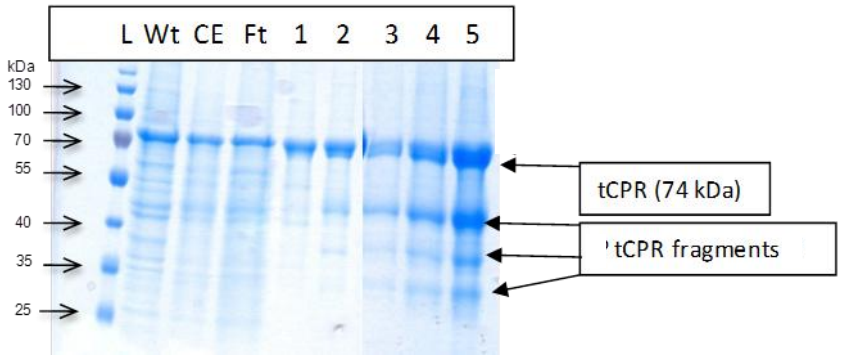


Figure 38.SDS-PAGE of tCPR purification

Table 60. Lane description of Figure 38

Lane	Sample	C(Imidazol)	Loaded volume
L	PageRuler™ Prestained Protein Ladder	/	5µl
Wt	Wild type	/	5µl
CE	Cell extract	/	1µl
Ft	Flow through	/	1µl
1	Wash 3	20 mM	5µl
2	Elution 2	200 mM	1µl
3	Elution 2, concentrated	200 mM	1µl
4	Elution 2, concentrated	200 mM	2µl
5	Elution 2, concentrated	200 mM	5µl

After purification and concentration also bands apparently smaller and bigger than 74 kDa occurred with significant bands at around 35 kDa, 40 kDa and 56 kDa. After concentration of the purified tCPR, protein concentrations of 5.5 mg/ml were determined using Bradford assay. Western blot analysis was performed with the purified tCPR to control if the proteins of the different sizes still contain the His-tag. Also Anti - AOX1 antibody was used to control if there was AOX1 protein in the purified sample.

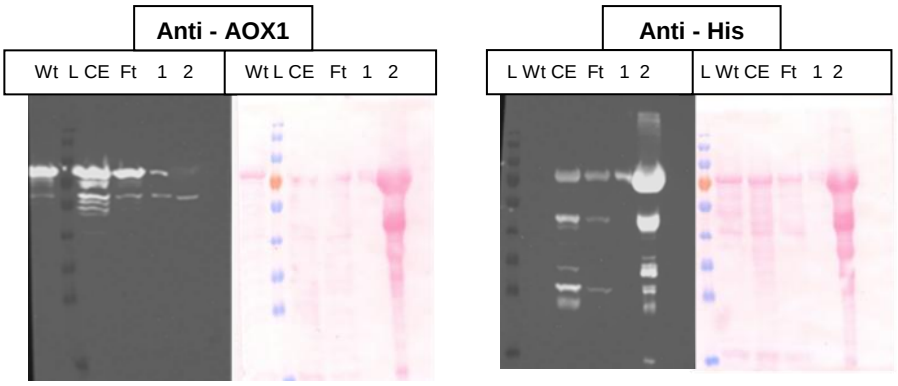


Figure 39. Western blot analysis of purified and concentrated tCPR with AOX and His antibody

Table 61. Lane description of Figure 39

Lane	sample	Imidazol concentration
L	PageRuler^{TM} Prestained Protein Ladder	/
CE	Cell extract	/
Ft	Flow through	/
1	Wash 3	20 mM
2	Elution 2 - concentrated	200 mM

The Western blots in Figure 39 showed, that in the purified and concentrated tCPR fraction (imidazol concentration of 200 mM) only a very weak band of AOX1 was visible. Most of the AOX1 protein was found in the washing fraction and in the Flow through. The strongest band in the anti-His blot was found at a size of 74 kDa, which was the expressed tCPR. Quite strong bands were detected at ~56 kDa and ~ 40 kDa. All fragments still contained the His-tag as the respective antibody detected the protein in the Western blot. To control if the different fragments in the SDS-PAGEs for the purifications of tCPR and thCPR shown above occurred due to fragmentation during expression or because of the denaturation with SDS during SDS-PAGE, a native PAGE was performed. The fragments of SDS-PAGE were analyzed with mass spectrometry.

Resulting protein concentrations of the purified and concentrated tCPR and thCPR:

Table 62. Cell cultures used for purification of tCPR and thCPR

Sample	Culture	Cell mass	Protein solution	C (protein) [mg/ml]	C (protein- purified) [mg/ml]	C (protein-concentrated) [mg/ml]
thCPR	600 ml (3 cultures)	17g	30 ml	22.9	0.7	6.1
tCPR	600 ml (3 cultures)	15 g	30 ml	24.5	0.6	5.6

The obtained protein amounts of 6.1 mg/ml for thCPR and 5.6 mg/ml for tCPR were high enough for crystallisation studies and were sent to the core facility of structural biology.

Native - PAGE

Native PAGE was performed as described in the Methods part. Samples were kept at 0°C while adding the sample buffer and loaded immediately on the gel. For a gel thickness of 0.75 mm and 15 wells, 0.5 to 5 µg for Coomasie Blue stain was used. For staining and destaining the same solutions as for SDS-PAGE were applied.

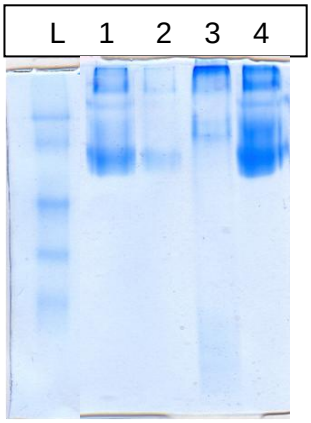


Figure 40. Native PAGE of purified tCPR and thCPR

Table 63. Lane description for Figure 40

Lane	Sample	C(protein) [mg/ml]	Loaded volume [µl]
L	Prestained MW marker	/	5
1	thCPR	4.5	5
2	thCPR	4.5	1
3	tCPR	0.55	10

In the native PAGE more than one band occurred. Thus, the bands of different sizes in the SDS-PAGE do most likely not occur during the denaturation step with SDS. Proteins of different sizes in SDS-PAGE propably were fragments of tCPR and thCPR accumulating during expression of the proteins. The fragmentation was also controlled by mass spectrometry as follows.

Mass spectrometry analysis:

Mass spectrometry analysis of the different bands, which occurred after purification of tCPR, was performed to control if all fragments contained sequences of tCPR. It was performed by Dr. Ruth Birner-Grünberger from the ZMF – center for medical research. Therefore, SDS-PAGE (NuPAGE) was done and different volumes of protein were loaded. The gel was Coomassie stained and the visible bands were cut out and stored in 10% EtOH solution at

-20°C and measurement was performed at the ZMF. Bands of 40 kDa, 56 kDa, 75 kDa and 85 kDa were tested (Figure 41 and Figure 42). For better separation of the two upper bands at 85 kDa and 75 kDa the SDS – PAGE was run for around 90 min. Different protein amounts were loaded onto SDS-gels (NuPAGE gel, 12% Bis-Tris).

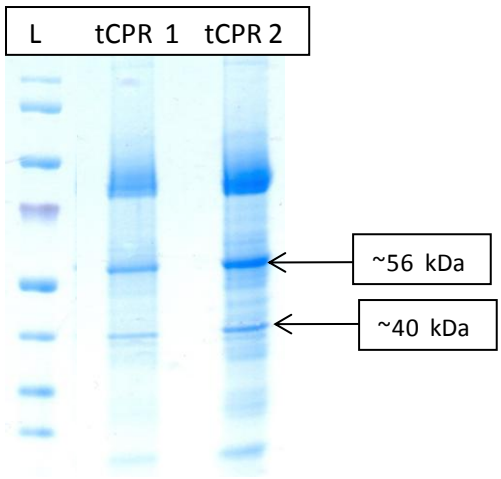


Figure 41. SDS-PAGE for mass spectrometry analysis of tCPR

Table 64. Lane description to Figure 41

Lane	Sample	Loaded volume [µl]
L	PageRuler™ Prestained Protein Ladder	5
tCPR 1	Purified tCPR	2
tCPR 2	Purified tCPR	5

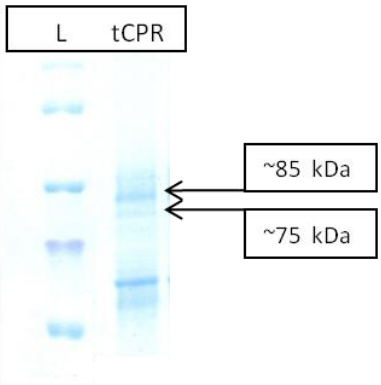


Figure 42. SDS-PAGE for mass spectrometry analysis of tCPR

Table 65. Lane description of Figure 42

Lane	Sample	Loaded volume [µl]
L	PageRuler™ Prestained Protein Ladder	5
tCPR	Purified tCPR	5

Mass spectrometry showed that all tested proteins contained sequences of the tCPR protein, and therefore, all fragments were part of tCPR. This fragmentation probably occurred because of hydrolysis of tCPR after cell disruption. TCPR appeared to be unstable after disruption and purification. With the use of NCBI BLAST the mass spectra were compared to the primary sequence of tCPR. Mass spectrometry suggested that for all tested proteins, C- and N-terminal amino acids were supposed by present in all sequences. TCPR has a nominal mass of 73.9 kDa and a calculated pI value of 5.35. Quite high sequence coverage values were achieved with (Table 66).

Table 66. Sequence coverage of mass spectras

Protein size	Sequence coverage
40 kDa	51 %
56 kDa	47 %
57 kDa	55 %
75 kDa	55 %
85 kDa	66 %

To get rid of the smaller bands and get highly pure tCPR and thCPR, the samples were purified by size exclusion chromatography. This sample was sent to the core facility structural biology to crystallize the protein.

