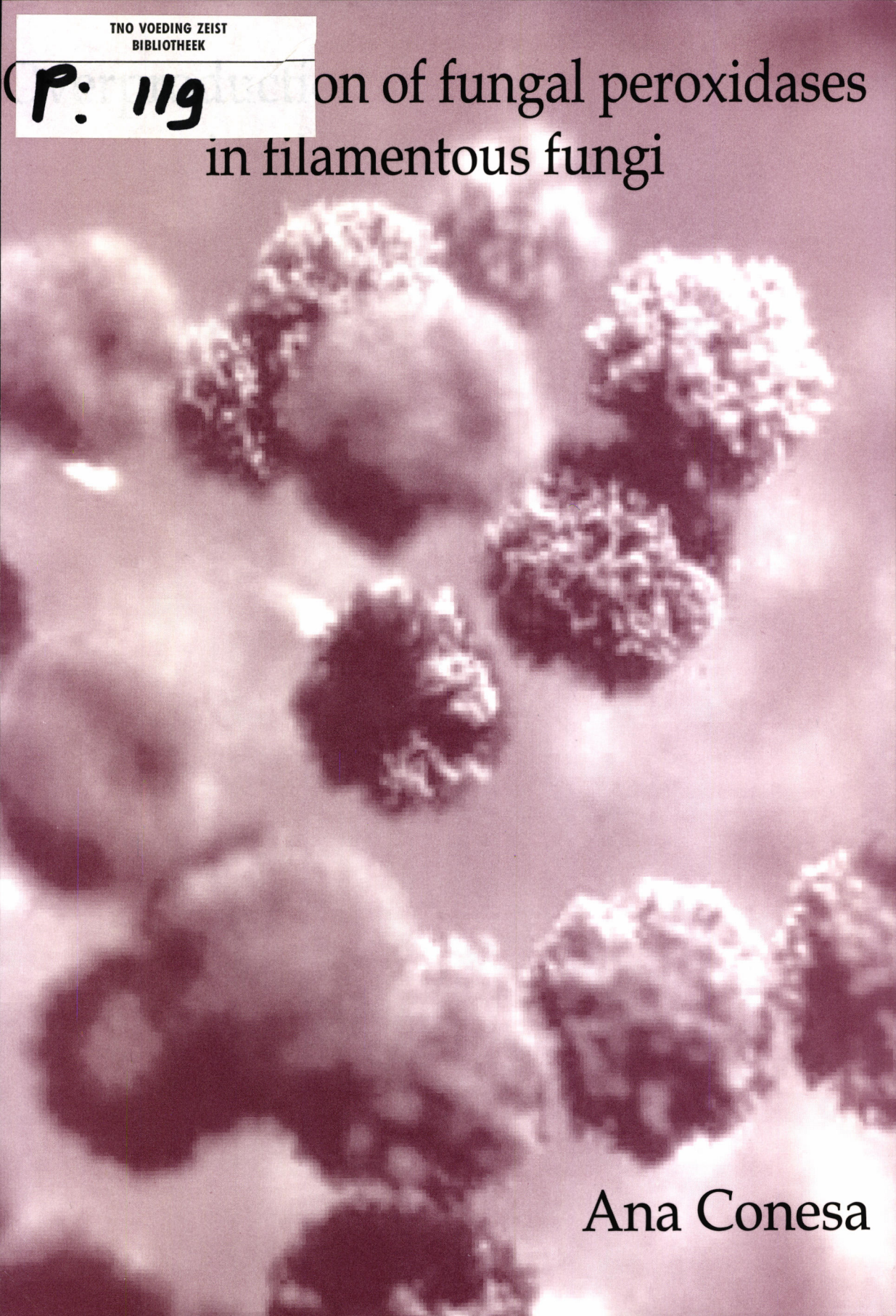
Characterization of fungal peroxidases in filamentous fungi



Ana Conesa

Overproduction of fungal peroxidases in filamentous fungi

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Overproduction of fungal peroxidases in filamentous fungi

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	Prof.dr. P.J.J. Hooykaas

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Al final del viaje está el horizonte,
Al final del viaje partiremos de nuevo,
Al final del viaje comienza el camino,
Otro buen camino que seguir descalzos
contando la arena.

Silvio Rodriguez

A mis padres

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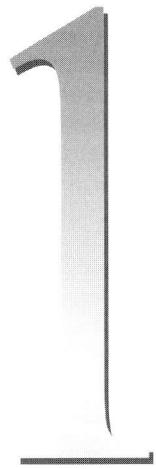
OUTLINE OF THE THESIS

The aim of the work described in this thesis was to study the factors involved in the production of haemoperoxidases in filamentous fungi and to identify bottlenecks for their overproduction. In **chapter 1** a literature review on the structural features and applications of fungal haemoperoxidases is presented. The interest in and difficulties for the heterologous production of this type of proteins are summarised. In **chapter 2** a review is presented on the secretion pathway of the filamentous fungal production hosts used in this study. Special attention is payed to recent research efforts for increasing the production of heterologous proteins in these organisms. For our studies on peroxidase production we have used *Aspergillus niger* as production host. The expression of three peroxidase enzymes was addressed: the manganese peroxidase (MnP) of the white-rot basidiomycete *Phanerochaete chrysosporium* (**chapter 3**), the lignin peroxidase of the same organism (**chapter 4**) and the chloroperoxidase (CPO) from *Caldariomyces fumago* (**chapter 5**). In **chapter 6**, the chloroperoxidase expression system is used to study aspects related to the post-translational modifications of CPO, namely the role of the C-terminal propeptide of CPO in the biogenesis of this protein. In the last two experimental chapters, limiting factors for the overproduction of fungal peroxidases are addressed using manganese peroxidase as a model system. **Chapter 7** deals with the role of haem availability in haemoprotein overproduction, whereas in **chapter 8** the effect of the overexpression of two chaperones in MnP overproduction is discussed. Finally, in **chapter 9**, the results of this thesis are discussed in more general terms with regard to resolving the bottlenecks in overproduction of fungal peroxidases in filamentous fungi.



Fungal peroxidases: molecular aspects and applications

Ana Conesa, Peter J. Punt and Cees A.M.J.J. van den Hondel

Submitted

Generalities

Peroxidases are enzymes that utilise hydrogen peroxide to catalyse the oxidation of a variety of organic and inorganic compounds. Peroxidases have been isolated from a broad variety of organisms and the great majority contains a protoporphyrin IX (haem) as prosthetic group. They have been divided into two superfamilies, namely, the mammalian peroxidase superfamily which includes enzymes such as lactoperoxidase and myeloperoxidase, and the plant superfamily (English and Tsaprailis, 1995; Welinder *et al.*, 1992). The members of the plant peroxidase superfamily are believed to be evolutionary related and have been classified into three classes (Welinder and Gajhede, 1993). *Class-I*: intracellular peroxidases of prokaryotic origin. These include the yeast cytochrome c peroxidase (CCP[1]), chloroplast and cytosol ascorbate peroxidases and gene-duplicated bacterial peroxidases. *Class-II*: extracellular fungal peroxidases, like the lignin (LiP) and manganese (MnP) peroxidase from *Phanerochaete chrysosporium* and other white-rot basidiomycete peroxidases, involved in lignin degradation. *Class-III*: extracellular plant peroxidases such as the classical horseradish peroxidase (HRP). Class-I peroxidases undergo little post-translational modifications, except for the removal of their targeting sequences, whereas Class-II and -III peroxidases contain a signal peptide, disulphide bridges, glycans and structural calcium.

Peroxidases not fitting within the classification above are the haloperoxidases. In nature, haloperoxidases catalyse the oxidation of halides by H_2O_2 resulting in the halogenation of organic compounds (Butler, 1998). They may contain haem, vanadium or no heterogroup at the redox active centre, the three types showing little sequence homology among them or with the peroxidases described above. Only one haem-containing haloperoxidase, the chloroperoxidase (CPO) from the fungus *Caldariomyces fumago,* has been biochemically characterised and is thought to be involved in the synthesis of halogenated compounds with antimicrobial activity (Morris and Hager, 1966). Vanadium haloperoxidases are found mainly in algae and terrestrial fungi (Butler, 1998; Vollenbroek *et al.*, 1995), whereas the third type is formed by bromo- and chloroperoxidases of bacterial origin (Burd *et al.*, 1995; Pelletier *et al.*, 1994; Rob *et al.*, 1995; Wolfframm *et al.*, 1993).

This review will focus on fungal haem peroxidases, i.e., white-rot basidiomycete peroxidases, especially the lignin and manganese peroxidases, and the haem-containing chloroperoxidases.

Reaction mechanism

The typical haemoperoxidase reaction is a redox process consisting of three distinct steps (Scheme 1) (Everse and Everse, 1991):

The first step is the reaction of the resting enzyme [(Fe^{3+})Pox] with H_2O_2 in a two electron transfer reaction which results in the formation of Compound **I**. Compound **I** has one reducing equivalent at the oxyl-ferric iron [Fe(IV)=O] and the other forms a cation radical [Pox*]. Compound **I** is then reduced by the substrate (A) in two sequential one-electron steps through Compound **II**.

[1] Abbreviations used are: LiP: Lignin peroxidase, MnP: Manganese Peroxidase, CPO: Chloroperoxidase, HRP: Horse Radish Peroxidase, ARP: *Arthromyces ramosus* Peroxidase, CiP: *Coprinus cinereus* Peroxidase CCP: Cytochrome *c* Peroxidase, VA: veratryl alcohol

$(Fe^{3+})Pox + H_2O_2$ --->$(Fe(IV)=O)Pox^{*}+ H_2O$
Compound **I**
$(Fe(IV)=O)Pox^{*} + A$ --->$(Fe(IV)=O)Pox + A^{*}$
Compound **I** Compound **II**
$(Fe(IV)=O)Pox + A$ ---> $(Fe^{3+})Pox + A^{*}$
Compound **II**

Scheme 1. Reaction mechanism for haem peroxidases.

The various types of fungal peroxidases differ in the nature of the reducing substrate.

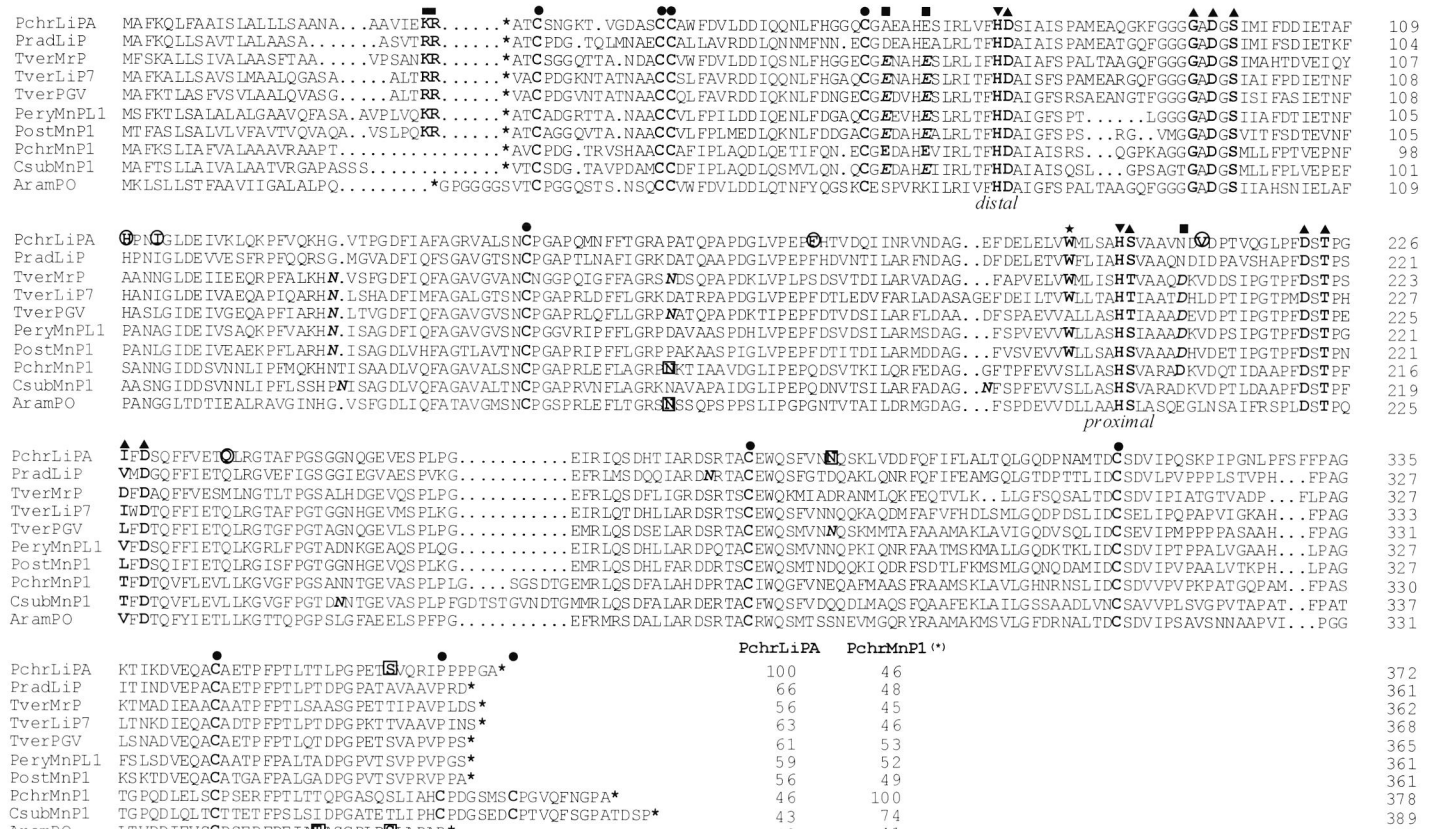
Lignin peroxidases catalyse the oxidation of a wide variety of aromatic nonphenolic lignin model compounds. Enzymatic reactions include benzylic alcohol oxidations, side-chain cleavages, ring-opening reactions, demethoxylations and oxidative dechlorinations (reviewed by Gold and Alic, (1993)). The model substrate for LiP is veratryl alcohol (VA), which is also secreted by the fungus. VA has been suggested to participate in the LiP reaction mechanism in protecting LiP from inactivation by H_2O_2 (Valli *et al.*, 1990) or by acting as a diffusible redox mediator between the enzyme and the substrates that cannot approach the redox centre (Harvey *et al.*, 1986). More recent work indicates that LiP **I** and VA react to form a LiP **II**-VA^{+} complex with a redox potential and lifetime in accordance with the reactions catalysed by LiP (Khindara *et al.*, 1996).

Manganese peroxidases catalyse the Mn-mediated oxidation of lignin and phenolic lignin model compounds. Mn^{2+} is oxidised both by MnP **I** and MnP **II** compounds, generating Mn^{3+}. Mn^{3+} is subsequently stabilised by chelation with organic acids such as oxalate and malonate, both chelators being also secreted by the fungus, and diffuses from the surface of the enzyme to oxidise the organic substrates (Glenn *et al.*, 1986).

Similarly to other haloperoxidases chloroperoxidase catalyses, apart from oxidative dehydrogenation reactions as in Scheme 1, oxygen transfer reactions, H_2O_2 disproportionations and oxidative chlorinations in which Cl^{-} is transferred to an organic substrate (reviewed by van Deurzen *et al.*, 1997).

Fig. 1. A: Multiple alignment of selected fungal peroxidases. Proteins were selected to include representatives of the different groups of peroxidases obtained in the clustering analysis shown in figure 3. The alignment was created with the program DNAMAN and manually optimised following structural information. PchrLiPA: *Phanerochaete chrysosporium* lignin peroxidase A (isozyme H8); PradLiP: *Phlebia radiata* lignin peroxidase; TverMrP: *Trametes versicolor* manganese-repressed peroxidase; TverLiP7: *T. versicolor* lignin peroxidase isozyme 7. TverPGV: *T. versicolor* peroxidase PGV; PeryMnPL1: *Pleurotus eryngii* manganese peroxidase 1. PostMnP1: *P. ostreatus* manganese peroxidase 1; PchrMnP1: *P. chrysosporium* manganese peroxidase 1; CsubMnP1: *Ceriporiopsis subvermispora* manganese peroxidase 1; AramPO: *Arthromyces ramosus* peroxidase. For GenBank accession numbers see Fig.3. (*) The percentage of sequence identity with PchrLiPa and PchrMnP1 is indicated. **B**: *Caldariomyces fumago* chloroperoxidase (CfumCPO) complete sequence (GenBank accession number AJ300448).
The sequence of the (putative) mature proteins is flanked by ✻. Dibasic motifs at the splicing site of prosequences are indicated by ▬. Relevant residues are highlighted in **bold**. Proximal and distal haem ligands are indicated in with ▼. S-S forming cysteines are indicated with ●. Cation-binding residues are indicated with ▲. The Mn^{2+} binding site of PchryMnP1 is indicated with ■; conserved Mn^{2+} binding sites are shown in *italics*. The VA-oxidising tryptophan residue of PchrLiPA (W171) is denoted by ★; conserved tryptophan residues are shown in *italics*. Putative substrate-interacting residues in PchrLiPA are circled. Glycosylation sites are boxed; putative N-glycosylation sites are shown in *italics*.

A


PchrLiPA
PradLiP
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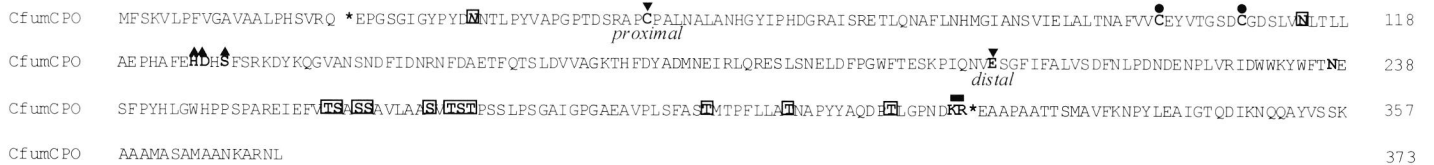
PchrLiPA 100 46
PradLiP 66 48
TverMrP 56 45
TverLiP7 63 46
TverPGV 61 53
PeryMnPL1 59 52
PostMnPl 56 49
PchrMnPl 46 100
CsubMnPl 43 74
AramPO 43 41

B


CfumCPO
proximal
distal

Structural features

The haem environment

The 3-D structure of a considerable number of fungal peroxidases has been resolved by crystallographic refinement (Kunishima *et al.*, 1994; Poulos *et al.*, 1993; Sundaramoorthy *et al.*, 1994; Sundaramoorthy *et al.*, 1995) or homology modelling (Hoffren *et al.*, 1993; Ruiz-Dueñas *et al.*, 1999). Figure 1 displays a multiple sequence alignment of several characteristic peroxidases (see also below), indicating relevant structural residues. Fungal peroxidases contain a single high-spin protoporphyrin IX (haem *b*) as prosthetic group. The haem group is sandwiched between an N-terminal and a C-terminal helix and rests at the bottom of the space formed by the surfaces of both structures. The iron coordination and the residues involved in the active site are conserved among most peroxidases. The Fe is pentacoordinated to the four pyrrole nitrogens of the haem and to the nitrogen in the

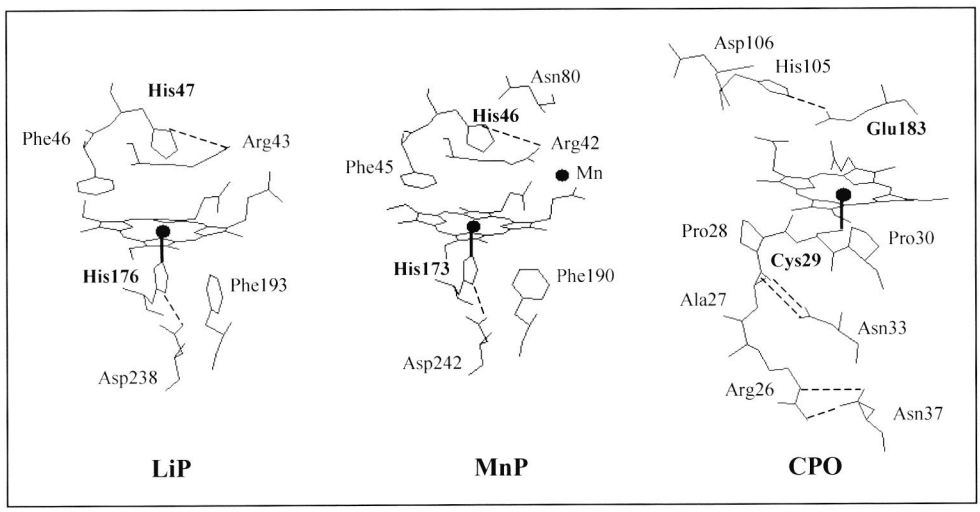


Fig. 2. The haem pocket of LiP, MnP and CPO. Proximal and distal ligand residues are indicated in **bold**. Haem coordination to the proximal ligands is indicated by a solid line. Hydrogen bonds are represented by *dashed lines*.

imidazole group of the proximal histidine. Another, distal, histidine, assisted by an asparagine residue, participates in the transfer of the oxidising equivalents from H_2O_2 to the haem. In contrast, CPO contains a thiolate rather than imidazole fifth ligand, which resembles the situation in the P450 cytochromes. Moreover, in the CPO distal pocket, though polar as in other peroxidases, a glutamic acid instead of a histidine is responsible for the cleavage of the peroxide O-O bond in the formation of Compound **I** (Sundaramoorthy *et al.*, 1995) (Fig.2).

It has been suggested that the length of the Fe-imidazolic nitrogen (Fe-Nε2) bond is related to the redox potential of the different peroxidases. A weaker, longer Fe-Nε2 bond, such as in LiP, would imply a higher basicity of the imidazole group, a more stable Compound **I** and a higher redox potential (Banci *et al.*, 1991), although not all peroxidases follow this rule (discussed by Choinowski *et al.*, 1999). This basicity is dependent on the electron withdrawal applied on the imidazolic nitrogen by the residues surrounding the proximal histidine (Sinclair *et al.*, 1995). In LiP and MnP, Ser177 and Asp201 have been pointed out as two residues involved in the weakening of the Fe-Nε2 bond. In CPO, however,

the proximal ligand, the cysteine sulphur atom, is stabilised by a positive electrostatic environment that, as for the other peroxidases, may be necessary to increase the haem iron redox potential (Sundaramoorthy *et al.*, 1995). Moreover, the redox potential is pH-dependent. Considering their respective pH optimum, CPO, LiP and MnP show higher redox potentials than other peroxidases such as ARP, HRP and CCP (Hammel *et al.*, 1986; Kersten *et al.*, 1990; Makino *et al.*, 1976; Millis *et al.*, 1989). In all cases, the rate of Compound **I** formation (oxidation of the enzyme by H_2O_2) is relatively insensitive to pH over a wide range (Marquez *et al.*, 1988; Wariishi *et al.*, 1991), but the reduction of Compound **I** (substrate oxidation) is pH-sensitive, with an increasing potential as the pH decreases. LiP, MnP and CPO have low optimal pHs: 3 (Tien and Kirk, 1985), 4.7 (Glenn and Gold, 1985) and 2.75 (Morris and Hager, 1966) respectively, and it has been hypothesised that the *pH optimum of these enzymes reflects the pH required to attain the redox levels necessary to oxidise high redox potential substrates*. The pH dependence of the redox potential could indicate the participation of a carboxylic acid side chain in the catalytic reaction. For LiP, several residues have been proposed to play this role (Poulos *et al.*, 1993): Asp183, which forms a unique carboxylate-carboxylate bond with one haem propionate and Glu146, a possible substrate interacting residue. In the case of MnP, three acidic residues are involved in Mn binding (Sundaramoorthy *et al.*, 1994), whereas CPO, as already discussed, utilises a glutamic acid for the distal Fe ligand (Sundaramoorthy *et al.*, 1995). None of these features are present in CCP, ARP or HRP, enzymes working at neutral pH.

Substrate binding

Another point of much study in the structure of these enzymes has been the interaction of the catalytic centre with the substrate. In most peroxidases direct access to the Fe^{4+}=O is restricted but the haem edge is available for substrate interactions (discussed by Smith and Veitch, 1998). In LiP the opening to the haem edge is much smaller than in other peroxidases but may still fit small molecules such as veratryl alcohol (Poulos *et al.*, 1993). An environment of hydrophobic residues at this opening (Ile-85, Val-184, Gln-222, Phe-148 and His-82) may provide the primary contacts between the aromatic substrate and the protein (Poulos *et al.*, 1993; Schoemaker *et al.*, 1994). Recently, a second substrate interaction site, located at Trp171 has been proposed (Doyle *et al.*, 1998). This residue, present in all LiPs but not in other peroxidases has been shown to be redox active and has been suggested to be an alternative binding site for VA and other aromatic substrates (Choinowski *et al.*, 1999). Replacement of this residue by site-directed mutagenesis resulted in loss of VA oxidising activity (Doyle *et al.*, 1998) and, conversely, engineering of a tryptophan at the corresponding site in MnP resulted in an enzyme which could oxidise VA (Timofeevski *et al.*, 1999). Moreover, a tryptophan residue is present at the homologous position of a novel *Pleurotus eryngii* peroxidase (Ruiz-Dueñas *et al.*, 1999), an enzyme which can oxidise both Mn(II) and VA (Ruiz-Dueñas *et al.*, 1999).

Similarly, determination of the crystal structure of MnP (Sundaramoorthy *et al.*, 1994) revealed the putative site for Mn^{2+}. Mn^{2+} was proposed to be hexacoordinated to the carboxylate oxygens of Glu35, Glu39 and Asp179, a haem propionate oxygen and two water oxygens. A similar site was also found in the modelled structure of the Mn-binding *P. eryngii* peroxidase (Ruiz-Dueñas *et al.*, 1999). The participation of these residues in Mn^{2+} binding by MnP was confirmed by site-directed mutagenesis (Kishi *et al.*, 1996; Whitwam *et al.*, 1997) and crystallographic analysis (Sundaramoorthy *et al.*, 1997). These studies showed that the three acidic residues are involved in both Mn^{2+} oxidation and Compound **II** reduction (Kishi

et al., 1996) but not in the reaction with phenolic substrates or with H_2O_2 (compound **I** formation) (Kishi *et al.*, 1996; Whitwam *et al.*, 1997). Furthermore, resolution of the crystal structures of a single (D179N) and double (E35Q, D179N) MnP mutants showed that the mutant proteins lack a cation at the Mn^{2+} binding site (Sundaramoorthy *et al.*, 1997).

Yeung *et al.* (1997) tried to engineer a Mn^{2+} binding site in CCP by constructing a site-directed triple mutant (Gly41->Glu, Val45->Glu, His181->Asp), which they named MnCcP. Although these changes resulted in a typically MnP Mn^{2+}-binding spectrum for MnCcP, little Mn^{2+} oxidising capabilities were created in the mutant enzyme. Factors which should contribute to the low activity of MnCcP include different pH optima, lower reduction potential of Compound **I** and the formation in Compound **I** of a ferryl tryptophan radical rather than the ferryl porphyrin IX cation radical of MnP. The possibility of creating a Mn^{2+}-binding site in LiP has also been subject of discussion (Poulos *et al.*, 1993). Of the three Mn^{2+}-binding residues in MnP one is conserved in LiP (Glu40); Asp179 is Asn182 and Glu35 is Ala36. Although it seems possible to accommodate an Asp residue in place of Asn in the LiP structure, the space occupied by the side chain of Glu35 in MnP is filled by the backbone structure of the C-terminus in LiP. This does not leave the necessary space in LiP for an acidic residue. Therefore, the creation of a Mn^{2+}-binding site in LiP appears more complicated than a few amino acid substitutions.

Finally, in CPO, the haem edge is not connected to the molecular surface via a channel as in other peroxidases, but there is a small opening above the haem which could allow direct access of the substrate to the Fe^{4+}=O (Sundaramoorthy *et al.*, 1995). In this characteristic CPO resembles once more the P450 cytochromes and differs from the classical peroxidases. Hydrophobic residues such as Phe103, Val182 and Phe186 surround the opening to the Fe^{4+}=O and may be involved in substrate binding. A clear binding site for Cl^- could not be identified in the crystal structure, however (Sundaramoorthy *et al.*, 1995).

Other post-translational modifications

As already pointed out in the first section, class II peroxidases contain glycans, disulphide bonds and structural calcium. These features are also present in the *C. fumago* chloroperoxidase. Four disulphide bonds have been shown to maintain the protein structure in LiP, ARP and *T. versicolor* peroxidases (Kunishima *et al.*, 1994; Limongi *et al.*, 1995; Poulos *et al.*, 1993) and a fifth SH bridge is present in MnP (Sundaramoorthy *et al.*, 1994). In contrast, only three cysteines are found in the sequence of CPO from which Cys29 forms the axial haem ligand, the other two remaining available for disulphide bond formation (Sundaramoorthy *et al.*, 1995).

Both N- and O-glycans are found in extracellular peroxidases, and differences in glycosylation extent are, in some cases, responsible for the appearance of isozymes (Kenigsberg *et al.*, 1987; Kjalke *et al.*, 1992). Deglycosylation studies and expression in *E. coli* (Dalton *et al.*, 1996; Doyle and Smith, 1996; Whitwam *et al.*, 1995; Zong *et al.*, 1995) have shown that glycan groups are not essential for activity, but they do improve the enzyme stability (Hiner *et al.*, 1995; Nie *et al.*, 1999) and solubility (Tams *et al.*, 1999).

The presence of structural calcium is also a crucial characteristic of extracellular peroxidases. Two highly conserved Ca^{2+}-binding sites are typically present in these proteins, which have been located at the proximal and distal domains (Kunishima *et al.*, 1994; Poulos *et al.*, 1993; Sundaramoorthy *et al.*, 1994). Reconstitution of aggregated apoforms of the in *E. coli* recombinantly produced peroxidases requires Ca^{2+} (Doyle and Smith, 1996; Whitwam and Tien, 1996; Zong *et al.*, 1995) and Ca^{2+} binding has been shown to be important for

enzyme stability (Sutherland and Aust, 1996). It is currently believed that Ca^{2+} may play an important role in maintaining the structural environment of the active site (reviewed by Banci, 1997).

Being extracellular enzymes, fungal peroxidases are synthesised with an N-terminal signal peptide which targets them to the secretory pathway and is later removed. Additionally, a 7-aa propeptide is found at the N-terminus of the *P. chrysosporium* LiP (Ritch *et al.*, 1991) which has also been proposed to be present in the *P. radiata* LiP (Saloheimo *et al.*, 1989) and the *P. ostreatus* MnP (Asada *et al.*, 1995) proteins. This propeptide is removed by cleavage after a dibasic site (K/RR, Fig.1). A propeptide in also found in the *C. fumago* chloroperoxidase, in this case, located at the C-terminus. Interestingly an identical motif (KR) is found at the site of processing of the CPO C-terminal propeptide (Sundaramoorthy *et al.*, 1995). The biological function of these prosequences is unknown.

Molecular genetics of fungal peroxidases

Gene organisation and homology analysis

Lignin-degrading peroxidases have been found in a large number of basidiomycetous fungi (Kimura *et al.*, 1990; Pelaez *et al.*, 1995; Varela *et al.*, 2000), and in species such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus spp.*, *Phlebia radiata*, *Coprinus spp.*, *Bjerkandera adusta, Ceriporiopsis subvermispora, Dichomitus squalens* and in the imperfect fungus *Arthromyces ramosus*, the proteins have been studied at the molecular level. Lignolytic peroxidases are often secreted as multiple isozymes that may originate from multiple genes and/or differential post-translational modifications. Typically, the secreted enzymes are classified as lignin peroxidases or manganese peroxidases based on their substrate specificity (see above). For example, in the best-characterised white-rot fungus, *P. chrysosporium*, at least ten structurally similar LiP (*lipA-J*) and three MnP (*mnp1-3*) genes are present. The ten LiP genes cluster in three linkage groups, whereas the three MnP genes are unlinked (reviewed by Cullen, (1997)). In *T. versicolor*, two LiP and one MnP encoding genes are found grouped within a 10 kb region (Johansson and Nyman, 1996). Also multiple peroxidase genes are found in *Pleurotus* and *Ceriporiopsis* species (Camarero *et al.*, 2000; Lobos *et al.*, 1998; Ruiz-Dueñas *et al.*, 1999; Tello *et al.*, 2000).

Homology analysis of the basidiomycete peroxidase sequences present in public databases shows a high degree of similarity (54%) within this class of enzymes. The dendrogram representation of the multiple alignment indicates a number of interesting features (Figure 3; see also Figure 1). The *Arthromyces ramosus* peroxidase (identical to the *Coprinus cinereus* peroxidase (Kjalke *et al.*, 1992)) does not cluster with the other lignolytic enzymes. Biochemically, this protein also shows different properties, such as the inability to oxidise either Mn or VA and a neutral, rather than acidic optimal pH (Farhangrazi *et al.*, 1994). The remaining peroxidases can be distributed basically within three groups. One clustering group comprises the manganese peroxidases from *P. chrysosporium*, *C. subvermispora* and *D. squalens*. These enzymes show a high degree (~75%) of homology with each other, which extends to the fifth cysteine pair and the Mn-binding residues proposed for the *P. chrysosporium* MnP (Sundaramoorthy *et al.*, 1994). A second group includes the lignin peroxidases from *P. chrysosporium*, *B. adusta* and *P. radiata*. They all conserve the putative substrate-binding residues, including the W171, revealed in the analysis of the crystal structure of the *P. chrysosporium* LiPA (Poulos *et al.*, 1993), as well as a dibasic-motif containing N-terminus which resembles the pre-pro structure described for the leader peptide of LiPA (Ritch *et al.*, 1991). Finally, the third cluster contains the peroxidases

from *T. versicolor*, and *Pleurotus spp*. These enzymes show some characteristics of the previous two groups. For example, they all contain the pre-pro-like structure at the N-terminus, eight cysteines and a shorter, "LiP-like" C-terminus. In addition, the manganese-dependent peroxidases of this group conserve the Mn-binding residues, and, except for the *P. ostreatus* MnP and *T. versicolor* PGV, also the tryptophan residue (W171 in LiP) involved in VA oxidising activity. Interestingly the manganese peroxidases from *P. eryngii*, also clustering in this group, were shown to oxidise both Mn^{2+} and veratryl alcohol (Camarero *et al.*, 1999; Ruiz-Dueñas *et al.*, 1999). Furthermore, a manganese peroxidase-lignin peroxidase hybrid enzyme was reported to be secreted by *Bjerkandera spp* (Mester and Field, 1998). Although the complete sequence of this protein is not known, its N-terminus shows most homology to the *T. versicolor* LiP7, an enzyme that contains the putative VA and Mn^{2+} binding residues. However, whether this last clustering group forms a class of Lip-MnP hybrid enzymes needs to be further investigated. The recently isolated *T. versicolor* manganese-repressed peroxidase and the G. *applatanum* MnP, although the homology analysis places them in an intermediate subgroup, also share LiP-like and MnP-like characteristics.

Concerning CPO-related sequences, database mining produces only two significant matches, namely with the gene products of the *A. nidulans stcC* (Brown *et al.*, 1996) and an *A. bisporus* ORF (accession number AJ293759). For both putative proteins, homology with CPO is restricted to the haem pocket. The *A. nidulans stcC* is a member of the sterigmatocystin biosynthetic gene cluster and the *A. bisporus* gene is reported to be involved in cellulose degradation. However, the catalytic characteristics of these proteins have not yet been determined.

Gene regulation

Typically, expression of the lignolytic genes is tiggered in situations of carbon, nitrogen and sulphur limitation (Gold and Alic, 1993). However, for *Bjerkandera adusta* and *Pleurotus ostreatus*, induction by high nitrogen has also been reported (Kaal *et al.*, 1995; Mester *et al.*, 1996). Transcription of MnP genes is, additionally, regulated by Mn^{2+} (Brown *et al.*, 1991), although the *P. chrysosporium mnp3* and the *C. subvermispora mnp3* genes were shown not to respond to Mn^{2+} induction (Gettemy *et al.*, 1998; Tello *et al.*, 2000). Recently, a manganese repressed, nitrogen induced peroxidase has been identified in *T. versicolor* (Collins *et al.*, 1999). The expression of this peroxidase gene was also insensitive to heat shock, and oxidative and chemical stress (Collins *et al.*, 1999), factors that have been shown to regulate the transcription of the *P. chrysosporium mnp* genes (Brown *et al.*, 1993; Li *et al.*, 1995) and to trigger lignin peroxidase production (Zacchi *et al.*, 2000). Furthermore, the expression pattern of the different peroxidase isozymes varies greatly depending on the strain, culture conditions, substrate and time course, and is not related to their genomic organisation (reviewed by Cullen, 1997). Although the reason for the multiplicity of lignolytic enzymes and the heterogeneity of their gene regulation found in white-rot basidiomyces is not fully understood, it might reflect the adaptation strategy of the fungus to ensure expression under different environmental situations and/or lignin substrates.

The *C. fumago* CPO is produced during primary metabolism. The protein is encoded by a single gene, which is strongly repressed by glucose and induced by fructose (Axley *et al.*, 1986). The physiological significance of this regulation is unknown.

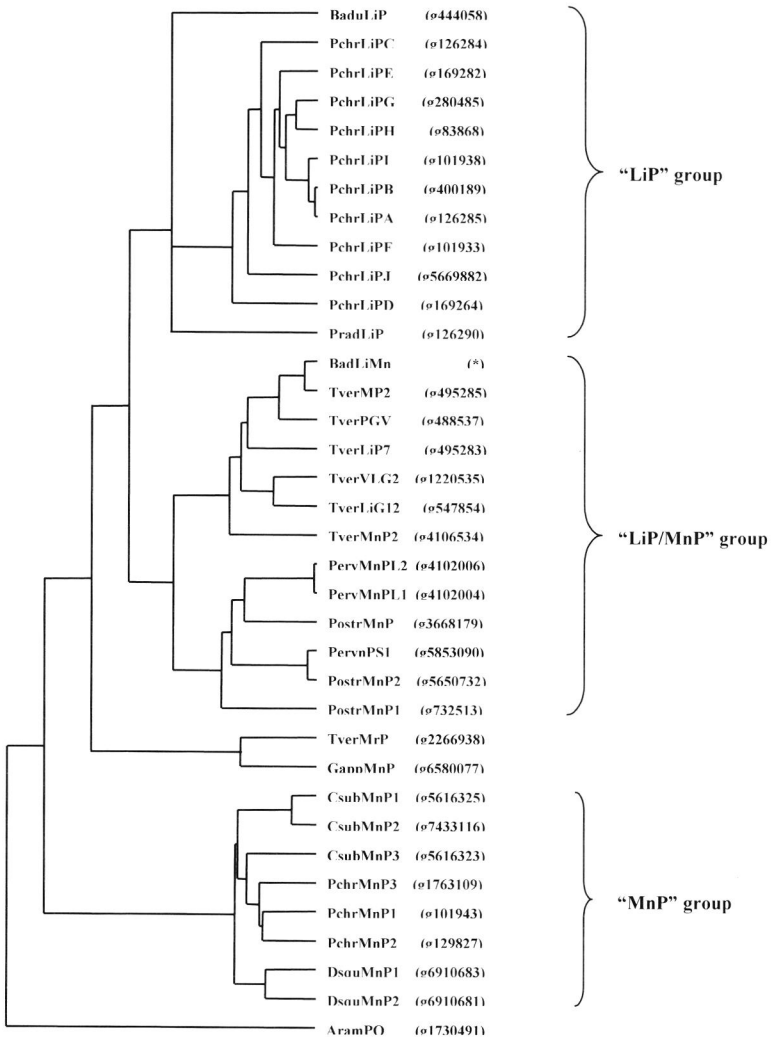


Fig. 3. Dendrogram representation of the multiple alignment of basidiomycete peroxidases. Clustering groups are indicated (see text).Organism abbreviations are: Pchr: *Phanerochaete chrysosporium*, Prad: *Phlebia radiata*; Badu: *Bjerkandera adusta*, Tver: *Trametes versicolor*, Post: *Pleurotus ostreatus*, Pery: *Pleurotus eryngii*, Csub: *Ceriporiopsis subvermispora*, Dsqu: *Dichomitus squalens* and Aram: *Arthromyces ramosus*. Gapp: *Ganoderma applatanum*. GenBank identification numbers are given in brackets. (*): N-terminal sequence only (Mester and Field, 1998).

