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DIPLOMARBEIT

Purification of heterologous membrane proteins Cytochrome P450 CYP81A9 and Homogentisate Solanesyltransferase

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Danksagung

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Abstract

The enzyme homogentisate solanesyltransferase (HST) of *Arabidopsis thaliana* is a member of the UbiA superfamily of intramembrane prenyltransferases and is naturally found in the plant chloroplast envelope and in the plastid membrane. These enzymes play a key role in prenylation and thus in the biosynthetic pathway of chlorophylls, vitamin E, heme, and various quinone derivatives. HST itself catalyzes a reaction leading to the immediate precursor of plastoquinone. Since plastoquinones act as mobile electron carriers in the light-reaction of plants, inhibition of their biosynthesis results in seedling-lethal phenotypes.

The second protein investigated belongs to the family of Cytochromes P450, which is known for metabolizing herbicides into non-toxic compounds. In eukaryotic cells, these are mostly located in the endoplasmic reticulum or the inner mitochondrial membrane. One member of this family is the protein Cytochrome P450 CYP81A9 (referred to as CYP81A9) of *Zea mays.* As most Cytochrome P450s in plants, it has a substrate specificity for a small number of herbicides, such as classes of sulfonylurea and triketones.

In order to gain more detailed knowledge about HST and CYP81A9, and to possible new substrates for them, it is necessary to obtain them in purified form. To enable this follow-up research, a strategy for purifying the membrane proteins was developed.

Both, the typically low concentration of membrane proteins in their native environment and the slow growth of plants represent reasons for heterologous production of plant membrane proteins in yeasts.. *Pichia pastoris* is a methylotrophic yeast widely used in biotechnological applications in both industry and in science to produce recombinant proteins. The relatively simple applicability of genetic manipulation and the stable integration of heterologous genes into the genome makes *P. pastoris* an attractive producer. Furthermore, it is possible to achieve comparatively high biomass concentration and high abundance of proteins during fermentation of *P. pastoris*.

This thesis focuses on developing a strategy for purification and recovery of the recombinant membrane proteins HST and CYP81A9 from *P. pastoris*, by optimizing the downstream steps of protein solubilisation and purification, using a combination of detergent-based solubilisation and column chromatography.

Zusammenfassung

Das Enzym Homogentisat-Solanesyltransferase (HST) aus *Arabidopsis thaliana* gehört zur UbiA-Superfamilie der intramembranen Prenyltransferasen und ist in den Chloroplastenund in der Plastidenmembran lokalisiert. Diese Enzyme spielen eine Schlüsselrolle bei der Prenylierung und damit im Biosyntheseweg von Chlorophyllen, Vitamin E, Häm und verschiedenen Chinonderivaten. HST selbst katalysiert eine Reaktion, die zur unmittelbaren Vorstufe von Plastochinon führt. Da Plastochinone als mobile Elektronenüberträger bei der Lichtreaktion von Pflanzen fungieren, kann eine Hemmung ihrer Biosynthese lethale Stecklinges-Phänotypen zur Folge haben.

Im Gegensatz dazu ist die Familie der Cytochrome P450 dafür bekannt, Herbizide in ungiftige Verbindungen umzuwandeln. In eukaryotischen Zellen sind diese meist am endoplasmatischen Retikulum oder der inneren Mitochondrienmembran gebunden. Ein Mitglied dieser Familie ist das membrangebundene Protein Cytochrom P450 CYP81A9 (CYP81A9) von *Zea mays*. Wie die meisten pflanzlichen CytochromP450 hat es eine hohe Substratspezifität für nur wenige Klassen, wie zum Beispiel die der Sulfonylharnstoffe und Triketone.

Um detailliertere Kenntnisse über HST und CYP81A9 zu gewinnen und auch mögliche neue Substrate für sie zu finden, ist es notwendig, sie in gereinigter Form zu erhalten. Um diese weiterführende Forschung zu ermöglichen, wurde eine Strategie zur Aufreinigung der Membranproteine durch Optimierung des Downstream-Prozesses entwickelt.

Sowohl die typischerweise geringe native Konzentration von Membranproteinen, als auch das langsame Wachstum von Pflanzen stellen Gründe für die heterologe Produktion von Pflanzenmembranproteinen in Hefen da. *Pichia pastoris* ist eine methylotrophe Hefe, die in biotechnologischen Anwendungen sowohl in der Industrie, als auch in der Wissenschaft zur Herstellung rekombinanter Proteine weit verbreitet ist. Die relativ einfache Anwendbarkeit von genetischen Methoden und die stabile Integration heterologer Gene in das Genom machen *P. pastoris* zu einem attraktiven Produzenten. Darüber hinaus ist es möglich, bei der Fermentation von *P .pastoris* eine vergleichsweise hohe Konzentration an Biomasse und eine hohe Abundanz an Proteinen zu erreichen.

Diese Arbeit konzentriert sich auf die Entwicklung einer Strategie für die Aufbereitung und Reinigung der rekombinanten Membranproteine HST und CYP81A9 aus *P. pastoris, wobei*

eine Kombination aus Detergenzien-basierter Solubilisierung und Reinigung mithilfe von Säulenchromatographie durchgeführt wurde.

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1 Introduction

Membrane proteins play crucial roles in various cellular processes, including transport, signaling, energy conversion and detoxification. However, their purification and isolation can be challenging due to their hydrophobic nature, complex folding patterns, and low abundance in cells [1]. To overcome these difficulties, heterologous expression has become a widely used method in molecular biology and biotechnology. The goal of this strategy is to provide high yields of functional protein, that can be purified in further steps for a variety of applications such as the production of vaccines, biotherapeutics, and diagnostic tools.

The development of efficient and scalable methods for the downstream processing of membrane proteins is crucial for the advancement of their industrial use. These methods include various purification techniques, such as ultracentrifugation, detergent-based solubilization and column chromatography. To accelerate the development, challenges and limitations of the downstream processing of membrane proteins should be examined too.

1.1 Production of heterologous proteins in *Pichia pastoris*

Production of heterologous proteins is one of the most important branches in modern biotechnology. Over the years, different expression systems, like bacterial, mammalian, or insect systems were developed for this purpose. Another important system for heterologous protein expression are yeasts. Yeasts are not only fast growing, but can perform posttranslational modifications and secration as well. One such yeast, with wide popularity due to its easy handling and wide range of applicability, is the methylotroph yeast *Pichia pastoris* [2, 3]. *P. pastoris* was originally used to metabolize cheap methanol and the biomass obtained was then fed to animals as a source of single cell protein. In 1995, it first performed as a host for heterologous, integral membrane protein production [3]. The success of *P. pastoris* is mainly owed to the high cell density up to 100 g/L that can be achieved, the strong and tightly regulated alcohol oxidase I (AOXI) promoter and the high protein expression in the gram per litre range that can be achieved [4, 5]. Heterologous genes are integrated into the genome under the control of the AOXI promoter, whereby methanol can be used for induction [4]. Yeasts, as eukaryotic expression systems, have the advantage over, for example bacteria, that post-translational modifications (PTM), such as glycosylation, disulfide isomerization and C-terminal methylation can be carried out in the course of protein expression. These modifications of the heterologous proteins are often essential for their biological activity and

stability [3]. Also, the proper folding of proteins in the endoplasmic reticulum (ER) and the possibility of protein secretion makes *P. pastoris* one of the most successful expression systems for recombinant protein production [6]. However, overexpression of heterologous proteins can also lead to intracellular stress and an overload of the folding- and translocation machinery of the cells [3]. This leads to degradation of proteins, resulting in heterogeneity of the recombinant proteins and lowers protein yield in general. As a further obstacle in the recovery of membrane proteins, yeast cells possesses a cell wall. Due to the harsh methods required for disruption of these, damage of the protein-of-interest can occur [3].

1.2 Recombinant Membrane Proteins

Membrane proteins play crutial roles in different biological processes. Among other things, they act as a part of metabolism, molecule-transport and bioenergetics [1]. In order to understand these biological mechanisms and consiquently perform biochemical and biophysical studies, large quantities of purified proteins are necessary [3, 7]. As membrane proteins are often expressed at a low level in their native hosts, their recombinant production is necessary to overcome this obstacle [3].

At first, the production of recombinant membrane proteins can lead to difficulties, since the correct protein folding repeatedly cannot be achieved. Furthermore, purification steps like solubilisation and chromatography represent a challenge, if soluble, purified membrane proteins shall be obtained [3].

1.2.1 Homogentisate solanesyltransferase (HST)

The Homogentisate solanesyltransferase is a member of the UbiA superfamily of prenyltransferases [8]. In plants, prenyltransferases are reported to be membrane proteins with multiple transmembrane domains and are separated into two groups; the 4-hydroxybenzonate prenyltransferases, and the homogentisate prenyltransferases (HPT). HPTs are located in the chloroplast membranes and are involved in plastoquinone and vitamin E biosynthesis. By prenylation of homogentisate, derived from shikimate pathway, with different substrates, like geranylgeranyldiphosphate (GGPP), phytyldiphosphate (PPP) or solanesyldiphosphat (SPP), thus various precursors are synthesised [9, 10]. Examples for the biocatalytic reactions of HPTs are depicted in Figure 1.

The Homogentisate solanesyltransferase (HST) itself does not only use SPP as a substrate for prenylation and decarboxylation of homogentisate, but also a wide range of other prenyl

diphosphates, such as geranyldiphosphate (GPP) and farnesyldiphosphate (FPP). The natural donor of HST, SPP, leads to the transfer of a solanesyl group and the formation of 6-methyl-solanesyl-benzoquinol (MSBQ) which is a precursor of Plastoquinone-9 [11].

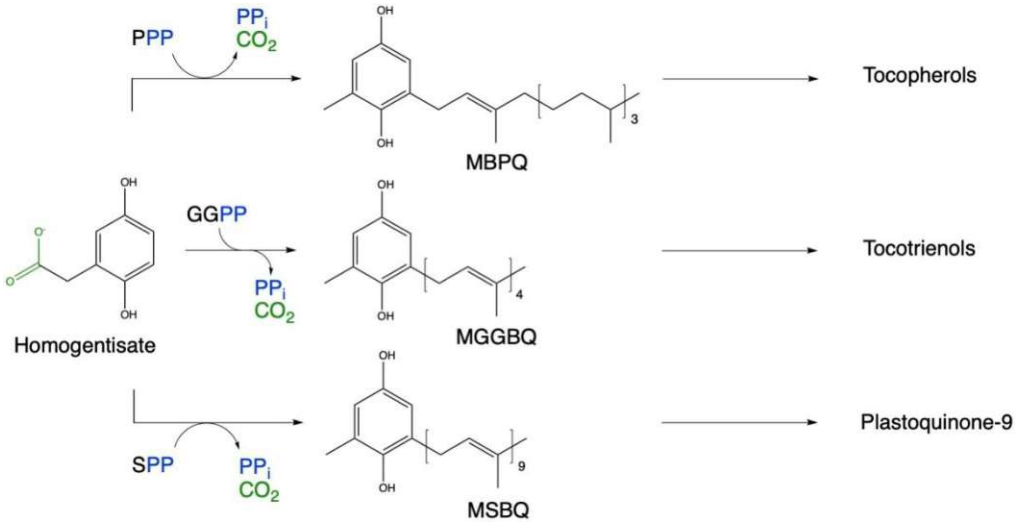

Figure 1 Prenylation and decarboxylation of homogentisate by homogentisate prenyltransferases during biosynthesis of tocopherol, tocotrienol and plastoquinone-9 (MBPQ: 2-methyl-6-phytyl-1,4-benzoquinol; MGGBQ: 2-methyl-6-geranylgeranyl-1,4-benzoquinol; MSBQ: 2-methyl-6-solasesyl-1,4-benzoquinol) [10, 12].

The native HST of *A. thaliana* belongs to the group of homogentisate prenyltransferases. It consists of 8 transmembrane domains and a signal peptide sequence for localisation in the chloroplast membrane. The whole protein has a molecular weight of 42.841 kDa [12]. In Figure 2, the predicted 3D structure of HST is depicted.

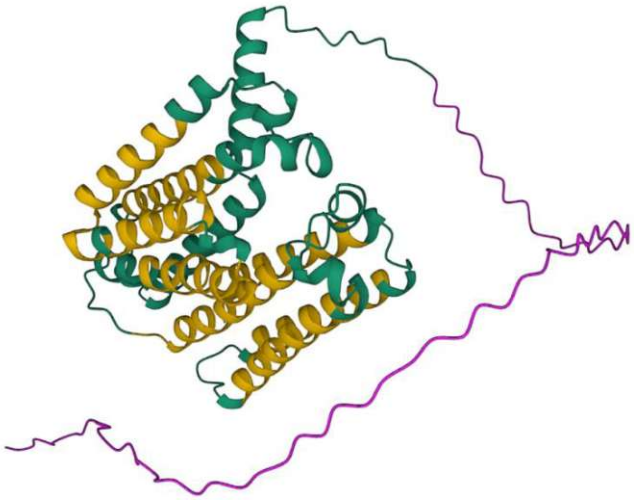

Figure 2 Predicted 3D structure of the native HST of *A. thaliana* (UniProt ID: Q1ACB3) [13, 14]. The transmembrane α-helix structures are depicted in yellow, the parts of the protein, which are not incorporated into the membrane in green and the signal sequence for the chloroplast membrane in purple. The figure was created with the RCSB Mol* 3D Viewer (https://www.rcsb.org/3d-view).

1.2.2 Cytochrome P450 CYP81A9

Cytochromes P450 are heme-dependent reductases, which transfer electrons from NADPH to its substrate. Thereby, Cytochrome P450 CYP81A9 (CYP81A9) plays a role in hydroxylation reactions and detoxification of herbicides in *Zea mays* [15, 16]. It was proven to be associated with sensitivity of *Z.mays* to herbicides like bentazone and nicosulfuron [16, 17].

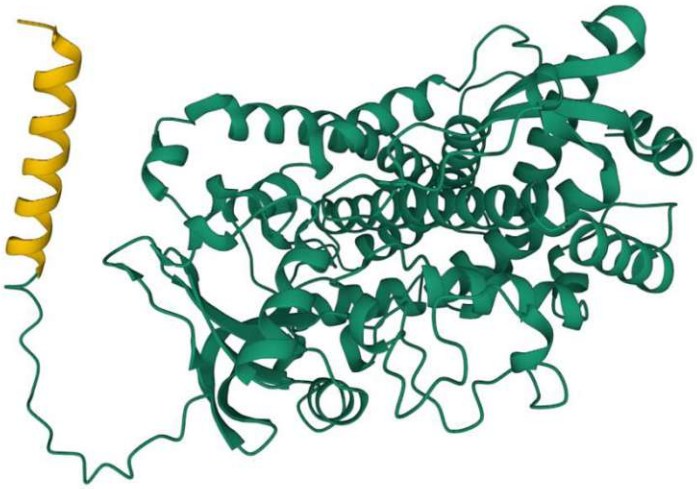

Figure 3 Predicted 3D structure of the native CYP81A9 from Z. mays (UniProt ID: B6ST45) [13, 14]. The transmembrane α-helix structure, which is predicted to be a signal sequence, is depicted in yellow. The rest of the protein sequence is coloured in green. The figure was created with the RCSB Mol* 3D Viewer (https://www.rcsb.org/3d-view).

The native CYP81A9 of *Z. mays* contains a membrane anchor and is therefore only membrane associated. The protein has a molecular weight of 56.991 kDa [18, 19].

1.2.3 Fusion tags

Fusion domains or "tags" are normally expressed together with the protein of interest and are most often used to facilitate protein purification [20]. Therefor either small peptide tags, like FLAG-, His-, Arg- or Strep-tag, or large protein tags, like the Glutathion-S-transferase-tag (GST-tag) can be located on the C-terminal or N-terminal end of the protein sequence [21, 22].

In a first step, the addition of tags to the protein of interest enables the purification of the recombinant protein. Furthermore, it also allows to monitor the success and efficiency of the individual purification steps. For the purification process, several different chromatography strategies, such as affinity chromatography for His-, FLAG- or GST-tag or cation exchange chromatography for Arg-tag, have been established [22, 23]. In order to detect the purified recombinant proteins, antibodies for the mentioned tags are available, which can be used in qualitative and quantitative analysis.

It is important to consider, that the type of fusion tag used may also influence the efficiency of the downstream process. This is especially relevant regarding membrane proteins. For the solubilisation of these, the use of detergents is a common strategy. For instance, one commonly used and well-working detergent is dodecyl-β-D-maltoside (DDM) [25]. Maltose-binding-protein (MBP)-tagged proteins, which are purified by affinity chromatography using a maltose resin, are not compatible with DDM. Since DDM is an analogue of maltose, it also binds to the MBP and therefor reduces the product yield [24, 25]. Coordination of the purification process and the fusion tags used therefore is essential.

1.3 Extraction and purification of membrane proteins

The challenge of purifying membrane proteins lies in the overall downstream process, which includes extraction, solubilization, and purification. Depending on the organism used for the heterologous production of the protein, the membrane in which the protein is located and the protein itself, different strategies can be applied to get a purified and active protein. The main obstacle in the process of membrane protein purification, however, is certainly the solubilisation of the protein, meaning the creation of a stable, artificial environment for the hydrophobic motifs and domains of the protein [25].

1.3.1 Membrane Protein Extraction

Most of the transmembrane domains extend the membrane bilayer as single α-helix, multiple, bundled ones or β-barrels. The protein can be integrated into the membrane, or just be anchored in it by a peptide sequence. All of these structures have in common, that the peptide membrane domains are amphipathic; hydrophobic on the outside, where it is in contact with the membrane and hydrophilic on its inside. Therefore, most of the amino acids of the intramembrane-sequence are carrying apolar side chains, such as alanine, methionine, isoleucine, leucine, phenylalanine, valine, tryptophan and proline [26].