Purification of t2D6:

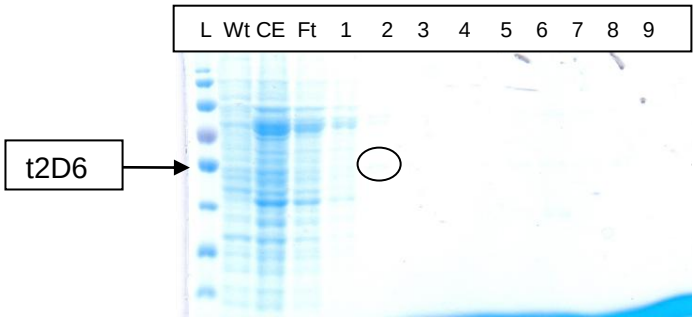


Figure 43. SDS- PAGE of t2D6 purification

Table 67. Lane description to Figure 43

Lane	Sample	C (Imidazol) [mM]	Lane	Sample	C (Imidazol) [mM]
L	PageRuler™ Prestained Protein Ladder	/	4	Wash 4	30
Wt	Wild type	/	5	Wash 5	50
CE	Cell extract	/	6	Elution 1	100
Ft	Flow through	/	7	Elution 2	200
1	Wash 1	10	8	Elution 3	300
2	Wash 2	15	9	Elution 4	500
3	Wash 3	20			

The protein was not purified under these conditions as all of the protein was washed out with the flow through and the first washing step at an imidazol concentration of 10 mM. The protein did not bind to the Ni-Sepharose beads. To purify t2D6, conditions were changed and 0.01% of SDS was added to the homogenate of t2D6 and incubated at 30°C for 1 h to slightly denature the protein for the His-tag to bind to the Ni-Sepharose beads. SDS-PAGE in Figure 44 shows the results of the purification with the new conditions.

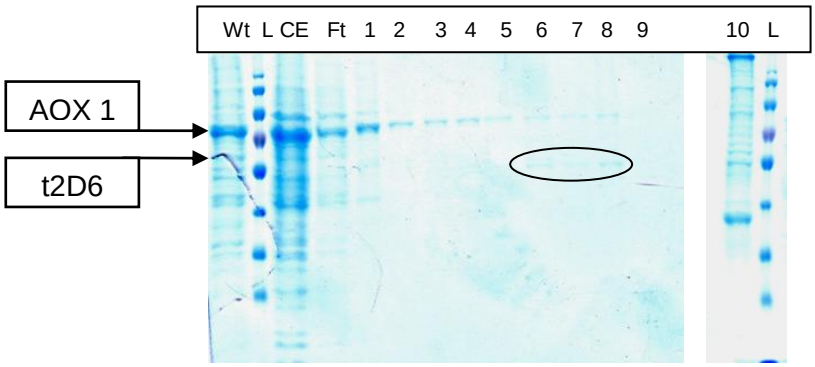


Figure 44. SDS- PAGE of t2D6 purification using 0.01% SDS

Table 68. Lane description to Figure 44

Lane	Sample	C (Imidazol) [mM]	Lane	Sample	C (Imidazol) [mM]
L	PageRuler™ Prestained Protein Ladder	/	4	Wash 4	30
Wt	Wild type	/	5	Wash 5	50
CE	Cell extract	/	6	Elution 1	100
Ft	Flow through	/	7	Elution 2	200
1	Wash 1	10	8	Elution 3	300
2	Wash 2	15	9	Elution 4	500
3	Wash 3	20	10	t2D6, concentrated	/

Only very small amounts of the protein bound to the beads and were purified and also a bigger band, which had the size of AOX1 protein, occurred. The elution fractions 1, 2 and 3, containing t2D6 were pooled and concentrated with Millipore concentrator (10 000 MWKO). The concentrated sample contained a crude mixture of proteins of very different mass (slot 10, Figure 44). Some of the smaller bands might indicate t2D6 fragmentation.

Purification of tHPO:

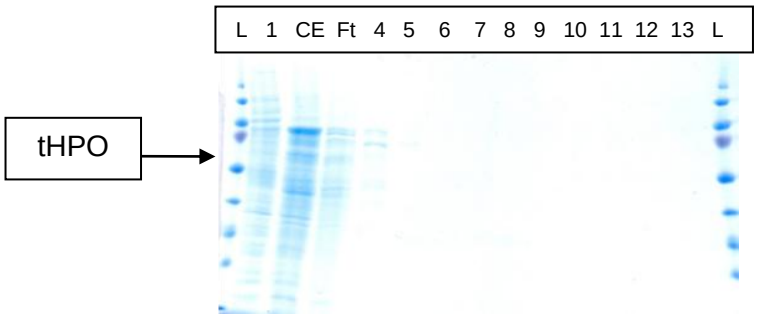


Figure 45. SDS- PAGE of tHPO purification

Table 69. Lane description to Figure 45

Lane	Sample	C (Imidazol) [mM]	Lane	Sample	C (Imidazol) [mM]
1	Wild type	/	7	Wash 4	30
L	PageRuler^{TM} Prestained Protein Ladder	/	8	Elution 1 – fraction 1	100
CE	Cell extract	/	9	Elution 1 – fraction 2	100
Ft	Flow through	/	10	Elution 2 – fraction 1	200
4	Wash 1	10	11	Elution 2 – fraction 2	200
5	Wash 2	15	12	Elution 3	300
6	Wash 3	20	13	Elution 4	500

The protein was not purified with the used conditions. All of the protein was washed out with the flow through and the first washing step at an imidazol concentration of 10 mM. The protein did not bind to the Ni-Sepharose beads. To purify tHPO, conditions were changed and 0.01% of SDS was added to the homogenate and incubated at 30°C for 1 h to slightly denature the protein, for the His-tag to bind to the Ni-Sepharose beads. SDS-PAGE in Figure 46 showed the results of the purification with the new conditions.

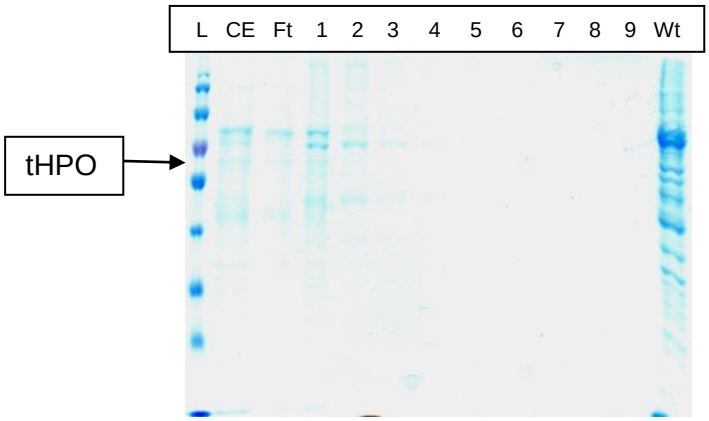


Figure 46. SDS- PAGE of tHPO purification using 0.01% SDS

Table 70. Lane description to Figure 46

Lane	Sample	C (imidazol) [mM]	Lane	Sample	C (imidazol) [mM]
L	PageRuler™ Prestained Protein Ladder	/	5	Wash 5	50
CE	Cell extract	/	6	Elution 1	100
Ft	Flow through	/	7	Elution 2	200
1	Wash 1	10	8	Elution 3	300
2	Wash 2	15	9	Elution 4	500
3	Wash 3	20	Wt	Wild type	/
4	Wash 4	30			

tHPO did not bind to the Ni-Sepharose beads and all of the protein was washed out with the first two washing steps.

Purification of tPM17:

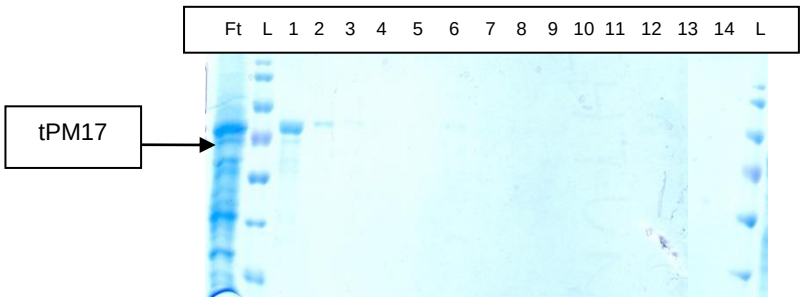


Figure 47. SDS-PAGE of tPM17 purification

Table 71. Lane description to Figure 47

Lane	Sample	C (imidazol) [mM]	Lane	Sample	C (imidazol) [mM]
L	PageRuler™ Prestained Protein Ladder	/	8	Elution 1 - fraction 3	100
Ft	Flow through	/	9	Elution 2 – fraction 1	200
1	Wash 1	10	10	Elution 2 – fraction 2	200
2	Wash 2	15	11	Elution 2 – fraction 3	200
3	Wash 3	20	12	Elution 3 – fraction 1	300
4	Wash 4	30	13	Elution 3 – fraction 2	300
5	Wash 5	50	14	Elution 4 – fraction 1	500
6	Elution 1 – fraction 1	100	15	Elution 4 – fraction 1	500
7	Elution 1 - fraction 2	100			

tHPO, t2D6 and tPM17 were not purified, because the protein did not binnd to the column and the whole protein was already eluted with the first washing steps. One reason for this observation can be that the His-Tag was located inside the protein, because the protein folding. The His-tag might therefore not reach the Ni- Sepharose beads and would not bind to

the column. Only 6 His residues were used to tag the protein on the C- terminal side. For membrane proteins it could be better to use 10 His residues and the protein could also be tagged on the C- and N-terminal side of the protein to enhance the chances that the protein binds to the beads for further experiments [34].

4.5. *IN VITRO* ACTIVITY ASSAYS

Western blot analysis was performed with all protein fractions used for the activity assays to control if sufficient protein was present. SDS-PAGE, Western blot and subsequent immunodetection of the proteins were done according to the protocol described in Materials and Methods. Different protein amounts were loaded onto SDS-gels (Self made gels, 12% Bis-Tris).

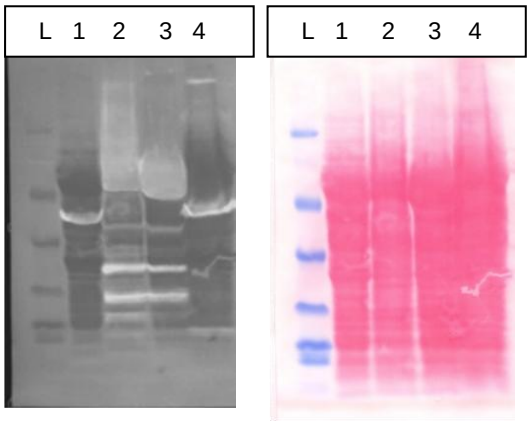


Figure 48. Western blot of expressed HPO, PM17 and tCPR, used for activity assays

Table 72. Lane description to Figure 48

Lane	Sample
L	PageRuler™ Prestained Protein Ladder
1	HPO
2	tCPR - 1
3	tCPR - 2
4	PM17

TCPR-1 and tCPR-2 were tCPR samples of two different protein expressions in different flasks. Lanes shown in Figure 48 were overloaded as 10 µl of protein samples were loaded on the gel. For tCPR also smaller fragments were visible, which was already discussed above in the purification part. Samples shown in Figure 48 and they were used for *in vitro* activity assays, i.e. limonene assay and valencene assay, described in the following part.

