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Para mí sólo recorrer los caminos que tienen corazón,
cualquier camino que tenga corazón. Por abi yo recorro,
y la única prueba que vale es atravesar todo su largo.
Y por abi yo recorro mirando, mirando, sin aliento.

(Voor mij is er alleen het reizen langs wegen met hart,
langs elke weg die maar hart heeft. Daar reis ik,
en de enige uitdaging die maar de moeite loont is,
hem over zijn hele lengte te bereizen.
En daar reis ik – kijkend, ademloos kijkend.)

- De lessen van Don Juan, Carlos Castaneda -

Voor mijn ouders

Contents

Chapter 1

General Introduction

INTRODUCTION

Biotechnology provides the opportunity to generate and use genetically modified microorganisms for the production of several compounds like antibiotics, enzymes for the food and chemical industry, organic acids, amino acids and flavour compounds. Currently, in biotechnology new technologies are used to produce all kinds of improved enzymes, leading to more stable enzymes or enzymes with enhanced activity (Cherry *et al.* 1999). For example, different variants of *Fusarium solani* f. sp. *pisi* cutinase have been constructed and were analysed for improved lipase activity (van Gemeren *et al.* 1996; Sagt *et al.* 1998). In addition to enzyme engineering, also novel enzymes with unexplored catalytic properties have been discovered. Examples are newly discovered cellulases and lipases (Falch 1991) and a novel type of dioxygenase (Kooter *et al.* 2002).

Enzymes are already frequently used for industrial applications, although many enzymes have a broad substrate specificity and their use can result in non-specific activity. Furthermore, certain applications demand that enzymes are available in large quantities during the catalytic processes. This requires a cost-effective large-scale production process for enzymes of interest. The construction of bifunctional proteins consisting of a substrate-recognising moiety and an enzyme (the effector molecule) is hypothesised to improve the enzyme's specificity for a certain substrate and to enrich the enzyme concentration at the target-site. If this bifunctional protein can subsequently be produced cheaply in large quantities by industrial relevant micro-organisms such as filamentous fungi, this may lead to improvement of established enzyme applications and to novel applications of enzymes, such as in consumer products.

THE "MAGIC BULLET" APPROACH

Antibodies are glycoproteins that have the unique ability to specifically react with foreign molecules (antigens) during an immune response in humans and mammals. As a result of this response, micro-organisms, larger parasites, viruses and bacterial toxins are rendered harmless. A particular antibody has the ability to bind to one or more specific antigens. This unique ability of antibodies to specifically recognise and bind with high affinity to virtually any type of antigen, makes them interesting molecules for medical and scientific research.

Coupling of antibodies to enzymes, leading to bifunctional proteins (in this thesis also called "Magic Bullets[*]" or fusion proteins) allows interesting applications due to the fact that antibodies can direct the coupled enzyme to the place where it should act. The "Magic Bullet" approach was in first instance developed in the medical field with the main purpose to direct anti-tumour agents towards sites of malignant growing cells by means of antibodies that recognise these cells (Reiter 2001; von Mehren and Weiner 1996). Application of antibodies and bifunctional proteins in other fields as *e.g.* consumer products like detergents, toothpaste, deodorants and shampoos has been less exploited yet. However, this type of application requires that antibodies or bifunctional proteins can be produced cheaply and in large

[*] Paul Ehrlich (1854-1915) envisioned the creation of "Magic Bullets", which are compounds that would have a specific attraction to disease-causing micro-organisms. These "Magic Bullets" would seek out these organisms and destroy them, avoiding other organisms and having no harmful effects on the bodies of patients. He used the expression "Magic Bullet" for the first time in his Harben Lectures.

amounts. To investigate whether large scale and cheap production is feasible, research has been initiated to study the production of antibodies and bifunctional proteins by industrial relevant micro-organisms, such as filamentous fungi.

BIFUNCTIONAL PROTEINS AND THE IMPROVEMENT OF DETERGENTS

Although much improvement of the performance of detergents has been achieved over recent years, removal of persistent spots or stains on laundry (blood, grass, tea, tomato, wine and fruit) is still a major problem and can only be achieved by bleaching methods, either chemically or enzymatically. As a consequence, relatively high amounts of chemicals have to be added to detergents, whereas the removal of persistent spots also requires temperatures of 40 °C or higher. Furthermore, a major disadvantage of the present detergents is the non-specific bleaching of the spots, which results in fading of the textile colour, transmission of colour and harmful effects on textile itself.

The main cause of persistent spots are heterocyclic aromatic compounds. Oxidation by bleaching components results in disruption of the ring structure, which in turn also causes bleaching of the colour. By using bleaching enzymes in detergents, which are already fully active at relatively low temperatures, less energy is required during the washing cycle (Falch 1991). Furthermore, relatively low doses of enzymes are sufficient to remove various stains and spots on laundry during the washing cycle.

Hydrolytic enzymes that are commonly used in detergents are cellulases, proteases, amylases and lipases. Cellulases prevent formation and remove fuzz and pills and provide colour brightness to cellulose-based fabrics, such as cotton. Proteases are effective in cleaning protein stains, like blood, grass and milk. Amylases remove starch-based stains such as chocolate, gravy, rice and pasta. For further information about enzymes in detergent see www.novozymes.com and www.genencor.com.

An example of an interesting application of bifunctional proteins could be the improvement of detergents by specific targeting of oxidising enzymes to persistent spots in order to remove them by their oxidising capacity. It was hypothesised that by genetically coupling of spot-recognising units to bleaching enzymes (so-called chimeric redox-enzymes) only bleaching of the spot would be accomplished, leaving the rest of the textile untouched. Enzymes are completely biologically degradable and are active at relative low temperature. Moreover, efficient targeting would result in relatively high concentrations of the effector molecule at the site of action, resulting in a lower required dose of effector molecules and subsequently a reduction of the consumed detergent. Furthermore, generation of detergents capable of removing difficult spots by the addition of bifunctional proteins will probably also result in the use of fewer chemical-bleaching components in detergents.

Specific targeting to certain spots requires a molecule that recognises and binds to it. Protein molecules that are exceptionally suited for this application are antibodies.

LAMA VARIABLE HEAVY-CHAIN ANTIBODY FRAGMENTS (V_{HH}s)

Conventional IgG antibodies contain two identical heavy (H) chains and two identical light (L) chains (see Figure 2 in Chapter 2), which are held together by a combination of non-covalent and covalent (disulfide) bonds (see for a review on antibody structure Padlan, 1994). The heavy and light chains themselves consist of variable and constant domains. At the N-terminal part of the antibody molecule the variable domains of the heavy and light chains (V_H and V_L, respectively) are located. These domains are extremely variable in amino acid sequence and form together the unique antigen-recognition site of the antibody. The amino acid sequences of the remaining C-terminal constant domains are more conserved between the different antibodies and are called C_H1, C_H2, C_H3 and C_L.

For many 'new' applications entire antibodies are not necessarily required and in many cases only their binding domains may be preferred because of their smaller size. The development of recombinant DNA technology and the increasing knowledge on the structure of antibody molecules have made it possible to clone and engineer smaller fragments of antibody genes (Winter *et al*. 1994) and subsequent alter their functions, for example to improve the affinity for their antigen. Besides that, recombinant DNA technology provides the possibility to generate bifunctional molecules.

A novel class of IgG antibodies was discovered in *Camelidae* (camels, dromedaries and llamas) by Hamers-Casterman *et al.* (1993). These antibodies are devoid of light chains and are referred to as 'heavy-chain' IgGs or HCAb (see Figure 2 in Chapter 2). Their binding domains consist only of the heavy-chain variable domains, called V_{HH}s (Muyldermans and Lauwereys 1999). These V_{HH}s are of great interest since they comprise the smallest possible recognition units of antibodies. They have a molecular mass of ~15 kDa (Sheriff and Constantine 1996; Muyldermans 2001) and are simple in structure. Furthermore, they were shown to posses excellent binding affinity for their antigen (Lauwereys *et al.* 1998; van der Linden *et al.* 1999). Besides that, V_{HH}s were shown to be highly soluble and stable under harsh conditions (Ghahroudi *et al.* 1997), even at temperatures as high as 90 °C (van der Linden *et al.* 1999). The thermal unfolding of V_{HH}s at high temperatures was shown to be (partially) reversible (Perez *et al.* 2001; van der Linden *et al.* 1999). In addition, the extended hypervariable regions of V_{HH}s are capable of penetrating deep into the cleft of active sites of enzymes, binding to novel epitopes that are not recognised by conventional antibodies (Desmyter *et al.* 1996; Transue *et al.* 1998; Decanniere *et al*. 1999). This encouraged the development of V_{HH}s as potent enzyme inhibitors (Lauwereys *et al.* 1998; Conrath *et al*. 2001).

V_{HH}s from llama antibodies are of great interest for biotechnological applications, because of their stability, which makes them for example extremely suitable for the envisaged application in detergents.

PEROXIDASES

Enzymes of major interest for application in detergents are for example oxidases, peroxidases, laccases and dioxygenases. Several of these enzymes are naturally secreted by fungi, for example laccase by *Pleurotus ostreatus* (POX2; Giardina *et al*. 1996), peroxidase by *Arthromyces ramosus* (ARP; Akimoto *et al.* 1990; Kjalke *et al.* 1992), *Acremonium murorum* phenol oxidase (AMO; Gouka *et al.* 2001) and *Aspergillus japonicus* quercetinase (QUER;

Kooter *et al.* 2002). As some peroxidases have good bleaching capacity (Kjalke *et al.* 1992), they are of main interest as components of bifunctional proteins in detergents. The research described in this thesis is focussed on the peroxidase from *Arthromyces ramosus* (ARP).

Peroxidases are heme-containing enzymes that use hydrogen peroxide (H_2O_2) to oxidise a broad range of substrates. They are produced and secreted by a number of organisms, including bacteria, fungi, plants and animals. Their broad substrate specificity, poly-functionality and availability from different sources allows the application of these enzymes in various industrial and biotechnological processes. Especially fungal peroxidases, such as manganese peroxidases (MnPs) and lignin peroxidases (LiPs), are of interest as biological and environmentally friendly alternatives for the highly polluting methods presently used for wood-pulping (pulp delignification; Paice *et al.* 1995) and processes in the paper industry (Sasaki *et al.* 2001). Other applications of fungal peroxidases are the use in detergents, bleaching applications in (textile) industries (Michel *et al.* 1991) and water detoxification or degradation of environmental pollutants (Hammel 1989). For a review on fungal peroxidases, we refer to Conesa *et al.* (2002b) and Martinez (2002).

ARTHROMYCES RAMOSUS PEROXIDASE (ARP)

Arthromyces ramosus peroxidase (ARP) is unrelated to other peroxidases from white-rot fungi (Conesa *et al.* 2002b). The mature protein consists of 344 amino acid residues (see Figure 1), has a molecular weight (MW) of 41 kilodalton (kDa) and contains one iron protoporphyrin IX as a prosthetic group. The heme-containing peroxidase is secreted into the medium under normal conditions in high yields (Morita *et al.* 1988) in contrast to MnPs and LiPs, which are produced under starvation conditions. The enzyme is interesting for industrial applications, such as bleaching (Sawai-Hatanaka *et al.* 1995) and removal of phenolic and anilinic compounds from waste water (Ikehata and Buchanan 2002).

The peroxidase from the ink cap mushroom *Coprinus cinereus* (CiP) is 99 % identical to ARP in amino acid sequence (Baunsgaard *et al.* 1993; Sawai-Hatanaka *et al.* 1995) but differs in its glycosylation (Kjalke *et al.* 1992). One N-linked glycan is present in CiP and for information about other post-translational modifications, see Limongi *et al.* (1995). The crystal structure of ARP has been resolved at 1.9 Å resolution (Kunishima *et al.* 1994) and that of CiP at 2.06 Å resolution (Petersen *et al.* 1994). A schematic drawing of the ARP molecule is given in Figure 2. In ARP two N-acetylglucosamine residues (at Asn^{163}) and the two calcium ions, typical for fungal peroxidases were found. The eight cysteine residues in ARP form four disulfide bridges (C32:C44, C43:C313, C63:C149 and C277:C342). The active site contains a proximal His^{204} and distal of this site the peroxide pocket is present, consisting of a catalytic His^{76} and Arg^{72}. The proximal His^{204} serves as the axial ligand for heme, and the distal residues are proposed to be involved in the charge stabilisation during the reaction of heme with H_2O_2. Although the sequence similarity to other fungal peroxidase is low, the overall folding of ARP is very similar to that of lignin peroxidases (Kunishima *et al.* 1994).

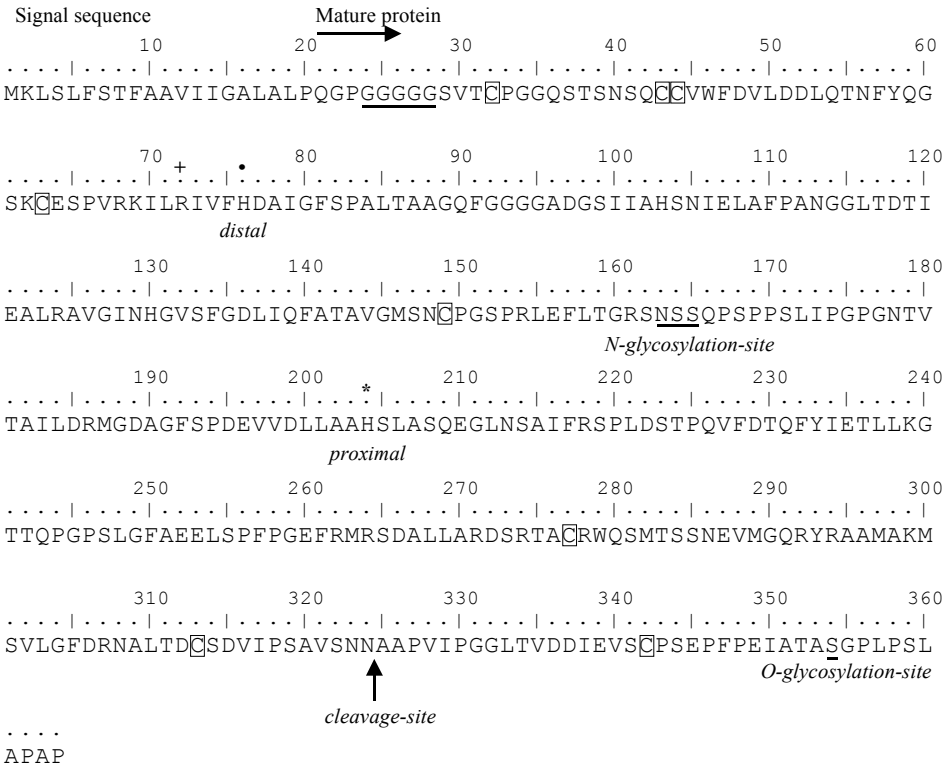

Figure 1. Amino acid sequence of the ARP protein from *A. ramosus* (Swiss-Prot accession number P28313). The sequence of the mature protein, which starts with Q (glutamate), is preceded by a 20 amino acid signal sequence. Disulfide-bond forming cysteines are boxed. The proximal (*) and distal (•) histidine residues and the Arg^{72} (+) residue, are marked. The N-glycosylation-site is indicated and is in *A. ramosus* modified with $Man_7GlcNAc_2$. Also a putative O-glycosylation site is indicated.

It was observed that during cultivation of *A. ramosus*, secreted ARP is cleaved at the C-terminal side of Asn^{324}. This observation was ascribed to the action of proteases that could cleave the bond between Asn^{324} and Ala^{325} in ARP because the side-chain of Asn^{324} is oriented outwards from the molecule (Kunishima *et al.* 1994). This was also observed in samples of commercial CiP, which contained 10-20 % CiP molecules cleaved between Asn^{323} and Ala^{324} (Kjalke *et al.* 1992). However, this cleavage had little effect on protein activity.

The substrate specificity of ARP is different but broader than that of MnPs and LiPs. The reason for this is probably due to the wider access channel to the heme group and the difference in amino acids that comprise this channel (Kunishima *et al.* 1994). This results in different biochemical properties such as the inability to oxidise either the MnP substrate Mn or the LiP substrate veratryl alcohol (Kunishima *et al.* 1994; Sawai-Hatanaka *et al.* 1995) and a neutral, rather than acidic optimal pH (Farhangrazi *et al.* 1994; Kunishima *et al*. 1996).

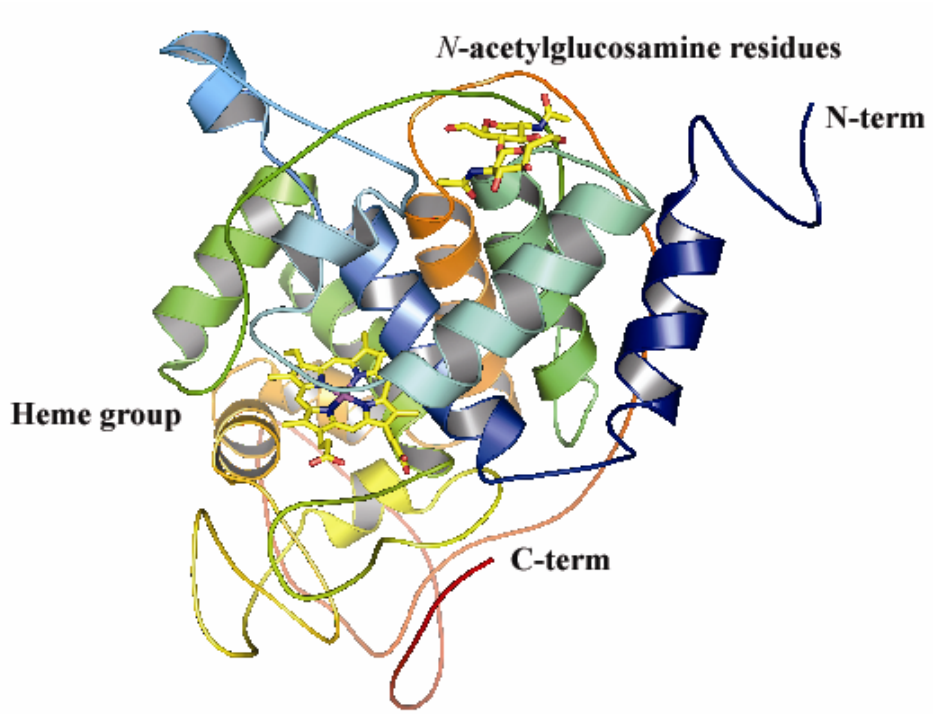

Figure 2. 3D-structure of *Arthromyces ramosus* peroxidase (ARP). The coordinates from the refined 2.06 Å structure (PDB IGZA; Kunishima *et al*. 1996) were used to generate this figure with PyMOL Open-Source software, version 0.97 (http://www.pymol.org).

FILAMENTOUS FUNGI AS CELL FACTORIES FOR HETEROLOGOUS PROTEIN PRODUCTION

Filamentous fungi have been used for ages in production of fermented food, especially in Asia. In addition, filamentous fungi, in particular species from the genera *Trichoderma* and *Aspergillus* have the capacity to secrete large amounts of proteins, metabolites and organic acids into their culture medium. This property has been widely exploited by the food and beverage industries where compounds secreted by these filamentous fungal species have been used for decades. The safe application of filamentous fungal products has led to the GRAS status (Generally Regarded As Safe) for some of their products. Their industrial importance has been proven by the production of a wide variety of important products that ranges from metabolites such as organic acids (*e.g.* citric acid by *A. niger*; reviewed by Bodie *et al.* 1994) and polyketides (*e.g.* statins by *A. terreus*; Manzoni and Rollini 2002) to homologous proteins (*e.g.* α-amylase by *A. oryzae* and glucoamylase by several *Aspergillus* species; Finkelstein 1987).

Since filamentous fungi have a very efficient protein-production capacity, they are suitable host organisms for the overproduction of commercially interesting homologous and heterologous proteins (Punt *et al.* 2002; Radzio and Kuck 1997). Important production species include *A. awamori*, *A. niger* and *A. oryzae*. Furthermore, large-scale fermentation technology for these organisms is well established and optimised fermentation protocols are available to

cultivate them in inexpensive media to high cell densities. There is extensive knowledge on downstream processing of their products and an extensive toolbox for genetic modification of filamentous fungi is available.

Despite the high levels obtained with the production of homologous proteins in filamentous fungi, the production of heterologous proteins is much lower, and in general does not exceed the level of 10-100 milligrams per litre in the culture medium (van den Hondel *et al.* 1991). Factors that can limit protein production are low transcriptional levels, mRNA instability, inefficient translation, post-transcriptional bottlenecks and finally degradation of the protein of interest (reviewed by Gouka *et al.* 1997). Several approaches are available to increase the level of heterologous protein production, for example the generation of protease-deficient-mutants of the fungal production strain or a fusion carrier strategy. The fusion carrier strategy is an approach to overcome limitations in the secretion process of filamentous fungi by fusion of the gene of interest to the 3' end of a gene of which the encoded protein is efficiently secreted. In general, the N-terminal protein is believed to serve as a carrier, improving the translocation of the protein into the Endoplasmic Reticulum (ER), to aid folding and to protect the heterologous protein from degradation. Further along the secretion pathway, in most cases the fusion protein is cleaved (due to the introduction of a proteolytic processing site between the carrier and the protein of interest, which is a target for a KEX2-type protein processing protease; Broekhuijsen *et al.* 1993; Contreras *et al.* 1991), resulting in the secretion of the separate proteins. A well-known example of a carrier protein is glucoamylase (GLA) which is efficiently secreted (Ward *et al.* 1990). A number of successful applications of the fusion carrier strategy to improve heterologous protein production has been described (Broekhuijsen *et al.* 1993; Contreras *et al.* 1991; Keränen and Penttilä 1995). More strategies to improve protein secretion by filamentous fungi are extensively reviewed by Archer *et al.* (1994), Gouka *et al.* (1997) and Verdoes *et al.* (1995).

Although several strategies to improve heterologous protein production in filamentous fungi have been developed, it is still of major importance to extend our knowledge and understanding of the molecular genetics of the fungal secretion pathway to solve remaining bottlenecks. At present, knowledge on the secretion pathway of filamentous fungi is still very limited, although much effort has been spent the last years to elucidate some major bottlenecks of protein secretion (reviewed by Conesa *et al.* 2001). Nowadays several powerful genetic, biochemical and molecular techniques are available for a more detailed analysis of cellular functions in filamentous fungi. For example, the introduction of directed genetic modification in *Aspergilli* may be used to design more efficient "microbial factories" through metabolic engineering (see David *et al.* 2003). The fast developments in the fields of Transcriptomics, Proteomics and Metabolomics will probably give answers on many of our questions concerning the secretion pathway. Recently the genome of *A. niger*, being almost three times larger than the *S. cerevisiae* genome (34.5 Mb), has been completely sequenced by the Dutch company DSM (Groot *et al.* 2002). The genome sequence will contribute to the identification of new gene functions and elucidation of important pathways in filamentous fungi (Archer and Dyer 2004).

Because of its numerous favourable biotechnological properties that have been validated over the years and the fast development in improvement of heterologous protein production, *Aspergillus* might be the preferred host for production of bifunctional proteins.