To get these hydrophobic domains and motifs solubilised, the formation of an artificial hydrophobic environment is necessary. This formation of a membrane-like environment is the bottleneck for studies on membrane proteins, if the native structure and functionality should be preserved. The stability of a solubilised protein is a particular challenge, when it comes to integral membrane proteins. Since the function of these membrane proteins depends on the conformation of the transmembrane structures, it is essential to maintain it [27].

1.3.1.1 *Detergents*

The most common approach to extract membrane proteins and creating a stable, artificial environment is through the use of detergents. The challenge within this strategy is to find a detergent that is capable of disrupting the native membrane structure to efficiently extract membrane proteins, while simultaneously preserving the protein structure.

Detergents are amphiphilic molecules that bind to the hydrophobic domains and motifs of membrane proteins, making them soluble in aqueous solutions. They generally consist of two parts: a charged or polar head group and a hydrophobic part. Both domains are highly variable, which leads to the major problem of membrane protein solubilization using detergents: there is no routine protocol for membrane protein extraction. The extractability of different types of membrane proteins depends strongly on the type of detergent used. Furthermore, not every detergent can guarantee the stability of every protein. The best detergent for a specific protein can only be found through trial and error [28, 29].

In general, detergents can be classified in three groups: ionic- (anionic and cationic), non-ionic and zwitter-ionic detergents. Each group has some basic attributes [28].

Non-ionic The head group consists of hydrophilic, uncharged groups, such as glucosides, maltosides, or polyoxyethylene, and therefore, they have no electrophoretic mobility or conductivity. Non-ionic detergents are mild detergents that typically do not disrupt protein-protein interactions, which is why they are commonly used for protein extractions [28].

Ionic The head group contains anionic or cationic groups, as well as a straight hydrocarbon or stiff steroid group as a tail. These detergents are generally very effective, but they also have a tendency to denature proteins [28].

Zwitter-Ionic Zwitterionic detergents have both positively and negatively charged groups as part of their head group, resulting in them having no net charge. Similar to non-ionic detergents, they lack electrophoretic mobility and conductivity, and do not bind to ion-exchange resin. However, like ionic detergents, they tend to denature proteins. Therefore, zwitterionic detergents possess properties of both ionic and non-ionic detergents [28].

Another important parameter, in relation to protein solubilization using detergents, is the critical micelle concentration (CMC). This refers to the concentration, at which detergent monomers start to form stable aggregates, known as micelles [28]. However, it is more of an approximate range than an exact value of concentration [29]. The CMC is individual for each detergent and is also influenced by various factors. These factors include pH, temperature, and the ionic strength of a buffer system. For ionic detergents the CMC is reduced by an increased salt concentration, whereas it is hardly affected by temperature changes. Zwitter ionic detergents, on the other hand, are less sensitive to changes in ion concentration than ionic detergents. The opposite is the case for non-ionic detergents compared to ionic detergents. Their CMC is clearly influenced by temperature, but the ionic strength of a solution has hardly any effect on it [28, 31].

1.3.1.2 *Solubilisation*

During solubilisation, the native membrane, in which the membrane protein is located, will be replaced with detergent molecules. (Figure 4)

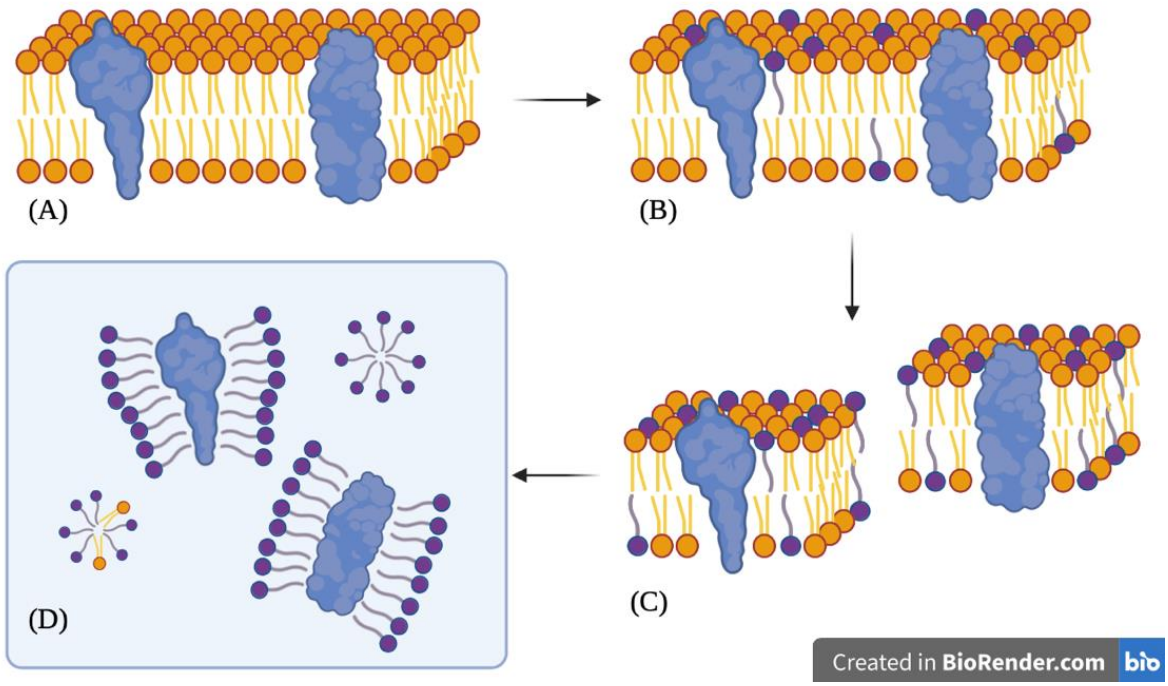

Figure 4 The schematic illustration of solubilization can be seen, depicting different stages or detergent concentrations. **(A)** The native membrane (shown in orange with double tails) in which the membrane proteins are embedded. At very low detergent concentrations, the detergent monomers (shown in purple with single tails) adhere to the membrane but are hardly incorporated into the membrane. **(B)** At higher detergent concentrations, the membrane is perturbed and the detergent monomers are integrated into the membrane structure. **(C)** If the detergent concentration is increased further, the membrane is destroyed by further incorporation of the detergent monomers. **(D)** At an even higher detergent concentration, complexes of detergent/protein and detergent/lipid begin to form. At this stage, the membrane proteins get solubilized and can be used for further downstream processes [29].

To find the best conditions for the solubilization of membrane proteins, additional factors besides the selection of the detergent must be considered. These primarily include temperature, time, concentration of detergent, and concentration of biomass used [31]. The overall goal is to find conditions under which membrane proteins are effectively solubilized without being denatured [32].

1.3.2 Membrane protein purification

For the purification of membrane proteins, conventional techniques can be applied. When the proteins are solubilised in detergent micelles, different chromatographic strategies such as ion exchange, affinity, or size exclusion can be used to isolate the protein of interest. However, interactions between detergents and chromatography resins may occur and need to be taken into account [33, 34]. Another point to be kept in mind, when using chromatography for membrane protein purification is, that membrane proteins tend to aggregate, particularly when the detergent concentration is too low during and after elution. As a result, it is necessary to add detergents in every buffer used for the chromatography procedure [32].

1.3.2.1 *Ion Exchange*

Since charged detergents (ionic detergents) interfere with the ion exchange resin by masking the charged groups of the resin, the protein can no longer bind to it. Therefore, only non-ionic or zwitterionic detergents without any net charge should be used for this type of chromatography. However, it is also possible that zwitterionic and non-ionic detergents cover the charge of the protein itself, resulting in weaker binding to the column. [33, 34].

1.3.2.2 *Size Exclusion Chromatography*

The use of detergents to create an artificial hydrophobic environment results in a detergent/protein complex, that can influence the migration behavior of membrane proteins in size exclusion chromatography (SEC). However, SEC is often used in connection with membrane proteins. For example, by creating elution profiles, the elution time of the detergent/protein complex can be determined, allowing for further analysis of sample quality, including homogeneity, purity, and stability of the product. With knowledge of the elution time, the desired protein can then be purified using SEC.[32, 35].

1.3.2.3 *Affinity Chromatography*

When it comes to membrane proteins, affinity chromatography is by far the most successful method for purification [33]. However, affinity chromatography can also present its own

challenges and limitations. As already mentioned in 1.2.2, detergents can interact with the affinity resin. Furthermore, similar to ion-exchange chromatography, detergents can partially or fully cover the affinity-binding sequence of the protein, resulting in weaker binding to the column [33, 34].

1.4 Motivation

Although membrane proteins play a crucial role in the metabolism of all organisms, their production and purification in significant quantities pose numerous challenges and obstacles. Membrane proteins with multiple transmembrane domains, in particular, can complicate the downstream process. However, to investigate the biotechnological relevance of the intermembrane protein HST and the membrane-associated protein CYP81A9, purified and soluble protein samples are necessary. Therefore, the objective of this master thesis is to develop a strategy for the purification of the aforementioned membrane proteins.

The main focus of this thesis is on the transmembrane protein HST, which is part of the plastoquinone pathway. Disruption or inhibition of HST results in degenerated roots and shoots, as well as bleached plants, making it a promising target for the development of new herbicides. With purified HST, research can be conducted to investigate the exact inhibition mechanism of HST with herbicides, such as cyclopyrimorate derivatives [36]. This knowledge will be valuable for the development of new HST inhibitors, through targeted rational design of herbicides.

The overall aim of this thesis is to successfully solubilize and purify the two heterologously produced membrane proteins, including HST, using detergent, affinity, and size exclusion chromatography techniques.

1.5 Scientific Questions

1. Is it possible to solubilise the membrane-bound protein Cytochrome P450 CYP81A9 and the integral membrane protein homogentisate Solanesyltransferase using detergents?

   Hypothesis

   With the help of the right detergents, it is possible to solubilise both proteins from the ER membrane of *P. pastoris*.

   Approach

   Finding the right detergent to solubilise a membrane protein is only possible by trying different detergents. Therefore, in the course of this work, different detergents are tested for both proteins.

2. Which factors, apart from the choice of detergent, influence solubilisation the most?

   Hypothesis

   In addition to choosing the right detergents, it is also important to determine the right conditions for solubilisation.

   Approach

   Different steps that are taken in the course of the solubilisation of the membrane proteins are varied and thus their influence on the membrane proteins is investigated.

3. Which strategies for protein purification can be usefully applied in this context?

   Hypothesis

   With the help of affinity and size exclusion chromatography, purification and concentration can be achieved.

   Approach

   Affinity chromatography is performed through the tags on the proteins of interest and the resulting samples are then further purified by size chromatography.

2 Material and Methods

2.1 Strains for recombinant protein production

All experiments performed for solubilisation and purification of membrane proteins were prepared with biomass from the following constructs for HST and CYP81A9. Heterologous expression was achieved by using the *P. pastoris* strain KM71H MutS. The corresponding constructs containing the target protein were integrated into the yeast genome using a pPICZ plasmid system. The biomass was stored in the freezer at -20°C. If necessary, an aliquote of biomass was thawed and used for further downstream process (DSP).

2.1.1 Homogentisate Solanesyltransferase

For the expression of HST, various *P. pastoris* strains were transformed with different gene constructs and used for small-scale expression. In all the constructs used, the signal sequence of the native protein was truncated. The first construct included a FLAG- and a GST-tag on the N-terminal end of the recombinant HST and a His-tag on the C-terminal end (FLAG-GST-HST-His). The second construct only included a FLAG- and a GST-tag on the N-terminal end of HST (FLAG-GST-HST). For both constructs, different colonies were chosen, and the resulting strains were analysed for the expression of HST using Western blot analysis. Based on this analysis, the strain resulting from colony FLAG-GST-HST-1 was used for target protein production and downstream analysis. The protein production was controlled by the AOXI promoter and induced by methanol feeding.

2.1.2 Cytochrome P450 CYP81A9

For the expression of CYP81A9, two different gene-constructs were used for transformation of *P. pastoris* and small scale expression screening. The first construct carries a His-tag on the C-terminal end (CYP81A9-His) and the second one has an additional Strep-tag on the N-terminal end (Strep-CYP81A9-His). Similar to HST, several colonies were picked for each construct, and the resulting strains were then analyzed for protein expression. Additionally, the activity of CYP81A9 was measured through LC-MS assays, where the conversion of bentazone to 6-hydroxybentazon by CYP81A9 was investigated. Based on the results of these assays, the strain resulting from the colony CYP81A9-His-4 was identified as the best candidate for protein production and downstream processing. The induction of protein production during fed-batch fermentation was carried out under the control of the AOXI promoter using methanol feed.

2.2 Solubilisation and Purification

2.2.1 Workflow

The DSP followed the workflow given in Figure 5.

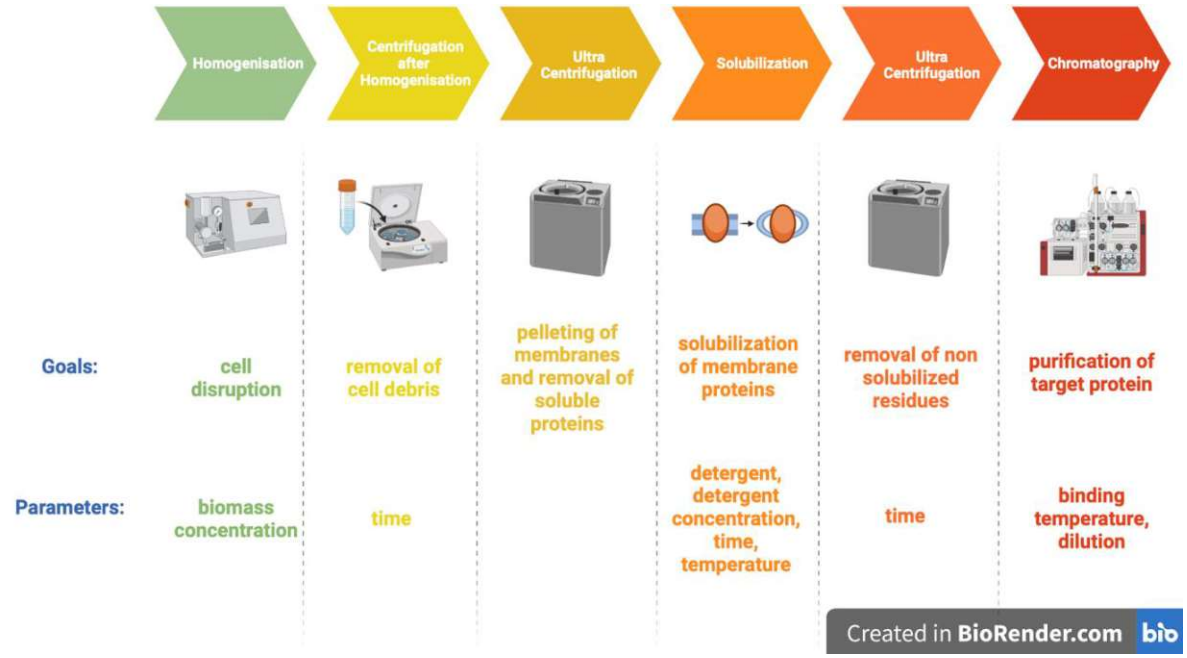

Figure 5 Unit operations for the DSP of HST and CYP81A9. The goal of each step and the investigated parameters are given.

Different protocols provided by the industrial partner were used and adapted for the solubilization of the target proteins CYP81A9 and HST. The biomass suspension used for the downstream process was kept on ice during the entire process.

2.2.2 Resuspension and Homogenisation

2.2.2.1 *Homogentisate Solanesyltransferase*

For resuspension, HST-biomass was stirred in at 4°C for 60 minutes using a magnetic stirrer. Either disruption buffer (20 mM Hepes, pH 7.5, 5 mM 1,4-Dithiothreitol (DTT), 2 mM Ethylenediaminetetraacetic acid (EDTA), 250 000 units benzonase per 100 mL buffer, Roche complete Mini EDTA-free Protease Inhibitor Cocktail 1/100 mL) or buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, 10% w/v glycerol, 0.5 mM Tris(2-chlorethyl) phosphate (TCEP)) was used for resuspension. A biomass concentration of 35 $g_{\text{wet cell weight (WCW)}}$/100 mL_{buffer} was used, as suggested by the industrial partner. Additionally, a concentration of 20 $g_{\text{wet cell weight (WCW)}}$/100 mL_{buffer} was tested, to check wether a better disruption of the yeast cells could be reached at lower concentrations. Homogenisation of the resuspended cells was performed at 1800 bar for 10 passages, using a PandaPLUS2000 homogeniser (GEA, Düsseldorf, Germany).

2.2.2.2 *Cytochrome P450 CYP81A9*

For CYP81A9, a pre-washing step was carried out by resuspending 20 g biomass (WCW) in 40 mL of either DTT/TES B buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 0.6M Sorbitol, 50 mM NaCl, 30 mM DTT) or buffer A, and stirring the suspension at room temperature (RT) for 10 minutes. Cells were centrifuged at 1000g for 10 min at 4°C in the refrigerated benchtop centrifuge Sigma 3-18K (Sigma Laborzentrifugen, Osterode am Harz, Germany). The supernatant was discarded, and the remaining cells were resuspended in TES B buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 0.6M Sorbitol, 50 mM NaCl) with a concentration of 20 g_{cells}/100 mL_{buffer}. The cell suspension was stirred at 4°C for 60 min. The homogenisation was performed analogously to the homogenisation of HST at 1800 bar for 10 passages using a PandaPLUS2000 homogeniser (GEA, Düsseldorf, Germany).