4.5.1. Limonene *in vitro* activity assay

In this activity assay, PM17 and tPM17 samples were tested for their ability to convert (-)-Limonene to (-) - trans- isopiperitenol (Figure 49).

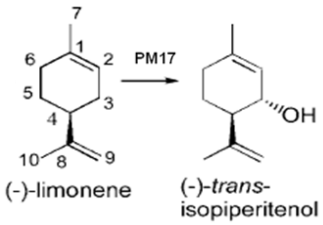


Figure 49. Conversion of (-)-Limonene to (-)-trans-isopiperitenol

All used volumes are listed in Table 74 and 75. Protein concentrations were estimated by Bradford assay and the fraction of PM17 proteins among total protein was assessed by SDS-PAGE. A GDH based cofactor regeneration system was used. For the GC-FID measurement 50 µl of sample were used directly after centrifugation and the rest was concentrated under N_2 steam, dissolved in 100 µl EtOAc and used for another GC measurement. EtOAc was also measured with GC-FID to define the background. The standards (-)-limonene, D-camphor and a standard mixture containing (-)-limonene, D-camphor and L-carvone were also measured. Wild type strain (Wt CBS 7437-his) samples were used for the activity assay to define the activity of the empty strain without PM17. Zero minute samples were measured as well. The samples at zero minutes were extracted directly after initiation of the reaction to define how much of the product had already been produced while adding the substrate. The blank included all reagents except the enzymes and was measured to define the background activity. Different protein fractions were tested including the homogenate of PM17 expression strains and the solubilized ER fraction of PM17 using detergents 7 (Polyoxyethylene 10 tridecyl ether) and 10 (n-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate). Concerning CPR the ER fraction and the whole homogenate of the respective expression strain were tested. Furthermore also the homogenate of tCPR expression strain was used.

Table 73. Stock solutions for the Limonene *in vitro* assay

Reagent	Concentration (Stock)	Mass [mg]	Volume [µl]	in Volume buffer [ml]
KCl	1 M	745.0	/	10.0
$MgCl_2$	500 mM	1500.0	/	10.0
(-)-limonene	200 mM (δ=0.84g/0.001l)	272.0	323.0	1.0
Glucose	10%	/	10.0	1.0
GDH	800 U	67.0	/	1.0
NADPH	50 mM	37.2	/	1.0
FAD	5 mM	39.3	/	10.0
FMN	5 mM	22.3	/	10.0

Table 74. Composition of the Limonene *in vitro* assay for wild type (WT CBS 7437-his) and the BLANK

Protein / Reagent	Concentration (Stock)	Concentration (final)	Wild type [µl]	BLANK [µl]
Wt	/	/	200	/
tCPR (homogenate)	45.65 mg/ml	500 pmol/1 nmol	200	/
KCl	1 M	250 mM	25	25
$MgCl_2$	500 mM	50 mM	100	100
(-)-limonene 3	200 mM	2 mM	10	10
10x Glucose	870 mM	87 mM	100	100
GDH	800 U	8 U	10	10
NADPH	50 mM	5 mM	100	100
FAD (Flavin- Adenin-Dinukleotid)	5 mM	500 µM	100	100
FMN (Flavinmononukleotid)	5 mM	500 µM	100	100
KP_i Buffer	100 mM		55	455

Table 75. Composition of the Limonene *in vitro* assay, 7…detergent Polyoxyethylene 10 tridecyl ether, 10… detergent n-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate

protein/reagent	Concentration (Stock)	Concentration (final)	A [µl]	B [µl]	C [µl]
PM17 (homogenate)	20 mg/ml	500 pmol/1 nmol	200	/	/
PM17 (solubilized – detergent 7)	2.27 mg/ml	500 pmol/1 nmol	/	250	/
PM17 (solubilized – detergent 10)	4.96 mg/ml	500 pmol/1 nmol	/	/	250
tCPR (homogenate)	45.65 mg/ml	500 pmol/1 nmol	200	200	200
KCl	1 M	250 mM	25	25	25
$MgCl_2$	500 mM	50 mM	100	100	100
(-)-limonene	200 mM	2 mM	10	10	10
10x Glucose	870 mM	87 mM	100	100	100
GDH	800 U	8 U	10	10	10
NADPH	50 mM	5 mM	100	100	100
FAD	5 mM	500 µM	100	100	100
FMN	5 mM	500 µM	100	100	100
KP_i Buffer	100 mM	/	55	5	5

After extraction and centrifugation, two phases were formed. The phase fraction was the EtOAc fraction containing the extracted products and the substrate. The yellow color of the samples indicated the FAD and FMN compounds, which have a characteristic yellow to light orange color. The EtOAc phase was used for GC-FID measurement.

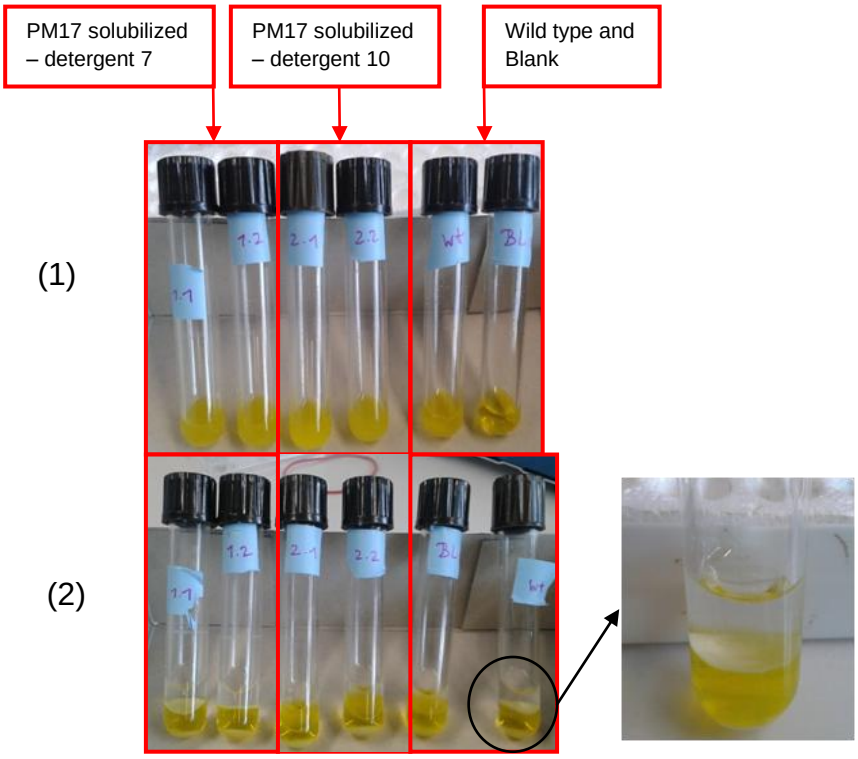


Figure 50. Pyrex tubes of Limonene *in vitro* activity assay, (1) Reaction after incubation and mixing (2) Reaction after Extraction and centrifugation

The chromatograms from the GC-FID measurements showed that no product was formed, which means that all tested PM17 proteins were inactive. Figure 51 shows the measurement of the wild type, the standard mix and the Blank to compare with the measurements of the samples. Figure 52 shows the chromatograms of the activity measurement of the homogenate of PM17 and zero point of PM17. No product peak was found after comparison with the standard and wild type measurement. Removal of solvent under N_2 steam did not improve the situation (Figure 53). Figure 54, 55 and 56 show the GC-FID measurements of the activity assays with PM17 protein after solubilization with two different detergents. With these measurements no products were formed either. One peak that occurred, turned out to be PMSF shown by mass spectrometry measurements (Figure 57).

Measurement of the PM17 homogenate:

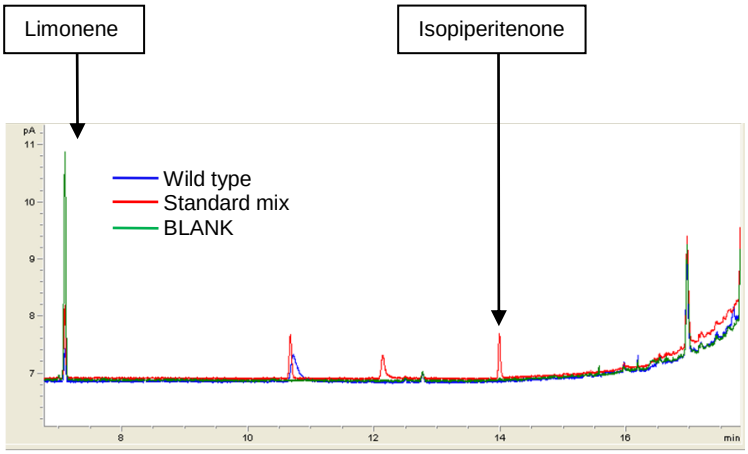


Figure 51. GC-FID measurement of samples of Limonene *in vitro* assay, wild type, Standard and Blank

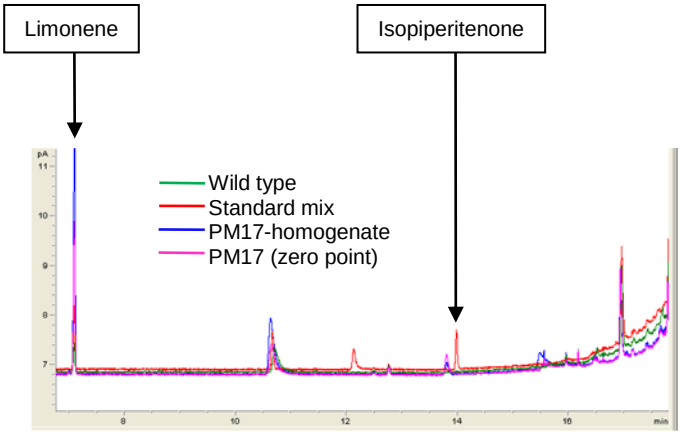


Figure 52. GC-FID measurement of samples of Limonene *in vitro* assay, wild type, Standard, homogenate of PM17 and zero point of PM17