The application of fungal peroxidases

Fungal peroxidases have been subject of considerable research in the last two decades for their possible industrial and environmental applications. Lignin and manganese peroxidases are part of the extracellular oxidative system developed by white-rot fungi to degrade lignin. Other components of this system are laccases, a H_2O_2 generating system and non-enzymatic agents such as oxalate and veratryl alcohol. Lignin is a highly complex, stable and irregular polymer consisting of non-repeating phenyl propanoid units linked by various carbon-carbon and ether bonds (Sarkanen and Ludwig, 1971). This structure of the lignin polymer implies that lignolytic enzymes possess the ability to oxidise substrates of high redox potential in a non-specific manner. Hence, white-rot fungi are also able to degrade a large number of environmental recalcitrant pollutants, such as polycyclic and chlorinated aromatic compounds, DDT and other pesticides, dyes, munitions, cyanides, azides and cross-linked acrylic polymers (Barr and Aust, 1994; Higson, 1991; Krcmar and Ulrich, 1998; Sutherland *et al.*, 1997). The use of white-rot fungi as a biological and environmentally friendly alternative to the highly contaminating pulping and bleaching treatments of the paper and pulp industries (for reviews see Breen and Singleton,1999 and Paice *et al.*, 1995) and in the degradation of coal tars (Aust and Bumpus, 1997; Ziomek *et al.*, 1991) has been extensively studied. In many cases, the direct involvement of lignin peroxidase and/or manganese peroxidase in these processes has been demonstrated. Purified preparations of lignin and manganese peroxidase showed to be effective in decolouring Kraft effluents (Ferrer *et al.*, 1991; Kondo *et al.*, 1994) and in oxidising a broad range of xenobiotic compounds (summarised by Field *et al.*, 1993). Hunt *et al.* (1998) reported the activity and optimisation of the Mn-peroxidase of *Trametes versicolor* in pulp delignification, and *in vitro* depolymerisation studies using LiP and MnP showed that the enzymes were able to degrade coal substrates (reviewed by Fakoussa and Hofrichter, 1999).

CPO has raised the interest of the fine chemicals industry for its wide substrate repertoire and the selectivity of its reactions. Apart from the natural halogenating activity of CPO, this enzyme catalyses synthetically useful (enantioselective) oxygen transfer reactions, e.g. asymmetric epoxidation of olefins epoxidation, allylic, benzylic, and propargylic hydroxylation, asymmetric sulfoxidation, and regio-selective oxidation of indoles (reviewed by van de Velde, (2000)).

Limitations to the application of fungal peroxidases

Despite the potential industrial use of fungal peroxidases, the actual application of these enzymes in industrial processes is hampered by two main constraints: the limited availability of the proteins and their rather low stability. In their natural hosts, lignolytic enzymes are produced during secondary metabolism in response to nutrient starvation and only limited amounts are produced (for a review see Reddy and D'Souza, 1994). Although CPO production does not have the problem of low secretion, (up to 500 mg/L can be obtained in fructose induced cultures of *C. fumago* (Pickard and Hashimoto, 1982), this protein suffers, similarly to other fungal peroxidases, from inactivation by H_2O_2 and elevated temperatures.

Haem peroxidases are readily inactivated by the oxidant. Enzyme inactivation may develop from the oxidative destruction of the porphyrin ring, the irreversible reaction with suicide inhibitors such as sodium azide and hydrazines or from the formation of the so-called Compound III or oxyperoxidase after reaction of enzyme intermediates with H_2O_2 as reductant (discussed by van de Velde, 2000). This last mechanism is believed to operate

when the enzyme is incubated in the absence of a reducing substrate and in excess of H_2O_2 and implies a serious constraint for the high-input applications of these proteins.

Several approaches have been applied in attempts to increase the stability of peroxidases, either by thoroughly regulating the H_2O_2 concentration in the reaction or by the intrinsic improvement of the enzymes by genetic engineering. The first approach includes methods such as the "feed-on demand" system or by *in situ* generation of H_2O_2 and have been extensively discussed elsewhere (van de Velde, 2000). Site-directed mutagenesis has proven to be a powerful tool to study and improve oxidative and thermal stability of various peroxidases. Welinder *et al.* (1993) analysed the effect on oxidative stability of single and multiple amino acid substitutions near the substrate channel or the active centre of the *Coprinus cinereus* Peroxidase (CiP). They observed that mutations resulting in the introduction of bulky amino acids in the proximity of the substrate channel (G154E+G146F and G154E+G156F+N157E) improved the oxidative stability of the enzyme. These mutants had, however, impaired catalytic activity (measured as dye bleaching), which could be recovered by introducing "accelerators" such as 7-hydroxycoumarin in the enzymatic reaction, suggesting that such mutations restricted accessibility of the dye but not of the smaller accelerator to the reactive centre of the protein. Cherry *et al.* (1999) significantly improved this result by applying random mutagenesis and *in vivo* shuffling to the stability improvement of the same enzyme. An improvement of 174 times the thermal stability and 100 times the oxidative stability was achieved by a combination of both techniques. The resulting improved enzyme had seven amino acid substitutions at positions that included oxidisable residues near the active site, charged destabilising residues, and residues that affected helix topology and protection of the proximal histidine from attack by H_2O_2. Curiously, this mutant, as in the previous work, had a considerably reduced substrate turnover compared to the wild type protein, although the overall performance was improved. Similarly, a triple amino acid substitution in cytochrome *c* completely prevented the enzyme from haem destruction by oxygen peroxide but entailed a significant loss of catalytic activity (Villegas *et al.*, 2000). The reasons for the apparently paired oxidative stabilisation and activity reduction observed in these studies were not clear. However, if inactivation by H_2O_2 was prevented by restricting its interaction with the haem, it should then not be surprising that the enzymatic activity is also reduced. A detailed analysis of the reaction mechanism in the mutant proteins is needed in order to resolve this question.

Production of peroxidases in recombinant systems

Overexpression of fungal peroxidase encoding genes in recombinant systems has been investigated in attempts to, increase production yields on one hand, and on the other, to provide a system for site-specific mutagenesis, gene evolution studies and improvement of the activity and stability of these oxidising enzymes. However, only limited success has been obtained so far (Table 1). Expression of fungal peroxidases in *E. coli* resulted invariably in the production of the apoenzymes in inclusion bodies and protein reconstitution to yield the active enzymes was only possible after tedious and commercially unattractive treatment with haem, Ca^{2+}, urea and/or high pressure (Doyle and Smith, 1996; Whitwam and Tien, 1996; Whitwam *et al.*, 1995; Zong *et al.*, 1995). Similarly, expression of CPO in insect cells resulted in the production of extracellular inactive enzyme. In this case, reconstitution to active protein was not possible (Sigle, 1993). In contrast, manganese peroxidase isozyme H4 and lignin peroxidase isozymes H2 and H8 were successfully produced as active proteins using the baculovirus expression system (Johnson and Li, 1991; Johnson *et al.*, 1992; Pease

Table 1. Production of fungal peroxidases in recombinant systems

Protein	Host	Result	Reference
CPO(*)	*E. coli*	Apoenzyme in inclusion bodies	(Zong *et al.*, 1995)
"	Baculovirus	Inactive CPO	(Sigle, 1993)
"	*S. cerevisiae*	No extracellular protein	(Sigle, 1993)
"	*C. fumago*	Mutant CPO	(Yi *et al.*, 1999)
PchrLiP	*E. coli*	Apoenzyme in inclusion bodies	(Doyle and Smith, 1996)
"	Baculovirus	0.4 mg/mL, hemin addition	(Johnson and Li, 1991)
"	Baculovirus	Active LiP	(Johnson *et al.*, 1992)
"	*S. cerevisiae*	No extracellular protein	(Pease and Tien, 1991)
"	*P. chrysosporium*	2 mg/L (gpd promoter)	(Sollewijn Gelpke *et al.*, 1999)
"	*A. niger*	1.125 nKat/mg extracellular protein	(Aifa *et al.*, 1999)
PradLiP	*T. reseei*	No extracellular protein	(Saloheimo and Niku-Paavola, 1991)
DsqutMnP	*P. chrysosporium*	1.0-1.5 mg/L	(Li *et al.*, 2001)
PchryMnP	*E. coli*	Apoenzyme in inclusion bodies	(Whitwam *et al.*, 1995)
"	Baculovirus	35.7 U/L, hemin addition	(Pease *et al.*, 1991)
"	*S. cerevisiae*	No extracellular protein	(Pease and Tien, 1991)
"	*P. chrysosporium*	2 µmol/mL/min (gpd promoter)	(Mayfield *et al.*, 1994)
"	*A. oryzae*	5 mg/L, haem addition	(Stewart *et al.*, 1996)
PerMnPL2	*A. nidulans*	Active protein, haem addition	(Ruiz-Dueñas *et al.*, 1999)
CiP/ARP	*S. cerevisiae*	0.02 U/mL	(Sawai-Hatanaka *et al.*, 1995)
"	*A. oryzae*	~ 1000 PODU/mL, haem addition	(Andersen *et al.*, 1992)

(*) CPO: *Caldariomyces fumago* chloroperoxidase, PchrMnP: *Phanerochaete chrysosporium* manganese peroxidase, PchrLiP: *P. chrysosporium* lignin peroxidase, PradLiP: *Phlebia radiata* lignin peroxidase, DsqutMnP: *Dichomitus squalens* thermostable manganese peroxidase. PerMnPL2: *Pleurotus eryngii* manganese peroxidase, CiP: *Coprinus cinereus* peroxidase. Approaches resulting in production of active enzymes are underlined.

et al., 1991). Unfortunately, this system suffers from low yields and high production costs, and therefore is inappropriate for industrial scaling-up. In *S .cerevisiae*, only the ARP/CiP has been produced with reasonable success (Sawai-Hatanaka *et al.*, 1995), whereas expression of CPO (Sigle, 1993) or the *P. chrysosporium* peroxidases (Pease and Tien, 1991) did not result in any detectable extracellular activity. As a further possibility, the overproduction in the homologous hosts was attempted. Since peroxidase production in white-rot fungi normally occurs during secondary metabolism, the recombinant production of these proteins has been addressed by expression of the encoding genes under control of the strong, constitutively expressed glyceraldehyde phosphate dehydrogenase (*gpd*) promoter. This approach resulted in the successful production of the *P. chrysosporium* manganese and lignin peroxidases, as well as of a thermostable manganese peroxidase from *D. squalens*, under nutrient-rich conditions (Li *et al.*, 2001; Mayfield *et al.*, 1994; Sollewijn Gelpke *et al.*, 1999). However, the production levels obtained were not much higher than those typical for the lignolytic conditions. Similarly, CPO has been recombinantly expressed in *Caldariomyces fumago* (Yi *et al.*, 1999). In this work expression of CPO site-directed mutants, and not increased protein production, was the main goal. However, as this system uses the native CPO promoter and gene replacement is required, identification of the recombinant CPO expressing strains is complicated and uncertain.

Although some of these approaches have succeeded in producing limited amounts of the recombinant proteins, which may be sufficient for fundamental and site specific mutagenesis studies, this does not resolve the problem of the large-scale production requirements. Another potential expression system which has proven to be suitable for high-level protein production is that of filamentous fungi such as *Aspergillus* and *Trichoderma*.

These fungi have an extraordinary protein production capacity and they have been exploited for the production of various homologous and recombinant proteins (van den Hondel *et al.*, 1992). However, initial studies on overproduction of fungal peroxidases in these hosts showed only limited sucess. Expression of both the *P. radiata* lignin peroxidase in *T. reesei* (Saloheimo and Niku-Paavola, 1991) and the *C. fumago* CPO in *A. nidulans* (Sigle, 1993) failed in producing any extracellular protein and only traces of extracellular peroxidase activity were detected upon expression of the *P. chrysosporium* lignin peroxidase in a tunisian *A. niger* strain (Aifa *et al.*, 1999). In contrast, the *C. cinereus* peroxidase, the *P. chrysosporium* MnP and the *Pleurotus eryngii* manganese peroxidase MnPL2 have been successfully produced as active proteins in *Aspergillus spp.* (Elrod *et al.*, 1997; Ruiz-Dueñas *et al.*, 1999; Stewart *et al.*, 1996). However, initial production yields obtained in these studies were low compared to those typical of other fungal proteins and, in some cases, the addition of large amounts of haem was required to improve production yields. Based on these results we have initiated a research project on the use of *Aspergillus spp.* for the production of fungal peroxidases. The results of this work will be described in the following chapters of this thesis.

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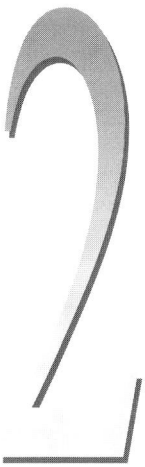
The secretion pathway in filamentous fungi: a biotechnological view



Ana Conesa, Peter J. Punt, Nicole van Luijk and Cees A.M.J.J. van den Hondel

ABSTRACT

The high capacity of the secretion machinery of filamentous fungi has been widely exploited for the production of homologous and heterologous proteins; however, our knowledge of the fungal secretion pathway is still at an early stage. Most of the knowledge comes from models developed in yeast and higher eukaryots, which have served as reference for the studies on fungal species. In this review we compile the data accumulated in recent years on the molecular basis of fungal secretion, emphasizing the relevance of these data for the biotechnological use of the fungal cell, and indicating how this information has been applied in attempts to create improved production strains. We also present recent emerging approaches that promise to provide answers to fundamental questions on the molecular genetics of the fungal secretory pathway.

INTRODUCTION

Filamentous fungi, such as species from the genera *Aspergillus* and *Trichoderma*, are extraordinary in their capability of secreting large amounts of proteins, metabolites and organic acids into the growth medium. This property has been widely exploited by the food and beverage industries where compounds secreted by filamentous fungi have been used for decades. This long tradition of utilisation has led to the conference of the GRAS status (Generally Regarded As Safe) to several of these species, which makes filamentous fungi attractive hosts for the synthesis of (new) products for human use. Since gene-transfer systems are available for these organisms, the possibility has arisen to use them as biological factories for the production of proteins of non-fungal origin. However, whereas large amounts of homologous proteins can be obtained in the industrial fermentation of production strains, production yields obtained with heterologous proteins are often rather low (Gouka *et al.*, 1997; Radzio and Kurk, 1997).

The commercial use of the fungal secretory machinery and the limitations encountered in the production of heterologous proteins has stimulated research into the genetics of protein secretion in filamentous fungi. Although much progress has been made in this field in the last two decades, our knowledge of the fungal secretory pathway is still limited. Nevertheless, it is generally accepted that the secretion pathway in fungi does not differ greatly from those in yeast and higher eukaryots, from which a more complete picture is available. Evidently, there are also significant differences. Particularly, the mycelial growth phenotype of filamentous fungi, which results from polar extension at the hyphal tips, is not found in either *Saccharomyces cerevisiae* or in higher eukaryots. Furthermore, the capacity for protein secretion in filamentous fungi is much higher than that in yeast. While yields up to 30 g/L of extracellular protein can be obtained in the fermentation of certain *Aspergillus* and *Trichoderma* strains (Durand *et al.*, 1988; Finkelstein *et al.*, 1989), only a few yeast species (*Pichia*, *Hansenula*) reach the gram-per-litre production level (Werten *et al.*, 1999; Wyss *et al.*, 1999).

In this paper, we review the present knowledge on the fungal secretion pathway, with special attention to those aspects relevant for the biotechnological application of these organisms. The models that have been developed for the yeast secretion pathway will be taken as a reference to assist in the discussion on data that have been obtained for filamentous fungal species. In specific cases, also data available from mammalian systems will be included in the discussion. However, a detailed review on either the yeast or the mammalian secretion pathway is beyond the scope of this paper and can be found elsewhere (Lazar *et al.*, 1997; Sakaguchi, 1997; Zapun *et al.*, 1999; Benham and Braakman, 2000).

THE SECRETORY PATHWAY

Overview

A schematic view of the fungal secretory pathway is given in Fig 1. Secretory proteins begin their journey to the extracellular medium by entering the endoplasmic reticulum (ER). In the ER proteins are folded and can undergo distinct modifications such as glycosylation, disulphide bridge formation, phosphorylation and subunit assembly. Subsequently, proteins leave the ER packed in transport vesicles and head to the Golgi compartment, where additional modifications can take place such as further glycosylation and peptide processing. Finally, again packed in secretory vesicles, proteins are directed to the plasma membrane from where they are secreted.

In some cases, the proteins will not reach the extracellular space, but are targeted to intracellular compartments such as the vacuole, either to become resident proteins or to undergo proteolytic degradation.

Although there has been some controversy, most studies indicate that protein secretion occurs at the apical or sub-apical hyphal regions (reviewed by Archer and Peberdy, 1997). Recent work has reinforced this hypothesis (Gordon *et al.*, 2000; Lee *et al.*, 1998). Using the novel glucoamylase::green fluorescent fusion protein (GLA::GFP) as secretion reporter to study protein secretion in *Aspergillus niger*, Gordon *et al.* (2000a/b) observed that GFP fluorescence was predominant at the hyphal apices and showed that this approach is a promising tool for further research in this field, as it allows *in vivo* monitoring of protein secretion.

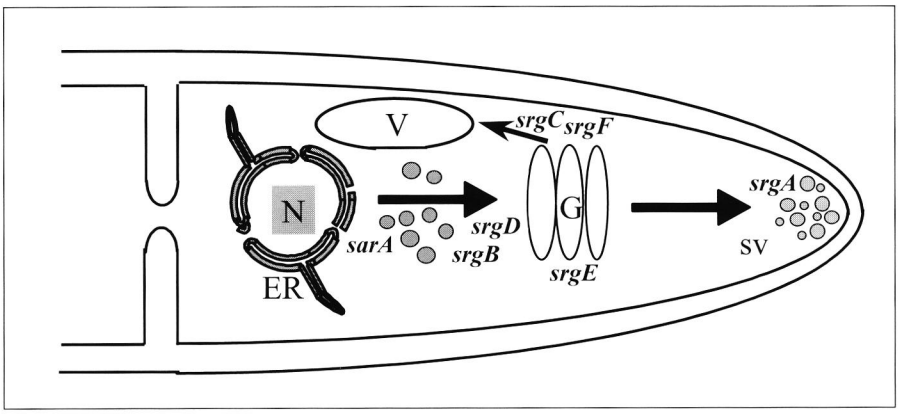


Fig. 1. The secretion pathway in filamentous fungi. Cloned secretion related GTPases (*srgA-F*), involved in vesicular trafficking, are indicated. N: nucleus, V: vacuole, ER: endoplasmic reticulum, G: Golgi apparatus, sv: secretion vesicles.

The apical localisation of protein secretion has led to the suggestion of employing morphological mutants displaying an increased apical surface, i.e. hyperbranching mutants, as "supersecretion" strains (Lee *et al.*, 1998). Moreover, hyperbranching strains often grow as compact pellets, which results in low-viscosity cultures and has additional technical advantages in the fermentation process. However, data on the actual secretion efficiency of these mutants is not yet conclusive. Although an increased production of glucoamylase was measured for *A. oryzae* hyperbranching mutants when grown in stirred batch cultures, this was not the case when other culturing techniques were employed, and no correlation was found between tip density and protein secretion (Bocking *et al.*, 1999). On the other hand, a UV mutagenesis approach to obtain *Trichoderma viride* mutants with an increased production of extracellular cellulase in some cases resulted in the recovery of overproducing strains which had a concomitant hyperbranching phenotype (Farkas *et al.*, 1981). Furthermore, a *Neurospora crassa* cAMP-dependent protein kinase conditional mutant defective in growth polarity and displaying an enlarged growth surface area (Bruno *et al.*, 1996), was shown to secrete more protein than the wild type strain when grown at the restrictive temperature (Lee *et al.*, 1998). However, disruption of the gene function of *myoA*, encoding a myosin I in *Aspergillus nidulans*, which also alters polarised growth, had a negative effect on the secretion levels of acid phosphatase (McGoldrick *et al.*, 1995). As different, often pleiotropic, mutations can alter hyphal morphology, (e.g. Kruger and Fischer,

1998; Momany *et al.*, 1999; Reynaga-Peña and Bartnicki-Garcia, 1997; Seiler *et al.*, 1997; Sone and Griffiths, 1999; Wendland and Philippsen, 2000), an unequivocal correlation between hyperbranching and secretion seems unlikely. This shows that further research is needed to establish the applicability of hyperbranching strains for production purposes.

In the following sections we will address the different steps of the fungal and yeast secretion pathway and discuss how the acquired knowledge has been used in attempts to improve protein secretion. Finally we will present future prospects and indicate topics for further research.

Targeting to the ER

Two routes have been described in *S. cerevisiae* for protein targeting to the ER membrane: the signal recognition particle (SRP) dependent pathway, in which translocation through the ER membrane occurs co-translationally and the SRP-independent pathway, which targets proteins to the ER post-translationally, involving the ER chaperone BiP (Fig.2.).

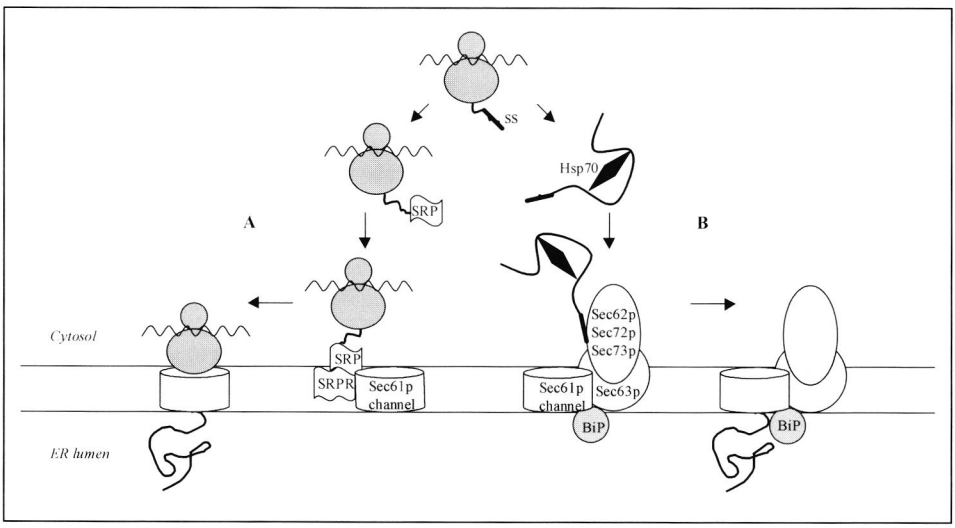


Fig. 2. Two pathways of protein targeting and translocation into the ER (Sakaguchi, 1997 and references therein). (A) SRP-dependent pathway: the signal sequence (ss) of the nascent polypeptide in a free ribosome is recognised by the signal recognition particle. Upon binding of the SRP to the signal sequence, translation is arrested and the nascent peptide-ribosome-SRP complex is targeted to the ER membrane where association with the SRP receptor (SRPR) occurs. The SRP is then released and the nascent polypeptide enters the ER through the Sec61p translocon complex. (B) SRP-independent pathway: by interaction with the cytosolic Hsp70 chaperone and co-chaperones, the nascent protein is maintained in an unfolded conformation. This complex is targeted to the ER membrane where it interacts with the Sec62p-Sec72p-Sec73p subcomplex, which functions as a membrane receptor. The lumenal ER chaperone BiP, interacting with membrane protein Sec63p, assists the translocation of the polypeptide chain, which occurs, as in the SRP-dependent pathway, through the Sec61p channel.

Ng *et al.* (1996) demonstrated that the hydrophobicity of the signal sequence determines the targeting route of each protein: proteins with a less hydrophobic signal sequence are targeted through the SRP-independent route, whereas both routes can be followed when a more hydrophobic signal is present. Although both targeting routes may be universal, the specificity of the system and the proteins that follow either route may not be interchangeable among

organisms, which could be of importance in studies on heterologous gene expression. Whether these two ER targeting mechanisms also operate in filamentous fungi remains to be determined. However, the identification in *A. niger* of a homologue of the *S. cerevisiae* signal recognition particle protein SRP54 (Thompson *et al.*, 1995) and of KAR2/BiP homologues in numerous fungal species (see below) suggests that both routes are also present in fungi.

Protein maturation in the ER

The translocation mechanism described above implies that secretory proteins enter the ER in an extended conformation. However, proteins have to fold and mature into their native forms to be functional and this process is assisted *in vivo* by helper proteins named chaperones and foldases (Table 1). Foldases catalyse slow, often rate limiting, covalent changes, such as disulphide bond formation and proline isomerisation, which are essential for obtaining a functional conformation. Molecular chaperones are not regarded as catalysts but as assisting proteins that transiently and non-covalently bind to non-native proteins to prevent non-productive protein-protein interactions and thus promote correct folding. Chaperones and foldases are ubiquitous: members of this class of proteins are not only present in the ER but also in the cytosol, mitochondria, chloroplasts and periplasm and are conserved between organisms (Gething and Sambrook, 1992).

BiP

BiP (also known as the glucose regulated protein GRP78) is a member of the heat shock 70 protein family (HSP70) of molecular chaperones which is localised in the lumen of the ER (Bole *et al.*, 1986; Munro and Pelham, 1986). In yeast, the protein is encoded by the essential gene *KAR2* (Nicholson *et al.*, 1990; Normington *et al.*, 1989; Rose *et al.*, 1989). *KAR2* homologues have also been cloned from a number of filamentous fungi (Hijarrubia *et al.*, 1997; Kasuya *et al.*, 1999; Techel *et al.*, 1998; van Gemeren *et al.*, 1997). The *A. awamori* gene showed complementation of a yeast *ts KAR2* mutation (Sagt, 2000), indicating functional similarities between the fungal homologues and *KAR2*. Other shared features of the fungal and yeast BiP proteins are the presence of a signal peptide and the carboxyl-terminal ER retention signal K/HDEL, corresponding to its ER localisation. BiP appears to be involved in a number of processes related to protein biogenesis (Haas, 1994 and references therein). BiP participates in ER-translocation of nascent polypeptides, protein folding and assembly in the ER, and in the degradation of proteins that do not reach their mature conformations (Fig.3). For further details on the BiP mode of action and regulation we refer to an excellent review in this field (Pedrazzini and Vitale, 1996).

In filamentous fungi, as in other organisms, the BiP encoding gene has a basal expression level under normal growth conditions and is overexpressed in situations of cellular stress such as glucose starvation, heat shock and conditions typical of the Unfolded Protein Response (UPR, see below) (Mori *et al.*, 1992; Ngiam *et al.*, 2000; Techel *et al.*, 1998; van Gemeren *et al.*, 1997). Enhanced *bipA* mRNA levels have been observed in various yeast and *Aspergillus* strains expressing recombinant extracellular proteins (Punt *et al.*, 1998; Sagt *et al.*, 1998). However, the correlation between BiP induction and secretion efficiency remains unclear. Overproduction of fungal proteins increased *bipA* mRNA levels in *A. niger* (Punt *et al.*, 1998). Moreover, inefficient secretion of single-chain antibodies in *A. awamori* coincided with an increased BiP production (Frenken *et al.*, 1998), and similarly, a two-fold induction in *bipA* mRNA levels was measured in two *A. niger* strains producing the hen egg white lysozyme (Ngiam *et al.*, 2000). However, BiP

Table 1. ER molecular chaperones and foldases

Protein	Category	Function	Gene		
			Mammalian	Yeast	Filamentous Fungi
BiP	Chaperone	Translocation Folding Quality control	*grp78*	*KAR2*	*bipA*
Cer1p	Chaperone	Folding Translocation	-na-	*CER1*	-na-
Protein disulphide isomerase and PDI-related proteins	Foldase Chaperone	Disulphide formation Disulphide isomerisation Folding	*pdi* *pdip; pdir; p5* *ER57; ER28*	*pdi* *Eps1; MPD2;* *EUG1*	*pdiA* *tigA; prpA*
Peptidyl-prolyl *cis-trans*-isomerase	Foldase	Interconversion of peptidyl-prolyl imide bonds	*cypB/C* *FKBP13*	*CPR2/4/5* *FPR2*	*cypB* *FKBP22*
Calnexin	Chaperone Lectin	Folding of glycoproteins	*IP90*	*CNE1*	*clxA*
Calreticulin	Chaperone Lectin	Folding of glycoproteins	*CALR*	-np-	-na-

-np- not present

-na- not available

levels remained unchanged when another non-fungal protein, interleukin-6, was produced in *A. niger* (Punt *et al.*, 1998). Although no clear relationship between BiP induction and a particular characteristic of the overproduced protein can be deduced from these data, they do suggest that protein overproduction may lead to increased levels of unfolded proteins, and thus result in *bipA* overexpression

Recently, Cer1p/Ssi1p/Lhs1p, a novel ER-located member of the Hsp70 has been identified in *S. cerevisiae* (Baxter *et al.*, 1996; Craven *et al.*, 1996; Hamilton and Flynn, 1996). This protein may have overlapping functions with BiP and be involved in protein biogenesis at low temperatures. Cer1p homologues have not been reported for filamentous fungi.

PDI

Protein disulphide isomerase (PDI) is a protein thiol-oxidoreductase that catalyses the oxidation, reduction and isomerisation of protein disulphides (Noiva, 1999). PDI is a member of the thioredoxin family. Thioredoxin is a ubiquitous protein involved in a variety of redox reactions via its active site consisting of two Cys residues in the sequence Cys-Gly-Pro-Cys (Holmgren, 1985; Holmgren, 1989). The typical structure of PDI consists of five domains (Ferrari and Soling, 1999): two domains (**a** and **a'**) have a high sequence similarity to thioredoxin, and contain double-cysteine redox-active sites. Domains **b** and **b'** show no sequence similarity to thioredoxin or the **a** domains but conserve the thioredoxin fold. The fifth (**c**) domain is not thioredoxin-related and contains a typical C-terminal XDEL ER retention signal, as well as putative Ca^{2+} binding sites. In addition to PDI, also PDI-related proteins with different domain organisations are found in the ER of many eukaryots.

PDI is the catalyst of disulphide bond formation and isomerisation during protein maturation in the ER and also displays typical chaperone-like functions (Ferrari and Soling, 1999; Gilbert, 1997; Wang, 1998). The disulphide bond formation and isomerisation activities of PDI are due to the reactivity of the N-terminal Cys residue in the two thioredoxin-like boxes (Ferrari and Soling, 1999). The chaperone activity of PDI is independent of the cysteine residues and may reside on the peptide binding capacity of a 51aa C-terminal stretch (Dai and Wang, 1997; Noiva *et al.*, 1993). However, more recent experiments have demonstrated a core role of the **b'** domain in peptide binding and the importance of all domains for the full-binding capacity of PDI. (Klappa *et al.*, 1998; Klappa *et al.*, 2000; Sun *et al.*, 2000). Furthermore, PDI has been shown to bind and promote folding of proteins where disulphide bonds are absent (Klappa *et al.*, 1998; Wang, 1998). PDI null mutations are lethal in *S. cerevisiae* (Farquhar *et al.*, 1991). The essential function of the protein in this organism was shown to be derived from its isomerase activity, rather than from its redox properties (Laboissiere *et al.*, 1995).

In filamentous fungi, genes encoding PDI have been cloned from *A. niger* (Malpricht *et al.*, 1996; Ngiam *et al.*, 1997), *A. oryzae* (Hjort, 1995; Lee *et al.*, 1996), *Trichoderma reesei* (Saloheimo *et al.*, 1999) and *Humicola insolens* (Kajino *et al.*, 1994). In *A. niger*, two other PDI-related genes, *tigA* (Jeenes *et al.*, 1997) and *prpA* (Wang and Ward, 2000), have also been cloned and characterised. The *tigA* and *pdiA* genes of *A. niger* were found to be moderately upregulated by tunicamycin treatment and by overexpression of heterologous proteins (Jeenes *et al.*, 1997; Ngiam *et al.*, 1997; Ngiam *et al.*, 2000). DTT treatment caused a stronger (8 to 10-fold) induction although this was still lower and delayed when compared to *bipA* induction (Ngiam *et al.*, 2000). These results may indicate that the *tigA* and *pdiA* genes in *A. niger* are not part of the primary stress response. Similar patterns of gene induction were found for the *pdiA* gene of *T. reesei* (Saloheimo *et al.*, 1999). However, whereas no UPR box could be identified in the promoter

region of the *tigA* and *pdiA* genes of *A. niger* (Jeenes *et al.*, 1997), two such elements were present in the promoter region of the *T. reesei pdiA* gene (Saloheimo *et al.*, 1999). Noticeable differences in the *T. reesei pdiA* expression levels were also observed under cellulose supported growth (high level of protein secretion) as compared to glucose supported growth (low level of protein secretion) (Saloheimo *et al.*, 1999). The authors suggested a mechanism of *pdi* regulation in response to variations in ER protein trafficking.

PPIase

Peptidyl prolyl isomerase (PPIase) catalyses the isomerisation of *cis* and *trans* peptide bonds on the N-terminal side of proline residues (reviewed by Gothel and Marahiel, 1999). They were discovered by their ability to bind to immunosuppressive drugs, and this property has remained a criterion for their classification. There are two major families of PPIases: the cyclophilins, which bind to cyclosporin A, and the FK-binding proteins (FKBPs), which bind to the FK506 compound. PPIases are ubiquitous proteins and are found in a wide variety of cellular compartments. They have been shown to accelerate protein folding *in vitro* (Freskgard *et al.*, 1992; Kops *et al.*, 1998) and to interact with other folding enzymes and chaperones (Schonbrunner and Schmid, 1992). However, their role in protein folding *in vivo* has not yet been elucidated, though gene deletion studies have shown that they are dispensable proteins (Dolinski *et al.*, 1997; Gothel and Marahiel, 1999).

In yeast, eight cyclophilins and four FKBPs have been identified. Three cyclophilins and one FKBP are presumably localised in the secretory pathway, as these proteins possess an N-terminal signal sequence and an ER retention signal. Furthermore, the expression of the corresponding genes is affected by factors known to regulate the expression of ER resident proteins, such as heat shock and tunicamycin treatment (Gothel and Marahiel, 1999). The two classes of PPIases also are found in filamentous fungi. A FKBP has been identified in the ER of *Neurospora crassa* (Solscheid and Tropschug, 2000) and homologues of the cyclophilin gene *cypB* have been cloned from *A. nidulans* (Joseph *et al.*, 1999) and *A. niger* (Derkx, 2000). Similar to the yeast gene, the fungal *cypB*'s are both non-essential and their expression is activated by heat-shock treatment (Joseph *et al.*, 1999) and heterologous protein expression (Wiebe *et al.*, unpublished).

Calnexin

Calnexin and calreticulin (the soluble homologue of calnexin, present in mammalian cells but not in yeast) are lectin-like chaperones. They specifically interact with partially trimmed monoglucosylated N-linked oligosaccharides and are an essential part of the maturation and quality control mechanism of glycoproteins. This mechanism, as described in Fig.3, has been well established in the mammalian cell and is assumed to also operate in the fission yeast *Schizosaccharomyces pombe* (Jakob and Burda, 1999; Parodi, 1999). In the mammalian system, a thioredoxin family member with isomerase activity, Erp57, has also been shown to complex with calnexin (and calreticulin) to assist protein folding (High *et al.*, 2000). However, the UDP-Glc:glycoprotein glucosyltransferase activity nor Erp57 homologues are found in *S. cerevisiae* (Jakob *et al.*, 1998). Furthermore, contrary to what is observed in *S. pombe*, disruption of the calnexin homologous gene (*cne1*) in *S. cerevisiae* is not lethal (Parlati *et al.*, 1995). This suggests that a different pathway of glycoprotein maturation occurs in the latter. The calnexin homologue has been cloned from *A. niger* (Jeenes *et al.*, personal communication), and database mining also resulted in the identification of calnexin homologues in *N. crassa* (Nelson *et al.*, 1997), *A.*

nidulans (http://www.genome.ou.edu/fungal.html, Roe *et al.*, 2000) and *Fusarium sporotrichioides* (http://www.genome.ou.edu/fsporo.html, Roe *et al.*, 1999). Also UDP-Glc:glycoprotein glucosyltransferase and Erp57 homologues can be found in these public fungal databases. Although little experimental data on the function of the fungal genes is available, the protein sequences are most similar to *S. pombe*, which suggests that the fungal calnexin cycle follows the *S. pombe*/mammalian model.

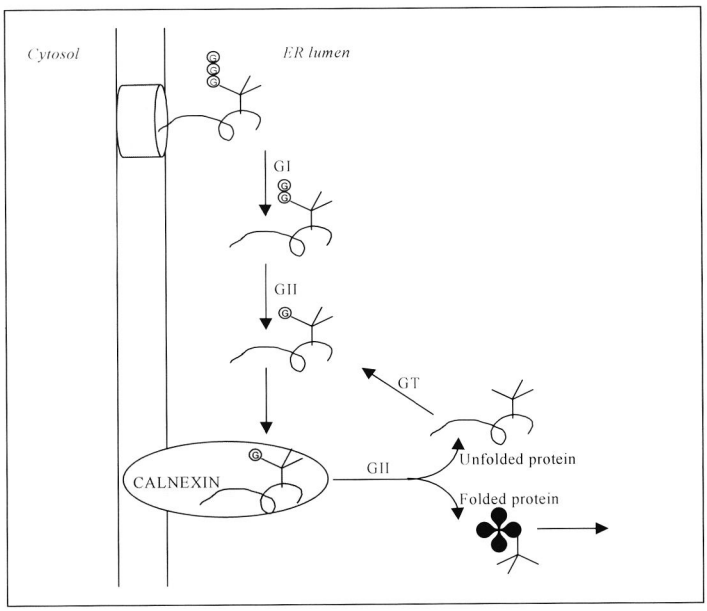


Fig. 3. Calnexin cycle. N-glycans ($Glc_3Man_9GlcNac_2$) are added to the side chain of asparagine residues in the consensus sequence N-X-S/T of translocating polypeptides by the oligosaccharyltransferase associated with the ER translocon. Removal of the glucose groups requires the sequential action of the glucosidase I (GI), which removes the terminal α1-2 linked glucose, and glucosidase II (GII), which eliminates the remaining α1-3 linked glucose in two successive reactions. Glycoproteins containing high mannose-type oligosaccharides are transiently reglucosylated in the ER by the action of the lumenal UDP-Glc:glycoprotein glucosyltransferase (GT) to generate monoglucosylated structures. Monoglucosylated polypeptides that arise from the stepwise removal of glucoses and GT reglucosylation are recognised by and bind to calnexin, allowing the chaperone to provide folding assistance. Upon GII action, the last glucose is trimmed off and glycoproteins are released from calnexin. Correctly and completely folded proteins will continue their journey to the Golgi whereas incompletely folded proteins are specifically recognised by GT and re-glucosylated to regenerate the monoglucosylated glycoprotein. This monoglucosylated glycoprotein will bind to calnexin and start a new cycle of folding and deglucosylation, reviewed by Helenius *et al.* (1997).

