University of South Bohemia in České Budějovice

Faculty of Science

MASTER THESIS 2009

# Expression and purification of *Synechocystis* ferrochelatase from *Escherichia coli*

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**Annotation**
Ferrochelatase (FeCH) is an ubiquitous enzyme producing heme, an essential pigment for all form of organisms. In photosynthetic organism, heme is synthesized together with the chlorophyll in one branched pathway and the FeCH enzyme appears to be important for regulation of both the chlorophyll and the heme biosynthesis. To understand regulatory role of this protein, an active recombinant FeCH from photosynthetic organism would be invaluable. The aim of this project is to express FeCH from cyanobacterium *Synechocystis* 6803 in *Escherichia coli* and to prepare a protocol for the purification of this protein as a highly active enzyme.

**Anotace**
Ferochelatáza (FeCH) je všudypřítomný enzym produkující hem, který je nezbytný pro všechny žijící organizmy. Fotosyntetické organizmy syntetizují heme a chlorofyl společnou dráhou, ve které hraje FeCH významnou roli jako klíčový enzym pro regulaci obou větví. Vytvoření aktivní rekombinantní FeCH by bylo neocenitelné pro pochopení role FeCH v regulaci biosyntetické dráhy chlorofylu a hemu. Cílem této práce je exprimovat FeCH ze sinice *Synechocystis* 6803 v *Escherichia coli* a připravit protokol pro purifikaci tohoto proteinu ve formě vysoce aktivního enzymu.

This project was supported by the Student Grant Agency of Faculty of Science, University of South Bohemia - SGA 2008/007 and by the Czech Academy of Sciences, Institutional Research Concept AV0Z50200510.

Prohlašuji, že svoji diplomovou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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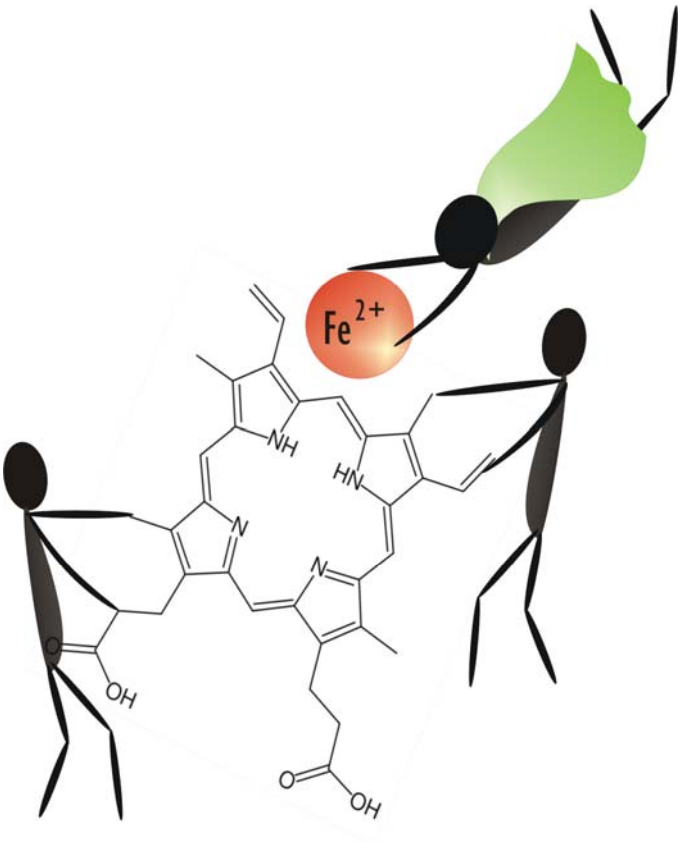



## Poděkování / Acknowledgement

Všichni, kdo mi pomohli dotáhnout tuto práci až do její tištěné podoby ví, že jsem jim hrozně vděčná. Přesto ještě jednou díky: Lachymu - za trpělivost při vytváření dalšího „molekulárníka“, všem z Opaťáku – především Martinovi, Evě, Janče, Markétě a Panterovi, mojí rodině a kamarádům... bez Vás by to nešlo !

Special thanks belong to Dan who was so kind and checked my English.

**CONTENT**

# 1. INTRODUCTION

## 1.1 Tetrapyrrole biosynthesis in photosynthetic organisms

Plants, algae and cyanobacteria are responsible for making our atmosphere breathable for humans and the majority of life-forms on Earth. Photosynthesis is the mechanism by which these organisms are able to produce oxygen. This important process is dependent on the tetrapyrrole molecule chlorophyll, subsequently the most abundant pigment on Earth, with an estimated $10^9$ tonnes synthesised per year. Chlorophyll shares part of its biosynthetic pathway with another important tetrapyrrole, heme, which is essential for almost all living organism as a cofactor for electron transfer reactions in the cell (Ferreira *et al.*, 1996; Tanaka and Tanaka, 2007).

The part of the pathway common to both chlorophyll and heme starts with glutamate. This molecule follows a three-step reaction producing 5-aminolevulinic acid (ALA), the universal precursor of tetrapyrrole biosynthesis (Fig. 1.1). For the first time this pathway divides at the point of the first closed macrocycle; uroporphyrinogen III, where the branch leading to siroheme re-routes (Fig. 1.1). However, protoporphyrin IX (PP IX) lies at the main branchpoint of this pathway. Here, PP IX can serve as a substrate for magnesium chelatase, which incorporates $Mg^{2+}$ ion and thus produce first precursor of chlorophyll, Mg-protoporphyrin IX. In the second route an $Fe^{2+}$ ion can be chelated into PP IX by ferrochelatase (FeCH) to form a protoheme molecule (heme *b*, Fig. 1). Heme oxygenase then oxidizes protoheme and disrupts its ring to produce biliverdin IXα, which is then converted to another member of tetrapyrrole family, phytochromobilin (Dailey and Dailey, 2003). The biosynthetic steps responsible for the synthesis of heme *a* and heme *c* have not yet been elucidated in plants.

Almost all tetrapyrrole biosynthesis in plants occurs in plastids, which is especially unsurprising for the chlorophyll and siroheme branches as both of these products function exclusively in this organelle (Tanaka and Tanaka, 2007). Nonetheless, exact localization of particular enzymatic steps remains unclear. Also, localization of the enzymes responsible for heme biosynthesis is as yet unknown. The two final enzymes in this pathway, PP IX oxygenase and FeCH, were reported to be targeted to both mitochondria and plastids, however, there is still no consensus among researchers regarding this issue (Lister *et al.*, 2001). Similarly in cyanobacteria, there is relatively little data about localization of the

chlorophyll/ heme biosynthesis in different cell compartments (e.g. stroma, lumen, thylakoid or cytoplasmatic membrane) (Vavilin and Vermaas, 2002).

Animals, fungi, yeast, and α-proteobacteria synthesize ALA by a distinct route involving the condensation of glycine and succinyl-coenzymeA by a specific ALA synthase enzyme (O'Brian and Thony-Meyer, 2002). Naturally, non-photosynthetic organisms do not have the chlorophyll synthesizing branch of the tetrapyrrole pathway. As will be discussed in more detail later, this makes control of tetrapyrrole metabolism much simpler.

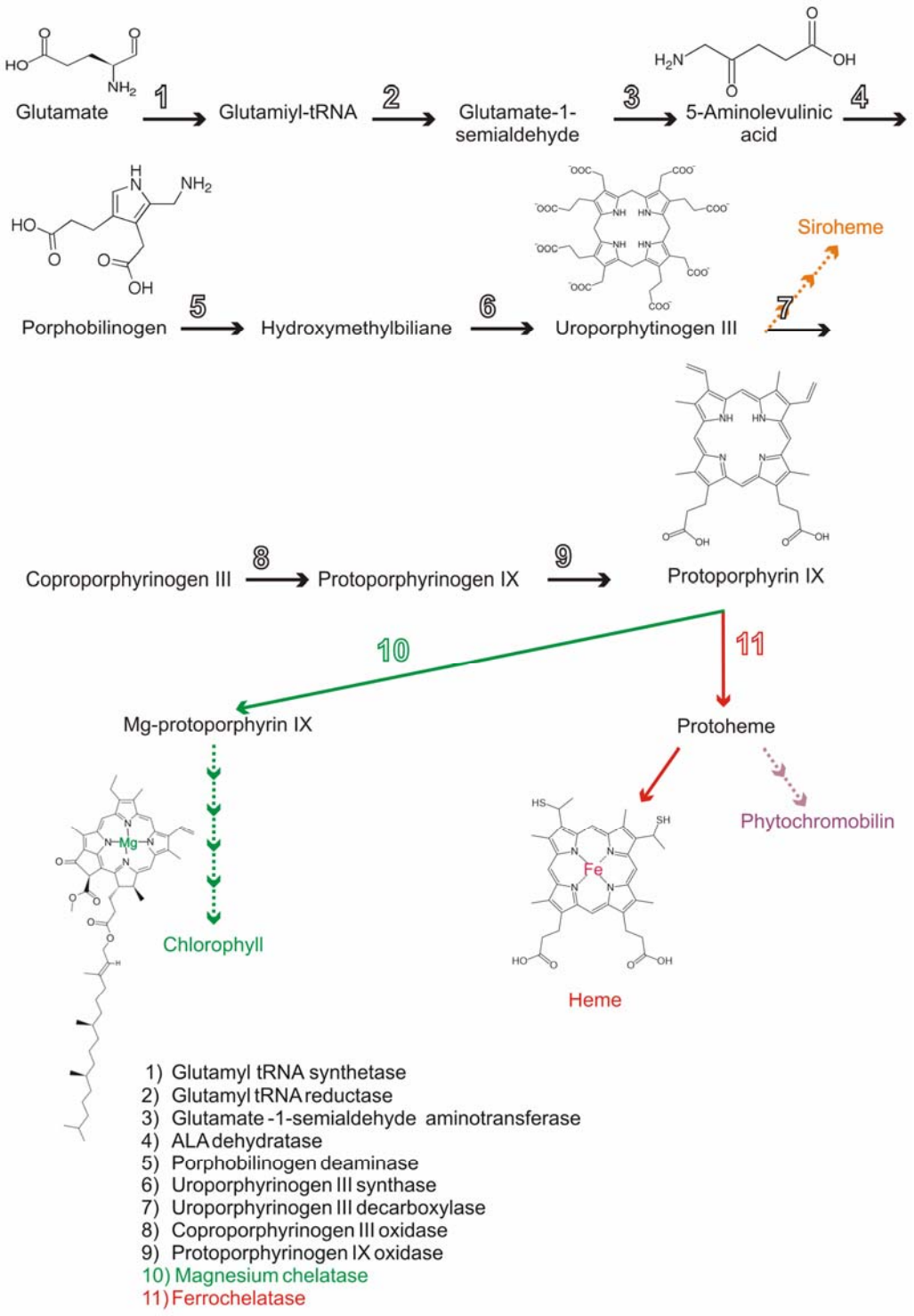



**Fig. 1.1:** The tetrapyrrole biosynthetic pathway in photosynthetic organisms. The complexity of this pathway suggests that sophisticated regulatory mechanisms must have evolved to ensure the required amounts of each end product in different conditions.
Important precursors are illustrated with structures above their names. Arrows symbolize steps catalyzed by enzymes with numbers indicating the name of the particular enzyme in the legend. Dashed arrows indicate more than one enzymatic step (one arrow in line corresponds to one step). Branchpoints are highlighted in color.

## 1.2 Regulation of the tetrapyrrole pathway – the emerging role of the ferrochelatase

Demand for the end products of the different tetrapyrrole biosynthetic branches can change according to the growth conditions of the culture e.g. light intensity. However, a photosynthetic cell cannot accumulate intermediates of chlorophyll/heme biosynthesis, as practically all of these molecules, even back as far as uroporhyrinogen III, are phototoxic compounds. They are readily excited by light and, under aerobic conditions, produce extremely toxic singlet oxygen – a powerful oxidant of amino acids, unsaturated fatty acids and other biomolecules. A high concentration of singlet oxygen inevitably damages cell structures by photo-oxidation (reviewed in Cornah *et al.,* 2003). Thus, mechanisms able to control the levels of any hazardous intermediates must exist to cope with the problem of photo-oxidation.

The formation of ALA was reported to be the first and key regulatory point of tetrapyrrole biosynthesis in mitochondria, as well as in bacteria, which makes sense given that ALA is the first common precursor for all tetrapyrroles and is involved exclusively in the biosynthesis of these compounds. It is well established that the ALA synthase enzyme is allosterically inhibited by heme (O'Brian and Thony-Meyer, 2002; Fig. 1.2 A). In contrast, in plants, algae and cyanobacteria, ALA is synthesized in three steps, one of which is catalysed and regulated by glutamyl-tRNA reductase (GluTR). This enzyme catalysis the first step unique to tetrapyrrole formation and it appears to be the most important regulatory step. Expression of this enzyme in plants was found to be stringently regulated by light, phytohormones and an endogenous circadian clock (Kumar *et al.,* 1996; Tanaka *et al.,* 1996; McCormac *et al.,* 2001; Yaronskaya *et al.,* 2006). Recent findings have confirmed that activity of GluTR is controlled by feedback inhibition. Failure to repress GluTR activity in the dark let to increased accumulation of protochlorophyllide in the *flu* mutant of *A. thaliana* (Goslings *et al.*, 2004). Algal and plant GluTRs are inhibited post-translationally by a specific FLU protein (Meskauskiene *et al.,* 2001). It was found that FLU, a small protein found in plastid membranes, is able to physically interact with GluTR and thus suppress its activity, specifically when an increased accumulation of chlorophyll precursors is detected (Meskauskiene *et al.*, 2001).