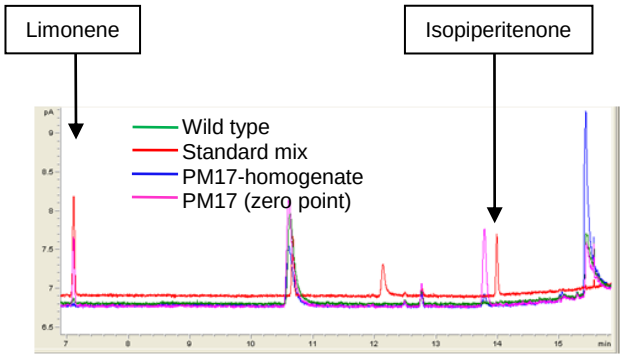


Figure 53. GC-FID measurement of samples of Limonene *in vitro* assay after concentration under N_2 steam, wild type, Standard, homogenate of PM17 and zero point of PM17

Measurement of PM17 solubilized in detergents 7 and 10:

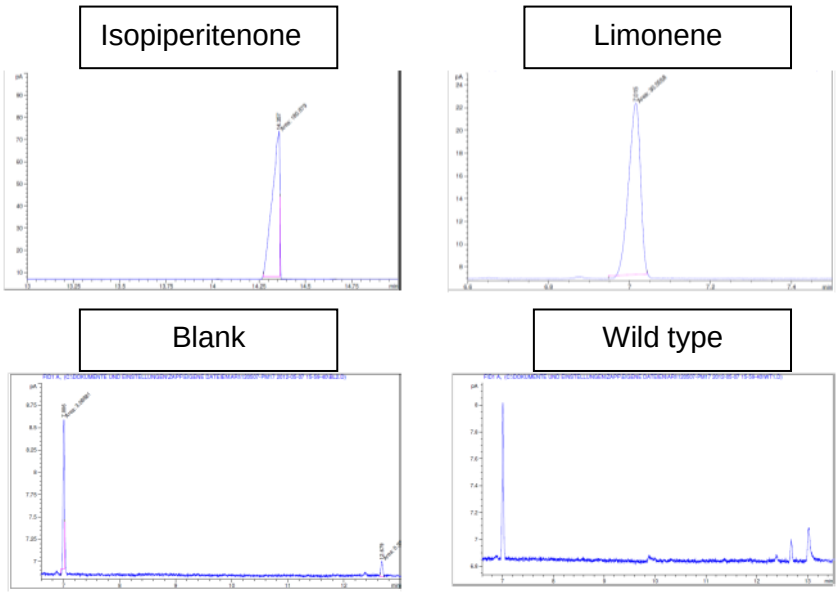


Figure 54. GC-FID measurement of samples of Limonene *in vitro* assay, Isopiperitenone, Limonene, Blank and wild type

PM17 solubilized using detergent 10:

For the assay the homogenate of CPR expression strain was used.

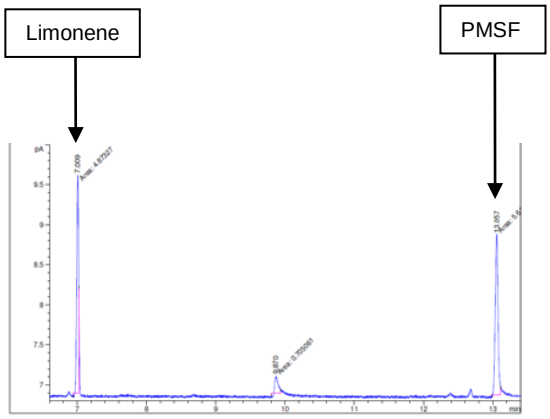


Figure 55. GC-FID measurement of samples of Limonene *in vitro* assay, PM17 solubilized using detergent 10

PM17 solubilized using detergent 7:

For the assay the ER fraction of CPR expression strain was used.

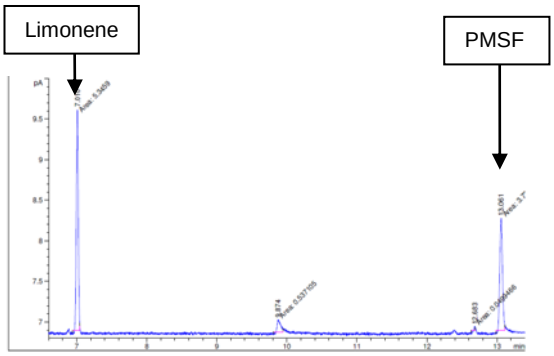


Figure 56. GC-FID measurement of samples of Limonene *in vitro* assay, PM17 solubilized using detergent 7

Mass spectrometry analysis was performed to define the different peaks. Therefore, 50 µl of sample were sent to the institute of analytical chemistry and mass spectrometry was performed by Prof. Erich Leitner. The two higher peaks turned out to be limonene and PMSF, which was used in the buffer for cell disruption and is present in all protein samples. The smaller peak at around 10 min is part of the solvents which were used.

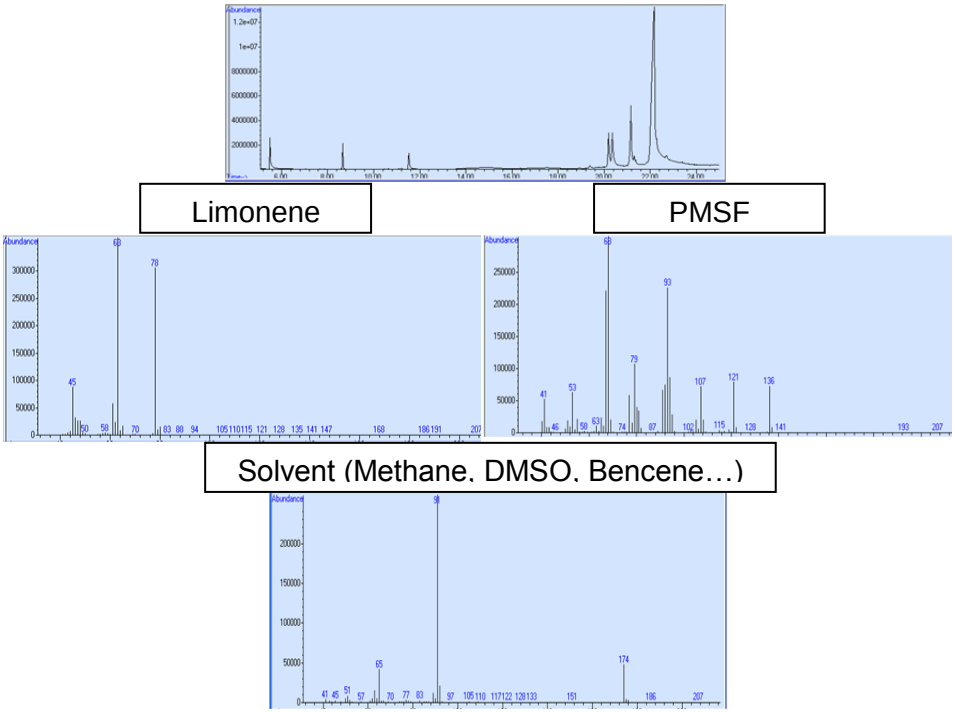


Figure 57. Mass spectra's of Limonene *in vitro* assay - PM17 solubilized using detergent 7,Limonene, PMSF and solvent

The Chromatograms and mass spectra confirmed that none of the tested enzyme samples formed product. No conversion of (-)-Limonene to (-)-trans-isopiperitenol took place and the tested PM17 proteins expressed in *P. pastoris* and solubilized using detergents were inactive.

4.5.2. Valencene *in vitro* activity assay

The composition and used amounts for the assay are listed in Table 77 and 77. Protein concentrations were estimated by Bradford assay and the fraction of HPO proteins among total protein was assessed by SDS-PAGE. EtOAc was measured with GC-FID to define the background of analysis and a standard mixture containing 20 ng/µl Nootkatone, 20 ng/µl cis-Nootkatol, 20 ng/µl trans-Nootkatol and 20 ng/µl Valencene were measured as well. The samples from wild type strain (Wt CBS 7437-his) are used for the activity assay to define the background activity of the empty strain. For all samples duplicate measurements were performed. A aliquote at zero minutes, was taken and measured. The sample at the zero minutes was extracted directly after initiation of the reaction to estimate the conversion while adding the substrate. Also a Blank was measured together with the other samples (Table 76). Blank was a reaction which included all reagents except the enzymes, and which was measured to define the background activity. Also a positive control (homogenate and ER fraction) was used for the assay, which was provided by T. Wriessnegger. Only about 1 % of the total ER fraction protein is HPO, which has to be considered for the calculation of the used amounts.

Table 76. Stock solutions for the Valencene *in vitro* assay

Reagent	Concentration (Stock)	Concentration (final)	Mass [mg]	in buffer volume [ml]	Dilution
NADPH	24 mM	2.4 mM	17.9	1	/
Valencene	10 mM	100 µM	20.4	10	/
FMN	5 mM	500 µM	22.3	10	1:10
FAD	5 mM	500 µM	39.3	10	1:10

Table 77. Composition of the Valencene *in vitro* assay

Reagent	Concentration (Stock)	Concentration (final)	Wild type [µl]	A [µl]	B [µl]	C [µl]	BLANK [µl]
Wt	21.7 mg/ml	5 mg	230	0	0	0	0
HPO (ER fraction)	17.5 mg/ml	5 mg	0	280	0	0	0
HPO (homogenate)	41 mg/ml	5 mg	0	0	250	0	0
HPO (homogenate, positive control)	14 mg/ml	5 mg	0	0	0	250	0
tCPR (homogenate)	45.65 mg/ml	5 mg	250	250	250	250	0
Me_2SO (DMSO)	100%	4%	40	40	40	40	40
NADPH	24 mM	2.4 mM	100	100	100	100	100
Valencene (1%Triton, DMSO)	100 mM	2 mM	20	20	20	20	0
FMN	500 µM	5 µM	10	10	10	10	10
FAD	500 µM	5 µM	10	10	10	10	10
100 mM KP_i, pH 7,4			340	290	320	320	840

Positive control obtained from T. Wriessnegger was measured indicating the product peak for trans-nootkatol suggesting that the proteins were active (Figure 58). The positive control was performed to test if the assay conditions work. Figure 59 shows the chromatograms of the wild type, Standard and Blank to compare with the samples. Figure 60 and 61 show the chromatograms of the tested HPO samples. However, no product peak was found. The tested HPO samples did not convert valencene.