2.2.3 Centrifugation after Homogenisation

Immediately after homogenisation, the biomass was centrifuged, unless stated otherwise, at 1000 x g for 15 minutes at 4°C in a Sorvall LYNX 6000 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) to remove cell debris. To check the results of homogenisation and centrifugation, the samples (pellets and supernatants) were analysed by Western blot.

2.2.4 Ultracentrifugation 1

After separation of the cell debris, the supernatant was ultracentrifuged to remove soluble proteins. This was performed using a Sorvall WX Ultra Series WX 80 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). A fixed angle rotor type 50.2 Ti (Beckman Coulter, Brea, CA, USA) was used. The homogenised and centrifuged supernatants were filled into OptiSeal polyallomer tubes (Beckman Coulter, Brea, CA, USA) and centrifuged at 4°C for 60 minutes at 40 000 rpm (approximately 190 000 x g). The ultracentrifugation vials have a capacity of 32.4 mL. To check the efficiency of the ultracentrifugation, the membrane pellet obtained, and the supernatant were analysed by Western blot.

As there was no protein of interest visible in the supernatant after ultracentrifugation, the parameters were set as described and no further experiments were carried out concerning ultracentrifugation.

2.2.5 Solubilisation

For the solubilisation of the membrane proteins, the membrane pellets obtained were resuspended in 40 mL buffer containing the detergent. Since the ultracentrifugation tubes have

a capacity of 32.4 ml, a total volume of 40 ml of solubilisation buffer was used to obtain enough sample.

For the resuspension of the membrane pellet, an Ultra Turrax IKA T10 basic instrument (IKA, Staufen, Germany) was used. The resuspended membrane pellets were swayed by using a PMR-30 compact Fixed-Angle platform rocker (Grant Instruments, Royston, UK).

2.2.5.1 *Homogentisate Solanesyltransferase*

For HST, HST resuspension buffer (20 mM Hepes, pH 7.5, 5 mM DTT, 10 % glycerol, Roche complete Mini EDTA-free Protease Inhibitor Cocktail 2/100 mL) or buffer A was used. Solubilisation was carried out at 4°C overnight (o/n). Detergents and testing conditions are given in Table 1.

Table 1 Detergents used for solubilisation of membrane protein HST, class of detergent, CMC, used concentration of detergent and biomass and used buffers

Detergent	**Class of detergent**	**Critical Micellar Concentration (CMC) [mmol/L]**	**Concentration Detergent [x *CMC]**	**Concentration of Biomass [g/100mL buffer]**	**Buffer used**
3[(3-Cholamidopropyl) dimethylammonio] promanesulfic acid **(CHAPS)**	Zwitter-Ionic	8.00	30	20	Resuspension buffer
Lauryldimethylamine oxide **(LDAO)**	Zwitter-Ionic	1.00	30	20,35	Resuspension buffer
Octaethylenglykol-monododecylether **(C12E8)**	Non-Ionic	0.11	30	20,35	Resuspension buffer
n-decyl-β-D-maltoside **(DM)**	Non-Ionic	1.80	30	20	Resuspension buffer
Lauryl Maltose Neopentyl Glycol **(LMNG)**	Non-Ionic	0.01	10, 30, 50	35	Resuspension buffer
n-Dodecyl-β-D-maltoside **(DDM)**	Non-Ionic	0.16	5, 15, 10,30, 50	35	Resuspension buffer, Buffer A
Fos choline-12	Zwitter-Ionic	1.5	5, 15, 30	35	Resuspension buffer

2.2.5.2 *Cytochrome P450 CYP81A9*

For CYP81A9 buffer A or TEG buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 20% (v/v) glycerol) were used. Different combinations of time and temperature (6h/4°C or overnight/RT) were applied to find the best conditions for solubilisation. The time/temperature experiment was carried out with the detergent LDAO.

Table 2 Detergents used for solubilisation of membrane protein CYP81A9, class of detergent, CMC, used concentration of detergent and biomass and used buffers

Detergent	Class of detergent	Critical Micellar Concentration (CMC) [mmol/L]	Concentration Detergent [x *CMC]	Concentration of Biomass [g/100mL buffer]	Buffer used
3[(3-Cholamidopropyl) dimethylammonio] promanesulfic acid (**CHAPS**)	Zwitter-Ionic	8.00	10	20	DTT/TES B, TES B & TEG buffer
Lauryldimethylamine oxide (**LDAO**)	Zwitter-Ionic	1.00	10, 20, 30	20	DTT/TES B, TES B & TEG buffer or Buffer A
Octaethylenglykol-monododecylether (**C12E8**)	Non-Ionic	0.11	10	20	DTT/TES B, TES B & TEG buffer
Fos choline-10	Zwitter-Ionic	11.00	10	20	DTT/TES B, TES B & TEG buffer
n-decyl-β-D-maltoside (**DM**)	Non-Ionic	1.80	10	20	DTT/TES B, TES B & TEG buffer or Buffer A

2.2.6 Ultracentrifugation 2

To remove the non-solubilised residues, a second ultracentrifugation step was carried out after solubilisation. The same instrument, rotor and vials were used as for the first ultracentrifugation step. Ultracentrifugation was performed at 40 000 rpm for 30 and for 60 minutes.

2.2.7 Chromatography

Based on the tags of the fusion proteins, an affinity chromatography was applied by using the supernatant after the second ultracentrifugation.

In case of CYP81A9, no chromatography was performed. This will be explained in further detail in the following section 3 Results and Discussion.

For HST an affinity chromatography for both tags of the fusion protein, GST- and FLAG-tag, was performed. Concerning the GST-tag, spin trap columns (GSTrap FF (Cytiva, Marlborough, MA, USA)) were used for the small-scale chromatography experiments. Additionally, GST GraviTrap colomns (Cytiva, Marlborough, MA, USA) were used. In both cases, samples were diluted 1:10 or 1:2 in PBS buffer (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) to reduce the concentration of detergents and also to stay below the binding capacity of the glutathione Sepharose resin of 10 $mg_{protein}$/1 mL_{resin}. PBS

buffer was used as binding buffer. The recombinant HST was eluted with GST elution buffer (50 mM Tris, pH 8.0, 10 mM reduced glutathione, 5 x CMC of detergent used for solubilisation).

For preparative affinity chromatography based on the FLAG-tag, spin trap columns Pierce™ Anti-DYKDDDDK Affinity Resin (Thermo Scientific, Waltham, MA, USA) were used. Again, samples were diluted in binding buffer, which is the same as HST resuspension buffer, 1:2 or 1:10 to reduce concentration of detergents and to stay below the binding capacity of the resin (3 $mg_{protein}$/1 mL_{resin}). The recombinant protein was eluted directly in Lämmli buffer (see 2.2.8 Western blots) by adding the washed resin to the Lämmli buffer und heat it up to 95°C for 5 minutes.

The spin trap columns and the GraviTrap columns were applied accordingly to the manual.

2.2.8 Western blots

The samples used for Western blotting were diluted in 2 x concentrated Lämmli Buffer (1 M Tris, pH 6.8, 10 % (w/v) SDS, 20 % (v/v) glycerol, 0.5 M EDTA, 0.006 mM bromphenol blue, 1.43 M β-Mercaptoethanol) and heated up to 95°C for 5 minutes and loaded to a 4-15% Mini-PROTEAN TGX precast gel (Bio-Rad, Hercules, CA, USA). For running the gels, the Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, Hercules, CA, USA) was used as electrophoresis chamber which was filled with SDS buffer (25.01 mM Tris, pH 8.3, 192.4 mM glycine, 3.467 mM SDS). First the gels were run at 120 V for 10 minutes, and then at 180 V for 25 minutes. After electrophoresis, a stain-free image was recorded of the gel using the ChemiDoc XR system Imager (Bio-Rad, Hercules, CA, USA) and ImageLab software (Bio-Rad, Hercules, CA, USA) to make visible the total protein on the gels. Afterwards the gel was blotted onto a nitrocellulose membrane (Amersham Protran Western blotting membrane, nitrocellulose 0.2 µm (GE, Boston, MA, USA) using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). For blotting and antibody (AB) incubation different protocols were used for CYP81A9 and HST. The total protein was initially detected by using Ponceau S staining on the nitrocellulose membrane. However, due to the insufficient quality of the visualization, the detection of the total protein was instead performed using stain-free gel imaging.

In the case of HST, the nitrocellulose membrane was blocked for one hour at RT in 5% milk/PBS buffer. After washing the membrane, AB incubation was performed with a 5% (m/v)

skim milk powder/PBS buffer solution containing monoclonal Anti-FLAG-HRP (Invitrogen, Waltham, MA, USA) or monoclonal Anti-GST-HRP-AB (Invitrogen, Waltham, MA, USA) at a dilution of 1:1000 was used. For CYP81A9, the nitrocellulose membrane was incubated in 1 x Roti®Block (Carl Roth, Karlsruhe, Germany) overnight at RT. After washing the membrane, it was incubated in an Anti-His-HRP solution (Thermo Fisher Scientific, Waltham, MA, USA) for two hours. It consisted of TBST buffer (24.7 mM Tris, pH 7.4, 136.9 mM NaCl, 0.5 % (v/v) Tween 20), 1x Roti®Block and AB diluted 1:1000.

After AB incubation, membranes were washed again und then incubated in SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes. For detection ChemiDoc XR system Imager (Bio-Rad, Hercules, CA, USA) and ImageLab software (Bio-Rad, Hercules, CA, USA) were used.

2.2.9 Protein Concentration

For total protein concentration measurement, Bradford assay was applied. As BCA assay in more stable in presence of detergents [28], Bradford and BCA Assay were compared. As the results were similar, Bradford assay was used due to faster implementation. Samples were diluted in PBS buffer and mixed with Bradford reagent (Pierce Coomassie Plus (Bradford) Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA)) in 96 well plates. Dilution was performed to stay in the working range of the assay. Measurements were conducted with the Tecan Spark (Tecan, Männedorf, Switzerland).

2.2.10 Mass Spectrometry Fingerprinting

To obtain samples for the mass spectrometrtry (MS) fingerprinting, the biomass was prepared as previously described. 3 x 25 µL of the supernatant after the 2nd ultracentrifugation step was loaded on SDS PAGE gel as described. Coomassie Brilliant Blue was used to visualize the protein bands, by shaking the gel for an hour at room temperature with an IKA Vibrax VXR orbital shaker (IKA, Staufen, Germany). Protein bands to be analyzed were excised from the gel, using a sterile scalpel and transferred into an Eppendorf vial.

3 Results and Discussion

The quantification of the target protein in the different process steps was determined systematically, by evaluation of Western blots in combination with the total protein amount, measured by Bradford Assay. Accordingly, the results for the determination of the downstream processing protocol were obtained by the relative comparison of different methods, reagents and concentrations for the same processing step. An absolute quantification of the target protein amount was not possible. For more details on this, see chapter 3.2.4.

The activity of both proteins was confirmed either by measurements at the TU Vienna (CYP81A9) or by the industrial partner (HST).

3.1 Cytochrome P450 CYP81A9

3.1.1 Resuspension, Homogenisation and Centrifugation

The biomass was homogenised using high pressure homogenisation (HPH). Mechanical methods, like HPH, lead to remarkable cell disruption and a high recovery of bioactive components in fungi [37]. A pressure of 1800 bar and a number of 10 passages are reasonable for homogenisation of yeasts [38].

For CYP81A9, a biomass concentration of 20 g biomass/100 mL buffer was used for homogenisation. Since the protein of interest was detectable on the Western blot at this concentration, no variation in biomass concentrations were used for the following experiments.

After centrifugation of the homogenised cells, there was still a considerable amount of His-tagged protein in the pellet. For further downstream processing, only the supernatant was used. As the conditions for centrifugation at 1000 x g were already very mild, an attempt was made to select a shorter centrifugation time in order to achieve a higher protein concentration in the supernatant. To investigate the influence of the centrifugation time, 15 minutes, instead of the initial 25 minutes, were chosen.

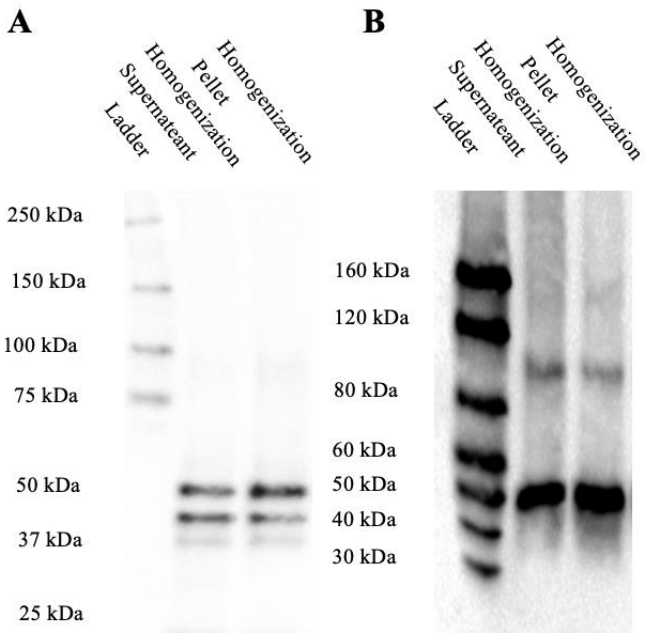

Figure 6 The Western blots show the results for different times of centrifugation. For both gels, the samples were diluted 1:5 in PBS buffer. In **A**, centrifugation time was at 25 minutes. Two intense protein bands are visible at 50 kDa and 42 kDa. The Precision Plus Protein Unstained Protein Standards (Bio-Rad, Hercules, CA, USA) was used. Imaging was done after 30 seconds. In **B**, centrifugation time was at 15 minutes. One intense band is visible at 50 kDa and a faint, second one at about 100 kDa. The BenchMark His-tagged Protein Standard (Invitrogen, Waltham, MA, USA) was used as a ladder. Imaging was done after 60 seconds.

As the recombinant protein carries a His-tag, which is about 2.5 kDa in size, the full-size fusion protein should have a size of 59.5 kDa. However, there are no protein bands visible at this respective size on the Western blots in Figure 6. Since the proteins are located in the *P. pastoris* membrane at this timepoint of the analysis, their mobility during the SDS-PAGE could be influenced by different factors. Among those are the effectiveness of solubilization and denaturation of SDS during sample preparation of SDS-PAGE samples. Additionally, the migration behavior of membrane proteins differs, compared to soluble proteins because of the highly hydrophobic areas [39]. Therefore, the protein band at a size of 50 kDa was assumed to be the protein of interest.

As, for both centrifugation times, the bands for pellet and supernatant are similar in intensity, it can be assumed, that it does not influence the amount of protein in the pellet significantly. This loss of protein in the pellet can hypothetically be caused by poorly disrupted cells [38]. However, using longer centrifugation time, a second band at a size of 42 kDa and a third, faint band at a size of 37 kDa can be observed. The band at 100 kDa, which is visible at a run time of 15 minutes, might show a dimer of the protein of interest. However, the occurrence of double bands should be prevented by adding Lämmli buffer and boiling the sample.

3.1.2 Ultracentrifugation 1

For removal of soluble proteins, it is recommended to ultracentrifuge the sample at 100 000 – 200 000 x g. [39, 40] For pelleting the membranes, a speed of 190 000 x g and a time of 60 minutes were used as initial conditions. Since no target protein was found in the supernatant, when using these parameters (Figure 9), conditions were set to 190 000 x g and 60 minutes for all upcoming experiments. Additionally, the resulting membrane pellet could be resuspended completely at these parameters. Longer centrifugation times result in more compact pellets, which negatively effects the resuspension process and subsequently the solubilisation.

3.1.3 Ultracentrifugation 2

To separate the insoluble components after solubilisation, ultracentrifugation was performed a second time. The speed was left at 40 000 rpm (190 000 x g) due to the good previous experience with ultracentrifugation 1. To investigate the time required for separation, centrifugation was carried out for 30 and 60 minutes. After 30 minutes, weak protein bands were detected in the supernatant, whereas no band was detected at 60 minutes. Consequently, 30 minutes, 190 000 x g and 4°C were chosen as parameters.

3.1.4 Solubilisation

The pellet, obtained from ultracentrifugation 1, was resuspended in a buffer/detergent mixture to solubilise the membrane proteins out of the membrane. Since the variety of detergents available for membrane protein solubilisation, the used ones were chosen by already successfully application related to *P. pastoris* or similar membrane proteins [15-19]. Additionally, the combability of the detergent with the chromatography strategy chosen for purification and protein crystallisation was considered [42, 43]. Furthermore, no heavily denaturing detergents, like SDS, were used to preserve the activity of the proteins [28].

During the investigation for the most fitting condition for protein solubilisation, multiple parameters have been tested. Besides detergents and their concentration, temperature and time of solubilisation and buffer composition have been tested as well. Initially, solubilisation was carried out with the detergents Chaps, LDAO, C12E8 and Fos-Choline 10 and DM. All were used at a concentration of 10 times their CMC (10 x CMC). For these first trials, TEG membrane prep buffer was used as a buffer system and solubilisation was performed at a time of 6 h and a temperature of 4°C. For none of the detergents used, a detectable amount of protein could be seen on the Western blots, after removal of the insoluble compounds. However, the

Bradford test showed that the protein concentration in these samples was highest when using LDAO, followed by DM. (Table 3)

Table 3 Total protein concentration of first solubilisation run measured by Bradford test

	Chaps	**LDAO**	**C12E8**	**Fos-Choline 10**	**DM**
Total protein amount	0.252 mg/mL	1.896 mg/mL	0.138 mg/mL	0.571 mg/mL	0.954 mg/mL

The influence of the buffer system used for solubilisation was therefore tested in combination with the two most successful detergents LDAO and DM. As an alternative, the buffer system buffer A was used, since its functionality in *P. pastoris* has already been proven, along with another membrane-associated protein of the Cytochrome P450 family of enzymes [44]. The detergent concentration, as well as the solubilisation parameters for time and temperature, were kept constant at 10 x CMC, 4°C and 6h.