The complexity of chaperone interaction

The process of protein folding must not be conceived as the sole sum of the folding activities of chaperones and foldases but as a complex network of interactions which is dependent on the characteristics of the folding proteins, the environment in the ER and the availability of specific co-factors. Chaperones may co-bind or act sequentially in protein folding. For example, Jannatipour *et al.* (1998) reported the co-interaction of calnexin and BiP in the folding of acid phosphatase in *S. pombe*. Also a co-operative action of BIP and PDI in protein folding (Gillece

et al., 1999; Mayer *et al.*, 2000) and the direct interaction of PDI with calreticulin has been suggested (Baksh *et al.*, 1995). Interestingly, Corbett *et al.* (1999) showed that the interaction between PDI and calreticulin was dependent on the concentration of Ca^{2+} ions, suggesting that the levels of Ca^{2+} in the ER participate in the regulation of the protein-protein interactions in this compartment. In other cases, the sequential action of chaperones is observed (Hammond and Helenius, 1994; Melinck *et al.*, 1994). Recently, Helenius and co-workers have shown that the sequence of chaperone binding depends on the position of N-glycosylation in the nascent protein. Proteins with N-terminal N-glycosylation bind first to calnexin/calreticulin and eventually later to BiP, whereas proteins having their glycans more COOH-terminally associate first with BiP (Molinari and Helenius, 2000). Finally, other more protein-specific factors may influence the specificity of chaperone binding. For example, Nausseef *et al.* (1998) observed the participation of both calreticulin and calnexin in myeloperoxidase biogenesis, but the association of only calnexin with the holoforms of this haemoprotein, which also suggests a specific role of this lectin at the stage of haem insertion.

Although most of these studies on chaperone interactions focus on the mammalian secretion pathway, they illustrate the complexity of the interactions that take place in the lumen of the ER and may also govern protein folding in fungal species. This complex behaviour may be of relevance when designing approaches to improve the use of these organisms as protein producers (see also below).

ER-quality control

The ER ensures that correctly folded proteins are delivered to subsequent cellular compartments. This protein quality function implies the participation of two major cellular mechanisms: the unfolded protein response (UPR) which detects the presence of unfolded proteins in the ER and induces the synthesis of folding enzymes, and the ER-associated protein degradation (ERAD) which degrades those proteins which fail to reach the correct conformation.

In *S. cerevisiae*, UPR intracellular signalling involves the action of the translation products of three genes, *HAC1*, *IRE1* and *RLG1* (Chapman *et al.*, 1998). In this model, the gene product of *IRE1* responds to the accumulation of misfolded proteins in the ER and, together with RLG1p, mediates the processing of the *HAC1* transcript. The hac1p, a bZIP transcription factor, travels to the nucleus and binds to the unfolded protein response element (UPRE) present in the promoter region of genes controlled by the UPR thereby inducing transcription. The UPR is induced by a variety of factors that alter the function of the ER, such as arrest of glycosylation by treatment with tunicamycin, inhibition of disulphide bridge formation by treatment with reducing agents, Ca^{2+} depletion in the ER, inhibition of protein degradation, expression of aberrant proteins and overexpression of normal proteins (Chapman *et al.*, 1998). Recently, it has been shown that UPR not only regulates ER-resident chaperones, but many other genes involved in secretion, and particularly those of the ERAD, which indicates that these two processes co-operate in maintaining the folding competence of the secretory pathway (Travers *et al.*, 2000).

The ERAD system eliminates misfolded proteins via degradation in the cytosol. Defective proteins are spotted in the ER, possibly by resident chaperones such as calnexin and BiP, retro-translocated to the cytosol through the Sec61p translocon complex and targeted to the 26S proteasome by ubiquitin-conjugating enzymes for proteolytic degradation (Brodsky and McCracken, 1999; Sommer and Wolf, 1997). To date, the only component of the ubiquitin-proteasome machinery that has been characterised in filamentous fungi is the *prs12* gene of *T. reesei*, which is homologous to the mouse regulatory subunit 12 of the 26S proteasome (Goller

et al., 1998). Proteasome homologues can also be found in the *N. crassa* and *A. nidulans* sequence databases (http://www.genome.ou.edu/fungal.html). The *prs12* gene was shown to be moderately up-regulated by treatments which cause cellular stress and accumulation of unfolded proteins in the ER (Goller *et al.*, 1998), indicating a co-ordinated regulation of the UPR and ERAD systems as suggested by Travers and co-workers (2000).

Post-ER routing

In the eukaryotic secretion pathway, correctly folded proteins exit the ER to be targeted to the Golgi apparatus. Although the classical dictyosome organisation of the Golgi compartment is not commonly seen in filamentous fungi (Markam, 1994), functions typically Golgi-associated are present in the fungal cell, and the term Golgi-like structure is normally used. A Golgi located peptidase (Kex2p) activity is found in filamentous fungi, and recently the gene of the *A. niger* homologue (*KEXB*) has been cloned (Jalving *et al.*, 2000 ; Punt *et al.*, unpublished). Furthermore, N- and O-glycosylation, modifications that develop in the Golgi compartment, are almost invariably present in fungal extracellular proteins. Oligomannose N- and O-glycans are predominant in filamentous fungi, whereas in *S. cerevisiae* hyperglycosylation often occurs (Archer and Peberdy, 1997). The presence of glucose, galactose, phosphate, sulphate and simple N-acetylglucosamine on the linked glycans has also been reported (Maras *et al.*, 1999b and references therein). Other more complex glycan structures typical of mammalian glycoproteins are not found in fungal proteins, presumably because filamentous fungi lack some of the glycosyltransferase activities present in higher eukaryots. This difference between the fungal and mammalian glycosylation machinery limits the applicability of the fungal cell for the synthesis of mammalian proteins when correct glycosylation is essential for activity and/or utilisation. Studies have been initiated to engineer the fungal glycosylation pathway to create strains that synthesise glycoproteins with the mammalian-like glycosylation patterns. The approaches used included the introduction of the genes encoding the missing glycosyltransferases into the fungal hosts. The cDNA encoding mammalian N-acetylglucosaminyltransferase I (GlcNAc-T1) has been successfully expressed in *A. nidulans* (Kalsner *et al.*, 1995) and *T. reesei* (Maras *et al.*, 1999a), although the *in vivo* transfer of GlcNAc residues to fungal glycans could be demonstrated only in the latter case. Further methodologies, such as disruption of typical fungal glycan structures or providing the required substrates for the engineered mammalian glycosyltransferases, may be required to model the fungal glycosylation to the mammalian type (Maras *et al.*, 1999b).

After passage through the Golgi compartment, proteins are targeted either to the plasma membrane for secretion or to the vacuole. Two different vacuolar routes operate in yeast: the carboxypeptidase Y (CPY) route which travels through an intermediary organelle, the prevacuolar compartment (PVC), and the direct route as followed by alkaline phosphatase (ALP) (Bryant and Stevens, 1998; Conibear and Stevens, 1998). Sorting signals have been identified which confer vacuolar targeting in both routes (Piper *et al.*, 1997; Valls *et al.*, 1987). The extent to which vacuolar targeting routes and signals coincide in yeast and filamentous fungi needs to be established, although the available data, once more, indicate similarities. Homologues of vacuolar sorting proteins operating in the two pathways can be found in the *A. nidulans* and *F. sporotrichioides* sequence databases (e.g. *vps1*, *vps45*, *vps29*, *vps15* homologues (CPY route); AP-3 complex, *vps39* homologues (ALP route)). Moreover, we have observed in *A. niger* intracellular retention of the otherwise secreted glucoamylase when the native GLA signal peptide was replaced by the vacuolar protease pepE prepropeptide (Punt, unpublished), whose yeast homologue, pep4, is known to carry vacuolar targeting information (Klionsky *et al.*, 1988).

Furthermore, in addition to specific vacuolar targeting, it is known in yeast that some heterologous proteins are directed and degraded in the vacuole without the presence of known vacuolar targeting signals (Hong *et al.*, 1996; Inoue *et al.*, 1997). In these cases, vacuolar targeting possibly relies on the recognition of extended secondary structures that may be present in misfolded proteins (Conibear and Stevens, 1998). However, to what degree vacuolar degradation hampers heterologous protein production in fungi is unknown.

MOLECULAR GENETIC MODIFICATION OF THE SECRETION PATHWAY FOR IMPROVEMENT OF HETEROLOGOUS PROTEIN PRODUCTION

In view of the secretion potential of the fungal cell and the many analogies among the secretory machinery of filamentous fungi, yeasts and higher eukaryots, the often low production yields obtained for heterologous proteins when employing the fungal production system pose an intriguing problem. Analysis of limiting factors that could be responsible for this phenomenon has shown that transcription of the heterologous genes and mRNA steady-state levels are mostly satisfactory, suggesting that bottlenecks are mainly present at the post-transcriptional stage, possibly along the secretion pathway (Gouka *et al.*, 1997). Different genetic approaches have been used to analyse and alleviate these putative blockages, with the aim of increasing the production yields of heterologous proteins.

Signal sequences

The N-terminal signal peptides are responsible for introducing secretory proteins into the secretion pathway. For the production of heterologous proteins, signal peptides from well-secreted homologous proteins are often fused to the mature recombinant protein to direct its secretion (van den Hondel *et al.*, 1991). When the heterologous protein is extracellular *per se*, the endogenous signal sequence can be used to target secretion. It is generally believed that signal sequences do not account for major differences in the production yields of the recombinant proteins they precede, although not many studies are available where this issue has been addressed in detail. The level of the *F. solani pisi* cutinase produced in *A. awamori* was not greatly affected by the replacement of the cutinase pre-sequence by the leader peptide of the endogenous *Aspergillus* endoxylanase (van Gemeren *et al.*, 1996). In contrast, the extracellular level of the *A. restrictus* restrictocin produced in both *A. nidulans* and *A. niger* was significantly higher when the restrictocin, rather than the glucoamylase signal sequence, was used (Brandhorst and Kenealy, 1995). Furthermore, not only signal peptides, but also prosequences may play a role in secretion efficiency. Prosequences are normally removed at a late stage in the secretion pathway and perform functions such as organelle targeting (Bening *et al.*, 1998; Klionsky *et al.*, 1988) and protein folding (Eder and Fersht, 1995; Wiederanders, 2000). Hence, in their work with restrictocin, Brandhorst and Kenealy (1995) also observed a markedly positive effect of the restrictocin prosequence on the secretion of this protein. However, addition of the glucoamylase prosequence at the N-terminus of cutinase (van Gemeren *et al.*, 1996) and chymosin (van Hartingsveldt *et al.*, 1990) reduced the secretion levels of these two heterologous proteins in *Aspergillus*.

Translational fusions

The production yields of heterologous proteins can be improved by expressing them as

translational fusions with an efficiently secreted homologous protein (reviewed by Gouka *et al.*, 1997). To date, this approach is probably the most successful modification to increase heterologous protein production. Although the molecular background behind the fusion strategy has not been fully elucidated, experimental data indicate that translational fusions alleviate (post)translational limitations in the production of the heterologous protein, for example by facilitating translocation and protein folding (see also below).

As a variation of the fusion strategy one can consider the use of synthetic leaders such as developed by Kjeldsen *et al.* (1997) to improve insulin secretion in *S. cerevisiae*. These synthetic leaders featured additional N-glycosylation sites and potential BiP binding sites, and were shown to prolong retention of the insulin precursor in the ER, thereby presumably providing additional time for correct folding of the heterologous protein. Moreover, the sole engineering of additional N-glycosylation sites in the preproinsulin sequence resulted in a significant increase in insulin secretion both in *A. niger* (Mestric *et al.*, 1996) and in yeast (Kjeldsen *et al.*, 1998). Similar results have been obtained with prochymosin in *A. awamori* (Ward, 1989) and hydrophobic cutinase in *S. cerevisiae* (Sagt, 2000). In this last work Sagt *et al.* also showed that the introduction of N-terminal rather than C-terminal glycosylation improved protein secretion, and that N-terminal glycosylated hydrophobic cutinase mutants did not aggregate in the ER, whereas the C-terminal glycosylated and not engineered cutinase variants did. These results suggest that additional N-terminal glycosylation increases the solubility of the engineered proteins in the ER, whereby protein folding could be favoured and secretion could be increased. Alternatively, the site of introduced glycosylation may change the choice of binding chaperone (see above), thus influencing aggregation and proper folding. Finally, it should be noticed that most translational fusions used also provide N-terminal glycosylation, which could (partially) explain the success of the fusion strategy.

Glycosylation

The capacity of the glycosylation machinery and the specific glycosylation requirements that heterologous proteins may have might also be of importance for the production of these proteins, although no experimental evidence of this is available so far. Kruszewska *et al.* (1999), showed that overexpression in *T. reesei* of the *S. cerevisiae* mannosylphosphodolichol synthase encoding gene, required for glycan synthesis, resulted in an increased level of secreted cellobiohydrolase, suggesting that glycosylation may limit the overproduction of glycoproteins. However, Wallis and colleagues (Wallis *et al.*, 1999) concluded the opposite when they found a similar glycosylation pattern in the glucoamylase produced by an *A. niger* overproducing strain and the wild type strain. Although at first glance contradictory, both research lines did observe a positive correlation between enzymatic glycosylation activities and protein secretion, indicating that in fungi protein overproduction requires a concomitant increase of the glycosylation capacity of the secretion machinery.

Modifying the levels of chaperones

Molecular chaperones promote protein folding and maturation. Moreover, when correct protein folding is impaired, association of the unfolded polypeptides with chaperones and/or foldases is commonly observed, as well as induction of the expression of the genes encoding the corresponding chaperones/foldases. These cellular responses are also frequently present in heterologous protein expression. It has been argued that in a heterologous system, proteins may encounter folding restrictions that limit their production efficiency and that this problem can

possibly be overcome by providing the cell with an increased level of the helper proteins. Bearing this idea in mind, the effect of chaperone levels on heterologous protein production has been assessed for a number of systems (Table 2). However, results are far from conclusive and frequently even seem contradictory.

Dorner and colleagues studied the effect of modified BiP levels on the secretion of recombinant proteins in mammalian CHO cell lines. They observed that BiP overexpression had a negative effect on the secretion levels of some proteins (Dorner *et al.*, 1992) and conversely, that reduced BiP levels resulted in an increased secretion of recombinant proteins (Dorner *et al.*, 1988). In contrast, Shusta *et al.* (1998) found a positive effect of *KAR2* (BiP) overexpression on the secretion titres of five single chain antibody fragments (scFv's) produced in *S. cerevisiae*. Similarly, Harmsen *et al.* (1996) measured a 20-fold increase in the amount of extracellular prochymosin when the *KAR2* gene was overexpressed in *S. cerevisiae*. Curiously, the secretion levels of another heterologous protein, the plant thaumatin, remained unchanged upon *KAR2* overexpression (Harmsen *et al.*, 1996). Robinson *et al.* (1996) found that reduction of kar2p levels diminished the production of three heterologous proteins in *S. cerevisiae*, but increased cellular BiP levels did not result in the opposite phenotype. In another study, these authors showed that constitutive overexpression of heterologous proteins reduced the levels of extractable kar2p and PDI and that *KAR2* overexpression could increase soluble cellular kar2p levels in wild type strains but not in strains overproducing heterologous proteins (Robinson and Wittrup, 1995). *bip* overexpression in the Baculovirus system led to increased levels of functional and soluble antibodies in cell lysates, although extracellular levels remained unchanged (Hsu *et al.*, 1994). In our laboratory, we have studied the effect of *bipA* overexpression on heterologous protein secretion in two recombinant strains expressing glucoamylase (*glaA*) fusion genes. Although the final amount of secreted recombinant proteins did not change significantly in strains with extra copies of the *bipA* gene, increased levels of unprocessed fusion protein were detected in the total protein extracts of these strains, indicating a role of BiP in the maturation of the fusion proteins (Punt *et al.*, 1998).

Similarly, the effect of overproduction of foldases on the expression of heterologous proteins has been addressed, mainly in yeast and especially for proteins where disulphide bridge formation was assumed to play an important role. In *S. cerevisiae, PDI* overexpression has proven to increase the secretion levels of heterologous proteins such as the human platelet derived growth factor B homodimer (PDGF), the *S. pombe* acid-phosphatase (Robinson *et al.*, 1994), antistasin (Schultz *et al.*, 1994) and human lysozyme (h-LZM) (Hayano *et al.*, 1995). However, although expression of antisense *PDIA* in *A. niger* reduced the levels of secreted glucoamylase (Ngiam *et al.*, 2000), overexpression of this foldase or of the PDI-related *prpA* (Wang and Ward, 2000) had no effect on the secretion of the homologous and heterologous proteins analysed. Similarly, overexpression of the cyclophilin gene *cypB*, the transcription of which was already increased in a human tissue plasminogen activator (t-PA) producing *A. niger* strain, had no effect on the secreted levels of this heterologous protein (Wiebe *et al.*, unpublished).

With regard to ER lectins, in insect cells both calnexin and calreticulin overexpression led to enhanced production of the tagged serotonin transporter (myc-SERT), an N-glycosylated membrane protein (Tate *et al.*, 1999). In contrast, a similar approach in mammalian cells had no effect on the titres of two hepatitis C virus (HCV) envelope proteins (Choukhi *et al.*, 1998).

Table 2. Effect of the modification of the levels of ER resident enzymes on heterologous protein production

Chaperone	Protein	Organism	Effect	Reference
BiP	Three human Proteins	CHO cells	BiP levels inversely correlated with protein production levels	Dorner *et al.*, 1992 Dorner *et al.*, 1988
BiP	Antibodies	Baculovirus	BIP overexpression increased intracellular but not secreted Ig protein levels	Hsu *et al.*,1994
BiP	GLA:IL6 GLA::scFV4715	*A. niger*	BIP overexpression increased intracellular but not extracellular levels of fusion proteins.	Punt *et al.*,1998
BiP	Cutinase	*A. awamori*	BIP overexpression had no effect	van Gemeren *et al.*, 1998
BiP	Three heterologous proteins	Yeast	Lower BiP levels resulted in lower secretion levels. Higher BiP levels did not increase secretion levels.	Robinson *et al.*, 1996
BiP	Chymosin TMT	Yeast	BiP overexpression increased level of secreted chymosin. TMT unaffected	Harmsen *et al.*, 1996
PDI	PDGF	Yeast	PDI overexpression increased PDGF secretion and lowered intracellular accumulation	Robinson *et al.*, 1994
PDI	Lysozyme	Yeast	PDI overexpression increased intracellular and secreted protein levels	Hayano *et al.*, 1995
PDI	Antistasin	Yeast	PDI overexpression increased protein production	Schultz *et al.*, 1994
PDI	Lysozyme, Glucoamylase	*A. niger*	*PDIA* overexpression did not influence the secretion levels of both proteins.	Ngiam *et al.*, 2000
PrpA	Chymosin	*A. niger*	*prpA* overexpression had no influence in chymosin extracellular levels.	Wang and Ward, 2000
CypB	tPA	A.niger	No effect of cypB overexpression on tPA production	Wiebe *et al.*, unpublished
BiP Calnexin	MnP	A.niger	Calnexin overexpression increases MnP production. BiP overexpression decreases MnP prodcution	Conesa *et al.* unpublished.
BiP Calnexin Calreticulin	HCV envelope proteins	Mammalian	The three chaperones interact, but when overexpressed, no effect on secretion levels	Choukhi *et al.*, 1998
BiP Calnexin Calreticulin Erp57	Myc-SERT	Baculovirus	Increased production with CNX, less with BiP and CLT. Erp57 no effect	Tate *et al.*, 1999

As results obtained with the various approaches are diverse and often disparate, general rules are difficult to formulate. However, it seems unlikely that fundamental differences exist in the function of the helper proteins in the organisms analysed. More likely we observe the consequence of the multiple cellular functions in which chaperones are involved and the different nature of the secreted proteins studied. As already mentioned, BiP participates in processes such as protein translocation, polypeptide solubilisation, protein folding, quality control, retrograde transport and degradation. Foldases may not only serve as catalysts but also have chaperone functions; lectins act in folding and quality control. In addition, all these helper proteins may have other as yet unknown functions. The extent to which each of these functions contributes to the efficient secretion of a certain protein may be protein-specific, and this may lead to different responses when the intracellular levels of the helper proteins are altered.

Furthermore, as already mentioned, one should be aware of the complexity of the molecular interactions behind the process of protein folding and of the consequences that this may have when manipulating strains for biotechnological applications. For example, we have seen opposite effects of *bipA* and calnexin (*clxA*) overexpression on the production of a fungal haemoperoxidase (MnP) in *A. niger*. Although both chaperones showed similar induction patterns in a MnP producing strain, *bipA* overexpression seriously reduced MnP production whereas *clxA* overexpression resulted in a 5-fold increase. However, when additional haem was provided to the growth medium of these strains in order to improve MnP production, this positive effect of *clxA* overexpression was no longer visible and MnP extracellular levels were even slightly reduced. In contrast, in the *bipA* overexpressing strain MnP production was enhanced (Conesa *et al.*, unpublished).

ANALYSIS OF VESICULAR TRAFFICKING: A SYSTEMATIC APPROACH

Although manipulating the ER environment has in some cases resulted in improved heterologous protein secretion, these approaches have not been able to resolve basic questions on the fate of heterologous proteins along the secretion pathway. This issue could possibly be better addressed by systematically analysing protein trafficking along the secretion route. In yeast and mammalian cells, many studies have been done on the molecular constituents of protein transport along the secretory pathway. Key components of cellular protein trafficking are small GTP-binding proteins of the Ras superfamily. These proteins can be divided into two groups: the ARF/SAR subfamily, involved in the formation of carrier vesicles from the donor organelle and in the preparation of vesicles for fusion with the acceptor organelle, and the SEC4/YPT/RAB subfamily which is required for vesicle targeting and/or fusion (for reviews see Novick and Brennwald, 1993 and Lazar *et al.*, 1997). Small GTP-ases, also referred to as molecular switches, perform their function by cycling between GTP-bound (active and membrane associated) and GDP-bound (inactive and soluble) states. In this cycle they are assisted by effector proteins such as GEFs (guanine nucleotide exchange factors), which catalyse GDP/GTP exchange and GAPs (GTPase-activating proteins) which accelerate GTP hydrolysis. A hallmark of these small GTPases is their specific subcellular distribution. Each GTPase is found at a particular stage of the membrane transport pathway, where they operate in a specific and directional way. This property makes them ideal targets for studies on the secretion pathway. By impairing the function of a certain GTPase, the secretion pathway can be halted at a specific stage, and this blockade can be used to identify bottlenecks occurring during the secretion of heterologous proteins.

A large number of transport GTPases has been identified and cloned from yeast and higher eukaryots, and, in many cases, the transport step in which they participate has been elucidated (Table 3). In our laboratory, heterologous hybridisation and PCR-based cloning approaches have led to the identification of seven putative secretion related GTPase-encoding genes (*sarA*, *srgA-F*) from the filamentous fungus *A. niger* (Punt *et al.*, unpublished; Table 3 and Fig.1). Although still at an early stage, functional analysis of these fungal proteins has again revealed similarities as well as differences with respect to yeast.

Table 3. GTPases involved in vesicular trafficking along the secretion pathway

Yeast homologue	Transport step	*A. niger* homologue	Reference
Sar1	ER to Golgi	*SarA*	Veldhuisen *et al.*, 1997
*RAB2**	ER to the Golgi	*SrgD*	Punt *et al.*, unpublished
Ypt1	ER to Golgi; cis- to medial-Golgi	*SrgB*	"
Ypt31 *Ypt32*	Intra Golgi	*SrgE*	"
Ypt6	Golgi to vacuole	*SrgC*	"
Ypt7	Late endosome to vacuole	*SrgF*	Montijn *et al.*, unpublished
Ypt51 *Ypt52* *Ypt53*	Plasma membrane to endosome/ early to late endosome	*na*	
Sec4	Golgi to plasma membrane	*SrgA*	Punt *et al.*, unpublished
Ypt10	?	*na*	
Ypt11	?	*na*	

* Human homologue
-na- no data available

The *SAR1* homologues of *A. niger* and *T. reesei* were the first fungal vesicular GTPases to be characterised (Veldhuisen *et al.*, 1997). In yeast and higher eukaryots, Sar1p is an essential protein involved in budding and docking of COPII vesicles, which transport cargo from the ER to the Golgi apparatus. In the two filamentous fungus species studied, the *sarA* gene is essential, and complementation and mutational analysis indicated a similar function of the gene product to that of the yeast counterpart.

All other cloned *srg's* are putative members of the *RAB/YPT/SEC4* family. The *srgA* gene is most homologous to the yeast *sec4*, whose gene product is involved in vesicle transport from the Golgi apparatus to the plasma membrane. However, whether *srgA* and *sec4* are also functional homologues is not clear. *srgA* showed to be nonessential in *A. niger*, whereas in *S. cerevisiae* the *sec4* null mutant is not viable. Furthermore, the *A. niger srgA* failed to complement a *sec4 S. cerevisiae* strain (Punt *et al.*, unpublished) Nonetheless, *A. niger srgA* strains did show temperature dependent secretion and morphological differences when compared with wild type strains, suggesting a role of this GTPase in protein secretion in *A. niger* (Punt *et al.*, unpublished).

The Ypt6p and Ypt7p in *S. cerevisiae* are dispensable proteins involved in vacuolar targeting. *YPT6* and *YPT7* deletion mutants in yeast show secretion of proteins otherwise located in the vacuole (Tsukada and Gallwitz, 1996). This property could be used to improve the secretion of heterologous proteins subjected to vacuolar degradation. Whether the cloned *A. niger*

homologues, *srgC* and *srgF* respectively (Punt *et al.*, unpublished), have similar functions remains to be established. Preliminary analysis of *srgC* disruption mutants showed an abnormal hyphal phenotype and temperature-sensitive growth (Montijn *et al.*, unpublished). Other cloned fungal GTPases include the *ypt1* homologues of *N. crassa* (Heintz *et al.*, 1992), *T. reesei* and *A. niger* var.*awamori* (Saloheimo *et al.*, 2000).

CONCLUSIONS AND FUTURE PROSPECTS

Research efforts in the last years have increased our specific knowledge of secretion in filamentous fungi, identifying similarities and differences with the secretory pathway of yeast and other eukaryots. This knowledge has been exploited to improve the biotechnological use of the fungal cell as a cell factory for protein synthesis. Bottlenecks for (heterologous) protein overproduction have been identified and clues for strain improvement have arisen from approaches to manipulate molecular components of the secretion pathway. However, at this stage, further research will require new tools and more rational approaches. The isolation of secretion related GTPases would allow the generation of specific blockages at different stages of protein secretion and facilitate the systematic study of the successive steps in the fungal secretory pathway. Furthermore, the new overall and high throughput technologies (genome sequencing, transcriptomics, proteomics, etc.) must and will be the tools to study the fungal secretion pathway in the near future. In *S. cerevisiae*, such approaches have proven to be powerful in identifying multiple secretion associated molecular responses and interactions (Sagt, 2000; Travers *et al.*, 2000), and initiatives in this direction are now emerging at several fungal laboratories. To analyse and evaluate the enormous amount of data that arise from these studies is the challenge the fungal biologist will face in the near future.

ACKNOWLEDGEMENTS

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Production and characterisation of the *Phanerochaete chrysosporium* manganese peroxidase in *Aspergillus niger*

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ABSTRACT

The expression of the cDNA encoding the *Phanerochaete chrysosporium* manganese peroxidase (MnP) has been analysed in *Aspergillus niger* by using different expression cassettes and a protease-deficient strain. High steady-state mRNA levels were observed for the recombinant gene and MnP was secreted into the culture medium as an active protein. The recombinant protein showed similar specific activity and absorption spectrum as the native enzyme. The protein was correctly processed at its N-terminus and had a slightly lower mobility on SDS-PAGE. In batch-fermentations at neutral pH, up to 500 mg/L rMnP was produced when the gene was placed under control of the *gpdA* promoter. Expression of the *mnp1* cDNA fused to the *A. niger* glucoamylase gene did not result in improved secretion of the recombinant protein.

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INTRODUCTION

Fungal heam-containing peroxidases (EC 1.11.1.7) are extracellular glycoproteins secreted by white-rot fungi as part of their unique lignin degrading machinery. Other known components of this lignolytic system are laccases, glyoxal oxidases and aryl alcohol oxidases. Fungal peroxidases, as some of these other oxidising enzymes, have been subject of extensive studies for their potential as biological alternative for chemical oxidative processes as wood-pulping, bleaching and degradation of xenobiotics (Aust, 1990; Higson, 1991; Karam and Nicell, 1997; Kirk and Farrell, 1987). In their natural hosts, these proteins are synthesised during secondary metabolism in response to nutrient limitation and only limited amounts are produced (for a review see Reddy and D'Souza, 1994). This limits their availability and hinders their industrial applicability.

Different strategies have been employed in attempts to increase the production yields of these proteins. In white-rot fungi, this has been addressed by either optimising culture parameters (Collins *et al.*, 1997; Feijoo *et al.*, 1995; Kaal *et al.*, 1995), screening for high producing strains (Dosoretz *et al.*, 1993) and genetic manipulation (Mayfield *et al.*, 1994; Sollewijn Gelpke *et al.*, 1999). Although improved fermentation and media conditions, as well as strains constitutively producing the lignolytic enzymes, have arisen from these studies, none of these approaches have resulted in an adequate large-scale production system for these proteins in *Phanerochaete chrysosporium*.

A different approach has been to produce fungal peroxidases recombinantly in a number of alternative expression systems. Expression in *E. coli* of the *P. chrysosporium* lignin and manganese peroxidases (Doyle and Smith, 1996; Whitwam and Tien, 1996), as well as of other haem-containing peroxidases (Dalton *et al.*, 1996; Smith *et al.*, 1990; Zong *et al.*, 1995) resulted in production of the inactive apoproteins in inclusion bodies, indicating that this prokaryotic expression system is not adequate for the expression of this type of proteins. Expression in the baculovirus system did result in the recovery of the active, extracellular enzymes (Johnson and Li, 1991; Johnson *et al.*, 1992; Pease *et al.*, 1991). However the baculovirus expression system suffers from high costs and low production yields, and therefore is not suitable for appropriate commercial scaling-up. Similarly, expression of another fungal peroxidase from *Arthromyces ramosus* in *S. cerevisiae*, resulted in low levels of extracellular active protein (Sawai-Hatanaka *et al.*, 1995).

Also the possibility of producing these fungal peroxidases in a filamentous fungal system has been explored. Filamentous fungi have a high protein secretion capacity and since DNA-transfer systems are available for these organisms, the necessary tools are present for the production of recombinant proteins. However, whereas high production yields can be obtained when homologous proteins are expressed, much lower amounts are obtained with the expression of heterologous proteins (van den Hondel *et al.*, 1992). Much research has been carried out to establish the reasons for this difference and although the problem has not yet been fully solved, some tools are now available to increase the production levels of heterologous proteins (reviewed by Gouka *et al.* 1997). Attempts to overproduce fungal peroxidases in filamentous fungi have so far shown that these proteins result in lower yields than normally obtained for fungal proteins (Aifa *et al.*, 1999; Saloheimo *et al.*, 1989; Stewart *et al.*, 1996). In most cases, no or little extracellular peroxidase activity could be detected.

We have initiated a research line to get more insight into the limiting factors for the production of haeme-containing peroxidases in filamentous fungi. As a start, we have studied

the expression of the *P. chrysosporium* manganese peroxidase in *Aspergillus niger*. For this study, different expression cassettes and a protease deficient strain have been used.

MATERIAL AND METHODS

Expression cassettes

MnP isozyme H4 (*mnp1*) cDNA was a gift from Dr. D.Cullen (Inst. Microbial and Biochem. Tech., Madison, USA). The *mnp1* cDNA was amplified by PCR using primers MNP15E/N (5'-GGAATTCCATGGCCTTCGGTTCT-3') and MNP13B/H (5'-CGGGATCCAAGCTTAGGCAGGGCCATC-3') to introduce appropriate cloning sites at both 3' and 5' ends. The amplified product was cloned into pUC19 at the *Eco*RI/*Hind*III sites to obtain pΔM5R. The MnP coding sequence was then excised from pΔM5R by *Nco*I/*Hind*III digestion and cloned at the same sites into pAN52-10Not (carrying the *A. niger glaA* promoter; Punt, unpublished), resulting in pMnp1.I, or into pAN52-5Not (carrying the *A. nidulans gpdA* promoter; Punt *et al.*, 1991), resulting in pgpdMnp1.I. To construct pGLA::MnP, the *mnp1* cDNA was amplified with primers MNP1E/B (5'-GCGAATTCGAAGACCTCGCGCAGTCTGTCCAGA-3') and MNP13B/H, the amplification product digested with *Bbs*I/*Bam*HI and cloned into pAN56-2 (Gordon *et al.*, 2000) at the *Nar*I/*Bgl*II sites using the linker *Nar*I-NVISKR-*Bss*HII (5'-CGCGCTTGGAAATCACATT-3', 5'-GAATGTGATTTCCAAG-3'), (Fig.1).

In all cases, PCR amplification products were checked by sequencing.

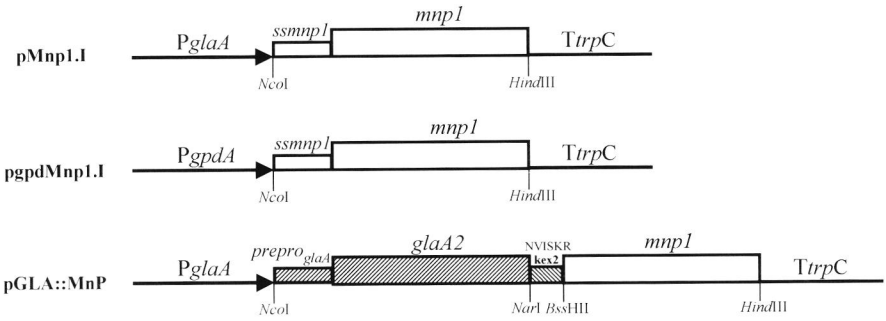


Fig. 1. Expression cassettes. *mnp1*: *P. chrysosporium* manganese peroxidase (isozyme H4) coding sequence. P*glaA*: *A. niger* glucoamylase promoter; P*gpdA*: *A. nidula*ns glyceraldehyde phosphate dehydrogenase promoter; T*trpC*: *A. niger* tryptophan C terminator; ssmnp1: *mnp1* signal sequence (24 aa); *preproglaA*: prepro sequence of the *A. niger* glucoamylase (24 aa); *glaA2*: glucoamylase catalytic domain coding sequence (514 aa) (Broekhuijsen *et al.*, 1993); kex2: KEX2-type processing signal (NVISKR) (Broekhuijsen *et al.*, 1993).

Strains and transformation procedures

Escherichia coli DH5α was used for construction and propagation of vector molecules.

A. niger MGG029 (*prtT*, *gla::fleo*^{r}, *pyrG*) was used as recipient strain in transformation experiments. MGG029 was obtained by para-sexual recombination *of A. niger* AB1.13#7 (*cspA1, fwnA, trpA, argB, leuA, nicA, prtT*), a derivative of AB1.13 (Mattern *et al.*, 1992) and *A. niger* AB6.4 (*cspA1*, *fwnA*, *pyrG*, *gla::fleo*^{r}), a fawn coloured mutant of AB6.1 (Broekhuijsen *et al.*, 1993). After crossing the two parental strains, the progeny was

screened for prototrophy and phleomycin resistance. From the resulting heterokaryotic progeny a diploid strain (MGG016) was isolated (Bos, 1986). Strain MGG029 was obtained after benomyl-induced haploidization of this diploid, selecting for phleomycin resistance, reduced milk-halo formation and *pyrG* (uridine requiring) phenotype.

Fungal co-transformation was basically carried out as described (Punt and van den Hondel, 1992) using each of the expression vectors and pAB4-1 (van Hartingsveldt *et al.*, 1987) containing the *A. niger pyrG* selection marker, in a 10:1 ratio. Transformants were selected for uridine prototrophy. Co-transformants containing expression cassettes were selected by colony PCR, as described by van Zeijl *et al.* (1997).

Screening for peroxidase activity

Colony PCR positive co-transformants were assayed for peroxidase activity using a modification of the plate assay method of Mayfield *et al.*, (1994): co-transformants were inoculated onto petri dishes containing *Aspergillus* minimal growth medium (AMM) (Bennett and Lasure, 1991), 5% maltose, 0.03% *o*-anisidine (Fluka, Switzerland) and 1,4% agar. The plates were incubated at 30°C for 3 days and then flooded with a solution of 50 mM Na-phosphate buffer pH=4.5 and 50 μM H_2O_2. Peroxidase producing transformants developed a purple halo upon incubation at 30°C.

Culture conditions

Shake-flask experiments were performed in 300 ml flasks containing 50 ml AMM supplemented with 0.5% casamino acids, using 5% maltodextrin as a carbon source (AMM-maltodextrin). Cultures were inoculated with $5x10^7$ conidia and incubated at 30°C/300 rpm for 72 hours. Samples were taken at different time points after inoculation. The mycelium was separated from the culture medium by filtration through Miracloth, washed with physiological salt and total protein extracts were prepared as described elsewhere (van Gorcom *et al.*, 1985). The filtered culture medium was dialysed overnight against 50 mM sodium succinate buffer pH=4.5.

Batch fermentations were performed in a Bioflow2 fermentor (New Brunswick Scientific). Fermentations were started from a 30 ml inoculum of a 24 hours pre-culture in potato dextrose and kept at a stirring speed of 400 rpm and a DO of 100 % (20% air oxygen). The airflow was set to 500 ml/min. The air was replaced by pure oxygen at a flow of 200 ml/min when the DO dropped below <30%. The fermentation medium contained 30g/L glucose, 20 g/L yeast extract, 7,2 g/L NH_4Cl, 2,5 g/L $KHPO_4$ and spore elements, and the pH was controlled with NaOH and H_3PO_4. 40 ml medium culture samples were taken during the fermentation to determine dry mycelium weight, total extracellular protein, glucose concentration and MnP activity.

Molecular methods

Molecular methods were carried out essentially as described (Sambrook *et al.*, 1989). Fungal DNA isolations were performed as described by Kolar *et al.*, (1988). Total fungal RNA was isolated using the RNAzol™ kit from CINNA/BIOTECX. Probes used for Northern analysis experiments were a 2 kb *Sfi*I-*Bam*HI fragment from pAN56-2 (Gordon *et al.*, 2000) containing a 72bp 5' untranslated region of the *glaA* mRNA which hybridises to P*glaA*-derived transcripts, and a 1.5 kb *Hind*III fragment from pAB5-2 (*A. niger gpdA* fragment; Verdoes *et al.*, 1994). For Southern analysis, chromosomal DNA was digested with *Mlu*I, which cuts at the *glaA* promoter and at the *trpC* terminator. A 0.7 kb *Bam*HI-*Xmn*I fragment

of the *glaA* promoter was used as a probe. Quantification of both Northern and Western analysis band intensities was performed with the GeneTools software (Syngene).

Protein methods

Total protein was determined using the Bradford assay, with BSA as standard. MnP activity was measured by monitoring the oxidation of diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) in the presence of 20 μM Na-oxalate as described by Glenn and Gold (1985). For Western blotting experiments, a polyclonal antibody against MnPH4 (kindly provided by Dr. D. Cullen, Madison) and an antiglucoamylase monoclonal antibody (Mab1.5, Mateo-Rosell, unpublished), were used in combination with 1% glucoamylase and 1%BSA, respectively, as blocking agents.