PRODUCTION OF ARP IN HETEROLOGOUS HOSTS

Analysis of the production level of ARP by its natural host *A. ramosus*, has revealed that it is too low for industrial applications. Therefore, attempts have been made to express ARP in heterologous systems. *E. coli* is very often envisaged as a suitable host for heterologous protein production, but expression of fungal peroxidases resulted in apo-enzymes that are only present in inclusion bodies. Therefore, to obtain active enzymes from inclusion bodies isolation of these enzymes has to be performed under harsh conditions and these conditions are not favourable for industrial production (Whitwam *et al.* 1995). Expression in insect cells resulted in successful production of active MnP but levels were too low and the costs of using this expression system are relatively high when exploited for commercial use (Pease *et al*. 1991). *S. cerevisiae* is also one of the possible candidates and ARP has been expressed in this system, although the production levels were low: 0.02 U/ml using the *gpdA* promoter, as reported by Sawai-Hatanaka *et al.* 1995 and < 0.5 mg/L active rARP using the GAL7 promoter (R.J. Gouka, pers. comm.). Expression of ARP in the methylotrophic yeast *Pichia pastoris* resulted in 21 mg/L of active ARP (R.J. Gouka, pers. comm.), which is still too low for commercial applications.

As described previously, filamentous fungi like *Aspergillus* and *Trichoderma* are suitable hosts for large-scale protein production. Several reports showed that for example MnP (MnP from *P. chrysosporium* and MnPL2 from *P. eryngii*) can be produced in active form in different *Aspergillus* species (Conesa *et al.* 2000; Ruiz-Duenas *et al.* 1999; Stewart *et al.* 1996). However, the levels were similar to that of the parental host.

ARP was successfully produced in *A. oryzae* (~1000 PODU /ml, by using the TAKA amylase promoter and addition of heme to the culture medium; Andersen *et al.* 1992) and *A. awamori* (Lokman *et al.* 2003, Chapters 4 and 7). Surprisingly, very high levels of ARP were produced by *A. awamori*, reaching up to 800 mg/L in the culture medium (Chapter 4). Even higher levels were obtained when heme was added to the culture medium (Chapter 7).

Recently it was shown that with optimising culture conditions (*e.g.* optimised medium and/or fermentation techniques) MnP protein production levels could be increased significantly, indicating that production levels of over grams per litre are within reach (Punt *et al*. 2002). Furthermore, *A. awamori* was shown to be an excellent host for the production of another redox-enzyme, the blue-copper phenol oxidase from *Acremonium murorum* (AMO). Using the GLA-fusion strategy, 600 mg/L AMO was secreted into the culture medium (Gouka *et al.* 2001). This suggests that *A. awamori* is the preferred host for the large-scale production of heterologous proteins such as redox-enzymes and heme-containing proteins like ARP.

One major concern for the production of heme-containing peroxidases in *Aspergillus* is the requirement of heme supplementation of the culture medium. MnP production studies in a protease-deficient *A. niger* strain yielded 10 mg/L, which could be increased up to 70 mg/L with hemin and even up to 100 mg/L with hemoglobin supplementation of the culture medium (Conesa *et al.* 2000). MnPL2 from *P. eryngii* (showing both MnP and LiP peroxidase activity) was produced in *A. nidulans* in amounts up to 150 U/L, but only in the presence of hemin in the medium these peroxidase production levels could be obtained in both minimal and complete media (Ruiz-Duenas *et al.* 1999). Another example where heme addition had a significant positive effect on produced peroxidase is that of *Calderomyces fumago* peroxidase (CPO). Hemin supplementation resulted for the first time in an active enzyme, reaching a level up to 10 mg/L (Conesa *et al.* 2001b).

BOTTLENECKS FOR OVERPRODUCTION OF HETEROLOGOUS PROTEINS BY FILAMENTOUS FUNGI

CHAPERONES AND THE UNFOLDED PROTEIN RESPONSE (UPR)

Proteins that are destined to be secreted enter the ER through a pore in the ER membrane (known as Sec61p) and are subjected to a series of post-translational modifications, including folding and maturation. In the lumen of the ER, several folding assistants are present, like folding enzymes and molecular chaperones (*e.g.* the molecular chaperone BiP; Pedrazzini and Vitale 1996; Gething 1999). When the nascent protein is properly modified it can leave the ER by means of vesicle budding and is transported to the Golgi apparatus for further modifications and subsequently secreted through vesicle fusion with the plasma membrane. The folding assistants are also involved in other processes such as translocation into the ER, Quality Control (reviewed by Sitia and Braakman 2003) and ER-associated degradation (ERAD; reviewed by Brodsky and McCracken 1999).

Accumulation of unfolded or misfolded proteins (due to inefficient folding capacity of the cell or stress-causing agents), an increased load of proteins destined for secretion, altered metabolic conditions and depletion of Ca^{2+} can activate an intracellular signalling pathway referred to as the Unfolded Protein Response (UPR; Kaufman 1999; Rutkowski and Kaufman 2004). As a consequence, expression of genes encoding chaperones and foldases is induced to increase the folding capacity (reviewed by Chapman *et al.* 1998; Conesa *et al*. 2001a; Welihinda *et al*. 1999) and thereby limiting the accumulation of unfolded or misfolded proteins in the ER (discussed later). Furthermore, also other genes involved in the secretion pathway are induced (Travers *et al.* 2000).

Most information available on UPR is derived from studies on *S. cerevisiae* and mammalian cells, although the last few years much effort has been put in understanding the UPR pathway of filamentous fungi (Conesa *et al*. 2001a and Figure 3).

In non-stressed cells BiP is bound to the transmembrane protein kinase/ribonuclease Ire1p, which acts as the stress-sensor (Cox *et al.* 1993; Mori *et al*. 1993; Bertolotti *et al.* 2000). When unfolded, unassembled or aberrant proteins accumulate in the ER, BiP is believed to bind to the exposed hydrophobic patches in unfolded nascent proteins (see Figure 3) and thereby prevents aggregation before proper folding or subunit assembly has occurred. The tight binding of BiP requires ATP hydrolysis. As a consequence of BiP dissociation, Ire1p is activated by oligomerisation and autophosphorylation (Okamura *et al.* 2000; Welihinda *et al.* 1996). Activated Ire1p then splices an unconventional intron from *HAC1* mRNA. *HAC1* mRNA encodes a basic leucine zipper transcription factor Hac1p (Cox and Walter 1996). After splicing, the exons are rejoined with the help of the tRNA ligase Rlg1p (Sidrauski *et al.* 1996). Hac1p subsequently binds to a consensus region in the promoters of UPR target genes and activates their transcription.

The *HAC1* homologues from *A. niger* (*hacA*), *A. nidulans* (*hacA*) and *Trichoderma reesei* (*hac1*) were recently cloned (Mulder *et al.* 2004; Saloheimo *et al*. 2003). In *A. niger* stress was shown to splice a 20 nucleotides-long intron from *hacA* mRNA and induced truncation of the 5' upstream region of the *hacA* mRNA. This truncation removes an upstream open reading frame from the *hacA* mRNA, which is involved in translational regulation of the gene.

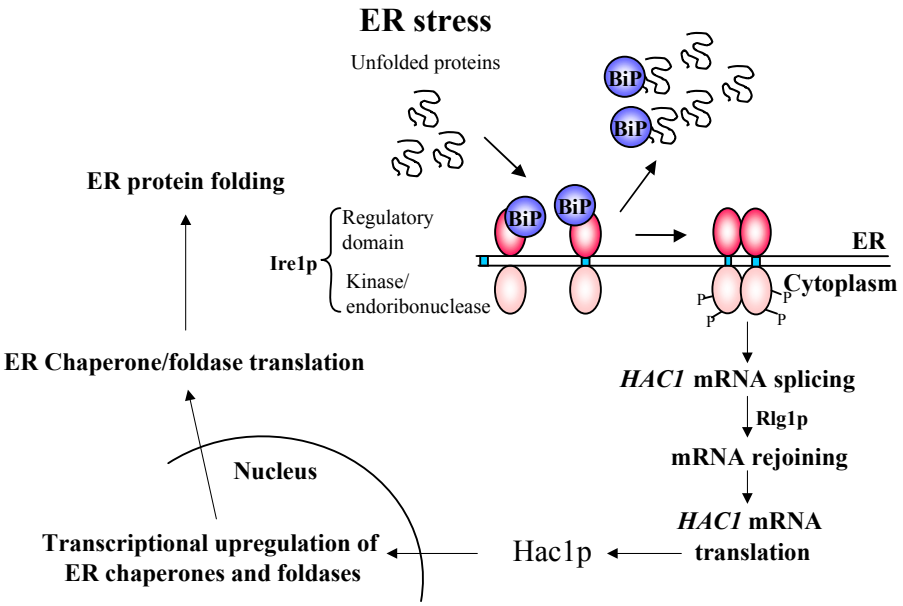

Figure 3. Schematical overview of the UPR. Upon accumulation of unfolded proteins in the ER, BiP dissociates from Ire1p, which induces the UPR. The Ire1p ER lumenal site is the ER stress 'sensor' domain and the effector domain is located on the cytosolic side of the membrane. The effector domain of Ire1p has kinase and endoribonuclease activity. ER stress results in release of BiP, which causes Ire1p to oligomerise, trans-autophosphorylate and activate its effector domain. The endoribonuclease activity of Ire1p is subsequently responsible for splicing of the *HAC1* mRNA, which encodes a basic leucine zipper-containing transcription factor. Upon ligation of its transcript by Rlg1p, Hac1p is synthesised and serves to activate UPR genes in the cell nucleus. For further details, see text.

After non-spliceosomal based translation of *HAC1* mRNA (Cox and Walter 1996; Mori *et al.* 1996), Hac1p directly activates UPR genes (such as ER chaperones) in the cell nucleus. The promoters of these UPR genes contain the typical UPRE sequence (Unfolded Protein Response Element). As a result of induction of UPR genes, several processes are initiated (*e.g.* up-regulation of protein folding, up-regulation of degradation pathways, inhibition of protein synthesis). The transcription of genes involved in ER-associated degradation is also upregulated by Hac1p (Casagrande *et al.* 2000; Friedlander *et al.* 2000).

Mulder *et al.* (2004) showed that *A. niger* HACA was able to bind to the UPRE in the promoter sequence of several ER chaperones and foldases. Furthermore, overexpression of the processed form of *hacA* in *A. niger* resulted in an induction of the genes encoding the ER chaperone BipA, the foldases peptidyl-prolyl cis-trans isomerase CYPB and protein disulfide isomerase PDIA, and HACA itself.

In filamentous fungi, expression of the genes encoding the molecular chaperone BiP and a PDI-related foldase was induced upon treatment with UPR-causing agents (Ngiam *et al.* 2000; Saloheimo *et al.* 1999; van Gemeren *et al.* 1997), such as tunicamycin and dithiothreitol (DTT). Tunicamycin disturbs the N-glycosylation process in the ER and DTT is a reducing agent, which prevents the formation of disulfide bonds. Moreover, overexpression of the heterologous protein tissue plasminogen activator (t-PA) and treatment of mycelium

with DTT or tunicamycin induced UPR in *A. niger* (Mulder *et al.* 2004; Wiebe *et al*. 2001). PDI was induced 2-3 fold upon Fab antibody fragment expression in *T. reesei* (Saloheimo *et al*. 1999). Concomitant to activation of the UPR pathway, Pakula *et al.* (2003) and Al-sheikh *et al.* (2004) reported a decrease in transcript levels of secreted proteins in *T. reesei* and *A. niger,* respectively, upon treatment with agents that inhibit protein transport.

Also *A. niger clxA*, encoding calnexin, was induced upon heterologous overexpression of bovine prochymosin (Wang *et al.* 2003) and MnP (Conesa *et al.* 2002a). The lectin-like proteins calnexin and calreticulin are chaperones that interact with nascent and newly synthesised glycoproteins in the ER and function in Quality Control by aiding folding (Parodi 1999). Calnexin specifically recognises mono-glycosylated glycoproteins in the ER, and is thus an essential component of the process that assesses the folded state of nascent secreted proteins (reviewed by Ellgaard and Frickel 2003). Furthermore, an important role is postulated for calnexin in ERAD (McCracken and Brodsky 1996).

As overexpression of heterologous proteins often induces a UPR, it seems likely that the folding capacity of the cell can not cope with the large amount of unfolded proteins in the ER (reviewed by Cudna and Dickson 2003). This may be the main reason for the low levels of heterologous proteins secreted by filamentous fungi (reviewed by Conesa *et al*. 2001a). An approach to increase the folding capacity of the cell is the overexpression of chaperones and foldases. However, this led to varying results in diverse heterologous hosts. Concerning filamentous fungi, most papers reported the failure of improvement of heterologous protein secretion (Punt *et al.* 1998; Wang and Ward 2000; Ngiam *et al.* 2000) although recently, Conesa *et al*. (2002a) reported increased secretion of MnP upon overexpression of *clxA*, encoding calnexin in *A. niger*. Although MnP overexpression resulted in induction of *clxA* and *bipA* transcript levels, *bipA* overexpression reduced MnP secretion (Conesa *et al.* 2002a). Even more, hemin addition reduced the effect of *clxA* overexpression. As hypothesised by Conesa *et al*. (2002a), a positive effect on heterologous protein secretion may be specific for the chaperone-protein system analysed. Furthermore, calnexin was suggested to be involved in the proper folding of MnP and heme incorporation.

HEME

A pre-requisite for the production of active heme-containing enzymes in heterologous hosts is that the host is able to incorporate heme, produced by the heme biosynthetic pathway, into the protein. Heme is important for both the conformation and activity of the enzymes. Fungal peroxidases contain protoporphyrin IX (heme *b*) as a prosthetic group. See Conesa *et al.* (2002b) for further information on the coordination of heme in fungal peroxidases.

Although little information is available on heme biogenesis in filamentous fungi, it is likely that a similar pathway is present as in yeast and higher eukaryotes. The heme biosynthetic pathway, which is tightly controlled by glucose, oxygen and heme itself, is reviewed by Padmanaban *et al.* (1989) and Dailey (1997). The formation of heme in all organisms starts with the synthesis of δ-aminolevulinic acid (ALA) in the mitochondria (see Figure 4). The enzyme 5-aminolevulinate synthase (ALAS) catalyses the condensation of succinyl-CoA and glycine to yield ALA in mitochondria of a variety of species, including fungi (reviewed by Ferreira and Gong 1995). The next step is the synthesis of a substituted pyrrole compound (porphobilinogen; PBG) from ALA, catalysed by ALA dehydratase. Four PBG molecules are then condensed to yield the porphyrin ring by the enzyme porphobilinogen deaminase. Finally, the side chains are modified and the ring system is

dehydrogenated yielding protoporphyrinIX (PpIX). PpIX is subsequently converted into heme by ferrochelatase by the addition of iron.

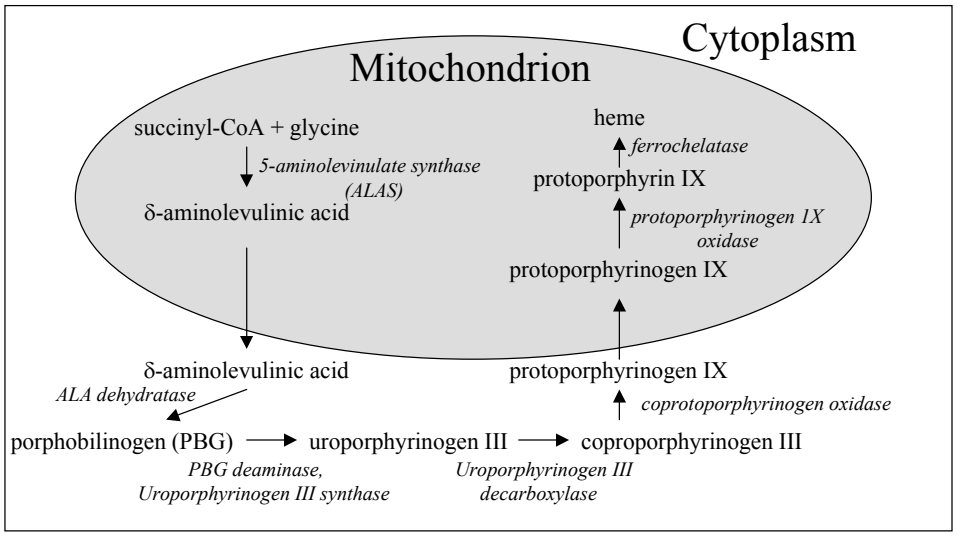

Figure 4. Heme biosynthetic pathway in the yeast *Saccharomyces cerevisiae*. For further details, see text and Labbe-Bois and Labbe (1990).

ALA synthase is the committed step in the heme biosynthesis pathway of bacteria and mammals, and is usually rate-limiting for the overall pathway. Intracellular levels of both heme and iron play critical roles in the tightly controlled feedback inhibition of ALAS, since heme-regulatory elements have been found within the ALAS sequence (Ades *et al.* 1987). In contrast, yeast *hem1* encoding ALAS is constitutively expressed and regulation takes place in the second step of the pathway through ALA dehydratase (Labbe-Bois and Labbe 1990). Concomitant, for *A. nidulans* it was found that *hemA* was transcriptionally unaffected by C-source repression, heat shock or oxygen levels (Bradshaw *et al.* 1993).

After its synthesis, heme is subsequently distributed to heme-proteins located in organelles or cytoplasm (reviewed by Smith 1990). Furthermore, for proteins that are targeted to the ER, it is expected that heme incorporation takes place in the ER. This requires translocation of heme to the ER (Pinnix *et al.* 1994). The intracellular transport of heme is still not well understood, but a role has been proposed for cytoplasmic heme carriers and also a direct transport of heme from mitochondria to the ER (Asagami *et al.* 1994).

Two solutions for heme limitations have been explored: 1) supplementation of the culture medium with heme or a heme intermediate and 2) genetic modification of the organisms in such a way that the metabolic heme biosynthesis is increased. Several studies indicate that heme supplementation of the medium increases heme-containing protein production (*e.g.* Andersen *et al.* 1992; Conesa *et al.* 2000; Ruiz-Duenas *et al.* 1999). Also supplementation of ALA could increase protein production (Herbaud *et al*. 2000) and activity (Yi *et al.* 2003). Furthermore, ALA addition rescues an *A. oryzae hemA* mutant (*hemA* encodes ALAS; Elrod *et al.* 2000).

Relative high concentrations of hemin are required to increase the yield of extracellular heme-peroxidases (500 mg/L). Lowering the hemin concentration decreased MnP yields in *A. oryzae* (Stewart *et al.* 1996). Although the addition of 500 mg/L hemin to the culture medium seems to be an effective approach for increasing ARP production (as will be shown in Chapter 7), for large-scale production of ARP supplementation with 500 mg/L hemin is unrealistic due to the high cost of hemin. Furthermore, there is confusion about the uptake of heme by *Aspergillus* as no hemin transport mechanism has been identified so far. On the other hand, supplementation experiments carried out indicate that heme increases the yield of heme-containing proteins. If only diffusion into cells takes place, this may be hindered by the impermeability of the fungal cell membrane.

Another approach is to increase heme synthesis in the host cell itself. Elrod *et al.* (1997) showed that overexpression of *hemA* (encoding ALAS) but not *hemB* (encoding ALA dehydratase) resulted in an increased production of heme-containing fungal peroxidase in *A. oryzae*. Even more, expression of both genes resulted in a synergistic positive effect on production, although these strains still responded to heme supplementation of the media, suggesting that additional limitations can also be present at later stages of the heme biosynthesis.

OUTLINE OF THIS THESIS

The aim of the work presented here is to study the feasibility of the production of llama variable heavy-chain antibody fragments (V_{HH}s) and bifunctional proteins by the filamentous fungus *A. awamori*. In **Chapter 2** a literature review concerning the production of antibody fragments and related fusion proteins by industrially relevant lower microorganisms is presented. The review deals with different antibody fragments, their production by different expression hosts and the envisaged applications, both in human therapeutics and in consumer goods. Finally, the review focuses on llama heavy chain antibody fragments and their future applications in fusion proteins. Analysis of expression and production of 'naked' antibody fragments in *A. awamori* is described in **Chapter 3**. Here it is shown that it is possible to secrete llama heavy-chain antibody fragments (V_{HH}s) in the *Aspergillus* culture medium, although the levels were lower than expected from their observed mRNA levels. Protease activity in the culture medium was partially responsible for the low levels observed, although it was hypothesised that intracellular degradation in the vacuole or proteasome is a more feasible explanation. In **Chapter 4** the results are presented of production of the *Arthromyces ramosus* peroxidase (ARP) by *A. awamori*. High levels of ARP were obtained in the culture medium, up to 800 mg/L, which was shown to be independent of heme supplementation of the medium. Therefore, it was hypothesised that *A. awamori* is the preferred production organism for peroxidases. In **Chapter 5** expression and production of the model fusion proteins ARP-R9 and R9-ARP was analysed. ARP was genetically fused to an antibody fragment recognising the azo-dye RR6 (V_{HH} R9) and the preferred orientation of V_{HH}-R9 (C-terminal or N-terminal linkage to ARP) was determined. It was shown that R9-ARP is the preferred orientation, probably because in ARP-R9 the N-terminal linkage of ARP shields the binding sites of the V_{HH} fragment. In **Chapter 6** V_{HH}-R9 in the ARP fusion proteins was replaced by V_{HH}s that specifically recognise tomato spots on textile. Production and binding was analysed, confirming the hypothesis that linkage of the N-terminus of ARP to the C-terminus of V_{HH}s (V_{HH}-ARP) is the preferred orientation. In **Chapter 7** the influence of heme

on production of heme-containing proteins by *A. awamori* is described. To this aim, production of ARP, fusion proteins and manganese-dependent peroxidase (MnP) was analysed under different cultivation conditions. The results of these experiments suggested that heme limitation was merely a consequence of cultivation conditions than a strain-specific problem. Finally, in **Chapter 8**, the results of the research described in this thesis are summarised and discussed.

ACKNOWLEDGEMENTS

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Chapter 2

The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi

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ABSTRACT

In this review we will focus on the current status and views concerning the production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. We will focus on single-chain antibody fragment production (scFv and V_{HH}) by these lower eukaryotes and the possible applications of these proteins. Also the coupling of fragments to relevant enzymes or other components will be discussed. As an example of the fusion protein strategy, the "Magic Bullet" approach for industrial applications will be highlighted.

INTRODUCTION

Antibodies (also called immunoglobulins) are glycoproteins, which specifically recognise foreign molecules. These recognised foreign molecules are called antigens. When antigens invade humans or animals, an immunological response is triggered which involves the production of antibodies by B-lymphocytes. By this immunological response, micro-organisms, larger parasites, viruses and bacterial toxins can be rendered harmless. The unique ability of antibodies to specifically recognise and bind with high affinity to virtually any type of antigen, made them interesting molecules for medical and scientific research.

In 1975 Köhler and Milstein developed the monoclonal antibody technology (Köhler and Milstein 1975) by immortalising mouse cell lines that secreted only one single type of antibody with unique antigen specificity, called monoclonal antibodies (mAbs). With this technology, isolation and production of mAbs against protein, carbohydrate, nucleic acids and hapten antigens was achieved. The technology resulted in a rapid development of the use of antibodies in diagnostics (*e.g.* pregnancy tests; Porter *et al.* 1988), human therapeutics and as fundamental research tools.

More applications outside research and medicine can be considered, such as consumer applications. Examples are the use of antibodies in shampoos to prevent the formation of dandruff (van der Linden, Thesis 1999) or in toothpaste to protect against tooth decay caused by caries (Frenken *et al.* 1998). For these purposes large quantities of antibodies are required. However, for these applications on a larger scale there were some major problems concerning the expensive production system based on mammalian expression, the difficulty of producing antibodies in bulk amounts and the low stability and solubility of some antibodies under specific (harsh) conditions.