It is highly likely that the heme feedback loop also operates in plants (Weinstein *et al.,* 1993; Goslings *et al.,* 2004) and cyanobacteria (Sobotka *et al.*, 2008). It has been reported that a strain of the cyanobacterium *Synechocystis* 6803 with a reduced activity of FeCH had greatly increased levels of ALA, suggesting a direct link between heme level and the

rate of ALA formation (Sobotka *et al.*, 2008). Direct control via heme is further supported by analysis of recombinant GluTRs. It has been shown that the addition of heme inhibits the activity of recombinant GluTRs from the green sulphur bacteria *Chlorobium vibrioforme* and barley, purified from *Escherichia coli*, and in each case the protein was purified in a heme-bound state (Vothknecht *et al.,* 1998; Srivastava and Beale, 2005). Therefore, it is likely that heme evolved as a universal inhibitor of tetrapyrrole biosynthesis in virtually all groups of organisms, despite the existence of two different targets of inhibition - GluTR and ALA synthase.

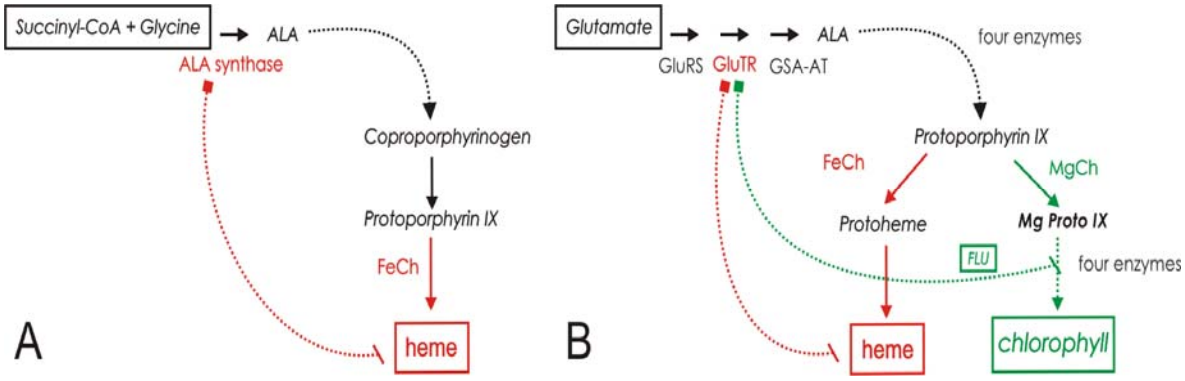



**Fig. 1.2: A**) Feedback inhibition of ALA synthase by heme in α-proteobacteria and mitochondria. **B**) The predicted feedback loops controlling the activity of glutamyl-tRNA reductase in photosynthetic organisms.

Another mechanism controlling metabolite flow through the pathway may involve the family of early light-induced proteins (called ELIP proteins in plants or HLIP/Scp in cyanobacteria). This family of proteins has a conserved region expected to contain one or several transmembrane helices with similarity to the chlorophyll–binding region of the LHC proteins (Light Harvesting Complex) in higher plants. They appear to have no light-harvesting functions but were found to be synthesized in higher levels in response to excess excitation energy stress such as chilling, nitrogen or sulphur deprivation and high light conditions (Vavilin and Vermaas 2002). DNA microarray data have also confirmed that *scp* genes are expressed under stress conditions such as high light, low temperature, hyper osmotic stress, salt stress, or the presence of inhibitors of photosynthetic electron transport (Promnares *et al.* 2006). It was recently reported that Scp proteins participate in the regulation of tetrapyrrole biosynthesis in cyanobacteria (Xu *et al.* 2002) and that the physiological function of ELIPs could be related to the regulation of chlorophyll concentration in higher plants (Tzvetkova-Chevolleau *et al.,* 2007). It has been speculated that the conserved chlorophyll-binding motif of ELIP/Scps could allow these proteins to

function as sensors of chlorophyll deficiency or over-accumulation (Vavilin and Vermaas 2002).

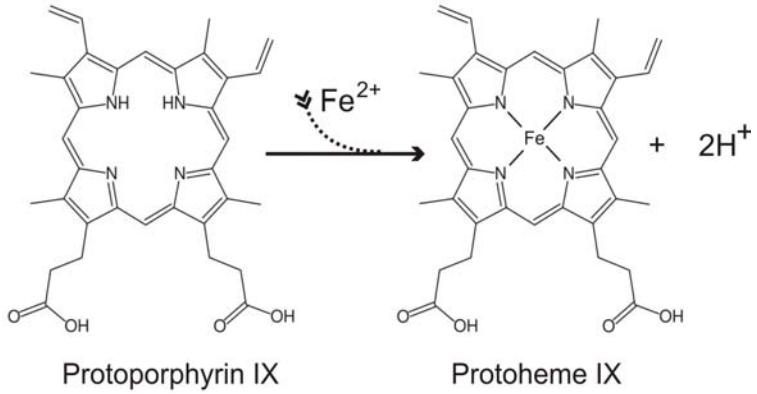



**Fig. 1.3:** The reaction catalysed by ferrochelatase.

FeCH has become an enzyme of particular interest regarding the regulation of the pathway over the last decade. Increased production of heme results in feedback control by inhibiting synthesis of ALA at the start of the pathway. The activity of the enzyme may regulate flux down the heme and Chl branches of the pathway by differential consumption of the common PP IX substrate (Sobotka *et al.,* 2005; Yaronskaya *et al.,* 2003). FeCH targeted to the plastid (plastid-type) has a segment with high similarity to ELIP/Scp proteins (Fig.1.4) which, as mentioned earlier, were found to modulate tetrapyrrole biosynthesis (Funk and Vermaas, 1999). Indeed, among the plant tetrapyrrole biosynthesis mutants only FeCH antisense plants showed a necrotic phenotype, which is in accordance with the expectation that ALA synthesis, the product of which regulates total flux through pathway, is inhibited by heme (Papenbrock *et al.*, 2001). Furthermore, it was reported recently that FeCH activity is involved in the regulation of chlorophyll biosynthesis, as decreased activity of this enzyme was followed by substantial increase in the level of chlorophyll precursors and in the accumulation of chlorophyll (Sobotka *et al.* 2005).

## 1.3 Structural and functional features of the plastid type ferrochelatase

As mentioned earlier and illustrated by figures 1.1 and 1.3, FeCH catalyzes the insertion of $Fe^{2+}$ into PP IX to form protoheme (heme *b*). Currently, the *Bacillus subtilis* and human FeCHs are most commonly used for biophysical and kinetic studies and FeCH crystal structures from these two organisms, as well as *Bacillus anthracis* (Muller *et al.*, to be published) and *Sacharomyces cerevisiae* are available (Wu *et al.,* 2001; Karlberg *et al.*, 2002). Although FeCH catalyses the same enzymatic reaction in all organisms, it varies substantially in amino acid sequence, molecular size, subunit composition, solubility, and the presence or absence of [2Fe-2S] cluster. However, we can predict some structural properties from our knowledge of the human, *Bacillus* and yeast enzymes (Dailey *et al.*, 2000).

Human FeCH is a homodimer attached to the membrane via a hydrophobic 12-residue region on one of the active site lips. However, this hydrophobic segment is absent in the water-soluble *B. subtilis* enzyme. It appears that all FeCHs lacking this hydrophobic segment from different sources exist in a soluble form (e.g. *Bacillus*, *Staphylococus*, *Mycobacterium,* etc.). In addition, the dimerization motif of the human enzyme is the carboxyl-terminal extension, missing in the monomeric FeCH from *B. subtilis*. Thus amino acid sequence information could tell us whether *Synechocystis* FeCH forms a dimer (Burden *et al.,* 1999; Al-Karadaghi *et al.,* 1997). If one assumes that a protein lacking both a hydrophobic segment and carboxyl-terminal extension is a water-soluble monomer, the enzymes from *Streptomyces*, *Propionibacterium*, and *Mycobacterium* would be expected to be soluble dimers and in bacteria, such as *Escherichia coli*, *Haemophilus* and *Rhodobacter,* FeCHs would be membrane-associated monomers (Dailey *et al.,* 2000).

In contrast to other groups of organisms, plants require heme in plastids as well as in mitochondria. It was found that higher plants possess two FeCH isozymes – one targeted to both mitochondria and plastids and the second targeted specifically to plastids (Dailey and Dailey 2003). The second isoform (plastid-type) is the only form found in cyanobacteria and algae and its expression is controlled by light (Papenbrock *et al.,* 1999). Plastid-type FeCH has a catalytic domain similar to other FeCHs, with a hydrophobic segment which binds the enzyme to the membrane (Fig. 1.5; Sobotka *et al.,* 2008). A specific feature of plastid-type FeCH, found only in organisms that synthesize chlorophyll, is a C-terminal extension with high similarity to a transmembrane motif found in LHC proteins or ELIP/Scp proteins. This so called CAB domain, with a putative chlorophyll binding motif,

is highly conserved throughout photosynthetic organisms and is connected to the catalytic domain of FeCH by a more variable spacer (Figs. 1.4 & 1.5). The CAB domain was first speculated to function in binding or targeting the enzyme to the thylakoid membrane (Jansson 1999). However, it has recently been shown that the *Synechocystis* mutant lacking the entire C-terminal extension is still a membrane-bound protein (Sobotka *et al.*, 2008). Interestingly, this mutant strain has strongly reduced FeCH activity both *in vivo* and *in vitro*, which resulted in dramatic changes to tetrapyrrole biosynthesis (Sobotka *et al.*, 2008).

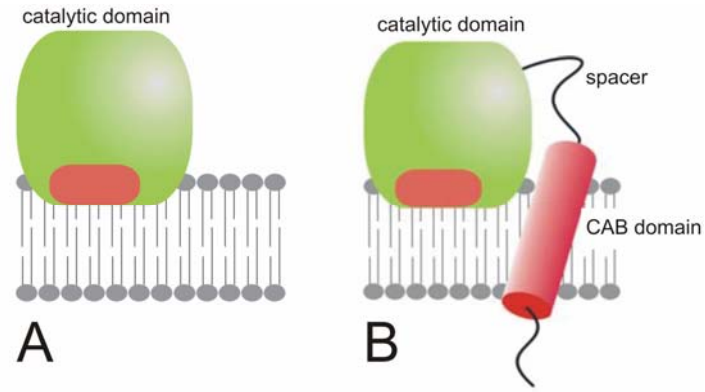



**Fig. 1.4: A)** Model of non-plastid-type FeCH attached to the membrane via a hydrophobic segment (red ellipse). **B)** Predicted model of plastid-type FeCH showing the C-terminal transmembrane CAB domain connected to the catalytic core by a spacer sequence (Sobotka *et al*., 2008).