Measurement of the positive control:

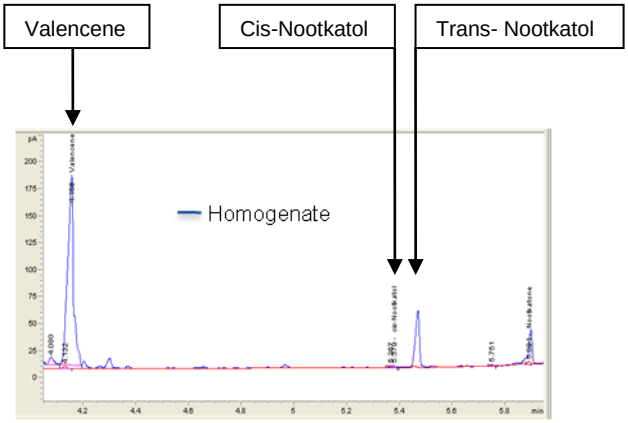


Figure 58. Valencene *in vitro* activity assay, positive control

Valencene assay:

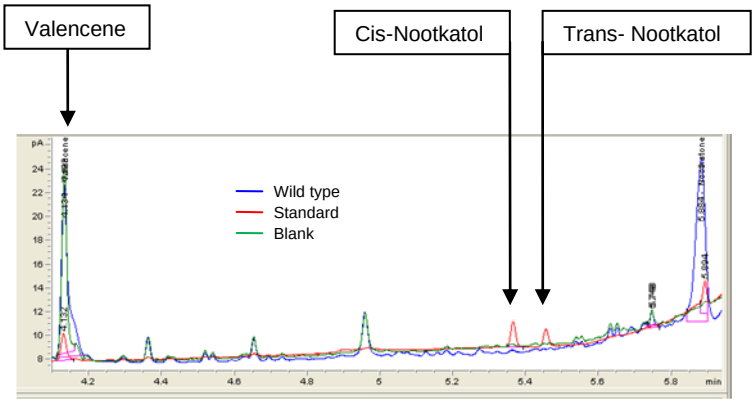


Figure 59. Valencene *in vitro* activity assay, wild type, Standard and Blank

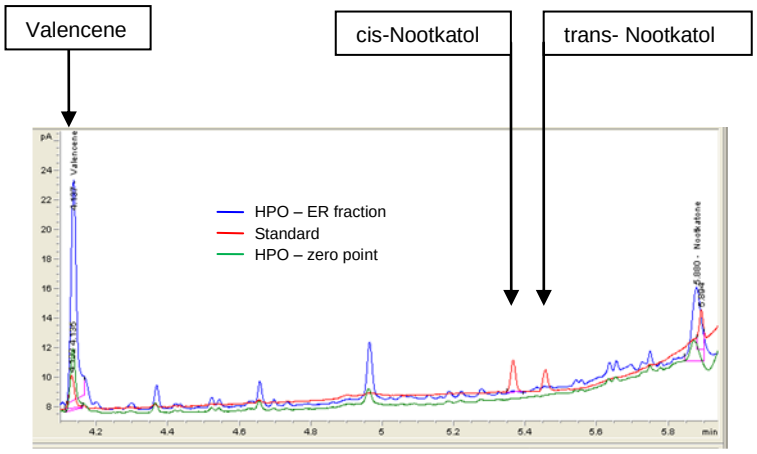


Figure 60. Valencene *in vitro* activity assay, HPO (ER fraction), Standard and HPO (ER fraction) at zero minute

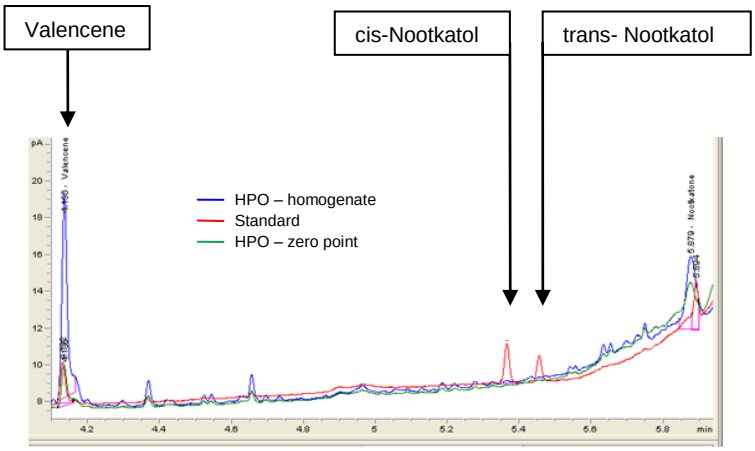


Figure 61. Valencene *in vitro* activity assay, HPO –homogenate, Standard and HPO at zero minute (homogenate)

4.5.3. Cytochrome c *in vitro* activity assay

Protein concentrations were determined by Bradford assay (Table 78). Components and amounts used for the assay are listed in Table 79. The assay was performed as described in the Methods section above. In Figure 62 the color change of the solution containing active protein is shown in comparison with the Blank.

Table 78. Protein determinations for Cytochrome c *in vitro* activity assay

Sample	C [mg/ml]
CPR (homogenate)	24.19
hCPR (homogenate)	19.81
tCPR (homogenate)	22.76
thCPR (homogenate)	21.19
hCPR, ER fraction	33.08
tCPR, purified	5.50
thCPR, purified	4.50
WT (homogenate)	21.17

Table 79. Components used for the Cytochrome c in vitro activity assay

Component	Stock solution	End concentration	Volume [µl]
Tris- HCl, pH 7.5 + 1 mM EDTA	50 mM	33.30 mM	500
Cytochrome c	0.3 mM	0.05 mM	125
KCN	50 mM	3.33 mM	50
Protein sample			25
NADPH	1.5 mM	0.10 mM	50

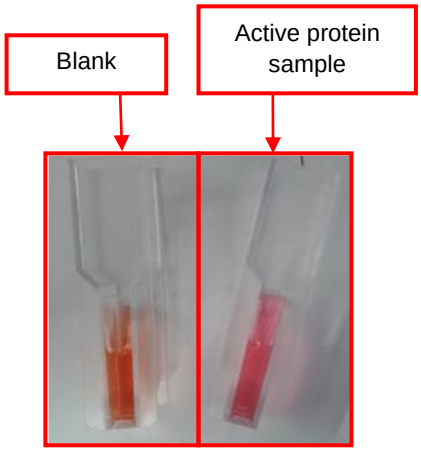


Figure 62. Samples from the cytochrome c *in vitro* activity assay after addition of NADPH and incubation

The activity of the protein was determined according to the calculation in Figure 63.

$$A = \frac{\frac{E_{550}}{t \times d \times V_R}}{\varepsilon \times V_S} \left[\frac{U}{ml}\right] \qquad A = \frac{\frac{E_{550}}{t \times d \times V_R}}{\varepsilon \times V_S \times c} \left[\frac{U}{mg}\right]$$

ε…molar extinction coefficient of NADPH, 21 $mM^{-1}*cm^{-1}$

d…layer thickness [1 cm]

V_R…reaction volume [0.75 mL]

V_S…sample volume [0.025 mL]

c…protein concentration [mg/ml]

If 1% of the whole protein concentration is reductase enzyme:

A(reductase) = A [U/mg] * 100

Figure 63. Calculation of the protein activity of cytochrome c *in vitro* activity assay