In this review we will discuss the possibilities of large-scale production of antibodies and fragments thereof by relevant expression systems. Requirements are that the system used for production is cheap, accessible for genetic modifications, easily scaled up for greater demands and safe for use in consumer applications.

First, structure and characteristics of antibodies and antibody fragments generated thereof will be discussed, followed by the impact of recombinant DNA technology and antibody engineering techniques on the generation and modification of antibodies and antibody fragments. The modification of antibodies is of major interest since changes in their functionality and physico-chemical properties will broaden their application area. For most applications only the antigen-binding site of the native antibody molecule is required and even preferred. By the development of recombinant DNA technology and the increasing knowledge on the structure of antibody molecules created the opportunity to clone and engineer smaller fragments of antibody genes (Winter and Milstein 1991; Winter *et al.* 1994) and subsequent alter their functions, for example improve the affinity for their antigen. Besides that, recombinant DNA technology provides the possibility to generate fusion proteins or “Magic Bullets”, consisting of an antibody fragment fused to an effector molecule.

In this review the various expression systems for this type of proteins will be outlined. We will detail on using yeasts and filamentous fungi as suitable expression systems for antibody fragments and antibody fusion proteins.

ANTIBODIES AND THEIR UNIQUE ANTIGEN BINDING DOMAINS

WHOLE ANTIBODIES

In vertebrates five immunoglobulin classes are described (IgG, IgM, IgA, IgD and IgE), which differ in their function in the immune system. IgGs are the most abundant immunoglobulins in the blood and these molecules have a molecular weight of approximately 160kDa. They have a basic structure of two identical heavy (H) chain polypeptides and two identical light (L) chain polypeptides (Figure 1). The H and L chains, which are all β-barrels, are kept together by disulfide bridges and non-covalent bonds (for a review about antibody structure see Padlan 1994). The chains themselves can be divided in variable and constant domains. The variable domains of the heavy and light chain (V_H and V_L) which are extremely variable in amino acid sequences are located at the N-terminal part of the antibody molecule. V_H and V_L together form the unique antigen-recognition site. The amino acid sequences of the remaining C-terminal domains are much less variable and are called C_H1, C_H2, C_H3 and C_L.

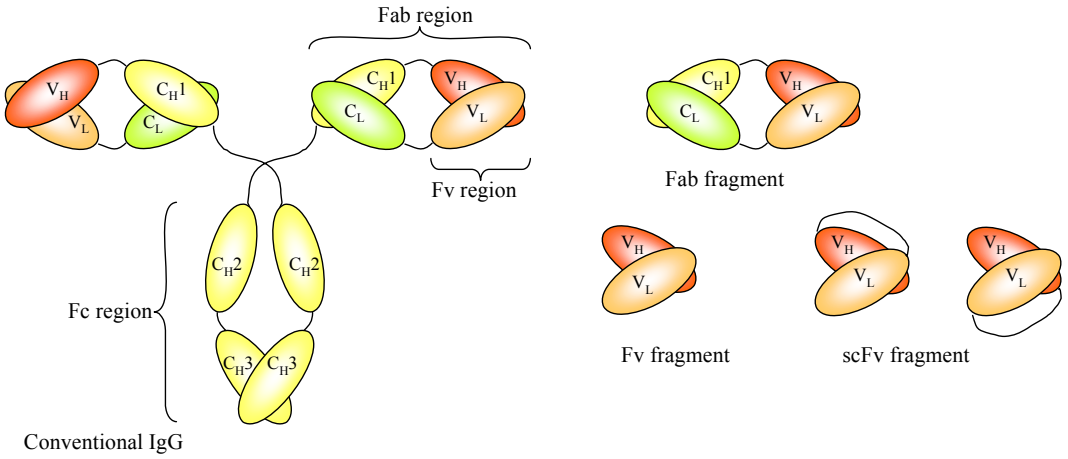

Figure 1. Schematical representation of the structure of a conventional IgG and fragments that can be generated thereof. The constant heavy-chain domains C_H1, C_H2 and C_H3 are shown in yellow, the constant light-chain domain (C_L) in green and the variable heavy-chain (V_H) or light-chain (V_L) domains in red and orange, respectively. The antigen binding domains of a conventional antibody are Fabs and Fv fragments. Fab fragments can be generated by papain digestion. Fvs are the smallest fragments with an intact antigen-binding domain. They can be generated by enzymatic approaches or expression of the relevant gene fragments (the recombinant version). In the recombinant single-chain Fv fragment, the variable domains are joined by a peptide linker. Both possible configurations of the variable domains are shown, *i.e.* the carboxyl terminus of V_H fused to the N-terminus of V_L and vice versa.

Fc fragment

The non-antigen binding part of an antibody molecule, the constant domain Fc mediates several immunological functions, such as binding to receptors on target cells and complement fixation (triggering effector functions that eliminate the antigen). The Fc domain is not essential for most biotechnical applications, relying on antigen binding. The Fc fragment,

which is glycosylated, can have different effector functions in the different classes of immunoglobulins.

Antigen binding region
The unique antigen-binding site of an antibody consists of the heavy and light chain variable domains (V_H and V_L). Each domain contains four conserved framework regions (FR) and three regions called CDRs (complementarity-determining regions) or hypervariable regions. The CDRs strongly vary in sequence and determine the specificity of the antibody. V_L and V_H domains together form a binding site, which binds a specific antigen.

ANTIBODY FRAGMENTS GENERATED THEREOF
Several functional antigen-binding antibody fragments could be engineered by proteolysis of antibodies (papain digestion, pepsin digestions or other enzymatic approaches), yielding Fab, Fv or single domains (Figure 1).

Fab fragments
Fab fragments (fragment antigen binding) are the antigen-binding domains of an antibody molecule, containing V_H + C_H1 and C_L + V_L. Between C_L and C_H1 an interchain disulfide bond is present. The molecular weight of the heterodimer is usually around 50 kDa (Better *et al.* 1988). Fab fragments can be prepared by papain digestions of whole antibodies.

Fv fragments
The minimal fragment (~30 kDa) that still contains the whole antigen-binding site of a whole IgG antibody is composed of both the variable heavy chain (V_H) and variable light chain (V_L) domains. This heterodimer, called Fv fragment (for fragment variable) is still capable of binding the antigen (Skerra and Plückthun 1988). Normally, native Fv fragments are unstable since the non-covalently associated V_L and V_H domains tend to dissociate from one another at low protein concentrations.

Single domains
Single domain antigen binding fragments (dAbs) or V_Hs were generated in the past (Cai and Garen 1996; Ward *et al.* 1989). They have good antigen-binding affinities, but exposure of the hydrophobic surface of the V_H to the solvent, which normally interacts with the V_L, causes a sticky behaviour of the isolated V_Hs. It turned out to be difficult to produce them in soluble form, although replacement of certain amino acids increased solubility of these single domains [see also "Llama heavy-chain antibody fragments (V_{HH}s)"]. Besides that, their affinity for the antigen was much less compared with other antibody fragments (Borrebaeck *et al*. 1992).

HEAVY-CHAIN ANTIBODIES IN *CAMELIDAE*
In 1993 Hamers-Casterman *et al.* discovered a novel class of IgG antibodies in *Camelidae* (camels, dromedaries and llamas). These antibodies are devoid of light chains and therefore called 'heavy-chain' IgGs or HCAb (for heavy-chain antibody; Figure 2). HCAbs have a molecular weight of ~95 kDa instead of the ~160 kDa for conventional IgG antibodies. Their binding domains consist only of the heavy-chain variable domains, referred to as V_{HH}s (Muyldermans and Lauwereys 1999) to distinguish them from conventional V_Hs. Since the first constant domain (C_H1) is absent (spliced out during mRNA processing due to loss of a

splice consensus signal; Nguyen *et al.* 1999; Woolven *et al.* 1999), the variable domain (V_{HH}) is immediately followed by the hinge region, the C_H2 and the C_H3 domains. Although the HCAbs are devoid of light chains, they have an authentic antigen-binding repertoire. The current knowledge about the genetic generation mechanism of HCAbs is reviewed by Nguyen *et al.* (2001 and 2002).

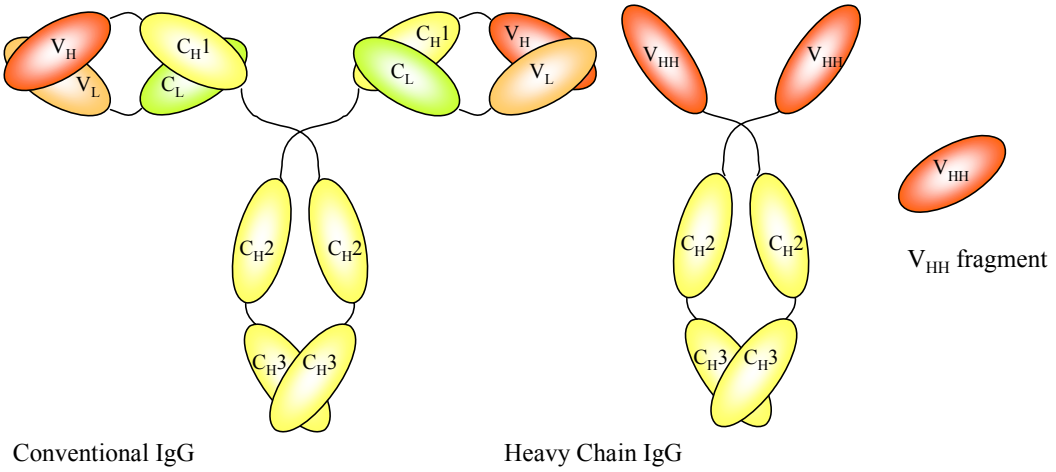

Figure 2. Schematical representation of the structure of a conventional IgG, a heavy-chain IgG antibody and the variable heavy-chain antibody fragment (V_{HH}) that can be generated of the latter. Heavy-chain antibodies found in llama and camel are only composed of heavy-chains and lack the light chain completely, as shown in this Figure. The antigen-binding domain consists of only the V_H domain, which is referred to as V_{HH} (variable heavy-chain antibody fragment), to distinguish it from a normal V_H. The constant heavy-chain domains C_H1, C_H2 and C_H3 are shown in yellow, the constant light-chain domain (C_L) in green and the variable heavy-chain (V_H or V_{HH}) or light-chain (V_L) domains in red and orange, respectively.

RECOMBINANT ANTIBODIES, ANTIBODY FRAGMENTS AND ANTIBODY FUSION PROTEINS

The development and applications of recombinant DNA technology led to the design of several new antibodies and antibody fragments. First, functionalities of these proteins may be altered resulting in novel and improved functions. One of the possible applications of recombinant whole antibodies is the use in human therapeutics. Second, smaller antibody fragments may be synthesised having the advantage over whole antibodies in applications requiring tissue penetration and rapid clearance from the blood or kidney. Moreover, the use of recombinant expression systems could also be the solution for large-scale production of antibody (fragments).

RECOMBINANT WHOLE ANTIBODIES

The development of human(ised) antibody molecules is mostly aimed at reduction of undesired immunological properties in medical applications (Colcher *et al.* 1998). Repeated doses of foreign (murine) antibody molecules could lead to an immune response in patients recognising the mouse antibody as foreign. This so-called HAMA (human anti-mouse antibody) response can lead to severe health problems.

Two strategies are developed to reduce the antigenicity of therapeutic antibodies (see also Kipriyanov and Little 1999). One of these strategies is chimerisation. In this case the constant murine domains are replaced by human constant domains (Neuberger *et al.* 1985; Riechmann *et al.* 1988). The second strategy is grafting of only the murine CDRs onto existing human antibody framework regions, which is called humanisation (Riechmann *et al.* 1988).

At present there are more than 10 recombinant antibodies approved by the US Food and Drug Administration (FDA) for use in medicine and many more are in a late stage of clinical trials. FDA approved recombinant mAbs are *e.g.* Herceptin™ (Genetech, San Francisco, CA), which targets and blocks the growth factor Her2 on the surface of breast cancer cells and Rituxan™ (IDEC Pharmaceuticals Inc., San Diego, CA) used against non-Hodgkin's lymphoma. See for more examples Brekke and Løset (2003); Gura 2002; Hollinger and Hoogenboom 1998). The use of recombinant antibodies for medical purposes does not require a cheap large-scale production process *per se*, since only a limited amount of pure preparations is needed.

PRODUCTION OF RECOMBINANT ANTIBODY FRAGMENTS BY *ESCHERICHIA COLI*

Much work on antibody fragment production has been focussed on *Escherichia coli* as an expression system (reviewed in Plückthun 1994 and Humphreys 2003). The advantage of this system is the ability to produce proteins in relative large amounts. Besides that, *E. coli* is easily accessible for genetic modifications, requires simple inexpensive media for rapid growth and they can easily be cultured in fermentors permitting large-scale production of proteins of interest. Several antibody fragments have been produced in functional form (*e.*g. Better *et al.* 1988; Skerra and Plückthun 1988; Le Gall *et al.*1998; Zhu *et al.* 1996) and expression of relevant gene segments also permitted the production of the recombinant antibody fragments. The problem of stability has been tackled by generation of single-chain Fv (scFv) or disulfide stabilised Fv (dsFv) fragments.

SELECTION OF ANTIBODY FRAGMENTS WITH IMPROVED FUNCTIONALITIES

In 1990 McCafferty *et al*. (1990) showed that antibody fragments could be displayed on the surface of filamentous phages, called phage-display. This technology is based on the fusion of the antibody variable genes to a phage coat protein gene (*e.g.* Kipriyanov and Little 1999). After displaying an antibody fragment on the protein surface of the phage, antigen specific phages can be selected and enriched by multiple rounds of affinity panning (*e.g.* reviewed in Hoogenboom and Chames 2000; Hoogenboom *et al*. 1998). This technique makes it possible to select phages that bind almost any antigen, including those previously considered to be difficult, such as self-antigens or cell surface proteins. Furthermore, antibody fragments can be isolated under adverse conditions, which can result in more stable fragments under for example washing conditions.

Libraries can be prepared from variable genes isolated from immunised animals, non-immunised sources (naïve libraries, thus avoiding the need for immunisation) or even (semi-) synthetic libraries can be constructed. The V genes can be subjected to random mutagenesis, chain or DNA shuffling methods (Marks *et al.* 1992), mimicking the natural hypermutation mechanism.

SINGLE-CHAIN F_V FRAGMENTS AND MULTIMERS

An attractive recombinant antibody fragment is the single-chain Fv (scFv) fragment (reviewed in Worn and Plückthun 2001; Raag and Whitlow 1995). It has a high affinity for its antigen and can be expressed in a variety of hosts (Verma *et al.* 1998). These and other properties make scFv fragments not only applicable in medicine (reviewed in Huston *et al.* 1993), but also of potential for biotechnological applications. In the scFv fragment the V_H and V_L domains are joined with a hydrophilic and flexible peptide linker, which improves expression and folding efficiency (Bird *et al.* 1988; Huston *et al.* 1988). Usually linkers of about 15 amino acids are used, of which the $(Gly_4Ser)_3$ linker has been used most frequently (Huston *et al.* 1993). Unfortunately, some scFv molecules have a reduced affinity compared to the parental whole antibody or Fab molecule (Borrebaeck *et al.* 1992; Glockshuber *et al.* 1990; Mallender *et al.* 1996). Besides that, scFv molecules can be easily proteolytically degraded, depending on the linker used (Whitlow *et al.* 1993). With the development of genetic engineering techniques these limitations could be practically overcome by research focussed on improvement of function and stability, as discussed in Worn and Plückthun (2001). An example is the generation of disulfide-stabilised Fv fragments where the V_H-V_L dimer is stabilised by an interchain disulfide bond (Glockshuber *et al.* 1990; Brinkmann *et al.* 1993; Reiter *et al.* 1994). Cysteines are introduced at the interface between the V_L and V_H domains, forming a disulfide bridge, which holds the two domains together (reviewed in Reiter *et al.* 1996).

Dissociation of scFvs results in monomeric scFvs, which can be complexed into dimers (diabodies), trimers (triabodies) or larger aggregates (Atwell *et al.* 1999, reviewed in Hudson and Kortt 1999). The simplest designs are diabodies that have two functional antigen-binding domains that can be either similar (bivalent diabodies) or have specificity for distinct antigens (bispecific diabodies). These bispecific antibodies allow for example the recruitment of novel effector functions (such as cytotoxic T cells) to the target cells, which make them very useful for applications in medicine (reviewed in Cao and Suresh 1998; Kriangkum *et al.* 2001).

LLAMA HEAVY-CHAIN ANTIBODY FRAGMENTS (V_{HH}s)

The other type of interesting antibody fragments are V_{HH}s (see Figure 2) comprising the smallest available intact antigen-binding fragment (~15kDa, 118-136 residues; Sheriff and Constantine 1996; Muyldermans 2001). The affinities found for V_{HH}s were in the nanomolecular range and comparable with those of Fab and single chain Fv (scFv) fragments (van der Linden *et al.* 1999; Lauwereys *et al.* 1998). Besides that V_{HH}s are highly soluble and more stable than the corresponding derivatives of scFv and Fab fragments (van der Linden *et al.* 1999; Ghahroudi *et al.* 1997). V_{HH}s carry amino acid substitutions that make them more hydrophilic and prevent the prolonged interaction with BiP (Immunoglobulin heavy-chain binding protein), which normally binds to the H-chain in the Endoplasmic Reticulum (ER) during folding and assembly, until it is displaced by the L-chain (Knarr *et al.* 1995). There are indications that this increased hydrophilicity improves secretion of the V_{HH}s from the ER. Hence, production of V_{HH}s in commercially attractive microorganisms may be favourable.

Several ways are described to obtain functional V_{HH}s: from proteolysed HCAb of an immunised camelid, direct cloning of V_{HH} genes from B-cells of an immunised camelid resulting in recombinant V_{HH}s or from naïve or synthetic libraries (Muyldermans 2001; Yau *et al.* 2003). V_{HH}s with desired antigen specificity could be selected by phage display. Using V_{HH}s in phage display is much simpler and more efficient as compared with Fabs or scFvs,

since only one domain needs to be cloned and expressed to obtain a functional antigen-binding fragment (Ghahroudi *et al.* 1997; van der Linden *et al.* 2000).

As already noted before (see paragraph "Antibody fragments generated thereof"), classical V_Hs were difficult to produce in soluble form. To improve their solubility and prevent non-specific binding, residues located on the V_L side of V_Hs were replaced by 'V_{HH}-like' residues, mimicking the more soluble V_{HH} fragments. This process has been termed camelisation (Davies and Riechmann 1994, 1996; Tanha *et al.* 2001) and these camelised V_H fragments, particularly those based on the human framework, are expected to have significant advantages for therapeutical purposes in humans (reviewed by Riechmann and Muyldermans 1999).

FUSION PROTEINS ("MAGIC BULLETS")

A completely new use of the binding capacity of antibody fragments is the design of a fusion approach, in which an effector protein is coupled to an antigen recognising antibody fragment. In human medicine this approach is referred to as "Magic Bullets". All kinds of molecules can be used as effector molecule, limited only by the imagination. The gene encoding the effector may be directly fused to the gene of the antibody fragment of interest, resulting in novel bifunctional proteins (Neuberger *et al.* 1984). Examples of the use of this approach will be described later. The effector molecule and antibody fragment can be coupled chemically, but this is labour intensive since it requires several purification steps. Therefore, genetically linkage by recombinant DNA technology is a much easier way to obtain fusion proteins.

APPLICATIONS OF ANTIBODY FRAGMENTS AND ANTIBODY FUSION PROTEINS

APPLICATIONS OF ANTIBODY FRAMENTS IN HUMAN MEDICINE

The smaller the better

Most applications of recombinant antibody fragments are related to diagnosis and therapy in human medicine, which is especially focussed on the use of antibodies as the ideal cancer-targeting reagent (reviewed in Colcher *et al.* 1998; Hazra *et al.* 1995; Hudson 1999; von Mehren and Weiner 1996). For some clinical applications small antibody fragments have advantages over whole antibodies. The small size permits them to penetrate tissues and solid tumours more rapidly than whole antibodies (Yokota *et al.* 1992), which recently also was shown for V_{HH}s (Cortez-Retamozo *et al*. 2002). Smaller antibody fragments have also a much faster clearance rate in the blood circulation, which leads to differences of selectivity (Yokota *et al.* 1992). Nowadays there are also promising pre-clinical and clinical trials with antibody fragments as diagnostic or therapeutical agents (Hudson 1999; Maynard *et al.* 2002). Another application of antibody fragments is to treat viral infections with so-called intrabodies, which are intracellular antibodies synthesised by the cell and targeted to inactivate specific proteins within the cell (Marasco 1995).

"Magic Bullets" in medicine

The use of bi-functional molecules in medicine is aimed at delivery of a protein drug, which is only active where it is required. It thereby limits the dose of the drug, resulting in less side

effects of the drug towards healthy tissue and/or less immunogenic response to the protein drug itself. Also the physical interaction between the target and the effector molecule increases the potency of the effector. Fusion proteins are ideal immuno agents for cancer diagnosis (Spooner *et al.* 1994) and cancer therapeutics. An example is the use of cancer-specific bi-functional antibodies targeting potent cytotoxic molecules to tumour cells and subsequently eliminate these tumour cells without harming healthy cells (Boleti *et al*. 1995).

POTENTIAL OF APPLICATION OF V_{HH}S

Specific applications of V_{HH}s are foreseen in the following direction:

V_{HH}s as drug carriers

It is expected that V_{HH}s are also applicable in diagnosis and therapy in human medicine, especially when an economically feasible production, small size and stability are required (reviewed by Muyldermans 2001). Cortez-Retamozo *et al*. (2002) recently showed that V_{HH}s specifically could be targeted to tumour cells, which together with the possibility of generation of bispecific V_{HH} constructs (Conrath *et al.* 2001a) and pentamerisation (Zhang *et al.* 2004) is of major interest for cancer therapy. Furthermore, targeting of specific V_{HH}s coupled to β-lactamase was observed to conserved cryptic epitopes of infectious agents such as VSG from trypanosomes (Stijlemans *et al.* 2004), making a more efficient drug against malaria within reach.

V_{HH}s as delivery carriers in the brain

Antibodies and many other water soluble compounds are excluded from the brain by the blood-brain barrier (BBB), thus making treatment of brain-related disease very difficult. Recently, Muruganandam *et al*. (2002) showed that V_{HH} were able to selectively bind to and transmigrate across the BBB in a human *in vitro* BBB model and partly *in vivo* in mice. This property can be exploited for the development of efficient antibody carriers suitable for delivery of macromolecules across the human BBB and subsequently for treatment of neurological diseases.

V_{HH}s as potent enzyme blockers and inhibitors

Hypervariable regions in V_{HH}s are on average longer than those of V_Hs (Vu *et al.* 1997; Harmsen *et al.* 2000). The extended hypervariable regions of V_{HH}s are capable of penetrating deep into the cleft of active sites of enzymes, binding to novel epitopes that are not recognised by conventional antibodies (Lauwereys *et al.* 1998; Desmyter *et al.* 1996; Transue *et al.* 1998). Because of this property V_{HH}s may act as better potent enzyme inhibitors (Lauwereys *et al.* 1998; Conrath *et al*. 2001b; Desmyter *et al.* 2002). That V_{HH}s are suitable targets for drug discovery is illustrated by recent published papers *e.g.* inhibition of the hepatitis C virus NS3 serine protease by a camelised V_{HH} (Dottorini *et al.* 2004). Even more exciting is the discovery that specific V_{HH}s could inhibit the formation of amyloid fibrils, which in the near future can possibly can as an agents directed against protein deposition diseases like Alzheimer's and Parkinson's (Dumoulin *et al.* 2003). Recently, Meddeb-Mouelhi and co-workers reported that immunised camels produced HCAbs against scorpion venom, which were able to neutralise the toxin (Meddeb-Mouelhi *et al.* 2003). Furthermore, enzyme-neutralising V_{HH}s could be expressed in plants and inhibit enzyme function (Jobling *et al.* 2003), which may be a new tool for immuno-modulation (disruption of the function of phytohormones or plant pathogens).