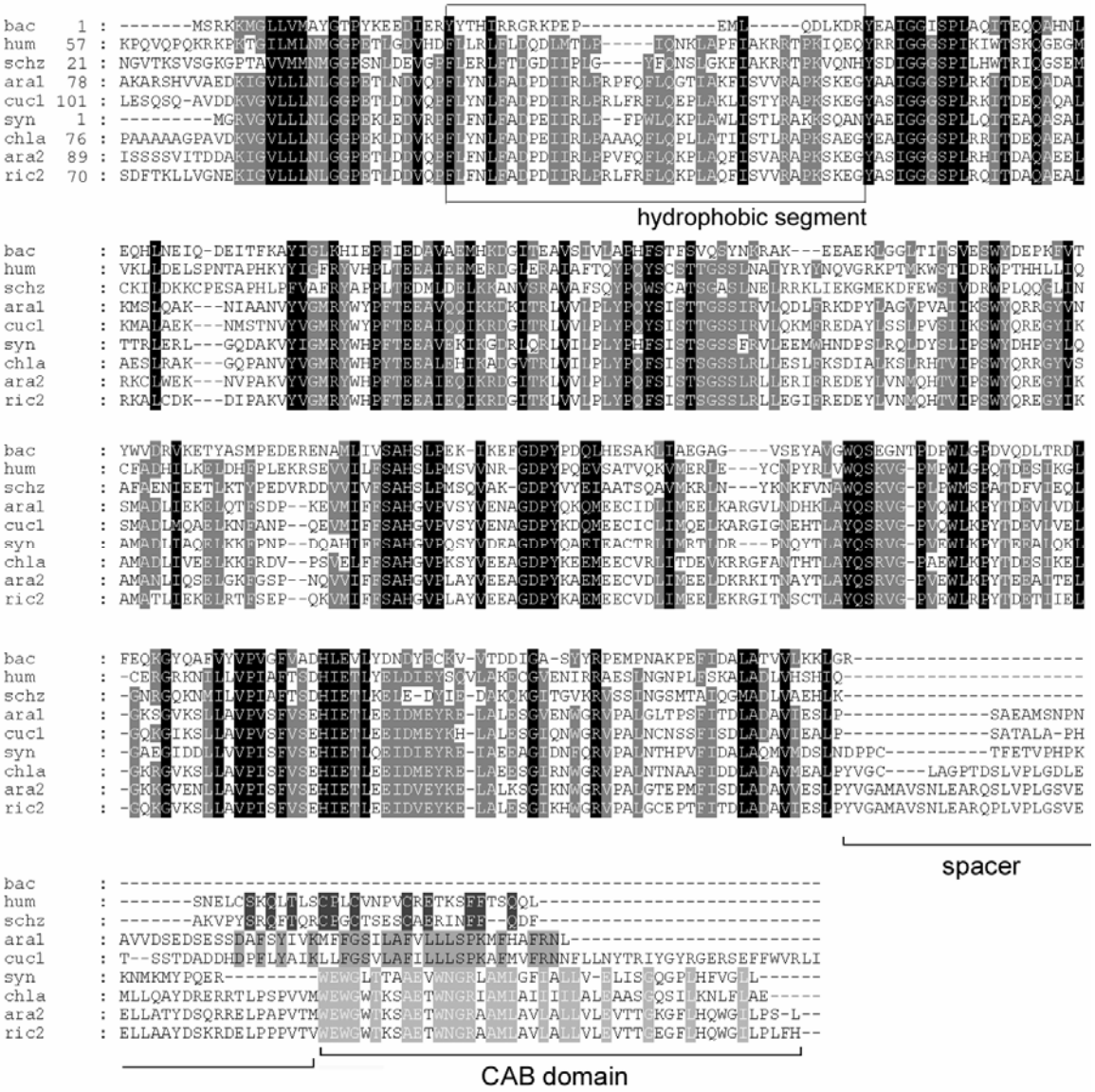



**Fig. 1.5:** Amino acid alignment of FeCH proteins from *Bacillus subtilis* (bac), human (hum), *Scizosaccharomyces pombe* (schz), Arabidopsis isoform I (ara1), cucumber isoform I (cuc1), *Synechocystis* (syn), *Chlamydomonas* (chla), Arabidpopsis isoform II (ara 2) and rice isoform II (ric 2). The targeting sequences in eucaryotic FeCHs have been omitted for clarity. The hydrophobic region at the N-terminus is boxed, the spacer region and the C-terminal CAB domain of Synechocystis FeCH are also shown.

## 1.4 Working hypothesis and aims of the project

As described in chapter 1.2, photosynthetic organisms need to exert stringent control of tetrapyrrole biosynthesis to avoid photo-oxidation. Based on accumulating data it is expected that FeCH plays a very important role in regulation and thus there may be some fast-acting mechanism(s) modulating FeCH activity on a post-translational level. It is logical to predict that the C-terminal extension of FeCH, unique to chlorophyll-producing organisms, may play a role in this regulation. It was found recently that FeCH purified directly from *Synechocystis* using a 3xFLAG tag is a dimer whereas the truncated enzyme lacking the C-terminal extension is strictly monomeric (Sobotka, unpublished data). Interestingly, the FeCH CAB domain in *Synechocystis* has a high sequence similarity with Scp's proteins, especially with the ScpB protein (Fig. 1.6). It is possible that, under stress conditions, accumulating Scps interact with the FeCH CAB domain, thus preventing dimerization. This could be part of a control mechanism; either directly influencing activity or triggering changes in localization or protein complex assembly (Fig. 1.7).

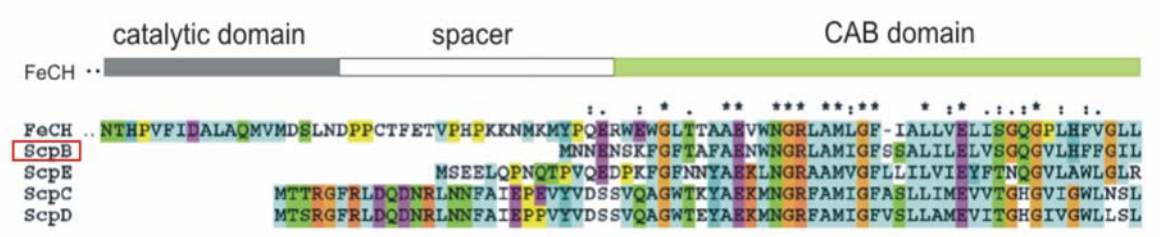



**Fig. 1.6:** Amino acid sequence alignment of the C-terminal extension of *Synechocystis* FeCH with four Scp proteins found in the genome of this cyanobacterium. The ScpB protein, most similar to the FeCH CAB domain, is boxed in red.

To test this hypothesis and to answer other questions concerning the function of FeCH in a photosynthetic cell, highly active recombinant *Synechocystis* FeCH would be invaluable. Therefore, the main aim of this thesis is to prepare active recombinant FeCH from the cyanobacterium *Synechocystis* 6803 in *Escherichia coli*. *Synechocystis* 6803 serves as a model organisms for studies on photosynthesis since it is unicellular photosynthetic organism which has a small (3.5 Mbp), fully sequenced genome (1996), is naturally transformable and is capable of homologous recombination. However, sufficient overexpression of a desired protein in this organism has not yet been possible.

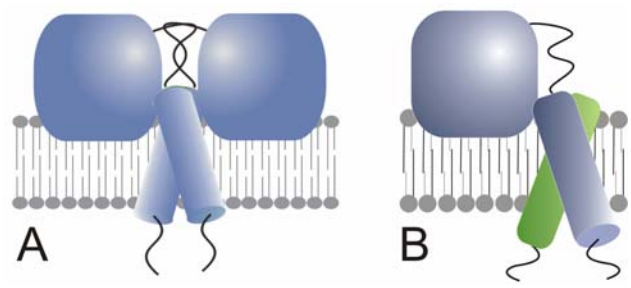



**Fig. 1.7: A)** Predicted model of dimeric FeCH as a typical active form in the cell. **B)** A model of FeCH forming a specific complex with the Scp protein(s) under stress conditions when Scp highly accumulate in the cell.

The aims of this project are:

- to obtain pure and active recombinant FeCH of *Synechocystis* 6803
- to compare the activities and oligomerization of purified recombinant FeCH with native 3xFLAG-FeCH purified directly from *Synechocystis*.
- to prepare an expression vector for the preparation of *Synechocystis* ScpB in *Escherichia coli* for a future studies on FeCH – Scp interactions.

A successful preparation of plastid-type FeCH (cyanobacterial, algal or plant) has not been reported yet indicating problem with expression/purification of this enzyme in *E. coli*. Indeed, preliminary data obtained by Roman Sobotka demonstrated difficulties in the preparation of recombinant *Synechocystis* FeCH. As apparent from Fig. 1.8 His-tagged FeCH tends to form high-mass inactive aggregates broken only by treatment with dithiothreitol (DTT). So, FeCH aggregates are probably cross-linked with disulphide bounds and moreover, even after a reduction of FeCH by DTT, this enzyme was found to be practically insoluble in commonly used detergents (Sobotka, unpublished).

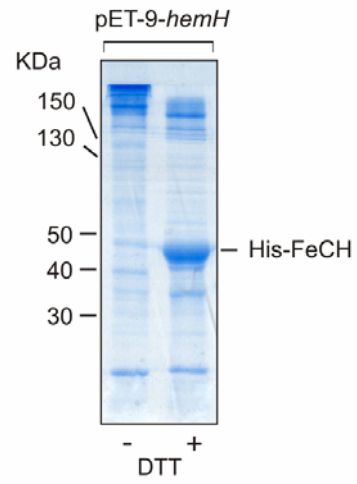



**Fig. 1.8:** Aggregation of the *Synechocystis* His-tagged FeCH expressed in *E. coli*. The same amount of membrane proteins from *E. coli* strain possesses pET-9- *hemH* expression vector was analyzed on the SDS-PAGE. DTT + indicates incubation of a sample with 100 mM DTT for 30 min.

## 2. MATERIALS AND METHODS

As the main aim of this thesis was the preparation of protocol for the purification of recombinant FeCH, progress in optimization will be described in result section and only a final version of this protocol will be described here.

### 2.1 Construction of a recombinant FeCH expression vector

The *hemH* gene coding for FeCH was amplified by PCR using gene-specific oligonucleotides with designed restriction sites for *Bgl*II and *Not*I restriction enzymes (Tab. 2.1) and chromosomal DNA from wild-type *Synechocystis* 6803 was used as a template. The resulting product was purified using the GenElute PCR Clean-up kit (Sigma-Aldrich, Germany) and subsequently digested with the relevant restriction enzymes (Fermentas, Germany). This insert was ligated into pGEX-6p (GE Healthcare, Germany) and pET-49 (Novagen, USA) plasmids via the same restriction sites. Another FeCH expression construct, pGEX-4T-*hemH*, was kindly provided by Prof. Neil Hunter (Sheffield University). All constructs are depicted in Fig. 2.1.

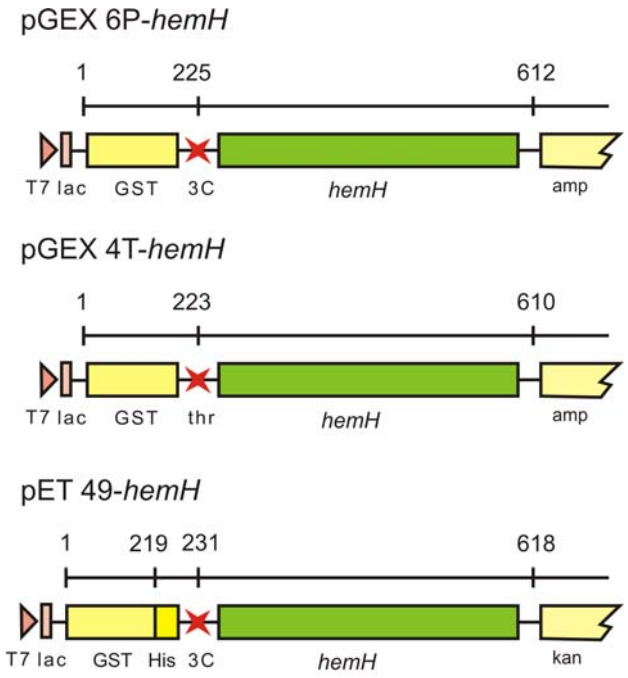



**Fig. 2.1:** List of constructs used for the expression of FeCH in *E. coli*. Values above each construct indicate the length of the fusion proteins in amino acids. T7 = T7 promotor; lac = lactose operon, GST = glutathion-S-transferase, His = 6xhistidine tag, 3C = HRV 3C protease cleavage site, thr = thrombine cleavage site, amp = ampicillin resistance cassette, kan = kanamycin resistance cassette.

All three constructs (pGEX-6P-*hemH*, pGEX-4T-*hemH*, pET-49-*hemH*) were transformed into electrocompetent *E.coli* BL21 (DE3) cells (Novagen, USA) and plasmid containing colonies were selected for on LB plates supplemented with ampicillin (100 μg $ml^{-1}$) for the pGEX constructs or kanamycin (30 μg $ml^{-1}$) for the pET vector. pGEX-6P-*hemH* and pET-49-*hemH* plasmids prepared for this project were verified by sequencing.

## 2.2 Expression and solubilization of the recombinant FeCH

To express recombinant FeCH 100 ml of LB media with appropriate antibiotic was inoculated with *E. coli* BL21 (DE3) cells containing an expression vector. Cells were grown at 37°C with constant shaking at 300 rpm until they reached an $OD_{600}$ ~ 0.6 when transcription of the gene was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was then left shaking overnight at 16°C. To reveal the best conditions for fractionating of the cells and solubilization of recombinant FeCH we tested several different buffers, detergents, the effect of sodium dodecyl sulfate (SDS), salts and urea (see Results section).

The Protocol described here provided the best yield of active soluble FeCH: A total of 100 ml of induced cells were harvested and washed with solubilization buffer containing 50 mM potassium phosphate pH 7.5, 5mM MgCl, 0.2 M NaCl, 10 % glycerol and 4mM dithiothretiol (K-Phos buffer) and resuspended in 1ml of the same buffer with Protease Inhibitor Cocktail (Roche, Germany). The cell suspension was broken in a Mini-BeadBeater and the resulting homogenate was centrifuged at 22,000 x g for 10 min at 4°C and the supernatant was discarded. Pelleted membranes were resuspended in the same volume of the K-Phos buffer containing 2 % Triton X-100 and 0.1 % SDS and solubilized overnight at 10°C. Solubilized membranes were centrifuged at 22,000 x g for 10 min and resulting supernatant was filtered through a 0.22 μm Spin-X filter (Corning Incorporated, USA). Solubilized protein was stored at -80°C.

## 2.3 Purification of recombinant GST-FeCH

Solubilized and filtered membrane proteins containing active FeCH were diluted 1:1 with K-Phos buffer to give a final concentration of 1% Triton X-100 and 2mM dithiothreitol in a final volume of 2 ml. The sample was mixed with 300μl of Glutathione Sepharose High Performance resin (GE Healthcare, Germany), equilibrated with K-Phos buffer, and

incubated at room temperature for 3 hours. The resin was transfered into a column, washed with 6 ml of K-Phos buffer containing 0.04 % dodecyl-β-maltoside (DDM). The washed resin was incubated overnight at 8°C with 800 µl of K-Phos buffer containing 0.04 % DDM, 1 mM DTT and 15 units of HRV 3C Protease (Novagen, USA) to cleave the GST-tag. The sample was then filtered using a 0.22 µm Spin-X filter and concentrated 4 times using a 50 KDa cut off microcon (Millipore, USA).