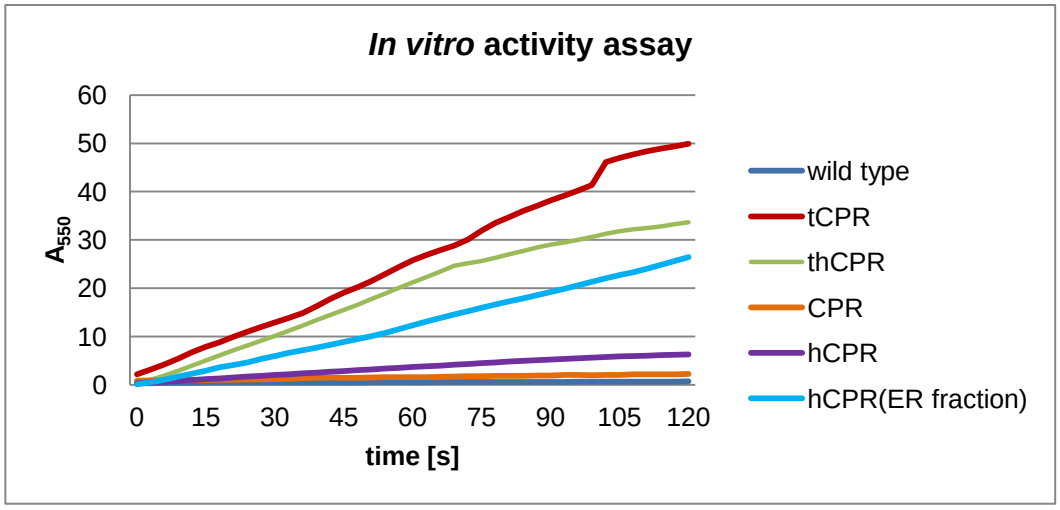


Figure 64. Extinctions of Cytochrome c *in vitro* activity assay

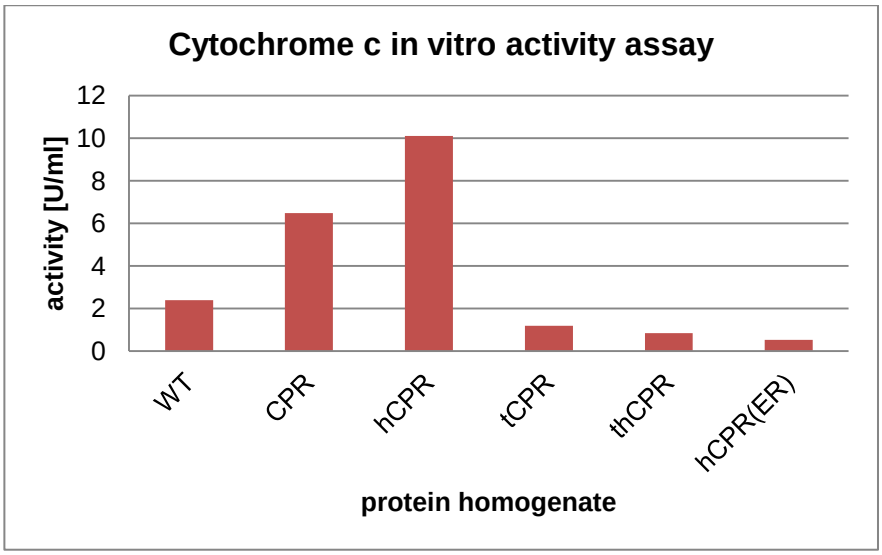


Figure 65. Activities of Cytochrome c *in vitro* activity assay

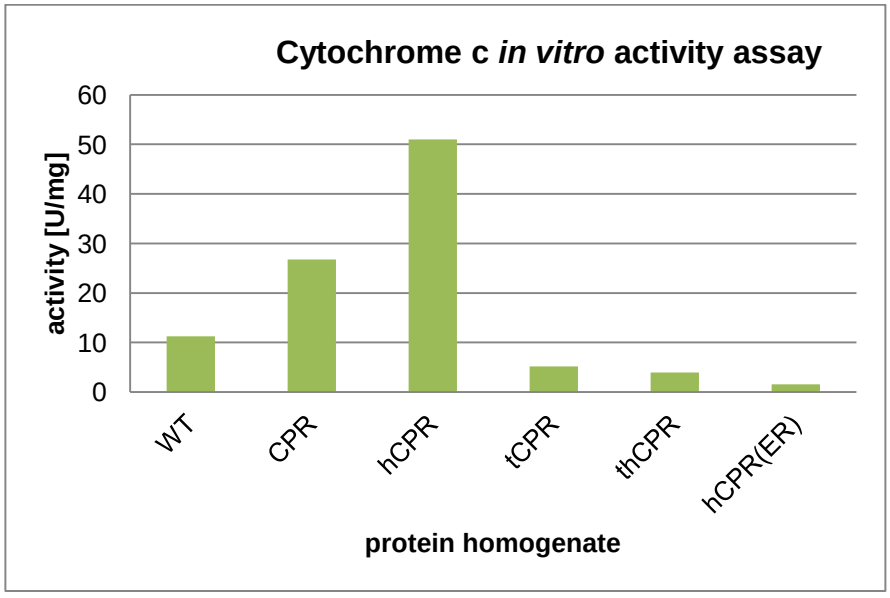


Figure 66. Activities of Cytochrome c *in vitro* activity assay, when 1% of the whole protein concentration is reductase, U/mg CPR

***In vitro* activity assay with purified and concentrated proteins:**

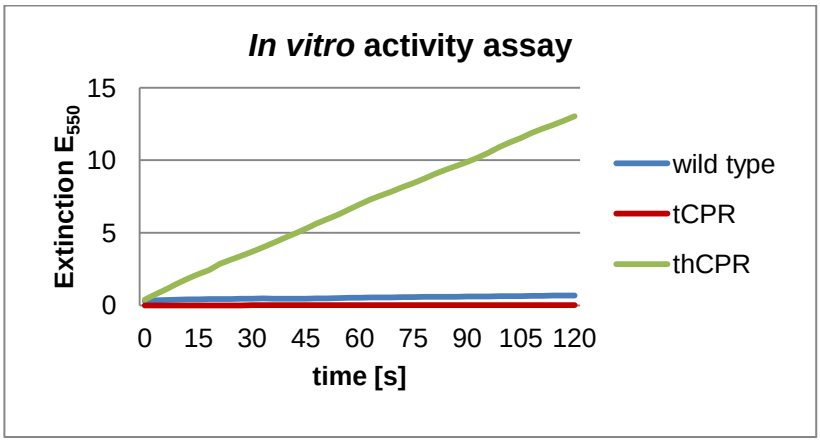


Figure 67. Extinctions of Cytochrome c *in vitro* activity assay of purified proteins

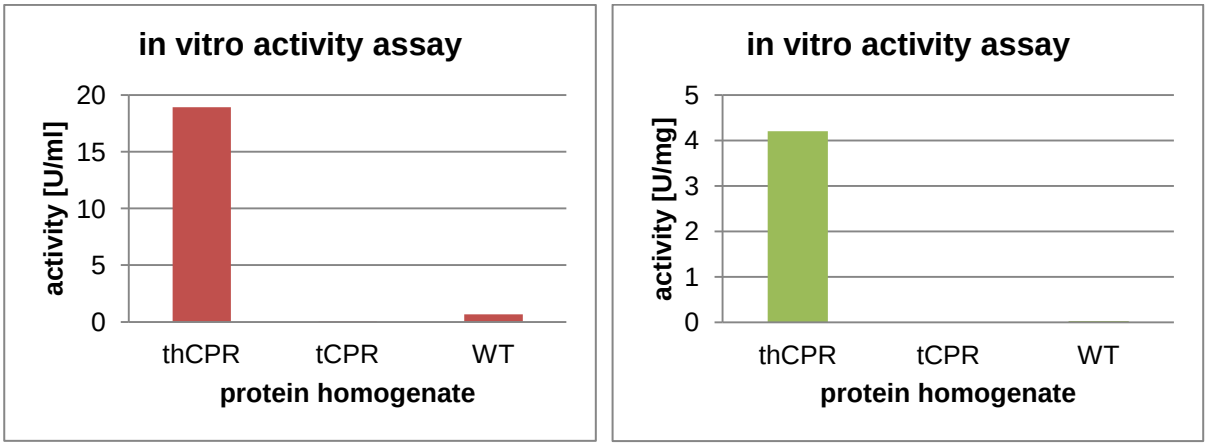


Figure 68. Activities of Cytochrome c *in vitro* activity assay of purified proteins

All tested proteins, except the purified tCPR, showed activity towards cytochrome c reduction, highest activity was found for the membrane-bound proteins CPR and hCPR. Truncated proteins showed clearly less activity. It was estimated that only around 1% of the whole protein in the solution was cytochrome P450 reductase, which was considered in the calculations for activity in U/mg (Figure 66).

4.6. CRYSTALLIZATION AND STRUCTURAL ANALYSES

tCPR and thCPR, containing 5.5 mg/ml and 4.5 mg/ml protein, were crystallized and analyzed by A. Lyskowski of the core facility of structural biology of University of Graz. The crystals grew under the conditions a01, h03, h08 and h10 of the JCSG screen. Condition C12 was also tested, but was optimized (Table 80). JCSG-plus HT-96 is a 96 reagent deep-well block, optimized sparse- matrix screen of classic and modern conditions, devised at the Joint Centre for Structural Genomics [22].

Table 80. Conditions for protein crystallization, Bis Tris…Bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane, PEG...Polyethylene glycol

Condition	Salt	Buffer	pH	Precipitant
A01	0.2 M lithium sulfate	0.1. M sodium acetate	4.5	50 % v/v PEG 400
C12	none	none	/	10% w/v PEG 1000/ 10% w/v PEG 8000
H03	none	0.1 M Bis Tris	5.5	25 % w/v PEG 3350
H08	0.2 M sodium chloride	0.1 M Bis Tris	5.5	25 % w/v PEG 3350
H10	0.2 M ammonium acetate	0.1 M Bis Tris	5.5	25 % w/v PEG 3350

All tests for the crystallization studies were performed by A. Lyskowski. Gel filtration (GF) was performed using Superose 6 10/300 (GE Healthcare) equilibrated with multicomponent buffer system (L-malic acid, MES, TRIS) (Newman, 2004) at 0.1x buffer concentration (N6, pH 6.5).

4.6.1. Crystallization screening

An aliquot of the samples thCPR and tCPR were concentrated to 10 mg/ml protein using Amicon Ultra 0.5 ml 10K MWCO centrifugal filters (Millipore). For crystallization, two macromolecular crystallization screens, Morpheus and JCSG (Molecular Dimensions), were performed. One µl drop was set up using Oryx 6 crystallization robot (Douglas Instruments) with 1:1 sample to screen ratio in SWISSCI-3 plates (sitting drop). The plates were stored at 20° C between inspections. ThCPR produced very thin crystal plates in condition C12 of JCSG screen. Testing the crystals was inconclusive as no diffraction pattern was obtained. The optimization was unsuccessful as only very thin and small needles were produced. tCPR produced a thin plate like crystalline in number of conditions in the Morpheus screen. A single plate was tested and a single protein diffraction image at beam line ID29 at the ESRF was produced (Figure 69). Conditions have to be optimized for further crystallizations [35].

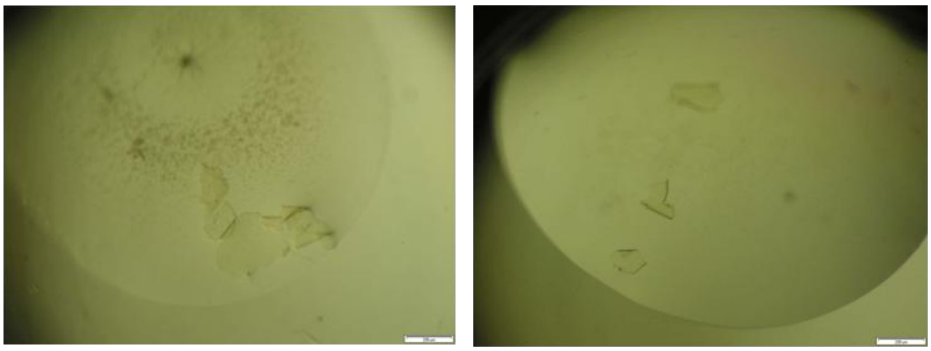


Figure 69. Crystal photos of tCPR crystal

5. CONCLUSIONS

All five membrane proteins (HPO, PM17, CPR, hCPR and 2D6) and truncated versions thereof (tHPO, tPM17, tCPR, thCPR and t2D6) were cloned into pPpB1 and expressed in *Pichia pastoris* wild type strain from the P_{AOX1}. Expression levels were confirmed with Western blot analysis and SDS-PAGE. Purification was attemped for the truncated proteins using affinity chromatography, however purification was only possible for tCPR and thCPR. tPM17, t2D6 and tHPO did not bind to the column and therefore were not purified. It is speculated that, the folding of the proteins and too few C-terminal His residues might have been the reason for unsuccessful purification. To purify the membrane-attached proteins, a detergent screening was performed. For all proteins tested, specific detergents were found to solubilize them. Based on valencene *in vitro* conversion assay, it was established that HPO was not active after solubilisation with detergents. Furthermore, also the unsolubilized ER fraction and the homogenate of HPO expression strains were not active under these conditions. Also the activity of PM17 (limonene hydroxylase) was tested, using limonene *in vitro* assay. Unfortunatly, also this membrane protein did not show any activity in the homogenate and ER fractions or for the solubilized PM17. The homogenate and the ER fractions of CPR, tCPR, thCPR and hCPR expression strains, as well as the purified thCPR protein were shown to be active as documented by cytochrome c reduction assay. Only the purified tCPR lacked activity. Highest activity was achieved with the membrane-bound hCPR and CPR. The purified truncated proteins (thCPR and tCPR) had lowest activity. Truncation of the reductase proteins (tCPR and thCPR) lead to loss of activity compared to the membrane bound versions. Purification of these truncated, soluble proteins leads to a loss of around 50% of the activity. The purified thCPR and tCPR proteins were handed over to the core facility of structural biology for crystallization studies. For thCPR, small crystals were produced, but for both truncated proteins the conditions have to be optimized. Moreover expression in *E. coli* had been performed with the truncated proteins to achieve higher expression yields. Cloning in *E. coli* appeared to be more difficult than in *P. pastoris.* Genes were cloned into pET-26b(+) vector with and without pelB sequence, which is responsible for the transport into the periplasm. Proteins were expressed by autoinduction and by induction with IPTG. Expression in *E. coli* did not lead to high protein amounts and did not work with autoinduction. Therefore, all expression experiments were furtheron performed in *P. pastoris*.