The Western blot in Figure 7 shows a faint, but visible band for the supernatant after solubilisation at a size of 50 kDa, when LDAO was used as a detergent and buffer A as a buffer system. The Bradford measurement confirmed the resulting assumption for the best working combination of detergent and buffer system, since the total protein concentration was highest as well. Therefore, a combination of LDAO and buffer A was identified as most successful.

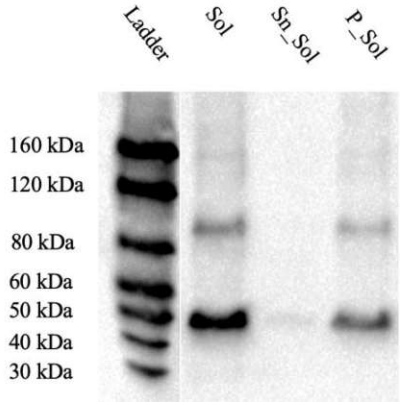

Figure 7 Western blot of Cyp81A9 (Sol: Solubilised sample, Sn_Sol: Supernatant of solubilised and centrifuged sample, P_Sol: Pellet of solubilised and centrifuged sample). BenchMark His-tagged Protein Standard (Invitrogen, Waltham, MA, USA) was used as a ladder. All samples are prepared with buffer A and solubilised with 10 x CMC of LDAO and were diluted 1 to 5 in PBS buffer for SDS-PAGE. The most intense band for each sample is visible at 50 kDa for the samples Sol and P_Sol. A faint band is visible for Sn_Sol.

Further adjustments of detergent concentration and solubilisation parameters led to higher solubilisation of CYP81A9.

The exact combination of parameters are listed in Table 4.

Table 4 Parameters which were used as combinations for the adjustment of solubilisation conditions (Sol_3)

Number of Run	Parameters (LDAO as detergent for all runs)		
Sol_3A	10 x CMC	o/n	RT
Sol_3B	20 x CMC	o/n	RT
Sol_3C	20 x CMC	6h	4°C
Sol_3D	30 x CMC	6h	4°C

Samples, which were ready earlier (6 hours solubilisation time), were snap frozen in liquid nitrogen and then stored at -80°C, until used for Western blot and Bradford analysis to ensure protein stability. As the protein band was visible only faintly in the supernatant after solubilisation and centrifugation, the sample preparation for Western blot analysis and Bradford test was adjusted as well. Samples after homogenisation, uncentrifuged solubilised samples and insoluble parts (pellet after solubilisation) were diluted 1 to 5 with PBS buffer. The supernatants of the solubilised samples were used undiluted. In Figure 8 the results of Sol_3 are depicted.

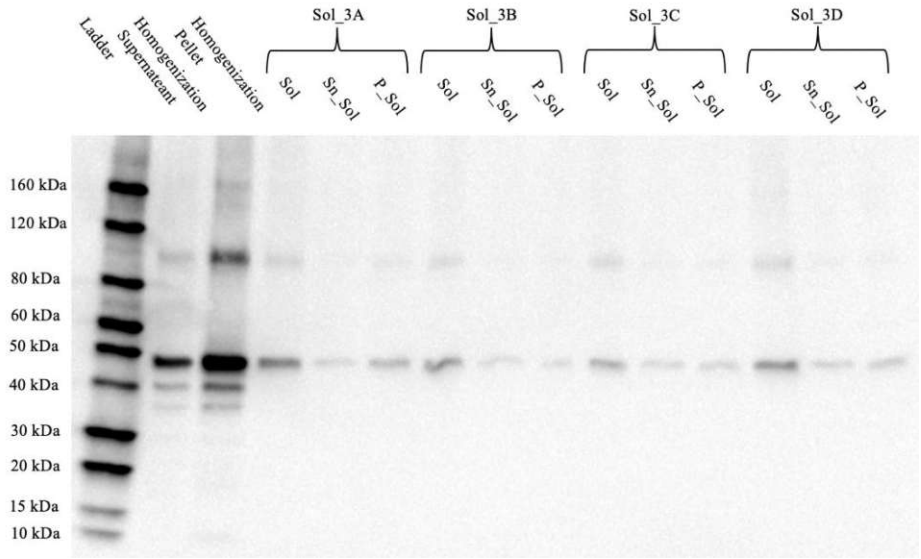

Figure 8 Western blot of Cyp81A9 (Sol: Solubilised sample, Sn_Sol: Supernatant of solubilised and centrifuged sample, P_Sol: Pellet of solubilised and centrifuged sample). BenchMark His-tagged Protein Standard (Invitrogen, Waltham, MA, USA) was used as a ladder. All sample preparation was done with buffer A and the samples were solubilised with conditions, given in Table 4. Samples after homogenisation, uncentrifuged solubilised samples and insoluble parts (pellet after solubilisation) were diluted 1 to 5 with PBS buffer. The supernatants of the solubilised samples were used undiluted The most intense band for each sample is visible at 50 kDa. For the intensity of the bands of samples, there is no clear difference between the different conditions.

Again, the Western blot analysis is not applicable for a quantitative statement about the amount of solubilised protein, due to the lack of a clear difference in band intensities between the different conditions.

However, the Bradford test resulted in highest concentration of total protein in the supernatant after solubilisation at 30 x CMC, 6h and 4°C. (Table 5)

Table 5 Results of Bradford total-protein measurement

	Conditions	Supernatant after Solubilisation	Pellet after Solubilisation
Sol_3A	10 x CMC, o/n, RT	1.295 mg/mL	0.249 mg/mL
Sol_3B	20 x CMC, o/n, RT	2.473 mg/mL	0.088 mg/mL
Sol_3C	20 x CMC, 6h, 4°C	2.467 mg/mL	0.066 mg/mL
Sol_3D	30 x CMC, 6h, 4°C	3.158 mg/mL	0.074 mg/mL

The amount of protein, at 20 x CMC LDAO, is approximately the same for both conditions (o/n, RT and 6h, 4°C). The lowest concentration is contained in the samples at 10 x CMC. This result was expected, since a higher concentration of detergent leads to a higher rate of solubilised protein automatically. However, since the Bradford assay determines the total amount of protein, it is only possible to draw a conclusion about the general solubilisation ability for the total protein concentration.

Based on the Bradford test results, it can therefore be assumed that neither the temperature nor the solubilisation time had any significant influence on the amount of solubilised protein. When looking at samples Sol_3B and Sol_3C, total protein concentrations are in the same range, although temperature and time used for solubilisation are different. However, samples with increasing detergent concentration show an increasing amount of total protein in the supernatant after solubilisation. Accordingly, the detergent concentration must have the greatest influence on the total protein amount.

Considering the results of the Bradford assay, an increase in band intensities at a higher detergent concentration would be expected for the samples on the Western blot. Since this is not the case, it can be assumed that although the total protein amount increases with rising detergent concentration, the concentration of protein of interest does not increase noticeably. More precise statements regarding the protein quantity of CYP81A9, by using the Bradford test, can only be made after purification of and separation of the not-targeted dissolved proteins.

As depicted in the Western blots given in Figure 8, even after the solubilisation, the position of the strong protein bands do not differ from those of the membrane preparations. Therefore, it can be said, that the *P. pastoris* membrane environment is not the reason for the size shift of the protein band.

3.1.4.1 *Truncated Cytochrome P450 CYP81A9*

However, the use of detergents can also lead to a change in the migration behaviour of proteins of up to 20% [45]. Nevertheless, the construct for CYP81A9 was re-examined, which showed that the amino acid sequence of the protein of interest was truncated. In the gene sequence of CYP81A9 in *P. pastoris*, a part was missing, which led to a loss of amino acids of approximately 10 kDa. This explains the shifted protein band on the Western blots. Due to this truncated version of CYP81A9, further experiments were discontinued.

3.2 Homogentisate Solanesyltransferase

3.2.1 Resuspension, Homogenisation and Centrifugation

At first, a biomass concentration of 20g biomass/100mL buffer was used for homogenisation. Since hardly any bands were visible on the Western blots, the biomass concentration was increased to 35 g biomass/100 mL buffer. In comparison to the strain, which expresses CYP81A9, the expression of the HST producing strain seems to be lower. This might be caused by a lower number of HST genes having been inserted into the *P. pastoris* genome.

The parameters for homogenisation (1800 bar, 10 passages) and for centrifugation after homogenisation (15 minutes, 1000 x g, 4°C) were kept the same as for CYP81A9. The results are depicted in Figure 9.

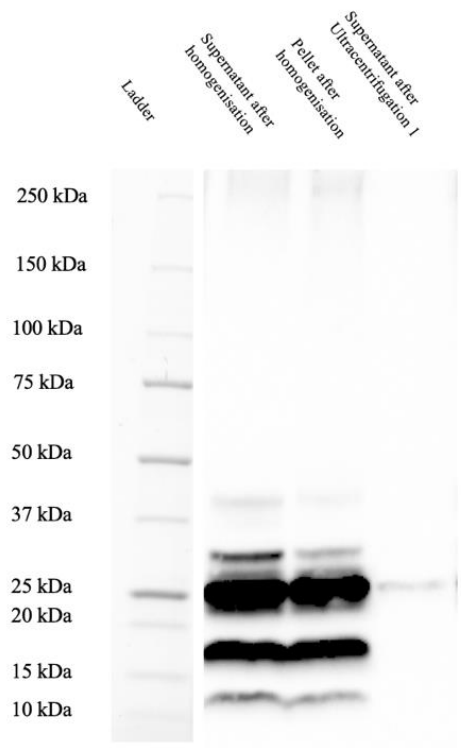

Figure 9 Western blot of the supernatant after homogenisation, the pellet after homogenisation and the supernatant after ultracentrifugation1 at a biomass concentration of 35 g biomass/ 100 mL buffer. The biomass used contained HST as protein of interest. Therefor the Western blot was incubated with a GST-HRP AB. As a ladder standard Precision Plus Protein Unstained Protein Standards (Bio-Rad, Hercules, CA, USA) was used.

As the truncated HST has a size of 35.81 kDa, the FLAG tag a size of 1.01 kDa and the GST tag a size of 26.41 kDa, the recombinant fusion protein FLAG-GST-HST should have a size of 63.23 kDa. As can be seen in Figure 9, there is no band visible at this size. Still, at a size of 40 kDa and lower, multiple bands in different intensities are visible on the blot. Due to the tendency of low expression of membrane proteins [23], the protein of interest may be expressed

at too low concentrations to be visible on the Western blot, at this point of the downstream process. On the other hand and as stated before, the intramembrane domains of HST can influence the migration during SDS-PAGE, and therefore lead to altered migration patterns on the Western blot [39]. This would also be an indicator of the origin of the small bands at smaller masses. This problem is addressed in more detail in chapter 3.2.6.

3.2.2 Ultracentrifugation 1 and 2

The ultracentrifugation was performed equally to the ultracentrifugation of CYP81A9.

3.2.3 Solubilisation

For HST five different detergents, DDM, LMNG, LDAO, C12E8 and Fos-Choline 12, were tested. Initially the detergents DDM, LMNG, LDAO and C12E8 were used with 30 times their CMC (30 x CMC). Fos-Choline 12 was added later as those first four detergents, which are depicted in Figure 10 and Figure 11, and did not result in clearly visible bands at the expected size of HST. The antibody-binding of the Western blots was performed with both, Anti-GST and Anti-FLAG, monoclonal antibodies, to investigate if one antibody leads to better results on the blots than the other. The antibody binding was performed o/n at 4°C for both Western blots. These conditions were considered reasonable and therefore adopted from the former experiments with CYP81A9, since lower temperatures are beneficial for protein stability and o/n solubilisation can be well implemented. For the sample preparation, HST resuspension buffer and HST disruption buffer were used. Additionally, a Bradford test was performed, to check the amount of total protein concentration in all the samples.

After the initial solubilisation of HST biomass, faint bands at the target protein size of 63 kDa are visible on the Western blots. Nevertheless, the small bands at a size lower than 40 kDa are significantly stronger. Since these small bands occur especially strong in the samples ‘supernatant after solubilisation’, an enrichment of those seems to have taken place during centrifugation after solubilisation.

As the lane for LDAO_Sn_Sol was blurred, this sample was re-done using the same conditions. As a result of this second attempt, the line-pattern was similar to the other samples. Nevertheless, the sample lane was very faint, and no band could be observed at 63 kDa. With this information, the assumption was made, that LDAO is not capable of solubilizing HST produced in *P. pastoris*. This shows the importance of trial and error of diverse detergents for different proteins even if the same host organism is used, as LDAO worked best for CYP81A9.

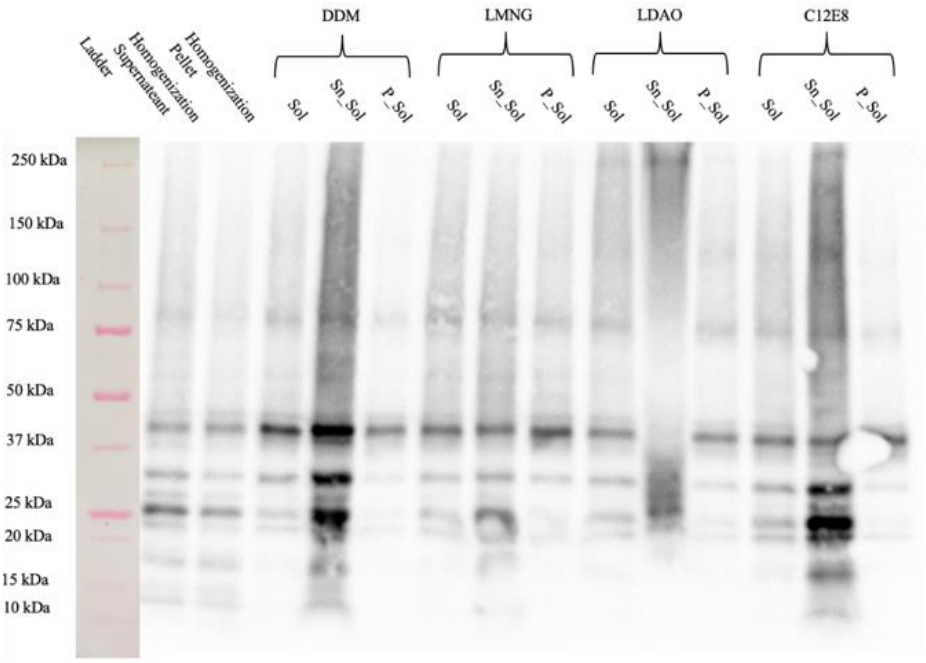

Figure 10 Western blot of HST (Sol: Solubilised sample, Sn_Sol: Supernatant of solubilised and centrifuged sample, P_Sol: Pellet of solubilised and centrifuged sample). Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-GST antibody for incubation. All samples were prepared with 30 x CMC of the according detergent in HST resuspension buffer and HST disruption buffer. For SDS-PAGE, the homogenisation samples, the samples Sol and Sn_P were diluted 1:10 in PBS-buffer. The samples Sn_Sol were used concentrated.

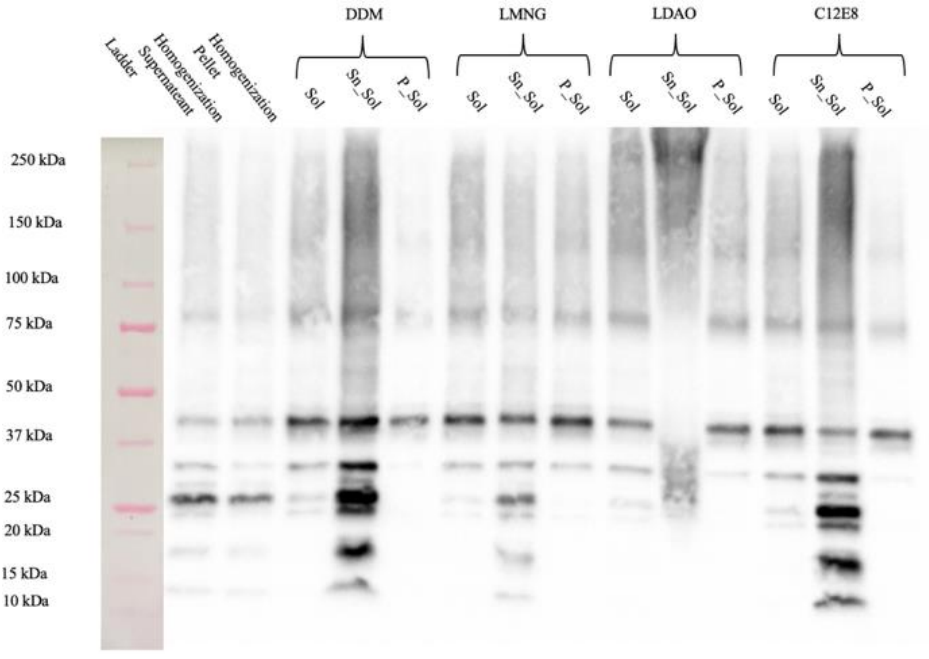

Figure 11 Western blot of HST (Sol: Solubilised sample, Sn_Sol: Supernatant of solubilised and centrifuged sample, P_Sol: Pellet of solubilised and centrifuged sample). Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation. All samples were prepared with 30 x CMC of the according detergent in HST resuspension buffer and HST disruption buffer. For SDS-PAGE, the homogenisation samples, the samples Sol and Sn_P were diluted 1:10 in PBS-buffer. The samples Sn_Sol were used concentrated.