The binding of GST-FeCH to the Glutathione Sepharose resin was found to be relatively weak (see Results), therefore an alternative purification procedure using polyclonal anti-GST antibodies (Sigma-Aldrich, Germany) was attempted. To prepare a specific resin anti-GST antibodies were coupled to AminoLink resin (Pierce, USA) using the ProFound Co-Imunoprecipitation Kit (Pierce, USA) following the manufacturer's instructions. The efficiency of coupling was measured as a decrease in absorbance of the antibody at 280nm after the coupling procedure. Anti-GST resin was washed with Hepes buffer (25 mM Hepes pH 7.4, 20 mM $MgCl_2$, 10 mM $CaCl_2$, and 20 % glycerol, 0.2 M NaCl) containing 1% Triton X-100. A solubilized, filtered sample, diluted to 4 ml was added to the anti-GST resin and incubated for 30 min at room temperature. The suspension was then loaded onto a column and washed with 10 ml of Hepes buffer containing 1% Triton X-100. The resin was then incubated for 3 hours at room temperature with 800 µl of the same buffer containing 15 units of HRV 3C protease and filtered to obtain a supernatant containing purified FeCH.

## 2.4 Measurement of FeCH activity

Enzyme activity was monitored spectrofluorometrically at 35°C by directly recording the rate of zinc-PP IX formation using an Aminco-Bowman Series 2 Spectrofluorometer (Thermo Scientific, USA). The reaction mixture (1.5 ml final volume) contained 0.3 M Tris/HCl pH 8.0, 0.03 % Tween 80, 2 µM PP IX and 2 µM $ZnSO_4$. Recombinant FeCH was assayed by adding of 3 µl of protein sample to the reaction mixture (Sobotka *et al.*, 2008).

## 2.5 SDS-elerophoresis and size-exclusion chromatography

Protein samples for SDS-electrophoresis were denatured by incubation with 2% SDS and 1% DTT for 30 min at room temperature. Precipitates in the sample were removed by centrifugation at 22,000 x g for10 min. Proteins were separated on the BioRad-MiniProtean system using 4% acrylamide-bis solution for the stacking gel and 12 % for the resolving gel. Electrophoresis was run in tris-glycine buffer at 4 mA for 30 min and then 12 mA for 2 hours. Proteins were stained with Commassie brilliant blue.

Gel filtration chromatography was carried out on the BioSep SEC-S2000 300 x 7.80 mm column (Phenomenex, USA) connected to an Agilent 1200 photodiode array detector (Agilent, USA). The column was equilibrated with 20 mM Hepes buffer pH 7.4, 0.1 M NaCl, 10 % glycerol and 0.04% dodecyl-β-maltoside. The flow rate was set to 0.15 ml $min^{-1}$.

## 2.6 Cloning and expression of the ScpB protein in *Escherichia coli*

The *scpB* gene was amplified by PCR using oligonucleotides with designed restriction sites for *Bgl*II and *Not*I enzymes and *Synechocystis* genomic DNA was used as the template. The forward primer included the DNA sequence of the Strep II tag (Tab. 1). The resulting product was cloned into the pGEX-6P (Fig. 2.2) vector as described for recombinant FeCH. Expression and solubilization of the recombinant GST-Strep-ScpB protein was carried out as described for recombinant FeCH.

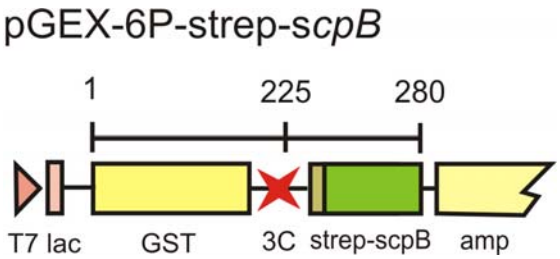



**Fig. 2.2:** The pGEX-6p-Strep-*scpB* vector used for the expression of the Strep-ScpB protein. Values above construct indicate length of resulting protein inamino acids. T7 = T7 promotor; lac = lactose operon, GST = glutathion-S-transferase, 3C = HRV 3C protease, amp = ampicillin rezistance cassette.

| gene | primer | orientation | sequence (5' – 3') |
|---|---|---|---|
| *hemH* | hemH-*Bgl*II | forward | CTTAACAAGATCTGGTCGTGTTGGGGTCTTAC |
| | hemH-*Not*I | reverse | GACTTTCAGCGGCCGCCTAAAGCAAGCCGACAAAATGC |
| *ScpB* | scpB-Strep-*Bgl*II | forward | GTTCTTAGATCT**TGGTCCCATCCCCAATTTGAAAAA**AACA ACGAAAACTCTAAATTTGGATT |
| | scpB-*Not*I | reverse | GAACTAGCGGCCGCTTACAGAATGCCGAAGAAGTGAAG |

**Tab. 2.1:** List of oligonucleotides used for the cloning of *hemH* and *scpB* genes into pGEX-6P and pET-49 expression vectors. Red letters indicate restriction sites. The StrepII tag sequence underlined in bold type.

## 2.7 Expression of the 3xFLAG-FeCH protein in *Synechocystis*

The *Synechocystis* FLAG-FeCH/Δ*hemH* strain expressing the FeCH enzyme fused with a 3xFLAG tag was prepared and kindly provided by Dr. Martin Tichy (Institute of Microbiology, Trebon). This strain was constructed using the pSK9-FLAG-*hemH* vector (Fig. 2.3) generously provided by Prof. Annegret Wilde (University of Giessen, Germany). Wild-type *Synechocystis* cells were transformed with the pSK9-FLAG-*hemH* plasmid and consequently the *hemH* gene, including the *petJ* promoter, was inserted into the chromosome via homologous recombination. The native *hemH* gene was then deleted using a PCR-based mutagenesis protocol (Sobotka *et al.* 2008, Martin Tichy, unpublished data). The *petJ* promoter is regulated by the presence of $Cu^{2+}$ in the growth medium. In practice, maximum expression of FLAG-FeCH was obtained when the strain was grown in medium containing 10mM glucose and completely lacking $Cu^{2+}$.

The FLAG-FeCH/Δ*hemH* strain was growth photoautotrophically in liquid BG-11 medium (Rippka *et al.*, 1979) supplemented with 10 mM TES (N/tris(hydroxymetyl)methyl-2-aminoethanesulfonic acid) at 30°C under normal light conditions (30 μmol photons $m^{-2}s^{-1}$) on a rotary shaker. To induce expression of FeCH, *Synechocystis* cells were transferred to BG-11 medium lacking $Cu^{2+}$, supplemented with 10 mM glucose.

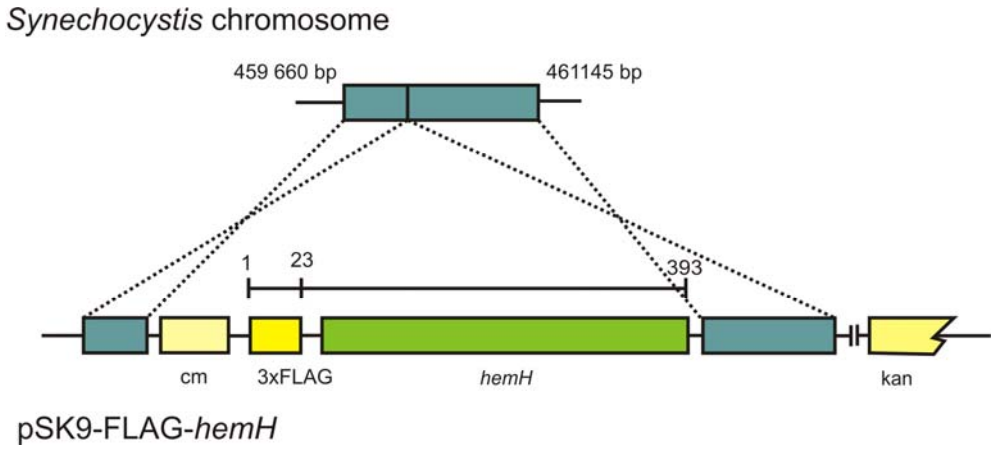



**Fig. 2.3:** Construction of the *Synechocystis* FLAG-*hemH* strain used for the expression and purification of 3xFLAG-FeCH directly from the cyanobacterium. Values above the pSK9 construct indicate amino acids. cm = chloramphenicol resistance cassette; 3xFLAG = repeated amino acid sequence [DYKDDDDK] of the protein tag, kan = kanamycin resistance cassette.

## 2.8 Purification of the 3xFLAG FeCH protein using affinity chromatography

For purification of the FLAG-FeCH a total volume of 4 l of *Synechocystis* cells with an $OD_{750}$ ~ 0.5-0.7 was harvested and washed in thylakoid buffer containing 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 20 % glycerol. Cells were resuspended in 8 ml of thylakoid buffer containing Complete Protease Inhibitor (Roche, Germany), mixed with glass beads and disrupted in a BeadBeater (BioSpec, USA). The resulting homogenate was centrifuged at 40,000 x g for 20 min at 4°C. The supernatant was discarded and pelleted membranes were resuspended in 20 ml of thylakoid buffer, transferred into a new centrifuge tube and pelleted again at 40,000 x g for 10 min at 4°C. The washed membranes were then resuspended in 8 ml of thylakoid buffer containing 1 % DDM and incubated on a rotor at 8°C for 30 min. The sample was then centrifuged at 40,000 x g for 10 min at 4°C to solubilized protein from the membranes and the supernatant was filtered through a 0.22µm Rotalibo filter (Roche, Germany).

The FLAG-FeCH protein was purified on a column containing 300 µl Anti-FLAG M2 Affinity Gel (Sigma-Aldrich, Germany) equilibrated with thylakoid buffer containing 0.04 % DDM. The solubilized protein fraction was passed through the column twice by gravity flow. The resin was washed with 12 ml of thylakoid buffer containing 0.04 % DDM. To elute the protein, the anti-FLAG-resin was incubated in 600 µl of elution buffer (thylakoid buffer, 0.04 % β-DDM, 100 µg $ml^{-1}$of the 3 x FLAG peptide, Sigma-Aldrich, Germany) for one hour at room temperature. The resulting slurry was filtered through a 0.22 µm Spin-X filter to remove the resin from the protein sample.

# 3. RESULTS

## 3.1 Description of vectors used in this project

The primary aim of my project was to develop a protocol for the preparation of active recombinant FeCH from *Synechocystis* by expression in *E. coli.* For this purpose three different expression vectors were tested: pGEX-4T-*hemH*, pGEX-6P-*hemH* and pET-49-*hemH* (Fig. 2.1). The pGEX-4T-*hemH* plasmid was provided as a generous gift from Prof. Neil Hunter (Sheffield University); pGEX-6P-*hemH* and pET-49-*hemH* were prepared during this project as described in the *Material and Methods* section.

All three vectors express FeCH fused to a glutathione S-transferase (GST) protein (Fig. 2.1). GST is a ubiquitous enzyme that catalyzes the addition of glutathione to various hydrophobic electrophilic molecules. The high affinity shown by GST to glutathione is utilised for the purification of GST-fused proteins by affinity chromatography with immobilized glutathione (Viljanen *et al.*, 2007). Another important advantage of GST-tags in protein purification is the hydrophilicity of the protein. This property can markedly improve the solubility of a linked hydrophobic protein when expressed (Promdonkoy *et al.*, 2008). This could be crucial for the preparation of proteins such as *Synechocystis* FeCH, already known to be almost completely insoluble and prone to aggregating when overexpressed in *E. coli* (Fig 1.8).

The pGEX-4T-*hemH* vector was first available for pilot expression experiments showing high and stable expression (Fig 3.1), however, throughout the project this plasmid was used primarily as a control for pGEX-6P-*hemH.* This second plasmid was considered to be more suitable for the purification of active FeCH since it contains the HRV 3C protease cleaving site between the tag and FeCH, instead of a thrombin cleavage site present in pGEX-4T-*hemH.* In contrast to thrombin – a slow enzyme active at room temperature, the HRV 3C protease demonstrates faster cleavage even at lower temperature (4-8°C) reducing any loss in activity of the purified protein (Cole and Marks, 1984).
Initial attempts to purify GST-FeCH using glutathione affinity chromatography were unsuccessful (see below), therefore another construct was prepared (pET-49-*hemH*). In contrast to pGEX-6P-*hemH,* pET-49-*hemH* also contains a 6xHistidine (His) tag. Expression from this plasmid leads to the production of GST-His-FeCH protein, thus allowing purification by metal-ion affinity chromatography.

