Chapter 7

General discussion and outlook

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The ability of peroxidases (and laccases) to degrade lignin from all kinds of plant biomass makes them enzymes of interest to biotechnology. There enzymes are naturally produced by white-rot fungi, but unfortunately in only limited amounts. Production in an extensively used fungus for protein production like *Aspergillus niger* therefore might provide an interesting alternative to scale up their production. Previous research however has indicated heme availability is a likely bottleneck in the heterologous production of heme-requiring peroxidases. Therefore, heme biosynthesis and its regulation was the scope of this research and the obtained results are discussed below in view of future research.

Considerations for future research on heme and/or hemoprotein production

The results obtained and described within this thesis demonstrate the ability of *A. niger* to utilize exogenous heme sources, but also demonstrate the involvement of heme in multiple processes essential for fungal growth. Furthermore, regulation of heme biosynthesis was shown to be tight, and a "simple" overexpression of pathway genes will not "just" result in increased heme synthesis. During this project heme biosynthesis and its regulation was studied in wild-type and deletion mutant strains to clearly identify key points in regulation of the pathway.

In future research several aspects would require further attention. One of these aspects concerns the utilization of exogenous heme and its regulatory effects. We have demonstrated in this thesis that hemin can be taken up and utilized as an intact molecule using the ferrochelatase deficient Δ*hemH* strain and that hemoglobin can be used as a source of heme as well. Furthermore, both hemin and hemoglobin were found to positively affect the expression of heme biosynthesis genes (chapter 4; Franken et al. 2012). These findings imply that the positive effect of heme supplementation during production conditions is at least two-fold: heme supplementation directly reliefs a potential heme limitation while simultaneously heme biosynthesis is upregulated, potentially generating more endogenously synthesized heme.

However, uptake of intact hemin molecules does not exclude the possibility of iron extraction from hemin prior to or after its uptake. Most organisms contain heme oxygenases able to degrade the heme molecule and subsequently extract and recycle iron (Pae et al. 2010; Pendrak et al. 2004; Protchenko and Philpott 2003; Santos et al. 2003). Interestingly however, no similar proteins to the currently known heme oxygenases were identified in genomes of *Aspergilli* as mentioned by Haas et al. (2008). It therefore remains unclear whether these fungi are able to degrade and reuse heme iron or possibly employ

different mechanisms of iron extraction from heme such as the possibility of two-way reactions of human ferrochelatase depending on its localisation, as described by Sakaino et al. (2009).

A first indication for actual heme degradation (and subsequent iron extraction) was provided by the Δ*hemF* strain. This strain is defective in coproporphyrinogen III oxidase resulting in termination of heme biosynthesis at coproporphyrinogen III. The resulting strain is therefore heme auxotroph and devoid of endogenous synthesis of protoporphyrinogen IX, protoporphyrin IX (PPIX) and heme. However after analysis of its porphyrin content, besides the accumulation of coproporphyrinogen III, also elevated levels of PPIX were measured which could only have arisen from the exogenously added hemin (unpublished result).

Iron is a major contributor in the regulation of heme biosynthesis and as is the case for heme, elevated levels of iron are toxic (Hamza 2006; Hortschansky et al. 2007; Hou et al. 2006; Krishnamurthy et al. 2007; Schrettl et al. 2008). Under normal environmental conditions, the biologically available iron is scarce and organisms compete for this available iron (Haas et al. 2008). However, iron uptake is tightly controlled through low affinity iron uptake, the synthesis of siderophores and reductive iron assimilation (RIA) to ensure sufficient uptake (Haas 2012; Haas et al. 2008). Elevated iron uptake could also be achieved in *A. niger* by deregulating siderophore synthesis and RIA, but the ability of heme uptake and potentially iron extraction from heme sources would be an important option in the battle for this scarcely available iron. At the same time, the potential iron extraction also raises new questions on how heme is imported and if heme import is under the control of iron uptake systems. Furthermore, despite increased iron uptake and upregulation of several heme biosynthetic genes in a regulatory mutant Δ*sreA*, accumulation of porphyrins, but no elevated levels of heme were measured (Chapter 6; Franken et al. submitted). This finding supports our earlier findings in the *hemA, hemF*, and *hemH* overexpression strains (Chapter 5; Franken et al. 2013) and indicates an important limitation at the level of ferrochelatase (*HemH*). Only overexpression of *hemH* resulted in increased heme levels while elevated levels of PPIX were observed in several other overexpression strains. As this increase in PPIX was also observed in strains with increased expression levels of *hemH*, this suggest (additional) post-translational regulation and/or feedback inhibition at the level of ferrochelatase. Therefore, in a future approach, a peroxidase producing strain combined with *hemH* overexpression may be used to a) increase ferrochelatase capacity and b) provide peroxidise as a sink for heme thereby also preventing potential feedback inhibition. Whether this kind of mechanism is in place in natural peroxidase producing white rot fungi is as yet unknown.