Purification of the rMnP

One of the MnP producing transformants, MGG029[pMnp1.I]#25, was used to purify recombinant MnP (rMnP). Six litres of a 3 days culture in AMM-maltodextrin were filtered through Miracloth and centrifuged at 20.000 rpm in a Beckman C5-6R Centrifuge to remove mycelial debris. The supernatant was concentrated to ~800 ml at 4°C using a hollow fibre filter system (5 kDa cut-off, 3500 cm^2, Omega Filtron) and dialysed against 10 mM Na-acetate (pH=6). The concentrate was applied to a 100 ml SourceQ column equilibrated with 10 mM Na-acetate pH=6 in a Biopilot system. The proteins were eluted with a linear gradient of 1M to 10 mM Na-acetate pH=6. Elution was followed both at 280 nm (total protein) and 405 nm (haem protein). Fractions containing ABTS oxidising activity were pooled, desalted and concentrated to 15 ml using a 8 ml SourceQ column eluted with a steep Na-acetate gradient. This concentrate was then applied to a 1800 ml Superdex75 column and proteins were eluted in 50 mM Na-succinate pH=4.5. Purity of the haem protein peak fractions were analysed on SDS-PAGE/silver staining using the Phast system (Pharmacia, Piscataway, N.J.), and by measuring their absorption spectra. Fractions containing the highest A_{407}/A_{280} ratio were dialysed against 10 mM Na-acetate pH=6 and concentrated.

RESULTS

pMnp1.I but not pGLA::MnP *A. niger* transformants produce active MnP

In a first co-transformation experiment strain MGG029 was transformed with a mixture of plasmids pAB4-1 and pMnp1.I or pGLA::MnP. Transformants were selected for their ability to grow in AMM plates without uridine. More than 300 uridine prototrophic transformants were obtained per plasmid pair. Co-transformants containing the corresponding *mnp1* expression vector were identified by colony-PCR using specific primers (data not shown). Three colony-PCR positive transformants per construct were analysed by Southern hybridisation and multi-copy integration of the expression cassettes was found in all cases (3-6 copies, data not shown).

Transformants were analysed for peroxidase production using an activity plate assay based on the oxidation of *o*-anisidine. Transformants containing the pMnp1.I expression cassette developed a purple halo, indicating extracellular peroxidase activity. However, no halo formation was visible for transformants containing the pGLA::MnP expression cassette (Fig.2).

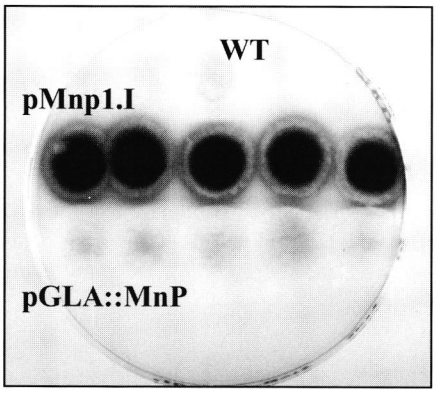


Fig. 2. Detection of *A. niger* MnP producing transformants by halo forming on the o-anisidine plate assay. WT: *A. niger* MGG029 transformed with pAB4.1. pMnp1.I: *A. niger* MGG029 transformants containing pAB4.1 and pMnP.I vectors. pGLA::MnP: *A. niger* MGG029 transformants containing pAB4.1 and pGLA::MnP vectors.

To further characterise MnP production in the pMnp1.I containing transformants and to analyse whether the absence of *o*-anisidine activity in the fusion transformants was a failure in MnP production or in the synthesis of active enzyme, shake-flask experiments were carried out. Two transformants per construct were grown for 72 hours in AMM-maltose, and samples were taken at 24 hour intervals. Medium samples were assayed for peroxidase activity as described in M&M and both samples of culture medium and mycelium extracts were subjected to Western analysis using MnP and GLA antibodies (Fig.3).

In agreement with the results obtained with the plate assay, the culture medium of pMnp1.I containing transformants showed ABTS oxidising activity, and Western analysis revealed the presence of a MnP cross-reactive protein band of approximately the size of the native MnP (nMnP; Fig.3a). Maximum rMnP yields (5-10 mg/L) were measured at the 48 hours time point, where the pH of the medium had dropped to 4. After this point, both pH and MnP activity decreased (data not shown). In contrast, in the medium of transformants containing the fusion expression vector pGLA::MnP, no MnP cross-reactive material could be detected, and accordingly, no peroxidase activity was found. However, these transformants did secrete the glucoamylase part of the fusion protein into the medium, which could be detected as a 70 kDa protein band and some degradation products (Fig.3b). Similar results were obtained when the mycelium extracts were analysed. Intracellular MnP was detected in pMnp1.I but not in pGLA::MnP containing transformants. In the latter, only a weak 120 kDa protein band was observed which cross-reacted both with MnP and GLA antibodies, possibly corresponding to the uncleaved GLA::MnP protein. (Fig. 3c and d, arrow).

No MnP or GLA was detected in the parent strain transformed with only a vector containing the selection marker, MGG029[pAB4-1] (Fig.3).

mnp1 is efficiently transcribed in *A. niger* MGG029

The MnP production yields obtained with the pMnp1.I expression cassette were about a factor 10 lower than those typical of other recombinant fungal proteins (Gouka *et al.*, 1997; Verdoes *et al.*, 1994). Furthermore, the absence of MnP cross-reactive material both in the extracellular medium and mycelial extracts of the GLA::MnP fusion transformants was surprising. These results raised the question whether efficient transcription from these constructs was occurring. Therefore we analysed transcription of the recombinant genes by

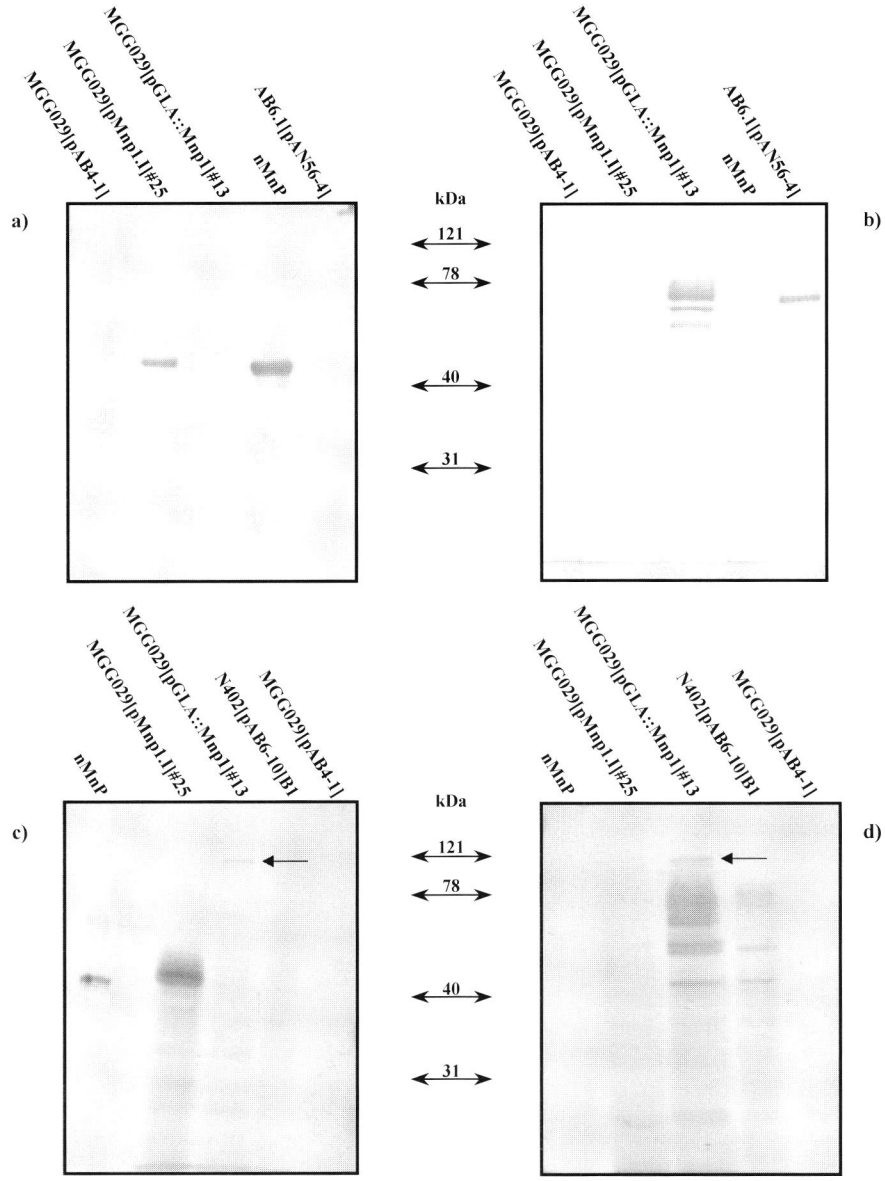
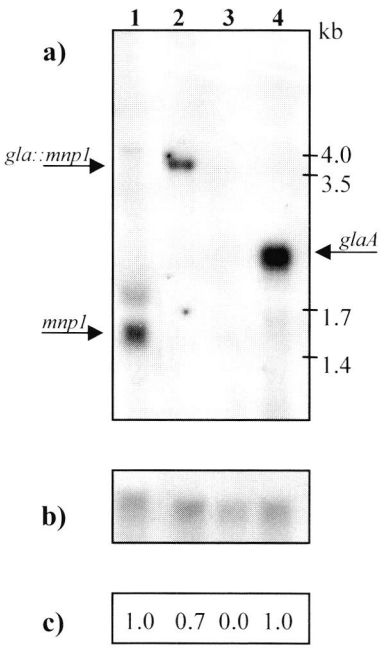


Fig. 3. Western blotting analysis of MnP transformants. 48 hour culture samples of one representative transformant per construct are shown. MGG029[pAB4.1]: wild type strain transformed with the selection marker pAB4.1. MGG029[pMnp1.I]#25: transformant containing pMnp1.I and pAB4.1 plasmids. MGG029[pGLA::MnP]#13: transformant containing pGLA::MnP and pAB4.1 plasmids. nMnP: native manganese peroxidase. N402[pAB6-10]B1: *A. niger* transformant producing the GLA-G2 form (lacking the starch-binding domain; Broekhuijsen *et al.*, 1993). a) and b): extracellular medium c) and d) mycelium extracts. Blots were probed either with a polyclonal MnP antibody [a) and c)], or a monoclonal GLA antibody [b) and d)].

Northern blotting. Strain MGG029[pMnp1.I]#25, which showed the highest MnP activity, and strain [MGG029[pGLA::MnP#13], a pGLA::MnP colony-PCR positive transformant, were selected for this analysis. N402[pAB6-10]B1, a multicopy (20 copies) GLA producing strain (Verdoes *et al.*, 1993), which produces up to 900 mg/L extracellular glucoamylase, was taken as reference. As shown in Fig.4a, Northern blot analysis revealed messenger RNA of the expected size both for pGLA::MnP and pMnp1.I containing strains. The double transcript band observed in the pMnp1.I sample is due to the two polyadenylation sites used in the *A. nidulans trpC* terminator (Mullaney *et al.*, 1985). Transcript signal intensities were measured and corrected for loading differences using the *pgdA* probe (Fig.4 b and c). Significant hybridisation signals were obtained for the *mnp1* and *gla::mnp1* transcripts and these were comparable to the *glaA* transcript level observed in the N402[pAB6-10]B1 strain. Therefore, we concluded that at the transcriptional level no major bottlenecks exist for the production of MnP in *A. niger* MGG029, neither when it is expressed as an unfused nor as a fusion protein.



Expected size mRNAs (kb)

pMnp1.I	~ 1.6
pGLA::MnP	~ 3.7
glaA	2.3

Fig. 4. Northern blotting analysis of total RNA isolated from one representative transformant per expression cassette after 48 hours culture on AMM-Maltose. 1: MGG029[pMnp1.I]#25; 2: [MGG029]pGLA::MnP#13; 3:[MGG029]pAB4-1; 4: N402[pAB6-10]B1. a) P*glaA* probe. b) *gpd* probe. c) Ratio between the intensity of the normalised *glaA* signal of each transformant and the normalised *glaA* signal of N402[pAB6-10]B1. *glaA* signals were normalised against *gpdA* signal shown in b). The expected messenger RNA sizes are indicated.

rMnP displays similar properties to those of nMnP

rMnP was purified from the culture medium of strain MGG029[pMnp1.I]#25 by a two step purification procedure. By using simultaneously 280 nm and 405 nm filters, the purification of rMnP could be easily monitored and showed that MnP was practically the only haem protein present in the culture medium. The purest rMnP fraction had a A_{407}/A_{280} ratio of 5.1,

comparable to that of the native enzyme, and its absorption spectrum (Fig.5A) was very similar to reported data (Fig. 5B; Stewart, *et al.*, 1996). The specific activity determined by ABTS oxidation was 0.44 ΔAbs/min/μg enzyme. Under the same conditions, a specific activity of 0.63 ΔAbs/min/μg was measured for a commercially available native protein preparate. Upon N-terminal sequencing of the extracellular rMnP, the sequence AlaValXxxProAsp was obtained which matches the native MnP N-terminus (AlaValCysProAsp). SDS-PAGE analysis of the purified recombinant protein confirmed the observed slightly slower mobility compared to nMnP (Fig. 3a and results not shown).

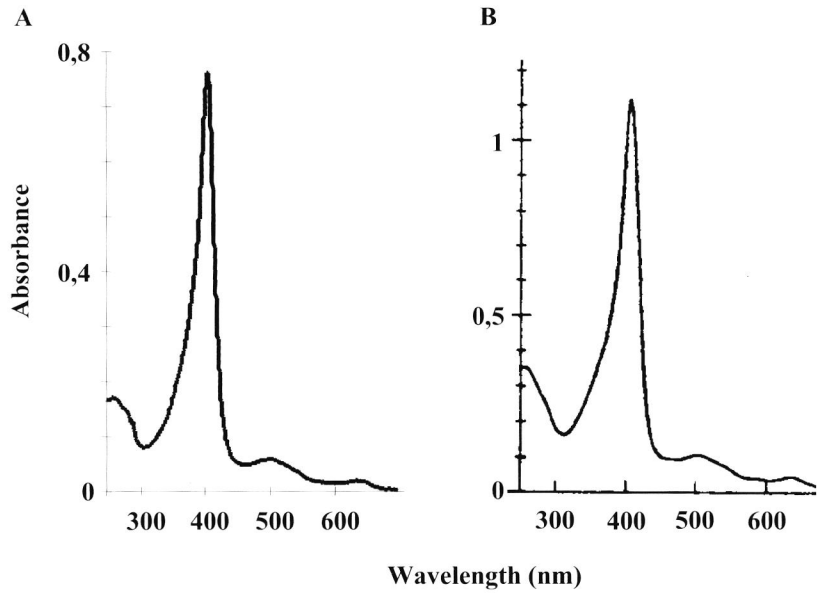


Fig. 5. Absorption spectra of rMnP (A) and nMnP (B) (Stewart *et al.*, 1996).

Controlled fermentation increases rMnP production

Controlled fermentations were carried out to determine rMnP production yields. When performing shake-flask experiments, we noticed that rMnP production diminished when the culture medium became strongly acidified. Therefore we decided to analyse MnP production under different pH conditions. Since the activity of the *glaA* promoter used in pMnp1.I is pH dependent (Withers *et al.*, 1998 ; Punt *et al.*, unpublished) and this would affect our analysis, we also constructed strains in which the *mnp1* gene was placed under control of the constitutively expressed, pH-independent, *gpdA* promoter (Punt *et al.*, 1991). Consequently, two strains MGG029[pMnp1.I]#25 (*glaA* promoter) and MGG029[pgpdMnp1.I]#13 (*gpdA* promoter) were subjected to batch fermentations at pHs ranging from 3 to 6. MnP activity and biomass production was monitored in time. Fig.6 shows the results of the fermentation experiments.

As shown in Fig. 6A both strains behaved similarly in growth development at the different pHs analysed, the biomass production decreasing as the pH became less acidic. However, rMnP production was almost a factor 10 higher in the MGG029[pgpdMnp1.I]#13 compared to the MGG029[pMnp1.I]#25 strain (Fig.6B). Moreover, the two strains differed in

their response to pH variations. MGG029[pMnp1.I]#25 showed maximum rMnP production at low pHs, which is in agreement with the pH dependence that characterises the *glaA* promoter. In contrast, strain MGG029[pgpdMnp1.I]#13 had similar rMnP production levels (250-500 mg/L) in the pH range analysed (Fig. 6B) and showed a significantly higher specific rMnP production at pH=6 (Fig. 6C), mainly due to the lower biomass production at this pH.

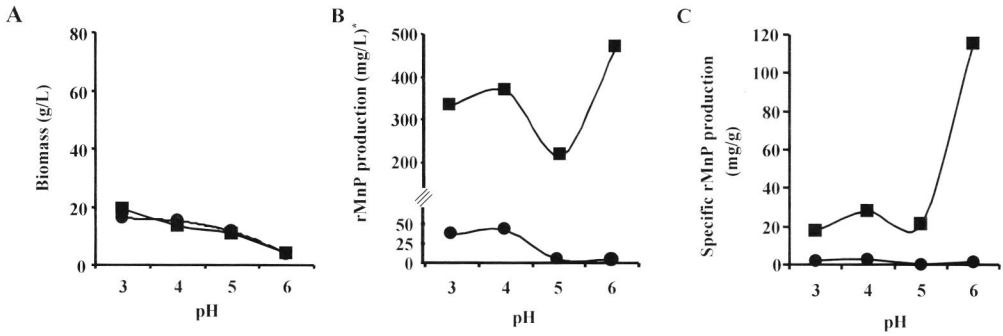


Fig. 6. Production of rMnP in batch fermentations at different pHs. Circles represent strain MGG029[pMnp1.I]#25, squares represent strain MGG029[pgpdMnp1.I]#13. A: Fungal biomass per volume fermentation medium. B: Production of rMnP per volume fermentation medium. C: Specific rMnP production in mg rMnP per gram fungal biomass. Values shown correspond to 48 hours samples. (*) rMnP production was calculated based on ABTS oxidising activity (see M&M).

DISCUSSION

To study the production of Mnp1 in *A. niger* we have used strain MGG029. This strain was constructed to combine in one strain various characteristics favourable for studies on the production of heterologous proteins. *A. niger* MGG029 is deficient in the expression of several protease genes due to a regulatory mutation (Mattern *et al.*, 1992; Schuren *et al.*, unpublished). Lacking the *glaA* gene, MGG029 produces no endogenous glucoamylase. This facilitates analysis when glucoamylase fusion constructs are employed, since detection of glucoamylase secretion confirms successful expression of the fusion gene. Finally, the strain is *pyrG*, which enables the use of the *pyrG* selection marker. As expression cassettes, we made constructs to express the *mnp1* cDNA either as an unfused or as a glucoamylase fusion protein. The use of a protein fusion strategy to improve the production of heterologous proteins in filamentous fungi has in many cases proved to be successful (Broekhuijsen *et al.*, 1993; Contreras *et al.*, 1991; Jeenes *et al.*, 1993; Keränen and Penttilä, 1995), and to date, has not yet been described for the expression of fungal peroxidases. Expression of the two constructs in *A. niger* MGG029 transformants was confirmed by Northern blotting (Fig.4). Steady-state *mnp1* mRNA levels were similar to the *glaA* transcript levels detected in a GLA efficient producer, and we therefore concluded that no major limitations at the transcriptional level were present in the production of MnP in *A. niger*.

Active MnP was secreted into the culture medium of transformants carrying the expression vector pMnp1.I, as was detected by both a colorimetric plate assay (Fig.2) and

Western analysis (Fig.3). Surprisingly, no MnP activity or MnP cross-reacting material could be detected in the culture medium of transformants carrying the GLA::MnP fusion construct (Fig.3). This was intriguing, since the glucoamylase counterpart was efficiently secreted. A possible explanation for this is that the presence of the glucoamylase part of the fusion could interfere with essential maturation events for the MnP protein, leading to the degradation of rMnP. This is, to our knowledge, the first reported case where a fusion approach failed. Interesting, similar results were obtained in our laboratory by Hessing and Schuren (unpublished) in their studies on the expression of a laccase gene in *Aspergillus spp.* These results suggest that the secretion of carrier-target fusion protein is more complex than thought before and indicate that the success of the fusion approach is not only dependent on an efficient carrier, but also on the nature of the cargo protein.

Analysis of purified rMnP showed similar spectral properties and specific activity to those of the native enzyme. Similar results were obtained by Stewart *et al.* (1996) in their studies on the expression of *mnp1* in *A. oryzae.* In our work we also show that correct processing of the MnP signal peptide occurs in *A. niger.* In contrast to Stewart's results, rMnP produced in *A. niger* showed a slightly slower mobility on SDS-PAGE than the nMnP. The slower mobility could be the result of a higher degree of glycosylation of the recombinant enzyme and apparently has no major effect on the activity of rMnP. MnP is both N- and O-glycosylated (Kenigsberg *et al.*, 1987), and Nie *et al.*, (1999) have shown that glycosylation is not essential for the enzyme activity of MnP. Overglycosylation has also been observed in the production of chloroperoxidase (chapter 5) and phytase (Wyss *et al.*, 1999) in *Aspergillus spp.*

Reports on the expression of peroxidases in filamentous fungi (Johnson and Li, 1991; Stewart *et al.*, 1996) have shown that the production yields normally reached are low in comparison to those typically obtained for other fungal proteins (Gouka *et al.*, 1997; Radzio and Kurk, 1997). This suggests that specific limitations are present for the overproduction of this type of oxidative enzymes. Our initial rMnP production yields of 5-10 mg/L obtained in shake-flasks cultures of strain MGG029[pMnp1.I]#25 are, although still low, slightly higher than others reported previously in *A. oryzae* (Stewart *et al.*, 1996), and may be the consequence of a more suitable strain used in this study. As a first approach to improve yields we studied the production of rMnP in batch fermentations. Compared to shake-flask cultures, in controlled fermentations conditions such as nutrient and oxygen sufficiency, or pH, are controlled, which is normally favourable for protein production. Accordingly, rMnP production increased up to 40 mg/L in batch fermentations of strain MGG029[pMnp1.I]#25 (Fig.6B). This result was obtained for a low pH condition, where the glucoamylase promoter of pMnp1 is active. However, extracellular proteases are known to operate in the culture medium of many *Aspergillus* strains at acidic pH (Archer *et al.*, 1990; Archer *et al.*, 1992). Although the host strain used in our study is protease deficient, we could not exclude that rMnP suffered of degradation by protease activity that might still be present at low pH. To further improve production levels at neutral pH strain MGG029[pMnp1.I]#25 was not appropriate and therefore we constructed strain MGG029[pgpdMnp1.I]#13 in which the constitutive, pH-independent *gpdA* promoter drives the expression of the *mnp1* gene. Compared to MGG029[pMnp1.I]#25, an overall five to ten fold increase in production level of rMnP was reached with MGG029[pgpdMnp1.I]#13 (Fig.6B). A reason for this significant difference could be the different promoter used (*glaA* versus *gpdA*), and the transcription level reached due to copy number or differences in integration site. Furthermore, rMnP production in strain MGG029[pgpdMnp1.I]#13 was the highest at pH=6 (480 mg/L). As

biomass production at this pH was minimal, this implies a major improvement in specific rMnP production at pH=6 compared to lower pHs, which could be explained by a lower protease activity at pH=6.

In conclusion, we have shown that by choosing adequate strains and culture conditions the production of peroxidases in *Aspergillus* can be significantly enhanced. Our initial yields of 10 mg/L in shake-flasks were improved upto ~500 mg/L in controlled batch fermentations at neutral pH. Other factors more related to the particular nature of these proteins such as haem availability may also be of importance and will be the subject of further research.

ACKNOWLEDGEMENTS

The authors thank R. van den Dool and Dr. W. van Hartingsveldt for their collaboration in rMnP purification, D. de Kloe for his assistance in the construction of *A. niger* MGG029, Dr. D. Cullen for providing the MnP clone and antisera and N. van Luijk for critical reading of this manuscript.

Expression of the *Phanerochaete chrysosporium* lignin peroxidase in *Aspergillus spp*

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ABSTRACT

To study the production of the *Phanerochaete chrysosporium* lignin peroxidase (LipA) in *Aspergillus*, different expression cassettes were constructed where efficient fungal expression signals were employed and LiP was produced as an unfused or a glucoamylase::LiP fusion protein. Expression of any of these constructs in an *A. niger* protease deficient strain resulted in the secretion of a truncated, inactive form of the LipA protein. Genetic approaches were undertaken in attempts to get more insight in and/or alleviate this degradation event. Our results suggest that LiP may undergo incorrect N-terminal processing at a late stage during secretion.

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INTRODUCTION

The lignin peroxidases (LiP's) of white-rot fungi form a family of extracellular enzymes involved in lignin degradation by these organisms (Boominathan and Reddy, 1992; Buswell and Odier, 1987). In the best studied white-rot species, *Phanerochaete chrysosporium*, LiP occurs as different isoenzymes (Tien and Kirk, 1985) encoded by multiple, differentially regulated genes (Cullen, 1997; Janse *et al.*, 1998; Reddy and D'Souza, 1994; Stewart and Cullen, 1999) normally induced under nutrient-limited conditions (Li *et al.*, 1994; Reiser *et al.*, 1993; Stewart and Cullen, 1999; Stewart *et al.*, 1992). *In vitro*, LiP can oxidise small aromatic compounds such as veratryl alcohol (VA; Tien and Kirk, 1984). VA is also secreted by the fungus and has been proposed to act as mediator in the reaction mechanism of LiP *in vivo* (Goodwin *et al.*, 1995; Koduri and Tien, 1994). Compared to most other microbial and plant peroxidases, LiPs are distinct in their ability to oxidise substrates of high redox potential and in their low pH optimum for activity (Banci, 1997; Millis *et al.*, 1989). Because of its interesting oxidative properties, LiP appears an attractive candidate for enzymatic applications in the paper, pulp and waste industries as reviewed by Breen and Singleton, (1999) and Karam and Nicell, (1997). The potential of these applications has stimulated research for a suitable, large-scale production system for these proteins. Research approaches included overproduction in the natural host (Dosoretz *et al.*, 1993; Feijoo *et al.*, 1995; Orth *et al.*, 1991; Zacchi *et al.*, 2000) and the overexpression in recombinant systems (Aifa *et al.*, 1999; Doyle and Smith, 1996; Johnson and Li, 1991; Johnson *et al.*, 1992; Saloheimo *et al.*, 1989). Although the first approach improved production yields, white-rot fungi remain unsuitable for industrial fermentation processes. Expression of the encoding genes in more traditional producing organisms has invariably resulted in low production yields (Aifa *et al.*, 1999; Doyle and Smith, 1996; Johnson and Li, 1991; Johnson *et al.*, 1992; Saloheimo *et al.*, 1989), though the reasons for this low production have not been elucidated yet.

Previously we have reported the efficient production in *Aspergillus niger* of another structurally related lignolytic enzyme, the *P. chrysosporium* manganese peroxidase (MnP; chapter 3). In this chapter we report our efforts in overproducing the *P. chrysosporium* lignin peroxidase in filamentous fungi. A similar approach to that developed for *mnp1* was followed.

MATERIAL AND METHODS

Strains

Escherichia coli DH5α was used for construction and propagation of vector molecules. Fungal strains used in expression experiments are shown in Table 1.

Table 1. Fungal strains used in expression experiments

Species	Strain	Genotype	Characteristics	Reference
A. niger	MGG029	*prtT, gla::fleo*^{r}, *pyrG*	Protease deficient	Conesa *et al.*, 2000
A. niger	D15	*prtT, phmA, pyrG*	Protease deficient, non-acidifying	Gordon *et al.*, 2000
A. niger	AB6.4-ΔpepE	*pepE::hph*^{r}, *gla::fleo*^{r}, *pyrG*	Deficient in vacuolar protease PepE	Broekhuijsen *et al.*, 1993 Kassow, upublished
A. niger	AB1.13-ΔpclA	*pclA::pyrG*^{mut*}, *prtT, pyrG*	Deficient in protein convertase PclA	Mattern *et al.*, 1992 Punt *et al.*, in prep.
A. awamori	AW4.20-*pyrG*	*pyrG*	Non-acidifying	Gouka *et al.*, 1995

* Mutated, non functional *pyrG*

Expression cassettes and plasmids

LiP isozyme H8 (*lipA*) cDNA was a gift from Dr. D.Cullen (Inst. Microbial and Biochem. Tech., Madison, USA).

Aspergillus expression vectors used to construct *lipA* expression cassettes were: pAN52-10Not, containing the *A. niger* glucoamylase (*glaA*) promoter (Punt, unpublished); pAN52-6Not, containing the *A. niger glaA* promoter fused to the *A. niger* glucoamylase (GLA) 24aa prepropeptide coding sequence (Punt, unpublished); pAN56-2 (Gordon, *et al.*, 2000), containing the *A. niger glaA* promoter fused to the DNA sequence encoding the first 514 N-terminal amino acids of GLA, and pAN52-12Not (Punt, unpublished), containing the *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter.

Seven different expression cassettes were constructed to study the expression of *lipA* in *Aspergillus spp.* (Fig.1). Table 2 lists the sequences of the oligonucleotides used for cloning. The *lipA* cDNA was amplified by PCR using primers LIPA5E/N and LIPA3B/H to introduce appropriate cloning sites at both 3' and 5' ends. The amplified product was cloned in pUC19 at the *Eco*RI/*Hind*III sites resulting in pΔL11R. To obtain pLipA.I, a 1 kb fragment containing the LiP coding sequence was excised from pΔL11R by partial-*Nco*I/*Hind*III digestion and cloned at the same sites in pAN52-10Not. pLipA.II was obtained by cloning a *Bss*HII/*Hind*III fragment from pΔL11R, comprising the DNA sequence encoding the mature LiP, into pAN52-6Not. To construct pGLA::LiP, a *Bss*HII/*Bam*HII fragment from pΔL11R was cloned in pAN56-2 at the *Nar*I/*Bgl*II sites using the linker *Nar*I-NVISKR-*Bss*HII (chapter 3). To construct pgpdLipA.I-AmdS, the 1 kb *Nco*I-*Hind*III fragment from pΔL11R was first cloned into pAN52-12Not at the *Nco*I/*Hind*III sites. Secondly, a 4.5 kb *Not*I fragment containing the *A. nidulans amdS* (Corrick *et al.*, 1987) gene was introduced at the unique *Not*I site of pAN52-12Not.

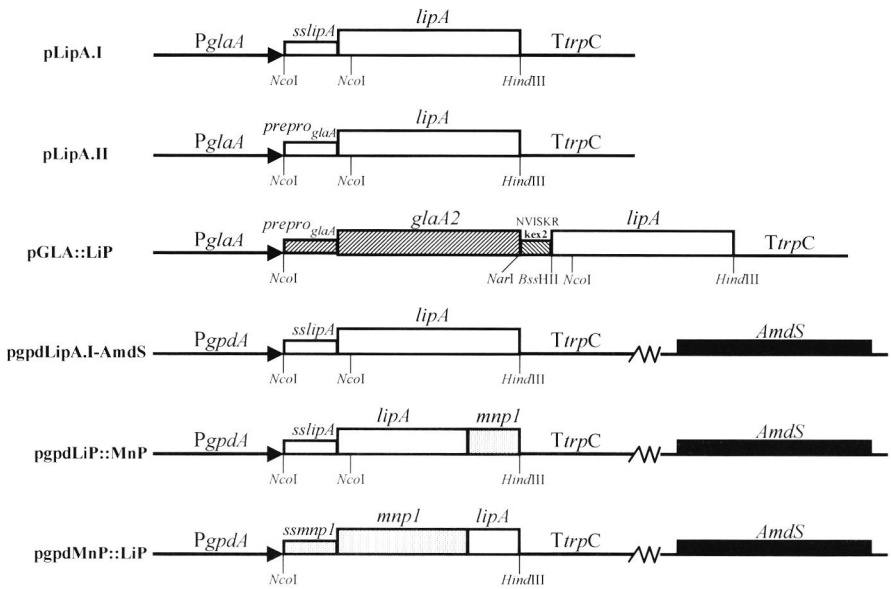

Fig. 1. Expression vectors. For description see text.

Fusion constructs between LiPA and MnP1 were made by fusion PCR. To construct pgpdLip::MnP, the DNA sequence encoding the first 272 N-terminal amino acids of LiP was amplified with primers LipA5E/N and L/Mrev, the DNA sequence encoding the last 110 C-terminal amino acids of MnP was amplified with primers L/Mfor and MNP13B/H (chapter 3), and the two amplification products were joined by fusion PCR using primers LipA5E/N and MNP13B/H. The fusion product was then digested with *Nco*I(partial)/*Hind*III and cloned into pAN52-12Not. Finally, as in pgpdLipA.I-AmdS, the *A. nidulans AmdS* selection marker was introduced at the unique *Not*I site. A similar approach was followed to make the reciprocal construct pgpdMnP::LiP, this time using primers MNP15E/N and M/Lrev to amplify the DNA sequence encoding the first 272 N-terminal amino acids of MnP, and primers M/Lfor and LIPA3B/H to amplify the DNA sequence encoding the last 100 C-terminal amino acids of LiP. Primers MNP15E/N and LIPA3B/H were used in the last fusion PCR reaction.

In all cases, amplification products were checked by sequencing.

Table 2. Primers used for cloning

Oligonucleotide	Sequence (5'-3')*
LIPA5E/N	*Eco*RI *Nco*I GGAATTCCATGGCCTTCAAGCAG M A F K Q
LIPA3B/H	*Bam*HI *Hind*III CGGGATCCAAGCTTAAGCACCCGGAGG Stop A G P P
L/Mfor	LiP MnP CTATCGCCCGCGACGAGCGCACGGCGTGC T I A R D E R T A
L/Mrev	GCACGCCGTGCGCTCGTCGCGGGCGATAG
M/Lfor	MnP LiP GCGCTCGCGCGCGACTCGCGCACGGCGTG A L A R D S R T A
M/Lrev	CACGCCGTGCGCGAGTCGCGCGCGAGCGC

*Restriction sites and amino acids encoded in the oligo sequences are indicated

Transformation procedures

Fungal strains were either transformed or co-transformed following the method described by Punt and van den Hondel (1992). Transformations were performed with vectors containing the *amdS* selection marker and using acetamide for selection (Kelly and Hynes, 1985). Co-transformations were carried out with either pLipA.I, pLipA.II or pGLA::LiP and pAB4-1 (van Hartingsveldt *et al.*, 1987) containing the *A. niger pyrG* selection marker, in a 10:1 w/w ratio. Here, transformants were selected for uridine prototrophy and co-transformants containing expression cassettes were selected by colony PCR, as described by van Zeijl *et al.* (1997).

Culture conditions

Shake-flask experiments were performed in 300 ml flasks containing 50 ml *Aspergillus* minimal medium (Bennett and Lasure, 1991) supplemented with 0.5% casamino acids, using 5% maltose as a carbon source (AMM-maltose). Cultures were inoculated with $5x10^7$ conidia

and incubated at 30°C/300 rpm for 72 hours. Samples were taken at different time points after inoculation. The mycelium was separated from the culture medium by filtration through Miracloth, washed with physiological salt and total protein extracts were prepared as described elsewhere (van Gorcom *et al.*, 1985). When indicated, the filtered culture medium was dialysed overnight against 10 mM sodium acetate buffer pH=6.

Molecular and protein methods

Unless otherwise stated, molecular and protein methods were carried out as described in chapter 3.In the *O*-anisidine-based plate assays for peroxidase activity 50 mM Na-phosphate pH=3 instead of 50 mM Na-phosphate pH=4.5 was used as reaction buffer. Lignin peroxidase activity was measured by monitoring the oxidation of veratryl alcohol (VA) at 310 nm (Tien and Kirk, 1984). In Western blotting experiments, two different polyclonal antibodies against LiPH8 were used: αLiP-Ma (kindly provided by Dr. D. Cullen, Madison) and αLiP-Fr, (kindly provided by Dr. E. Record, INRA, Marseille). 1%GLA was used as blocking agent.

Purification of the rLiP

rLiP was concentrated and partially purified from a 5 L , 48 hours AMM-maltose culture of a pLipA.I containing transformant. The rLiP medium sample was filtrated, concentrated, dialysed and SourceQ fractionated as described for rMnP (chapter 3) but using 20 mM Bis-Tris pH=6.5 as dialysis buffer and a 100 mM to 1 M Bis-Tris pH=6.5 elution gradient. SourceQ fractions were analysed by Western blotting and concentrated with a 5kDa cut-off FILTRON Omegacell^{TM} at 4°C.

RESULTS

LiP is produced in *A. niger* MGG029 as a truncated, inactive form

A. niger MGG029 was co-transformed with pAB4.1 and either pLipA.I, encoding the full-length LiP, pLipA.II where the LiP prepro-sequence was replaced by the GLA prepro, and pGLA::LiP, expressing a Glucoamylase::LiP fusion protein. Twenty uridine prototrophic transformants per construct were analysed for the presence of the integrating expression vectors by colony PCR and for peroxidase activity with the *o*-anisidine plate assay. Although in all three cases, co-transformants were identified by PCR, none of them showed peroxidase activity in the plate assay (data not shown).

To further analyse LiP production in the various transformants, strains were grown in maltose-containing medium where the glucoamylase promoter is induced. RNA and the intracellular protein fraction were extracted from the mycelium, and the culture medium was analysed for extracellular LiP production. Northern-blotting analysis revealed, regardless of the expression cassette used, significant amounts of *lipA* transcript comparable to the transcript signal of the related strain MGG029[pMnp1.I].#25 (chapter 3; Fig.2), which secretes active manganese peroxidase. This result indicates that, as for *mnp1*, no major bottlenecks for LiP production were present at the transcriptional level.

The extracellular culture medium of these transformants grown for 24, 48 and 72 hours was dialysed and examined for veratryl alcohol oxidation. As observed in the plate assay, no LiP activity was detectable in any of the samples analysed. Only upon a 50-fold concentration of the culture medium, traces of VA oxidising activity could be measured in some experiments. However, when the medium samples were analysed by Western blotting,

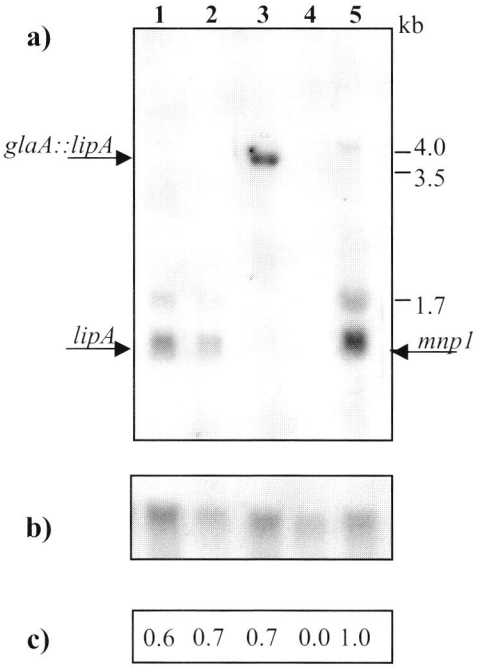

Fig. 2. Northern blotting analysis of *lipA* expression in *A. niger*. Total RNA isolated from one representative transformant per expression cassette after 48 hours culture on AMM-Maltose.
1: MGG029[pLipA.I]#5;
2: MGG029[pLipA.II]#10
3: MGG020[pGLA::LiP]#13;
4: MGG029[pAB4-1];
5: MGG029[pMnp1.I]#25
a) *glaA* probe. b) *gpdA* probe. c) Ratio between the intensity of the normalised *glaA* signal of each transformant and the normalised *glaA* signal of MGG029[pMnp1.I]#25. *glaA* signals were normalised against *gpdA* signal shown in b).