## 3.2 Expression of recombinant FeCH

The BL21 (DE3) strain of *E. coli* (Novagen) was used for overexpression from the three plasmids. BL21 (DE3) possesses a highly specific viral T7 polymerase under the control of the lac operon T7 promoter. Expression was induced by adding IPTG to a final concentration of 0.4 mM to 100 ml of *E. coli* cell culture at $OD_{600}$ ~ 0.6. The recombinant protein was expressed overnight at 16°C; cells were then harvested and fractionated as described in the *Material and Methods* section. Cell lysates were separated into soluble- and membrane-protein fractions by centrifugation and these fractions were analyzed by SDS-PAGE. Expressed GST-FeCH was exclusively localized in the membrane fraction, consistent with the fact that *Synechocystis* FeCH is a highly hydrophobic, membrane-bound protein (Fig. 3.1 A, Sobotka *et al.*, 2008). However, a portion of FeCH was also localized in inclusion bodies (not shown) but, for simplicity, all proteins pelleted by centrifugation at 14 000 g will be marked as 'membrane fraction' in the following text. Interestingly, the addition of DTT appears to have no effect on the membrane samples (Fig. 3.1 B) suggesting that there is no formation of intermolecular disulphide bonds between the GST-tagged proteins, unlike the observed results after DTT treatment of His-FeCH (Fig. 1.8).

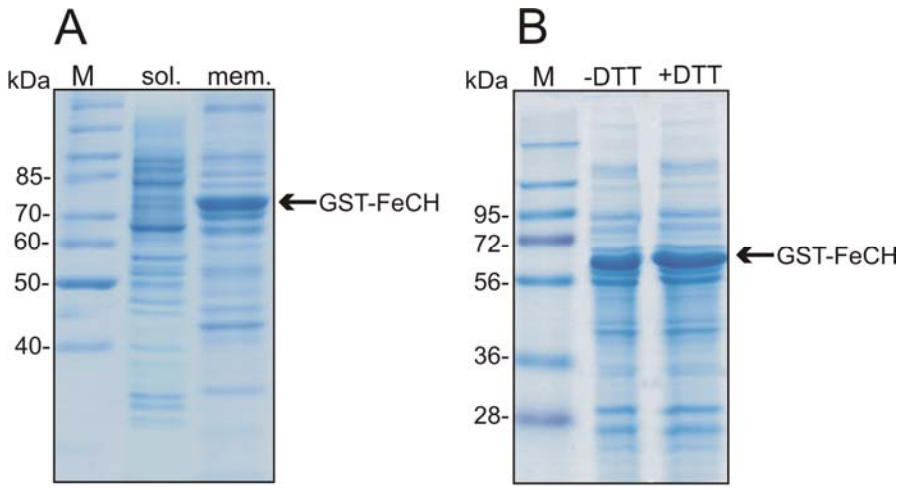



**Fig. 3.1:** Expression and localization of recombinant GST-FeCH in the pGEX-4T-*hemH* strain. **A)** Separation of soluble (sol.) and membrane-protein (mem.) fractions by SDS-PAGE. M = marker. **B)** Comparison of membrane-protein fractions with and without the addition of 100 mM DTT. The protein content from ~100 µl of lysed cells were separated on SDS-PAGE and stained in coomassie blue. The size of recombinant GST-FeCH (~70 kDa) is indicated.

As a next step, expression of FeCH from different vectors was compared. FeCH was expressed as described above and the amount of recombinant protein was analyzed by SDS-PAGE with a non-induced pGEX-4T-*hemH* strain used as a control. FeCH expressed from all three plasmids was present as a major band in each membrane-protein fraction (Fig 3.2). The sizes of the GST-tagged proteins were approximately 70 kDa and slightly higher mass was observed for the GST-His-FeCH protein, as expected. As depicted in Fig. 3.2, GST-His-FeCH expression was significantly lower than the levels observed for the pGEX-expressed proteins.

To reveal whether GST-FeCH is produced in an active form, the enzyme's activity was measured in an *in vitro* FeCH assay. A simple yet sensitive assay was attempted in which zinc, rather than iron, is used as the substrate for FeCH. Trace activity of FeCH can be measured as even a small increase in fluorescence of zinc-protoporphyrin can be detected using specific excitation and emission wavelengths. The observed activity corresponds well to the total amount of FeCH in the assay (Sobotka *et al.*, 2008). The GST-FeCH proteins were both found to be highly active enzymes; however, the GST-His-FeCH protein demonstrated only very low activity (Fig 3.2 B).

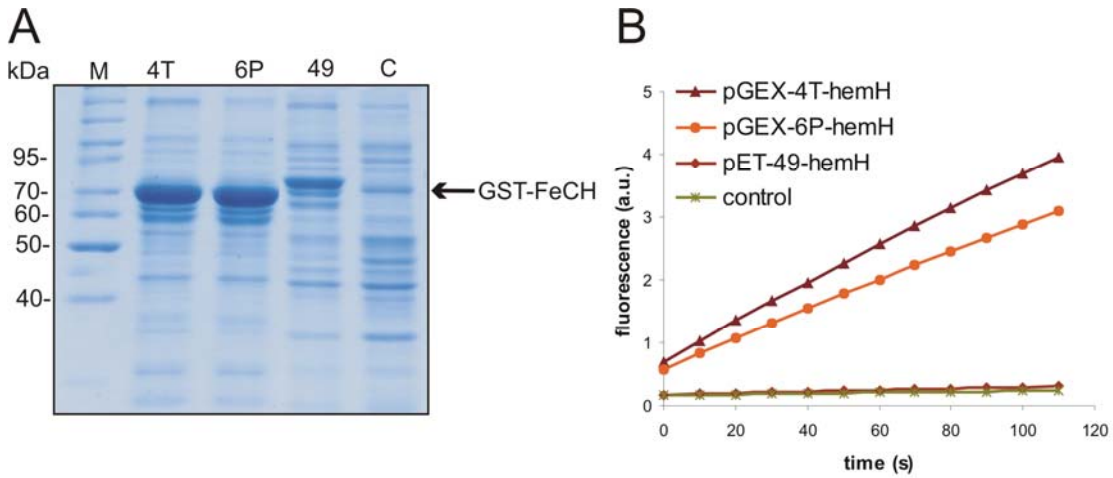
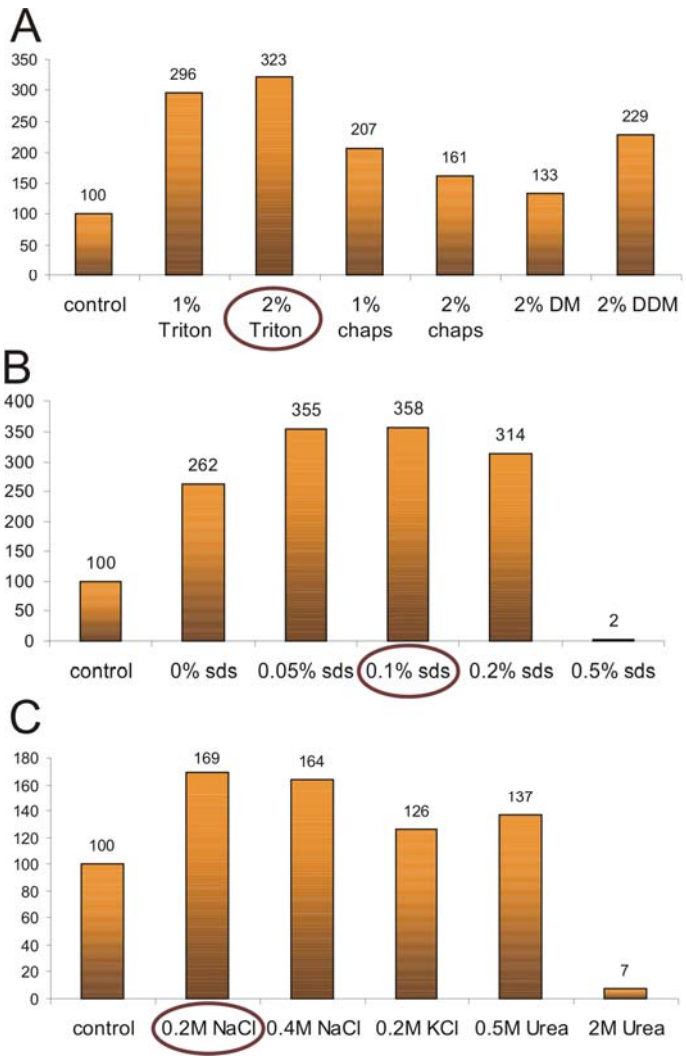



**Fig 3.2:** Accumulation and *in vitro* activity of recombinant FeCH expressed in *E.coli* cells using pGEX-4T-*hemH* (4T), pGEX-6P-*hemH* (6P), pET-49-*hemH* (49). **A)** Membrane proteins from ~100 µl of cells at $OD_{600}$ ~1.2 were separated by SDS-PAGE and stained with coomassie blue; M = marker; C = non-induced pGEX-4T-*hemH* strain. The expected mass of GST-FeCH (~70 kDa) is indicated. **B)** *In vitro* activities of each of the FeCH samples. Assays were conducted using the same volume of protein as loaded onto the gel in A). Continuous spectrofluorometric assays were performed in which an increase in the fluorescence of zinc-protoporphyrin IX corresponded to FeCH activity. Excitation and emission wavelengths of 420 and 590 nm, respectively, were used for the measurements.

## 3.3 Solubilization of recombinant FeCH

To obtain pure recombinant FeCH, it was first necessary to find optimal conditions for the solubilization of this protein. As described in the *Introduction* section, FeCH possesses two hydrophobic domains which make the solubilization of this protein difficult. Thylakoid buffer (20mM Hepes pH 7.4, 10mM $MgCl_2$, 5mM $CaCl_2$, 20 % glycerol, 4mM DTT, protease inhibitor) was found to be suitable for the purification of active FeCH directly from *Synechocystis* using a 3xFLAG tag (Sobotka, in preparation). This buffer was used to test the ability of various detergents to solubilize GST-FeCH (Fig. 3.3 A). The effects of sodium dodecyl sulfate (SDS), different salts and urea on the activity of FeCH were also tested (Fig. 3.3 B, C). After overnight incubation at 8°C on a rotor the yield of solubilized protein in the filtered supernatant was monitored using the FeCH *in vitro* assay.

As K-phosphate buffer is recommended for both the HRV 3C protease and the gluthatione sepharose resin, the solubilization of recombinant GST-FeCH in thylakoid buffer and K-Phosphate buffer (50mM K-Phosphate, pH 7.5, 15% glycerol, 5mM $MgCl_2$, 4mM DTT, protease inhibitor, 0.2M NaCl, 2% Triton X-100) was compared under conditions described above. As illustrated in Fig 3.4 K-Phosphate buffer (K-Phos+) was found to improve GST-FeCH solubilization when compared with the thylakoid buffer. Based on these data K-Phos+ was used as the buffer for the following parts of this project.




**A**: 20mM Hepes pH 7.4, 10mM $MgCl_2$, 5mM $CaCl_2$, 20% glycerol, 4mM DTT, protease inhibitor

**B**: 20mM Hepes pH 7.4, 10mM $MgCl_2$, 5mM $CaCl_2$, 20% glycerol, 4mM DTT, protease inhibitor, **2% Triton**

**C**: 20mM Hepes pH 7.4, 10mM $MgCl_2$, 5mM $CaCl_2$, 20% glycerol, 4mM DTT, protease inhibitor, **2% Triton and 0.1% SDS**

**Fig. 3.3:** The effect of buffer components on solubilization of recombinant GST-FeCH as monitored by *in vitro* activity. **A)** Comparison of different detergents; **B)** Comparison of SDS concentrations; **C)** Comparison of different concentrations of NaCl, KCl and urea. The composition of the buffers used for each comparison are boxed on the right of each graph. The control values for each data set were obtained using the same volume of membrane proteins from an uninduced strain. All samples were solubilized overnight at 8°C and pelleted. FeCH activities of filtered supernatants were then measured. Values above each bar indicate relative FeCH activity in assay in comparison to the control sample. Conditions that were adopted for the final protocol are circled.

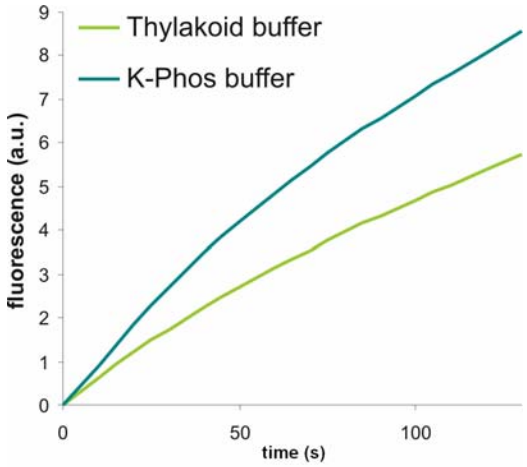



**Fig. 3.4:** Comparison of Thylakoid and K-Phos buffer. *In vitro* activities measured in the sample after solubilization with protease inhibitor, 0.2M NaCl, 2% Triton X-100 and 0.1% SDS. Same amount of protein was used for analysis of both samples.