V_{HH}s in (bio)-technical and immunological applications
V_{HH}s could be used as a crystallisation aid (*e.g*. the short-lived MazE, which was difficult to isolate due to its instability; Lah *et al.* 2003) or for immunoaffinity purification and immuno-perfusion chromatography (Verheesen *et al.* 2003). More applications are foreseen in the field of diagnostics, for example in agriculture and food industry where V_{HH}s can be used for monitoring pesticides, environmental contaminants or toxic substances in the environment (Yau *et al*. 2003). Another application is in diagnosis/proteomics studies of human diseases, as was previously described by van Koningsbruggen *et al*. (2003).

V_{HH}s in consumer products
Since llama V_{HH}s are very stable, even at high temperature, applications can be envisaged in which a high temperature step is involved (*e.g.* pasteurisation), without losing antigen-binding properties (van der Linden *et al.* 1999). Recently it was shown that V_{HH}s could be used to prevent phage infection in cheese production processes (Ledeboer *et al.* 2002), by recognising a structural protein of the phage, which is involved in recognition of the host *Lactococcus lactis*.

ANTIBODY FRAGMENTS AND ANTIBODY FUSION PROTEINS FOR LARGE-SCALE APPLICATIONS AND CONSUMER PRODUCTS
Many additional applications can be envisaged if an inexpensive and simple production system, yielding large amounts of antibody fragments that can be purified easily. The highly specific antigen-binding ability could be used for inactivating bacteria or specific enzymes that can cause spoilage of food. Other suggested applications are the use in biosensors (detection/monitoring pollution and contamination) and removal of environmental pollution (Harris 1999), treatment of wastewater (Graham *et al.* 1995), industrial scale separation processes such as separation of chiral molecules (Got and Scherrmann 1997), purification of specific components (proteins) from biological materials or the use as abzymes (Janda *et al.* 1997; Wade and Scanlan 1997). They have also been considered as components of novel consumer goods with new improved functionalities, in oral care and personal hygiene (*e.g*. in toothpaste or mouthwashes; Beggs *et al.* 1995). For dental applications antibody fragments can be coupled to enzymes to increase the concentration of antimicrobials like hypothiocyanate and hypohalites, for example glucose oxidase (GOX; Hill *et al.* 1997), galactose oxidase (GaOX; Lis and Kuramitsu 1997) or lactate oxidase (LOX; Hayes 1996). Other examples are targeted bleach in laundry washing (*e.g.* detergents containing antibodies coupled to molecules that specifically remove difficult stains) or the use in shampoos where antibodies act to prevent dandruff by inhibiting growth of specific microorganisms causing this (van der Linden, Thesis 1999).

SUITABLE EXPRESSION SYSTEMS FOR THE LARGE-SCALE PRODUCTION OF ANTIBODY FRAGMENTS AND ANTIBODY FUSION PROTEINS

To be able to use antibody fragments and antibody fusion proteins in these large-scale applications, a suitable expression system has to be chosen. Several expression systems are available, both from prokaryotic (Table 1) and eukaryotic (Table 2) origin. Our main interest

goes out to these systems that are able to economically produce large amount of proteins into the culture medium. Several of these systems can be considered as suitable (both from prokaryotic and eukaryotic origin). Here after several of these systems will be discussed, with an emphasis on yeast and fungal systems.

Table 1. Extracellular production of antibody fragments in prokaryotic expression systems.

Strains	Characte-ristics	Food grade	Production yields	References
Lactobacillus zeae	$Gram^+$	yes	scFv , ND* (secreted/cell-bound)	Kruger *et al*. 2002
Bacillus subtilis	$Gram^+$	yes	10 to 15 mg/L scFv (secreted)	Wu *et al.* 1993; Wu *et al.* 2002
Streptomyces lividans	$Gram^+$	yes	1 mg/L Fv fragment (secreted)	Binnie *et al*. 1997; Ueda *et al.* 1993
Staphylococcus carnosus	$Gram^+$	yes	5-10 mg/L V_H domain (secreted)	Pschorr *et al*. 1994
Proteus mirabilis	$Gram^-$	no	40 to 200 mg/L scFv (secreted)	Kujau *et al.* 1998; Rippmann *et al.* 1998
Escherichia coli	$Gram^-$	no	Several fragments (inclusion bodies/periplasmic space)	Plückthun 1994; Verma *et al.* 1998

*ND = not determined.

DRAWBACKS USING *E. COLI* AS A HOST FOR ANTIBODY FRAGMENT PRODUCTION

As described previously, *E. coli* has shown to be a potential expression host for antibody fragments and fusion proteins. Although the general production yields in shake-flask cultures are low (several mg/L), in fermentation processes several g/L could be obtained (reviewed by Harrison and Keshavarz 1996). There are two possibilities of antibody fragment production in *E. coli*, either by secretion of the fragments into the culture medium and/or periplasmic space (the compartment between the inner and outer membrane) or preparation of inclusion bodies with subsequent *in vitro* folding. However, both strategies have disadvantages that make the use of this prokaryote not attractive for the large-scale production of antibody fragments and antibody fusion proteins. Firstly, the secretion of folded and fully assembled fragments in the medium or periplasmic space is often accompanied with cell lysis and subsequent product loss. Secondly, 'toxicity' of the antibody sequence and concomitant plasmid loss is frequently observed, which hamper high production levels (reviewed by Plückthun 1994). Thirdly, expression of the fragments in inclusion bodies, which often results in insoluble protein aggregates (Skerra 1993), demands laborious and cost-intensive *in vitro* refolding (denaturation and renaturation) and purification steps. Hence, the final yield of fragments is only a small percentage of the protein that was initially present in the inclusion bodies even though purification steps are nowadays facilitated by affinity chromatography using C-terminal polypeptide tails, like poly-His_6 or FLAG (Kortt *et al.* 1994; Casey *et al.* 1995).

Recently, production of soluble and functional scFv by *E. coli* could be increased by improving disulfide bond formation activity in the cytoplasm, using mutants and overexpression of disulfide-bond isomerase (Jurado *et al*. 2002). Finally, *E. coli* is unable to carry out eukaryotic post-translational modifications and is therefore not suitable when glycosylation of antibody fragments or more importantly the fusion proteins is required.

ALTERNATIVE PROKARYOTIC EXPRESSION SYSTEMS

E. coli is not the only available prokaryotic expression system, although it is rather dominant in the field. Alternative prokaryotic expression systems are available for antibody fragment production (Table 1). However, these will encounter similar limitations as *E. coli*, even though most organisms described in Table 1 secrete the investigated antibody fragment into the culture medium. A field where production of antibody fragments in prokaryotic cells could still be interesting is in food grade organisms used for delivery passive immunisation in humans, by means of functional foods. In a recent article, Kruger *et al*. (2002) reported the production of scFv antibody fragments against *Streptococcus mutans* by the Gram positive food-grade bacteria *Lactobacillus zeae*. In experimental animals a decrease of *S. mutans* and reduced development of caries was observed.

EUKARYOTIC EXPRESSION SYSTEMS

Also several eukaryotic systems can be envisaged for large-scale production of antibody fragments and antibody fusion proteins (see also Verma *et al.* 1998), like mammalian cells, insect cells, plants, transgenic animals and lower eukaryotes (see Table 2).

The production of therapeutical whole antibodies is well established in mammalian cells. However, large-scale production is expensive and time-consuming. Furthermore, there is a high risk of contamination of the final product with viruses, prions and oncogenic DNA.

Table 2. Higher eukaryotic expression systems for heterologous protein production and possible advantages and disadvantages of the expression system.

Expression systems	Ease of cloning	Scale-up capacity	Economic feasibility[1]	Pathogenic contaminants[2]	References[3]
Mammalian cells	+	+/-	+	+	Neuberger *et al*. 1984; Neuberger 1985; King *et al* 1993; Riechmann *et al.* 1988; Dorai *et al.* 1994; Jost *et al.* 1994
Insect cells	++	+	+	+	Ailor and Betenbaugh 1999; Bei *et al*. 1995; Carayannopoulos *et al.* 1994; Hasemann and Capra 1990; Kretzschmar *et al.* 1996; Mahiouz *et al*. 1998
Plants	++	+++	++	++	Conrad and Fiedler 1998; Peeters *et al.* 2001; Stöger *et al.* 2000; Hiatt *et al*. 1989; Fischer *et al.* 1999
Transgenic Animals*	+/-	+++	+/-	+/-	Kuroiwa *et al.* 2002; Little *et al*. 2000; Pollock *et al.* 1999; Young *et al.* 1998
Yeasts	+++	+++	+++	+++	See references in next paragraph
Filamentous fungi	+++	+++	+++	+++	Frenken *et al*. 1998; Nyyssönen *et al.* 1993; Frenken *et al.* 1994

+++ = excellent, ++ = good, + = sufficient, +/- = poor.

* With transgenic animals in this context is mentioned the production of antibodies or antibody fragments in the milk of transgenic animals, for example rabbits, sheep, goats or cows.

[1] With economical feasibility is mentioned the time and cost of molecular cloning, upscaling and downstream processing (purification).

[2] Pathogenic contaminants like viruses or pyrogens.

[3] Articles dealing with production of antibodies, antibody fragments and antibody fusion proteins.

'Plantibodies' can be produced in several plant target organs (reviewed by Conrad and Fiedler 1998). Roots, storage organs (seeds and tubers) and fruiting bodies can be suitable for mass oral (edible) applications (see Peeters *et al.* 2001 and references therein). Expression of scFv in transgenic plants has been proposed as a way to produce and store pharmaceutical antibodies (Stöger *et al.* 2000; Yuan *et al.* 2000) and as means to block physiological processes in the plant itself (de Jaeger *et al.* 2000) or establish plant pathogen resistance (Ziegler and Torrance 2002). Plants show several advantages as large-scale antibody production systems (reviewed by Ma *et al.* 2003), like the ease and low costs of growing plants, even in large quantities. However, the generation of transgenic plants that express antibodies is a time consuming process and the downstream processing to isolate the expressed antibodies from the plant parts is relatively expensive and laborious.

PRODUCTION OF ANTIBODY FRAGMENTS BY LOWER EUKARYOTES

An attractive possibility for the cost-effective large-scale production of antibody fragments and antibody fusion proteins are yeast or fungal fermentations. Large-scale fermentation of these organisms is an established technology already used for bulk production of several other recombinant proteins and extensive knowledge is available on downstream processes. Besides that, yeasts and filamentous fungi are accessible for genetic modifications and the protein of interest may be secreted into the culture medium. In addition, some of their products have the so-called GRAS (Generally Regarded As Safe) status and they do not harbour pyrogens, toxins or viral inclusions.

Methylotrophic and other yeasts

The methylotrophic and other yeasts like *Candida boidinii*, *Hansenula polymorpha*, *Pichia methanolica* and *Pichia pastoris* are well known systems for the production of heterologous proteins (reviewed by Gellissen 2000). High levels of heterologous proteins (milligram-to-gram quantities) can be obtained and scaling up to fermentation for industrial applications is possible (Gregg *et al.* 1993; Faber *et al.* 1995; Fischer *et al.* 1999).

Especially the *P. pastoris* system is used in several industrial-scale production processes (Cregg *et al.* 2000). Ridder *et al.* (1995) were the first to report the expression of a scFv fragment by *P. pastoris*. From then on several papers reported about the use of *P. pastoris* for the production of recombinant antibodies and fragments thereof (Andrade *et al.* 2000; Pennell and Eldin 1998). In shake-flask cultures a level of 250 mg/L scFv was obtained (Eldin *et al.* 1997) and Freyre *et al.* (2000) were able to obtain even an expression level of 1.2 g/L scFv fragment under fermentation conditions. However, Cupit *et al*. (1999) also showed that the production of antibody fragments by *P. pastoris* is not always a success story.

Based on the described results the commercial recombinant antibody production by *P. pastoris* is promising. However, products currently obtained from *P. pastoris* are not regarded as GRAS, which may limit its use.

Wood *et al.* (1985) were the first to report the production of mouse IgM by the baker's yeast *S. cerevisiae*, although only unassembled chains were detected in the culture medium. However, the production of Fab fragments was possible as was first shown by Horwitz *et al.* (1988). Although the obtained levels were low, functional Fab fragments were secreted in the culture medium. Davis *et al.* (1991) expressed scFv antibody fragments in *Schizosaccharomyces pombe.* Studies on the scFv production in the non-conventional yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis* resulted in 10-20 mg/L functional and soluble anti-Ras scFv (Swennen *et al*. 2002).

Filamentous fungi: Trichoderma reesei and Aspergillus spp.
Filamentous fungi, in particular species from the genera *Trichoderma* and *Aspergillus* have the capacity to secrete large amounts of proteins, metabolites and organic acids into their culture medium. This property has been widely exploited by the food and beverage industries where compounds secreted by these filamentous fungal species have been used for decades. This has led to the GRAS status for some of their products. Filamentous fungi like *A. awamori*, *A. niger* and *A. oryzae* are therefore suitable organisms for the production of commercially interesting homologous and heterologous proteins (Radzio and Kuck 1997; Punt *et al*. 2002; van den Hondel *et al.* 1991). Strategies to improve protein secretion by filamentous fungi are extensively reviewed in (Archer *et al.* 1994; Gouka *et al.* 1997; Verdoes *et al.*1995; Ward *et al.* 1990).

Production strains of *Trichoderma reesei* (*Hypocrea jecorina*) have an exceptional secretion capacity up to 35 g protein/L, where half of the secreted protein consists of the cellulase cellobiohydrolase I (CBH1; Durand *et al.* 1998). Therefore, *Trichoderma* is considered as an excellent host for the production of heterologous proteins (reviewed by Keränen and Penttilä 1995; Nevalainen *et al.* 1994). Nyyssönen *et al*. (1993) reported a production of 1 mg/L in shake-flasks of Fab antibody fragments by *T. Reesei* Rut-C30. More strikingly, when the Fab antibody fragment chain was fused to the core-linker region of CBH1, a production level of 40 mg/L in shake-flasks and 150 mg/L in bioreactor cultivations was obtained (Nyyssönen *et al*. 1992 and 1993).

THE USE OF *S. CEREVISIAE* AND *A. AWAMORI* FOR THE LARGE-SCALE PRODUCTION OF ANTIBODY FRAGMENTS AND FUSION PROTEINS

In our own laboratory at TNO Nutrition and Food Research in Zeist (The Netherlands) and in collaboration with Unilever Research Vlaardingen (The Netherlands) research on antibody fragment production in *S. cerevisiae* and *A. awamori* has been carried out (Hessing *et al.* 1994; Hamers *et al.* 1994). The aim of this project was a detailed comparison of both expression systems, in relation to their possible large-scale production process of antibody fragments and fusion proteins. In the framework of this collaboration also a new *A. awamori* expression system, based on D-xylose induction was developed (Gouka *et al.* 1996).

THE USE OF *S. CEREVISIAE* AND *A. AWAMORI* FOR THE LARGE-SCALE PRODUCTION OF $ScFv$

To investigate the feasibility of a large-scale cost-effective process for the extracellular production of (functionalised) scFv fragments initially *S. cerevisiae* was used. However, it was shown that *S. cerevisiae* was a poor host for the production of scFv, since the secretion of scFv was hampered by improper folding of the fragments, because large aggregates were formed in the ER and vacuolar-like organelles. It was hypothesised that the exposure of the hydrophobic surfaces on the V_L and V_H chains of scFv plays an important role in the accumulation of scFv in the cell (Frenken *et al.* 1994). Shusta *et al.* (1998) reported the increase of scFv production up to 20 mg/L in *S. cerevisiae* by optimising the expression system by overexpression of two ER resident chaperones and reduction of growth temperature. Kauffman *et al.* (2002) showed that overexpression of scFv in *S. cerevisiae*

resulted in cellular stress, displayed by decreased growth rates and induction of the Unfolded Protein Response (UPR). It was hypothesised that a functional UPR was required to decrease the malfolded scFv in the ER, leading to a recovery from cell stress.

As further improved levels were desired also a fungal expression system was considered (Frenken *et al.* 1998). In shake-flask cultures a production level of 10 mg/L was achieved by using *A. awamori* as production host. As secretion of a heterologous protein can be greatly enhanced by fusing it to a "carrier" protein such as glucoamylase (GLA; Gouka *et al.* 1997; Ward *et al.* 1990), also this fusion-approach was employed. Analysis of the culture medium of transformants carrying the fusion construct revealed a production of approximately 50 mg/L scFv in the culture medium (Frenken *et al.* 1998). Several commercially interesting scFv fragments were investigated for their ability to be produced by *A. awamori* using the GLA-fusion strategy. The results showed that the production levels differed significantly between the different scFv transformants. Interestingly, in some cases increased levels of scFv detected in the culture medium corresponded to an increase of transcription level of the ER chaperone BipA (Punt *et al.* 1998), indicating that the antibody fragments, like in *S. cerevisiae,* may have problems with correct folding and aggregate in the fungal cell.

To increase production levels, successful 10 L and 15,000 L scale fermentations were carried out resulting in 200 mg/L scFv under optimal conditions. However, variable amounts of scFv dimers and other multimers were observed. Recent fermentation experiments performed by Sotiriadis *et al.* (2001) showed that the highest scFv level was observed when induction was started in the late exponential phase. An increase of the carbon and nitrogen source concentrations and a decreased of the concentration of the inducer resulted in increased product yields.

Recently, Ward *et al.* (2004) reported the production of full-length IgG1, Fab' and F(ab')2 in *A. niger*. The secreted antibody (fragments) were correctly assembled and were functional in binding to their antigen.

PRODUCTION OF LLAMA V_{HH} ANTIBODY FRAGMENTS BY *S. CEREVISIAE* AND *A. AWAMORI*

Although the production of scFv fragments by *S. cerevisiae* and *A. awamori* was successful, levels up to several g/L were not achieved. Possibly the hydrophobic regions of the scFv, responsible for keeping the variable regions of the heavy and light chains together, could also interact with other molecules in the cell. Aggregation of scFv in *S. cerevisiae* may result in accumulation and subsequent degradation (of a part) of the antibody fragment molecules (Frenken *et al*. 1994) as frequently observed when expressing heterologous proteins that exhibit hydrophobic surfaces (Sagt *et al*. 1998). Interestingly, antibody fragments devoid of these hydrophobic surfaces could be obtained from camels, dromedaries and llamas (V_{HH}s; Hamers-Casterman *et al.* 1993), providing an option to improve production levels in relevant microorganisms (Hamers *et al*. 1994).

V_{HH}s could be produced in *E. coli* up to levels of 6 mg/L, were found to be extremely stable, highly soluble and reacted specifically and with high affinity with antigens (Ghahroudi *et al.* 1997). V_{HH}s were produced in *S. cerevisiae* at levels over 100 mg/L in shake-flask cultures (Frenken *et al.* 2000), although considerable amounts of V_{HH}s were detected intracellularly. From a 15,000 L fed-batch fermentation, 1.3 kg of V_{HH}s was obtained, which clearly showed that these fragments could be produced in this host more efficiently than scFv fragments (Thomassen *et al.* 2002). For a cost-effective large-scale process for the production

of V_{HH}s in *S. cerevisiae* further improvement is required. Van der Linden *et al.* (2000) showed that production of V_{HH}s by *S. cerevisiae* could be improved by DNA shuffling techniques, in which three homologous V_{HH} genes were randomly fragmented and reassembled subsequently.

Based on the fact that *A. awamori* performed superior for scFv also the possibility of V_{HH} production by *A. awamori* was investigated. As a model V_{HH}s against the hapten RR6 were chosen (Frenken *et al.* 2000). Gene fragments coding for anti-RR6 V_{HH}s were cloned in an expression vector containing the highly inducible endoxylanase promoter. As will be described in Chapter 3, functional V_{HH}s could be produced in the culture medium during shake-flask cultivations, albeit at relatively low levels. For further optimisation a carrier strategy and controlled fermentations can be carried out.

PRODUCTION OF "MAGIC BULLETS" BY *A. AWAMORI*

A major research interest is the production of fusion proteins or "Magic Bullets", consisting of an antibody fragment (scFv or V_{HH} fragment) fused to an enzyme of interest. In our laboratory research has been carried out with a few examples of scFv fragments coupled to glucose oxidase (GOX). GOX is already for many years an interesting enzyme for coupling to antibodies for killing cells (Verhoeyen *et al.* 1995). A scFv, which recognises for example oral *Streptomycetes*, when fused to GOX, which is an antimicrobial enzyme, may kill bacteria by generation of the bactericidal hydrogen peroxide. In activity assays it was shown that the fusion protein produced by *A. awamori* was functional, both in binding to the antigen and GOX activity (Frenken *et al*. 1998).

In the detergent industry enzymatic bleaching may be a good alternative to the current chemical bleaching used. To make these laundry-cleaning products more effective, the production of "Magic Bullets" by filamentous fungi or yeasts is of interest. An enzyme coupled to an antibody fragment recognising persistent stains on textile can result in a more directed bleaching process, resulting in lower amounts of required detergent, reduction of harmful effects of the enzyme to the textile and lower environmental burden (see Figure 3).

Currently we are investigating the feasibility of production of V_{HH}-enzyme fusions by *A. awamori*. One of the V_{HH}s used is a model llama V_{HH}, recognising the azo-dye Reactive Red 6 (RR6; Frenken *et al.* 2000). As a bleaching enzyme, the *Arthromyces ramosus* peroxidase (ARP; Akimoto *et al.* 1990; Fukuyama *et al.* 1995) was genetically linked to the V_{HH} fragment. This peroxidase utilises hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds, which makes the enzyme suitable for use in bleaching processes (Kjalke *et al.* 1992). ARP alone could be produced in high amounts by *A. awamori* (800 mg/L; Lokman *et al.* 2003). As will be described in Chapter 5, our results showed the feasibility of fusion protein production by *A. awamori*. Both ARP activity and azo-dye binding activity of the fusion protein R9-ARP could be demonstrated.

In future experiments V_{HH}s fragments can be replaced by other more relevant antibody fragments, for example those binding tomato or blood spots. Also the peroxidase part of the fusion can be optimised further.

