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Chapter 1

General introduction

Renewable plant polymers

The utilization of renewable materials from non-feedstock sources (white biotechnology) is an extremely active area of research these days for various purposes such as the production of renewable chemicals and materials (Martínez et al. 2009). One of these abundant renewable materials is cellulose. Cellulose is extensively used in the paper and pulp industry, and has demonstrated its value as a source for bio-ethanol. Although cellulose can be found in plant species in various degrees (Pauly and Keegstra 2010), the isolation of cellulose from plant biomass is complicated by the presence of lignin. This aromatic polymer cements cellulose and hemicellulose fibers in plant biomass providing strength and pathogen protection to the plant (Harris and DeBolt 2010). Lignin is predominantly composed of the *p*-hydroxycinnamyl alcohol monomers *p*- couamyl, coniferyl, and sinapyl alcohol (Simmons et al. 2010). Via oxidative coupling through laccases and peroxidases with other related molecules, the lignin monomers are polymerized in the cell wall to form the aromatic structure in so-called lignocellulose (Simmons et al. 2010)

The utilization of cellulose requires the removal of lignin. However, its complex structure in combination with a low porosity makes the lignin highly recalcitrant towards degradation (Hammel and Cullen 2008). The current approach to remove lignin therefore involves harsh pre-treatments and/or chemicals such as uncatalysed steam explosion, ammonia fiber/freeze explosion, acid- or base-catalyzed hydrolysis and liquid hot water pretreatment (Harris and DeBolt 2010). Despite the potential of lignin to be used in various applications e.g. as phenol replacement in phenol–formaldehyde resins (Harris and DeBolt 2010), the lignin residue is often considered as waste and burned for energy supply (Martínez et al. 2009; Pauly and Keegstra 2010) making these pretreatment methods not only costly but also environmentally unfriendly.

Alternative methods to deal with lignin and possibly utilize it for different applications are needed. Several approaches in plant breeding such as decreasing the lignin content, altering the composition or change the structure of lignin (Harris and DeBolt 2010; Pauly and Keegstra 2010; Simmons et al. 2010) are possible. However, it is not uncontroversial to release genetically modified lignin mutant plants in intense field trials (Simmons et al. 2010) and the modifications could easily result in altered growth and lower pest resistance (Martínez et al. 2009).

Biocatalysts: peroxidases

White rot fungi can efficiently utilize lignin in an oxidative process by various peroxidases and therefore provide an interesting alternative for dealing with lignin in plant biomass. These peroxidases are provided with extracellular hydrogen peroxide (H_2O_2) by a multitude of enzymes like glyoxal oxidase, pyranose-2 oxidase, and aryl-alcohol oxidase (Martínez et al. 2009). Comprising a large and diverse group, the peroxidases can be classified into two superfamilies: peroxidase-cyclo-oxygenases (Class I: animal peroxidase) and non-animal peroxidases (Class II). The fungal peroxidases belong to the latter class being secreted and often glycosylated enzymes capable to oxidize a large range of substrates and all requiring heme as cofactor (Hofrichter et al. 2010). The various peroxidases follow a similar catalytic cycle. Initially, the resting Fe (III)-peroxidase reacts with H_2O_2 , which results in the formation of Fe (VI)-peroxidase known as compound I. Compound I oxidizes the substrate in two single electron reactions. The first reaction yields compound II plus a radical product. The second reaction brings the peroxidase back to its resting state with the release of another radical (Conesa et al. 2002b; Hiner et al. 2002; Hofrichter et al. 2010). The class II fungal peroxidases are mainly comprised of lignin peroxidases (LiPs EC 1.11.1.14), manganese peroxidases (MnPs EC 1.11.1.13), versatile peroxidases (VPs EC 1.11.1.16) and *Coprinopsis cinereus* peroxidases (CiP's EC 1.11.1.7) and their characteristics and role in biological ligninolysis have recently been extensively reviewed (Hammel and Cullen 2008; Hofrichter et al. 2010; Martínez et al. 2009). Based on substrate affinity, LiP, MnP and VP are classified as high redox potential peroxidases and are functional lignin modifying enzymes. LiP is catalytically the most powerful fungal peroxidase and is able to directly oxidize dimeric lignin model compounds. Characteristic for LiP is an invariable tryptophan (Trp171 in the isozyme LiP H8) (Hofrichter et al. 2010). The Trp-171 is involved in the long range electron transfer of substrates that cannot make direct contact with the oxidized heme. However, veratryl alcohol, a metabolite secreted by *Phanerochaete chrysosporium* and substrate for LiP, is required for larger lignin derived compounds (Hammel and Cullen 2008).