The solubilization of GST-FeCH in K-Phos+ buffer is shown in Fig 3.5. Unsolubilized membranes were pelleted and the resulting supernatant was filtered through a 0.22 µm filter and analyzed by SDS-PAGE and in a FeCH assay. No notable difference in amount of protein between pGEX-4T-*hemH* and pGEX-6P-*hemH* after expression and solubilisation was found, however, clearly only a limited amount of recombinant protein was solubilized (Fig. 3.5 A). According to the results of the FeCH activity measurement the overnight solubilization increased total FeCH activity indicating that some amount of 'active' GST-FeCH can be freed from protein aggregates/inclusion bodies. This effect was repetitively more intensive for the GST-FeCH possessing a thrombin cleavage site (Fig. 3.5 B)

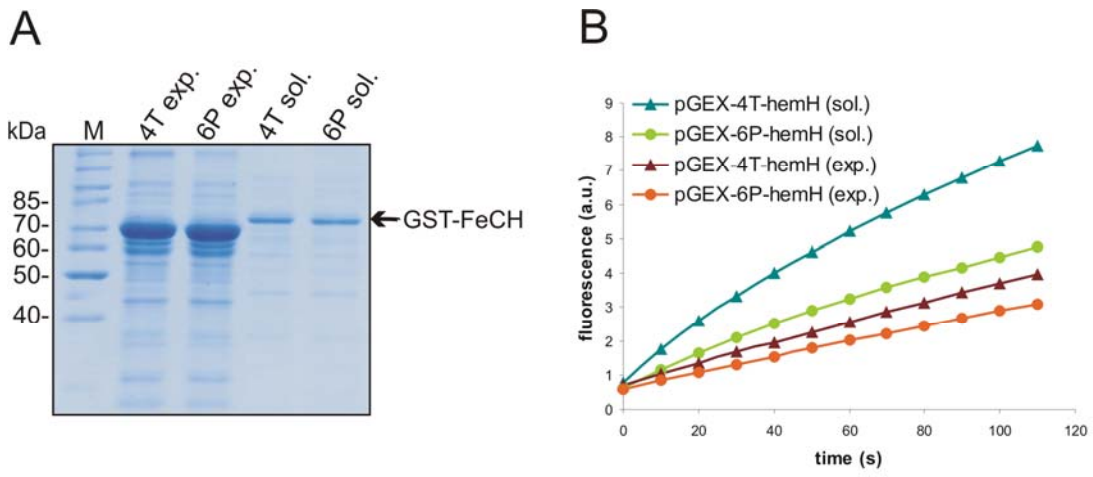



**Fig 3.5:** Solubilization and *in vitro* activity of recombinant GST-FeCH. **(A)** The same volume of membrane protein fraction from pGEX-4T-*hemH* (4T) and pGEX-6P-*hemH* (6P) strains before (exp.) and after (sol.) overnight solubilization in K-Phos+ separated by SDS-PAGE. M = marker. **(B)** Comparison of *in vitro* activities of GST-FeCHs in membrane-protein fractions before and after solubilization. The same volume of sample loaded on the gel was used in the assay.

## 3.4 Purification of recombinant FeCH using glutathione sepharose

Purification of GST-FeCH using glutathione sepharose resin was attempted by following the manufacturer's instructions (GE Healthcare, Germany). 300 µl of glutathione sepharose was loaded into a Biorad column and equilibrated with K-Phos+ buffer containing 2 % Triton X100. Solubilized membrane-proteins from both pGEX-4T-*hemH* and pGEX-6P-*hemH* strains were diluted to obtain 1 % Triton X-100 (recommended concentration for glutathione sepharose). Samples were flowed through column by gravity. To wash unspecificaly-bound proteins from the column the resin was washed with 6 ml of K-Phos+ buffer with 1 % Triton X-100. The resin was then incubated overnight in 300 µl of K-Phos+ buffer with 15 units of the relevant protease at cleavage temperature (e.g. HRV RC at 8°C for pGEX-6P-*hemH* and thrombin at room-temperature for pGEX-4T-*hemH*). To obtain pure FeCH, samples were passed through a 0.22 µm Spin-X filter to remove the resin. Elution of the proteins from the resin using 10mM glutathione rather than cleavage using a protease resulted in the immediate precipitation of GST-FeCH, followed by the total loss of its enzymatic activity (data not shown).

Analysis of eluates revealed practically no purified FeCH suggesting very weak binding of the GST-FeCH to the glutathione sepharose (Fig 3.6). In order to ascertain the reason for this, the biding of GST alone to the glutathione sepharose was tested, as well as the cleavage of GST-FeCH by the HRV 3C protease in the K-Phos+ buffer. As no differences between the two pGEX vectors were found, it was decided to continue using only the pGEX-6P-*hemH* strain.

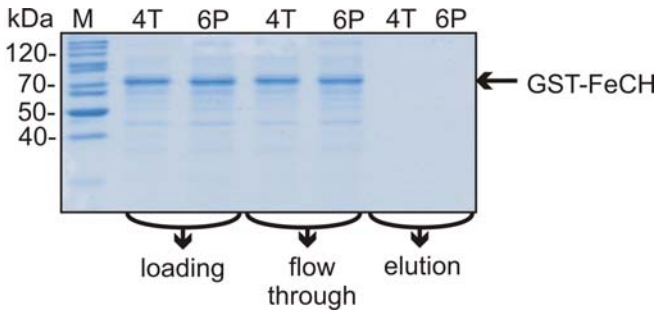



**Fig. 3.6:** The different steps of recombinant FeCH purification separated by SDS-PAGE. Solubilized membrane-proteins from pGEX-4T-*hemH* (4T) and pGEX-6P-*hemH* (6P) strains were loaded on a glutathione sepharose column and washed. The FeCH protein was eluted after cleavage of the GST from FeCH by the appropriate protease. However, no significant amount of FeCH protein in elutions was detected on the coomassie stained gel when 10% of the total elution volume (400 µl) was loaded. M = marker, the size of the GST-FeCH protein is indicated by an arrow.

To test the effect of the protease, membrane-proteins solubilised in 1% Triton X100 K-Phos+ buffer were incubated with HRV 3C for 2 hours (recommended in manufacturer's protocol). Interestingly, after HRV 3C treatment, FeCH activity in the sample significantly increased (Fig. 3.8) suggesting that the GST tag inhibits overall activity of the enzyme. A partial digestion of the GST-FeCH was confirmed by SDS-PAGE as new band corresponding to mass of the FeCH (43 kDa) was visible on the gel. However, some undigested GST-FeCH, running at 70 kDa was still present in the sample. 100 µl of the protein sample was then mixed with 50 µl of glutathione sepharose resin, incubated for 2 hours at room temperature and passed through a Spin-X filter. As shown in Fig. 3.7, GST alone sufficiently bound to the resin as the GST band almost completely disappeared after incubation with the gluthatione sepharose. Each of the purification steps was analyzed for activity using the FeCH assay described earlier (Fig. 3.8).

As weak binding of the GST-FeCH to the glutathione sepharose was revealed, alternative approaches to purify this enzyme were attempted. First, a home-made anti-GST resin was prepared by coupling a commercial polyclonal anti-GST antibody (Sigma-Aldrich, Germany) to aminolink resin (Pierce) by a protocol described in the *Material and Methods* section. Successful coupling of the antibody to the resin was confirmed by spectroscopy. Surprisingly, the binding of the GST-FeCH to this new resin was again found to be quite weak, with only a low concentration of FeCH eluted (not shown). Finally, the pET-49-*hemH* vector was constructed to express GST-His-FeCH, a recombinant protein that should be possible to purify by metal-affinity chromatography. As shown earlier, the activity of this enzyme was very low and this activity was completely lost upon solubilisation, indicating a problem with protein stability (not shown).Therefore, purification of this protein was not attempted.

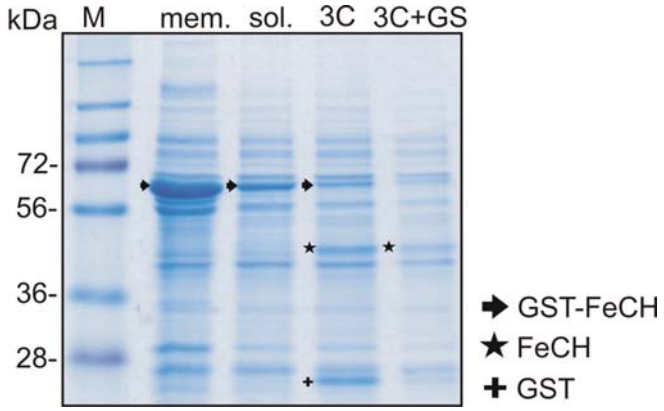



**Fig. 3.7:** Cleavage of GST-FeCH by HRV 3C protease and binding of GST to the glutathione sepharose. Membrane-proteins from an induced pGEX-6P-*hemH* strain (mem.) were solubilized (sol.) and incubated with the HRV 3C protease for 2 hours at room temperature (3C). Finally, this fraction was mixed with glutathione sepharose, incubated for a further 2 hours and the filtered supernatant was analyzed (3C+GS). The GST-FeCH protein is indicated by an arrow, cleaved FeCH is marked by a star and the GST tag by a cross. The same volume of the each sample was loaded in all lanes. M = marker.

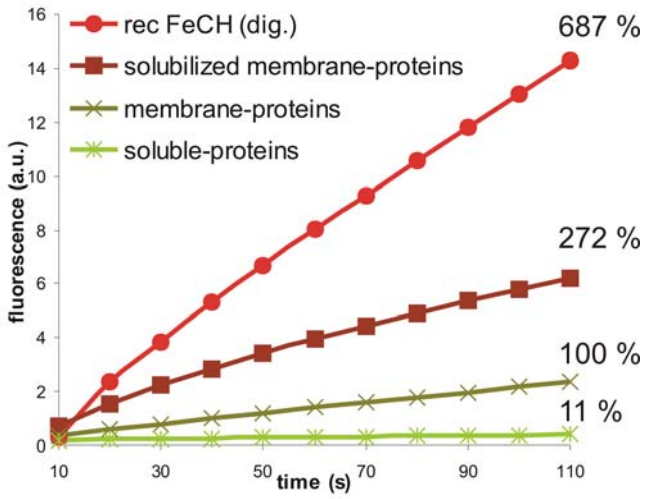



**Fig. 3.8:** *In vitro* activities of recombinant FeCH in different steps of its purification. Cell-extract from induced pGEX-6P-*hemH* strain was separated into soluble and membrane-protein fractions. Membrane-proteins were solubilized and the resulting supernatant was cleared by centrifugation and filtration. Finally, the GST-FeCH was cleaved with HRV 3C; this step is marked as: rec FeCH (3C). The fluorescence value of 100 % was assigned to the membrane-protein fraction, as FeCH is a membrane-bound protein. The same volume of each sample was assayed in all cases.

## 3.5 Optimized protocol for the purification of GST-FeCH using glutathione sepharose

As described above, GST protein alone was able to bind to the glutathione sepharose when incubated for two hours at room temperature in K-Phos+ buffer with 1% Triton X-100 (Fig. 3.7). For this reasons, similar conditions for GST-FeCH were also tested. However, in this case the column was washed with K-Phos buffer containing 0.04 % DDM rather than Triton X-100 as this detergent is not suitable for gel filtration due to its strong absorption at 280 nm and its large micelle size (80 kDa), close to the expected size of recombinant FeCH. Finally, the resin with bound protein was incubated overnight at 8°C with HRV 3C protease to ensure full cleavage of the GST-FeCH protein. During the elution step 0.04 % DDM was again used instead of Triton X-100. As is apparent from both SDS-PAGE analysis and activity measurements, shown in Fig. 3.9, a portion of GST-FeCH was successfully bound to the resin and an active recombinant FeCH was eluted. The eluted protein fraction is still contaminated by other proteins (Fig. 3.8 A) and its activity appears to be decreased during the purification procedure (see below), however it should be possible to improve this by further optimization.

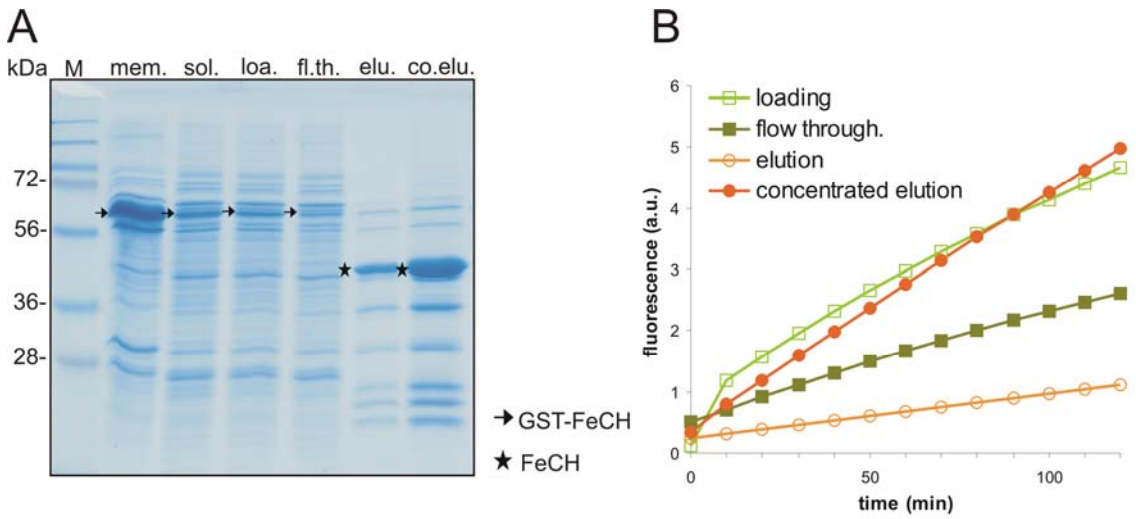



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**Fig. 3.9:** Purification of recombinant FeCH using an optimized protocol. **A)** Analysis of each purification step by SDS-PAGE; (mem.) membrane-proteins from ~100 µl of cells of the pGEX-6P-*hemH* strain at $OD_{600}$ ~1.2; (sol.) membrane fraction after solubilization; (loa) the same protein solution diluted 1:1 in K-phos+ buffer without Triton X100 and loaded onto the column; (fl.th) proteins that did not bind to the column, (elu); 3 µl from 600 µl of total elution volume prepared as described in the text; (co.elu) 3 µl of elution concentrated 8 times in a 50 kDa cut off microcon (Millipore); M = marker. **B)** *In vitro* activities of the same samples; the amount of protein assayed was proportional to those loaded on the gel in (A).