αLiP cross-reactivity was observed (Fig.3a). Most cross-reactive material was found as two protein bands migrating at a lower position than native LiP, and only a small amount was detected at the position of the nLiP control. These cross-reactive bands were also observed in the mycelium extracts (Fig.3b). This result was found regardless of the construct used for expression of the *lipA* gene. In the case of the GLA::LiP producing strain, additional high molecular weight cross-reacting material was detected intracellularly, which presumably corresponds to unprocessed fusion protein, and some degradation products. Furthermore, analysis of medium and mycelium extracts of the GLA::LiP fusion with the αGLA antibody showed synthesis and secretion of the glucoamylase counterpart (data not shown). To confirm that the cross-reactive bands observed in the Western analysis were lignin peroxidase protein fragments, the culture medium of transformant MGG029[pLipA.I]#5 was concentrated and proteins were fractionated and purified as described in M&M. Three αLiP-Ma cross-reacting polypeptides, of aproximately 42, 30 and 15 kDa were submitted to N-terminal amino acid sequencing. Unfortunately, no interpretable sequence resulted from this analysis, probably due to N-terminal blockage of the polypeptides. Neither was VA oxidising activity detectable for any of the fractions analysed. However, all three protein bands cross-reacted with a second polyclonal antibody raised against LiPH8 (Fig.3c and results not shown), which supports the LipH8 nature of these polypeptides.

Taken together, these results suggest that, although LiP is synthesised in MGG029, most of the protein produced is incorrectly processed to lower molecular weight fragments whereby peroxidase activity is lost.

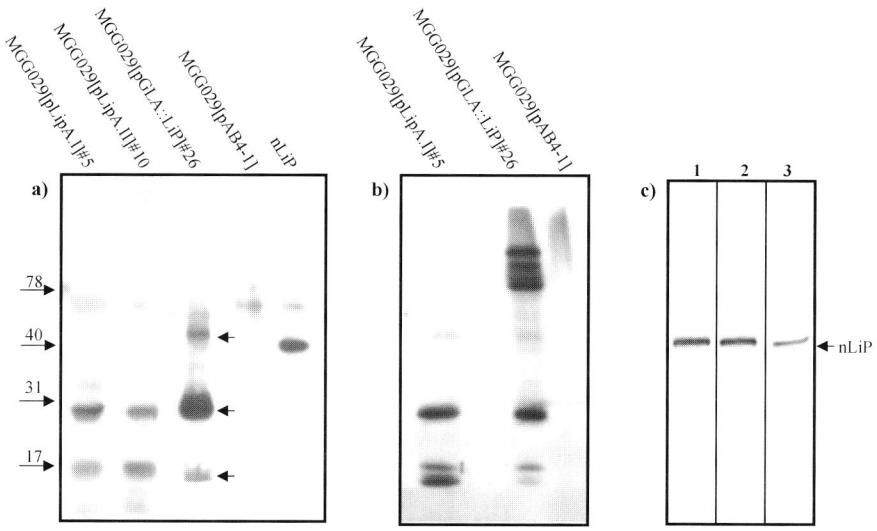

Fig. 3. Western blotting analysis of LiP transformants. a) medium and b) protein extracts: 48 hours culture samples of one representative transformant per construct are shown. MGG029[pAB4.1]: wild type strain transformed with selection marker pAB4.1. MGG029[pLipA.I]#5: transformant containing pLipA.I and pAB4.1 plasmids. MGG029[pLipA.II]#10: transformant containing pLipA.II and pAB4.1 plasmids; MGG029[pGLA::LiP]#26: transformant containing pGLA::LiP and pAB4.1 plasmids. nLiP: native lignin peroxidase. Blots were probed with αLipA-Ma. The position of the three αLipA-Ma cross-reactive bands is indicated in lane 3 with arrows. c) Immunodetection of rLiP. Purification fraction containing a αLiP cross-reactive 42kDa protein band was detected both with αLiP-Ma polyclonal antibody (1) and with αLiP-Fr (2) polyclonal antibody. (3) *P. chrysosporium* LiPH8 control probed with αLiP-Fr polyclonal antibody.

Analysis of LiP processing

To get more insight in the possible causes for the observed incorrect processing of the LiP protein produced in *A. niger* MGG029, we analysed the production of this protein in other fungal hosts differing in their genetic background. First, we studied the possible role of two intracellular proteolytic activities in the incorrect processing of LiP by expressing the *lipA* gene from vector pLipA.I in *A. niger* AB6.4-ΔpepE and *A. niger* AB1.13-ΔpclA. *A. niger* AB6.4-ΔpepE lacks the major vacuolar protease PepE whereas *A. niger* AB1.13-ΔpclA is deficient in the Golgi located KEX2-like protein convertase PclA. Expression of the *lipA* gene in the two strains resulted in the production of truncated forms of LiP as observed previously in MGG029 (Fig 4, lane 1 to 3), indicating that none of the corresponding mutated proteases were responsible for the observed processing of LiP. Curiously, when construct pGLA::LiP was expressed in the AB1.13-ΔpclA strain, in which PclA-mediated splicing of the fusion products at the KEX2-like site is impaired, only GLA::LiP fusion products, and no LiP processing bands, were observed in the extracellular medium (Fig 4, lane 4). This suggests that the fused GLA protected LiP from degradation.

The possible role of extracellular acidic proteases in the incorrect processing of LiP was studied by expressing the *lipA* gene in two non-acidifying *Aspergillus* strains: *A. niger* D15, a pH mutant derivative from AB1.13 and *A. awamori* AW4.20. To study production of

LiP in these strains extra expression cassettes were required, since the glucoamylase promoter used in the initial constructs is not active at neutral or basic pH. Therefore, plasmid pgpdLipA.I-AmdS was constructed where the expression of the *lipA* gene is under control of the constitutive, pH-independent, *gpdA* promoter (Punt *et al.*, 1991). In pgpdLipA.I-AmdS the selection marker *AmdS* is included, which allows the generation of multicopy transformants. *A. niger* D15 and *A. awamori* AW4.20 and the reference strain *A. niger* MGG029 were transformed with pgpdLipA.I-AmdS and multicopy transformants were selected by their ability to grow on acetamide plates. Similarly to the other *Aspergillus* strains, no *o*-anisidine or veratryl alcohol oxidising activity could be measured in the extracellular medium of the AW4.20, D15 and MGG029 transformants expressing the *lipA* gene under control of the *gpdA* promoter (data not shown). Western analysis revealed the presence of degraded forms of the LiP protein in the extracellular medium of these transformants, (Fig. 4 lanes 6 to 8). Curiously, the major degradation band observed in *A. awamori* migrated at a lower position on SDS-PAGE as compared to the *A. niger* strains.

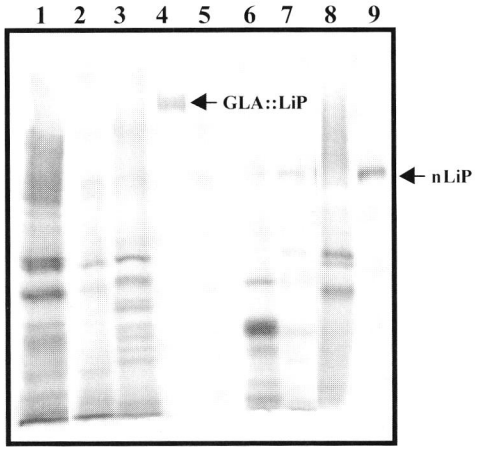

Fig. 4. Western blotting analysis of LiP production in *Aspergillus* spp. 48 hours culture samples of one representative transformant per construct are shown. 1: *A. niger* MGG029[pLipA.I]#5 2: *A. niger* AB6.4 ΔpepE[pLipA.I], 3: *A. niger* AB1.13 ΔpclA[pLipA.I], 4: AB1.13 ΔpclA[pGLA::LiP], 6: *A. awamori* AW4.20[pgpdLipA.I-AmdS], 7: *A. niger* D15[pgpdLipA.I-AmdS], 8: *A. niger* MGG029[pgpdLipA.I-AmdS]. The blot was developed with αLiP-Ma polyclonal antisera. The positions of native LiP and the fusion protein GLA::LiP are indicated with an arrow.

Analysis of the LiP deduced amino acid sequence revealed the presence of several basic amino acid motifs (SR, SK) at the C-terminal part of the protein, which could be potential recognition sites for protease cleavage. Comparison of the LiP amino acid sequence with the deduced sequence from the closely related manganese peroxidase (MnP), produced as an active protein in *A. niger* MGG029 (chapter 3), showed that none of the mentioned basic amino acid motifs found in LiP were present at the corresponding position in MnP. To study whether processing at any of these sites was the cause of the degradation observed for the LiP protein, LiP/MnP fusion constructs were made where the N- and C- terminal parts of the two proteins were exchanged. Hence, in pgpdLip::MnP the last 110 C-terminal amino acids of LiP, containing the basic sites were replaced by the corresponding sequence of the MnP protein. Reciprocally, in pgpdMnP::LiP, the last 100 C-terminal amino acids of MnP were replaced by the corresponding sequence of the LiP protein, containing the putative protease recognition sites. Expression of the pgpdMnP::LiP vector in *A. niger* MGG029 resulted in the production of the same degradation bands as observed for the pgpdLipA.I construct (data not shown) indicating that these C-terminal basic sites were not responsible

for the observed processing of the LiP protein. Expression of the reciprocal vector pgpdMnP::LiP in *A. niger* failed in producing any detectable fusion protein in the culture medium (data not shown).

DISCUSSION

To study the production of the *P. chrysosporium* lignin peroxidase in *A. niger*, we used a similar approach as developed for manganese peroxidase (chapter 3). Expression of the *P. chrysosporium mnp1* under the control of the glucoamylase promoter in *A. niger* MGG029 resulted in the production of significant amounts of active MnP. However, in contrast to the situation with *mnp1*, no LiP activity could be detected in the extracellular medium of any of the *lipA* containing transformants analysed, and only traces of VA oxidising activity could be measured when the culture medium was concentrated. Western blotting analysis showed synthesis and secretion of the LiP protein in these transformants, although most of the extracellular LiP cross-reacting material appeared as discrete bands migrating at a lower molecular weight position than the native LiP band. Only a small fraction, presumably the full-length recombinant protein, co-migrated with nLiP. Unfortunately, although attempts were done to purify this last fraction, we did not succeed in obtaining enough material to unequivocally determine the activity of the recombinantly produced LiP. These results suggest that, although LiP is synthesised in MGG029, most of the protein is incorrectly processed to lower molecular weight fragments which have no peroxidase activity.

In other studies on the expression of lignin peroxidase in filamentous fungi (Aifa *et al.*, 1999; Saloheimo *et al.*, 1989; Sollewijn Gelpke *et al.*, 1999), the major bottlenecks for the production of this protein have been shown to occur at the protein level. In these previous works, no or little extracellular LiP protein could be detected, although sufficient mRNA was available. In our case, we also observe significant amounts of *lipA* transcript (Fig.2) and we do observe extracellular protein production (Fig.3), but no measurable activity, presumably due to incorrect processing of the LiP protein.

In attempts to get more insight in the causes of this incorrect processing different genetic approaches were undertaken. It is known that production of heterologous proteins can be seriously hampered by proteolytic degradation by acidic extracellular proteases (Archer *et al.*, 1990; Archer *et al.*, 1992). The *A. niger* strain we used in our study is strongly reduced in extracellular protease activity (Mattern *et al.*, 1992), but we could not rule out that residual proteolytic degradation was still present. However, expression of *lipA* in two different low protease, non-acidifying strains, i.e., *A. niger* D15 and *A. awamori* AW4.20 failed in preventing LiP from degrading, suggesting that degradation by acidic proteases was not the major cause for the observed incorrect processing of LiP.

As the truncated LiP bands were also detected in mycelial extracts, we questioned whether the putative incorrect processing could occur intracellularly. Analysis of the LiP deduced amino acid sequence revealed the presence of several C-terminal basic motifs that could be recognition sites for intracellular proteases such as PclA, a KEX2-like protein convertase (Punt *et al.*, in preparation). These residues were not present at the corresponding position of the related MnP, which is correctly processed in *A. niger* MGG029. However, expression in a ΔpclA strain did not impede the incorrect processing of LiP. Also removal of the C-terminal basic motifs of the LiP sequence by replacement with the corresponding MnP sequence had no positive effect. This suggests that proteolysis at these sites was not

responsible for the observed incorrect processing of the LiP protein, and also suggests that the processing occurs on the N-terminal half of the protein. This last possibility is also supported by the results obtained with the GLA::LiP fusion construct. In this construct the catalytic domain of the *A. niger* glucoamylase is fused to the N-terminus of LiP through a linker sequence containing the PclA splicing site. When such a construct is expressed in a wild type strain, where PclA is active, splicing of the fusion counterparts occurs intracellularly and the two proteins are secreted separately (Punt *et al.*, in preparation). Accordingly, we detected spliced GLA and LiP in the extracellular medium, the LiP protein being, as with the non-fusion constructs, incorrectly processed (Fig. 3 and results not shown). However, when this fusion construct is expressed in a ΔpclA strain, intracellular splicing of GLA and LiP is abolished and the fusion product is secreted into the culture medium. In this situation we do not observe incorrect processing of the LiP counterpart, suggesting that the GLA N-terminal fusion somehow protects LiP from incorrect processing. Moreover, since PclA is presumably located in the Golgi apparatus, our results also suggest that this aberrant LiP processing occurs at a post-Golgi location.

Although we have not succeeded in producing active LiP in *Aspergillus* and in avoiding the incorrect processing, we believe this processing is related to the LiP sequence and not for instance the requirement for the haem cofactor, since it was not observed for other haem peroxidases expressed in *A. niger* such as MnP (chapter 3) or the chloroperoxidase (CPO) from *Caldariomyces fumago* (chapter 5). Furthermore, addition of haem to the culture medium of LiP producing strains did not protect LiP from incorrect processing (data not shown). A number of features have been described for the LiP protein, which could be of importance for the secretion of this protein. It has been suggested that, in *P. chrysosporium,* secretion of LiP occurs via the vacuole (Kuan and Tien, 1989). Different vacuolar protease specificities in other hosts could hamper the heterologous production of this protein. We have shown in this work that the major vacuolar protease PepE is not responsible for the incorrect processing of the LiP produced in *Aspergillus*, since this is still observed when the protein was produced in a ΔpepE strain (Fig.4). However, incorrect processing by other vacuolar-located proteases can not be excluded. Furthermore, in contrast to MnP and CPO, a pro-sequence is present at the N-terminus of LiP (Ritch *et al.*, 1991). N-terminal pro-sequences are known to be involved in intracellular targeting and protein maturation (Bening *et al.*, 1998; Eder and Fersht, 1995; Klionsky *et al.*, 1988; Wiederanders, 2000) and, in some cases, were shown to be important for protein secretion (Brandhorst and Kenealy, 1995). However, the function of the LiP pro-sequence is completely unknown. Finally, cell fractionation experiments in *P. chrysosporium* showed accumulation of LiP in small-sized vesicles whereas MnP co-fractionated with the ER-derived membrane fraction, suggesting a differential routing of the two peroxidases in the natural host (M. Asther, personal communication). Whether any of these features really implies a specific secretion route for LiP in *P. chrysosporium* or are related to the difficulties encountered to produce active LiP recombinantly, remains to be investigated.

ACKNOWLEDGEMENTS

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5

Expression of the *Caldariomyces fumago* chloroperoxidase in *Aspergillus niger* and characterisation of the recombinant enzyme

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ABSTRACT

The *Caldariomyces fumago* chloroperoxidase was successfully expressed in *Aspergillus niger*. The recombinant enzyme was produced in the culture medium as an active protein and could be purified by a three-step purification procedure. The catalytic behaviour of recombinant chloroperoxidase (rCPO[1]) was studied and compared with that of native CPO. The specific chlorination activity (47 U/nmol) of rCPO and its pH-optimum (pH 2.75) were very similar to those of native CPO. rCPO catalyses the oxidation of various substrates in comparable yields and selectivities to native CPO. Indole was oxidised to 2-oxindole with 99 % selectivity and thioanisole to the corresponding R-sulfoxide (e.e. > 98 %). Incorporation of ^{18}O from labelled $H_2{}^{18}O_2$ into the oxidised products was 100% in both cases.

INTRODUCTION

Chloroperoxidase (CPO; E.C. 1.11.1.10) is a heavily glycosylated monomeric haemoprotein, with a sugar content of 18 % of its molecular weight of 42 kDa (Kenigsberg *et al.*, 1987). The chloroperoxidase is secreted by the filamentous fungus *Caldariomyces fumago* and was first purified and described in 1966 by Morris and Hager. *In vivo,* CPO catalyses oxidative chlorination. *In vitro*, in the absence of Cl^-, CPO catalyses a variety of synthetically useful (enantioselective) oxygen transfer reactions (Adam *et al.*, 1999; Hager *et al.*, 1998; van Deurzen *et al.*, 1997) e.g. asymmetric epoxidation of olefins (Allain *et al.*, 1993; Dexter *et al.*, 1995; Lakner and Hager, 1996), allylic, benzylic, and propargylic hydroxylation (Hu and L.P., 1999; Miller *et al.*, 1995; Zaks and Dodds, 1995), asymmetric sulfoxidation (Colonna *et al.*, 1997; Colonna *et al.*, 1992; Kobayashi *et al.*, 1987; van Deurzen *et al.*, 1996) and oxidation of indoles to the corresponding 2-oxindoles (Corbett and Chipko, 1979; van Deurzen *et al.*, 1996). In catalysing these oxygen transfer reactions CPO behaves more like the P-450 cytochromes than like classical peroxidases such as the peroxidases from horseradish roots, soy beans and the fungus *Coprinus cinereus*, which mostly catalyse one electron oxidations, e.g. polymerisation of phenol and anilinic compounds (Dordick, 1992; Kobayashi *et al.*, 1995). Moreover, the iron protoporphyrin in CPO is ligated to the active site through a cysteine residue (Blanke and Hager, 1988; Dawson and Sono, 1987; Liu *et al.*, 1995), as characteristic of P-450 cytochromes, whereas the axial ligand in peroxidases normally is a histidine residue (Smith and Veitch, 1998). Interestingly, the *C.fumago* CPO shows no sequence similarity to other extracellular haem peroxidases (Black and Reddy, 1991; Godfrey *et al.*, 1990; Mayfield *et al.*, 1994; Sawai-Hatanaka *et al.*, 1995; Zhang *et al.*, 1991) or to known microbial vanadium haloperoxidases (Bantleon *et al.*, 1994; Burd *et al.*, 1995; van-Schijndel *et al.*, 1993) but is most similar to the *Aspergillus nidulans stcC* (Brown *et al.*, 1996) a member of the sterigmatocystin biosynthetic gene cluster and shows also significant sequence similarity to a *Agaricus bisporus* cellulolytic gene (Accession number AJ293759).

Site-directed mutagenesis has proved to be a powerful tool in exploring structure-function relationships in classical peroxidases (Smith and Veitch, 1998); especially horseradish peroxidase (HRP) has been studied in great detail by Morishima and co-workers (Mukai *et al.*, 1997; Nagano *et al.*, 1996; Tanaka *et al.*, 1996; Tanaka *et al.*, 1997) and by Smith and co-workers (Rodriguez Lopez *et al.*, 1996; Rodriguez-Lopez *et al.*, 1996; Sanders *et al.*, 1994; Smulevich *et al.*, 1994). Furthermore, Ortiz de Montellano and co-workers have used site-specific mutagenesis to engineer HRP with oxygen transfer catalytic properties, suitable for enantioselective sulfoxidation and epoxidation reactions (Newmyer and Ortiz de Montellano, 1996; Ozaki and Ortiz de Montellano, 1994; Ozaki and Ortiz de Montellano, 1995; Savenkova *et al.*, 1996). The use of such an approach for the *C. fumago* CPO could help in revealing the structural basis of the unique properties of this enzyme and to explore further possibilities.

For site-directed mutagenesis studies an efficient expression system for the *cpo* gene is required. As CPO is a protein with several post-translational modifications, i.e. N- and O-glycosylation, disulphide-bridge formation, cleavage of N-terminal and C-terminal sequences and prosthetic group incorporation (Kenigsberg *et al.*, 1987), prokaryotic hosts appear unsuitable for synthesising the active protein. Indeed, Zong *et al.* (1995), reporting the expression of *cpo* in *E. coli*, showed that the non-glycosylated enzyme was secreted into the periplasm in its apoform and only after a tedious high-pressure assisted reconstitution process

limited amounts of the active holoenzyme could be recovered. Therefore, other, eukaryotic, expression systems have been considered. Expression of the *cpo* using the baculovirus system resulted in the production of extracellular inactive CPO, which could not be reconstituted to active protein (Sigle, 1993). Similarly, attempts to produce CPO in *Saccharomyces cerevisiae* and *Pichia pastoris* have been unsuccessful (Sigle, 1993; Zong, 1997). Recently, the genetic transformation of *Caldariomyces fumago* and the expression of mutant forms of CPO in the parental host have been reported (Yi *et al.*, 1999). However, this system has the inconvenience of the presence of native CPO background, which hampers the screening for recombinant CPO producing strains, and has failed in providing specific CPO mutant proteins (Hager, personal communication).

We have explored the possibility of producing CPO in another filamentous fungal expression host, namely *Aspergillus niger*. Filamentous fungi are capable of secreting large amounts of proteins into the extracellular medium. Since versatile DNA-transfer and gene expression systems are available for these organisms, the necessary tools are available for the production of recombinant proteins. Furthermore, *A. niger* has no detectable extracellular peroxidases, and therefore, opposite to the *C. fumago* system, no interference of endogenous oxidising activities when screening for CPO producing transformants. To date several reports on the expression of fungal metalloproteins in filamentous fungi have been published (Aifa *et al.*, 1999; Conesa *et al.*, 2000; Saloheimo and Niku-Paavola, 1991; Stewart *et al.*, 1996). However, although production of active recombinant enzymes was found in most cases, yield levels were still far from those obtained for less complex fungal proteins, making the secretion of metalloproteins an intriguing subject of study. Here, we describe the expression of the *C. fumago cpo* gene in *Aspergillus niger*. Fully active recombinant CPO was produced and purified. Its catalytic properties were compared with those of the native CPO from *Caldariomyces fumago*.

MATERIAL AND METHODS

Strains

Escherichia coli DH5α was used for construction and propagation of vector molecules. *A. niger* MGG029 (*prtT*, *gla::fleo*^{r}, *pyrG*; Conesa *et al.*, 2000) was used as recipient strain in transformation experiments.

Reagentia

Native chloroperoxidase from *Caldariomyces fumago* was obtained from Chirazyme Labs (Urbana, IL) and used without further purification. The enzyme solution contained 11.4 mg/ml CPO with R_z 1.23 (R_z = purity standard = A_{400}/A_{280} = 1.44 for pure enzyme) and an activity of 22.8 kU/ml (standard monochlorodimedon (MCD) assay as described by Morris and Hager 1966). *O*-anisidine was purchased from Fluka and hemin from Sigma. The gel filtration low molecular weight calibration kit was purchased from Pharmacia. Indole, 5-bromoindole, 5-chloroindole, 5-methoxyindole, thioanisole, ethyl phenyl sulphide, and methyl *p*-methoxyphenyl sulphide, were purchased from Aldrich Chemical Company. The corresponding sulfoxides were prepared by chemical oxidation according to Drabowicz *et al.* (1990). ^{18}O labelled hydrogen peroxide ($H_2{}^{18}O_2$; 90% ^{18}O) was obtained from Campro Scientific.

Analysis and Equipment

UV measurements were performed on a Cary 3 spectrophotometer from Varian. A Megafuge 2.0R from Heraeus Instruments was used for centrifugation. A Metrohm Dosimat 665 was used for continuous addition of H_2O_2.

Enzyme purification was performed with a Waters Delta Prep 4000 HPLC system equipped with a Pharmacia fast flow column (d=5 cm; 750 ml DEAE Sepharose) and a Waters fraction collector.

Gel filtration chromatography was done using a Superose 12 HPLC column (Pharmacia, 10 x 300 mm) with a Waters 590 programmable HPLC pump with detection on a Waters 486 tunable absorbance detector at 280 nm or 400 nm with Waters Millennium^{32} software. Fractions were collected using a Waters fraction collector.

Samples for analysing the enantioselective oxidation of sulphides were quenched with sodium sulphite, diluted with a hexane/isopropyl alcohol mixture of 75:25 (v:v) and dried over Na_2SO_4. After centrifugation, the samples were analysed on chiral HPLC using a Chiralcel OD column (Daicel Chemical Industries, Ltd., 250 x 4.6 mm), eluent flow 0.6 ml min^{-1}, and detected on a Waters 486 tunable absorbance detector at 220 nm with Waters Millennium^{32} software. A hexane/isopropyl alcohol mixture of 75:25 (v:v) was used as eluent. 1,2,3-trimethoxybenzene was used as internal standard.

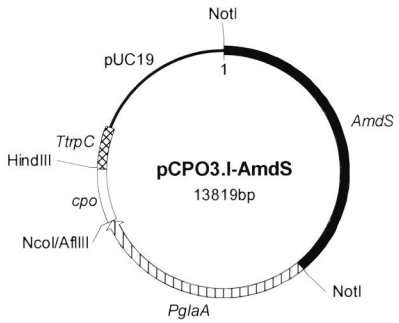
Samples for analysing indole oxidation were quenched with a saturated sodium sulphite solution and diluted with methanol. After centrifugation, the samples were analysed with reversed phase HPLC using a custom-packed Symmetry C_{18} cartridge (Waters Radial-Pak, 8 x 100 mm, 7 µm) contained in a Waters RCM 8x10 compression unit, with simultaneous detection on a Waters 410 differential refractometer and a Waters 486 tunable absorbance detector at 254 nm with Waters Millennium^{32} software. *tert*-butyl alcohol was used as internal standard. A methanol/water mixture of 70:30 (v/v) at 1.0 ml min^{-1} was used as eluent for all indole derivatives.

GS-MS analysis was performed on a CP SIL5CB MS column (25m x 0.25 mm) and a VG 70-SE mass spectrometer.

Chloroperoxidase activity was determined by the standard chlorination method as described by Morris and Hager (1966), defining one unit of chloroperoxidase activity as the amount of enzyme that catalyses the formation of 1 µmole of dichlorodimedon in one min. Total protein content was determined by the method of Bradford using bovine serum albumin as a standard. To obtain the ferrous-CO complex of CPO (Yi *et al.*, 1999), the Fe(III) was first reduced to Fe(II) with dithionite and then incubated for two minutes with CO. The reactivity of the recombinant enzyme to the Ellmann reagent for free SH-groups was carried out as described by Ellmann (1959).

Construction of cpo expression vector

pCf6, a plasmid containing a cpo genomic clone, was a gift from Dr. Hager (University of Illinois at Urbana-Champaign). Primers CLP15E/A (5'-GGAATTCACATGTTCTCCAAGG TCC-3') and CLP1CTERM3 (5'-CGCGCGGATCCAAGCTTAAAGGTTGCGGG-3') were used to amplify the DNA sequence encoding the full-length CPO precursor (GenBank Accession number AJ300448) from pCf6 and introduce suitable cloning sites. The resulting PCR product was *Eco*RI/*Bam*HI digested and cloned into pUC19 to render pCPO3. The amplified *cpo* fragment was checked by sequence analysis, excised from pCPO3 as an *Afl*III/*Hind*III fragment and cloned into the pAN52-10Not *Aspergillus* expression vector (Punt, unpublished) at the *Nco*I/*Hind*III cloning sites, which resulted in pCPO3.I. In vector



pCPO3.I the CPO coding sequence is placed under control of the *A. niger* glucoamylase promoter and *A. nidulans trpC* terminator. Finally, the A. *nidulans AmdS* selection marker (Kelly and Hynes, 1985) was introduced in pCPO3.I at a unique *Not*I site to obtain the *cpo* expression vector pCPO3.I-AmdS (Fig. 1).

Fig. 1. The *cpo* expression vector pCPO3.I-AmdS.

Transformation procedures

Fungal co-transformation was carried out as described (Punt and van den Hondel, 1992), using pCPO3.I-AmdS and pAB4-1 (van Hartingsveldt *et al.*, 1987) plasmids. Transformants were selected on fructose minimal medium plates without uridine and containing acetamide as sole nitrogen source. Transformants were selected for multicopy integration of the expression cassettes on acrylamide plates (Verdoes *et al.*, 1993) and for extracellular peroxidase activity on *o*-anisidine plates as described by Conesa *et al.* (2000) using 0.05% H_2O_2 in 0.1 M Na-phosphate buffer pH=2.7 as developing buffer.

Molecular and protein methods

Molecular methods were carried out essentially as described by Sambrook *et al.* (1989). Total fungal RNA was isolated using the RNAzol^{TM} kit from CINNA/BIOTECX. For Northern analysis experiments a 1 kb. *Sty*I fragment from pCF6 containing most of the *cpo* coding region was used as a probe. SDS-PAGE was performed with a BioRad MiniprotII system using the Tris-glycine method and 10% polyacrylamide gels. N-terminus determination of rCPO was performed by Edman degradation after SDS-PAGE of the purified protein and blotting onto a PVDF membrane. For deglycosylation experiments, proteins were treated with EndoH endoglycosidase (New England Biolabs) following manufacturer's instructions.

Polyclonal antisera

For preparation of polyclonal antibodies, CPO from *C. fumago* IMI 089362 was purified according to van Deurzen *et al.* (1994). A 3 mg aliquot of the purified CPO was treated with acetone-0.3% HCl to remove the haem group (Nakahara and Shoun, 1996) and both holo and apochloroperoxidase were used for rabbit immunisation. Immunisations were done in duplo using 100 µg protein in Freund's Complete Adjuvant-H_2O_2 (1:1). Boosters were done after two and sixteen weeks after immunisation using 100 µg protein in Freund's Incomplete Adjuvant. Rabbits were bled one week after the last booster and optimal sera dilution was determined by ELISA.

Production and purification of rCPO

Fungal culturing was carried out in two-liter Erlenmeyer flasks containing 500 ml *Aspergillus* minimal growth medium (AMM; Bennett and Lasure, 1991) with 5% maltodextrin and supplemented with 0.5% casein amino acids and 500 mg/L hemin. Cultures were inoculated

with $5 \cdot 10^8$ conidia and grown for 48 h at 30°C or 22°C in a rotary shaker revolving at 300 rpm. Medium samples were obtained by filtering the fungal cultures through a Miracloth.

To the filtered medium (1300 ml) cold acetone (1000 ml; 45 % v:v; -20°C) was slowly added at 4°C, and after one hour incubation at -20°C, precipitated impurities were removed by centrifugation (4400 min^{-1}; 20 min; 0°C). Cold acetone was then slowly added to the supernatant (1000 ml; final concentration 60 % v:v; -20°C) and CPO precipitation occurred overnight at -20°C. The supernatant was removed by decanting and the precipitated protein was dried for 10 min. The protein pellet was dissolved in phosphate buffer (300 ml; 10 mM; pH 5.2), adjusted to pH 5.8 with 10 mM H_3PO_4 and brought onto a DEAE Sepharose (Pharmacia, 750 ml) fast flow column in phosphate buffer (20 mM; pH 5.8; flow 10 ml min^{-1}) The column was washed with phosphate buffer (20 mM; pH 5.8; 10 ml min^{-1}) for 1 hour. The enzyme was eluted with a 20-200 mM phosphate buffer gradient (pH 5.8; 10 ml min^{-1}) during 4 hours. Fractions having peroxidase activity (MCD assay) above 0.25 U/ml were pooled, adjusted to pH 5.2 and concentrated over a 30 kDa membrane (Centriprep-30 concentrator, Amicon) at a speed of 1800 rpm. Further purification was done by gel filtration on a Superose 12 HPLC column (Pharmacia, 10 x 300 mm; phosphate buffer pH 5.2; 200 mM; 0.5 ml min^{-1}).

Oxidation of sulphides

For sulphide oxidation reactions, 50 µmol sulphide was dissolved at room temperature in 1.0 ml solvent (0.2 M phosphate buffer pH 5.2). 24 U chloroperoxidase were added to the reaction mixture and stirred for 5 min. The reaction was started by the continuous addition of H_2O_2 (0.15 M) at a rate of 1eq./2h to a total of 1.1 eq. H_2O_2. The reaction was quenched after 2.5 h by the addition of an excess of Na_2SO_3. The reaction mixture was homogenised by the addition of isopropyl alcohol (400 µl) and analysed by chiral HPLC.

Oxidation of thioanisole with $H_2{}^{18}O_2$ was performed at 0.5 ml scale. Oxidation was started with the stepwise addition of $H_2{}^{18}O_2$ (1.0 %; 5 µl each minute to a total of 95 µl). 5 minutes after the last addition the reaction mixture was extracted with dichloromethane and the reaction products were analysed with GC-MS.

Oxidation of substituted indoles

Oxidation of substituted indoles was performed at room temperature in 1.0 ml aliquots containing 10 µmol indole derivative dissolved in *tert*-butyl alcohol/0.2 M phosphate buffer pH 5.2 (50:50, v/v). 8 U chloroperoxidase were added to the reaction mixture, stirred for 5 min., and the reaction was started by the continuous addition of H_2O_2 (0.15 M) at a rate of 1eq./h, to a total of 1.1 eq. of H_2O_2. The reactions were monitored by removing aliquots and analysing by HPLC.

The oxidation of indole with $H_2{}^{18}O_2$ was performed at 0.5 ml scale. Oxidation was started with the stepwise addition of $H_2{}^{18}O_2$ (0.4 %; 5 µl each minute to a total of 55 µl). 5 minutes after the last addition the reaction mixture was extracted with dichloromethane and the reaction products were analysed with GC-MS.

RESULTS

Isolation of *Aspergillus niger* transformants producing rCPO

In a co-transformation experiment, *A. niger* strain MGG029 was transformed with a mixture of plasmids pCPO3.I-AmdS and pAB4-1. Several uridine prototrophic, acetamide utilising transformants were obtained and were transferred to both acrylamide and *o*-anisidine containing plates. Efficient growth and sporulation on acrylamide plates reflects multicopy integration of the transforming vector (Verdoes *et al.*, 1993), and coloured halo formation on *o*-anisidine plates indicates extracellular peroxidase activity (Conesa *et al.*, 2000). Four transformants growing vigorously on acrylamide and developing an intense halo with the *o*-anisidine test were selected. These four strains were cultured on maltose minimal medium for 48 hours and analysed for *cpo* mRNA synthesis by Northern blotting and extracellular CPO production by Western analysis (data not shown). From this analysis the best producing transformant, strain MGG029[pCPO3.I]#5, was selected for production and purification of recombinant CPO (rCPO).

Production and purification of rCPO

Extracellular production of rCPO could be readily detected in shake-flask cultures of strain MGG029[pCPO3.I]#5 without the need of extra haem supplementation. However, rCPO production levels could be increased by 10-fold upon hemin addition to the culture medium at a concentration of 500 mg/L. An additional 5-fold increase was achieved by switching the culturing temperature from 30°C to 22°C. Under these conditions up to 10 mg/L rCPO could be produced from strain MGG029[pCPO3.I]#5.

rCPO was purified to electrophoretic homogeneity by acetone precipitation and column chromatography as reported for CPO from *C. fumago* (van Deurzen *et al.*, 1994; Zong, 1997). The figures corresponding to the purification of rCPO are given in Table 1.

Table 1. Purification of rCPO

	Volume (ml)	Activity (U/ml)	Enzyme (U)	Yield (%)	R_z (A_{400}/A_{280})
Crude	1300	1.6	2080	100	n.d.
Acetone precipitation	300	n.d.	-	-	< 0.05
DEAE Sepharose	9	74	666	32	0.31
GPC	6	39	234	11	0.54

Molecular characterisation of rCPO

Figure 2 shows the UV-spectra of purified rCPO and native CPO (nCPO, commercial preparation with R_z 1.23). As can be seen, the ratio between A_{400} (indicating haem-containing protein) and A_{280} (indicating total protein), or R_z-value, is lower for rCPO (0.54) in comparison to nCPO. Homogeneous CPO from *C. fumago* has a R_z of 1.44. This suggests that rCPO is only partly (~40 %) occupied with haem. Similarly to nCPO, the absorption spectrum of the ferrous-CO complex of rCPO showed a Soret peak at 450 nm (data not shown), indicating the correct formation of the haem thiolate ligand Cys29 (Stern and Peisach, 1974).

To further characterise rCPO we compared the behaviour on SDS-PAGE of the native and recombinant proteins. Two major protein bands could be detected in the nCPO preparate, possibly corresponding to isozymes A and B. These two CPO forms have the same amino

acid composition and specific activity but they differ in the carbohydrate composition (Kenigsberg *et al.*, 1987). rCPO migrated as a single band at a position 5-10 kDa, respectively, higher than the native isozymes. As we suspected that this difference in size was due to overglycosylation of the recombinant enzyme, we treated both rCPO and nCPO with endoglycosidase H (EndoH) to remove N-linked glycans. As previously reported (Kenigsberg *et al.*, 1987), EndoH digestion of nCPO produced two species of reduced molecular weight.

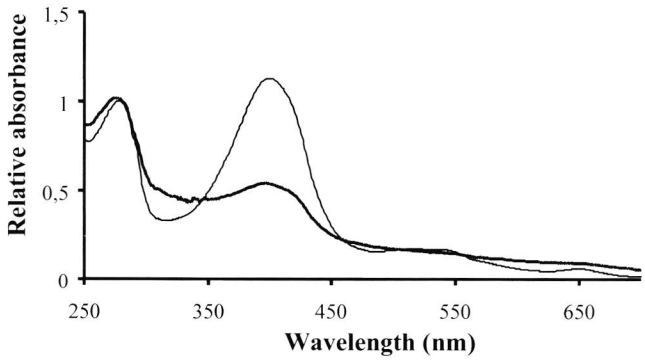


Fig. 2. UV-spectra of rCPO (thick line) and nCPO (thin line).

Both deglycosylated rCPO and nCPO shifted to a similar position on SDS-PAGE (Fig.3), indicating that the differences in size could indeed be attributed to overglycosylation of the recombinant enzyme. Furthermore, similarly to nCPO, the recombinant enzyme was not reactive to the Ellmann reagent (Ellmann, 1959), indicating a correct formation of the single disulphide bridge present in chloroperoxidase (Sundaramoorthy *et al.*, 1995).

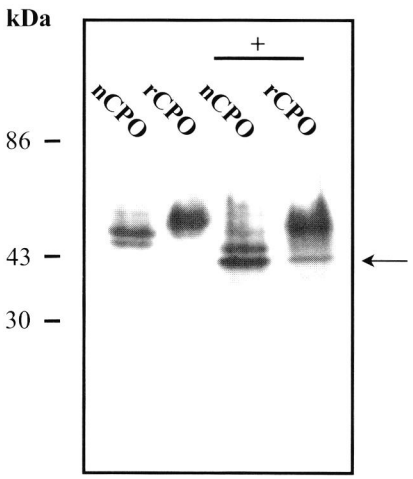


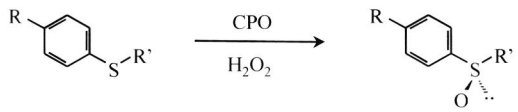
Fig. 3. Western blotting analysis of recombinant chloroperoxidase (rCPO) and native chloroperoxidase (nCPO). Proteins were detected with an antiCPO polyclonal antiserum (see M&M). Proteins were partially deglycosylated by treatment with EndoH (+), the deglycosylation protein bands are indicated by an arrow.

To analyse whether the CPO signal sequence was correctly processed in *A. niger*, the purified extracellular rCPO was submitted to sequencing of its N-terminus. However, no amino acid sequence could be recovered from this analysis, suggesting that the recombinant enzyme was blocked at its N-terminus.