The lane patterns were similar when using Anti-FLAG or Anti-GST-antibodies. For both, the bands at 63 kDa are visible and so are the bands at molecular weight smaller than 40 kDa.

In general, the lanes in which DDM or C12E8 were used as detergents, are the most intense, and solubilised most of the protein.

On the other hand, the total protein concentration was highest for the sample, in which LDAO was used as a detergent. (Table 6) When combining this information with the faint bands on the Western blot, it can be said, that LDAO is a good detergent in general, but in the particular case of HST, it is not capable of replace the native membrane environment of the protein.

Table 6 Total protein concentration of solubilisation run measured by Bradford test

	DDM	**LMNG**	**LDAO**	**C12E8**
Total protein amount	5.18 mg/mL	2.69 mg/mL	5.71 mg/mL	5.35 mg/mL

Samples, in which C12E8 and DDM were used as detergents, provided similar results in the Bradford test to the ones using LDAO. The protein concentration of the sample LMNG_Sn_Sol was lowest.

So far, non-ionic detergents (DDM, LMNG, C12E8) worked best for solubilization of the target protein HST. For further investigations, DDM and LMNG were used as detergents. Both worked out well for solubilization. Even if the total protein concentration was lower when using LMNG, the intensity of the 63 kDa bands on the Western blot was similar to those with DDM or C12E8. It has been suggested by the industrial partner that LMNG solubilised HST effectively. Since the bands on the Western blot and the total protein concentration of DDM and C12E8 were similar, DDM was chosen since C12E8 was significantly more expensive.

Since two suitable detergents for further solubilisation runs were identified, the next parameter to be investigated was the buffer system. Since buffer A (BA) achieved best results in the previous experiments with the target protein CYP81A9, this buffer was also tested for HST. Both selected detergents were tested with both buffer systems. In addition, the concentrations of detergents were increased to 50 x CMC in this run. As neither the increased CMC nor the alternative buffer system BA led to more intense bands at 63 kDa, the initially used buffer system was used for further investigations. Additionally, the containing protease inhibitor tablets in the Syn-buffer system are beneficial for target protein protection. The Western blot results for this are given in the appendix.

Since the 63 kDa was not clearly visible with any of the detergents used so far, another detergent, Fos-Choline-12, was tested to improve the solubilisation conditions for the target protein. For reference, a simultaneously solubilisation with DDM was performed. Since the use of the high CMC (50 x CMC) in the previous run had not led to stronger 63 kDa bands, different detergent concentrations between 5 and 30 x CMC were tested. In regard to purification by chromatography, lower detergent concentrations will be beneficial. In this third run, a protein standard prepared by the industrial partner was also applied to the SDS-PAGE to determine whether migration of the band on the gel is influenced by the detergents. The Western blots of the third run are given in Figure 12 (DDM) and Figure 12 (Fos-Choline-12).

The comparison of the HST standard and the samples showed, that also in the standard, three additional protein bands at 75 kDa, 40 kDa and 25 kDa are visible. Even though they also occur in the samples prepared, there are more bands smaller than 40 kDa in the solubilisation samples. Thus, it was assumed that the band visibly at approximately 60 kDa was the target protein.

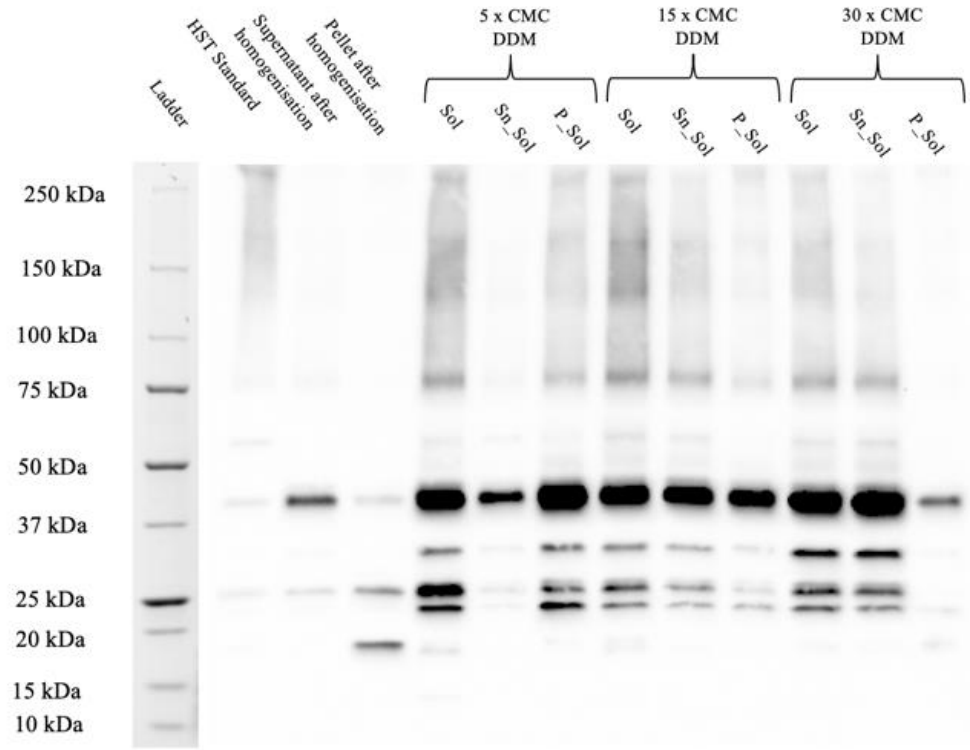

Figure 12 Western blot of HST (Sol: Solubilised sample, Sn_Sol: Supernatant of solubilised and centrifuged sample, P_Sol: Pellet of solubilised and centrifuged sample). Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG Antibody for incubation. For SDS-PAGE, HST standard and the homogenisation samples were diluted 1:10 in PBS-buffer. The samples Sol, Sn_Sol and P_Sol were used concentrated.

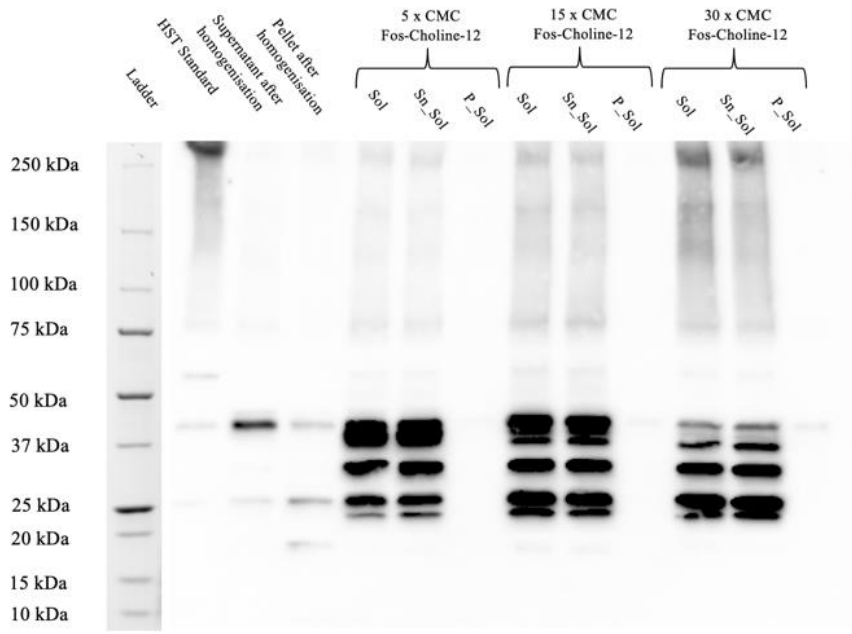

Figure 13 Western blot of HST (Sol: Solubilised sample, Sn_Sol: Supernatant of solubilised and centrifuged sample, P_Sol: Pellet of solubilised and centrifuged sample). Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation. For SDS-PAGE, HST standard and the homogenisation samples were diluted 1:10 in PBS-buffer. The samples Sol, Sn_Sol and P_Sol were used concentrated.

The increasing concentrations of DDM lead to more intense bands in the supernatants after solubilisation and a decrease in the intensity of the bands in the pellets after solubilisation. Thus, a higher concentration of DDM leads to a better solubilisation of the membrane-bound target protein from their native membrane environment. In the case of Fos-Choline-12, no significant difference between the different concentrations was found. Even at the lowest concentration (5x CMC) no bands are visible in the pellet after solubilisation. The pellets after solubilisation also differ from DDM to Fos-Choline 12. As for DDM, a membrane pellet was visible in the ultracentrifugation vial after centrifugation of the solubilised sample, while hardly any pellet was visible after solubilisation with Fos-Choline-12. This already indicated an almost complete solubilisation of the entire membrane components, with all the proteins contained. Fos-Choline-12 is a zwitter-ionic detergent, which disproves the theory that non-ionic detergents work better in the case of HST.

Due to the extremely strong dissolving capacity of Fos-Choline-12, this detergent is less suitable for the solubilisation of the target protein, as many other biocomponents are also dissolved. In addition, the protein bands smaller than 40 kDa are significantly stronger when using Fos-Chloline-12 than when using DDM.

DDM, LMNG and C12E8 showed the highest solubilization capacity. None of the detergents was able to influence the solubilization yield of the band at around 60 kDa, which was assumed to be the target protein. Instead, bands smaller than 40 kDa are most prominent on the Western blots. Therefore, affinity chromatography was performed in order to separate the target protein from the other peptides detected.

3.2.4 Quantification of Target Proteins

Before chromatography was carried out, an attempt was made to quantify the bands in the sample.

In order to quantify and compare the optimization process of protein solubilization from biomass, it is necessary to discuss the concentration and enrichment of the target protein in each process step. To establish a standard series, a GST-labeled control protein (Bovine Serum Albumin (BSA)) was used. GST-BSA (Origene, Rockville, MD, USA) was loaded onto an SDS-PAGE gel along with HST samples, and antibody incubation was performed using Anti-GST antibody conjugated to HRP. The resulting Western blots were analysed using ImageJ software [46].

The exact size of the BSA-GST fusion protein is not specified in the product data sheet. Therefore, the size of the native BSA from *Bos Taurus* (Uniprot ID: P02769; Size: 69.293 kDa) and the size of the GST tag (Size: 26.99 kDa) are used to assess the size of the control protein. This results in 96.283 kDa for the BSA-GST control protein.

Despite using different concentration ranges, it was not possible to generate a linear standard curve including all concentrations, as depicted in Figure 14. The distinct and clear bands for the highest concentration of BSA-GST could not be visualized. As a result, the highest concentration was excluded from the analysis for both blots in Figure 14.

For blot Figure 14/A, testing the calibration curve was not possible due to non-visible sample bands of the lowest concentration of BSA-GST on the blot and non-delineable bands of the highest BSA-GST concentration. Longer visualization times would have been necessary to visualize the band with the lowest concentration and a shorter time for the highest concentration. Therefore, alternative concentrations in a smaller range had to be tested, which are given in Figure 14/B.

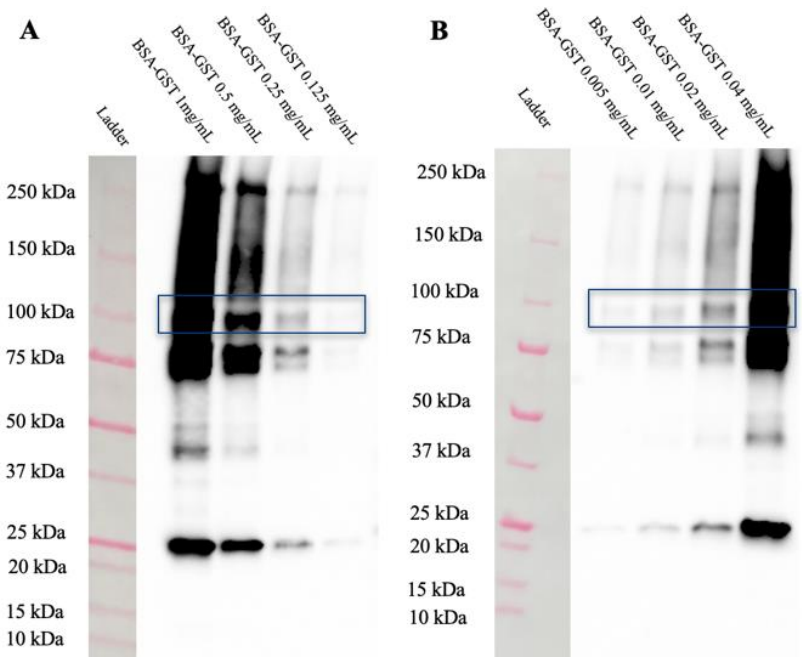

Figure 14 To obtain a standard series of the control protein BSA-GST, the protein was loaded onto the SDS-PAGE gel at the concentrations **A** 0.005 mg/mL, 0.01 mg/mL, 0.02 mg/mL and 0.04 mg/mL **B** 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL. The bands just below the 100 kDa standard correspond to the BSA-GST protein, which is marked in the blue square. The band at a level of 25 kDa could be free GST-tag.

The calibration curve obtained from Figure 15 was used to calculate the amount of protein of the target protein at about 60 kDa. The integrated density of the blue marked band in Figure 15 was determined and its concentration was calculated using the regression curve obtained from the BSA-GST reference protein. The results are presented in Table 7.

Since the highest concentration of the BSA-GST could not be differentiated from the lane background, it was not included in the calculations.

Table 7 Protein concentration of target protein HST for testing the viability of the calibration curves obtained

Calibration curve (B)	**Calculated concentration for diluted sample (1:2 in Lämmli buffer)**	**Original concentration in sample**
Lower three BSA-GST concentrations	0.0232 mg/mL	0.0466 mg/mL

The calibration curve obtained from BSA-GST concentrations of 0.005 mg/mL, 0.01 mg/mL, and 0.02 mg/mL can be used to approximate the protein amount in the 60 kDa band.

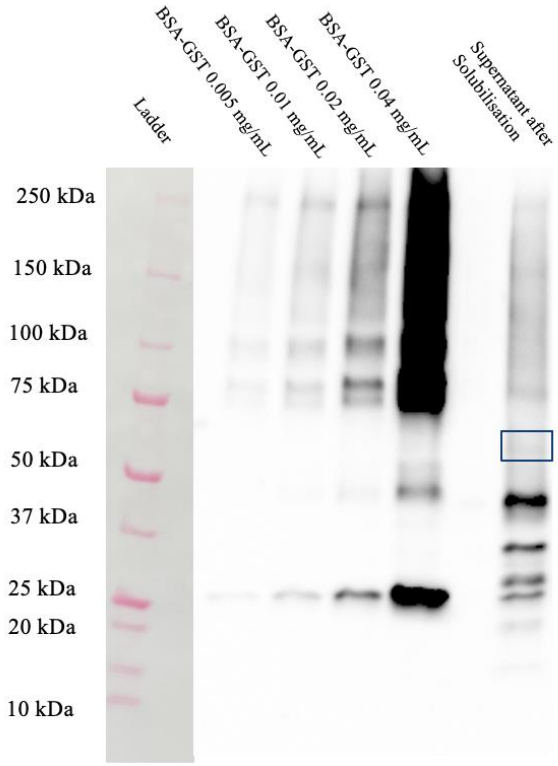

Figure 15 Western blot of BSA-GST dilutions and HST sample (solubilised in DDM with a concentration of 10 x CMC). The blue square marks the presumed HST band at about 60 kDa which was used to test the calibration curves obtained.

The calibration curve can be found in the appendix.

3.2.5 Chromatography

For the purification of the FLAG-GST-tagged intramembrane protein HST was loaded on both FLAG- and GST-affinity chromatography columns. Since detergents form an artificial environment around the membrane protein, they can influence the performance of the chromatography. In addition to the effects already described, detergents can also (partially) shield the tags of the protein, which can lead to poor binding to the column material. Particularly high concentrations of detergents can be problematic for chromatography [46].

The samples 'Supernatant after Solubilisation' were used for affinity chromatography, which were prepared by using detergents DDM and LMNG for solubilising HST.

To compare the results, flow-through- (FT) and eluate samples (E) of each chromatography run are depicted in Figure 16.

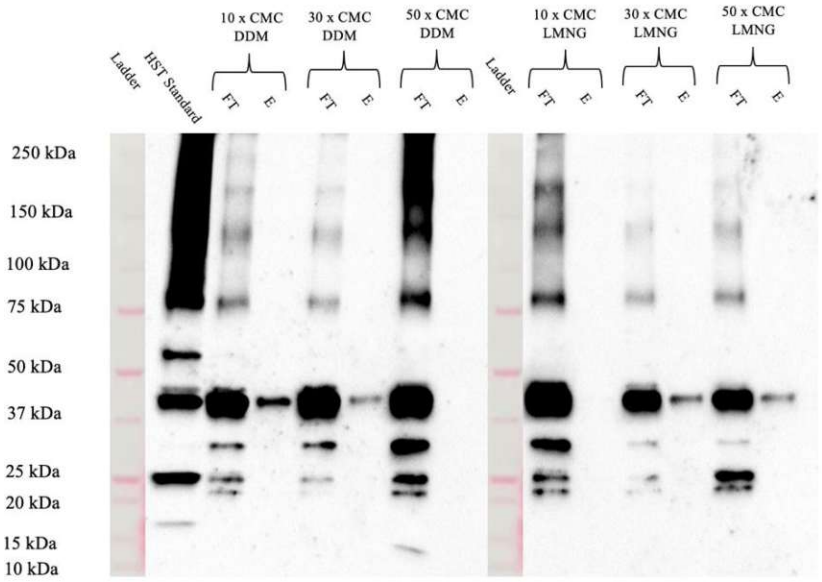

Figure 16 Western blot of HST affinity-chromatography using GST-affinity columns (FT: Flow Through, E: Eluate). Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation. For SDS-PAGE, HST standard was diluted 1:8 in PBS-buffer. The samples FT and E were used concentrated.