In many organisms, *hemA* is determined to be the rate-limiting enzyme of the heme biosynthesis pathway. Upregulation of the gene or overexpression of the protein therefore leads to an increase in heme biosynthesis. Exceptions are formed by *Saccharomyces cerevisiae* and *Neurosporra crassa*, where the second enzyme (aminolevulinic acid dehydratase) was found to be rate-limiting (Hoffman et al. 2003; Muthukrishnan et al. 1972). The observed limiting effect of ferrochelatase and the lack of increased heme in our *hemA* overexpressing strains therefore allow to speculate on the putative rate-limiting enzyme of the pathway in *A. niger*. The *hemA* overexpressing strains did not produce more heme, they rather demonstrated an additional bottleneck to be solved when elevated heme levels are desired. Upon overexpression of *hemA,* porphyrins prior to coproporphyrinogen III oxidase (CPO) accumulated (Chapter 5; Franken et al. 2013). This finding suggests that CPO activity is insufficient to deal with the additional uroporphyrinogen III and coproporphyrinogen III synthesis derived from *hemA* overexpression. An alternative possibility to explain limitations observed in the heme biosynthesis pathway derives from the predicted localization of CPO. As in mammals, but unlike yeast, the *A. niger* CPO is predicted to be mitochondrially localized (Franken et al. 2011). Coproporphyrinogen III therefore needs to be transported into the mitochondria for further processing towards heme. As found in many pathways requiring metabolite transport the actual transport step (Douma et al. 2010; Hanley et al. 2005; Pandak et al. 2002) might alternatively be insufficient/rate limiting, possibly under feedback control by heme (Susa et al. 2002), to support the increase in heme synthesis. Possibly a combined overexpression of *hemA* and *hemF* and/or *hemH* could provide more insight in the cause of porphyrin accumulation.

Also a control point for heme biosynthesis after the branchpoint for siroheme synthesis at CPO and/or ferrochelatase, is not unlikely. Under the commonly used nitrate containing growth conditions, *A. niger* requires active nitrate and nitrite reductases in order to be able to utilize this nitrate as nitrogen source (Chang et al. 1996). Whereas nitrate reductase required the cofactor heme, nitrite reductase required siroheme as cofactor and siroheme synthesizes derives from the heme biosynthetic pathway at the level of uroporphyrinogen III formation (Crane and Getzoff 1996; Raux et al. 2003; Schubert et al. 2002a). Consequently, both heme and siroheme synthesis are dependent on ALAS activity. Feedback inhibition by heme on ALAS to prevent a large increase in heme synthesis, through binding on heme regulatory motifs, would therefore not only reduce heme but also siroheme biosynthesis and might therefore reduce growth and/or protein production. Thus, by not using nitrate but e.g. ammonium as N-source, the need for siroheme is abolished while it simultaneously reduces the need for heme in basic cellular processes.

The results obtained on altering media composition to improve growth of Δ*hemF* and Δ*hemH* demonstrate that the requirement for heme in cellular processes can actually be reduced by simple supplementations like ammonium and cysteine. As heme pathway mutant strains are extremely impaired in growth, any relief of heme requirement will result in an improved growth. This feature makes these strains valuable to identify and verify processes that are heme-dependent. Consequently Δ*hemF* and Δ*hemH* can be useful to fine-tune culture media for heme-protein production by minimizing heme requirement for house-keeping processes. Together with generating knowledge on regulation of heme synthesis and enhancing endogenous heme biosynthesis, understanding the house-keeping processes that require heme might provide an additional alternative to enhance the available heme for peroxidase production.

The data generated during this project indicates a number of improvements still have to be achieved, together with optimal culture conditions in order to potentially achieve higher production levels of these peroxidases. Moreover, heme biosynthesis is not the only known bottleneck in heterologous proteins production. Factors like e.g. protease production (Braaksma et al. 2009; Hagag et al. 2012; Punt et al. 2008; Sharon et al. 2009), protein misfolding and their subsequent degradation through the unfolded protein response and ER associated degradation (Carvalho et al. 2011; Guillemette et al. 2007) are also known to play an important part and are therefore intensively studied as well. Taken all this information together, it is still an unanswered question whether *A. niger* would indeed be the best organism for the production of peroxidases derived from white-rot fungi, or if a more efficient approach could be found in optimizing protein and heme production in these very useful basidiomycetes.