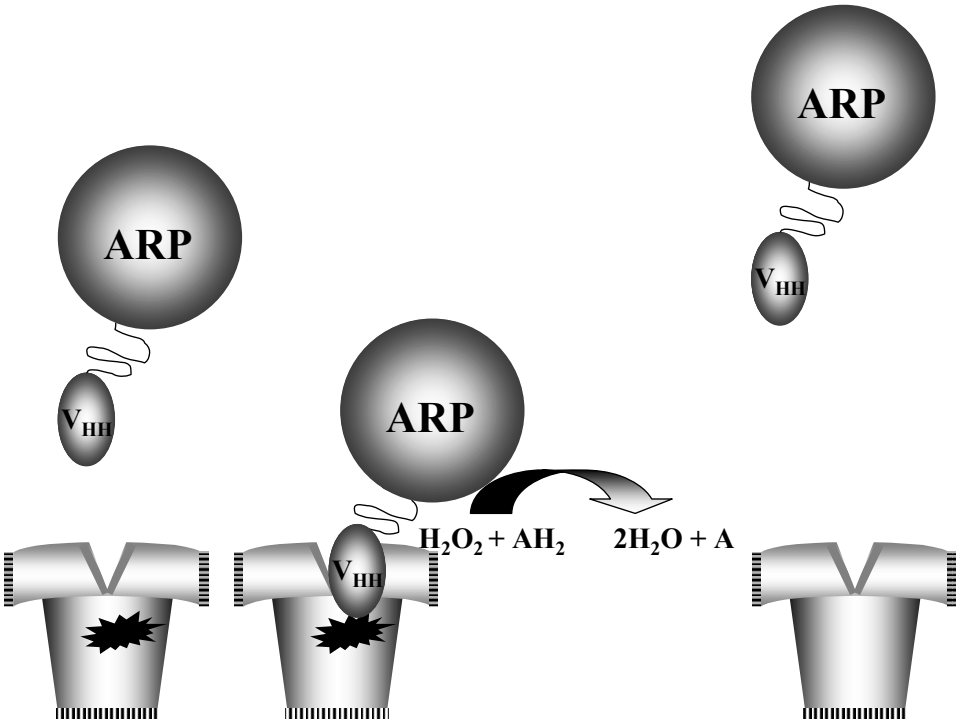

Figure 3. Schematical example of the '"Magic Bullet" approach in consumer applications, where an antibody fragment (in this case a llama variable heavy-chain antibody fragment; V_{HH}) recognising a spot on textile, is coupled to an effector molecule (in this case *A. ramosus* peroxidase; ARP). ARP is a peroxidase, which utilises hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic substrates (AH_2), which makes the enzyme suitable for use in bleaching processes (Kjalke *et al.* 1992).

CONCLUSION AND DISCUSSION

Recent developments in the fields of antibody engineering and expression systems have enabled the engineering and production of antibodies and antibody fragments for a wide variety of applications. A lot of examples are already mentioned, but presumably more applications can be envisaged. The development of the "Magic Bullet" approach will even increase the interest in antibodies and their related products, also for applications in human medicine. A recently envisioned application that is of much interest, is the use of antibody fragments in micro-arrays. Antibody arrays can be used for proteomic analysis by comparing the differences in presence of proteins in healthy and diseased cells. For this purpose antibody fragments derived from large phage-antibody libraries can be used as probes to capture proteins on chips in a high-throughput system (reviewed by Borrebaeck 2000; Holt *et al.* 2000). In this respect, V_{HH}s fragments are of great interest, due to their simple and stable structure.

In this review we evaluated whether the yeast *S. cerevisiae* and the filamentous fungus *A. awamori* are suitable expression systems for the large-scale production of antibody fragments and antibody fusion proteins. Although *A. awamori* is not the best expression

system for the production of V_{HH} fragments (as will be shown in Chapter 3) or scF_v, in particular for the production of antibody fusion proteins filamentous fungi offer significant potential. In particular in those cases where specific post-translational modifications (*e.g.* N-glycosylation) are required for functional expression of the effector protein (also in relation to pharmaceutical applications). In contrast to *S. cerevisiae*, filamentous fungi do not show extensive hyperglycosylation (Maras *et al.* 1999b). Considerable progress has been made the last years with engineering of the glycosylation pathway in filamentous fungi and yeast to obtain strains that show a more mammalian-type of glycosylation. By genetically modifying glycosylation pathway, sequences of enzymatic reactions could be carried out that mimic (although far from optimal yet) the processing of glycoprotein in humans (Maras *et al.* 1999a; Maras *et al.* 2000; Hamilton *et al*. 2003).

Both the scFv-GOX as well as results from ARP-V_{HH} fusion proteins showed that the filamentous fungal system is a promising candidate for the production of antibody fusion proteins. In the future production of other fusion proteins can be investigated in this or other fungal expression systems, allowing a potential breakthrough for antibody technology in producing large amounts of specific recognition units coupled to effector molecules for consumer applications.

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Chapter 3

Expression and production of llama variable heavy-chain antibody fragments (V_{HH}s) by *Aspergillus awamori*

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ABSTRACT

We report the expression and production of llama variable heavy-chain antibody fragments (V_{HH}s) by *Aspergillus awamori.* Fragments encoding V_{HH}s were cloned in a suitable *Aspergillus* expression vector and transformants secreting V_{HH} fragments were analysed for integrated gene copy-numbers, mRNA levels and protein production. Functional V_{HH}s were detected in the culture medium, indicating the feasibility of producing this type of proteins in a fungal expression system. Secreted V_{HH}s were susceptible to (extracellular) degradation, which could be partially prevented by the addition of BSA to the culture medium.

INTRODUCTION

Antibodies and fragments thereof are proteins able to bind specifically and with high affinity to their corresponding antigens and are therefore applied in human therapy and diagnostics (Von Mehren *et al*. 1996; Porter *et al*. 1988). New applications can be considered, such as use in consumer products like detergents (for spot-specific bleaching) or personal care products like toothpaste and anti-dandruff shampoo (Beggs *et al*. 1995; Frenken *et al*. 1998). For these purposes, inexpensive production of large amounts of these molecules is necessary and therefore cheap large-scale production systems are required (Joosten *et al*. 2003).

Hamers-Casterman *et al.* (1993) discovered a novel class of IgG antibodies in *Camelidae* (camels and llamas). These antibodies are devoid of light chains and therefore called 'heavy-chain' IgGs. Their binding domains consist only of the heavy-chain variable domains, referred to as V_{HH}s (Muyldermans *et al.* 1994). V_{HH}s are of great interest since they comprise the smallest possible recognition units of antibodies (Sheriff *et al*. 1996) and are simple in structure. Moreover, affinities found for V_{HH}s are in the nanomolecular range and are comparable with those of Fab (fragment antigen binding) and single chain Fv (scFv) fragments (van der Linden *et al*. 1999). Furthermore, V_{HH}s were shown to be highly soluble and more stable than the corresponding derivatives of scFv and Fab fragments (Ghahroudi *et al*. 1997), even at temperatures as high as 90 °C (van der Linden *et al*. 1999), and thermal unfolding was shown to be (partially) reversible (Perez *et al*. 2001). In addition, the extended hypervariable regions of V_{HH}s are capable of penetrating deep into the cleft of the active sites of enzymes, binding to novel epitopes that are not recognised by conventional antibodies (Desmyter *et al*. 1996; Transue *et al*. 1998; Decanniere *et al*. 1999). Because of this latter property, V_{HH}s may act as potent enzyme inhibitors (Lauwereys *et al*. 1998; Conrath *et al*. 2001).

In V_{HH}s, hydrophobic amino acids are substituted by more hydrophilic ones in the region where, in conventional V_H domains, a hydrophobic patch involved in binding of the V_L domain is normally formed (Padlan 1994; Desmyter *et al*. 1996; Spinelli *et al*. 1996; Muyldermans *et al*. 1994). These substitutions make V_{HH}s more soluble (Davies and Riechmann 1994) and thermostable (Davies and Riechmann 1996; Perez *et al*. 2001). The more hydrophilic properties of V_{HH}s probably increase their secretion from the ER when produced in industrially relevant microorganisms such as the bakers yeast *Saccharomyces cerevisiae* and filamentous fungi like *Aspergillus awamori*. Characteristics of V_{HH}s have been extensively reviewed by Muyldermans (2001).

V_{HH}s were produced in *S. cerevisiae* at levels over 100 mg/L in shake-flask cultures (Frenken *et al*. 2000), although considerable amounts of V_{HH}s were detected intracellularly. For a cost-effective large-scale process for the production of V_{HH}s in *S. cerevisiae*, further improvement is required (Thomassen *et al*. 2002).

Filamentous fungi have the capability of secreting large amounts of protein into their culture medium. Therefore these organisms are widely used for the production of commercially interesting proteins (reviewed by van den Hondel *et al*. 1991; Verdoes *et al*. 1995; Punt *et al*. 2002). In our study we investigate whether the filamentous fungus *A. awamori* is a suitable host for production of V_{HH} fragments raised against the azo-dye RR6 (van der Linden *et al*. 1999).

MATERIALS AND METHODS

Strains and media

A. awamori pyrG mutant strain AWC4.20 (Gouka *et al*. 2001), used as a recipient for transformation, is a derivative of *A. awamori* ATCC 11358 (CBS 115.52). *Escherichia coli* strain DH5α (Sambrook *et al*. 1989) was used for propagation of plasmids.

For shake flask cultivations, pre-culture medium consisted of *Aspergillus* minimal medium (MM; Bennett and Lasure 1991) supplemented with 1 % sucrose as non-inducing carbon source (MMS) and 0.1 % yeast extract (Difco). For induction, *Aspergillus* MM was supplemented with 5 % D-xylose as inducing carbon source (MMX) and 0.1 % yeast extract (Gouka *et al*. 1996a). To prevent proteolytic activity in the culture medium, 0.25 % bovine serum albumin (BSA; Sigma), 1 mM Pefabloc SC (Sigma) or Complete™ Protease Inhibitor Cocktail (Roche) was added, respectively.

Construction of the V_{HH}-expression vector

Molecular cloning procedures were performed according to Sambrook *et al*. (1989).
S. cerevisiae expression vectors containing the genes encoding anti-RR6 V_{HH}s R2, R5, R7 and R9, described by Frenken *et al*. (2000) were obtained from Unilever Research Vlaardingen (URV). These V_{HH}s differ only slightly in their amino acid composition, as shown in Figure 1.

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                                        CDR1                        CDR2
R2      QVQLQESGGGLVQAGGSLRLSCAASGRATSGHGHYGMGWFRQVPGKEREFVAAIRWS.GKETWYKDSVKGRFTISRDNAK     79
R5      QVQLQESGGGLVQAGGSLRLSCAASGRTSHGYGGYGMGWFRQVPGKERELVAAIRWS.GTSTYYADSVKGRFTISRDNVK     79
R7      QVQLQESGGGLVQAGDSLRLSCAASGRTSHGYGGYGMGWFRQIPGKERELVAAIRWS.GRNTYYADSVKGRFTISRDNVK     79
R9      QVQLQESGGGLVQAGESLKLSCAASGNTFSG.G.F.MGWYRQAPGKQRELVATIN.SRGI.TNYADFVKGRFTISRDNAK     75
Cons    qvqlqesggglvqag sl lscaasg    g g   mgw rq pgk re va i  s g  t y d vkgrftisrdn k
                                      ★      ★★ ★

                                CDR3
R2      TTVYLQMNSLKPEDTAVYYCAARPVRVDDISLPVGFDYWGQGTQVTVSS                                  128
R5      NMVYLQMNSLKPEDTAVYHCAARTVRVVDISSPVGFAYWGQGTQVTVSS                                  128
R7      DMLYLQMNSLKPEDTAVYTCAVRTVRVVDISSPVGFAYWGQGTQVTVSS                                  128
R9      KTVYLEMNSLEPEDTAVYYCYTHYFR....S......YWGQGTQVTVSS                                  114
Cons       yl mnsl pedtavy c     r    s      ywgqgtqvtvss
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Figure 1. Sequence alignment of the four anti-RR6 variable heavy-chain antibody fragments (V_{HH}s) used in this study. Hypervariable regions or complementarity-determining regions (CDRs) are boxes (Kabat *et al*. 1991). Amino acids involved in interaction with the V_L domains in conventional antibodies are indicated (∗). The consensus amino acid sequence (Cons) is given. Sequences taken from EMBL databank [accession numbers AJ236100 (R2), AJ236103 (R5), AJ236105 (R7), AJ236107 (R9)] and Frenken *et al*. (2000).

For expression in *A. awamori*, the genes were placed under the control of the strong, inducible *A. awamori* 1,4-β endoxylanase A (*exlA*) promoter (Gouka *et al*. 1996a). The integration vector was derived from the *A. awamori* expression plasmid pAW14B12 (van Gemeren *et al*. 1996). The 5'-end of the four V_{HH} genes encodes the sequence QVQLQ and comprises a *Pst*I-site. The 3'-end encodes the sequence VTVSS and comprises a *Bst*EII-site. *Pst*I-*Bst*EII V_{HH} DNA fragments were cloned into a pBluescript SKII (Stratagene) vector, named pBLUE-V_{HH} in which a part of the *exlA* promoter sequence, the *exlA* signal sequence and the identical 5'

and 3' ends of the V_{HH}s were introduced by PCR. The final integration plasmid was constructed by ligation of the *Xho*I/*Afl*II fragments from the pBLUE-V_{HH} subcloning vectors into the *Xho*I/*Afl*II site of plasmid pAW14B12. The resulting V_{HH}–expression vectors were designated pAWR2, pAWR5, pAWR7 and pAWR9. The expression cassette present in these vectors is schematically depicted in Figure 2.

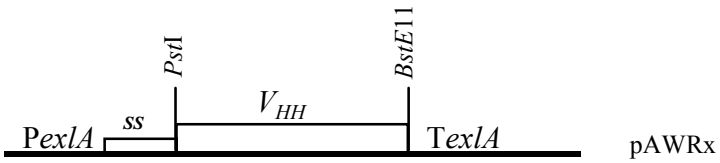

Figure 2. Schematic representation of the V_{HH} expression cassette, in which Rx is V_{HH} fragment R2, R5, R7 or R9, respectively. For an explanation, see Materials and methods.

Transformation and selection of transformants

For transformation of *A. awamori* AWC4.20, *Aspergillus* MM with 0.5 % yeast extract, 1 % glucose, 0.2 % casein amino acids, 10 mM uridine (Serva) and 10 mM uracil (Sigma) was inoculated with 1 x 10^6 spores/ml. After overnight growth, the mycelium was diluted 1:10 and cultivated for another 16-18 h. Transformation of *A. awamori* protoplasts was carried out essentially as described by Punt and van den Hondel (1992). Since no *Aspergillus* selection marker was present on the V_{HH} - expression vectors, a co-transformation experiment was carried out with the co-transforming plasmid pAmdS/pyrG, containing the *A. niger pyrG* gene (coding for orotidine-5'-phosphate decarboxylase; Gouka *et al*. 1995) and the *A. nidulans amdS* gene (coding for acetamidase; Hynes *et al*. 1983). 2 µg co-transforming plasmid (pAmdS/pyrG) was used together with 10 µg of the V_{HH}–expression vectors pAWR2, pAWR5 pAWR7 or pAWR9, respectively. Selection of *A. awamori pyrG*$^+$ (co-) transformants was performed on osmotically stabilised (1.2 M sorbitol) bacteriological agar (Oxoid) plates containing *Aspergillus* MM with 1 % glucose.

DNA/RNA isolation

Aspergillus chromosomal DNA was isolated as described by Kolar *et al*. (1988). For RNA isolation, mycelium was grinded in liquid N_2 with a mortar and pestle and total RNA was isolated using Trizol (Life Technologies), according to the manufacturer's instructions.

Southern and northern analysis

Samples for Southern and northern blot analysis were treated according to Sambrook *et al*. (1989). For Southern blot analysis, chromosomal DNA was digested with both *Nco*I and *Xho*I, which have restriction sites in the promoter and terminator sequence of both the V_{HH} expression cassette and endogenous *exlA*. Hybridisation was performed under homologous conditions using the EXLA probe - a 400 bp DNA fragment containing the 5' and 3' *exlA* non-coding sequences (Gouka *et al.* 1996b). A 1.5 kb *Hin*dIII fragment from pAB5-2 (*gpdA* probe) was used as a loading control (Verdoes *et al*. 1994). Probes were labelled with ^{32}P using the Rediprime ™ II kit (Amersham Pharmacia). Quantification of hybridising bands on

Southern and northern blot was performed with Genetools software, version 2.10.03 (Syngene Synoptics).

Induction of the *exlA* promoter in shake flask cultures

Induction of the *A. awamori exlA* promoter was carried out essentially as described by Gouka *et al*. (1996a), with minor modifications. Pre-culture medium (MMS; 100 ml) was inoculated with $1x10^6$ spores/ml (see Materials and methods). After 24 h of cultivation in an air-incubator (250 rpm, 30 °C) mycelium was harvested by filtration using a Miracloth filter (Calbiochem) and washed with MM without a carbon source. Mycelium was transferred to a shake flask containing 50 ml *Aspergillus* induction medium (MMX) and culture samples were collected after different periods of time. Medium and mycelium were separated by filtration over Miracloth and mycelium was frozen in liquid N_2.

Protein analysis methods

After induction of the *exlA* promoter, medium samples of *A. awamori* were taken and subjected to a 8-16 % gradient Tris-glycine gel (Pager™ Gold Precast Gels, BMA) under reducing conditions, followed by blotting onto a nitrocellulose membrane (Protran, Schleicher & Schuell).

The amount of cell-wall-associated V_{HH} or intracellular V_{HH} was determined from samples taken 24 h after induction according to van Gemeren *et al*. (1996) with minor modifications. Transformants were cultivated in 100 ml MMS for 24 h, followed by transfer of mycelium to 50 ml MMX. After 24 h induction, mycelium was separated from the culture medium by filtration over Miracloth. Half the mycelium was resuspended in 25 ml MM and the other half was resuspended in the same medium containing 1 % SDS. The latter treatment was carried out to remove (wash) secreted protein adhering to the mycelium. The cultures were shaken at 4 °C, 75 rpm for 1 h. Subsequently, the mycelium was filtered, frozen with liquid N_2 and powdered with a mortar and pestle. After disruption, the cells were resuspended in extraction buffer (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 1 mM EDTA), and centrifuged at 4 °C. The supernatant was named the 'soluble fraction'. The pellet was washed once with extraction buffer containing 1 % (v/v) Triton X-100 and a second washing step was performed with extraction buffer containing 1 % (w/v) SDS. These treatments were performed in order to distinguish between intracellular V_{HH} loosely or tightly associated with the pellet fraction. During the intracellular extraction procedure, protease inhibitor cocktail tablets (Complete; Roche) were added to all buffers used, according to the manufacturer's instructions. All fractions were subjected to western blot analysis.

Blots were blocked with 1 % Protifar (Nutricia) in PBS with 0.05 % Triton X-100 for 60 min. Polyclonal rabbit anti-llama V_{HH} was obtained by injecting 8 different antibody fragments, including V_{HH} R2, R5 and R7, but not R9. Blots were incubated with 1:2000 diluted polyclonal rabbit anti-llama V_{HH} or anti-endoxylanase, obtained from URV. In the second step blots were incubated with goat-anti-rabbit IgG alkaline phosphatase conjugate (Dako, D0487). Blots were developed with nitroblue tetrazoliumchloride (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) according to Blake *et al*. (1984) and amounts of extracellular V_{HH} were quantified with Genetools software, version 2.10.03 (Syngene Synoptics).

Functional analysis (ELISA)
Polysorb microtiter plates (Nalgene Nunc, 475094) were coated overnight at 37 °C with 0.5 mM RR6 (Reactive red 6, Procion Rubine MX-B, ICI) in coupling buffer (0.1 M *di*-sodium tetraborate decahydrate, 0.15 M NaCl, pH 8.5). Washing between the different steps was performed with washing buffer (20 mM Tris-HCl, 10 mM NaCl, 60 mM citric acid, 0.05 % Tween-20 pH 7.4). Plates were blocked with blocking buffer [3 % Protifar (Nutricia) in PBS pH 7.4] followed by incubation of medium samples obtained from induction experiments. Rabbit anti-llama V_{HH} 1: 2000 in buffer (PBS, 0.15 % Tween-20, 0.1 % BSA, pH 7.4) was used in the next step followed by incubation with polyclonal goat anti-rabbit IgG-HPO conjugate 1:2000 (Dako, P0448) to detect the bound V_{HH} fragments. As a substrate OPD·HCl (1,2-phenylenediamine dihydrochloride, Fluka) was used to detect bound conjugate.

D-xylose concentration measurements
D-Xylose concentrations in *Aspergillus* induction medium (MMX) were determined by measuring the production of newly liberated reducing groups using the DNS (2-hydroxy-3,5-dinitrobenzoic acid; Merck) method, as described by Gouka *et al*. (1996a).

RESULTS

Transformation and screening
In a co-transformation experiment, the *A. awamori pyrG* mutant strain AWC4.20 was transformed with both plasmid pAmdS/pyrG and one of the V_{HH}-expression vectors (see Materials and methods). Transformants were selected for their ability to grow on agar plates containing MM without uridine. Several transformants showed increased colony growth on acetamide and acrylamide plates, indicating that multiple copies of the co-transforming plasmid pAmdS/pyrG were integrated in the *A. awamori* genome. Transformants were screened for integration of the V_{HH}-expression vector by Southern blot hybridisation using the EXLA probe (results not shown). Based on the presence of a single-copy of the endogenous *exlA* gene, the number of integrated V_{HH} expression cassettes could be estimated. Transformants with different integrated gene copy-numbers were observed and for further studies, one single and one multi-copy transformant for each V_{HH}-expression vector were chosen (as indicated in Figure 3).

Northern blot analysis
To determine the expression of V_{HH} genes at the transcription level, mRNA from both single and multi-copy transformants isolated 24 h after induction was analysed on a northern blot. The EXLA probe was used, which hybridises both with the V_{HH} mRNA transcript provided with the *exlA* expression signals and with *exlA* mRNA of the host strain (Gouka *et al*. 1996b). Figure 3 shows results from the northern blot analysis of the different single and multi-copy V_{HH} transformants. In both single and multi-copy V_{HH} transformants, *exlA* and V_{HH} mRNA of the expected size was detected (~1.0 and 0.7 kb, respectively). After correction for loading differences (*gpdA* as a control; Verdoes *et al*. 1994), in most of the single-copy transformants the V_{HH} mRNA signal was comparable with the *exlA* mRNA signal (see Figure 3C). In multi-copy transformants higher levels of V_{HH} mRNA were observed (except for AWC R7#21, possibly a consequence of the integration sites of the vector). Furthermore, a decrease in the amount of *exlA* mRNA was observed in the multi-copy transformants compared with the

wild-type strain AWC4.20. This effect can be attributed to titration of the xylanase transcription activator XlnR (van Peij *et al*. 1998), which has three binding sites in the *exlA* promoter. Although in the multi-copy transformants the amount of transcription activator(s) might be limited, we conclude that, in view of the V_{HH} mRNA levels detected by northern blot analysis, no other bottlenecks at the transcription level exist.

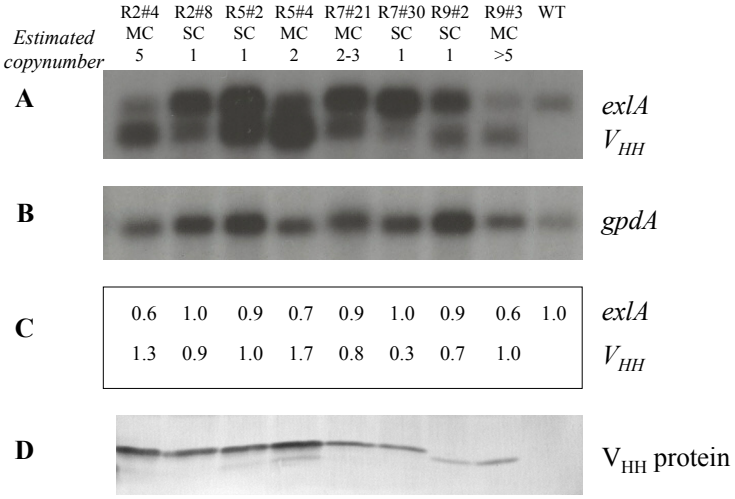

Figure 3 a, b Northern blot analysis of single-copy (SC) and multi-copy (MC) V_{HH} transformants under inducing conditions. Estimated gene copies are indicated and varied from one to more than five. The gene copy number was estimated based on the presence of a single copy of the endogenous *exlA* gene. **a** EXLA probe. **b** *gpdA* probe. **c** Ratio between intensity of the normalised V_{HH} and *exlA* signal of each transformant and the normalised *exlA* signal of the wild type strain. V_{HH} and *exlA* signals were normalised against the *gpdA* signal shown in **b**. *ExlA* and V_{HH} mRNA is indicated. **d** Western blot analysis of extracellular V_{HH} production in culture medium samples of the V_{HH} transformants taken 24 h after D-xylose induction. As a negative control, a medium sample of the wild-type strain AWC4.20 was used. Blots were probed with the anti-llama V_{HH} polyclonal antibody.