Although LiPs constitute the catalytically most active peroxidases, they are not present in a multitude of white-rot fungi. The MnPs are so far the most commonly occurring class II peroxidases. Despite being strongly oxidizing, MnPs are not capable to oxidize non-phenolic lignin-related structures directly. Instead compound I oxidizes Mn^{2+} to Mn^{3+} . Mn^{3+} is subsequently stabilized by the chelators oxalate or malonate which are produced by the fungus. The chelated Mn^{3+} in turn could diffuse into the lignified cell wall and attack the phenolic structures in lignin (Hammel and Cullen 2008; Hofrichter et al. 2010). VPs

identified in *Pleurotis* and *Bjerkandera* species, share catalytic properties with both LiPs and MnPs.

CiP on the other hand is a low redox potential peroxidase rather than a lignin modifying peroxidase. It lacks the invariable Trp-171 required for LiP activity, as well as the Mn²⁺ binding site specific for MnP and VP (Hofrichter et al. 2010). Introduction of the invariable Trp-171 and its negatively charged environment (D197W+R258E+R272D) however does provide CiP with LiP activity (Smith et al. 2009). Although CiP is not directly applicable in the modification of lignin, it is important in industrial processes like the oxidation of dyes (Kim et al. 2010) and removal of phenolic compounds from aqueous solutions and waste waters (Hofrichter et al. 2010). Also, CiP is already commercially produced (Hofrichter et al. 2010). Therefore, the generation of LiP activity in CiP could render this multi-functional enzyme even more important for white biotechnology.

Heterologous production of peroxidases

Filamentous fungi like *Aspergillus* spp. are considered preferred hosts for production of biocatalysts for industrial use due to their high secretion capacity of homologous and heterologous proteins. The production of heterologous proteins however, is often relatively low compared with the amount of homologous protein produced (Punt et al. 2002). Several mechanisms are responsible for this low level of protein production which, among others, includes the unfolded protein response (UPR) and ER associated degradation (ERAD) (Guillemette et al. 2007), protease activity and limitations at the level of co-factor incorporation (Punt et al. 2002).

Cofactor availability and incorporation is suggested to be a limiting factor in the production of fungal peroxidases. Peroxidase production can usually be increased by the supplementation of a heme source to the medium (Conesa et al. 2000; Elrod et al. 1997). However, uptake of heme by filamentous fungi has not been demonstrated yet and therefore the underlying mechanism is not clearly understood. Furthermore heme-supplementation is costly and difficult to implement and therefore not suited for industrial purposes (Elrod et al. 1997). Thus alternative methods are required to reduce the potential limitation of heme for the production of peroxidases. Elevation of endogenous heme biosynthesis could provide a potential solution for heme supplementation. Therefore we studied heme biosynthesis in *A. niger* with the final aim to further understand and resolve of bottlenecks in hemoprotein synthesis in this biotechnologically important organism.

Outline of the thesis

Chapter 1 provides a general introduction towards peroxidases derived from white-rot fungi and their production in *Aspergilli*, together with potential bottlenecks. **Chapter 2** describes a literature review of the current understanding of heme biosynthesis and its regulation and demonstrates the complexity in regulation in this highly conserved pathway. **Chapter 3** describes attempts to produce heterologous peroxidase proteins in *A. niger*. We demonstrate the limitations encountered to succeed in this and propose the relation to heme limitation. In **Chapter 4**, regulation of the heme biosynthetic pathway was studied also using a *hemA* deletion strain defective in one of the first steps of the heme biosynthetic pathway. In **Chapter 5** two enzymes late in the heme biosynthetic pathway were studied by means of deletion to generate more insights in pathway regulation and the siroheme branchpoint. Also, overexpression of these genes was analyzed with respect to heme production. The role of iron in the regulation of heme biosynthesis in *A. niger* was examined in **Chapter 6**. This thesis is finalized in **Chapter 7** with a general discussion on options for future research to improve heme biosynthesis for peroxidase production and is concluded with a **Summary**.