First and foremost, it must be stated that no band is visible at 63 kDa in the eluate samples. The target protein could therefore not be concentrated by GST-tag dependent affinity chromatography. Only bands of about 45 kDa are visible. Additionally, even no 63 kDa band is visible at the FT samples, beside the samples prepared by using LMNG/10 x CMC. The lane patterns of the flow through samples are similar to the patterns of the supernatants after solubilisation.

Nevertheless, these initial results show how differently the various detergents and detergent concentrations influence affinity chromatography. DDM produced the strongest visible bands in the eluates at the lowest detergent concentration of 10 x CMC, while at a detergent concentration of 50 x CMC no band can be seen in the eluate. Therefore, it can be concluded that a high concentration of DDM has a negative influence on the affinity of the GST-tag to the resin.

This is different in the case of LMNG. Here, the protein bands at 40 kDa are equally strong at a detergent concentration of 30 x and 50 x CMC. With 10 x CMC, no band can be detected. Therefore, it can be assumed, that higher detergent concentrations of LMNG impair the accessibility of the GST-tag less than high detergent concentrations of DDM.

3.2.5.1 *Stepwise separation of fragments smaller than 40 kDa*

To separate the visible proteins in the mass range below 40 kDa, an attempt was made to purify the sample step by step. It was assumed that certain peptides may bind more strongly to the resin than others, possibly due to incomplete tagging or tags shielding by detergents. The peptides that bind more strongly should be bound to the resin first, while the peptides that bind more weakly may not bind due to the resin's limited binding capacity, and hence, they should be found in the flow-through. For this purpose, the flow through of the first column was used as a sample for the next purification step by affinity chromatography (scheme Figure 17). Despite using only approximately 20% of the column's loading capacity (calculated based on the total protein concentration of the sample, measured by Bradford assay), there was no significant reduction of the bands below 40 kDa.

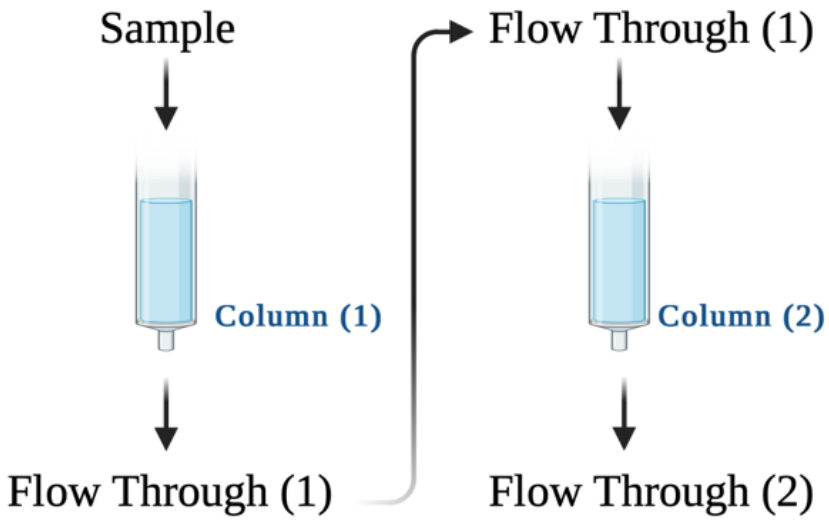

Figure 17 Scheme for stepwise purification of HST sample

Moreover, FLAG-tag affinity chromatography was used to purify the samples instead of GST-tag affinity chromatography. To obtain a protein concentration of approximately 20% of the affinity resin's loading capacity, as suggested by the resin supplier, the sample was diluted 1:4 in binding buffer. The results are depicted in Figure 18. For the stepwise purification six columns were used in total.

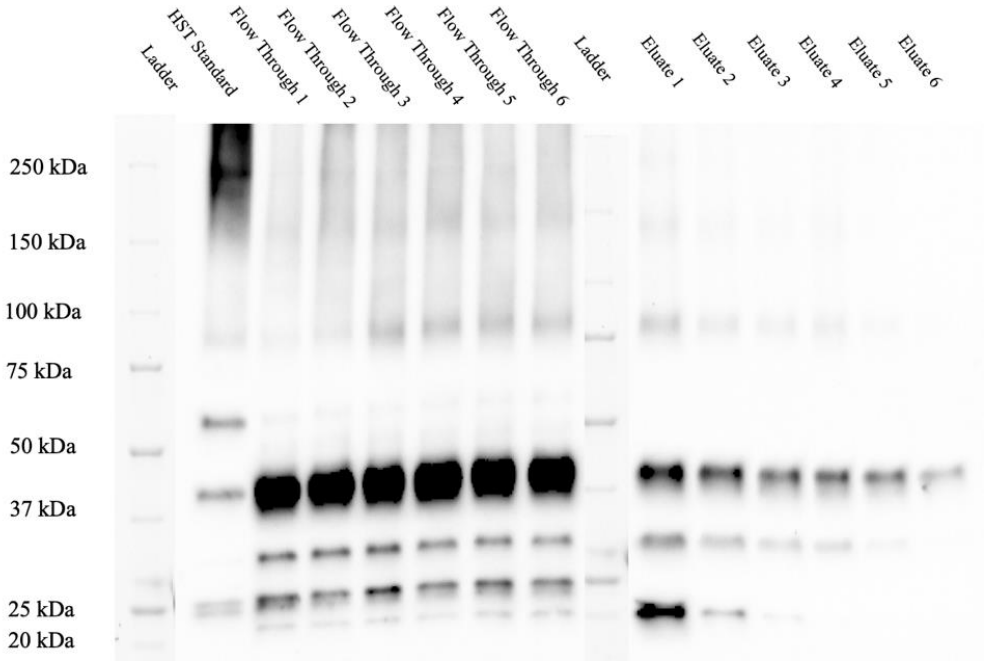

Figure 18 Western blot of stepwise purification of HST using DDM as a detergent in a concentration of 30 x CMC. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation of the membrane. For SDS-PAGE all samples were used concentrated, beside HST Standard, which was diluted 1:10 in PBS buffer.

Compared to GST-affinity chromatography, more bands of different sizes were observed in the Eluate samples performing purification by FLAG-affinity chromatography. However, as in the previous experiments by using GST-affinity chromatography, purification based on the FLAG-tag did not result in an enrichment of the assumed target protein at approximately 60 kDa. While the band intensities of the low-molecular-weight bands in the eluate samples decreased after each purification step, no concentration of the target protein was observed. Additionally, there was no decrease in band intensity visible in the Flow Through samples. This could be due to the detergents, which may cause a masking effect on the tags, hindering a constant percentage of the target protein from binding to the affinity resin [47]. The attempt to purify the target protein band stepwise over several columns was therefore not successful.

3.2.6 Low-molecular-weight protein bands in HST biomass

3.2.6.1 *Solubilisation process evaluation*

As additional protein bands with masses smaller than 40 kDa were visible in each Western blot, it was suspected that the target protein HST had been degraded. To eliminate the possibility of HST fractionation during the solubilization process, samples were taken after each main processing step and compared to each other on a Western blot (Figure 19).

Even in the first samples taken after homogenization, which occurred only 1.5 hours after thawing of the biomass, the small weight fragments were present. This indicates that the peptides showing up as low-molecular-weight bands were present from the beginning of the solubilization process and did not result from enzymatic degradation during sample preparation or solubilization. For even more confirmation, a sample taken directly after thawing the biomass should have been analysed as well.

The changes, in the band patterns of the different samples during the downstream process, are caused by the separation of soluble components and the concentration of membrane-bound. Since some of the bands in the homogenization samples decreased in intensity or disappeared completely, it can be assumed that some free tags or parts of the tags are present in the biomass.

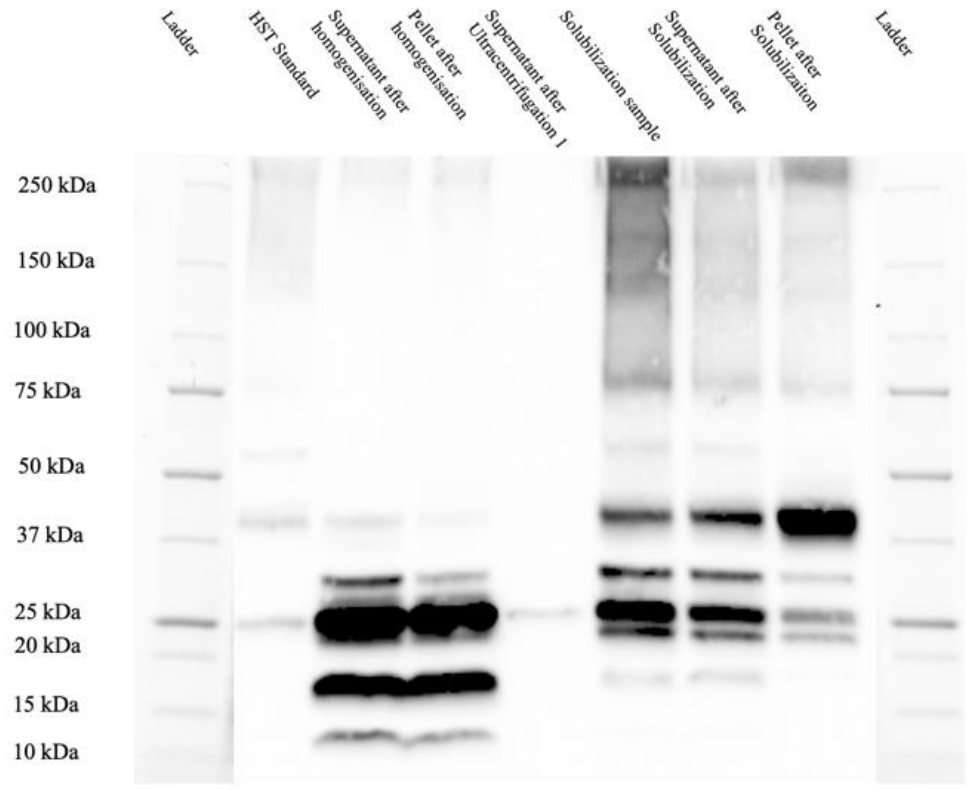

Figure 19 Western blot of the solubilisation process of HST using DDM as a detergents in a concentration of 15 x CMC. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation of the membrane. For SDS-PAGE HST Standard, supernatant after homogenisation and pellet after homogenisation were diluted 1:10 in PBS buffer. Supernatant after ultracentrifugation I, solubilisation sample, supernatant after solubilisation and pellet after solubilisation were used concentrated.

3.2.6.2 *Sample preparation validation*

Heating up membrane proteins in preparation for SDS-PAGE is not always advantageous, as they tend to aggregate at high temperatures. However, there are cases in which membrane proteins can only be detected on a Western blot by heating the sample during sample preparation. Therefore, when detecting membrane proteins by Western blots, different conditions must be tested to obtain a working detection method [48] .

When it comes to temperature based peptide bond breaks, the aspartic acid (D)-proline (P) bond is particularly susceptible to cleavage by heat. [49] Four D-P bonds can be found in the amino acid sequence of the target protein. One of it lies amidst the protein sequence at position 254/255. When calculating the protein mass of the protein part, which contains the GST- and FLAG-tags, a mass of about 30 kDa results. Another P-D bond, near the N-terminus (Position 459/450) results in a mass of about 51 kDa.

To determine whether HST fractionation occurred due to the heating process at 95°C during sample preparation for SDS-PAGE, different temperatures were used to process the supernatant and pellet samples after homogenization, the solubilized sample, and the supernatant after solubilization. For each sample, three parameters were tested. One without heating, one heated up to 60°C, and one to 95°C. The results are given in Figure 20.

Figure 20 illustrates that all three preparations of one sample display differences in their band patterns. Nevertheless, the band of approximately 60 kDa, which might be the target protein, is clearly visible only in the sample labelled as ‘supernatant after solubilisation’ when the sample is heated up to 95°C. Furthermore, an aggregation of sample material was observed in the samples which were incubated at RT and heated up to 60°C. This leads to the assumption that only the samples heated up to 95°C is accurate.

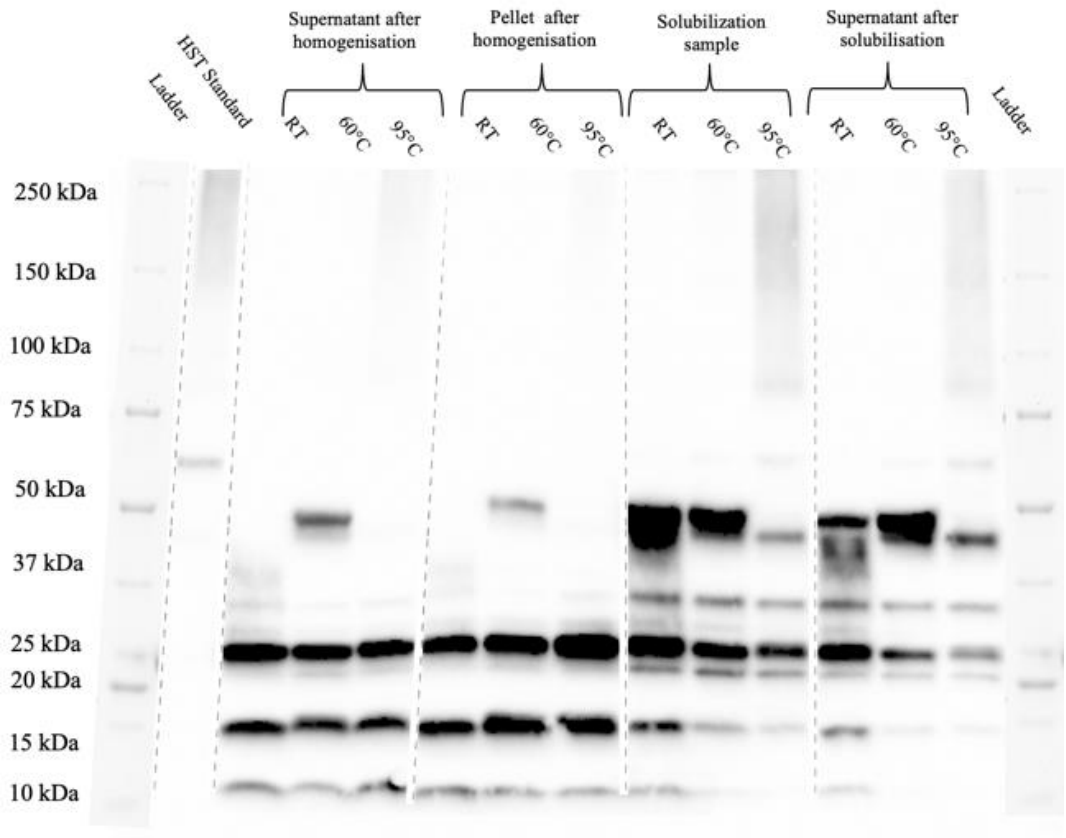

Figure 20 Western blot of different temperatures in sample preparation for SDS-PAGE. As a detergent, DDM at a concentration of 30 x CMC was used. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation of the membrane. For SDS-PAGE HST Standard, supernatant after homogenisation and pellet after homogenisation were diluted 1:10 in PBS buffer. Solubilisation sample and supernatant after solubilisation were used concentrated.

Comparing the 95°C samples, it can be concluded that the 60 kDa band is detectable in all samples after solubilisation, but it is significantly more visible in the 'supernatant after solubilisation' sample. In this sample, the soluble components of the *P.pastoris* cells, the membrane residues and cell debris of the *P. pastoris* biomass were separated. On the other hand, no 60 kDa band can be observed in the samples after homogenisation, indicating that the concentration of this band is too low compared to the other visible bands at this stage of the DSP, as can be detected on the Western blot. After solubilisation, the concentration of the 60 kDa band increased relative to the low molecular weight bands.

It is possible, that a protein with a size of 60 kDa is also present in the samples incubated at room temperature and at 60°C. However, it cannot be detected due to protein aggregation and inadequate dissolution of the secondary and tertiary protein structures. This is evident from the SDS-PAGE gel shown in Figure 21.

Since the bands at a lower molecular weight than 60 kDa also appear in the not-heated and 60°C samples, it is evident that the sample preparation at 95°C is not the reason for the presence of these smaller proteins. Moreover, it can be concluded that the heating process is crucial for the proper separation of proteins on the SDS-PAGE gel.