Catalytic properties

To analyse whether the recombinant CPO was fully active some of its catalytic properties were measured. The specific chlorination activity (MCD assay as described by Morris and Hager, 1966) was determined. The specific chlorination activity of purified rCPO was 47 U/nmol haem. The pH optimum for the chlorination of monochlorodimedon was measured for rCPO and native CPO. rCPO and nCPO showed the same pH-profile with a pH-optimum at pH 2.75.

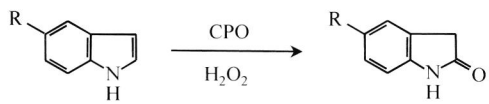
The enantioselective sulfoxidation of thioanisole and derivatives (see Scheme 1) was used to monitor the enantioselective properties of the enzyme. Although, as shown in Table 2, results obtained in 1 ml scale experiments differed slightly from the results published for 50 ml scale experiments, similar to the native CPO, recombinant CPO produced predominantly the R-sulfoxide in up to 99 % e.e. Experiments with labelled $H_2{}^{18}O_2$ showed 100 % incorporation of ^{18}O into thioanisole sulfoxide for both nCPO and rCPO (data not shown).



R-Sulfoxide

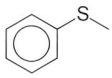
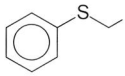
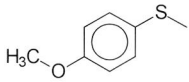
Scheme 1. Oxidation of sulphides. R=H or -OCH_3; R'=-CH_3 or -CH_2-CH_3

The regioselectivity of rCPO was studied by means of the oxidation of indole and derivatives (see Scheme 2). As shown in Table 3 the conversions obtained with rCPO was slightly lower than those obtained with native CPO. However, both rCPO and native CPO yield the corresponding 2-oxindoles in virtually quantitative yield. Experiments with labelled $H_2{}^{18}O_2$ showed 100 % incorporation of ^{18}O into 2-oxindole for both nCPO and rCPO (data not shown).



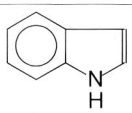
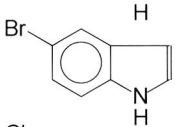
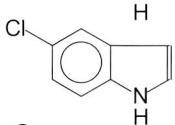
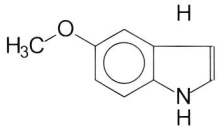
Scheme 2. Oxidation of substituted indoles. R=-Br, -Cl, or -OCH_3

Table 2. Oxidation of sulfides by native and recombinant CPO

Sulfide	rCPO Conversion (%)	rCPO e.e. (%)	Native CPO[a] Conversion (%)	Native CPO[a] e.e. (%)	Native CPO[b] Conversion (%)	Native CPO[b] e.e. (%)
	77	98	65	98	100	99
	91	99	94	98	83	99
	58	94	62	97	53	99

[a]results obtained in a 1 ml scale experiment with native CPO from Chirazyme Labs
[b]results reported by Van Deurzen *et al.* (1996) for a 50 ml scale experiment

Table 3. Oxidation of substituted indoles by native and recombinant CPO

Indole derivative	rCPO Conversion (15 min) %	rCPO Conversion (60 min) %	Native CPO[a] Conversion (15 min) %	Native CPO[a] Conversion (60 min) %	Native CPO[b] Conversion (15 min) %	Native CPO[b] Conversion (60 min) %
	24	78	24	83	25	96
	n.d.	24	n.d.	37	9	19
	14	35	16	45	19	47
	5	5	5	5	9	10

[a]results obtained in a 1 ml scale experiment with native CPO from Chirazyme Labs
[b]results reported by Van Deurzen *et al.* (1996) for a 50 ml scale experiment

DISCUSSION

Chloroperoxidase from the filamentous fungus *Caldariomyces fumago* is an enzyme of unique versatility as a catalyst for synthetically useful oxygen transfer reactions. Structurally, the enzyme shares characteristics of the P450 cytochromes and the haem peroxidases. These features make CPO a very attractive example for function-structure relationship studies of oxidative enzymes. To make this possible, an efficient recombinant expression system for the

cpo gene is required. Recently we reported the expression of two fungal haem-containing peroxidases in the filamentous fungus *Aspergillus niger* (Conesa *et al.*, 2000). Production of the recombinant proteins was achieved by placing the peroxidase coding sequences under control of efficient *Aspergillus* expression signals. Using a similar approach, the *Caldariomyces fumago cpo* gene has been efficiently expressed in *A. niger* and the recombinant enzyme was secreted into the culture medium as an active protein.

The production of rCPO could be increased by haem addition to the culture medium. Similar results have been obtained in previous studies by our and other groups on the expression of fungal peroxidases in *Aspergillus* species (Conesa *et al.*, 2000; Elrod *et al.*, 1997; Stewart *et al.*, 1996). However, our results show that despite haem supplementation, rCPO was only partially (40%) incorporated with haem. This is in contrast to our observations on the production of *Phanerochaete chrysosporium* manganese peroxidase (MnP) in *A. niger* (Conesa *et al.*, 2000), where the recombinant enzyme could be produced with the same haem content as the native protein. A possible reason for this different behaviour may be the different nature of haem attachment in the MnP (imidazole axial ligand) and CPO (thiolate axial ligand) protein.

EndoH treatment and SDS-PAGE analysis revealed a higher molecular weight of rCPO in comparison to nCPO as a result of overglycosylation of the recombinant enzyme. Overglycosylation has been reported for the expression of other heterologous proteins in *Aspergillus* spp. (Conesa *et al.*, 2000; Wyss *et al.*, 1999). In these reports, it was shown that the excess of glycosyl groups did not have a major effect on the properties of the recombinant enzymes. Our results on the characterisation of the recombinant chloroperoxidase indicate that this is also the case for rCPO. This is in agreement with the observation that CPO isozymes, differing in glycosylation pattern maintain the same specific activity (Kenigsberg *et al.*, 1987). Furthermore, Zong *et al.* (1995) in their studies on the expression of chloroperoxidase in *E. coli* showed that glycosylation is not an essential requirement for the activity of this enzyme.

The N-terminus of rCPO appeared to be blocked. This was not completely surprising, since native CPO is known to posses a N-terminal glutamic acid residue, which is mostly cyclized into a pyrrolidone carboxylic acid (Kenigsberg *et al.*, 1987). Such molecules, whose formation is induced in acidic environments, are unreactive to the Edman's reagent. As the culture medium of *A. niger* reaches a pH=2, this may explain the N-terminal blockage of rCPO in case the *A. niger* produced protein would have the native N-terminus.

To further validate the *Aspergillus niger* production system for CPO, we have assessed whether the structural and catalytic properties of rCPO were comparable with those of native CPO. Experimental data showed the correct formation of the haem thiolate ligand as well as the single disulphide bond in the recombinant CPO. The specific chlorination activity of rCPO (47 U/nmol haem) was in agreement with the activities reported by Morris and Hager (1966): 70 U/nmol; Van Deurzen *et al* (1996): 53 U/nmol; and Libby *et al.* (1996): 59 U/nmol. Also the pH-optimum for this chlorination reaction (pH 2.75 for rCPO) coincided with that of the native CPO and with the pH of the MCD assay as described by Morris and Hager (1966). From these results we conclude that the natural chlorination activity of CPO is completely present in the recombinant enzyme.

Similarly, the oxygen transfer properties of CPO were not changed upon expression of the enzyme in *Aspergillus*. Recombinant CPO showed an enantioselectivity of 99% for the sulfoxidation of thioanisole derivatives (the R-sulfoxide being predominantly formed) and a regioselectivity of 99 % for the oxidation of indole derivatives to the corresponding 2-

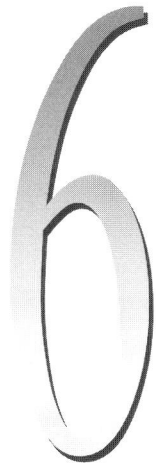
oxindoles. In aqueous buffer solutions (sulfoxidation reaction, Table 2) the yields obtained with rCPO were comparable with those obtained with native CPO. However when a mixture of *tert*-butyl alcohol and aqueous buffer (50:50 (v/v)) was used (oxidation of indoles, Table 3) rCPO resulted in a slightly lower yield than native CPO. Although the reasons for this result are not clear, it is possible that different glycosylation of rCPO has an influence on the stability of the enzyme in mixtures of *tert*-butyl alcohol and aqueous buffer.

Regio- and enantioselective oxidation reactions catalysed by CPO are known to be oxygen transfer reactions in which the oxygen atom from CPO compound I is directly transferred to the substrate molecule. For rCPO we found 100 % incorporation of ^{18}O from labelled $H_2{}^{18}O_2$ into thioanisole sulfoxide and 2-oxindole. This is in agreement with the results of labelling studies with native CPO, as reported for sulfoxidations (Doerge *et al.*, 1991) and oxidation of indole (van Deurzen *et al.*, 1996). Hence, we conclude that both the chlorination activity and the oxygen transfer properties of CPO are fully retained in the recombinant enzyme.

To our knowledge, this is the first report of the production of fully active chloroperoxidase in a heterologous expression system. We have shown that the catalytic properties of the enzyme remained basically unchanged, which makes the *A. niger* expression system a suitable system for mechanistic and mutagenesis studies of this unique enzyme.

ACKNOWLEDGEMENTS

The authors thank Dr. L. Hager for providing the *cpo* genomic clone, Prof. Dr. H. Duine and B. W. Groen for their collaboration in the purification of CPO, and G. van Duijn for assistance in obtaining the CPO antisera.



C-terminal propeptide of the *C. fumago* chloroperoxidase: an intramolecular chaperone?

Ana Conesa, Gerri Weelink, Cees A.M.J.J. van den Hondel and Peter J. Punt

ABSTRACT

The *C. fumago* chloroperoxidase (CPO) is synthesised as a 372 amino acid precursor which undergoes two proteolytic processing events: removal of a 21-aa N-terminal signal peptide and of a 52-aa C-terminal propeptide. The *A. niger* expression system developed for CPO was used to get insight in the function of this C-terminal propeptide. *A. niger* transformants expressing a CPO protein from which the C-terminal propeptide was deleted failed in producing any extracellular chloroperoxidase activity, although the CPO polypeptide was synthesised. Expression of the full-length gene in an *A. niger* strain lacking the KEX2-like protease PclA also resulted in the production of CPO cross-reactive material into the culture medium, but no chloroperoxidase activity. Based on these results, the possible function of the C-terminal propeptide in CPO maturation is discussed.

Submitted

INTRODUCTION

Chloroperoxidase (CPO) is a glycosylated haemoprotein secreted by the fungus *Caldariomyces fumago* at levels up to 500 mg/L (Hashimoto and Pickard, 1984; Pickard, 1981). *In vivo* this enzyme is involved in the synthesis of caldariomycin, a halogenated compound with antimicrobial function (Morris and Hager, 1966). *In vitro*, CPO catalyses a number of useful chemical reactions such as enantioselective oxygen-transfer reactions, H_2O_2 disproportionations and oxidative halogenations (reviewed by van de Velde 2000).

The molecular genetics of the *C. fumago* CPO has been extensively characterised by Hager and co-workers. The protein is encoded by a single-copy gene (Nuell *et al.*, 1988) and CPO synthesis is controlled at the mRNA level, fructose being an inducing and glucose a repressing carbon-source (Axley *et al.*, 1986). The gene product undergoes a number of post-translational modifications (Kenigsberg *et al.*, 1987). A 21-aa signal sequence is removed from the CPO precursor and the resulting N-terminal glutamic acid is converted into a pyroglutamic acid. Carbohydrate moieties are incorporated at N- and O-glycosylation sites. Different isozymes are found in the extracellular medium of *C. fumago* cultures (Pickard and Hashimoto, 1982; Sae and Cunningham, 1979), which are indicated to be glycosylating variants of the same polypeptide (Hashimoto and Pickard, 1984; Kenigsberg *et al.*, 1987). In the major CPO isozyme, the most heavily glycosylated, three N- and eleven O-glycosylation sites are occupied with a total of 21 sugar groups (Sundaramoorthy *et al.*, 1995). Other modifications include deamidation of three amidic residues to the corresponding acids (Kenigsberg *et al.*, 1987), one disulfide bond formation and the incorporation of a protoporphyrin XI (haem b) molecule (Sundaramoorthy *et al.*, 1995). Resolution of the crystal structure of the mature protein has shown that the haem pocket in CPO shares features of both classical peroxidases and cytochrome P450s. At the proximal site of the haem pocket, the haem is coordinated via a thiolate ligand to a cysteine, resembling the cytochrome P450, whereas at the distal site polar residues are found similar to other haem peroxidases (Sundaramoorthy *et al.*, 1995). The crystal structure also revealed that the last 52-aa of the primary translation product are lacking in the mature protein, suggesting that CPO also undergoes proteolytic processing at the C-terminus. This processing apparently occurs downstream of a dibasic (KR) processing site.

C-terminal propeptides are found in intracellular and secreted proteins and, although in many cases no function has been ascribed to these sequences, they have been suggested to participate in processes such as protein folding, targeting and secretion (for examples see Bulleid *et al.*, 1997; Feller *et al.*, 1998; Hiraiwa *et al.*, 1999; Kim *et al.*, 1997; Klionsky and Emr, 1989; Ohnishi and Horinouchi, 1996; Villanueva *et al.*, 1998). The function of the C-terminal propeptide of CPO is completely unknown. Here, we describe approaches to get more insight in the function of the CPO C-terminal propeptide using the *A. niger* expression system recently developed for CPO (Chapter 5).

MATERIAL AND METHODS

Expression cassettes

Figure 1 shows the expression cassettes used in this study. Construction of pCPO3.I-AmdS is described elsewhere (chapter 5). Expression vector pCPOΔ-AmdS which contains the *cpo* translation product lacking most of the C-terminal propeptide, was constructed by

amplifying the corresponding sequence in the *cpo* gene present in pCf6 (kindly provided by Dr. Hager, University of Illinois at Urbana-Champaign) with primers CLP15E/A (5'-GGAATTCACATGTTCTCCAAGGTCC-3') and CLP3B/H (5'-CGGGATCCAAGCTTCC CATGGAGGTGGTGG-3'). The amplification product was digested with *Afl*III/*Hind*III and cloned into the pAN52-10Not *Aspergillus* expression vector (carrying the *A. niger glaA* promoter; Punt, unpublished) at the *Nco*I/*Hind*III cloning sites. After checking the amplified fragment by sequencing, the *A. nidulans Amd*S selection marker (Kelly and Hynes, 1985) was introduced as a 4.5 kb *Not*I fragment.

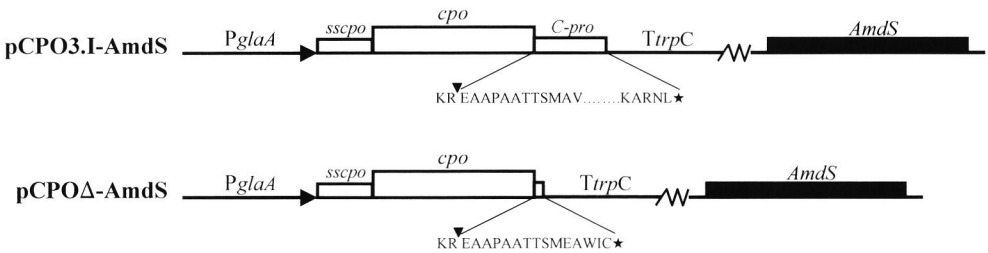


Fig. 1. Expression cassettes. P*glaA*: *A. niger* glucoamylase promoter; T*trpC*: *A. nidulans* tryptophan terminator, *cpo*: chloroperoxidase, *sscpo*: signal sequence chloroperoxidase (21 amino acids); *C-pro*: C-terminal propeptide (52 amino acids); KR: splicing site for the C-terminal propeptide; *AmdS*: *A. nidulans* acetamidase selection marker.★: Stop codon.

Fungal strains and transformation procedures

A. niger MGG029 (*prtT*, *gla::fleo*^{r}, *pyrG*; chapter 3) and *A. niger* AB1.13-ΔpclA (*pclA::pyrG*^{mut*}, *prtT, pyrG;* Punt *et al.*, in preparation) were used a recipient strains in transformation experiments. *AmdS*/*pyrG* based fungal transformations were carried out as described in chapter 5.

Plate assays

Transformants were analysed for chloroperoxidase production by means of the *o*-anisidine plate assay (chapter 5) and *in situ* immunodetection using sandwich cultures as described by Moukha *et al.* (1993): strains were grown between two polycarbonate filters on plates containing *Aspergillus* minimal medium (Bennett and Lasure, 1991). After 24 hours incubation at 30°C, a PVDF membrane, previously activated with methanol and rinsed with sterile distilled water, was placed between the agar surface and the polycarbonate sandwich and the plates were incubated for another 24 hours. The PVDF membrane, harbouring secretion proteins, was then removed, rinsed with water and subjected to immunodetection.

Shake-flask experiments

Strains were grown from conidial inocula in 300 ml shake-flasks containing 50 ml AMM-Maltose (chapter 3). Medium samples were taken at 24, 48 and 72 hours after inoculation and analysed for CPO activity using the standard monochlorodimedone halogenating reaction (Morris and Hager, 1966) and for CPO protein production by Western blotting (Sambrook *et al.*, 1989) using a CPO polyclonal antiserum (chapter 5).

RESULTS AND DISCUSSION

For our initial work on the production of the *C. fumago* chloroperoxidase in *A. niger* we constructed the expression vector pCPOΔ-AmdS based in the sequence information present in the GenBank (Accession number X04486). Later sequencing data revealed a mistake in the published *cpo* sequence (an extra C at position 1030, close to the putative stop codon). Removal of the extra nucleotide caused a shift in the reading frame, resulting in a translational product 52 amino acids longer than previously deduced (Accession number AJ300448). Comparison of the newly deduced sequence with that available from the determination of the CPO crystal structure (Sundaramoorthy *et al.*, 1995) showed that the last 52 amino acids were not present in the mature protein, indicating that these form a C-terminal propeptide which is removed somewhere during the route of the protein in the secretion pathway. As pCPOΔ-AmdS lacks most of the C-terminal propeptide (41 aa), we used this construct to study the function of this prosequence using the *A. niger* expression system developed for the full-length protein (chapter 5). Further analysis of the C-terminal propeptide sequence showed the presence of two basic amino acid residues (KR) at the place of splicing. As this motif resembles the recognition sequence for cleavage by Golgi-located protein convertases such as the KEX2 protease (Fuller *et al.*, 1989), we also studied the production of CPO in an *A. niger* strain in which the gene encoding this type of protease, termed PclA, was deleted. This strain, *A. niger* AB1-13-ΔpclA, although it shows a compact growth phenotype, retains the wild-type secretion capacity (Punt *et al.*, in preparation) and was shown to secrete active manganese peroxidase (MnP) when transformed with the MnP expression vector pMnP1.I (chapter 3 and results not shown).

A. niger MGG029, the production strain used for expression of the full-length CPO (chapter 5), was transformed with pCPOΔ-AmdS, encoding the C-terminal deletion form of CPO, and *A. niger* AB1.13-ΔpclA was transformed with the full-length CPO construct pCPO3.I-AmdS. Transformants were selected by their ability to grow and sporulate on acetamide containing agar plates and were screened for secretion of active CPO using the *o*-anisidine plate assay. *A. niger* MGG029 transformants expressing the full-length CPO construct develop a purple halo in this assay (chapter 5; Fig.2.a). No colour formation was visible for any of the analysed MGG029[pCPOΔ-AmdS] (Fig.2.a) and AB1.13-ΔpclA[pCPO3.I-AmdS] transformants (Fig.2.b), indicating the absence of extracellular peroxidase activity in these strains. To study whether these transformants secreted any CPO protein at all, we used the sandwich mode of culture as described by Moukha, *et al.* (1993) to monitor protein secretion *in situ*. This method has shown to be useful in detecting secretion of heterologous proteins that are rapidly degraded in the extracellular medium (Asgeirsdottir *et al.*, 1999). Using this technique, αCPO cross-reactive material could be detected at the place of colony growth of MGG029[pCPO3.I-AmdS], and most of the MGG029[pCPOΔ-AmdS] and AB1.13-ΔpclA[pCPO3.I-AmdS] transformants (Fig.2.b and c). No cross-reactivity was observed for the parental strains MGG029 and AB1.13-ΔpclA. From this result we concluded that the CPO was synthesised and secreted in the three types of transformants and we selected strains MGG029[pCPO-AmdS]#3 and AB1.13-ΔpclA[pCPO3.I-AmdS]#A3 together with the previously characterised MGG029[pCPO3.I-AmdS]#5 (chapter 5) to further analyse CPO production in liquid medium.

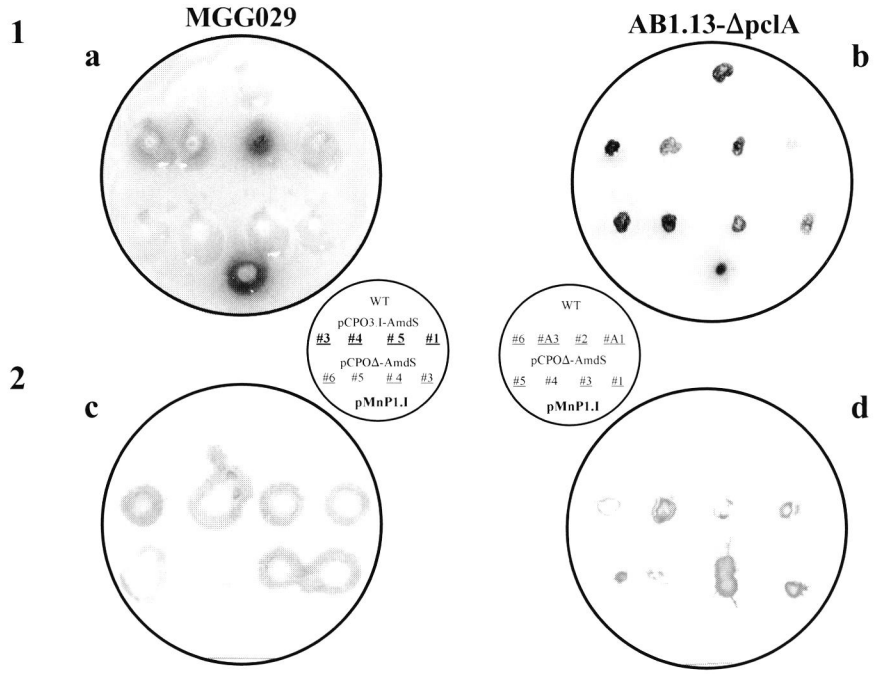


Fig. 2. Plate assays for analysis of CPO production. **1**: *o*-anisidine plate assay. Production of active peroxidase is detected by purple halo formation around the colony. Positive strains are indicated in template dishes in **bold**. **2**: *In situ* immunodetection of CPO secretion. Production of extracellular CPO is detected by reaction with a αCPO antibody at the site of colony growth. Positive strains are indicated in template dishes by underlining. **a** and **c**: *A. niger* MGG029 transformants containing either pCPO3.I-AmdS, pCPOΔ-AmdS, pMnP1.I (chapter 3) or only the selection markers (WT). Strain position is indicated in the adjacent template dish. **b** and **d**: *A. niger* AB1.13-ΔpclA transformants containing either pCPO3.I-AmdS, pCPOΔ-AmdS, pMnP1.I (chapter 3) or only the selection markers (WT). Strain position is indicated in adjacent template dish.

Under inducing conditions, strain MGG029[pCPO3.I-AmdS]#5 secretes active recombinant CPO (rCPO) into the extracellular medium (chapter 5). Accordingly, when grown in shake-flasks containing maltose as carbon source, chloroperoxidase activity (MCD halogenation; Morris and Hager, 1966) was observed in the culture broth of strain MGG029[pCPO3.I-AmdS]#5 and rCPO was readily detected by Western blotting as a strong band, migrating slightly slower that the CPO (nCPO; chapter 5) (Fig.3, lanes 1 and 6). Confirming the results of the plate assays, no MDC halogenating activity was present in the extracellular medium of strains MGG029[pCPOΔ-AmdS]#3 and AB1.13-ΔpclA[pCPO3.I-AmdS]#A3. However, both strains showed multiple αCPO cross-reactive bands when analysed by SDS-PAGE (Fig.3, lanes 2 and 4). In particular, in strain AB1.13-ΔpclA[pCPO3.I-AmdS]#A3 the CPO cross-reactive material appeared as an intense smear (Fig.3. lane 4). As the CPO protein synthesised in MGG029[pCPOΔ-AmdS]#3 lacks most of the C-terminal propeptide, we concluded that this sequence is required to ensure the production of active rCPO in *A. niger*.

Also a non-active CPO polypeptide is secreted in transformant AB1.13-ΔpclA[pCPO3.I-AmdS]#A3. Strain AB1.13-ΔpclA lacks the KEX2-like protease PclA, which in *A. niger* is known to be responsible for cleavage of the glucoamylase propeptide (Punt *et al.*, in preparation). Expression of the full-length CPO construct in this strain resulted in secretion of an inactive CPO, suggesting that the PclA protease is also involved in the maturation of the CPO polypeptide. As a dibasic site (KR), similar to the PclA recognition motif is present at the site of processing of the C-terminal propeptide, we postulate that PclA is directly involved in the cleavage of this sequence. A possible explanation for the results obtained in these experiments is that the C-terminal propeptide in CPO is required to ensure correct protein maturation, whereas its removal at a later stage during secretion is required to yield an active CPO protein. The C-terminal sequence may thus be considered to have a chaperone-like function for the chloroperoxidase protein. The SDS-PAGE migrating behaviour of CPO in MGG029[pCPOΔ-AmdS]#3 and AB1.13-ΔpclA[pCPO3.I-AmdS]#A3 as multiple or diffuse bands is not exactly understood. These multiple bands could originate from aggregation, heterogeneous glycosylation and/or incorrect processing of the incorrectly maturated CPO polypeptide. It should be noted that addition of hemin, which increases the yield of CPO in wild type strains producing full-length CPO (chapter 5), did not result in secretion of an active CPO in either MGG029[pCPOΔ-AmdS]#3 or AB1.13-ΔpclA[pCPO3.I-AmdS]#A3.

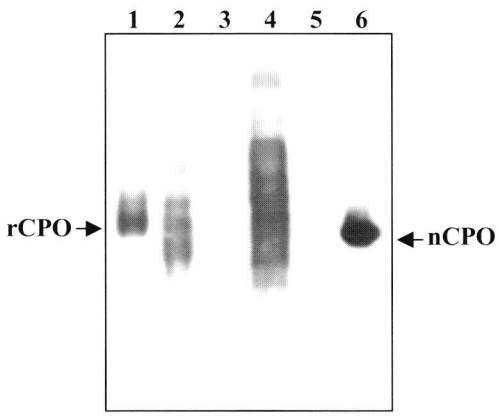


Fig. 3. Western blot analysis of CPO production. Medium samples of a AMM-maltose culture 48 hours after inoculation. Lane 1: MGG029[pCPO3.I-AmdS]#5; lane 2: MGG029[pCPOΔ-AmdS]#3; lane 3: MGG029 transformed with selection markers *pyrG* and *AmdS*; lane 4: AB1.13-ΔpclA[pCPO3.I-AmdS]#A3; lane 5: AB1.13-ΔpclA transformed with selection markers *pyrG* and *AmdS*; lane 6: *C. fumago* chloroperoxidase. Migrating positions of recombinant chloroperoxidase (rCPO) and native chloroperoxidase (nCPO) are indicated.

C-terminal propeptides have been identified for a number of secretory proteins. For example, bacterial proteases such as the *Serratia marcescens* Serine Protease (SSP) and the *Thermus thermophilus* aqualysin I are synthesised as large precursors with removable sequences at both N- and C-termini. The C-terminal pro-region in the *S.marcescens* SSP has shown to have different functional domains with functions in protein folding and secretion (Ohnishi and Horinouchi, 1996), whereas the aqualysin I C-terminal propeptide may have a role in stabilising an unfolded conformation to facilitate translocation across the cell membrane (Kim *et al.*, 1997). These C-terminal sequences, however, are much longer than that of the *C. fumago* chloroperoxidase and no structural relation is evident from sequence comparison. Interestingly, C-terminal propeptides have been reported for the horseradish peroxidase (Fujiyama *et al.*, 1988; Welinder, 1976) and several fungal laccases (Berka *et al.*, 1997 and references therein; Yaver *et al.*, 1999) and also the existence of a C-terminal

propeptide in the *Curvularia inaequalis* vanadium chloroperoxidase has been postulated (Barnett *et al.*, 1997). The biological function of these sequences has not been investigated at an experimental level, although for the vanadium chloroperoxidase propeptide a role in secretion has been suggested by Barnett *et al.* (1997). Also in these cases, the CPO C-terminal propeptide shows no sequence homology with any of these peptides. Our results however, indicate a role of this C-terminal sequence in providing CPO with its active conformation. To establish the mechanism in which this would operate and whether there is any interaction with the incorporation of the haem prosthetic group needs further research.

7

Analysis of haem limitation in the production of haem peroxidases by *Aspergillus niger*

Ana Conesa, Jeffrey Bartels, Cees A.M.J.J. van den Hondel and Peter J. Punt

ABSTRACT

In this work, we present a detailed analysis of the process of haem limitation as bottleneck for the production of haem-containing fungal peroxidases in filamentous fungi. Supplementation of the culture medium of an *Aspergillus niger* manganese peroxidase (rMnP) producing strain either with hemin or haemoglobin resulted in a seven and ten fold, respectively, increase in the extracellular rMnP activity. The increase in activity was due to a specific increase in rMnP production. Interestingly, rMnP competed for the available cellular haem with two differently located intracellular haem-containing proteins, nitrate reductase and eburicol 14α-demethylase. Our results indicate the overproduction of haem proteins in *Aspergillus niger* is limited by the cellular capacity for haem biosynthesis.

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INTRODUCTION

Fungal peroxidases are extracellular enzymes that utilise hydrogen peroxide to catalyse the oxidation of a wide variety of organic and inorganic substrates (Everse and Everse, 1991). One type of fungal peroxidases is the one secreted by white-rot basidiomycetes in response to nutrient depletion, which are involved in lignin degradation (Hatakka, 1994). These are haem containing proteins and catalyse the oxidation of organic compounds by two sequential one-electron transfer reactions (reviewed by van Deurzen *et al.*, 1997). White-rot fungi peroxidases are structurally related to other bacterial, organelle and secretory plant peroxidases (Welinder *et al.*, 1992). The iron in the haem prosthetic group is coordinated to the nitrogen in the imidazole of the so-called proximal histidine, while another, distal, histidine transfers oxidising equivalents from the H_2O_2 to the haem.

Lignolytic peroxidases are interesting enzymes for industrial applications. White-rot fungi peroxidases have potentials in chemical processes involving bleaching and bioremediation (Karam and Nicell, 1997; Paice *et al.*, 1995). For the actual development of these applications an inexpensive, large–scale production system for these proteins is required. Several groups have studied the overproduction of these enzymes in filamentous fungi (Aifa *et al.*, 1999; Elrod *et al.*, 1997; Saloheimo and Niku-Paavola, 1991; Stewart *et al.*, 1996). Filamentous fungi have a large protein secretion capacity, and fermentation technology is developed and has successfully been used for the production of a great variety of proteins (reviewed by Gouka *et al.*, 1997 and Radzio and Kurk, 1997). However, in most cases, expression of fungal peroxidases in filamentous fungi has resulted in low production yields. In the previous chapters, we have reported the successful production of fungal peroxidases in *Aspergillus niger* (chapters 3 and 5). We have already shown that factors such as adequate production strain and culturing conditions are important production determinants. However, other, more specific requirements for this type of proteins may also control production yields. In general, bottlenecks for heterologous protein production are believed to operate mainly at the post-translational level and especially for haem-containing peroxidases, the amount of available haem has shown to have a major effect on production yields (Weber *et al.*, 1992; Andersen *et al.*, 1992; Fowler *et al.*, 1993; chapter 5). However, the role and characteristics of limited haem availability in peroxidase overproduction has not been studied in detail.

In this work we have set out experiments to get more insights in the process of haem limitation in the production of haem peroxidases in filamentous fungi. For this, the production of manganese peroxidase in *Aspergillus niger* has been taken as a model.

MATERIAL AND METHODS

Strains

A. niger MGG029, a Δ*glaA,* protease deficient strain, and two derivative strains, MGG029[pMnp1.I]#25, where the *P. chrysosporium mnp1* gene is expressed under the control of the *A. niger* glucoamylase (*glaA*) promoter, and strain MGG029[pgpdMnp1.I]#13, where the *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter drives the expression of the *mnp1* gene (chapter 3) were used in this study.

Chemicals
Hemin and haemoglobin were purchased from Sigma. Apoheamoglobin was prepared according to Nakahara *et al.*, (1996). The 14α-demethylase inhibitor etaconazole was a generous gift of Dr. M. de Waard (Wageningen University, The Netherlands).

Protein methods
MnP activity was measured by monitoring the oxidation of diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) in the presence of 20 μM Na-oxalate at 415 nm (Glenn and Gold, 1985). Western blot analysis was carried out as described (Sambrook *et al.*, 1989) using a αMnP polyclonal antibody (kindly provided by Dr. D. Cullen, Madison) and a αGLA polyclonal antibody (Punt *et al.*, 1991), in combination with 1% glucoamylase and 1%BSA, respectively, as blocking agents. Quantification of Western analysis band intensities was performed with the GeneTools software (Syngene).

Culturing conditions
For analysis of haem limitation in liquid medium, strains were grown from conidial inocula in 300 ml shake flasks containing 50 ml *Aspergillus* minimal medium (AMM; Bennett and Lasure, 1991) having 0.5% cas amino-acids and 5% maltodextrin, supplemented with either 500 mg/L hemin, 5 g/L haemoglobin, 5 g/L apohaemoglobin or 0.02 g/L $FeSO_4.7H_2O$. Cultures were incubated at 30°C and 300 rpm, medium samples were taken at different time points after inoculation and dialysed overnight against 50 mM sodium succinate buffer pH=4.5.

Competition plate assays
Analysis of the intracellular competition for haem availability was done by monitoring differences in fungal radial growth on Petri dishes with defined media. To study competition between nitrate reductase and manganese peroxidase, plates contained *Aspergillus* minimal growth medium supplemented with sodium chlorate at different concentrations and containing either 10 mM sodium glutamate or 10 mM sodium nitrate as sole nitrogen source. For competition studies between eburicol 14α-demethylase and manganese peroxidase, Petri dishes contained MM supplemented with the fungicide etaconazole at different concentrations. Plates were inoculated with fresh spore suspensions, incubated at 30°C for 3-5 days and fungal radial growth was monitored.

RESULTS

Haem supplementation increases MnP production in *A. niger* MGG029
The role of limited haem availability in the production of rMnP by *Aspergillus* was studied in shake-flasks cultures. Two *A. niger* MGG029 pMnp1.I containing transformants, and the wild type control MGG029[pAB4-1] (wt) were cultured in AMM-maltodextrin supplemented with either hemin, haemoglobin, apohaemoglobin or a 4-fold $FeSO_4$ excess in comparison to AMM. Medium samples were taken at 12 hour intervals up to 72 hours, dialysed and analysed for ABTS oxidising activity. Although absolute activity values varied, both pMnP.1 strains showed similar behaviour. Maximum activity was reached after 36 hours. At this point, hemin and haemoglobin supplemented media showed respectively a seven and ten fold increase in activity when compared to the non-supplemented medium. Medium supplemented

with apohaemoglobin showed a three-fold increase in activity, whereas $FeSO_4$ supplementation had no significant effect (Table 1). Transformants containing only the pAB4-1 vector showed no activity, regardless of the culture medium (data not shown).

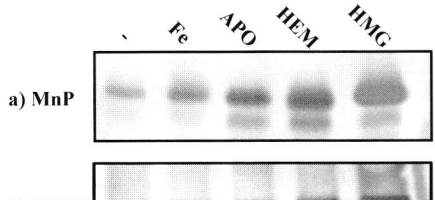
Table 1. Extracellular rMnP production of strain MGG029[pMnp1.I]#25 after 36 hours growth in differently supplemented AMM-maltodextrin media

Medium	Activity[a] (Abs/min/mL)	Extracellular rMnP (mg/L)
AMM-Maltodextrin	6.0±0.8[b]	9.6±1.2
AMM-Maltodextrin + 0.02 g/L $FeSO_4$	6.7±0.9	10.7±1.4
AMM-Maltodextrin + 500 mg/L hemin	41.8±0.8	66.9±1.2
AMM-Maltodextrin + 5g/L apohaemoglobin	19.7±0.6	31.5±0.9
AMM-Maltodextrin + 5g/L haemoglobin	66.2±12.1	105.9±19.4

[a] Activity was measured by ABTS oxidation and protein values were calculated based on rMnP specific activity (chapter 3)

[b]The values presented are the average of at least two measurements in two different experiments

Western blot analysis revealed that the observed differences in activity corresponded to different amounts of rMnP in the culture medium (Fig. 1 a). To verify that these differences in rMnP production were not merely the consequence of a general effect on protein productivity, the secretion of a non haem-related protein was analysed in parallel by Western blotting. For this purpose we used a 70 kDa extracellular amylase, which can be detected with a polyclonal αGLA antibody (Punt, unpublished; Fig. 1b). Protein bands were quantified and signal intensities were related to the non-supplemented medium (Fig.1c). This experiment showed that extracellular amylase levels were similar in all five media (protein signal variations less than 20% related to the non-supplemented medium) whereas MnP signals varied according to the measured activities. To verify that the rMnP produced in the different media had similar specific activity, it was shown that when equal amounts of ABTS oxidising activity (in U/ml) of the five different medium samples were analysed by Western blotting, similar amounts of rMnP were observed (data not shown).



c)	-	Fe	APO	HEM	HMG
R_{Mnp}	1	1.3	3.0	7.2	9.5
R_{AMY}	1	0.9	1.0	1.2	1.2

Fig. 1. Western blotting analysis of the extracellular protein production of strain MGG029[pMnp1.I]#25 cultured in differently supplemented AMM-maltodextrin media. - : no supplementation; Fe: FeSO4; APO: apohaemoglobin; HEM: hemin; HMG; haemoglobin. a) Production of manganese peroxidase (MnP): blot was probed with a αMnP polyclonal antibody. b) Production of amylase (AMY): blot was probed with a cross-reacting αGLA polyclonal antibody. c) Relative band signal intensities related to the non-supplemented medium.

Haem competes with nitrate reductase for the available cytosolic haem

The results of the supplementation experiments suggested that the amount of available haem was a limiting factor for the overproduction of MnP. To get more insight on haem limitation in the fungal cell, we analysed the competition for haem between MnP and other haem-containing proteins present in different cellular compartments. For this, we used nitrate reductase, which would give an indication of the competition for the cytosolic pools of haem, and eburicol 14α-demethylase, an ER located cytochrome P450 enzyme.

Nitrate reductase is the first component of the assimilatory nitrate reduction pathway, which is the conversion of nitrate to nitrite. This haem-containing enzyme is also able to reduce chlorate to chlorite, which is toxic to the fungus. Hence, when nitrate reductase activity is present, chlorate inhibits fungal growth. In a situation where another haem-requiring protein is overproduced resulting in an extra demand of haem, the amount of available haem may become limited. This could result in a reduced activity of nitrate reductase and, concomitantly, a reduced sensitivity for chlorate toxicity. A MnP overproducing strain would then be more resistant to growth on chlorate containing media than the wild type strain, but equally resistant if haem sufficiency is restored by, e.g., haem supplementation. To test this hypothesis, two *A. niger* rMnP producing strains MGG029[pMnp1.I]#25 and MGG029[pgpdMnp1.I]#13, an the wild type strain transformed only with the selection marker, MGG029[pAB4-1], were grown on chlorate-containing AMM plates, with or without extra haem. As nitrate is the regular nitrogen component of the AMM, and this could interfere in our determinations, glutamate instead of nitrate was used as a nitrogen source in the chlorate plates.