Analysis of protein production

Medium samples of transformants 24 h after induction were analysed by western blot (Figure 3d). V_{HH}s could be detected in the medium samples as a major band of ~14 kDa (R2, R5 and R7). The polyclonal serum used to detect V_{HH}s in the culture medium was not raised against V_{HH} R9 specifically. Nevertheless, a cross-reactive band of ~12 kDa was observed. Extracellular V_{HH} R2 levels were determined by comparing the amount of V_{HH} R2 secreted by AWCR2#4 with serial dilutions of purified V_{HH} R2 derived from a yeast transformant. AWCR2#4 secreted 1.5 mg/L V_{HH} R2 into the culture medium, determined after 24 h of growth on induction medium.

Functionality of V_{HH}s (ELISA)

To determine whether the V_{HH}s produced were functional in binding to their antigen RR6, medium samples of the transformants AWCR2#4, AWCR5#4, AWCR9#3 and the wild type strain AWC4.20 were analysed in an ELISA using a microtiter plate coated with the azo-dye RR6. Detection was performed with polyclonal rabbit anti-llama V_{HH} IgG as described in Materials and methods (Figure 4). No binding was observed when medium samples of the

wild-type strain AWC4.20 were applied to wells coated with RR6. This demonstrated that the reaction observed with the medium samples from the transformants is the result of specific binding of the V_{HH} fragment to its antigen RR6. Therefore, V_{HH}s produced by *A. awamori* are functional in binding to their antigen.

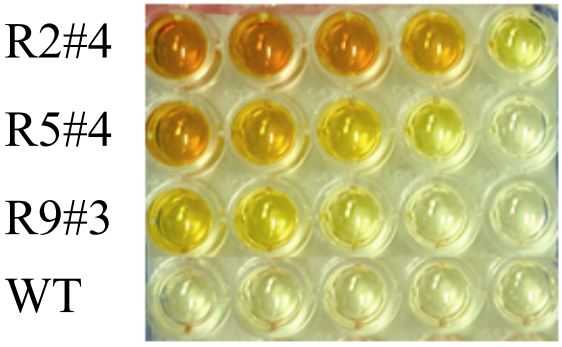

Figure 4. Functionality of V_{HH} fragments. Serial 2-fold dilutions of medium samples of transformants AWCR2#4, AWCR5#4 and AWCR9#3 were applied to an RR6- coated microtiter plate. Medium of the wild-type strain AWC4.20 was taken as a negative control. The ELISA procedure was performed as described in Materials and methods.

Effect of proteases on V_{HH} R2 production

As no major problem was observed on the mRNA level and overall protein production levels of V_{HH}s were low, we decided to select the best V_{HH} producer, multi-copy transformant AWCR2#4, for a more detailed study on V_{HH} production by *A. awamori*. One of the major factors affecting the final yield of heterologous protein production in filamentous fungi is thought to be the action of proteases. Proteases are abundantly produced by most fungal strains (Archer *et al*. 1992; van den Hombergh *et al*. 1997). Therefore, we determined whether the low levels of V_{HH} observed were due to the specific action of proteases. Experiments were carried out to inhibit putative extracellular proteases in the *Aspergillus* culture medium. Medium samples were taken at several time-points after transfer of transformant AWCR2#4 to MMX medium with or without the addition of protease inhibitors [1 mM Pefabloc (a serine protease inhibitor) or Complete™ Protease Inhibitor Cocktail] or 0.25 % BSA. BSA is supposed to act as an alternative substrate for proteases to prevent degradation of the protein of interest. Samples were analysed by western blot and the results after 4 h (lanes 1 to 4) and 7 h (lanes 5 to 8) after induction are shown in Figure 5. The addition of Pefabloc (lanes 2 and 6) to MMX medium did not increase the yield of V_{HH} R2 in the culture medium compared with the initially amount of V_{HH} R2 produced (lanes 1 and 5). When Complete™ was added to MMX, a slight increase was observed after 7 h. However, when 0.25 % BSA was added to MMX medium the amount of V_{HH} R2 was twice as much as that observed without any addition. Furthermore, the addition of BSA resulted in a 5-fold increase in V_{HH} R2 production after 24 h of induction (Figure 6 and results not shown). When Complete™ and BSA were added together, no additional increase in V_{HH} R2 was found, suggesting that proteases that might be inhibited by Complete™ also use BSA as an alternative substrate.

The observation that extracellular proteases were partially responsible for the low levels of V_{HH} R2 was further confirmed by experiments with the addition of purified R2 from yeast to the culture medium. Degradation was observed in the culture medium under different conditions (results not shown).

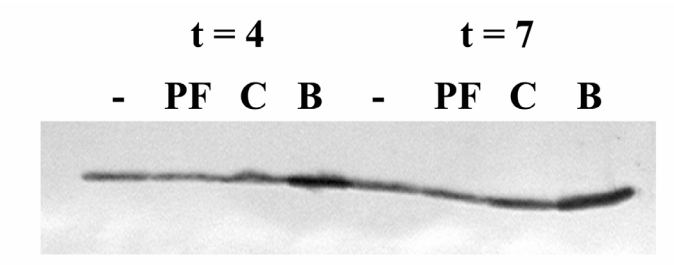

Figure 5. Secretion of V_{HH} R2 into the extracellular medium in the presence of protease inhibitors or 0.25 % BSA. Medium samples were taken 4 and 7 h after transfer to MMX. Medium was supplemented with 1 mM Pefabloc (PF), Complete™ Protease Inhibitor Cocktail (C) or 0.25 % BSA (B); - no supplement. Medium samples were subjected to SDS-PAGE and western blotting.

Heterologous production of V_{HH} R2 compared to EXLA

To analyse the heterologous production capacity of V_{HH} R2 by AWCR2#4, a time curve was constructed by taking medium samples every two hours after transfer of mycelium to MMX, measuring protein levels by means of western blotting and subsequent quantification of the signals.

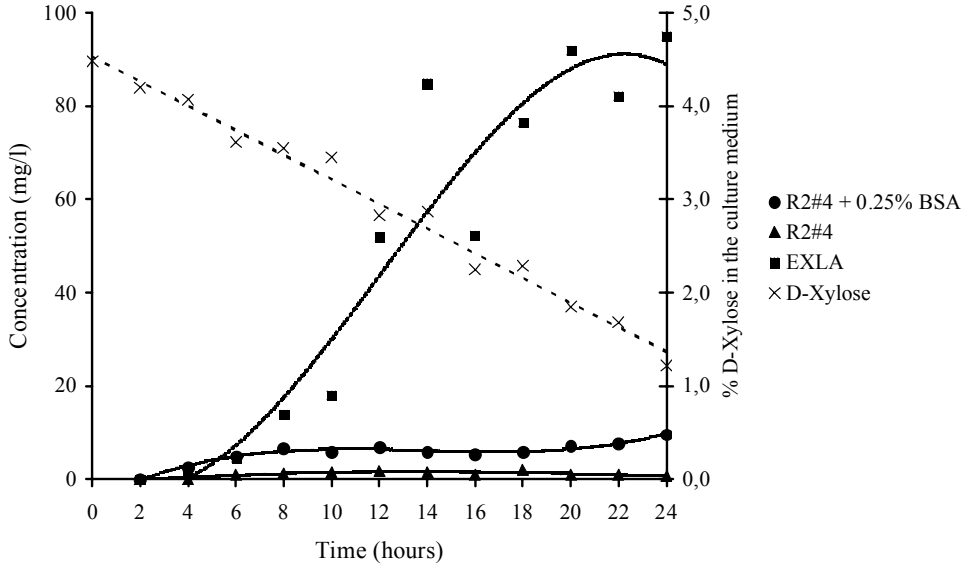

Figure 6. Amounts of extracellular V_{HH} R2 with (●) and without (▲) the addition of 0.25 % BSA and extracellular EXLA (■) after induction of AWCR2#4 with 5 % D-xylose (experiment performed in duplicate). Amounts were calculated from western blot, using α-V_{HH} or α-EXLA polyclonal antibodies. Percentage of D-xylose (x) present in the culture medium is given on the secondary axis. Concentration of D-xylose was measured using 2-hydroxy-3,5-dinitrobenzoic acid (DNS).

As shown in Figure 6, a concentration of approximately 1.5 mg/L V_{HH} R2 was reached in the culture medium (Figure 5; ▲). V_{HH} R2 was already detected in the culture medium after two hours. The addition of 0.25 % BSA to MMX increased the level of V_{HH} R2 in the culture medium, reaching a level of 7.5 mg/L after 24 h of induction (Figure 5; ●). This indicates that addition of BSA leads to a 5-fold increase in V_{HH} R2 levels, presumably by capturing (partially) the action of extracellular proteases in the culture medium. Besides the production levels of V_{HH} R2, the production levels of the endogenous EXLA protein by AWCR2#4 were also measured under the same conditions. Secretion levels of the endogenous EXLA increase rapidly, reaching an amount of approximately 75 mg/L in the culture medium (Figure 5; ■). As explained above (northern analysis), the gene encoding V_{HH} R2 is under the control of the *exlA* promoter, which leads to a titration effect in transformant AWCR2#4. As a result EXLA levels were lower than in the wild-type strain (Figure 3).

Induction of the *exlA* promoter is regulated at the transcriptional level by the presence of D-xylose in the culture medium. The initial D-xylose concentration was 5 %, although lower levels were detected at t = 0 (immediately after the transfer of mycelium to MMX), possibly by the dilution of mycelium in the medium. D-xylose levels declined rapidly although more than 1 % was still present at the end of the experiment. This indicates that depletion of D-xylose can be excluded as a bottleneck during V_{HH} production.

Localisation of V_{HH}s

Western blot analysis of detergent-treated and disrupted mycelium samples of transformant AWCR2#4 was carried out to determine whether the low level of V_{HH} R2 secreted into the culture medium was due to cell wall adherence or intracellular accumulation of V_{HH} R2 (Figure 7). After washing of whole mycelium in MM, some V_{HH} R2 is detected in this medium (Figure 7A, lane 4). This portion of V_{HH} R2 is probably loosely attached to the cell wall and is washed off in MM. Another possibility is that there is still some secretion of V_{HH} R2 during the 1 h washing of mycelium in MM. When mycelium is incubated in MM with 1 % SDS, a large amount of V_{HH} R2 is released from the cell wall (Figure 7A, lane 5), which is comparable to the amount detected in the medium (lane 2). This fraction of V_{HH} R2 probably adheres to the mycelium.

To determine whether V_{HH} R2 was accumulating intracellular, disrupted mycelium washed in MM with 1 % SDS, was treated with 1 % Triton X-100 and subsequently with 1 % SDS. The remaining pellet was resuspended in sample buffer. As shown in Figure 7A (lanes 6-9) hardly any V_{HH} R2 was detected in these fractions, indicating that no intracellular accumulation occurs. As a control for the experiment described above, the endogenous endoxylanase (EXLA) level was determined (Figure 7B). EXLA was detected in both culture medium and in medium samples from washed mycelium (Figure 7B, lane 2-5). No difference in EXLA level is observed between mycelium treated with MM or MM with 1 % SDS. This indicates that EXLA is loosely bound to the cell wall and does not adhere to the cell wall as shown in this experiment for V_{HH} R2. Furthermore, no EXLA was detected intracellularly, demonstrating that the protein is efficiently secreted (Figure 7B, lanes 6-9).

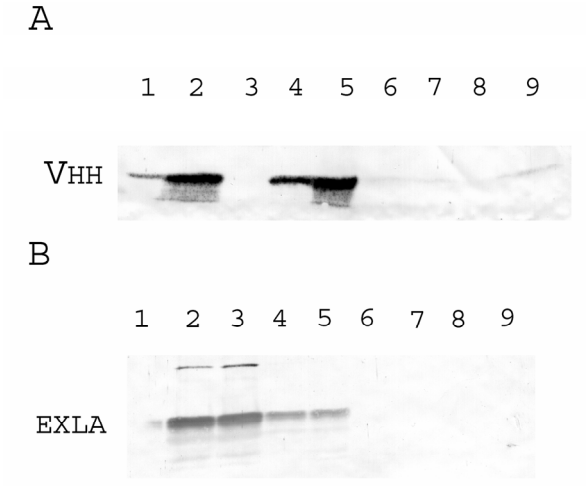

Figure 7 a, b. Western blot analysis of culture medium and different mycelial fractions of transformant AWCR2#4 after 24 h of induction. Lanes: 1, purified R2; 2, medium AWCR2#4; 3, medium wild type *A. awamori*; 4, wash with minimal medium of whole mycelium; 5, wash with minimal medium and 1 % SDS of whole mycelium; 6, soluble fraction of disrupted mycelium; 7, supernatant after 1 % Triton X-100 extraction of disrupted mycelium; 8, supernatant after 1 % SDS extraction of disrupted mycelium; 9, pellet of disrupted mycelium. Detection with **a** polyclonal rabbit anti-llama V_{HH}, and **b** polyclonal rabbit anti-endoxylanase.

CONCLUSIONS AND DISCUSSION

In this study we analysed the feasibility of V_{HH} production by *A. awamori*. We showed that, albeit with low amounts (up to 7.5 mg/L V_{HH} R2, with BSA addition), anti-RR6 V_{HH} fragments were secreted into the culture medium. Furthermore, ELISA plate assay demonstrated that the V_{HH}s produced were functional in binding to their antigen, the azo-dye RR6.

Interestingly, in multi-copy transformants, reduced levels of endogenous *exlA* mRNA were detected. As observed by Gouka *et al*. (1999) and Lokman *et al*. (2003), introduction of several copies of the *exlA* promoter results in binding (titration) of the transcription factor XlnR (van Peij *et al*. 1998), which is present in limiting amounts. This results in less binding of the factor to the promoter element upstream of the endoxylanase gene and thus a reduced *exlA* mRNA level (titration effect). The titration effect was also visible on a Coomassie-stained SDS-PAGE. It is therefore expected that increasing the copy-number of the V_{HH}-expression vector will have only a minimal effect on the level of V_{HH} mRNA and V_{HH} protein.

It has been observed that bottlenecks can occur at different levels of protein production, such as transcription, translation, secretion and/or extracellular degradation (reviewed by Gouka *et al*. 1997). As highly inducible fungal transcription-control regions are used to express heterologous genes in general, mRNA levels in are not generally considered as limiting factors. This was also observed in our studies, where expression of V_{HH} genes was under control of the strong, inducible *A. awamori exlA* promoter (Gouka *et al*. 1996). mRNA levels were more or less comparable with those of the endogenous *exlA* gene, which suggested that no major bottleneck exists at the transcriptional level. Little information is available on the stability of heterologous mRNA and it has been shown that codon

optimisation can lead to significantly higher expression in filamentous fungi (Gouka *et al*. 1997; Te'o *et al*. 2000). Since preliminary results in fermentation experiments showed that with a V_{HH} fused to *Arthromyces ramosus* peroxidase (ARP) under the control of the *exlA* expression signals, levels of 200 mg/L of the fusion protein could be achieved (B.C. Lokman, unpublished results), codon usage is not regarded as a major problem.

Based on our results, proteolysis is one of the reasons for the low levels of V_{HH} in the culture medium. Moreover, we showed that adherence of V_{HH} to the fungal cell wall also effects secreted yields. Washing of the mycelium from transformant AWCR2#4 with SDS-containing wash buffer resulted in removal of an amount of V_{HH} equal to that present in the culture medium. Localisation of heterologous proteins to the fungal cell wall has been observed before (Gordon *et al*. 2000; van Gemeren *et al*. 1996) and can contribute to the low level of protein of interest in the culture medium.

If extracellular protease activity is blocked by BSA, ~7.5 mg/L V_{HH} R2 is secreted in the culture medium. The capacity of the system under the same conditions is 75 mg/L EXLA. V_{HH} R2 secretion is then only 10 % of that expected based on extracellular EXLA levels. However, a correction factor of 2 is necessary for the difference in molecular weight (14 kDa for V_{HH} R2 versus 30 kDa for EXLA). So V_{HH} R2 secretion is only 20 % compared to that of EXLA. We therefore assumed that 80 % of the V_{HH} R2 protein initially produced is degraded. This suggests a post-translational problem in V_{HH} production, which probably acts intracellular and/or at the cell wall level.

Thomassen *et al*. (2002) reported production of the same anti-RR6 V_{HH} fragments by *S. cerevisiae*. Extracellular levels ranged between 0.07 mg/L for V_{HH} R5 to 0.12 mg/L for V_{HH} R7. In this *S. cerevisiae* expression system considerable amounts of V_{HH}s were detected inside the cell. This is in contrast with *A. awamori* where no accumulation of V_{HH} fragments was observed. This suggests that, in *A. awamori* V_{HH} fragments might be subjected to proteolytic degradation in the vacuole or via ERAD (ER-Associated Degradation; reviewed by Conesa *et al*. 2001).

At present, V_{HH} production levels are too low for industrial large-scale applications, where levels over 1 g/L are required. Taking into account that the production levels described in this paper were obtained without optimisation of the system, higher levels must be within reach. Several strategies have been developed to improve heterologous protein yields from filamentous fungi (reviewed by Archer *et al*. 1994; Verdoes *et al*. 1995). An approach to overcome limitations in the filamentous secretion process is fusion of the gene of interest to the 3' end of a highly expressed gene. A well-known example is *glaA* of which the gene product glucoamylase is efficiently secreted (Ward *et al*. 1990). Other examples of translational fusions with carrier proteins are extensively reviewed by Gouka *et al*. (1997). We are studying currently the possibility of using the peroxidase of *Arthromyces ramosus* (ARP; Sawai-Hatanaka *et al*. 1995) as a carrier protein for the production of V_{HH}s since ARP can be produced in high amounts by *A. awamori* (Lokman *et al*. 2003).

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Chapter 4

Efficient production of *Arthromyces ramosus* peroxidase by *Aspergillus awamori*

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ABSTRACT

The heterologous production of *Arthromyces ramosus* peroxidase (ARP) was analysed in the filamentous fungus *Aspergillus awamori* under control of the inducible endoxylanase promoter. Secretion of active ARP was achieved up to 800 mg/L in shake flask cultures. Western blot analysis showed that an ARP product of the correct molecular weight was produced. In contrast to several other studies about heterologous production of heme-containing peroxidases, our results suggest that in *A. awamori* no heme limitation exists during overproduction of ARP.

INTRODUCTION

Peroxidases are enzymes that utilise hydrogen peroxide to catalyse the oxidation of a range of organic and inorganic compounds. A wide variety of microbial organisms (in particular fungi), plants and animals produce them. The specificity and biological functions of these peroxidases vary with the source of the enzyme. In relation to their potential biotechnical applications in paper and pulp, and textile industries, several of these fungal peroxidases have been intensively studied (for a review, see Conesa *et al.* 2002).

Arthromyces ramosus peroxidase (ARP) is one of the peroxidases that are of industrial interest (Akimoto *et al.* 1990; Fukuyama *et al.* 1995). Sequence analysis of this 41 kDa monomeric glycoprotein revealed 99 % identity to the amino acid sequence of *Coprinus cinereus* peroxidase (Sawai-Hatanaka *et al.* 1995; Baunsgaard *et al.* 1993). ARP contains protoheme IX as a prosthetic group and has a broad specificity for phenolic and anilinic hydrogen donors that make the enzyme suitable for use in bleaching processes (Kjalke *et al.* 1992). To make industrial applications feasible, an efficient and cost effective production process is needed.

In this context we decided to evaluate the potential of the heterologous production of ARP in the filamentous fungus *Aspergillus awamori. Aspergilli* have a long history in industrial enzyme production (Archer and Peberdy 1997). However, a limited set of data is available for heterologous production of heme containing proteins in these organisms. In several studies it was shown that in particular the availability of heme is a factor which limits yields (Pease *et al.* 1991; Whitwam and Tien 1996; Stewart *et al.* 1996; Conesa *et al.* 2000). Therefore we determined whether in *A. awamori* the production of ARP is also limited by the availability of heme.

MATERIALS AND METHODS

Strains, media and transformation procedures

Escherichia coli strain DH5α was used for construction of recombinant DNA plasmids (Sambrook *et al.* 1989). *A. awamori* AWC4.20 (*pyrG*$^-$), used as the recipient strain in transformation experiments, is a derivative of *A. awamori* ATCC 11358 (CBS 115.52) (Gouka *et al.* 2001).

Aspergillus Minimal Medium (MM; Bennett and Lasure 1991), supplemented with 0.1 % yeast extract (Difco), 10 mM uridine and 1 % sucrose as C-source, was used for cultivation of *A. awamori* AWC4.20. Induction of the *A. awamori exl*A promoter was carried out in induction medium (MM + 0.1 % yeast extract) with 5 % D-xylose as C-source as described by Gouka *et al.* (1996a). Shake flask cultures were incubated at 30 ºC and 300 rpm, and samples were taken at different time points after induction. For studies on heme availability MM was supplemented with 500 mg hemin/L according to Conesa *et al.* (2000).

Transformation of *A. awamori* AWC4.20 was performed as described by Gouka *et al.* (1995) with a mixture of the peroxidase expression vector pARP (10 µg) and plasmid pAmdS/pyrG (2 µg), containing the *A. nidulans amd*S gene (Hynes *et al.* 1983) and *A. niger pyr*G gene (Gouka *et al.* 1995). Transformants were selected for uridine prototrophy. Co-transformants containing the peroxidase expression vector were selected by screening for peroxidase activity.

Recombinant DNA techniques

Enzymes for molecular cloning were purchased from Boehringer or Bethesda Research Laboratories and were used according to the specifications of the manufacturer. Transformation of *E. coli*, plasmid DNA isolation from *E. coli*, DNA and RNA transfer to Hybond N filters and filter hybridisations were performed according to Sambrook *et al.* (1989). *Aspergillus* chromosomal DNA and RNA isolations were performed according to Kolar *et al*. (1988).

For Southern and northern blot analysis a 400 bp DNA fragment comprising the 5'- and 3'- non-coding *exlA* sequences was used as a probe (EXLA probe) (Gouka *et al.* 1996b). Quantification of band intensities from Southern and northern blot analysis was performed with Gene Tools software (Syngene).

For western blotting experiments (Sambrook *et al.* 1989), polyclonal antibodies against ARP (α-ARP) were used. Detection was performed with alkaline phosphatase conjugated secondary antibodies (Promega).