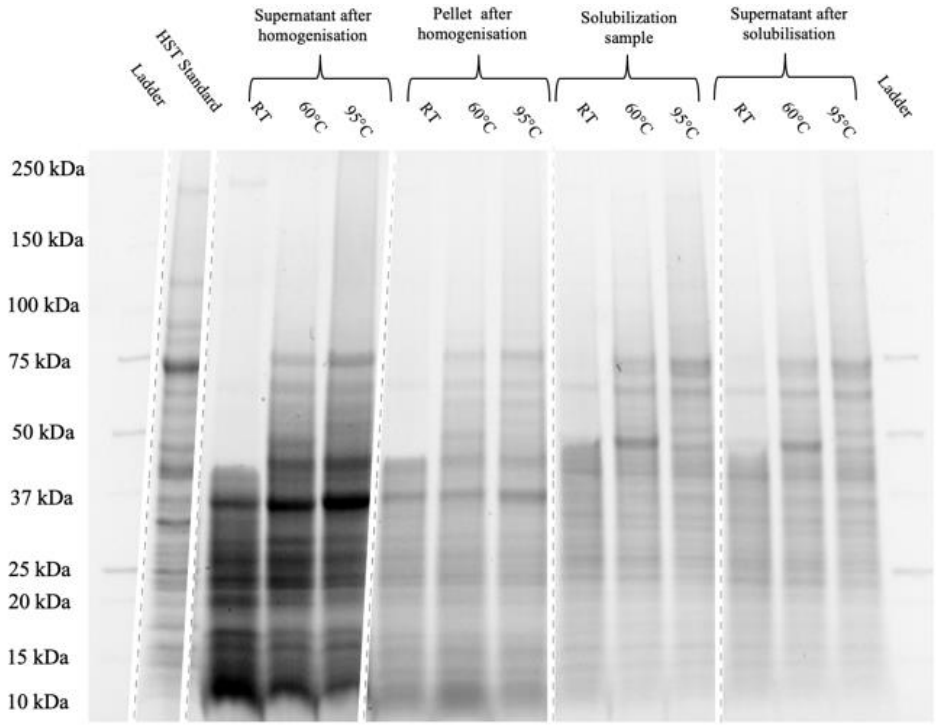

Figure 21 Stain free gel imaging of different temperatures in sample preparation for SDS-PAGE. As a detergent, DDM at a concentration of 30 x CMC was used. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder. For SDS-PAGE HST Standard, supernatant after homogenisation and pellet after homogenisation were diluted 1:10 in PBS buffer. Solubilisation sample and supernatant after solubilisation were used concentrated.

3.2.6.3 *MS/MS Fingerprint*

Consequently, the question still arises, as to where the bands smaller than 40 kDa on the Western blots result from. To gain certainty, a mass spectrometry fingerprint of some protein bands was conducted. In order to avoid ruling out the possibility of a shift in the migration behavior of the target protein, the band at 75 kDa was also examined.

For this purpose, the supernatant after solubilization (30 x CMC DDM) sample was loaded six times (2x3) on an SDS-PAGE gel, which was then stained with Coomassie blue. The bands to be analysed, were cut out with a scalpel, with three bands of a certain mass forming one MS/MS sample (Figure 22). These samples were then frozen in an Eppendorf vessel and forwarded for MS/MS fingerprinting analysis.

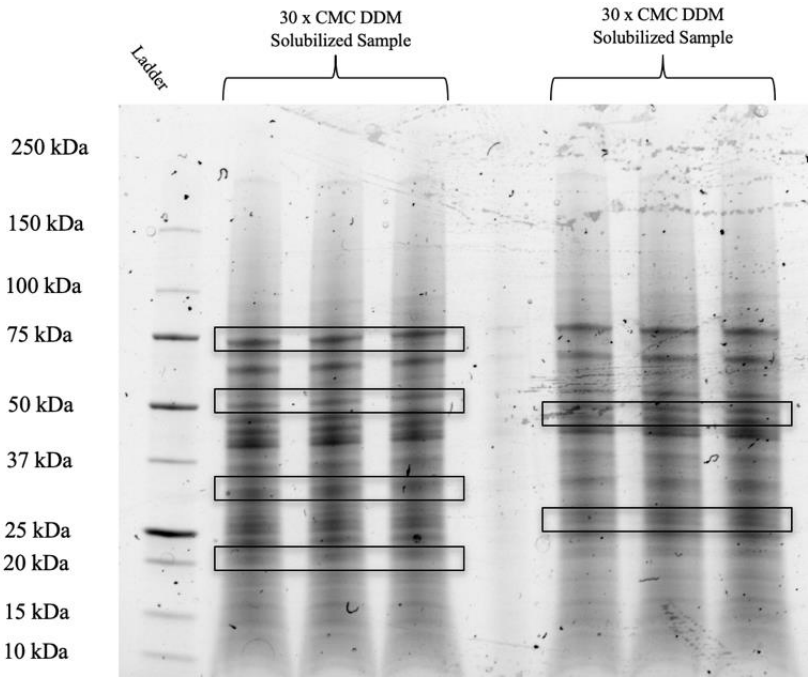

Figure 22 Coomassie blue protein staining of supernatants after solubilisation on a SDS-PAGE. As a detergent, DDM at a concentration of 30 x CMC was used. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder. For SDS-PAGE the sample was used concentrated. The black boxes show the bands, which were cut out of the gel and used for MS/MS fingerprinting analysis.

As all the protein bands, that were cut and analysed, showed a signal on the Western blots, when incubated with either Anti-GST or Anti-FLAG antibodies, it can be concluded that both tags are at least partially present in these bands. Therefore, the results of the MS/MS measurement were compared with the protein sequence of the entire synthetic FLAG-GST-HST protein (Protein ID: HST). The main results of the sequence coverage of the measured peptides are shown in Table 8. Trypsin was used as protease for cutting the contained peptides during the sample preparation. Trypsin cleaves peptide bonds C-terminally to arginine or lysine residues. Peptides containing less than 5 amino acids were not identified by the MS/MS analysis.

Table 8 MS/MS measurement of different protein bands (75 kDa, 50 kDa, 40 kDa, 31 kDa, 25 kDa, 15 kDa) of the solubilised HST sample

Protein Bands	Peptide counts (razor + unique)	Peptide counts (unique)	Unique Peptides	Unique Sequence Coverage [%]	Total HST Intensity
75 kDa	51	51	51	50.4	$2.33*10^8$
50 kDa	97	97	97	63.1	$6.96*10^8$
40 kDa	116	116	116	70.3	$9.44*10^8$
31 kDa	44	44	44	45.3	$2.46*10^8$
25 kDa	89	89	89	65.7	$4.32*10^8$
15 kDa	57	57	57	51.9	$1.09*10^8$

Table 8 shows that all measured samples contain fragments of HST. The 40 kDa band has the most unique peptides and is also the most prominently visible band on the Western blots of the supernatant after solubilisation. The total intensity of HST peptides in MS/MS is also highest for this band. Additionally, this band has the highest sequence coverage of the HST sequence at 70.3%.

Therefore, it can be assumed that all investigated bands contain at least some parts of HST. However, since peptides with fewer than 5 amino acids were not detected, a sequence coverage of 100% is not possible. For further investigation, the HST peptides of each sample were aligned. An example is shown in Figure 23, where the alignment of the 40 kDa band is presented.

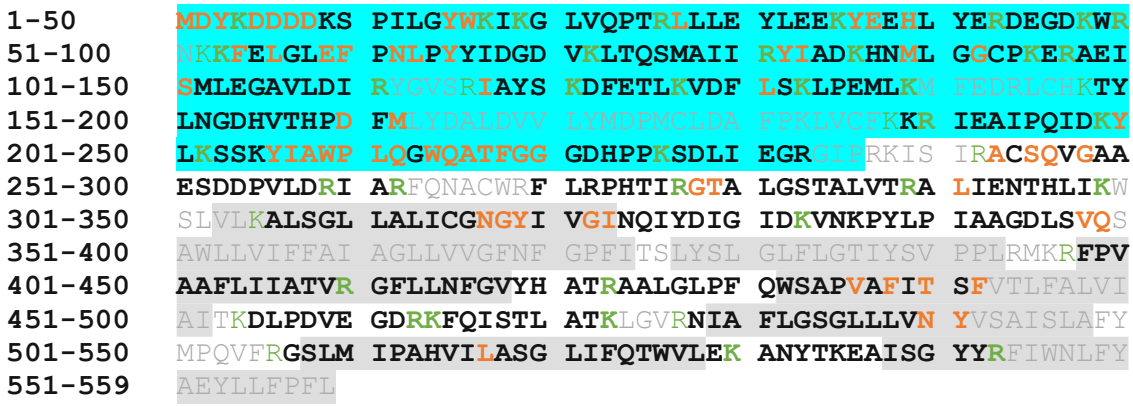

Figure 23 Alignment of the peptides to the HST amino acide sequence. The amino acids with a blue background show the sequence of the FLAG- and GST-tag, while those with a grey background form the transmembrane domains. The bold amino acids represent amino acids found in peptides in the MS/MS fingerprint analysis. The green and orange amino acids mark the cutting sites of the peptides found in the MS/MS fingerprinting. Green ones mark arginine and lysine, the cutting sites for Trypsin, while orange amino acids mark cutting sites that do not originate from Trypsin.

3.2.6.4 *Evaluation MS/MS fingerprint alignment*

To better compare the coverage of the amino acid sequence among the different samples, the bands were aligned with each other (see appendix). Due to the distribution of peptide artifacts on the SDS-PAGE gel and the mass spectrometer's high sensitivity, it is not possible to map which peptides are present in each band accurately. Additionally, some peptide fragments may not be as easily ionizable as others, which can lead to their undetectability by the mass spectrometer.

Peptide cleavage sites, not resulting from trypsin, were found throughout the HST sequence, including the tag-sequence, intramembrane sequences, and connecting loops between the

intramembrane helices. This leads to the assumption that HST was degraded by proteolytic cleavage during the fermentation process of *P. pastoris*.

As the 40 kDa band showed the highest sequence coverage and peptide intensity in the MS/MS measurement and appeared most intensely on the Western blot after solubilization, it is likely that this band contains the largest part of HST.

The peptides that do not have arginine or lysine as C-terminal amino acids contain different C-terminal amino acids such as tryptophan, histidine, methionine, phenylalanine, or glutamine. However, tyrosine and leucine occur most frequently at the C-terminal end of the peptides, which do not result from cleavage by trypsin.

The degradation of heterologous proteins is a well-known issue during the fermentation of *P. pastoris*. Yeasts typically contain three main types of peptidases: cytosolic proteases, vacuolar proteases, and proteases associated with the secretory pathway. An example of cytosolic proteases is the proteasome, a large 650 kDa structure composed of two subcomplexes that also exist in the nucleus. The proteasome is responsible for efficient and selective degradation of intracellular proteins [46, 47].

Proteases of the secretory pathway also play an important role in the degradation of heterologous proteins. They are mainly located in the plasma membrane or the Golgi apparatus and are responsible for processing protein precursors [51].

However, the heterologous HST is located in the membrane of the endoplasmic reticulum (ER) of the cell. In addition to glycosylation and protein folding, the ER is also a cellular compartment responsible for protein quality control. The accumulation of misfolded or unfolded proteins in a cell can be a consequence of overexpression. This leads to the unfolded protein response, which further activates genes for protein refolding or degradation, such as ER-associated protein degradation (ERAD) [52]. ER membrane proteins can be degraded in ERAD through three different pathways, depending on the location of the misfolded domain: cytosolic, ER-luminal, or transmembrane. The Hrd1 ubiquitin ligase complex is responsible for degrading proteins with intramembrane or ER-luminal misfolding, while the Doa10 ubiquitin ligase complex is responsible for degrading proteins with cytoplasmic misfolding [51, 52]. Another regulation for degradation of membrane proteins and the release of transmembrane fragments is intra-membrane proteolysis. Two intramembrane proteases

involved in this process are Ypf1 (aspartate protease) or Re1 (glutamate protease). Both are present in the ER of budding yeasts and function in conjunction with ERAD [55].

4 Conclusion

Recombinant production of membrane proteins remains a major challenge, as does the search for suitable conditions for downstream processes, particularly solubilization and the creation of an artificial environment for integral membrane proteins, without interfering with subsequent analytics and purification.

In this study, efforts were made to find conditions for the purification of two membrane proteins. However, experiments related to the membrane protein cytochrome P450 CYP81A9 were postponed due to a faulty gene sequence and will be continued at a later time, which is not part of this work. Nonetheless, the preliminary results suggest that solubilization of the protein using detergents, especially LDAO, is possible. Further research is required to determine the feasibility of further protein purification. The following steps would involve purification and concentration of the protein using affinity and size exclusion chromatography.

Unfortunately, the research on the membrane protein Homogentisate solanesyltransferase did not yield conclusive results. Based on previous activity measurements, the protein's activity, when produced heterologously in *P. pastoris*, was assessed. However, purification of the protein proved to be challenging due to proteolytic cleavage during fermentation, resulting in small and inseparable fragments. To address this issue, a low-producing *P. pastoris* strain carrying the HST gene could be developed to investigate, whether the degradation is a result of overproduction, as discussed in Chapter 3.2.6.4. Solubilisation and purification of HST would then need to be re-evaluated. The detergents DDM and LMNG, which showed promising results in the performed experiments, could serve as a starting point for further investigations. However, a comprehensive reassessment of the solubilization and chromatography processes is necessary.

5 Results Scientific Questions

1. Is it possible to solubilise the membrane-bound protein Cytochrome P450 CYP81A9 and the integral membrane protein homogentisate Solanesyltransferase using detergents?

For cytochrome P450 CYP81A9, solubilization was successfully achieved using detergents, with the best results obtained using lauryldimethylamine oxide. However, on the Western blot analysis, it was difficult to determine the optimal detergent concentration. Bands were observed at both detergent concentrations tested: 20 times the critical micelle concentration (CMC) and 30 times the CMC. Considering that higher detergent concentrations can significantly impact downstream processes [56], lower concentrations would be preferable to achieve similar results. However, since the protein is a truncated variant with missing amino acids, further investigation is required. Nevertheless, since the truncation does not affect the membrane anchor, the results obtained can serve as a reference for future investigations.

For Homogentisate solanesyltransferase, the most favorable results were obtained using the detergents n-dodecyl-β-D-maltoside and lauryl maltose neopentyl glycol, at concentrations of 15 x CMC and 30 x CMC. Under these conditions, a protein band of approximately 60 kDa was observed, which corresponds to the expected size of the target protein. However, considering the proteolytic cleavage of HST during fermentation, further steps are necessary before proceeding to downstream processes. The expression of HST using *P. pastoris* as a host needs to be reengineered to address this issue.

2. Which factors, apart from the choice of detergent, influence solubilisation the most?

Each step of the solubilisation process was thoroughly evaluated, and potential improvements were discussed. For cytochrome P450 CYP81A9, minor improvements were observed by varying different conditions during the process such as resuspension, homogenisation, centrifugation, and ultracentrifugation. However, a significant improvement was achieved by changing the buffer system. It can be concluded that the choice of buffer has a clear influence on the solubilisation efficiency. Buffer A, which contains glycerol, showed better results compared to DTT/TES B buffer. Glycerol prevents protein aggregation by interacting with

hydrophobic regions of the proteins [57]. This may support solubilisation efficiency of the detergents when buffer A is used.

For Homogentisate solanesyltransferase, the most significant factor that improved the results was the biomass concentration. Increasing the biomass concentration resulted in clearer visualization of bands on the Western blot. Other factors in the solubilisation process that were varied during the course of this work had only a minor impact on the solubilisation efficiency. However, due to the fragmentation, it was not possible to identify the most influential factors specifically related to HST solubilisation.

Although biomass concentration and selection of the buffer system were found to be the most influential factors in these specific cases, it is important to evaluate all process and preparation steps for membrane protein solubilisation in order to find the best conditions for a particular protein. Each protein may have unique requirements and sensitivities, and a comprehensive evaluation of the entire workflow is necessary to optimize solubility and preserve protein integrity.

3. Which strategies for protein purification can be usefully applied in this context?

In the case of cytochrome P450 CYP81A9, chromatography was not performed due to the priority of creating a strain that carries the complete, non-truncated gene.

Affinity chromatography was performed for HST using both the GST tag and the FLAG tag for purification. Because of the fragmentation of HST during fermentation, it was not possible to obtain a definitive answer regarding the purification of the specific 63 kDa protein. The presence of protein fragments hindered the purification process, making it impossible to isolate the desired protein fragment.

A second purification step, namely size exclusion chromatography, was not performed in this study. Instead, the focus was shifted to investigating the origin of the small, intense bands observed on the Western blot. In generall, the presence of detergent molecules that form a shell around the hydrophobic regions of the membrane protein can affect the retention time in the size exclusion matrix. This altered retention behaviour can vary depending on the detergent used, as both the size of the detergent molecule and its critical micelle concentration (CMC) can influence the performance of size exclusion chromatography (SEC) [58].

6 References

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Appendix

Sequenze of GST-tag used for the BSA-GST fusion protein (Origene, Rockville, MD, USA) for quantitative analysis:

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPN
LPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSR
IAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVV
LYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHP
PKSDLVPRGSPGIHRD

Comparison Buffer A and Syn-Buffer System

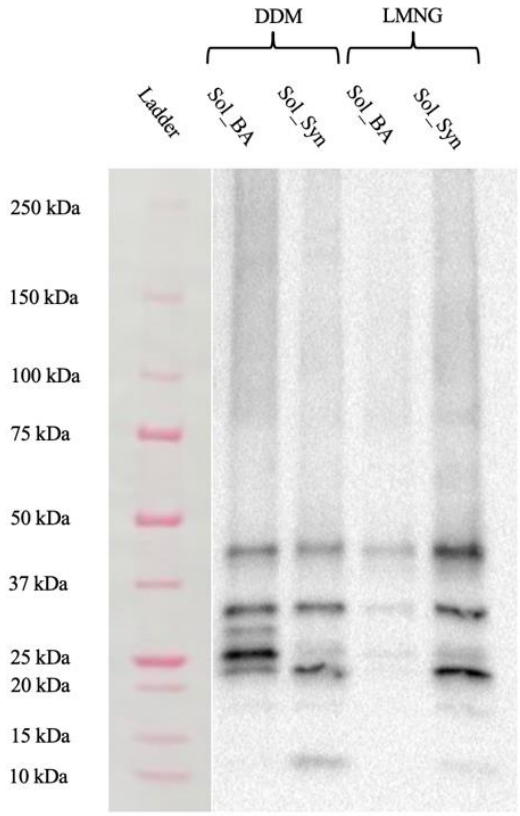


Figure 24 Western blot of HST (Sol_BA: Solubilised sample using buffer A-buffer system; Sol_Syn: Solubilised sample using HST disruption buffer/HST resuspension buffer-buffer system;). Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-GST antibody for incubation. All samples were prepared with 50 x CMC of the according detergent in the according buffer system. For SDS-PAGE the samples were used concentrated.