Compared to the chlorate-free control, reduction of fungal radial growth on chlorate-containing plates was observed in all three strains and correlated with the concentration of sodium chlorate (Fig.2a). As shown in Fig 2a, chlorate toxicity was less pronounced for the rMnP-producing strains than for the wild type strain. This was most noticeable at a concentration of 2.5 mM sodium chlorate and was more marked for strain MGG029[pgpdMnp1.I]#13 than for MGG029[pMnp1.I]#25. These results agree with our hypothesis on haem limitation and with the higher MnP production capacity observed for strain MGG029[pgpdMnp1.I]#13 in fermentation experiments (chapter 3). When external haem was added in the chlorate-containing plates (Fig.2b), the differences in growth among the three strains were annihilated, further indicating that the observed effect was indeed related to haem availability. To confirm that these growth differences were due to the activity of nitrate reductase, we analysed the effect of employing nitrate as nitrogen-source in the chlorate plates. As nitrate reductase has a higher affinity for nitrate than chlorate, the presence of nitrate in chlorate plates reduces the susceptibility of the fungus for chlorate and thus any effect of MnP overproduction would only become visible at a higher chlorate concentration. Accordingly, when nitrate was used, differences in growth between the MnP producing strains and the wild type strain became visible at a chlorate concentration of 5–10mM (Fig.2c) in contrast to a chlorate concentration of 2.5 mM when nitrate was absent (Fig.2a). Taken together these results indicate that MnP and nitrate reductase compete for the available cellular haem.

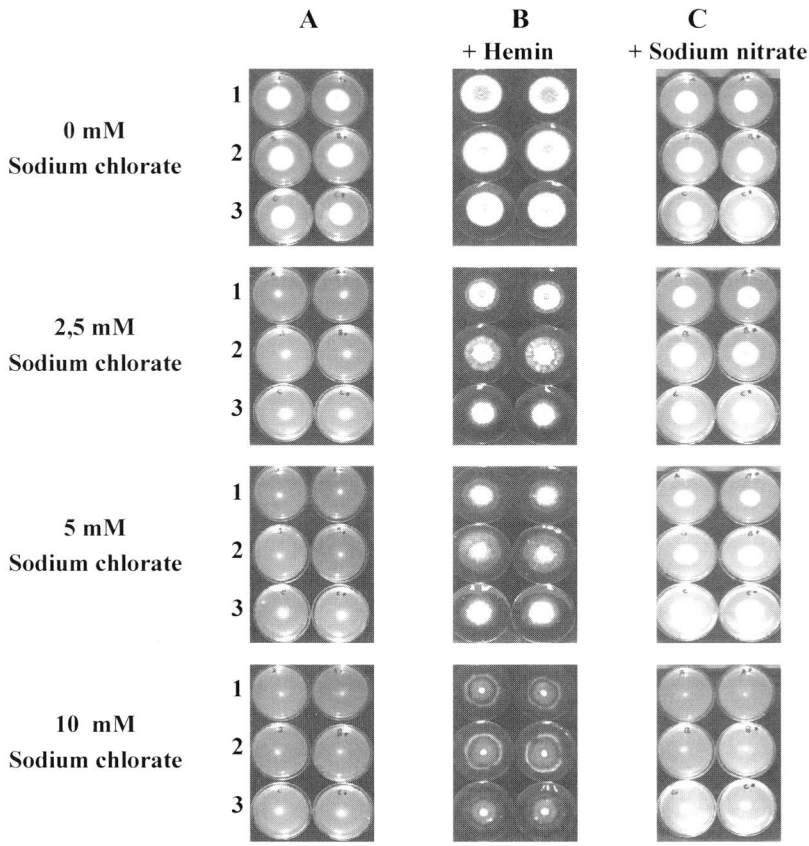


Fig. 2. Effect of MnP overproduction on the susceptibility of *A. niger* for chlorate. 1: MGG029[pAB4-1]; 2: MGG029[pMnp1.I]#25; 3: MGG029[pgpdMnp1.I]#13. Strains were inoculated, in duplo, on plates containing AMM with increasing concentrations of sodium chlorate. A: Nitrogen source is sodium glutamate. B: Nitrogen source is sodium glutamate and plates are supplemented with hemin at a final concentration of 500 µg/mL. C: Nitrogen source is sodium nitrate. Plates were incubated at 30°C for three (A) or five (B,C) days.

MnP competes for haem with the ER located eburicol 14 α-demethylase

As a secretory protein, MnP is expected to enter the secretory pathway at the endoplasmic reticulum (ER). We studied haem limitation in this cellular compartment by analysing the competition for haem between MnP and eburicol 14α-demethylase, a haem-containing, ER-resident P_{450} cytochrome. This protein is involved in ergosterol biosynthesis and target for 14α-demethylase inhibitors (DMIs), which are used in agriculture as fungicides (van den Bossche, 1988). Previously, van den Brink *et al.* (1996) have shown that eburicol 14α-demethylase activity can be indirectly measured by determining fungal resistance to DMIs in a plate assay. Inhibition of fungal growth on plates containing DMIs such as etaconazole is negatively correlated to the eburicol 14α-demethylase activity. Hence, following a similar reasoning as for nitrate reductase, if MnP overproduction results in a limitation of the available haem in the ER, MnP overproducing strains would display a higher susceptibility to DMIs than the wild type strain, and therefore show a stronger reduction in radial growth on

etaconazole-containing plates. As can be seen in Fig.3A, upon etaconazole addition reduction fungal growth was observed for all three strains and correlated with the amount of DMI added. MnP producing strains showed a stronger reduction in growth which became more clear as the fungicide concentration increased. This was especially noticeable for the higher MnP producing strain MGG029[pgpdMnp1.I]#13. As for in the chlorate assay, addition of haem annihilated these differences (Fig.3B). Again, these results support the hypothesis of competition for haem in the ER between MnP and eburicol 14α-demethylase.

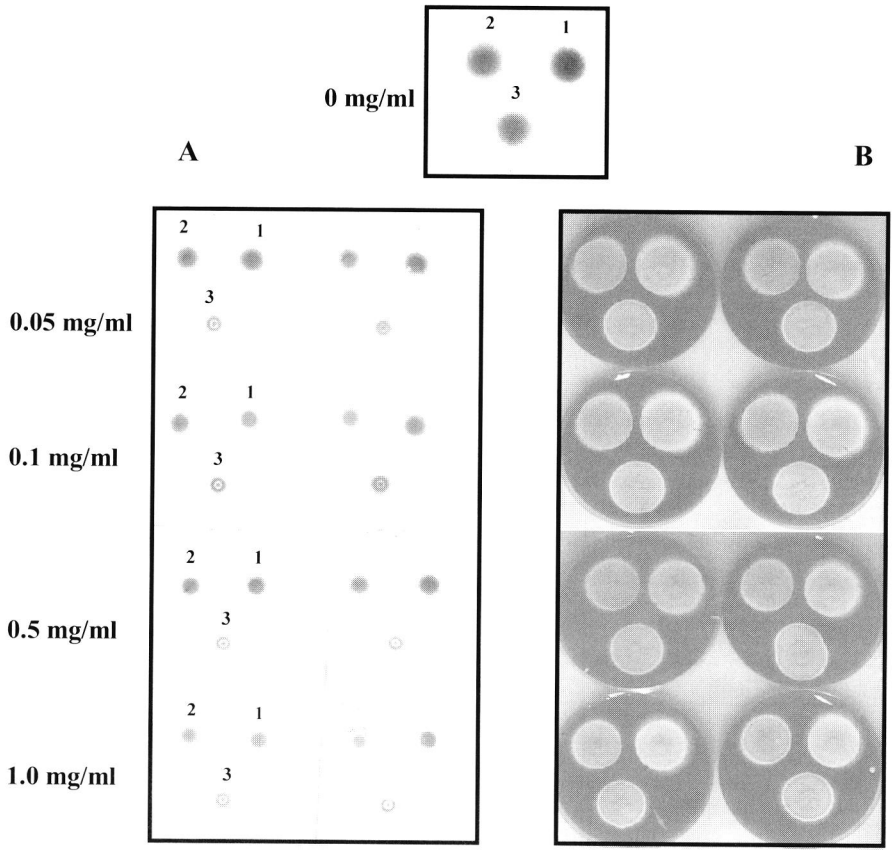


Fig. 3. Effect of MnP overproduction on the susceptibility of *A. niger* for etaconazole. 1: MGG029[pAB4-1], 2: MGG029[pMnp1.I]#25; 3: MGG029[pgpdMnp1.I]#13. Strains were inoculated, in duplo, on plates containing AMM with increasing concentrations of etaconazole. A: Plates without hemin supplementation. B: Plates are supplemented with hemin to a final concentration of 500 μg/mL. Plates were incubated at 30°C for three (A) or five (B) days.

DISCUSSION

In chapters 3 and 5, we have reported the production of active fungal peroxidases in *Aspergillus*. However, the production yields achieved were low in comparison to those obtained for other fungal proteins and lower than expected in view of the amount of transcript produced. Reduced haem availability has been suggested to be a limiting factor for the production of haem proteins in different expression systems (Andersen *et al.*, 1992; Fowler *et al.*, 1993; Weber *et al.*, 1992). However, a detailed analysis of this limitation is still missing. To get more insight in the role of haem in the overproduction of haem proteins in filamentous fungi we have analysed haem protein production in *A. niger* strains expressing the *Phanerochaete chrysosporium* manganese peroxidase (MnP).

In shake-flask cultures, haem supplementation in the form of haemoglobin or hemin resulted in a significant increase of extracellular rMnP activity. Similarly, a ~ 3 fold increase in the production of C. *cinereus* peroxidase by *A. oryzae* was observed when a haem source was provided in the growth medium (Andersen *et al.*, 1992). In our study we have shown that the increase in peroxidase activity was due to a concomitant increase in protein production (Fig.1), and not to an increase in the specific activity of rMnP. As only the holoprotein accumulates in the extracellular medium, this indicates that apoforms of rMnP may be unstable during or after secretion. Instability of the apoforms has also been observed for other haem-containing enzymes (Dumont *et al.*, 1990; Yu *et al.*, 1997). The increase in rMnP production was not due to the additional Fe^{2+} supplied by hemin and haemoglobin, since Fe^{2+} supplementation alone had no effect on the peroxidase activity. We observed that haemoglobin supplementation resulted in a higher rMnP production than the addition of hemin. The reason for this could be that haemoglobin may play a role not only as haem source but also in providing an excess of protein in the culture medium. This protein excess, as is also suggested by the fact that apohaemoglobin or BSA addition has a positive effect on rMnP yields (Figure 1 and data not shown), may protect rMnP from proteolytic degradation. Similar to MnP, hemin supplementation resulted in a significant increase in the production of the *Caldariomyces fumago* chloroperoxidase in *A. niger* (chapter 5). Consequently, our results in the haem supplementation experiments support the hypothesis of limited haem availability as a bottleneck for the production of haem proteins in filamentous fungi.

Little is known about haem biogenesis in filamentous fungi, although it can be expected that similar pathways as in yeast and in higher eukaryots are followed. Haem is synthesised in the mitochondria (for reviews see Dailey, 1997 and Padmanaban *et al.*, 1989) and distributed to haemoproteins located at different cellular compartments, such as mitochondria, microsomes, peroxisomes and the cytosol. The available data suggest that, in the case of proteins with ER targeting, haem incorporation occurs within the ER, and therefore, translocation of haem to the ER lumen is also required (Pinnix *et al.*, 1994). The mechanism of intracellular transport of haem is largely unknown. There is evidence supporting the model of cytoplasmic haem carriers and also direct transfer from the mitochondria to the ER has been proposed (Asagami *et al.*, 1994). Related to the biogenesis of haem proteins, haem limitation can be conceived in terms of insufficient haem biosynthesis, shortcoming of the haem at the required cellular location or restrictions at the stage of haem incorporation. The results of competition experiments presented in this study showed that MnP overproduction, which would increase the requirements of haem at the ER, resulted in a reduction in the activities of two differently located haem proteins, the cytosolic nitrate reductase, and the ER-located eburicol 14α-demethylase. Similarly, Weber *et al.*

observed competition for the cellular haem between lanosterol 14α-demethylase and cytochrome *c* in lanosterol 14α-demethylase overproducing yeast strains (Weber *et al.*, 1992). These results suggest that haem proteins compete for the general cellular haem pool and that haem limitation occurs at the level of biosynthesis. Furthermore, as addition of extracellular haem increased the activity of the various proteins considered in our study, it is suggested that haem transport and incorporation play a lesser role in the observed limitation. However, restrictions at these levels cannot be completely ruled out. Studies on the regulation of haem biosynthesis indicates that haem is very tightly regulated by glucose, oxygen and haem itself (Labbe-Bois and Labbe, 1990). In bacteria and mammals, the first enzyme of the haem biosynthetic pathway, aminolevulinate synthase (ALAS) is one of the main regulatory enzymes in haem biosynthesis (Padmanaban *et al.*, 1989). In contrast, in yeast, ALAS is expressed constitutively and not rate-limiting, and regulation occurs at the second step, controlled by aminolevulinate dehydratase (Labbe-Bois and Labbe, 1990). Lack of transcriptional regulation of the *hemA* (ALAS) gene to oxygen, carbon source and heat shock was also observed in *A. nidulans* (Bradshaw *et al.*, 1993). Interestingly, Elrod *et al.*, (1997) showed that *hemA* but not *hemB* (encoding the ALA dehydratase) overexpression in *A. oryzae* led to an increased yield of a secreted haem-containing fungal peroxidase, and that co-expression of the two genes had a synergistic effect. In the *hemA*/*hemB* overproducing strains uroporphyrin, a downstream intermediate of the haem biosynthetic pathway, accumulated and haemoprotein production still responded to haem supplementation (Elrod *et al.*, 1997), suggesting rate-limiting steps at later stages in the haem biosynthesis.

In conclusion, we have shown that in fungi, haem protein overproduction becomes limited by the cellular capacity for haem biosynthesis. However, production yields obtained for rMnP in situations of haem supplementation are still below the values typical of homologous protein production (Gouka *et al.*, 1997). Furthermore, the haem input provided in this study exceeded by far the haem recovered as rMnP. This indicates that factors other than haem limitation may be bottlenecks in the overproduction of this type of proteins.

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Calnexin overexpression increases manganese peroxidase production in *Aspergillus niger*

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ABSTRACT

Haem-containing peroxidases from white-rot basidiomycetes, in contrast to most proteins of fungal origin, are poorly produced in industrial filamentous fungal strains. Factors limiting peroxidase production are believed to operate at the post-translational level. In particular, insufficient availability of the prosthetic group, which is required for peroxidase biosynthesis has been proposed to be an important bottleneck. In this work, we have analysed the role of two components of the secretion pathway, the chaperones calnexin and BiP, in the production of a fungal peroxidase. Expression of the *Phaneochaete chrysosporium* manganese peroxidase (MnP) in *Aspergillus niger* resulted in an increase in the expression level of the *clxA* and *bipA* genes. In a haem-supplemented medium, where MnP was shown to be overproduced to higher levels, induction of *clxA* and *bipA* was also higher. Overexpression of these two chaperones in a MnP producing strain was analysed for its effect on MnP production. Whereas *bipA* overexpression seriously reduced MnP production, overexpression of calnexin resulted in a 4 to 5-fold increase in the extracellular MnP levels. However, when additional haem was provided in the culture medium, calnexin overexpression had no synergistic effect on MnP production. The possible function of these two chaperones in MnP maturation and production is discussed.

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INTRODUCTION

Filamentous fungi have a large protein secretion capacity and are therefore exploited for the industrial production of endogenous and recombinant proteins (Archer and Peberdy, 1997). However, whereas secreted homologous protein yields can reach several grams per litre, production of proteins of mammalian or avian origin usually remains in the range of mg/L (Gouka *et al.*, 1997; Radzio and Kurk, 1997). Fungal metalloproteins, such as laccases, haem peroxidases and other oxidases, though of fungal origin, are also normally produced in limited amounts (Aifa *et al.*, 1999; Berka *et al.*, 1997; Huang *et al.*, 1995; Kersten *et al.*, 1995; Ruiz-Dueñas *et al.*, 1999; Saloheimo and Niku-Paavola, 1991; Stewart *et al.*, 1996). Recently, we have reported the expression of two haem-containing peroxidases from the white-rot basidiomycete *Phanerochaete chrysosporium* in *A. niger* (Conesa *et al.*, 2000). We showed that production of the *P. chrysosporium* manganese peroxidase (MnP) could be significantly increased by haem supplementation of the culture medium of the producing strains. This suggests that limitation at the level of co-factor availability is a bottleneck for the overproduction of this type of proteins in filamentous fungi. Also in other examples of heterologous protein production in filamentous fungi limitations at post-transcriptional stages are believed to be responsible for the low secretion yields (Archer and Peberdy, 1997). During passage through the ER, secretory proteins are assisted by an array of proteins, i.e., chaperones and foldases, which modulate protein folding and maturation. These proteins are also involved in processes such as translocation, intramolecular bonding, quality control and ER associated degradation (ERAD) (reviewed in Chapter 2). The expression of chaperone and foldase encoding genes is induced by situations of stress in the ER that result in the accumulation of unfolded proteins, through the so-called Unfolded Protein Response (UPR) pathway (for reviews see Chapman *et al.*, 1998 and Welihinda *et al.*, 1999). In filamentous fungi, UPR inducing agents have been shown to provoke a rapid and strong increase in the expression of the major ER resident chaperone BiP (Ngiam *et al.*, 2000; van Gemeren *et al.*, 1997), whereas induction of the expression of foldases of the PDI-family occurred in a delayed and/or less intense fashion (Ngiam *et al.*, 1997; Ngiam *et al.*, 2000; Saloheimo *et al.*, 1999).

Similarly to stress causing agents, the expression of heterologous proteins often correlates with an enhanced transcript level of chaperones and foldases (reviewed in Chapter 2). This may indicate that heterologous proteins cause a UPR through folding restrictions in the ER. This UPR stress could possibly be the cause for the low secretion yields. We and other groups have analysed the possibility of increasing heterologous protein production in fungi by overexpressing chaperones and foldases. In other expression systems, this approach has in some cases been successful (Harmsen *et al.*, 1996; Tate *et al.*, 1999), although examples can also be found where overexpression of chaperones and/or foldases had no or a negative effect on protein secretion (Choukhi *et al.*, 1998; Dorner *et al.*, 1992; Hsu *et al.*, 1994; Robinson *et al.*, 1996). Overexpression of chaperones or foldases in filamentous fungi has so far failed to increase the extracellular levels of the heterologous proteins analysed (Punt *et al.*, 1998; Wang and Ward, 2000; Ngiam *et al.*, 2000; Wiebe *et al.*, unpublished). This variety of results suggests that a positive effect on heterologous protein secretion may be specific to the chaperone-protein system analysed.

In this chapter we report the differential effect of increasing the expression level of BiP and the recently isolated calnexin on the production of manganese peroxidase in

Aspergillus niger and discuss the possible interactions of these chaperones with the maturation of this metalloprotein in the ER.

MATERIAL AND METHODS

Strains and plasmids

Escherichia coli DH5α was used for construction and propagation of vector molecules. *A. niger* MGG029[pgpdMnp1.I]#13 (Chapter 3) was used as a recipient strain in transformation experiments. Plasmids pAN7-1, containing the hygromycin selection marker (Punt *et al.*, 1987), pGLABiP/hph, carrying the *A. niger bipA* gene under control of the *A. niger glaA* promoter and the *hph* gene (Punt *et al.*, 1998) and pGLACLX (see below) were used as transforming vectors.

Molecular methods

Vector pGLACLX was constructed by amplifying the *A. niger* calnexin gene, *clxA*, from pCLX2.5 (Jeenes *et al.*, unpublished) with a vector primer and CALN1201 (5'-GCTATCCATCATGAGGTTCAACGCTGCTTTGAC-3'). The resulting amplification fragment was digested with *Bsp*HI and *Eco*RV and cloned into pAN52-12Not (a derivative of pAN52-7Not, carrying the *A. niger* glucoamylase *glaA* promoter; Punt, unpublished) at the *Nco*I/*Eco*RV sites. The sequence of the amplification product was confirmed by sequencing.
Total fungal RNA was isolated as described by Kolar *et al.* (1988) using the RNAzol^{TM} kit from CINNA/BIOTECX. Probes used for Northern analysis were a 1.6 kb *Eco*RI-*Hind*III fragment from pABiP1-22, a pABiP1 subclone (van Gemeren *et al.*, 1997), containing the *A. niger bipA* gene; a 2kb *Bsp*HI-*Eco*RV fragment from pGLACLX (calnexin probe); a 1.1 *Nco*I-*Hind*III fragment from pMnP1.I (*mnp1* probe; Conesa *et al.*, 2000); and a 1.5 kb *Hind*III fragment from pAB5-2, (*gpdA* probe; Verdoes *et al.*, 1994). Northern hybridisation signals were measured with Cyclone^{TM} Storage Phosphor (Packard) and quantified with OptiQuant 3.0 software (Packard Instruments Co).

Fungal transformations

Fungal co-transformation was basically carried out as described (Punt *et al.*, 1987) using either pGLABiP/hph or pGLACLX in combination with pAN7-1. Transformants were selected for growth on hygromycin-containing plates.

Protein methods

MnP activity was measured by monitoring the oxidation of diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) in the presence of 20 μM Na-oxalate at 415 nm (Glenn and Gold, 1985). MnP protein levels were also analysed by Western blotting as described previously by Conesa *et al.* (2000). Total protein was determined according to the method of Bradford using bovine serum albumin as standard.

Culturing conditions

MnP production was analysed in shake-flasks cultures. 500 ml flasks containing 100 ml *Aspergillus* maltodextrin minimal medium (AMM-Maltodextrin, (Conesa *et al.*, 2000)) were inoculated with $5x10^7$ fresh conidia and grown at 30°C and agitation at 300 rpm. After 24 hours of growth, mycelium was harvested by filtering through a Miracloth and washed with

physiological salt. Equal amounts of mycelium were then transferred to 300 ml flasks containing either 50 ml AMM-Maltodextrin or 50 ml AMM-Maltodextrin supplemented with 500 mg/L hemin. Strains were grown at the same conditions for another 24 hours, and medium and mycelium were sampled at various intervals.

RESULTS

Induction of *clxA* and *bipA* transcript levels upon MnP production

The levels of *clxA* and *bipA* transcripts were analysed in strain MGG029[pgpdMnp1.I]#13, which contains multiple copies of the *P. chrysosporium mnp1* gene under control of the strong and constitutively-expressed *gpdA* promoter. Transcriptional analysis was performed with samples taken from shake-flask cultures grown in the presence and absence of additional haem. Supplementation of haem in the culture medium increases extracellular MnP production (Conesa *et al.*, 2000), while the *mnp1* transcript levels remain unchanged (data not shown).

Transcriptional analysis showed a similar induction pattern for both chaperones. Compared to the wild type strain, *clxA* and *bipA* mRNA levels increased by approximately a factor 1.5 in MGG029[pgpdMnp1.I]#13 when grown in the inducing medium AMM-Maltodextrin. Upon haem addition to the culture medium of this strain, a 3.6 and 3.0-fold induction was measured for the *clxA* and *bipA* transcripts, respectively. In the wild type strain, haem supplementation had no significant effect on the transcript level of either chaperone (Fig.1).

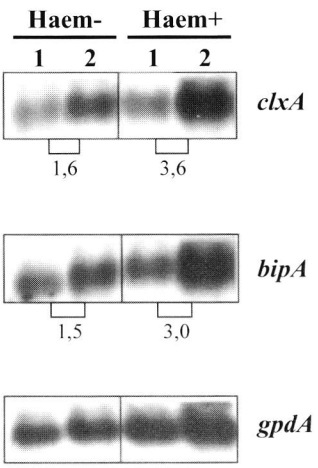

Fig. 1. Induction of *clxA* and *bipA* gene expression by MnP production. The Northern blot was prepared with RNA from the wild type strain MGG029 (**1**) and MnP producing strain MGG029[pgpdMnp1.I]#13 (**2**). Strains were pre-grown for 24 hours in AMM-Maltodextrin medium and then transferred to either AMM-maltodextrin (**Haem-**) or AMM-maltodextrin supplemented with 500mg/L hemin (**Haem+**). After 24 hours of additional growth, samples were harvested. The signal of the *gpdA* was used as loading control. Induction factors, calculated after correction for loading differences (*gpdA* signal), are indicated.

Effect of calnexin and BiP overexpression on MnP production

To study the effect of calnexin and BiP overexpression on the production of MnP by *A. niger* we used expression cassettes where the *clxA* and *bipA* genes were placed under control of the strong and regulated glucoamylase promoter (P*glaA*). This approach has shown to be successful in providing increased intracellular chaperone levels (van Gemeren *et al* ., 1998). As a different promoter, P*gpdA*, controls the expression of the *mnp1* gene in strain

MGG029[pgpdMnp1.I]#13, no interfering transcriptional titration effects are expected between expression of the chaperone and *mnp1*.

Strain MGG029[pgpdMnp1.I]#13 was supertransformed either with pGLABiP/hph, containing the *A. niger bipA* and the hygromycin (*hph*) selection marker in one vector or with pGLACLX in combination with pAN7-1, containing the *A. niger clxA* and the *hph* gene respectively. Hygromycin resistant transformants were obtained and analysed for overexpression of the *clxA* and *bipA* genes by Northern blotting. From this analysis, two *clxA* (MGG029-pgpdMnp1.I#13[pGLACLX]#1 and MGG029-pgpdMnp1.I#13[pGLACLX]#4) and two *bipA* (MGG029-pgpdMnp1.I#13[pGLABIPA]#1 and MGG029-pgpdMnp1.I#13[pGLABIPA]#2) overexpressing strains (Fig.2) were selected to study MnP production in shake-flask cultures.

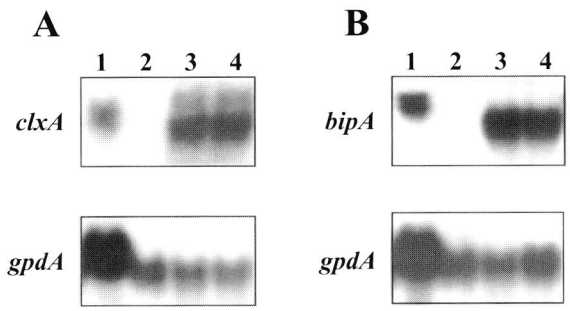

Fig. 2. Northern blot analysis of *clxA* and *bipA* overexpression. RNA was extracted after 24h. + 24h. culture in AMM-Maltodextrin medium.
A) Overexpression of *clxA*.. 1: MGG029[pgpdMnp1.I]#13, concentrated RNA sample; 2:MGG029[pgpdMnp1.I]#13; 3:MGG029-pgpdMnp1.I#13[pGLACLX]#1; 4:MGG029-pgpdMnp1.I#13[pGLACLX]#4.
B) Overexpression of *bipA*. 1: MGG029[pgpdMnp1.I]#13, concentrated RNA sample; 2: MGG029[pgpdMnp1.I]#13; 3:MGG029-pgpdMnp1.I#13[pGLABIPA]#1, 4:MGG029-pgpdMnp1.I#13[pGLABIPA]#2. The signal of the *gpdA* was used as loading control.

MnP production was monitored in AMM-Maltodextrin medium. To minimise the effects of differences in growth among the various strains, cultures were started from equal amounts of mycelium obtained from a 24 hours preculture. MnP activity was measured after 10 and 24 hours of additional growth. An increase in extracellular MnP activity was observed in both *clxA* overexpressing strains compared to the MGG029[pgpdMnp1.I]#13 strain or this strain transformed only with the hygromycin selection marker. This increase was weak but already visible after 10 hours induction (data not shown) and reached a 4 to 5-fold value after 24 hours (Fig.3a, empty bars). In contrast, both *bipA* overexpressing strains showed a considerable reduction in extracellular MnP activity to almost undetectable levels at 24 hours after induction (Fig. 3a, empty bars). At each time point, the amount of total extracellular protein was similar among the six strains (data not shown). Western blot analysis showed that the observed differences in MnP activity corresponded with similar differences in the extracellular levels of the MnP protein (Fig.3b). These results indicate that overexpression of *clxA* results in an increase in the production of MnP, whereas *bipA* overexpression reduces MnP secretion.

Growth in the presence of haem excess eliminates the effect of calnexin overexpression

We have previously shown that addition of a haem source to the culture medium of MnP producing *A. niger* strains results in an improvement in the production level of MnP (Conesa *et al.*, 2000). To analyse whether this effect was additive to, or could counteract, the observed effects of *clxA* and *bipA* overexpression, we studied MnP production in the various strains

grown under conditions of haem supplementation. Strains were cultivated from mycelial inocula in maltodextrin medium supplemented with 500 mg/L hemin and MnP activity was measured after 24 hours growth.

Under these conditions a 3.5-fold increase in MnP extracellular activity was observed in the MGG029[pgpdMnp1.I]#13 strain compared to the level observed in the unsupplemented medium (Fig.3a, filled bars). MnP production in both *bipA* overexpressing strains also increased but still reached less than 10% of the value obtained for MGG029[pgpdMnp1.I]#13. In contrast, MnP production in both *clxA* overexpressing strains did not increase further upon hemin supplementation, and stayed at the level obtained for MGG029[pgpdMnp1.I]#13 in the haem-supplemented medium (Fig.3a, filled bars). As with the results obtained in non-supplemented medium, Western blot analysis confirmed the increase in extracellular MnP protein levels (Fig. 3b).

DISCUSSION

In previous studies, we have shown that limited haem availability is a bottleneck for the overproduction of haem-containing peroxidases in filamentous fungi. In this paper we present our continued work on the analysis of the factors affecting the secretion levels of this type of proteins. We have studied the role of two ER resident chaperones, the lectin-like calnexin, and the Binding Protein BiP, in the secretion of the *P. chrysosporium* manganese peroxidase (MnP) in *A. niger*. Calnexin is involved in the folding of glycosylated proteins and is a major component of the ER quality control system (Helenius *et al.*, 1992). BiP is a well-studied member of the heat-shock protein family with multiple functions in the secretory pathway(for reviews see Pedrazzini and Vitale, 1996 and Gething, 1999).

Overexpression of MnP in *A. niger* resulted in a moderate (1.6-fold) increase in the transcript levels of both *clxA* and *bipA*. Induction was higher (~ 3-fold) in the haem-supplementation condition which, in this case, corresponds to a 3.5-fold higher MnP production level, compared to the non-supplemented medium. Many studies on chaperone induction by expression of heterologous proteins in filamentous fungi report induction values of the same magnitude. Expression of the hen egg lysozyme in *A. niger* resulted in a 1.8 to 2-fold increase in the *bipA* mRNA level and a 2-3 fold increase the levels of *pdiA* and the PDI-related *tigA* transcripts (Ngiam *et al.*, 2000). Likewise, t-PA expression in *A.niger* increased *bipA*, *pdiA* and the cyclophilin *cypB* transcript levels by a factor 1.5-2 (Wiebe *et al.*, 2001). In *T. reesei,* a 2 to 3-fold enhancement in *pdiA* mRNA level was observed in a Fab antibody fragment overexpressing strain (Saloheimo *et al.*, 1999). BiP is considered a major component of the primary stress response to the accumulation of unfolded proteins in the ER (for a recent review see Gething, 1999). The induction of BiP expression in the MnP producing strain may indicate, therefore, a limitation at the stage of folding for the biogenesis of this haemoprotein in *A. niger*. The *clxA* steady-state mRNA levels were increased by MnP overexpression, and also by overexpression of chymosin (Wang *et al.*, submitted), indicating that *clxA* responds to signals generated by the secretion of heterologous proteins.

Compared to the non-supplemented medium, induction of the *bipA* and *clxA* transcripts in MGG029[pgpdMnp1.I]#13 was higher in the haem-supplemented condition. This increase was not due to the additional haem, since the *clxA* and *bipA* mRNA levels were not changed in the wild-type strain. More likely, the higher *bipA* and *clxA* induction could be related to the higher MnP production level reached upon haem addition.

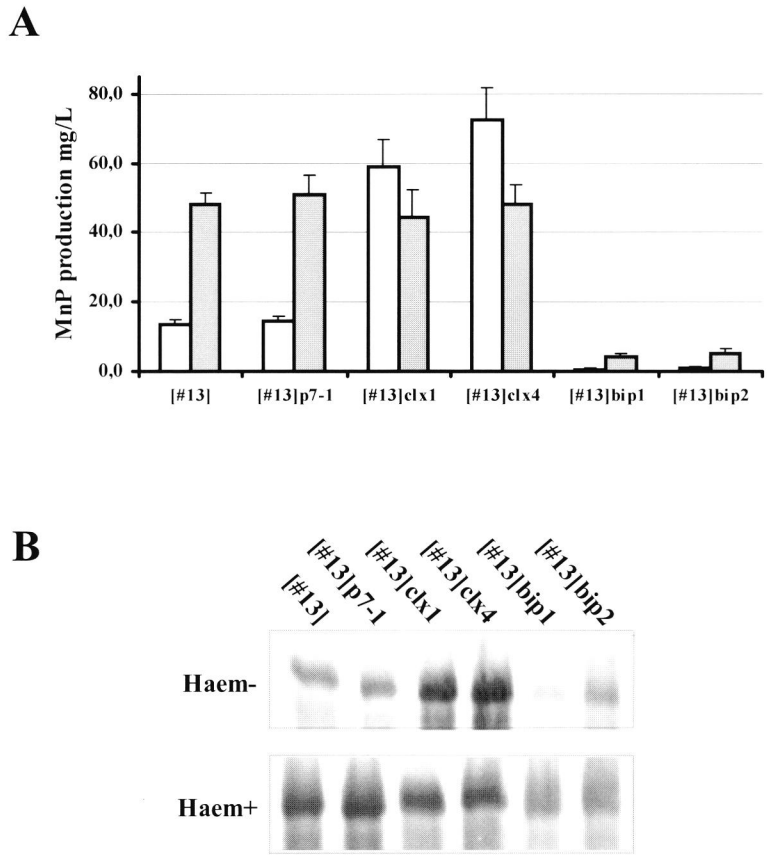

Fig. 3. Effect of *clxA* and *bipA* overexpression on MnP production by *A. niger*. Cultures were inoculated with equal amounts of mycelium obtained from a 24 hours pre-culture in AMM-Maltodextrin medium. [#13]: MGG029[pgpdMnp1.I]#13; [#13]p7-1: MGG029-pgpdMnp1.I#13[pAN7-1]; [#13]clx1: MGG029-pgpdMnp1.I#13[pgpdGLACLX]#1; [#13]clx4: MGG029-pgpdMnp1.I#13[pgpdGLACLX]#4; [#13]bip1: MGG029-pgpdMnp1.I#13[pgpdGLABIPA]#1; [#13]bip2: MGG029-pgpdMnp1.I#13[pGLABIPA]#2. **A)** MnP activity determined in the culture broth of strains grown in AMM-Maltodextrin (empty bars) or in AMM-Maltodextrin supplemented with 500 mg/L hemin (filled bars) (n=2). **B)** Detection of MnP production by Western blotting.

Although MnP production induced *bipA* expression, when a high expression level of this chaperone was provided by introduction of multiple *bipA* gene copies under control of a strong promoter, the production of MnP was severely reduced. A similar inverse correlation of BiP transcript levels with heterologous protein production was observed by Dorner and colleges in CHO cells (Dorner *et al.*, 1988; Dorner *et al.*, 1992). In contrast, BiP overexpression increased the production levels of chymosin and the cocaine-sensitive serotonin transporter (SERT) in yeast and insect cells, respectively (Harmsen *et al.*, 1996; Tate *et al.*, 1999). In other studies where the role of BiP in heterologous protein production has been addressed, BiP overexpression did not affect the extracellular levels of the proteins

analysed (Hsu *et al.*, 1994; van Gemeren *et al.*, 1998). Overexpression of the *bipA* gene in *A. niger* failed to improve the extracellular levels of two heterologous proteins, although the intracellular levels were increased (Punt *et al.*, 1998). The reason for this variety of results is not clear, but could be related to the multifunctionality of the BiP chaperone. BiP is involved in several functions in the ER (Gething, 1999). Some processes, such as translocation into the ER and protein folding, could be classified as favourable for protein production, whereas others, such as ERAD, the ER-associate degradation of misfolded proteins, can be considered as unfavourable. The participation of each process in the secretion efficiency of heterologous proteins may be protein-specific and lead to different results when BiP is overproduced. We suggest that, under conditions of BiP overexpression, the unfavourable interactions predominate for MnP, increasing degradation and diminishing the production level of this haemoprotein.

In contrast to the results obtained with the BiP overproducing strains, overexpression of *clxA* in strain MGG029[pgpdMnp1.I]#13 resulted in a 4 to 5-fold increase in MnP production after 24 hours. Calnexin is a lectin-like chaperone involved in the folding of glycosylated proteins and is part of the ER quality control system (Helenius *et al.*, 1997; Jakob and Burda, 1999). MnP is glycosylated (Paszczynski *et al.*, 1986), and therefore, a potential substrate for calnexin. Calnexin co-overexpression also enhanced the levels of functional serotonin transporter produced using the baculovirus expression system, whereas co-overexpression of other chaperones (BiP, calreticulin and Erp57) had a lower or no effect (Tate *et al.*, 1999). In contrast, co-overexpression of calnexin did not increase the formation of hepatitis C virus (HCV) envelope proteins complexes in mammalian cells (Choukhi *et al.*, 1998). On the other hand, calnexin disruption in *S. cerevisiae* resulted in a 2.5-fold increase in the secretion of unstable lysozyme mutants (Arima *et al.*, 1998). Also for calnexin, this variety of results suggests different specificities for different protein-chaperone combinations. The positive effect of calnexin co-overexpression in MnP production suggests its participation in the maturation of MnP. Interestingly, Naussef and co-workers, studying the biosynthesis of another haem-containing protein, the human myeloperoxidase (MPO), observed a specialised role for calnexin in MPO maturation (Nauseef *et al.*, 1998). They showed that two ER resident lectins, calreticulin and calnexin, associated with apoproMPO, but only calnexin interacted with holoproMPO and haem insertion was required to release holoproMPO from calnexin binding. This suggests a function of calnexin at the stage of incorporation of the prosthetic group into this haemoprotein. Furthermore, Fayadat and co-workers showed that calnexin overexpression increased the initial folding steps of the human thyroperoxidase (Fayadat *et al.*, 2000), and that haem insertion was required for secretion of this haemoprotein (Fayadat *et al.*, 1999).

Considering all these results, we postulate also a role for calnexin during haem incorporation into MnP. We have shown that haem is limiting for MnP production and apo-forms of MnP do not accumulate extracellularly (Conesa *et al.*, 2000). Under these haem-limiting conditions calnexin overexpression may help MnP maturation, e.g., by facilitating or providing sufficient time for haem insertion and therefore, increasing MnP secretion. However, under conditions of haem supplementation the limitation at this maturation step is alleviated, and consequently calnexin overexpression would not have a further positive effect.

In conclusion, our results suggest a differential role of calnexin and BiP in the maturation of MnP. Although both chaperones are induced by *mnp1* overexpression, indicating a folding limitation for MnP in the ER, calnexin has a positive role, possibly by facilitating proper folding and haem incorporation, whereas BiP may act mainly in the degradation of misfolded MnP molecules. Additional work on the kinetics and specificity of these interactions is required to further confirm these observations.