5.1. FINAL DISCUSSION / SUMMARY

Before I started my Master`s thesis already a lot of research had been done in this area by Tamara Wriessnegger and Anita Emmerstorfer. T. Wriessnegger started with the expression of membrane proteins (HPO, CPR, PM17, hCPR and 2D6) in *Pichia pastoris*, codon-optimized the genes, constructed several different expression plasmids and showed for the first time functional heterologous expression of both proteins. She also established several methods, assays and tools to monitor valencene conversion, measure protein performance and screen for best activity, which were adapted and used in this work.

The native HPO, PM17, CPR, hCPR and 2D6 N-terminal transmembrane domain, which was truncated in case of expression of soluble proteins was desired. When I started my Master`s thesis, expression of the membrane proteins in *P. pastoris* had already been optimized by T. Wriessnegger and A. Emmerstorfer yielding quite good expression levels. Thus, I have chosen the *P. pastoris* WT CBS7437 strain for expression of the His-tagged membrane-associated proteins. For easier purifications, also truncated (= soluble) versions of these proteins were expressed in *P. pastoris*. For crystallisation, high amounts of the proteins were needed. I have decided to express the truncated proteins also in *E. coli* to achieve higher expression yields. Therefore, I cloned the genes for the truncated proteins into the pET-26b (+) vector and expressed them using T7 promoter. The Master thesis of A. Emmerstorfer had shown that expression of these proteins in *E. coli* is difficult, because inclusion bodies were formed. To circumvent this problem, the truncated proteins were expressed by autoinduction or by using minimal amounts of IPTG for induction in order to provide the proteins with more time for correct folding. All cloning steps were performed in *E. coli* Top10F' cells to generate sufficient amounts of plasmid DNA for the individual transformations. For expression *E. coli* BL21 star (DE3) was used.

To purify the proteins, genes were tagged using PCR methods. A 6xHis-tag was C-terminally fused to all genes, coding for the membrane proteins and for the truncated proteins. Expression of tagged proteins was successfully detected by Western blot analysis, using antibodies for recognition of the tag-epitopes and secondary antibodies carrying horseradish peroxidase as reporter. Peroxidase activity yielded a chemoluminescence signal when incubated with commercially available peroxide substrates.

5.1.1. Summary-*P. pastoris*

Table 81.Summary of the results for expression in *P. pastoris* and purifications

Experiment	Used vectors / promoters / reagents	Conditions	Comment
Cloning	pPpB1 vector	Insert at *Eco*RI, *Not*I restriction sites	✓
Transformation in *E. coli*	*E. coli* Top10 F`	Plasmid DNA was transformed, Selection on LB-Zeo plates at 37°C, o.n.	✓
Transformation in *P. pastoris*	*P. pastoris* WT CBS 7437-his	Linearized plasmid was transformed, Selection on YPD-Zeo plates at 28°C for 2-3 days	✓
Expression in *P. pastoris*	P_{AOX1} → Induction with MeOH	Cell growth: 60 h, 28°C, 120 rpm in BMGY medium Induction: 60-72 h, 28°C, 120 rpm with BMMY / MeOH every morning and evening	✓ Highest expression levels were achieved for tCPR, CPR, thCPR and hCPR
Detergent screening	Detergents: CHAPS, ASB-14, n-Dodecyl-β-D-maltoside, OTG, SB3-10, OG, Polyoxyethylene 10 tridecyl ether, C7BzO, 3-08, 3-16, Tween 20, SDS	5 mg protein, 1% - 2% detergent (above CMC), 100 mM NaCl, 1 mM PMSF, 25°C, 3 h incubation	✓ All proteins were solubilized with specific detergents. Also ER fractions were used for solubilization studies.
Purification optimisation of conditions	Ni-sepharose beads Fast™ Flow	Incubation of Beads with protein solution o.n. at 4°C. Buffer = 20 mM KP_i, pH 7.4, 0.5 M NaCl,10-500 mM imidazol Washing: 10 CV (column volumes) 10-50 mM imidazol Elution: 5 CV 100-300 mM imidazol, 10 CV 500 mM imidazol ➔ Storage at 4°C or -20°C (+5% Glycerol)	~ Only tCPR and thCPR were purified. tPM17, t2D6, tHPO did not bind to the column and were not purified.
SDS treatment	0.01 % SDS	Incubation of protein solution (tHPO, tPM17) with 0.01% SDS at 30°C for 1 h.	X Proteins were partially denatured to bind to the column, but tHPO and tPM17 were degraded and washed out.

Purification	Ni-sepharose beads Fast^{TM} Flow	Final conditions for tCPR and thCPR purification: Washing: 10-20 CV 20 mM imidazol Elution: 5-10 CV 200 mM imidazol	~ Proteins were purified, but fragments of different sizes occurred after concentrating.
Desalting and	PD-10 desalting column	Purified protein solution passed through the column.	✓ To get rid of imidazol and other salts. Was performed directly after purification.
Concentration	Millipore concentrator (30 000 MWCO)	Centrifugation at 4000 rpm (table top centrifuge), 4°C for ~ 20 min	✓ Viva Spin concentrators were also tested, but did not work, because the protein bound to the filter material of the columns.
Limonene *in vitro* activity assay	Conversion of limonene to trans-isopiperitenol: PM17 (membranes and solubilized protein), CPR (ER fraction) or tCPR (homogenate or purified)	Assay buffer: 100 mM KP_i, pH 7.4 PM17 protein, reductase protein, KCl, $MgCl_2$ →Incubation at RT for 30 min Initiation: (-)-limonene, G-6-P, G-6-P dehydrogenase, NADPH, FAD, FMN up to 1 ml → incubation 2 h, 30°C Extraction: EtOAc for 30 min	X No conversion of limonene to the product, no activity of PM17, expressed in *P. pastoris*.
Valencene i*n vitro* activity assay	Conversion of valencene to nootkatol: HPO (ER and homogenate fractions), tCPR (homogenate)	Assay buffer: 100 mM KP_i, pH 7.4 HPO protein, tCPR, DMSO, assay buffer → Incubation for 5 min at 30°C Initiation: NADPH, valencene, TritonX-100, FMN, FAD up to 1 ml → incubation o.n. at 30°C Extraction: EtOAc for 30 min	X No conversion of valencene to the product, no activity of the tested HPO. Positive control was active → assay conditions worked.
Cytochrome c *in vitro* activity assay	Reduction of cytochrome c: CPR, hCPR, tCPR (homogenate, purified), thCPR (homogenate, purified), hCPR (ER fraction)	Assay buffer: 50 mM Tris-HCl buffer, pH7.5 + 1 mM EDTA Cytochrome C, KCN, NADPH, diluted protein solution (reductase) → measurement at 550 nm for 2 min	✓ All proteins, except the purified tCPR showed activity compared to the wild type strain. Highest activities achieved with membrane-bound proteins.

5.1.2. Summary-*E. coli*

Table 82. Summary of the results of expression in *E. coli*

Experiment	Used vectors / promoters/ reagents	Conditions	Comment
Cloning	pET- 26b (+) vector	Cloning with pelB leader sequence (periplasmic transport) at restriction sites *Eco*RI, *Not*I and cloning without pelB leader sequence at restriction sites *Nde*I, *Hind*III.	✓
Transformation	*E.coli* BL21 star (DE3)	Plasmid DNA was transformed ane selected on LB-Kan plates, o.n. at 37°C	✓
Expression	Autoinduction	Cell growth: ZY medium, 120 rpm, 28°C / 37°C, o. n. Induction: ZYP-5052 medium (+ Kan, δ-ALA, $FeCl_3$), 120 rpm, 20°C / 37°C, 16-20 h	X No or very little protein was expressed.
	Induction with IPTG	Cell growth: LB medium (+ Kan), 20°C / 27°C, 250 rpm, 80%, o. n. Induction: LB medium (+ Kan, δ-ALA, $FeCl_3$), 20°C, 250 rpm, 80%, ~24 h 0.05 to 0.5 mM IPTG	X No or very little protein was expressed.

5.1.3. Expression of membrane attached and truncated, soluble proteins

5.1.3.1. Expression in *P. pastoris*

The expression of various proteins, membrane-bound (HPO, CPR, hCPR, PM17, 2D6) and corresponding truncated versions (tHPO, tCPR, thCPR, tPM17, t2D6), was driven from P_{AOX1}. The membrane-bound proteins contain an N-terminal TMD and are attached to the ER membrane. The separation of growth phase and induction phase of *Pichia pastoris* cultivation, using P_{AOX1}, made cell growth and induction more reproducible and easier to control. Transformants were always selected for antibiotic resistance on adequate plates. Protein expression was verified with Western blot technique targeting the His-tags, using the respective antibodies and chemoluminescent detection in a bio-imager (G:Box). Comparison of the expression levels, as tested with Western blot analysis, of the different membrane-attached and respective truncated proteins showed that generally the truncated proteins showed higher expression levels. The reductase proteins (tCPR, thCPR, CPR and hCPR) showed highest expression levels in *P. pastoris* as compared to cytochrome P450 proteins.