## 3.6 Activities and oligomerization of recombinant FeCH *versus* FLAG-FeCH purified from *Synechocystis*

Before this project was undertaken the only known method to prepare pure active plastid-type FeCH was based on direct purification of this enzyme from *Synechocystis* under native conditions using 3xFLAG tag (Sobotka *et al*., in preparation). To assess the properties of recombinant FeCH prepared during this project, its activity and oligomerization was compared with those of the FLAG-tagged enzyme purified from *Synechocystis.* FLAG-FeCH was isolated from 4 l of FLAG-FeCH/Δ*hemH Synechocystis* culture and purificated on a column with M2 anti-FLAG resin (Sigma, Germany) as described in the *Material and Methods* section. A pure and active protein sample can be obtained using this protocol (Fig 3.10).

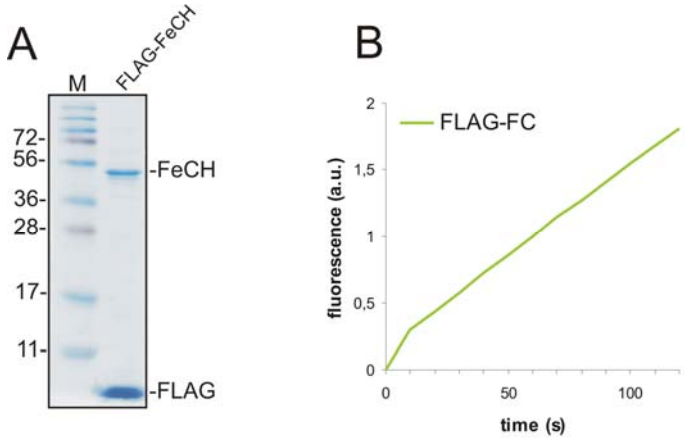


**Fig 3.10:** Purification of FLAG-FeCH from *Synechocystis* under native conditions. FLAG-FeCH (FLAG FC) was purified on an M2 anti-FLAG resin column and eluted with 3xFLAG peptide as described in the *Material and Methods* section. **A)** Purified FLAG-FeCH analyzed by SDS-PAGE. FLAG-FeCH (~45 kDa) and the 3xFLAG peptide (~2.5 kDa) used in the elution step are indicated. **B)** *In vitro* activity of purified FLAG-FeCH (FLAG FC), the same amount of protein as loaded on the gel was assayed.

To compare the activities of FeCHs prepared via different methods, samples containing the same concentration of FeCH were prepared to be used in an *in vitro* assay (~250ng, see Fig. 3.11) For this experiment, the solubilized membrane-protein fraction containing GST-FeCH cleaved with HRV 3C was also included to estimate any loss in FeCH activity during the purification procedure. Apparently, there is a dramatic decrease in FeCH activity during purification of the recombinant enzyme. On the other hand, the activity of

the purified recombinant enzyme is still comparable with the FLAG-FeCH protein, which is surprising when the loss of activity during purification is taken into account (Fig. 3.11 B).

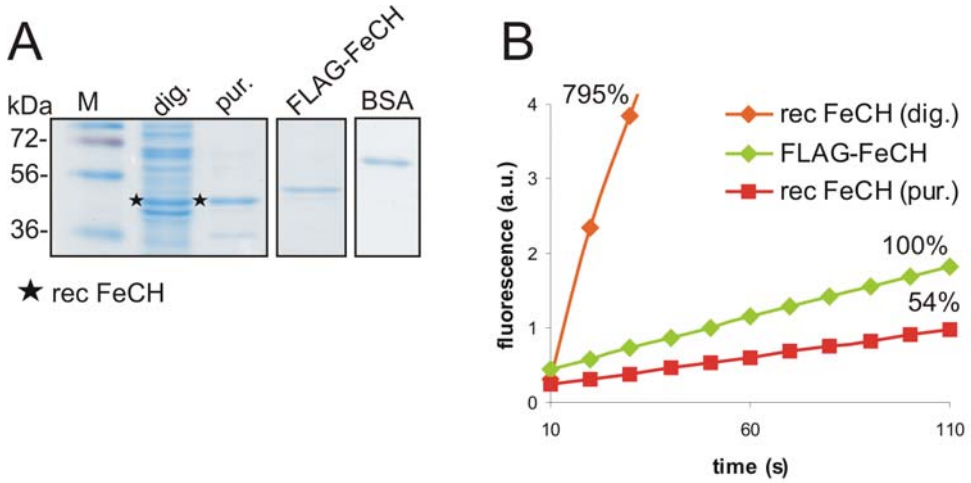


**Fig. 3.11:** Activities of recombinant FeCH purified from *E. coli* and FLAG-FeCH purified from *Synechocystis*. **A)** Concentration of FeCHs prepared using different methods were estimated by SDS-PAGE; (M) = marker, (dig.) = solubilized membrane proteins from the pGEX-6P-*hemH* strain after cleavage by HRV 3C protease, (pur.) = recombinant FeCH purified using an optimized protocol described in chapter 3.5, (FLAG-FeCH) = FLAG-FeCH purified directly from *Synechocystis* on anti-FLAG resin, (BSA) = 250 ng of bovine serum albumin loaded on the same gel. **B)** *In vitro* activities of purified FeCHs; the same samples and the same protein concentrations were assayed as loaded on the gel in (A).

FeCH is active as a dimer in many organisms (Grzybowska *et al.*, 2002; Ohgari *et al.* 2005) and the FLAG-FeCH purified from *Synechocystis* appears to be in a dimeric form (Sobotka *et al.*, in preparation). To reveal any oligomerization of recombinant FeCH prepared in this project the enzyme was analysed by gel filtration on a BioSep SEC-S2000 column with the FLAG-FeCH included as a control (Fig. 3.12). The column was also calibrated using a protein standard with sizes from 29 kDa of carbonic anhydrase to the void volume of blue dextran (Fig. 3.11 B).

During gel filtration, FLAG-FeCH migrated in two forms: the major peak eluted in 42 min with the shoulder in 44 min (Fig. 3.11 A). Following the calibration, these peaks correspond to proteins of 180 and 90 kDa, respectively. As the size of β-DDM micelles, which were present in the sample, is 50 kDa (Rosevear *et al.*, 1980), the size of the main peak corresponds to a dimer and the shoulder appears to be monomeric FeCH. It should be noted that the dimerization of FLAG-FeCH was confirmed by native gel electrophoresis (Sobotka *et al.*, in preparation).

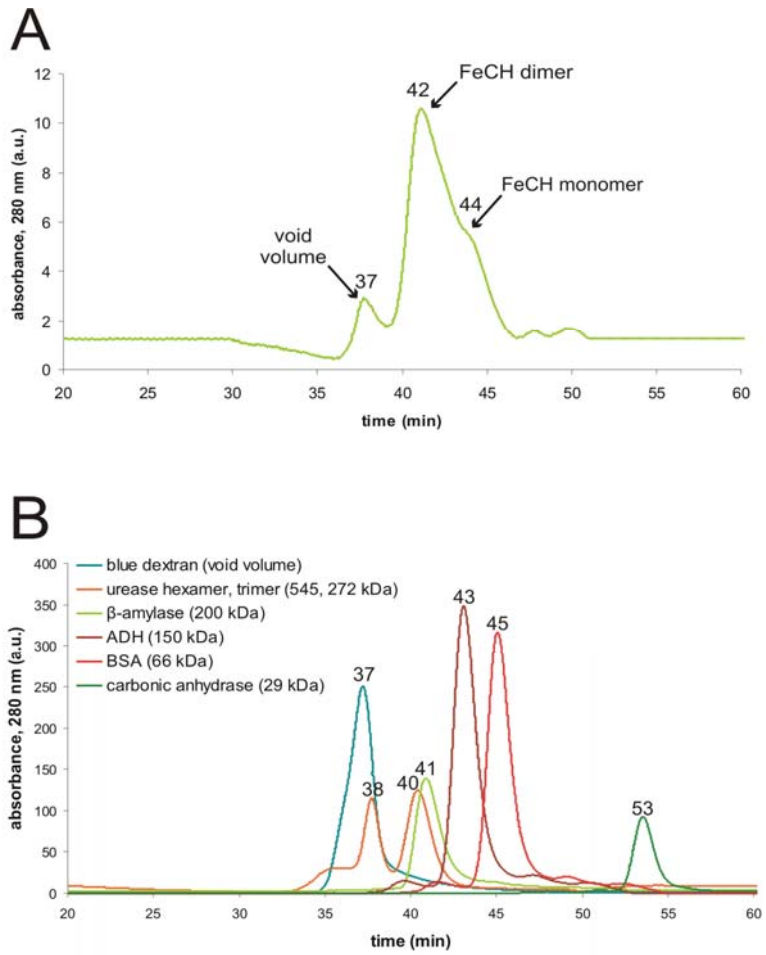


**Fig. 3.12:** Oligomerization of FLAG-FeCH purified from *Synechocystis*. **(A)** ~2.5 µg of purified FLAG-FeCH was loaded onto a Phenomenex SEC2000 column and absorbance at 280 nm was recorded. Void volume was eluted after 37 minutes, the main protein peak eluted at 42 minutes with a shoulder at 44 minutes which, according to the calibration, corresponds to proteins with sizes 180 and 80 kDa, respectively. **(B)** Calibration of the same column with protein standards. Abbreviations used: Alcohol dehydrogenase (ADH), bovine serum albumin (BSA). The value above each peak indicates time (min) when protein was eluted.

As the purified recombinant FeCH fraction contained contaminating proteins (Fig. 3.9) the interpretation of protein absorption on gel filtration could be difficult. Therefore the molecular mass of recombinant FeCH was analysed using its activity profile in minute fractions collected during gel filtration. Importantly, using this approach, information about a molecular mass of *active* enzyme can be obtained that may differ from total protein absorbance. For this analysis filtered membrane proteins containing the cleaved GST-FeCH were used as here the FeCH activity was much better than for purified FeCH (Fig. 3.11).

For this approach fractions were collected from after gel filtration from elutions between the void volume (38 min) and 49 min. The activity of FLAG-FeCH peaked at 43 min,

which correlates very well with the main peak in the absorption spectra (Fig. 3.12 A) taking into account a delay (~30 s) between the detector and sample collector. A similar activity profile was obtained for recombinant FeCH, most of which eluted between 42-44 minutes as an active dimer, as confirmed by SDS-PAGE (Fig. 3.13). This demonstrates that recombinant FeCH can be prepared as an active enzyme with similar structural properties to the native enzyme, key for future enzymology, structure and regulation studies of plastid-type FeCH.

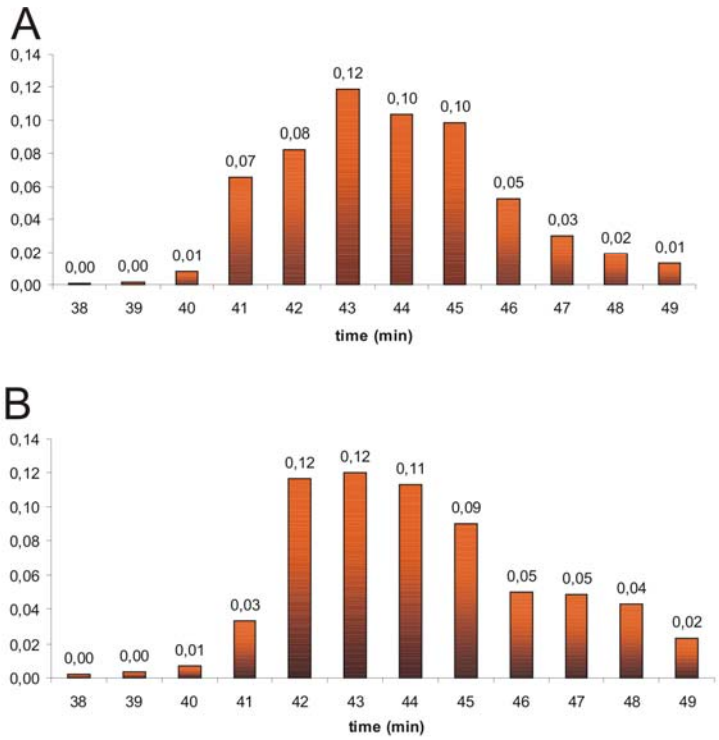


**Fig. 3.13:** Molecular mass of active FLAF-FeCH **(A)** and recombinant FeCH **(B)** as determined by analysis of the activity profile samples collected during gel filtration. The major fraction containing active protein was eluted at 43 min; however there is ~30s delay in comparison to calibration in Fig. 3.12B. Numbers above each bar express a linear regression of increase in fluorescence of zinc-protoporphyrin IX.