Detergent Screening at 15 x CMC (DDM, LMNG, C12E8, DM)

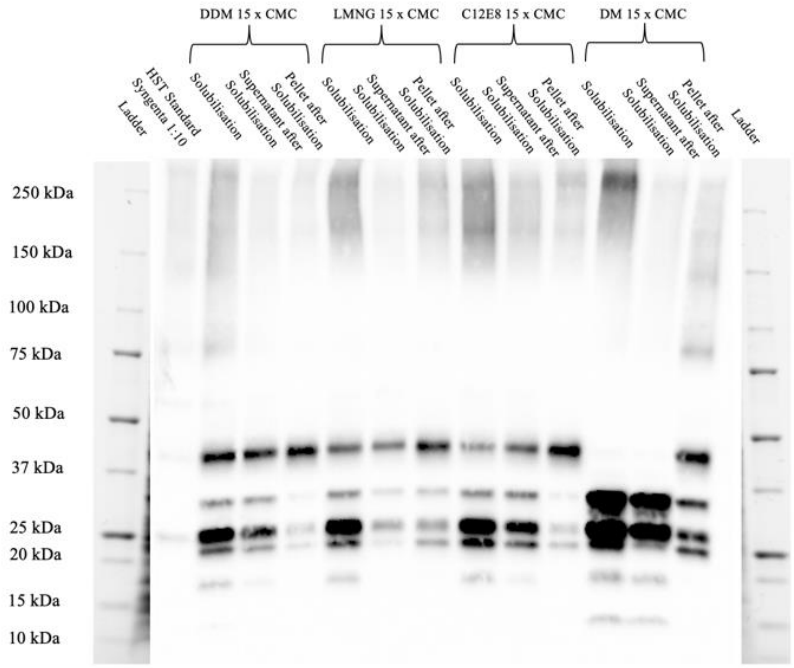


Figure 25 Western blot of HST using different detergents in a concentration of 15 x CMC. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation. For SDS-PAGE all samples were used concentrated, beside HST Standard, which was diluted with PBS buffer 1:10.

Stepwise Purification

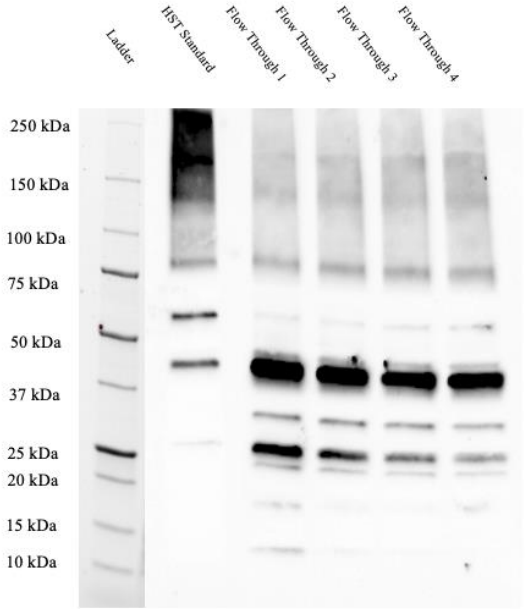
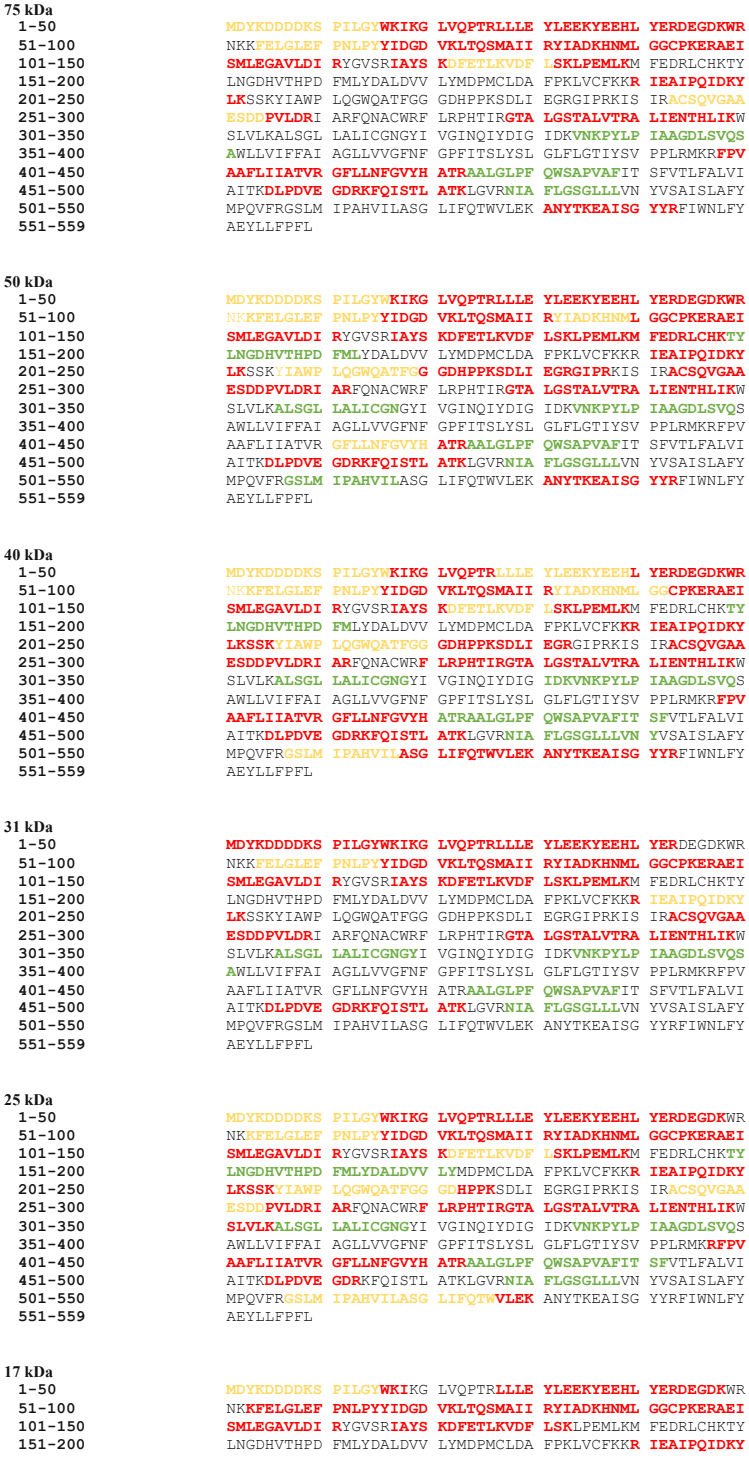


Figure 26 Western Blot of Stepwise Purification of HST using DDM as a detergents in a concentration of 15 x CMC. Precision Plus Protein Unstained Protein Standard (Bio-Rad, Hercules, CA, USA) was used as a ladder and Anti-FLAG antibody for incubation. For SDS-PAGE all samples were used concentrated, beside HST Standard, which was diluted with PBS buffer 1:10.

Alignment of the MS/MS Data

The amino acids in red represent those amino acids, that results from peptides produced by C-terminal digestion with trypsin. They, therefore, have an arginine or lysine at the C-terminal end of their sequence. The green amino acids come from peptides, which have neither arginine nor lysine at their C-terminal end. Yellow amino acids occur in peptides that have arginine or lysine at the end or another amino acid.



```
201-250          LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA
251-300          ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW
301-350          SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS
351-400          AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV
401-450          AAFLIIATVR GFLLNFGVYH ATRAALGLPF QWSAPVAFIT SFVTLFALVI
451-500          AITKDLPDVE GDRKFQISTL ATKLGVRNIA FLGSGLLLVN YVSAISLAFY
501-550          MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY
551-559          AEYLLFPFL
```

Comparison of Alignments

The amino acids in red represent those amino acids, that come from peptides produced by C-terminal digestion with trypsin. They, therefore, have an arginine or lysine at the C-terminal end of their sequence. The green amino acids come from peptides that have neither arginine nor lysine at their C-terminal end. Yellow amino acids occur in peptides, that have arginine or lysine at the end or another amino acid.

```
1-100
 75 KDA     MDYKDDDDKS PILGYWKIKG LVQPTRLLLE YLEEKYEEHL YERDEGDKWR NKKFELGLEF PNLPYYIDGD VKLTQSMAII RYIADKHNML GGCPKERAEI
 50 kDa     MDYKDDDDKS PILGYWKIKG LVQPTRLLLE YLEEKYEEHL YERDEGDKWR NKKFELGLEF PNLPYYIDGD VKLTQSMAII RYIADKHNML GGCPKERAEI
 40 kDa     MDYKDDDDKS PILGYWKIKG LVQPTRLLLE YLEEKYEEHL YERDEGDKWR NKKFELGLEF PNLPYYIDGD VKLTQSMAII RYIADKHNML GGCPKERAEI
 31 kDa     MDYKDDDDKS PILGYWKIKG LVQPTRLLLE YLEEKYEEHL YERDEGDKWR NKKFELGLEF PNLPYYIDGD VKLTQSMAII RYIADKHNML GGCPKERAEI
 25 kDa     MDYKDDDDKS PILGYWKIKG LVQPTRLLLE YLEEKYEEHL YERDEGDKWR NKKFELGLEF PNLPYYIDGD VKLTQSMAII RYIADKHNML GGCPKERAEI
 17 kDa     MDYKDDDDKS PILGYWKIKG LVQPTRLLLE YLEEKYEEHL YERDEGDKWR NKKFELGLEF PNLPYYIDGD VKLTQSMAII RYIADKHNML GGCPKERAEI


101-200
 75 KDA     SMLEGAVLDI RYGVSRIAYS KDFETLKVDF LSKLPEMLKM FEDRLCHKTY LNGDHVTHPD FMLYDALDVV LYMDPMCLDA FPKLVCFKKR IEAIPQIDKY
 50 kDa     SMLEGAVLDI RYGVSRIAYS KDFETLKVDF LSKLPEMLKM FEDRLCHKTY LNGDHVTHPD FMLYDALDVV LYMDPMCLDA FPKLVCFKKR IEAIPQIDKY
 40 kDa     SMLEGAVLDI RYGVSRIAYS KDFETLKVDF LSKLPEMLKM FEDRLCHKTY LNGDHVTHPD FMLYDALDVV LYMDPMCLDA FPKLVCFKKR IEAIPQIDKY
 31 kDa     SMLEGAVLDI RYGVSRIAYS KDFETLKVDF LSKLPEMLKM FEDRLCHKTY LNGDHVTHPD FMLYDALDVV LYMDPMCLDA FPKLVCFKKR IEAIPQIDKY
 25 kDa     SMLEGAVLDI RYGVSRIAYS KDFETLKVDF LSKLPEMLKM FEDRLCHKTY LNGDHVTHPD FMLYDALDVV LYMDPMCLDA FPKLVCFKKR IEAIPQIDKY
 17 kDa     SMLEGAVLDI RYGVSRIAYS KDFETLKVDF LSKLPEMLKM FEDRLCHKTY LNGDHVTHPD FMLYDALDVV LYMDPMCLDA FPKLVCFKKR IEAIPQIDKY


201-300
 75 KDA     LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW
 50 kDa     LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW
 40 kDa     LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW
 31 kDa     LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW
 25 kDa     LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW
 17 kDa     LKSSKYIAWP LQGWQATFGG GDHPPKSDLI EGRGIPRKIS IRACSQVGAA ESDDPVLDRI ARFQNACWRF LRPHTIRGTA LGSTALVTRA LIENTHLIKW


301-400
 75 KDA     SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV
 50 kDa     SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV
 40 kDa     SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV
 31 kDa     SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV
 25 kDa     SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV
 17 kDa     SLVLKALSGL LALICGNGYI VGINQIYDIG IDKVNKPYLP IAAGDLSVQS AWLLVIFFAI AGLLVVGFNF GPFITSLYSL GLFLGTIYSV PPLRMKRFPV


401-500
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 50 kDa     AAFLIIATVR GFLLNFGVYH ATRAALGLPF QWSAPVAFIT SFVTLFALVI AITKDLPDVE GDRKFQISTL ATKLGVRNIA FLGSGLLLVN YVSAISLAFY
 40 kDa     AAFLIIATVR GFLLNFGVYH ATRAALGLPF QWSAPVAFIT SFVTLFALVI AITKDLPDVE GDRKFQISTL ATKLGVRNIA FLGSGLLLVN YVSAISLAFY
 31 kDa     AAFLIIATVR GFLLNFGVYH ATRAALGLPF QWSAPVAFIT SFVTLFALVI AITKDLPDVE GDRKFQISTL ATKLGVRNIA FLGSGLLLVN YVSAISLAFY
 25 kDa     AAFLIIATVR GFLLNFGVYH ATRAALGLPF QWSAPVAFIT SFVTLFALVI AITKDLPDVE GDRKFQISTL ATKLGVRNIA FLGSGLLLVN YVSAISLAFY
 17 kDa     AAFLIIATVR GFLLNFGVYH ATRAALGLPF QWSAPVAFIT SFVTLFALVI AITKDLPDVE GDRKFQISTL ATKLGVRNIA FLGSGLLLVN YVSAISLAFY


501-559
 75 KDA     MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY AEYLLFPFL
 50 kDa     MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY AEYLLFPFL
 40 kDa     MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY AEYLLFPFL
 31 kDa     MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY AEYLLFPFL
 25 kDa     MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY AEYLLFPFL
 17 kDa     MPQVFRGSLM IPAHVILASG LIFQTWVLEK ANYTKEAISG YYRFIWNLFY AEYLLFPFL
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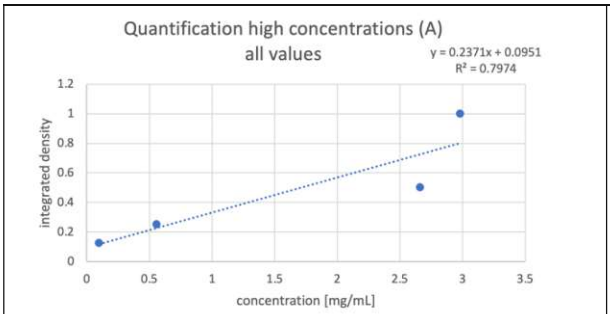
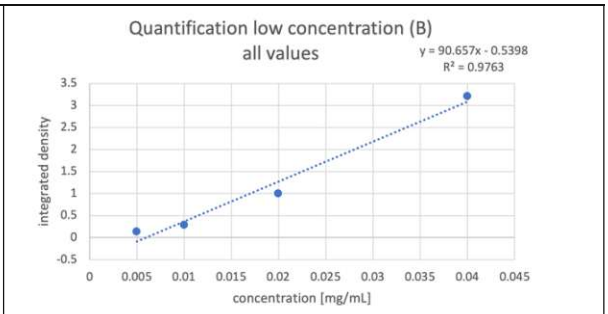
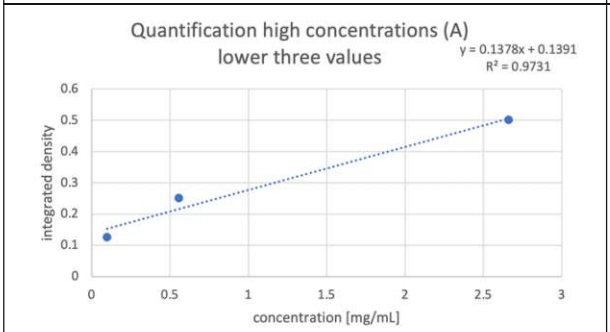
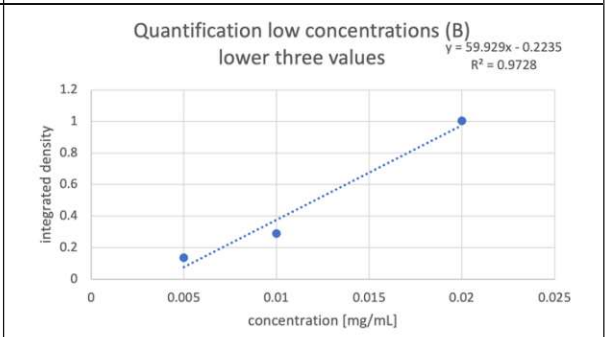
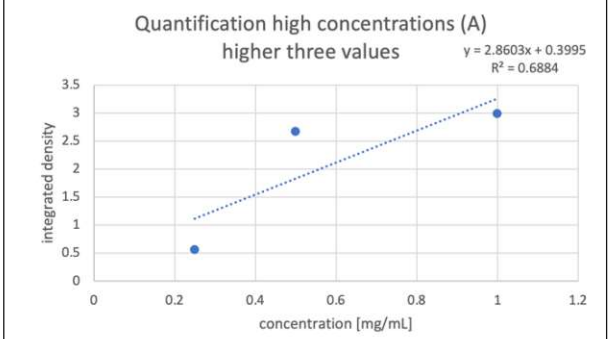
Quantification with BSA-GST

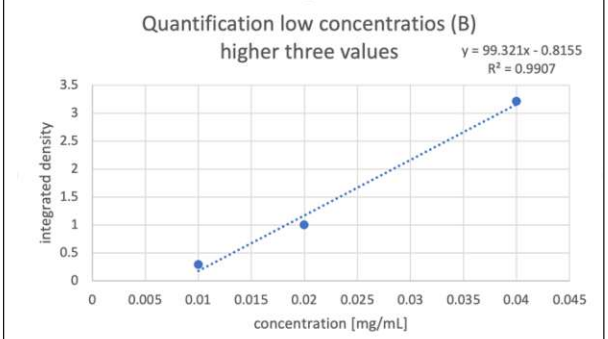












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