ACKNOWLEDGEMENTS

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General discussion and summary

INTRODUCTION

Peroxidases (EC. 1.11.1.-) are enzymes that utilise hydrogen peroxide to catalyse oxidation reactions (Everse and Everse, 1991). These proteins are widely found in nature and are involved in a great diversity of biological processes. The proteins considered in this thesis are haem-containing, extracellular enzymes of fungal origin. One type of fungal peroxidases is that secreted by white-rot basidiomycetes as part of their unique lignin degrading machinery (Hatakka, 1994). Lignin and manganese peroxidases belong to this class of lignolytic enzymes. A different type is formed by the haloperoxidases. To date, only one haem-containing haloperoxidase, the chloroperoxidase from the mould *Caldariomyces fumago*, has been biochemically characterised (Morris and Hager, 1966).

Extracellular fungal peroxidases are attractive enzymes for their potential industrial applications. Lignolytic peroxidases are able to oxidise compounds of high redox potential in a nonspecific manner and are therefore interesting as biological alternatives in processes such as bleaching, pulping and degradation of recalcitrant pollutants (Barr and Aust, 1994; Field *et al.*, 1993). Chloroperoxidase is a versatile enzyme with useful properties for the industry of fine chemicals because of the wide range of reactions it catalyses and its enantio- and regio-selectivity (van Deurzen *et al.*, 1997). In **chapter 1** of this thesis a review is presented on the molecular aspects and applications of fungal haemoperoxidases.

The use of peroxidases in industrial processes is hampered by two main problems: the limited availability of these proteins and their low thermal and oxidative stability. An efficient recombinant expression system for these proteins would provide the means, on one hand, to increase production yields, and, on the other, to apply site-directed mutagenesis to improve enzyme performance. Expression in commonly used hosts such as *E. coli*, baculovirus and yeast has not succeeded in providing an adequate production system for these proteins (reviewed in **chapter 1**). An alternative candidate expression system is that of filamentous fungi. Filamentous fungi are used industrially for the production of homologous and heterologous proteins. Although high production levels are commonly obtained in the production of homologous proteins, much lower yields are normally reached with heterologous proteins (Gouka *et al.*, 1997; Radzio and Kurk, 1997). Until recently, fungal haemoperoxidases, though of fungal origin, were also produced in only limited amounts (reviewed in **chapter 1**). Much work was carried out in attempts to elucidate the reasons for this difference in production levels and to develop approaches for increasing recombinant protein production. Although the biochemical and molecular background for the differences in yields obtained with homologous and heterologous proteins remains largely unknown, successful approaches to improve (heterologous) protein production have been developed. Examples of this are the use of protease-deficient strains, the introduction of multiple copies of the expression cassettes and the fusion to carrier proteins (Gouka *et al.*, 1997; Jeenes *et al.*, 1991). Since heterologous proteins often provoke an unfolded protein response in the ER (reviewed in **chapter 2**), it has been suggested that these proteins encounter limitations at the stage of protein folding. However, most of the approaches undertaken to solve this misfolding problem by overexpressing molecular chaperones have not been successful. A detailed review on the fungal secretion pathway and strategies to improve heterologous protein production is given in **chapter 2**.

The aim of the work described in this thesis was to study the factors involved in the production of haemoperoxidases in filamentous fungi and to identify bottlenecks in their overproduction. We have used *Aspergillus niger* as production host and three peroxidases:

the lignin and manganese peroxidases of *Phanerochaete chrysosporium*, as two representatives of lignolytic peroxidases, and the chloroperoxidase from *Caldariomyces fumago*.

Expression of peroxidases in *A. niger*

Expression constructs were made in which the LiP, MnP and CPO coding sequences (*lipA*, *mnp1* and *cpo*, respectively) were placed under control of strong *Aspergillus* promoters, the *glaA* and the *gpdA* promoters.

Expression of the *mnp1* and *cpo* genes under control of the *glaA* promoter resulted in secretion of active protein into the extracellular medium (**Chapters 3 and 5**). Positive transformants were easily selectable using a plate assay based on the oxidation of *o*-anisidine (Mayfield *et al.*, 1994). In contrast, expression of the *lipA* gene following the same strategy failed in producing any extracellular LiP activity (**Chapter 4**). Analysis of the LiP transformants by Western blotting showed that this was probably due to an incorrect processing of the lignin peroxidase protein. It is suggested that this incorrect processing is related to a property of this protein other than being a haemoprotein, since it was not observed for MnP and CPO. Genetic studies suggested that this incorrect processing occurred at a late stage in the secretion pathway and might take place at the N-terminal half of the protein (**chapter 4**). As this incorrect processing is probably an enzyme-linked event, it would be interesting to analyse the expression of other LiP isozymes or isozymes of the LiP-MnPL2 "hybrid" class (**chapter 1**) in *Aspergillus*. Interestingly, a representative of this last type, the *Pleurotus eryngii* MnP, was produced in *Aspergillus nidulans* as an active protein (Ruiz-Dueñas *et al.*, 1999).

Initial yields obtained for MnP and CPO were 10mg/L and 0.5 mg/L respectively. Production of inactive LiP was also estimated at a few milligrams per litre. These values are low when compared to the yields typically obtained for homologous proteins (Radzio and Kurk, 1997), but significantly higher than those obtained in other studies on peroxidase production (see **chapter 1**, table 1). Particularly for CPO, this is the first report on the successful production of this protein in a recombinant system. Since high levels of mRNA were present in our peroxidase expressing strains, we concluded that no major bottlenecks for the overproduction of these proteins occurred at the transcriptional level. Limitations in heterologous protein production are generally observed at the post-translational level (Gouka *et al.*, 1997; Jeenes *et al.*, 1991). The reasons why CPO production was significantly less than MnP production are not clear. In contrast to the situation with MnP, haem attachment in CPO requires the formation of a thiolate ligand (Sundaramoorthy *et al.*, 1995). Furthermore, CPO is synthesised from a precursor and a proteolytic event at the C-terminus of the polypeptide is required to produce the mature protein (Sundaramoorthy *et al.*, 1995). The results presented in **chapter 7** suggest that the C-terminal propeptide of CPO plays an important role in the maturation of this enzyme. It is possible that limitations at these or other specific post-translational modifications of chloroperoxidase are responsible for the lower production yields as compared to those obtained for MnP.

Characterisation of the recombinant proteins

An important step in studying the expression of fungal peroxidases in *Aspergillus* is the characterisation of the recombinant proteins.

Both active rMnP and rCPO were purified and their structural and catalytic features were analysed (**chapters 3 and 5**). Experimental data indicated the correct formation of the

haem thiolate ligand as well as of the single disulphide bond in the recombinant CPO, while N-terminus determinations showed correct processing of the signal peptide in rMnP. Spectral analysis of rCPO suggested partial haem incorporation in this recombinant haemoprotein. In contrast, the absorption spectra of rMnP and nMnP were almost indistinguishable, indicating full haem insertion for rMnP. A possible explanation for the different efficiency in haem incorporation of the two peroxidases may be the different nature of haem attachment in MnP (axial ligand histidine) and CPO (axial ligand cysteine).

The SDS-PAGE mobility of both rMnP and rCPO was slightly lower compared to that of the native enzymes, possibly due to a higher degree of glycosylation of the recombinant proteins. Why the recombinant peroxidases appeared more glycosylated than their native counterparts is not known. Overglycosylation of heterologous proteins is often observed in yeast but less frequently in *Aspergillus* (discussed by Maras *et al.*, 1999), although some examples of hyperglycosylation in *Aspergillus* species can be found (Wyss *et al.*, 1999). In nMnP, only one of the two putative glycosylation sites is occupied (Sundaramoorthy *et al.*, 1994), and it is possible that glycosylation at the second site occurs in *Aspergillus*. nCPO is glycosylated at its three putative N-glycosylation sites, and has numerous O-glycosylation sites where side-chain variations are possible. Alternatively, a higher carbohydrate content may be the result of a longer extension of carbohydrate chains in *Aspergillus* as compared to the natural hosts. A third possibility is that overglycosylation results from a longer retention of the recombinant enzymes in the ER as a consequence of possible limitations at the stage of protein maturation.

As shown, this slight overglycosylation had no major effect on the catalytic behaviour of the recombinant proteins. Specific activity in both rMnP and rCPO was similar to that of the native enzymes. Furthermore, we showed that rCPO fully retained a number of the catalytic properties which are relevant for the industrial application of this protein, such as enantioselectivity and regio-selectivity in oxygen-transfer reactions.

These results show that active fungal peroxidases can be produced in *Aspergillus* as enzymes retaining the catalytic properties of their native counterparts, which makes this expression system a valuable tool for molecular studies of this type of proteins.

Analysis of approaches for production improvement

The initial production yields obtained in *Aspergillus* with MnP and CPO were lower than is normally achieved with homologous proteins and also clearly lower than that required for industrial applications. In this thesis we describe the use of manganese peroxidase as a model to study several factors which influence these production yields.

Strain selection

To study the expression of fungal peroxidases in *A.niger* we chose, in a first approach, the protease deficient strain MGG029, a derivative of *A. niger* AB1.13 (Mattern *et al.*, 1992). Strain MGG029 has a regulatory mutation resulting in a reduced secretion of numerous extracellular proteases (Mattern *et al.*, 1992) and has previously shown to be a suitable fungal strain for the production of human interleukin 6 (hIL6) (Punt *et al.*, in preparation). The higher production yields obtained for rMnP and rCPO, when compared to previously reported data (see **chapter 1,** table 1), may be the result of using a more appropriate host in our studies. MnP production was significantly enhanced when transformants harbouring multiple copies of the expression cassette were selected (**chapter 3**). Altogether, these results show

that considerable improvements can be obtained by adequately selecting the production strain.

Haem limitation

The role of haem availability in peroxidase production is addressed in **chapter 7.** Addition of a haem source to the culture medium of a MnP producing strain resulted in an increase in the extracellular peroxidase activity. This effect was also observed for rCPO (**chapter 5**) and other recombinantly produced peroxidases (Elrod *et al.*, 1997; Stewart *et al.*, 1996) and suggests that haem is a limiting factor in the production of this type of proteins. The work presented in **chapter 7** provides some details on the characteristics of this limitation. We have shown that the increase in rMnP extracellular activity measured upon haem supplementation was due to a concomitant increase in the enzyme production level and not to an increased specific activity. This suggests that only holoMnP accumulated in the extracellular medium and that apoforms may not be stable.

Using a series of plate assays, we have shown that MnP competes for the available cellular haem with at least two other intracellular haem proteins, the cytosolic nitrate reductase and an ER-located cytochrome P450. This competition was no longer observable when additional haem was provided in the growth medium. Since competition for haem was found to occur among proteins with different cellular locations and addition of haem could abolish this competition phenomenon, we concluded that a limitation was present at the level of haem biosynthesis. However, our results do not exclude additional limitations at the stage of haem transport or haem ligand formation. Furthermore, as the sole addition of extra Fe to the culture medium did not have a positive effect on MnP production, it is suggested that a factor other than reduced Fe availability limited haem biosynthesis.

Recently Elrod and co-workers showed that haem supplementation was not able to complement an ALAS null mutant *A. niger* strain (Elrod *et al.*, 2000). ALAS is the first enzyme of the haem biosynthesis pathway (Padmanaban *et al.*, 1989). This result suggests that haem is poorly taken up from the extracellular medium. This is in disagreement with the results presented in this thesis. Haem addition increased MnP production. In the plate assays for haem competition we saw a clear effect of haem addition on the activity of intracellular proteins. We also observed a response of two molecular chaperones (intracellularly located) to haem addition (see below). ALAS is involved in cellular pathways other than haem biogenesis, such as ergosterol and vitamin B12 biosynthesis and there may be other as yet unknown processes. Blockades in any of these pathways, which are not complemented by haem addition, could be lethal. However, the question of how a relatively large compound such as haem penetrates into the cell remains. There are some examples of biological mechanisms for active haem uptake, such as the bacterial haemophore-dependent haem acquisition system (Ghigo *et al.*, 1997), or the mammalian haemopexin mediated haem endocytosis system (Eskew *et al.*, 1999). Whether similar mechanisms exist in filamentous fungi, or whether haem uptake occurs via an alternative route, is currently unknown.

Chaperones

ER located chaperones and foldases are proteins involved in the folding of proteins which enter the secretion pathway. Since factors hampering heterologous protein production are believed to occur mainly at the post-translational level, the possible role of limitations at the stage of chaperone-assisted protein maturation has been addressed in several studies (reviewed in **chapter 1**). Although in most studies an effect of heterologous protein

production on the expression of chaperone encoding genes was observed, the overproduction of these helper proteins did not result in an increase in the extracellular levels of the recombinant proteins analysed.

In **chapter 8** we present novel data on the role of two ER resident chaperones, the lectin-like calnexin, and the Binding Protein BiP, on heterologous protein production. MnP production resulted in increased expression of both chaperones. However, overexpression of the corresponding chaperone genes had different effects on MnP production. *bipA* overexpression seriously reduced MnP production. BiP is involved in different functions in the ER (Gething, 1999). Processes such as translocation into the ER and protein folding, are favourable for protein biogenesis, whereas others, such as ER-associated degradation (ERAD) act to dispose of incorrectly folded proteins. We suggest that for MnP, under conditions of BiP overexpression, the unfavourable interactions predominate, resulting in increased degradation and reduced levels of secreted protein.

In contrast to the situation with BiP, overexpression of calnexin in a MnP producing strain resulted in a 4 to 5-fold increase of the production of MnP. Interestingly, upon addition of haem to the culture medium, the positive effect of calnexin on MnP production was no longer observed. Previously, the participation of calnexin at the stage of haem incorporation in a mammalian peroxidase has been shown (Nauseef *et al.*, 1998). Our results also suggest a role of calnexin in MnP biosynthesis. Under haem-limiting conditions calnexin overexpression may help MnP maturation, e.g., by facilitating or providing sufficient time for haem insertion, thereby increasing secretion of MnP. However, under conditions of haem supplementation the limitation at this maturation step is alleviated, and consequently calnexin overexpression would not have a further positive effect.

In conclusion, the data provided in **chapter 8** suggest that the effect of chaperone overexpression on heterologous protein production is specific for the protein analysed and that experimental determinations are required in each case.

Culture conditions

In controlled fermentation production physiological conditions for protein secretion can be optimised to improve production yields. In our case, a 5 fold increase in MnP production was obtained in batch fermentations when compared to shake-flask cultures (**chapter 3**). These experiments also showed that MnP production was most efficient at a neutral pH, provided that a pH-independent promoter was used to drive *mnp1* expression. The higher production at neutral pH may be related to protein degradation at low pH by acidic proteases. Although the strain we use in our study has reduced levels of extracellular proteases, residual protease activity is still present and may negatively influence peroxidase production. In general, production of the three peroxidases was reduced as soon as the pH of the medium dropped, suggesting degradation by extracellular proteases.

CONCLUDING REMARKS AND PROSPECTS

The research described in this thesis has highlighted several factors influencing the overproduction of fungal peroxidases in *Aspergillus* and has provided means for increasing production yields (figure 1). Whereas high steady-state levels of the corresponding messenger RNAs are found, major bottlenecks occur at the post-translational level. At this level, peroxidase overproduction is limited by the cellular capacity for haem biosynthesis.

Limitations at the stage of protein folding may occur in the ER. The chaperone BiP may participate in the degradation of misfolded molecules, whereas calnexin may assist peroxidase maturation at the step of haem incorporation. After secretion, production yields may be reduced by the action of extracellular proteases. Additionally, as shown for lignin peroxidase, production of the active enzymes can be hampered by incorrect processing at a late stage in the secretion pathway.

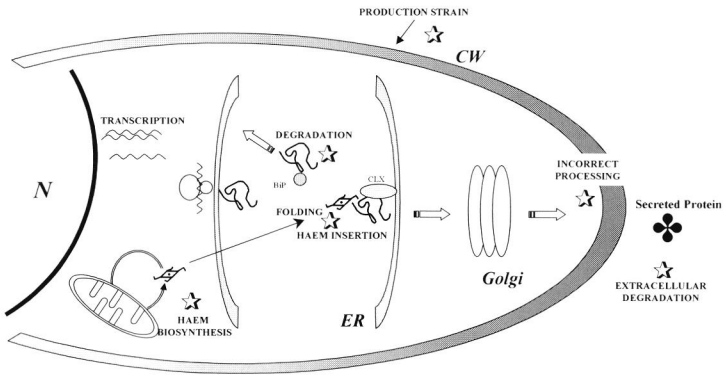

Fig. 1. Model for haemoperoxidase biogenesis and secretion in *Aspergillus*. N: nucleus; ER: Endoplasmic Reticulum, CW: cell wall, CLX: calnexin. Factors affecting peroxidase overproduction are highlighted with ☆. For explanation see text.

Approaches presented to increase peroxidase production yields include adequate strain selection, haem supplementation, overexpression of calnexin and controlled fermentations. The improvement factors achieved with these strategies are indicated in table 1.

Table 1. Approaches for improvement of MnP production in *Aspergillus niger*

Strategy	Improvement factor
Strain selection	x5
Haem supplementation	x7-10
Calnexin overexpression	x4-5
Fermentation	x5

Further improvement in production yields may be obtained by adequately combining the described approaches. However, also more holistic approaches based on new genomics tools may be used to study (other) complex processes influencing peroxidase production such as the UPR/ERAD responses, haem availability or the role of vacuolar degradation, and to identify targets for further strain improvement.

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Nederlandse samenvatting

Peroxidases zijn enzymen die waterstof peroxide gebruiken om oxidatie reacties te catalyseren. Dit soort enzymen komt in veel verschillende organismen voor. Er zijn voorbeelden van intra- en extracellulaire peroxidases. De meeste peroxidases bevatten een ijzer-houdende prostetische heem groep in het actieve redox-centrum. Een aantal soorten schimmels is in staat om dit type heem-bevattende peroxidases in het kweekmedium te secreteren. Met deze gesecreteerde peroxidases zijn een aantal interessante industriële toepassingen mogelijk. Uitgescheiden peroxidases die houtrot schimmels gebruiken bij de afbraak van de in hout voorkomende verbinding lignine hebben een hoge oxidatieve capaciteit en zijn niet erg specifiek met betrekking tot de te oxideren substraten. Deze eiwitten kunnen gebruikt worden als biologisch alternatief voor chemische processen zoals bleken, pulping in de papierproduktie en de afbraak van verschillende toxische stoffen. Chloroperoxidase, een ander type heem-bevattend peroxidase dat geproduceerd wordt door de schimmel *Caldariomyces fumago,* is in staat een aantal zeer specifieke chemische reacties te catalyseren. Dit enzym kan gebruikt worden in de bereiding van fijnchemicaliën.

Industriële toepassing van schimmel peroxidases wordt beperkt door een tweetal factoren; de kleine hoeveelheden die tot nu toe kunnen worden geproduceerd en de lage oxidatieve en thermische stabiliteit van deze enzymen. Deze beperkingen kunnen wellicht worden verholpen door schimmel peroxidases te synthetiseren in een geschikt recombinant systeem. Een dergelijke aanpak geeft namelijk de mogelijkheid om zowel hogere productieniveaus te bereiken, als ook om de eiwitten door mutagenese te modificeren om betere eigenschappen te introduceren.

Het in dit proefschrift beschreven onderzoek gaat over het gebruik van recombinante filamenteuze schimmels voor de overproductie van heem-bevattende schimmel peroxidases. In hoofdstuk 1 wordt een overzicht gegeven van recente literatuur omtrent de algemene moleculaire aspecten en industriële toepassingen van heem-peroxidases. Hoofdstuk 2 geeft via uitgebreid literatuuronderzoek inzicht over de wijze waarop eiwitten uit schimmels in het medium gesecreteerd worden en de strategieën om heterologe eiwitproductie te verhogen. De daaropvolgende hoofdstukken (Hoofstuk 3 t/m 8) beschrijven praktisch onderzoek naar de mogelijkheden voor overproductie van drie specifieke heem-eiwitten: lignine peroxidase (LiP) en mangaan peroxidase (MnP), beide afkomstig uit de houtrot schimmel *Phanerochaete chrysosporium*, en chloroperoxidase (CPO) uit *Caldariomyces fumago.* De filamenteuze schimmel *Aspergillus niger* is hierbij gekozen als gastheer organisme.

Wanneer de genen coderend voor MnP en CPO onder controle van een sterke *Aspergillus* promoter in de protease deficiënte *A. niger* stam (MGG029) tot expressie worden gebracht, leidt dit tot de secretie van actieve eiwitten in het medium (Hoofdstuk 3 en 5). Expressie van het gen coderend voor LiP in dezelfde stam leidde evenwel niet tot secretie van lignin peroxidase activiteit (Hoofdstuk 4). Analyse van *Aspergillus* transformanten verkregen met het LiP coderende gen wees uit dat het ontbreken van extracellulair lignine peroxidase activiteit waarschijnlijk veroorzaakt werd door een incorrecte splitsing van het heterologe eiwit. Nadere studies met mutante versies van het LiP gen en expressie in andere *Aspergillus* stammen liet zien dat deze incorrecte spiltsing waarschijnlijk pas in een laat stadium van de secretieroute plaatsvindt en dat het N-terminale deel van het eiwit hierbij betrokken is (Hoofdstuk 4).

De wel geproduceerde actieve peroxidase eiwitten MnP en CPO werden gezuiverd zodat een aantal structurele en catalytische eigenschappen bepaald konden worden (Hoofdstuk 3 en 5). Opvallend was dat, in tegenstelling tot wat werd gevonden voor MnP, uit

spectroscopische analyse bleek dat in het geval van CPO slechts een deel van het eiwit een heem molecuul bevatte. Verder bleek uit het electroforetisch gedrag van zowel MnP als CPO in SDS-PAGE gels dat de recombinante eiwitten waarschijnlijk een uitgebreidere glycosylering hadden dan de natieve eiwitten.

Voor industriële toepassingen van de peroxidase eiwitten is het van belang dat de catalytische eigenschappen van de recombinant eiwitten niet anders zijn geworden dan zoals bekend is van de natieve eiwitten. In hoofdstuk 3 en 5 hebben we laten zien dat de specifieke activiteit van recombinante en natieve eiwitten onderling vergelijkbaar is. Het geproduceerde rCPO behield een aantal van de voor de industrie belangrijke catalytische eigenschappen, zoals enantioselectiviteit en regioselectiviteit in oxidatie reacties.

De in eerste instantie behaalde eiwitproductieniveaus voor MnP en CPO waren 10 mg/l, respectievelijk 0,5 mg/l. De productie van het inactieve LiP werd geschat op enkele milligrammen per liter. Deze niveaus zijn hoger dan eerder beschreven waarden in de literatuur (zie Hoofdstuk 1, tabel 1). Voor CPO is dit zelfs de eerste keer dat de succesvolle productie in een heteroloog systeem is gerapporteerd. Dat we in staat zijn geweest dergelijke niveaus te bereiken zou verklaard kunnen worden doordat er in dit onderzoek gebruik is gemaakt van een *Aspergillus* stam die deficiënt is in de productie van een aantal extracellulaire proteases. Bovendien kon de MnP productie worden verhoogd door stammen te selecteren die meerdere copieën van de *mnp* expressiecassette bevatten en door fermentatie bij een neutrale pH (Hoofstuk 3). In het algemeen kan gesteld worden dat een geschikte stamselectie en kweekcondities van belang zijn voor de efficiënte productie van heem-bevattende peroxidases in *Aspergillus*. Toch zijn de hier gehaalde productiewaarden voor heterologe peroxidases duidelijk lager dan die voor soorteigen (homologe) eiwitten. Ook voor industriële toepassingen zijn duidelijk hogere productieniveaus gewenst.

Hoofdstuk 7 beschrijft hoe ook de toevoeging van heem de peroxidaseproductie in *Aspergillus* beïnvloedt. Door het toevoegen van heem aan het kweekmedium (in de vorm van de hemine of het eiwit hemoglobine), kon de productie van recombinant MnP en CPO met een factor 7 tot 10 worden verhoogd. Indien alleen ijzer-ionen (zoals die ook in heem aanwezig zijn) of een niet heem-bevattend eiwit aan het medium werd toegediend, bleef de productie laag.

Door middel van *in vivo* assays kon worden aangetoond dat heteroloog geproduceerd MnP kan concurreren voor het in de cel beschikbare vrije heem met homologe intracellulaire heem-bevattende eiwitten. Deze waarnemingen geven aan dat de overproductie van heem peroxidases in *Aspergillus* onder andere gelimiteerd kan worden door onvoldoende aanwezigheid van heem in de cel. Factoren zoals heem-synthese, transport in de cel of de incorporatie van heem in het peroxidase zouden hierbij een rol kunnen spelen.

Heterologe productie van MnP leidt tot de inductie van twee genen die coderen voor de moleculaire chaperone-eiwitten calnexin en Binding Protein (BiP) (Hoofdstuk 8). Moleculaire chaperones komen onder andere voor in het endoplasmatisch reticulum (ER) van eukaryote organismen en spelen daar een rol in de translocatie, vouwing en kwaliteitscontrole van de eiwitten die de secretieroute ingaan, maar ook in het verwijderen van niet of slecht gevouwen eiwitten. Indien de productie van heem peroxidases in *Aspergillus* gelimiteerd wordt door problemen in de vouwing van deze eiwitten in het ER, zou de heterologe peroxidaseproductie kunnen worden verhoogd door middel van overexpressie van één of meer van de genoemde cellulaire chaperones. Uit onze studie blijkt dat de overexpressie van BiP in een MnP producerende stam leidt tot een afname van extracellulair MnP, wat

suggereert dat BiP een effect heeft op de degradatie van (ongevouwen) MnP moleculen. Calnexin overexpressie daarentegen resulteerde in een 4-5 maal hogere productie van MnP. Dit duidt op een mogelijk rol van calnexin in de MnP biosynthese. Het positieve effect van calnexin overexpressie op de MnP productie werd echter niet waargenomen indien extra heem aan het medium werd toegevoegd. Op basis van deze resultaten veronderstellen wij dat onder heem gelimiteerde condities extra calnexin kan helpen bij de incorporatie van de heem groep in het peroxidase, terwijl bij voldoende heem extra calnexin geen effect meer heeft.

Het onderzoek beschreven in dit proefschrift heeft een aantal aspecten belicht die van invloed zijn op de overproductie van peroxidases in *Aspergillus*. Adequate stamselectie, heem supplementatie, calnexin overexpressie en gecontroleerde fermentatie bleken methoden om de productie van peroxidases te verhogen. Elk van de methoden afzonderlijk levert een 4 tot 10 keer hogere productie op. Door enkele van bovengenoemde methoden te combineren zal naar alle waarschijnlijkheid een verdere productieverhoging bereikt kunnen worden. Het valt verder te verwachten dat ook de ontwikkeling van genoom-brede benaderingen ("genomics"), zoals die momenteel voor schimmels worden ontwikkeld, een bijdrage kanleveren om verschillende processen die van invloed zijn op de productie van peroxidases te leren begrijpen en om nieuwe aangrijpingspunten te identificeren voor de verbetering van de productie.

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CURRICULUM VITAE

Op 9 oktober 1968 ben ik, Ana Conesa, in Cartagena (Spanje) geboren. Na afloop van mijn middelbare schoolopleiding aan het Instituto Isaac Peral te Cartagena ging ik in 1986 naar de Technische Universiteit van Valencia om Landbouwwetenschappen te studeren. Gedurende mijn 7-jarige opleiding heb ik stage gelopen bij het Instituto Valenciano de Investigaciones Agrarias (Valencia), en bij de Vakgroep Plantenveredeling van de Wageningen Landbouwuniversiteit te Wageningen. Als deel van mijn afstudeeropdracht heb ik binnen het veredelingsbedrijf INTERLEGSA (Valencia) gewerkt aan de veredeling van snijbonen voor virusresistentie. In september 1993 studeerde ik af in de richting Plantenproductie en Veredeling. Daarna ben ik, in het kader van het Europese Comett Project, voor hegen maanden naar het toenmalige DLO Instituut CPRO te Wageningen gegaan, waar ik onderzoek heb verricht naar de ontwikkeling van een moleculaire merkers systeem voor lelie, gebaseerd op microsatellite sequenties. Aansluitend heb ik ruim een jaar gewerkt op het Laboratorium voor Monoklonale Antistoffen (Wageningen) aan het "Plantibody" project voor ontwikkeling van resistentie in aardappel tegen het aardappelcystaaltje. Van maart 1996 tot februari 2000 was ik als BSDL AIO verbonden aan de afdeling Moleculaire Genetica en Gen Technologie van TNO Voeding, onder begeleiding van prof.dr. C.A.M.J.J. van den Hondel en dr. P.J. Punt. Het aldaar verrrichte onderzoek heeft geleid tot dit proefschrift. Vanaf januari 2001 ben ik werkzaam als post-doc bij de afdeling Verklarende Toxicologie van TNO Voeding te Zeist.

noemen die staan voor de mooie herinneringen van deze AIO-tijd. Egbert, bedankt voor de gezelligheid binnen en buiten het werk en het gezelschap tijdens de extra uren. En zet 'm op, voordat je het weet ben je ook bezig met het schrijven van je eigen dankwoord. Margreet, ik zal nooit vergeten dat jij degene bent die me heeft geleerd om *Aspergillus* te transformeren. Peter Pouwels, *gracias señor profesor* por salvarme de la pesadilla de los stellingen. Arthur, bedankt voor je hulp bij het zoeken van literatuur voor hoofdstuk 2. Ook de hartelijke groeten aan de rest van de Leidse club. Jeroen, je werd een soort stagiair met terugwerkende kracht. Bedankt, ook Alwin en Nick, voor het fermentatiewerk dat beschreven staat in hoofdstuk 3. Eric, thank you very much for your help and tips during the purification of rLiP. Annette, you became one of my "native English speakers" and gave me back the faith in my written English. Eichii, ik wacht nog op die paëlla van je. Christien, Cora en Marian, bedankt voor de fijne werksfeer en jullie bijdrage aan mijn zwanger- en moederschapsuitzet. En natuurlijk Jan, Heleen, Rob, Paula, Hans, Robert, Anneke, Renate, Roy, Bernhard, Joanne, Marion, Wietske, Annemiek, Frank, Hanny, Mariët, eigenlijk iedereen die ik heb meegemaakt bij MGG, of AMGT, of wat voor naam die de afdeling ooit zal krijgen. Jullie met z'n allen hebben voor stelling 10 gezorgd. Ik vertrouw erop dat deze bijzondere sfeer jullie zal blijven vergezellen door alle mogelijke reorganisaties en verhuizingen heen, en dat ik er af en toe nog eens van mag genieten. *Gracias, compañeros.*

Buiten de afdeling wil ik ook een aantal mensen bedanken. Prof. Hans Duine, hoewel ons contact zich heeft beperkt tot het begin van mijn promotieonderzoek, zal ik de leerzame discussies over peroxidases niet vergeten. Ik wil u en Barend Groen graag bedanken voor de samenwerking tijdens de zuivering van CPO. Fred van de Velde, beste Fred, je kwam naar ons toe omdat je een ruimte nodig had voor het kweken van je *Caldariomyces* mutanten en dit leidde tot een zeer fijne samenwerking. Zonder jouw bijdrage was hoofdstuk 5 en de bijhorende publicatie zeker niet mogelijk geweest. Ook Prof. Roger Sheldon en Dr. Fred van Rantwijk hartelijke dank voor het mogelijk maken van deze samenwerking. Ron van Dool wil ik ook graag bedanken voor zijn enthousiaste hulp bij het zuiveren van rLiP en rMnP. Aan jullie allen: *gracias colegas.*

Y por supuesto, mis padres. Esta tesis está dedicada a vosotros. Sin la capacidad de trabajo y el sentido de la responsabilidad que desde siempre me habeis inculcado no hubiera sido posible llevarla a buen término. Sé que se os hace difícil el que todo esto ocurra en un país tan lejos. Me consuela saber que, pese a la distancia, cada vez estamos mas cercanos. *Gracias papás.*
Julia, si no hubieras sido una bebé tan requetebuena y tranquila, aún estaría desesperándome en alguna parte de los capítulos anteriores. Perdóname, además, las para mí muchísimas horas que esta tesis nos ha robado de estar jutnas. Ahora nos toca a nosotras. *Gracias, mi vida.*
En tenslotte Peter. *Gracias, xiki* voor je grote steun, de continue zorg en lastige vertalingen naar het Nederlands. We hebben twee promoties overleefd. We kunnen alles aan.

Utrecht, 26 april 2001

NAWOORD

Het nawoord is het gedeelte van een proefschrift dat met de meeste voldoening wordt geschreven, ten minste, zo is het bij mij. Niet alleen omdat hiermee het schrijven zijn voltooiing nadert, maar vooral omdat dit de mogelijkheid geeft om te voorzien in een grote behoefte iedereen je dankbaarheid te betonen. Als je bovendien je dankwoord zit te schrijven na een weekendje Parijs, loop je misschien het risico dat het hele stuk behoorlijk sentimenteel wordt. Maar ja, daarom juist het losse velletje!

Als eerste wil ik mijn promotor bedanken. Beste Cees, toen ik in de winter van 1996 bij jou binnenkwam, geloofde je dat ik dit promotieonderzoek aankon. Dat heeft toen voor mij heel veel betekend (misschien zelfs dat ik nu hier zit en niet zuidelijker). Gedurende deze vier jaren wist je altijd een gaatje te vinden in je drukke agenda om me weer op het juiste pad te zetten, me te stimuleren in het onderzoek, en me van genoeg "ideeën" te voorzien tot het volgende gaatje. Je wens dat ik, na afloop van mijn AIO-tijd, bij TNO zou blijven, was voor mij weer van veel betekenis. En hoe dan ook, het is je toch weer gelukt! *Gracias jefe.*
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Nicole, je mag je met dit boekje -en niet zo maar een beetje- ook gepromoveerd voelen. Bijzonder en overvloedig is je bijdrage geweest, niet alleen aan de technische en letterkundige inhoud van dit proefschrift, maar vooral aan het psychologische welzijn van de promovenda. Ik ben heel blij dat we het auteurschap van een hoofdstuk hebben kunnen delen, ook al onthult dit slechts het puntje van de ijsberg van al je hulp. Er is geen taal die voldoende uitdrukking kan geven aan mijn dankbaarheid. Neem dan maar deze eenvoudige woorden: *gracias, amiga mía.*
Ik wil ook Vief ontzettend bedanken voor de fijne babbeltjes (wetenschappelijk of niet), de computertips en het helpen met de proefjes toen ik "niet meer werkte". Het werd dus snel duidelijk wie mijn andere paranimf zou moeten worden. *Gracias, Fifita.*
Y por supuesto no me olvido de tí, Bea. ¡Qué suerte el haberte tenido a mi lado durante la major parte de la tesis! Y no sólo por el poder desahogarme en cristiano contigo cuando los experimentos se me atascaban, si no, sobre todo, por lo pedazo de ejemplo científico y humano que eres. Espero que el contacto nunca lo perdamos y que sigamos viéndonos de vez en cuando en cualquier parte del Europa, o del extranjero. *Gracias, paisana.*
Ook een speciaal dankwoord voor "mijn" stagiaires, Gerri en Jeffrey. Wat heb ik met jullie geboft, zeg! Slim, ijverig, leuk... Gerri, ik moet je bedanken voor je grote inzet bij het moeilijke C-terminus onderzoek en voor de zorgvuldige analyse van de vele CPO trafo's. Jeffrey, hoewel ik weet dat je de voorkeur (en de magische vingers) had voor het DNA werk, heb je ook een uitstekende bijdrage weten te leveren bij de analyse van de heem-limitatie. *Gracias, pupilos.*

Als promovendus werk je, gelukkig, binnen een groep. Van de afdeling waarin ik het genoegen heb gehad om mijn promotieonderzoek te kunnen uitvoeren, wil ik graag een aantal mensen

8. Een voorwaarde voor de succesvolle toepassing van nieuwe, *holistische* benaderingen bij het bestuderen van biologische processen is dat wetenschappers niet alleen krachtige analytische technieken ontwikkelen maar bovenal weer op het niveau van het volledige organisme leren te denken.

Kanehisa and Goto (1997) Nucleic Acids Res ***28****, 27-30*
Nakao et al. (1999) Genome Inform Ser, Workshop Genome Inform ***10****, 94-103*

9. Het gebruik van pindakaas tijdens de zwangerschap of gedurende de eerste drie levensjaren van het kind verhoogt het risico voor het kind om voedselallergie op te lopen en dient, daarom, te worden ontraden.

Frank et al. (1999) Pediatr Allerg Immunol ***10****, 27-32*

10. Succesvolle integratie in een buitenlandse samenleving hangt met name af van het vermogen van collega's op het werk om een thuisgevoel te creëren.

11. Het verschil tussen de bevalling van een kind en die van een proefschrift is dat de tweede het einde is en de eerste juist het begin van vele slapeloze nachten. *Moraal: wie een promotieonderzoek laat aansluiten op het ouderschap zorgt voor een langdurig slaapgebrek.*

12. En este mundo traidor nada es verdad ni es mentira, todo es según el color del cristal con que se mira.

Gila (Spaanse cabaretier)

STELLINGEN

behorende bij het proefschrift

"Overproduction of fungal peroxidases in filamentous fungi"

1. De overproductie van heem-bevattende peroxidases in filamenteuze schimmels wordt beperkt door de cellulaire beschikbaarheid van heem.
   Dit proefschrift

2. De verscheidenheid aan effecten op de productie van heterologe eiwitten als gevolg van een verhoogde synthese van chaperone eiwitten, weerspiegelt de complexiteit van de chaperone functies en de specificiteit van de chaperone-eiwit interacties.
   *Molinar and Helenius (2000) Science **288,** 331-333*
   *Harmsen et al. (1996) Appl Microbiol Biotechnol **46**, 365-370*
   Dit proefschrift

3. De conclusies van Rai *et al.* met betrekking tot de biochemische eigenschappen van CPO mutant eiwitten geproduceerd in *Caldariomyces fumago* zijn voorbarig, omdat niet is aangetoond dat het endogene CPO afwezig is in de geanalyseerde preparaten.
   *Rai et al. (2000) Israel J Chem **40**, 63-70*

4. De conclusie van Erold *et al.* dat een *A. niger* ALAS null mutatie niet kan worden gecomplementeerd door het toevoegen van heem aan het kweekmedium omdat heem slecht wordt opgenomen, is voorbarig aangezien er geen rekening wordt gehouden met mogelijke pleiotrope, lethale effecten van een ALAS mutatie. De conclusie is bovendien in strijd met de resultaten beschreven in dit proefschrift.
   *Elrod et al. (2000) Curr Genet **38**, 291-298*
   Dit proefschrift

5. De uitdrukking "efficiënte eiwitproductie" zegt vaak weinig over het feitelijke productierendement, en meer over de productieniveaus behaald door concurrerende laboratoria.

6. Voor een goede *Aspergillus* transformatie heb je een beetje PEG nodig.

7. Het gebruik van "*metabolomica*" -dit is de biochemische analyse van het volledige repertoire van cellulaire metabolieten- biedt nieuwe en interessante mogelijkheden voor functionele genoom analyse: Raamsdonk *et al.* hebben laten zien dat de rol van genen waarvan uitschakeling geen merkbaar effect heeft op de celgroei toch kan worden onthuld door het vaststellen van het effect van dergelijke mutaties op de concentratie van twee of meer metabolieten.
   *Raamsdonk et al. (2001) Nature Biotechnol **19**, 45-50*
   *Cornish-Bowden and Cárdenas (2001) Nature **409**, 571-572*