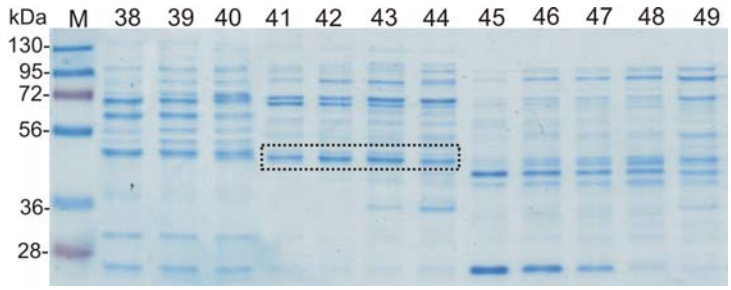


**Fig. 3.14:** Collected fractions from gel filtration of recombinant FeCH by minute, analyzed by SDS-PAGE. A band corresponding to FeCH is boxed (43 kDa). The values above each sample indicate time (min) when the fraction was eluted from the column. The same volume of each fraction was loaded in each lane. The samples correspond to those from Fig. 3.13 B.

## 3.7 Expression and localization of GST-ScpB protein in *E. coli*

*Synechocystis* ScpB belongs to the Scp family of small, single helix proteins that were found to have a putative chlorophyll binding motif and are expressed under various stress conditions (Promnares *et al.* 2006). As described in the *Introduction* section, it is speculated that Scp protein(s) interact with FeCH and modulate its activity. To obtain recombinant ScpB I prepared a pGEX-6P-*scpB* vector able to express GST-StrepII-ScpB in *E. coli*. The StrepII tag was introduced for a subsequent immobilization of the ScpB e.g. in a pull-down assay. This tag can also be used for immunodetection of this protein using anti-Strep antibodies, as no specific antibodies against this very small (~6 kDa) and hydrophobic protein are currently available. The GST-ScpB protein was expressed overnight and its accumulation and localization analyzed as described for GST-FeCH. After separation of soluble and membrane fractions by SDS-PAGE, the GST-ScpB protein (with size corresponding to 33 kDa) was identified as a membrane protein even though it is fused to a much larger hydrophilic GST (Fig. 3.15). One possibility for this is that the GST-ScpB protein is completely contained in inclusion bodies, which have yet to be analyzed. The GST-ScpB protein will be purified and used for future studies on interactions between FeCH and Scp's (see *Discussion*).

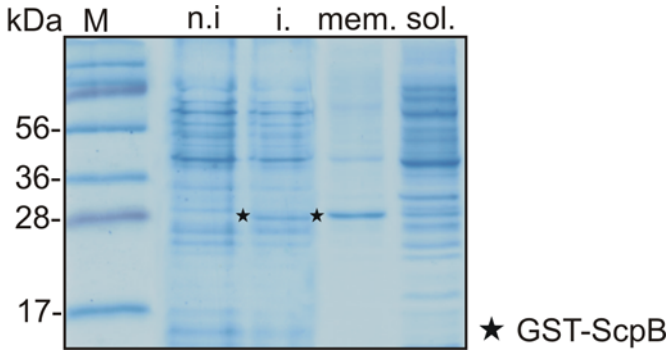


**Fig. 3.15:** Localization and expression of recombinant GST-ScpB. *E.coli* cells expressing the GST-ScpB protein were lysed and the whole-cell extract was fractionated by centrifugation to membrane-protein and soluble-protein fractions. The band with a size corresponding to GST-ScpB is marked by star. Proteins from ~ 100 µl of cells were loaded on the SDS-PAGE in each lane, M = marker, n.i = non-induced cells used as a control, (i) = induced cells; (mem) = membrane-protein fraction; (sol.) = soluble proteins.

## 4. DISCUSSION

FeCH seems to be a key enzyme in the regulation of tetrapyrrole biosynthesis pathway in photosynthetic organisms (Sobotka *et al.,* 2008), however there are many remaining questions, about its regulation, exact location in the cell or about its connection to intracellular signalling,network (Tanaka and Tanaka, 2007). To elucidate some of these questions an active plastid-type recombinant FeCH would be invaluable.

Several attempts were reported to purify FeCH from different sources, but low levels of enzyme activity, instability of protein during purification, and fact that the FeCH is often membrane associated generally makes those efforts complicated. In 1994 relatively high expression of recombinant human and mouse FeCH were obtained and later FeCH from *Xenopus, Gallus* and *Drosophilla* were also purified, which enabled pilot biophysical studies of pure animal protein (Dailey and Dailey, 2003). On the other hand, till now there is no successful preparation of recombinant FeCH from photosynthetic organisms, despite several laboratories spent an effort on this task (Sobotka, personal communication). Only known exception is recently reported preparation of the recombinant FeCH from cyanobacterium *Thermosynechococcus elongatus*, nonetheless, a specific activity of purified enzyme is not clear from this paper (Masoumi *et al.,* 2008).

The problem with preparation of the plastid-type FeCH, a hydrophobic membrane protein, is well apparent from a recent attempt to express and purify this enzyme with His-tag. Using this approach, Sobotka *et al,* (2008) had to cope with problem of aggregation, low activity and instability of FeCH. For preparation of a sufficient amount of the enzyme to assess the activity of a truncated FeCH mutant, pure plasmatic membrane containing the His-FeCH had to be first prepared on sucrose gradient from larger volumes of *E. coli* and even from pure membranes, only a quite limited amount of low active FeCH was finally obtained. (Sobotka, personal communication). However, to understand enzymology or structure of this enzyme, a mass of highly active protein is necessary.

To negotiate a strong tendency of the FeCH to aggregate in watery environment (Kiefhaber *et al.*, 1991) is thus probably crucial for the preparation of this enzyme in *E. coli*. Other problem is related to formation of inter-molecular disulfide covalent bonds (see Fig.1.8) between FeCH proteins further cemented protein aggregates (Cromwell *et al.,* 2006). The *Synechocystis* FeCH contains two cysteines only, so these residues are probably very reactive. To keep enzyme in reduced form, I purified this protein always in presence of 4 mM DTT, a strong thiol-reducing agent (Pestov and Rydström, 2007). Importantly, I have

found that the GST tag with molecular mass about 27 kDa (Viljanen *et al.*, 2007) probably served as a barrier between FeCH molecules as I have never observed a significant 'cross-linking' of GST-FeCH via disulphide bounds (Fig. 3.1 B). Also this tag clearly improved solubility and activity of the *Synechocystis* FeCH, al least based on experince with His-FeCH protein.

The GST tag thus appears to be very useful for the purification of FeCH and all vectors I tested contained this tag. From the beginning of project the pGEX 4T-*hemH* construct was available, however for long cleavage time by thrombin protease I did not plan to use this construct for a fine FeCH purification. Simply, I expected a substantial decrease in FeCH activity during protein cleavage and, indeed, time of procedure was found later to be very important for the total activity of purified protein (Fig. 3.11 B) As a 'better' equivalent of the pGEX-4T-*hemH* I have prepared the pGEX-6P-*hemH* vector to use the HRV 3C protease instead thrombin. Finally, I constructed also the pET-49-*hemH* vector that was similar to the pGEX-6P-*hemH* just with the 6xHis tag inserted between FeCH and the GST anchor. I decided to try this vector due to problems with binding of the GST-FeCH to glutathione sepharose resin. His-tag has been already successfully used for purification of recombinant *Synechocystis* FeCH (Sobotka *et al.*, 2008). Unexpectedly, the resulting GST-His-FeCH protein displayed very low activity even expression level was sufficient (Fig. 3.2 B). It is possible that histidine residues inserted between the GST and FeCH caused a protein destabilization, which is supported by an observation that the rest of GST-His-FeCH activity completely disappeared during overnight solubilization (not shown). This is in clear contrast to the GST-FeCH found to be 'more active' after solubilization (Fig. 3.5 B).

Solubilization of the FeCH was tested in different buffers and under different conditions. To evaluate efficiency of tested conditions, filtrated soluble proteins were analyzed by measurement of the FeCH *in vitro* activity (chapter 2.4) and using SDS-PAGE. Based on these data I proposed a protocol, which yielded a highly active solubilized FeCH (Fig. 3.5 B). Also, the stability of this enzyme appears to be sufficient as no significant lost of activity was detected when stored at -80°C for more then 14 days (data not shown).

Purification of the GST-FeCH on a glutathione sepharose column with protein flow through by gravity completely failed with no binding of the GST-FeCH to resin (Fig. 3.6). Surprisingly, also when I tested to purify the GST-FeCH using immobilized polyclonal anti-GST antibodies (Sigma-Aldrich, Germany), I eluted only traces of enzyme from total protein loaded (not shown). It is possible that observed low interaction between the GST

and glutathione or specific antibodies, are caused by a folding of the GST-FeCH. This protein very probably exists as a dimer and a its final conformation could keep the GST protein or at lest its active side partially burned inside the GST-FeCH complex. This corresponds with my finding that GST alone binds glutathione even in the presence of essentially the same buffer with detergent used for the GST-FeCH (Fig. 3.7). Moreover, the FeCH is apparently more active when GST tag is cleaved arguing for structurally close interaction between GST and FeCH (Fig. 3.7).

Using a prolonged time of the incubation I observed a binding of the GST-FeCH to glutathione sepharose. Once bound on resin the GST-FeCH protein was cleaved by the HRV 3C protease and eluted as an active enzyme. There was still a contamination by other proteins (Fig. 3.9), however, I expect that this problem can be easily solved by more intensive washing, increase in salt concentration or possibly by another purification steps e.g. ion exchange chromatography (Cole and Mars, 1984). To avoid a substantial decrease in the FeCH activity during purification, incubation of proteins with glutathione sepharose and especially treatment with HRV 3C protease can be reduce to a minimal required time.

Gel filtration was used to estimate oligomerization of the active form of recombinant FeCH. For this experiment I analyzed solubilized membranes containing GST-FeCH only treated with HRV 3C to obtain highest possible FeCH activity. This measurement was compared with purified FLAG-FeCH already known to form active dimer (Sobotka et al., in preparation). Importantly, the activity profiles were very similar for both native FLAG-FeCH and recombinant enzyme suggesting that is possible to prepare active dimeric FeCH in *E. coli* using a combination of detergent like Triton X-100 and DDM. In addition, activities of the purified recombinant FeCH and FLAG-FeCH were comparable (Fig. 3.13). However, when I checked activity of the recombinant FeCH before purification, just cleaved with the HRV 3C protease, it was almost one fold more active than the FLAG-FeCH. It means that it is possible to improve a final activity of recombinant protein substantially by a further optimalization.

Based on data discussed above I believe that our laboratory is very close to produce the desired amount of active recombinant *Synechocystis* FeCH for crystallography and for biochemical studies. Moreover, pure FeCH produced in *E. coli* that naturally does not contain any photosynthetic proteins, could be used to identify proteins interacting with the FeCH and controlling its activity in the *Synechocystis* cell (Vavilin and Vermaas, 2001). For such study, FeCH can be immobilized for a pull down assay to screen interacting proteins in *Synechocystis* membranes or cytosolic fraction. Another interesting question is

whether the plastid-type FeCH with a putative chlorophyll binding motif can be reconstituted with photosynthetic pigment(s). Finally, our experience with the isolating of recombinant FeCH will be useful for preparation of other membrane proteins, like Scp proteins, or other enzymes of chlorophyll biosynthesis.

ScpB protein is one of candidate proteins for a factor controlling FeCH activity and consequently all tetrapyrrole pathways (Xu *et al.,* 2002). As the ScpB protein shows the highest similarity to the CAB domain of FeCH I decided to prepare the GST- ScpB as a 'test' construct for subsequent purification and also for a future preparation of all four *Synechocystis* Scp proteins to analyse proposed specific interactions with FeCH. This protein appears to be successfully expressed; however a detail analysis of expressed protein e.g. using anti-strep antibodies, its proper localization in the cell and its purification was out of scope of my thesis.

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## 5. CONCLUSION

Preparation of recombinant membrane proteins in native form is rather complicated, which is especially true for enzymes. This project was focused on the preparation of cyanobacterial FeCH, an interesting enzyme that probably plays a key role in the regulation of tetrapyrrole biosynthesis. Regarding its C-terminal CAB domain, this enzyme is often speculated to bind chlorophyll, which would be very unusual in nature. I have successfully expressed *Synechocystis* FeCH in *Escherichia coli* as a GST-FeCH protein and prepared a protocol for its purification. Using this approach I have obtained the recombinant FeCH as an active enzyme in a quite pure form. I have also found that the molecular mass of protein in an active form corresponds to the size of dimeric FeCH, a native form of this enzyme. So, I expect that the FeCH preparation based on my protocol will lead to an enzyme pure and active enough to resolve questions about its enzymology, structure and interaction with other proteins. Finally, I designed a construct for the purification of recombinant ScpB protein, a small single helix protein with an elusive role in the *Synechocystis* cell. The experience we have gained from the preparation of FeCH will be directly utilised for other membrane protein including the ScpB.

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