Expression vector construction

A synthetic copy of the *A. ramosus* peroxidase was constructed encoding the mature form of ARP (Swiss-Prot accession number P28313), optimised for frequently used yeast codons (*i.e*. CTC was replaced by CTG), with minor modifications to incorporate unique restriction enzyme recognition sites without affecting the encoded amino acid sequence. For expression analyses the synthetic gene was cloned into a vector derived from the *Aspergillus* expression vector pAW14B12 (van Gemeren *et al.* 1996). In the resulting expression vector, pARP, expression is under control of the endoxylanase expression signals as described previously by Gouka *et al.* (1996a) (Figure 1).

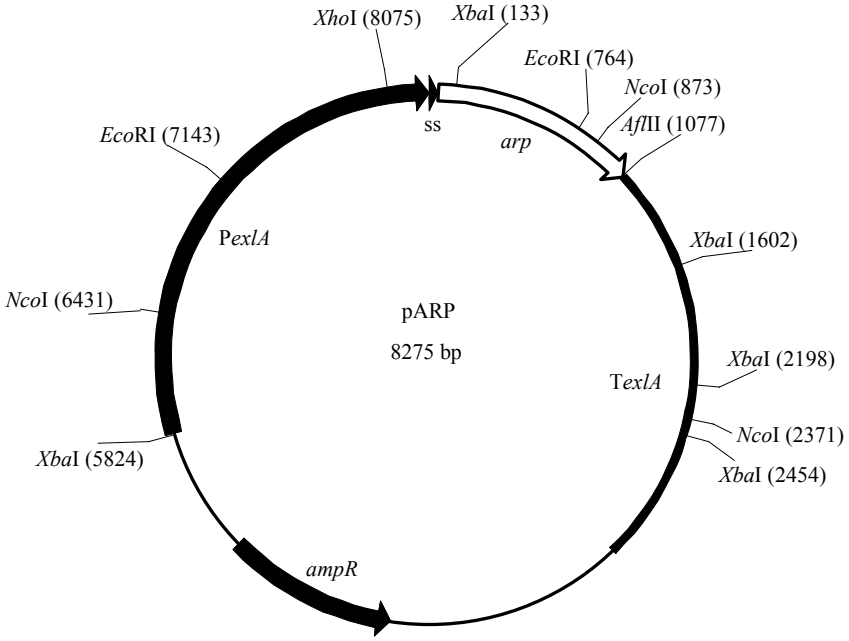

Figure 1. Schematic representation of expression vector pARP. *arp*: peroxidase gene of *A. ramosus*; P*exlA*: endoxylanase promoter of *A. awamori* (inducible by D-xylose); ss: endoxylanase signal sequence of *A. awamori*; T*exlA*: endoxylanase termination region of *A. awamori*.

Peroxidase activity assays

Transformants were assayed for peroxidase activity according to Conesa *et al*. (2000) with the following modifications. *A. awamori* transformants were inoculated onto Petri dishes containing *Aspergillus* MM (Bennett and Lasure 1991) supplemented with 5 % D-xylose, 0.03 % *O*-anisidine as a substrate (Fluka, Buchs, Switzerland) and 1.4 % agar. Plates were incubated at 30 ºC for 2 days and then flooded with an overlay containing 50 mM Na-phosphate buffer (pH 7.0), 0.015 % H_2O_2 and 1 % agarose. Peroxidase producing transformants developed a purple halo after 5-60 sec.

ARP activity in liquid cultures was measured by monitoring the oxidation of 0.5 % diammonium 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) in the presence of 50 mM Na-phosphate buffer (pH 7.0) and 0.015 % H_2O_2 at 415 nm (Glenn and Gold 1985). Purified ARP from Fluka (3280 U/mg) was used as a standard.

RESULTS

ARP producing transformants

Co-transformants of *A. awamori* AWC4.20 were analysed in a peroxidase activity plate assay to test their ability to produce active ARP (see Materials and methods). Three out of 12 uridine prototrophic transformants developed a clear purple halo, indicating that these transformants secrete active ARP into the medium (see Figure 2). The transformants were designated ARP#a1, ARP#1 and ARP#4, respectively. To verify if ARP secreted by *A. awamori* is similar in molecular weight to ARP produced by *A. ramosus,* medium samples of the three transformants from shake flask cultures using D-xylose induction were compared with purified ARP (Fluka) on a 10-15 % SDS PAGE gel. As indicated in Figure 3 protein bands representing amylase and endoxylanase are visible on a Coomassie stained gel, both in wild type and transformants. Furthermore, the 41 kDa ARP protein is clearly visible in the three transformants. From activity assays (see Materials and methods) we concluded that between 45 and 635 mg/L active ARP was produced in the respective strains (Figure 4A). The three transformants were further studied in Southern and northern analyses.

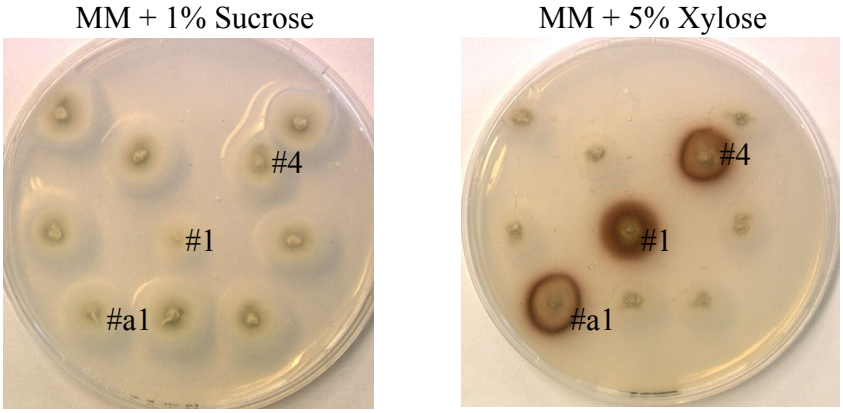

Figure 2. *O*-Anisidine plate assay of *A. awamori* co-transformants. Transformants were analysed on plates containing MM with *O*-anisidine and either sucrose or the inducer D-xylose. Under inducing conditions red halos are visible around the colonies of transformants ARP#a1, ARP#1 and ARP#4 indicating the production of active peroxidase.

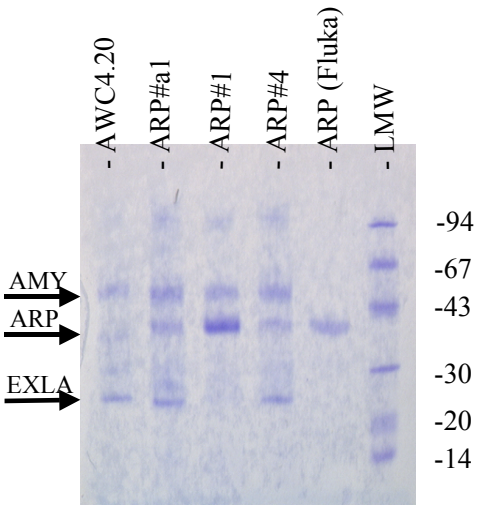

Figure 3. Analysis of medium samples on a 10-15 % SDS PAGE gel (Coomassie staining) of *A. awamori* ARP producing transformants ARP#a1, ARP#1, ARP#4 using host strain *A. awamori* AWC4.20 as a control. Relevant protein bands are indicated with an arrow. AMY: amylase; ARP: *A. ramosus* peroxidase; EXLA: endoxylanase. ARP (Fluka): 250 mg/L. The LMW protein marker is indicated in kDa.

Southern and northern analyses of ARP producing transformants

Chromosomal DNA of the three peroxidase producing transformants was isolated, digested with *Nco*I [which has restriction sites in the *exlA* promoter, the *exlA* terminator and the *arp* gene (Figure1)] and analysed in a Southern blot hybridisation using the EXLA probe (see Materials and methods). With this probe also the endogenous *exlA* gene could be detected in both, transformants and wild type strain allowing copy number determinations. Quantification of the band intensities revealed 5-10 copies of the *arp* gene in transformant ARP#1, 2 copies in ARP#a1, and 1 copy in ARP#4 (Figure 4B and C).

The *arp* mRNA levels of the *A. awamori* transformants were analysed after 24 hours induction with D-xylose using the EXLA probe. At this time point a high level of *arp* mRNA was observed in the multi-copy transformant ARP#1 whereas much lower levels were detected in the transformants ARP#a1 and ARP#4 (Figure 4D). Furthermore, mRNA derived from the endogenous *exlA* gene could be detected as well.

Heme limitation?

In several studies on heterologous expression of peroxidases it was shown that heme availability is a bottleneck for the overproduction (Pease *et al.* 1991; Whitwam and Tien 1996; Stewart *et al.* 1996; Conesa *et al.* 2000). To investigate whether the production of ARP in *A. awamori* could be even further increased by heme addition, we have cultivated *A. awamori* transformants with and without additional hemin (Conesa *et al.* 2000), in the induction medium and analysed ARP production after 24, 30 and 48 hours of induction. Analysis of medium samples on Coomassie stained SDS PAGE gels (not shown) and an activity assay using ABTS as substrate demonstrated that, under the conditions used here, additional heme did not result in increased ARP production in all three transformants. This result suggests that there might be no heme limitation during the overproduction of ARP in *A.*

awamori. In Figure 5 the heme-limitation experiment of the multi-copy transformant ARP#1 is depicted, demonstrating that in this experiment a production level of 800 mg/L was achieved.

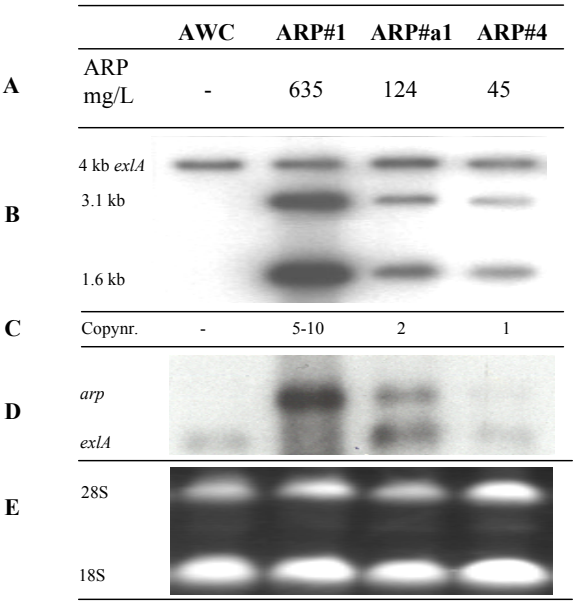

Figure 4. Analysis of *A. awamori* ARP producing transformants ARP#1, ARP#a1 and ARP#4. *A. awamori* AWC4.20 (AWC) was used as a negative control. (**A)** Production levels determined with an ABTS activity assay using purified ARP (Fluka) as a standard. (**B)** Southern blot analysis of ARP producing transformants using the EXLA probe. Sizes of the hybridising *Nco*I fragments are indicated. *ExlA* represents the hybridising fragment that harbours the endogenous *exlA* gene. **(C)** Copy number determination of the integrated expression cassettes based on quantification of the band intensities presented in the Southern blot analysis (**B**). (**D)** Northern blot analysis of ARP producing transformants after 24 h of induction using the EXLA fragment as a probe. *Arp* and *exl*A mRNAs are indicated. (**E)** Ethidium bromide-stained formaldehyde gel as loading control for the northern blot analysis.

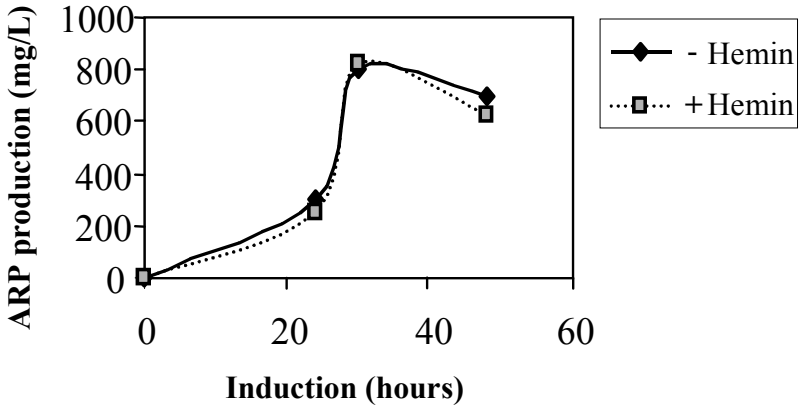

Figure 5. Analysis of heme limitation. Transformant ARP#1 was cultivated in minimal medium with 5 % D-xylose or 5 % D-xylose + 0.5 g/L hemin. Medium samples were analysed after 24, 30 and 48 hours of induction. *X*-axis: induction time (h) and *Y*-axis: production levels of active ARP (mg/L). Production levels of medium samples were analysed with an ABTS activity assay.

CONCLUSIONS AND DISCUSSION

In our research we demonstrated that *A. awamori* is a suitable host for the production of ARP. Among a very small sample of transformants already one transformant (ARP#1), harbouring 5-10 copies of the *arp* expression cassette, was identified that produced up to 800 mg/L active ARP.

Production of *A. ramosus* peroxidase has recently also been analysed in two commonly used yeast species: *Saccharomyces cerevisiae* and *Pichia pastoris*. In *S. cerevisiae arp* expression under control of the Gal7 promoter resulted in production of less than 0.5 mg/L of active ARP (R.J. Gouka, pers. comm.). Heterologous expression of *A. ramosus* peroxidase in *S. cerevisiae* has been studied before under control of the glyceraldehyde 3-phosphate dehydrogenase promoter (Sawai-Hatanaka *et al.* 1995). The results were comparable with the previous observation, meaning that active peroxidase could be produced in this organism, although at a very low level. Improvement of ARP production was obtained when *P. pastoris* was used as a host strain instead of *S. cerevisiae.* In the *arp* expression vector of *P. pastoris* the expression was driven by the promoter of *P. pastoris* alcohol oxidase, P_{aox1} (Cereghino and Cregg 2000). However, the production of 21 mg/L of ARP with *P. pastoris* was also much lower than that in *A. awamori* (R.J. Gouka, pers. comm.). These observations support that in several cases filamentous fungi are more effective secretors of proteins than yeast strains, like *S. cerevisiae* and *P. pastoris* (van Gemeren *et al.* 1997; Gouka *et al.* 1997; Archer and Peberdy, 1997). On the other hand production levels of heme containing proteins in heterologous systems are in general much lower (Conesa *et al.* 2002) than what we have demonstrated in our study with ARP. Moreover, *A. awamori* has also demonstrated to be a suitable host for the production of another redox-enzyme, *i.e.* phenol oxidase from *Acremonium murorum*. In shake flasks cultures this phenol oxidase was overproduced by *A. awamori* as a fusion protein with glucoamylase (processed in vivo) with levels of 0.6 g/L (Gouka *et al.* 2001). This suggests that up to now *A. awamori* might be the most preferred host for the heterologous production of redox-enzymes and heme containing proteins like ARP.

To further increase ARP production levels in *A. awamori* we investigated the role of heme. Reduced heme availability has been suggested to be a limiting factor for the production of heme proteins in different systems (Stewart *et al.* 1996; Conesa *et al.* 2002). Conesa *et al.* showed that hemin supplementation resulted in a significant increase in the production of *Phanerochaete chrysosporium* manganese peroxidase (MnP) and *Caldariomyces fumago* chloroperoxidase (CPO) in *A. niger* (Conesa *et al.* 2000; Conesa *et al.* 2002). Amino acid sequence comparison between MnP and ARP demonstrated an overall similarity of 51 % and moreover a conserved heme-binding region, indicating that as during the overproduction of MnP in *A. niger* also overproduction of ARP in *A. awamori* might be limited by the availability of heme. However, in contrast to the other studies the results here presented suggest that under our conditions heme availability might not be a bottleneck for the overproduction of ARP in *A. awamori* (Figure 5). Whether the availability of heme is dependent on the host strain that is used for production or on the particular heme protein that is produced remains to be established.

Northern blot analysis of the *A. awamori* ARP producing transformant ARP#1 demonstrated that high *arp* mRNA levels were obtained with this transformant. Analysis of medium samples of this strain on SDS PAGE gels revealed an ARP protein of a similar size as the native protein produced by *A. ramosus*. Upon analysis of multi-copy transformant

ARP#1 on northern blot and SDS PAGE gel, reduced levels of *exlA* mRNA and EXLA protein, respectively, were observed (Figure 4D and 3). This is probably a consequence of a titration effect caused by limited amounts of endoxylanase II transcription regulator XlnR (van Peij *et al.* 1998; Gouka *et al.* 1999). Due to this effect it is expected that a further increase in *arp* copy number will not result in much higher ARP levels (see for a similar case Gouka *et al.* 1999; Verdoes *et al.* 1994). As a consequence, this probably makes further transformation experiments to obtain more multi-copy transformants futile. To realise further increase of ARP production levels in *A. awamori* we suggest either the overproduction of XlnR and possible other associated xylanase transcription activator(s) or the use of a different strong promoter that is independent of transcription activators. This, in combination with a large-scale fermentation process brings industrial applications within reach.

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Chapter 5

Production of fusion proteins by *Aspergillus awamori*: llama variable heavy chain antibody fragment (V_{HH}) R9 coupled to *Arthromyces ramosus* peroxidase (ARP)

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ABSTRACT

Arthromyces ramosus peroxidase (ARP) was genetically fused to either the N- or C-terminal ends of the llama heavy-chain antibody fragment V_{HH} R9, resulting in the fusion expression cassettes ARP-R9 or R9-ARP. *Aspergillus awamori* transformants were obtained which produced up to 30 mg/L fusion protein in the culture medium. Both fusion proteins showed peroxidase activity in an ABTS activity test. Considerable amounts of fusion protein were detected intracellularly, suggesting that the fungus encounters problems in secreting these kind of proteins. ELISA experiments showed that ARP-R9 was less able to bind its antigen, the azo-dye RR6, as compared to R9-ARP. Furthermore, in contrast to R9-ARP, ARP-R9 bound to RR6 did not show peroxidase activity anymore. These results indicate that fusion of ARP to the C-terminus of the antibody fragment V_{HH} R9 (R9-ARP) is the preferred orientation.

INTRODUCTION

Genetically coupling of enzymes and antibodies (in this manuscript referred to as fusion proteins) allows interesting applications due to the fact that antibodies can direct the coupled enzyme to the place where it should perform its enzymatic function. This can result in an enhanced efficiency for the usage of the enzyme, since it is brought closer to the site of action and also less of the protein is required. This so-called "Magic Bullet" approach has already been developed in the medical field where for example anti-tumour agents are directed towards the site of malignant growing cells by means of antibodies that recognise these cells (Hudson 1998; Reiter 2001; von Mehren and Weiner 1996). The use of fusion proteins in consumer products like detergents, toothpastes and shampoos is a less exploited area, but with the help of modern biotechnology the applicability of these molecules is within reach (Szynol *et al*. 2004; reviewed in Joosten *et al.* 2003). We are interested in the application of fusion proteins for the improvement of detergents. Enzymes, like peroxidases that are able to bleach persistent spots on laundry, can be directed to these spots by coupling them to specific antibodies (Beggs *et al.* 1998). The idea is that the enzyme-part of the fusion protein will be enriched at the target site and therefore mainly act at the spot. This would leave the "clean" textile untouched, which is believed to be less harmful for the textile. The generation of detergents capable of removing difficult spots by the addition of fusion proteins will result in the use of less chemical bleaching components in detergents, thereby diminishing environmental pollution.

For the application of antibodies in detergents they should be stable under the harsh conditions of laundry washing and simple in structure to permit their production by relevant industrial micro-organisms. Hamers-Casterman *et al.* (1993) discovered a novel class of IgG antibodies in *Camelidae* (camels and llamas) that are devoid of light chains. Their binding domains consist only of the heavy-chain variable domains, called V_{HH}s (Muyldermans *et al.* 1994). V_{HH}s are of great interest since they comprise the smallest possible recognition units of antibodies (Sheriff and Constantine 1996) and are very simple in structure. More importantly, V_{HH}s were shown to be highly soluble and very stable at high temperatures (Ghahroudi *et al.* 1997; van der Linden *et al.* 1999). Denaturation of V_{HH}s through unfolding was shown to be a reversible process, mainly due to the absence of required V_H/V_L association, present in conventional antibodies or fragments thereof (Perez *et al.* 2001).

One of the peroxidases that is of major interest for improvement of detergents is the fungal *Arthromyces ramosus* peroxidase (ARP; Akimoto *et al.* 1990; Sawai-Hatanaka *et al.* 1995). Peroxidases are enzymes that utilise hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds and are produced by a variety of microbial organisms (in particular fungi), plants and animals. ARP is a 41 kDa monomeric glycoprotein that contains protoheme IX as a prosthetic group. It has a broad specificity for phenolic and anilinic substrates that make the enzyme suitable for usage in bleaching processes (Kjalke *et al*. 1992).

The application of fusion proteins in consumer products requires that the proteins can be produced cheaply, in large amounts and that the final product can be isolated easily from the culture medium. Filamentous fungi have the capability of secreting large amounts of protein into their culture medium. Therefore, these organisms are widely used for the production of commercially interesting proteins (reviewed by Punt *et al.* 2002; van den Hondel *et al.* 1991; Verdoes *et al.* 1995). Especially species from the genus *Aspergillus* are attractive candidates for the production of fusion proteins, comprising ARP fused to a llama

heavy-chain antibody fragment (V_{HH}). Recently, we have demonstrated that in *A. awamori* under control of the 1,4-β endoxylanase A (*exlA*) promoter (Gouka *et al.* 1996a) secretion of active ARP was achieved up to 800 mg/L in shake-flask cultures (Lokman *et al.* 2003). In a previous report we also showed that functional V_{HH}s raised against the azo-dye RR6 could be produced by *A. awamori* (Joosten *et al.* 2005a).

In this paper we studied the production of fusion proteins by *A. awamori*. For this purpose *A. ramosus* peroxidase was genetically fused to heavy-chain antibody fragment V_{HH} R9. Produced V_{HH}-ARP fusion proteins were tested in assays to determine the preferred orientation (N-terminal or C-terminal linkage to the antibody fragment) for potential applications of these "Magic Bullets" in detergents.

MATERIALS AND METHODS

Strains and Media

A. awamori pyrG$^-$ mutant strain AWC4.20 (Gouka *et al*. 2001), used as a recipient for transformation, is a derivative of *A. awamori* ATCC 11358 (CBS 115.52). *Escherichia coli* strain DH5α (Sambrook *et al.* 1989) was used for propagation of plasmids.

For shake flask cultivations pre-culture medium consisted of *Aspergillus* minimal medium (MM; Bennett and Lasure 1991) supplemented with 1 % sucrose as non-inducing carbon source and 0.1 % yeast extract (Difco). For induction of the 1,4-β endoxylanase A (*exlA*) promoter, *Aspergillus* MM was supplemented with 5 % D-xylose as inducing carbon source and 0.1 % yeast extract (Gouka *et al.* 1996a). The procedure for transfer of *A. awamori* cultures to induction medium was described previously (Joosten *et al.* 2005a). To test whether proteolytic activity is present, 0.25 % Bovine Serum Albumin (BSA; Sigma) was added to the culture medium (Joosten *et al.* 2005a).