5.1.3.2. Expression in *E. coli*

The limited membrane space, membrane integration or attachment of proteins and the complex interaction between the cytochrome P450 and CPR subunits for optimal functionality make them difficult to express in high abundance. Therefore expression experiments were also performed in *E. coli* to achieve higher expression levels. Cultivation conditions were improved for correct folding of the protein. Therefore, expression temperature was lowered to 20°C and the concentration of the inducing agent IPTG was reduced. These conditions influenced the fitness and growth characteristics of the cells. The expression of various truncated proteins (tHPO, tCPR, thCPR, tPM17, t2D6) was driven from the *T7* promoter in the *E. coli* BL21 star (DE3) strain. *E. coli* BL21 (DE3) had been shown to be the best strain for these applications in the previous work of A. Emmerstorfer. Addition of δ-aminolevulinic acid (δ-ALA) and $FeSO_4$ to the culture medium had increased the yields, as determined by A. Emmerstorfer [33]. Transformants were selected for antibiotic resistance on adequate plates. Protein expression was verified with SDS - PAGE. The proteins were expressed by autoinduction and induction with small amounts of IPTG. Unfortunately expression in *E. coli* did not work with this strategy and only very minor amounts of protein were expressed. SDS-PAGE of the wild type strains with or without empty vector, pET-26b (+) in BL21 star (DE3), showed the same band pattern as the expression strains. Furthermore, no inclusion body formation was observed in the cell debris fraction. The *T7* promoter obviously was not the proper promoter for the expression of these truncated proteins in *E. coli*. This part of the project was terminated and all proteins were expressed in *P. pastoris* for further experiments, because expression worked well with this strategy.

5.1.4. Protein purification

Detergent screenings, performed for the purification of the membrane proteins, showed high solubilization levels. For all five membrane proteins, different detergents were found to solubilize them (Table 49). The different solubilization levels were compared by Western blot analysis. After detergent screening, ER fractions of expression strains where solubilized with the best solubilizing detergents. Only nonionic and zwitterionic detergents were chosen for further experiments, because anionic detergents, like SDS, are very strong detergents and can also inactivate or destabilize the proteins. For all five membrane proteins, two detergents were chosen for further experiments (Table 52). PM17 was solubilized with Polyoxyethylene (10) tridecyl and detergent 3-16, CPR with Octyl-β-D-1-Glucopyranoside and Tween 20, hCPR with Octyl-β-D-1-thioglucopyranoside and Octyl-β-D-1-Glucopyranoside, 2D6 with n-Dodecyl-β-D-maltoside and detergent 3-16 and HPO was solubilized with Polyoxyethylene (10) tridecyl and CHAPS.

tCPR and thCPR were purified with affinity chromatography using Ni-sepharose beads. Purification of tPM17, tHPO and t2D6 was not possible with the tested conditions. For protein purification, 6xHis-tags had been added C-terminally to all membrane and truncated proteins. Purification of membrane proteins turned out to be difficult, because in several cases they did not bind to the affinity chromatography column, propably because of their folding. The purification tests of the different proteins suggested that the tag should have better contained 10 His residues, instead of 6 Histidins for more effective purification. Furthermore, the proteins could also be tagged on the C- and N-terminally of the protein to achieve a higher chance that the proteins bind to the column and, therefore can be purified. Furthermore, soft denaturation of the proteins, using SDS did not lead to better results. The purification of of tPM17, tHPO and t2D6 was terminated and only tCPR and thCPR were purified for the crystallization studies. tCPR and thCPR, as well as CPR and hCPR, were fragmented during the expression/purification of the protein and each fragment still carried the His-tag, which was confirmed by Western blot analysis and mass spectrometry. Mass spectrometry analysis of the purified tCPR fragments of variable size showed that every fragment obviously carried both N- and C-terminal peptides of tCPR. There is no valid explanation as to how apparently rather short tCPR fragments can contain very N- and C-terminal tryptic peptides at the same time. Native PAGE suggested that fragmentation was not necessarily triggered by the reducing agents used for SDS-PAGE, because also in the native PAGE more than one band occurred after purification of thCPR and tCPR (Figure 40).

5.1.5. *In vitro* activity assays

In vitro activity assays were performed in this project, because proteins were extracted and used for purifications and crystallization. For the crystallization studies the proteins ideally should be in their active form. The purified and solubilized proteins were tested for substrate conversion. Cell homogenates for conversion always had to be freshly prepared, because freezing and thawing destroyed the sensitive enzymes. For *in vitro* assays, a cofactor regeneration system was implemented, because good results were achieved using them in the past as A. Emmerstorfer shown in previous work [33]. Before starting the assay, Western blot analysis was performed and expression of the used proteins was controlled (Figure 48).

5.1.5.1. Limonene *in vitro* activity assay

The conversion of limonene to (-)-trans-isopiperitenol involves two proteins in the model reaction. The cytochrome P450 protein PM17 needs a reductase enzyme, like CPR, for regeneration and, thus, efficient conversion of limonene. However PM17 showed no activity towards limonene. This might be related to the PM17 protein, because all used reductase enzymes were active as the cytochrome c *in vitro* assay showed. As an alternative, interaction of membrane-bound PM17 with soluble CPR might be inadequate to facilitate efficient conversion of the rather hydrophobic substrate limonene. Furthermore, PM17 might have been inactivated during cell disruption or have been expressed already as inactive protein. Control measurements of the wild type strain (Wt CBS 7437-his) and background activity were performed to compare with the measurements of the PM17 samples. The compound identified in GC-FID measurements were subjected to a mass spectrometry analysis. With these measurements it was estimated whether the peaks did or did not relate to the product which should be formed. None of the peaks indicated product formation. The peaks were related to limonene, PMSF and solvent (Figure 57). PMSF had been used as protease inhibitor in the previous experimental steps.

5.1.5.2. Valencene *in vitro* activity assay

The *in vitro* conversion of valencene involves two proteins in the model reaction. The cytochrome P450 HPO is not able to oxidize valencene to the final product nootkatone, but rather forms the intermediate *trans*-nootkatol. All reductase enzymes, expressed and purified in this work, were used for HPO regeneration in this assay. HPO showed no activity for conversion of valencene to trans-nootkatol *in vitro*. This might be related to the HPO, because the reductase enzymes used *in vitro* were all active as shown by cytochrome c assay. The protein might have been inactivated during cell disruption or had already been expressed as inactive protein. Alternatively, reconstitution of membrane-associated HPO and reductase enzymes into functional complexes might not have worked out under assay conditions. Both enzymes had been expressed separately and only minor amounts of

detergent were used in the assay to supply the substrate valencene. It is possible that HPO and diverse CPR enzymes resided in separate vesicle populations, rendering them unable to interact with one another. Consequently, detergent addition should be considered for further activity assays involving the protein fractions described in this work. Such detergents should be wisely selected to avoid potential inactivation of membrane-attached HPO and CPR proteins. The tested HPO samples were compared to the wild type strain (Wt CBS 7437-his) and to a positive control provided by T. Wriessnegger. Apart from the positive control fractions, GC-FID analysis showed that all other HPO proteins were inactive and no trans-nootkatol was formed. The positive results for membranes, harboring the co-expressed HPO and CPR enzymes, indicates that reconstituting HPO/CPR interaction from separate membranes might require addition of detergent.

5.1.5.3. Cytochrome c *in vitro* activity assay

For the two assays above, the limonene and valencene *in vitro* activity assay, the tCPR, thCPR, CPR and hCPR proteins, expressed and purified in this work were used. To make sure that these proteins were active, cytochrome c reduction assay was performed. All tested reductase proteins turned out to be active. Highest activities were achieved with the membrane bound CPR and hCPR proteins of around 30 and 50 U/mg. Truncation leads to a loss of activity, tCPR and thCPR had activities of around 3.5 and 5 U/mg. Furthermore, purification leads again to a loss of activity. Purified tCPR had no activity and purified thCPR had an activity of 4 U/mg. Protein concentrations were determined for purified proteins, but also for crude homogenate or ER fractions of expression strains. In the latter case, abundance of recombinant proteins among total proteins was assassed by SDS-PAGE. It was estimated that only around 1% of the whole protein was related to the cytochrome P450 reductase. Thus, quantification of specific enzyme activities in crude fractions will not be precise. Purification was shown to negatively influence the activity of the CPR protein. All tested proteins, except the purified tCPR, were reducing cytochrome c. Membrane-attached proteins showed less activity than the truncated, soluble proteins.

5.1.6. Crystallization

The purified and active tCPR and thCPR proteins were used for crystallization analysis, which was performed by A. Lyskowski at the core facility of structural biology. A very thin crystal plate was produced of thCPR, but after testing the crystals no diffraction pattern could be obtained. After optimization, only very thin and small needles were produced. tCPR produced a thin, platelike crystalline (Figure 69). The crystallization of tCPR also has to be optimized.

5.1.7. Closing words / Prospect

After finishing the work on this thesis, several loose ends still remained. The purification of the truncated proteins tPM17, tHPO and t2D6 has to be improved and optimized. It is suggested to construct enzyme variants containing more than 6 His residues as tag and to perform the tagging both C- and N-terminally. The detergent screenings for the membrane bond proteins CPR, hCPR, PM17, 2D6 and HPO were completed during this work. As next step solubilized proteins still have to be purified using affinity chromatography. Another major issue to be clarified is whether cytochrome P450s and CPRs expressed separately can be reconstituted into a functional complex, thus characterising the separate entities as functional in crystallization trials. In future projects, also the other three truncated proteins and the membrane-bound proteins should be crystallized.

6. SUPPLEMENTARY MATERIAL

6.1. DNA AND PROTEIN LADDERS

(1) (2)

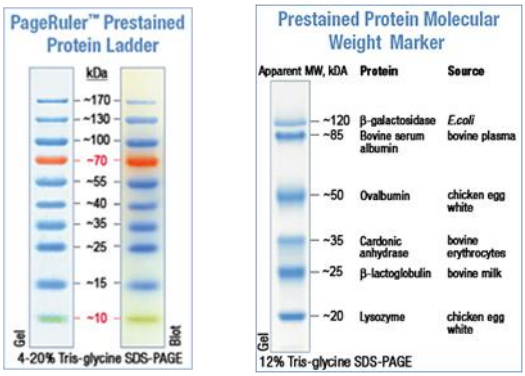


Figure 70. (1) PageRuler Prestained Protein Ladder and (2) Prestained Protein Molecular weight Marke for SDS-PAGE and Western blot

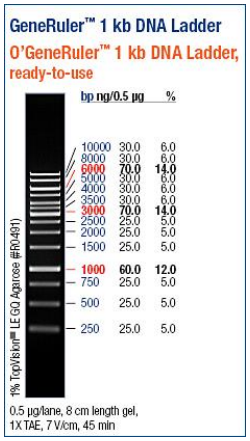


Figure 71. GeneRuler 1kb DNA Ladder for gel electrophoresis

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