Construction of the V_{HH}-ARP and ARP-V_{HH} expression vectors

Molecular methods were carried out as essentially described by Sambrook *et al.* (1989). For construction of the fusion expression vectors, *Saccharomyces cerevisiae* plasmids pUR7784 and pUR7788 [Unilever Research Vlaardingen (URV), The Netherlands], containing the ARP-R9 and R9-ARP fusion fragments, were used [ARP; *A. ramosus* peroxidase (Lokman *et al*. 2003) and V_{HH} R9; Llama variable heavy-chain antibody fragment recognising azo-dye RR6 (Frenken *et al.* 2000)]. In the fusion proteins, ARP and V_{HH} R9 are separated by a "flex-linker" (GTSGS) in order to allow proper and independent folding of both proteins (Frenken *et al.* 1999). A PCR cloning approach was used to introduce 5' and 3' ends on the fusion fragments compatible with the *Aspergillus* expression vector. In the fusion expression vector that derived from pAW14B12 (van Gemeren *et al*. 1996), expression is under control of the strong and inducible *A. awamori* 1,4-β endoxylanase A (*exlA*) promoter (Lokman *et al*. 2003) and contains the *exlA* signal sequence for efficient targeting to the secretory pathway of *A. awamori*. The resulting expression cassettes ARP-R9 and R9-ARP are depicted in Figure 1.

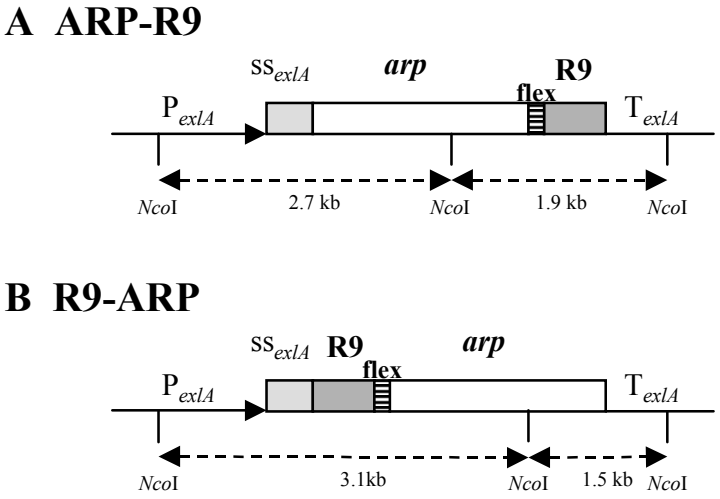

Figure 1. Schematic representation of the fusion expression cassettes. *arp*: *A. ramosus* peroxidase coding sequence; R9: llama variable heavy-chain antibody fragment R9 coding sequence; P*exlA: A. awamori* 1,4-β endoxylanase A (*exlA*) promoter; T*exlA*: *A. awamori exlA* terminator; ss*exlA*: *A. awamori exlA* signal sequence (16 amino acids); Flex: flex linker (amino acid sequence GTSGS). The *Nco*I restriction-sites are indicated.

Transformation and selection of transformants

For transformation of *A. awamori* AWC4.20 protoplasts we refer to Joosten *et al.* (2005a). Since no *Aspergillus* selection marker was present on the pARP-R9 and pR9-ARP expression vectors, a co-transformation experiment was carried out with the co-transforming plasmid pAmdS/pyrG. This plasmid contains the *A. niger pyrG* gene (coding for orotidine-5'-phosphate decarboxylase; Gouka *et al.* 1995) and the *A. nidulans amdS* gene (coding for acetamidase; Hynes *et al*. 1983). 1 µg co-transforming plasmid (pAmdS/pyrG) was used together with 10 µg of the ARP-R9 or R9-ARP expression vectors. Selection of *A. awamori pyrG*$^{+}$ (co-) transformants was performed on osmotically stabilised (1.2 M sorbitol) bacteriological agar (Oxoid) plates containing *Aspergillus* MM. Multi-copy transformants were obtained by double selection for uridine prototrophy and growth on acetamide as sole nitrogen source.

Southern and northern analysis

Aspergillus chromosomal DNA was isolated as described by Kolar *et al.* (1988) and digested with *Nco*I. After separation on gel, followed by Southern blotting according to Sambrook *et al.* (1989), blots were hybridised with the EXLA probe (Gouka *et al*. 1996b), a 400 bp DNA fragment comprising the 5'- and 3'- non-coding *exlA* sequences, which hybridises both with the *exlA* expression signals of the fusion constructs as with the expression signals of the endogenous *exlA* gene.

For RNA isolation, mycelium was powdered in liquid N_2 with mortar and pestle and total RNA was isolated using Trizol™ (Life Technologies), according to the manufacturer's instructions. Samples for northern blotting were treated according to Sambrook *et al.* (1989). Hybridisation was performed under homologous conditions using the EXLA probe (Gouka *et*

al. 1996b). A 1.5 kb *Hin*dIII fragment from pAB5-2 (*gpdA* probe) was used as a loading control (Verdoes *et al*. 1994). Probes were labelled with ^{32}P using the Rediprime ™ II kit (Amersham Pharmacia Biotech). Quantification of hybridising bands on Southern and northern blot was performed with ImageJ 1.33k (http://rsb.info.nih.gov/ij/).

Protein analysis methods

After induction of the *exlA* promoter, medium samples of *A. awamori* were taken and subjected to a 8-16 % gradient SDS-PAGE gel (Pre-cast Gold, Sanvertech) under reducing and denaturing conditions, followed by blotting onto nitrocellulose membrane (Protran, Schleicher & Schuell). Blots were blocked with 1 % Protifar (Nutricia) in PBS with 0.05 % Triton X-100 for 60 min. Subsequently, the blots were incubated with polyclonal rabbit anti-ARP (k-69613) or anti-endoxylanase (87011, k-21) diluted 1:2000, obtained from URV (Unilever Research Vlaardingen, The Netherlands). As a second antibody goat-anti-rabbit Immunoglobulin G alkaline phosphatase conjugate was used (Dako, D0487). Blots were developed with nitroblue tetrazoliumchloride (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) as substrate. Amounts of extracellular protein were quantified with Genetools software, version 2.10.03 (Syngene Synoptics Ltd.) using purified V_{HH} R2 (URV) or ARP (Fluka) as standard.

For deglycosylation experiments, proteins were treated with EndoH (Endoglycosidase H, New England Biolabs) to remove their mannose N-chain. Therefore, medium samples (36 µl) were denatured in 4 µl 10 x denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) at 100 °C, followed by overnight incubation with 1000 units Endo H at 37 °C in 150 µl 50 mM sodium citrate pH 5.5 in the presence of protease inhibitors (Complete™, Roche). Deglycosylated proteins were concentrated by precipitation in cold acetone and subjected to western analysis.

Determination of cell wall-associated and/or intracellular localised protein

The amount of cell wall-associated or intracellular V_{HH} was determined from samples taken 24 h after induction as described previously (Joosten *et al.* 2005a). Transformants were cultivated in 100 ml MM with 1 % sucrose for 24 h, followed by transfer of mycelium to 50 ml MM with 5 % D-xylose. After 24 h induction mycelium was separated from the culture medium by filtration over Miracloth. Half the mycelium was resuspended in 25 ml MM and the other half was resuspended in the same medium containing 1 % SDS. The latter treatment was carried out to remove secreted protein adhering to the mycelium. The cultures were shaken at 4 °C, 75 rpm for 1 h. Subsequently, the mycelium was filtered, frozen with liquid N_2 and powdered with mortar and pestle. After disruption, the powdered mycelium was resuspended in extraction buffer (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0; 1 mM EDTA), and centrifuged at 4 °C. The supernatant was named the 'soluble fraction'. The pellet was washed once with extraction buffer containing 1 % (v/v) Triton X-100 and a second washing step was performed with extraction buffer containing 1 % (w/v) SDS. These treatments were performed in order to distinguish between intracellular V_{HH} loosely or tightly associated with the pellet fraction. During the intracellular extraction procedure, protease inhibitor cocktail tablets (Complete™, Roche) were added to all buffers used, according to the manufacturer's instructions. All fractions were subjected to western blot analysis. Since extraction volumes were kept equal and equal amounts of sample were loaded on SDS-PAGE gel, signals in the different fractions are comparable.

Detection of RR6-binding activity of both fusion proteins by Enzyme-Linked Immuno Sorbent Assay (ELISA)
Polysorb microtiter plates (Nalgene Nunc, 475094) were coated overnight at 37 °C with 0.5 mM RR6 (Reactive red 6, Procion Rubine MX-B, ICI) in coupling buffer (0.1 M *di*-sodium tetraborate decahydrate, 0.15 M NaCl, pH 8.5). Washing between the different steps was performed with washing buffer [0.5 % Protifar (Nutricia) in PBS pH 7.4 with 0.05 % Triton X-100 (PBS-T)]. Residual protein-binding sites were blocked with blocking buffer (3 % Protifar in PBS-T) for 1 h at room temperature. Diluted medium samples (in PBS-T) were added to the wells and incubated at room temperature for 1 h. Binding of the V_{HH} moiety of the fusion proteins to the antigen RR6 was detected with rabbit α–ARP (1: 2000 in 0.5 % Protifar PBS-T), followed by incubation with alkaline phosphatase-conjugated goat α-rabbit IgG (Dako, D0487) and *p*-nitrophenyl phosphate (pNPP; Sigma) as a substrate. Plates were read at 405 nm in a micro-plate reader (Biorad Laboratories, model 3550-UV).

Peroxidase activity assays
Transformants were assayed for peroxidase activity according to Lokman *et al.* (2003) with the following modifications. *A. awamori* transformants were inoculated onto Petri dishes containing *Aspergillus* MM supplemented with 5 % D-xylose and 1.5 % agar. Plates were incubated at 30 °C for 2 days and then flooded with an overlay containing 0.5 % diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 50 mM Na-phosphate buffer (pH 7.0), 0.015 % H_2O_2 and 1 % agarose. Peroxidase-producing transformants developed a green halo after 5-60 s. For detection of ARP activity on native gels, 10-15 % gradient PHAST gels (Amersham Pharmacia Biotech) were used, according the manufacturers instruction, using native buffer strips. After running, the gels were flooded with an overlay containing 5 mg/ml ABTS, 50 mM Na-phosphate buffer (pH 7.0), 0.015 % H_2O_2 and 1 % agarose.

RESULTS

Generation of peroxidase-producing transformants
A. awamori AWC4.20 (*pyrG*$^-$) transformation was performed with vectors containing the expression cassettes required for the production of ARP-R9 or R9-ARP fusion proteins (see Figure 1) and the co-transforming plasmid pAmdS/pyrG. Both AWC ARP-R9 and AWC R9-ARP transformants were tested on acetamide or acrylamide plates as sole nitrogen source and analysed for difference in growth and sporulation on these plates, indicating a putative single- or multi-copy nature of the transformants. Transformants were subjected to an ABTS plate assay and peroxidase-positive transformants were analysed further (results not shown).

Determination of gene-copy number by Southern blot analysis
To compare expression and production levels between the two types of *A. awamori* transformants, the copy-number of the fusion constructs in both AWC ARP-R9 and AWC R9-ARP transformants was determined. As a positive control AWC ARP#4 was used, which contains a single copy of the *arp* gene (Lokman *et al*. 2003).

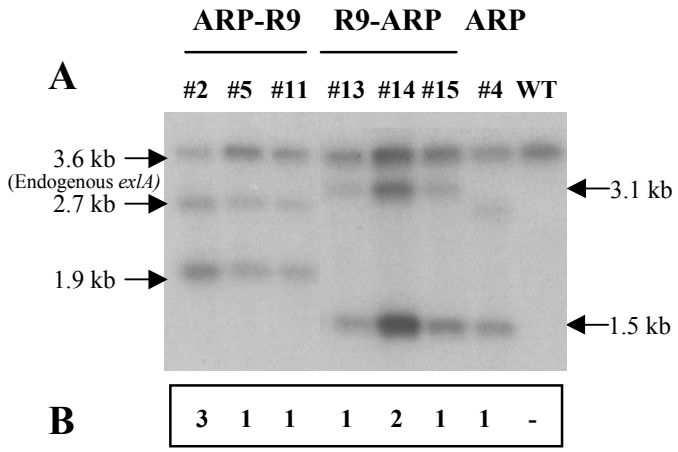

Figure 2A. Southern blot analysis of both AWC ARP-R9 and AWC R9-ARP transformants. Chromosomal DNA of putative transformants was digested with *Nco*I, separated on an agarose gel and subjected to Southern blot analysis. The wild-type (WT) strain AWC and AWC ARP#4 (Lokman *et al.* 2003) were used as a reference. Hybridisation was performed under homologous conditions using the EXLA probe, which is a 400 bp DNA fragment containing the 5' and 3' *exlA* non-coding sequences (Gouka *et al.* 1996b). The EXLA probe hybridises both with the *exlA* expression signals of the heterologous constructs as with the expression signals of the endogenous *exlA* gene. **B**. Copy numbers of the integrated fusion constructs, as determined by comparison of (both) band intensities with the endogenous *exlA* signal intensity as a loading control and ARP#4 as a single-copy situation. The *exlA* gene is present in a single-copy in the *A. awamori* genome.

Figure 2 shows a Southern blot of a selection of single-copy and multi-copy transformants that were obtained. Hybridising bands of 3.6 kb are observed that correspond to the endogenous *exlA* gene, which is present in all strains in a single copy. In AWC ARP-R9 and AWC R9-ARP transformants, hybridising bands are visible of 2.7 and 1.9 kb, and of 3.1 kb and 1.5 kb, respectively (as predicted in Figure 1). In AWC ARP#4 also the 1.5 kb and 2.7 kb bands are observed. Based on the presence of only a single copy of the *arp* gene in *A. awamori* AWC ARP#4 (Lokman *et al.* 2003), the integrated copy number of the expression cassette could be determined. As shown in Figure 2B, both single- and multi-copy transformants of AWC ARP-R9 and AWC R9-ARP were obtained. AWC ARP-R9#2 and R9-ARP#14 were designated multi-copy transformants to distinguish them from the single-copy transformants. Transformants were studied further by northern blot analysis.

Northern blot analysis

Fusion mRNA levels of the different transformants were determined by northern blot analysis. Therefore, total RNA was isolated from cultures of different ARP-R9 and R9-ARP transformants that were grown for 24 h on D-xylose medium. In Figure 3 the results of the northern blot are shown for the single copy transformants AWC ARP-R9#5 and AWC R9-ARP#13. As a positive control single-copy transformant AWC ARP#4 (Lokman *et al*. 2003) was used. Hybridising bands were quantified and signal intensities were corrected for loading differences and subsequently related to the wild-type strain (Figure 3C).

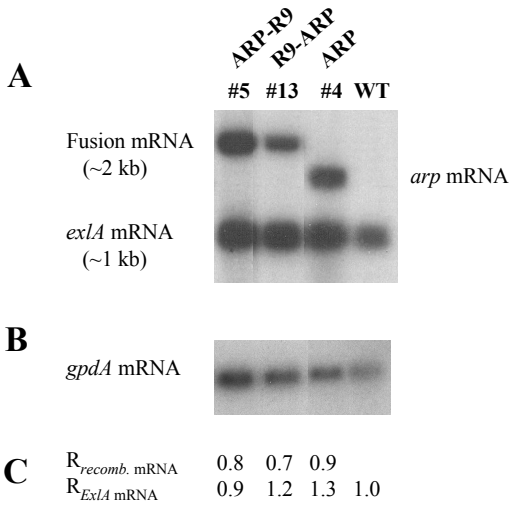

Figure 3. Northern blot analysis of total RNA samples taken from the single-copy transformants AWC ARP-R9#5, R9-ARP#13, ARP#4 and the wild-type (WT) strain. Blots were hybridised with the EXLA probe (A), which hybridises with the non-coding region of the *exlA* expression signals, present in both fusion mRNAs, ARP mRNA and the endogenous *exlA* mRNA, and (B) with the *gpdA* probe as a loading control (Verdoes *et al*. 1994). In (C) the relative band intensities ($R_{recomb.}$ and R_{exlA}) related to the wild-type strain are indicated. Hybridising signals were corrected for loading differences as determined with the *gpdA* probe (B).

As shown in Figure 3A, for both fusion transformants a band was found of approximately 2 kb, which corresponds to the expected size of mRNA derived from a fusion between ARP and V_{HH} R9 (see Figure 1). With this probe also a lower band (~1 kb) is detected that corresponds to the endogenous *exlA* mRNA (Gouka *et al*. 1996b). The same results were obtained with multi-copy transformants AWC ARP-R9#2 and R9-ARP#14 and with single-copy transformants AWC ARP-R9#11 and R9-ARP#15 (results not shown). After correction for loading differences using *gpdA* as an internal control (Figure 3B), the relative band intensities ($R_{recomb.\ mRNA}$ and $R_{exlA\ mRNA}$) related to the wild-type strain were calculated (Figure 3C). The differences in relative band intensities are considered equal since the relative small deviations are presumably due to natural variations within the experiment.

For further analysis both multi-copy transformants (AWC ARP-R9#2 and R9-ARP#14) and single-copy transformants (AWC ARP-R9#5 and R9-ARP#13) were chosen.

Analysis of the production of both fusion proteins by *A. awamori*

To study the production and secretion of fusion proteins in the culture medium, transformants were pre-cultured in *Aspergillus* minimal medium with sucrose and subsequently transferred to D-xylose induction medium. Medium samples were taken with intervals of 2 h and subjected to western blot analysis.

In Figure 4A the results are shown of the western blot analysis of AWC ARP-R9#2 medium samples. A clear increase of the amount of fusion protein up to 18 h after induction is observed where after it reaches a steady-state level. In Figure 4B the western blot analysis is shown of medium samples of transformant AWC R9-ARP#14. The amount of R9-ARP fusion protein in the medium increased up to 12 h after induction and then reached a steady-state

level. At the same time a band was observed with almost a similar molecular weight as purified ARP (~41 kDa). This protein band shows peroxidase activity (see next paragraph), indicating that it represents ARP, which is cleaved off from the fusion protein. For the single-copy transformants AWC ARP-R9#5 and R9-ARP#13 the same results were obtained as for the multi-copy transformants AWC ARP-R9#2 and R9-ARP#14, respectively (results not shown). For both AWC ARP-R9#2 and R9-ARP#14 the amount of intact fusion protein in the culture medium after 24 h was estimated. Based on serial dilutions of purified ARP, the amount of fusion protein in the culture medium was calculated to be 30 mg/L for ARP-R9 and 10 mg/L for R9-ARP, indicating that the amount of ARP-R9 is about 3-fold higher than R9-ARP.

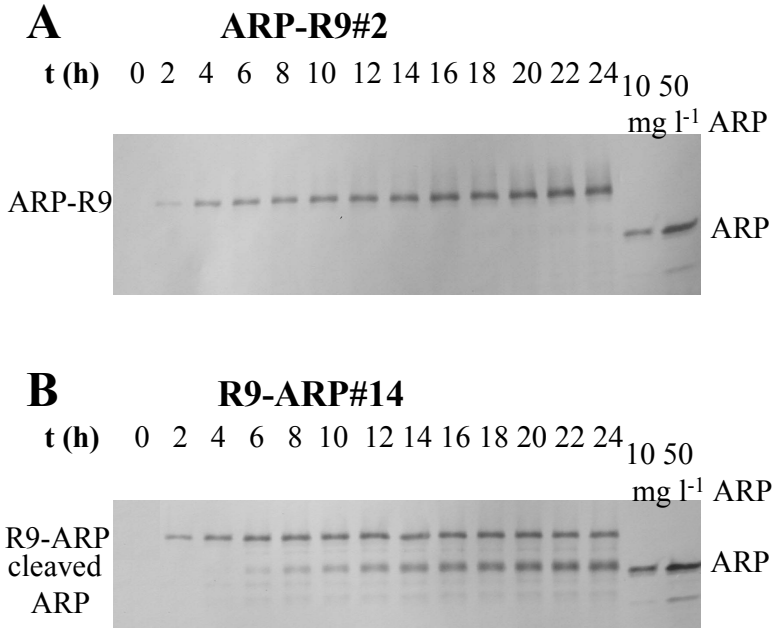

Figure 4. Western blot analysis of medium samples (10 µl) taken with intervals of 2 h after transfer to induction medium of multi-copy transformants AWC ARP-R9#2 (panel A) and AWC R9-ARP#14 (panel B). The position of the fusion proteins (~53 kDa) is indicated on the left and the position of ARP on the right. 10 µl of 10 and 50 mg/L purified ARP (~41 kDa) was loaded as a standard. Blots were incubated with α-ARP.

Detection of RR6 binding activity of both fusion proteins by ELISA

The ability of the fusion protein, produced by *A. awamori* transformants, to bind its target antigen azo-dye RR6 was investigated. For this purpose an ELISA binding assay was developed to detect fusion proteins that bind to a RR6 coated microtiter plate. Medium samples from cultures taken 4 and 24 h after transfer to D-xylose induction medium (see also Figure 7) were added to a RR6-coated microtiter plate. Bound fusion protein was detected with α-ARP. The results are presented in Figure 5A.

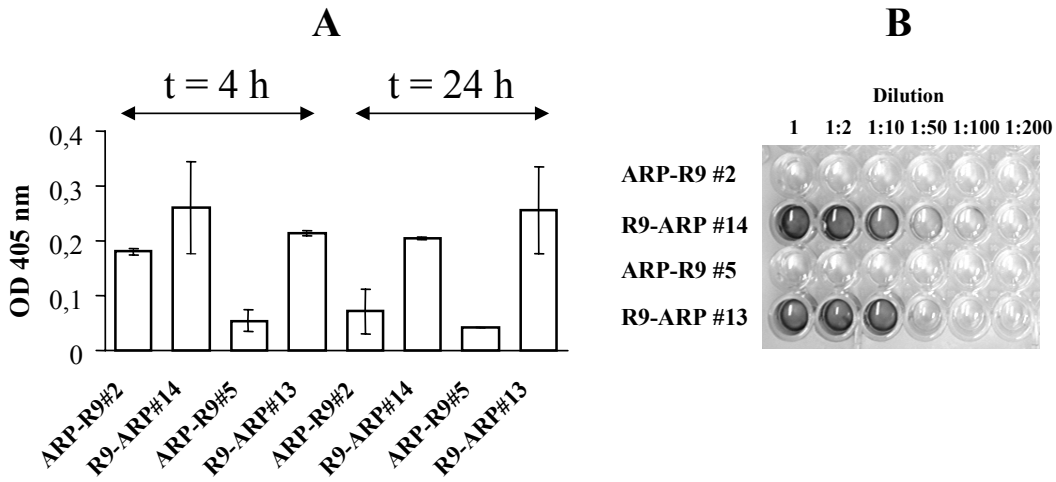

Figure 5A. RR6 binding activity of both fusion proteins was tested by ELISA by means of binding of the V_{HH} R9 fragment in the fusion protein to its antigen RR6. Microtiter plates were coated with RR6 and subsequently diluted medium samples of both multi- and single- copy transformants were loaded onto the plates. Bound fusion protein was detected with α-ARP and goat anti-rabbit alkaline phosphatase, subsequently. Plates were developed using pNPP as a substrate and the OD at 405 nm was measured, which is an indication of the binding capacity of the fusion proteins. Signals were obtained from two independent growth experiments that were corrected for background signals with medium samples from the wild type strain AWC. Here the results are graphically presented of 1/50 diluted medium samples, taken 4 and 24 h after induction. Standard deviations are indicated with error bars. **B.** Peroxidase activity of bifunctional protein bound to the RR6 coated microtiter plate. Medium samples 24 h after induction were loaded on a RR6 coated microtiter plate and after binding the plates were immediately assayed for peroxidase activity with ABTS as a substrate.

As shown in Figure 5A, fusion proteins are able to bind to the antigen RR6. However, it was observed that R9-ARP binds more efficiently than ARP-R9. For AWC ARP-R9#2 more binding of the fusion protein was observed after 4 h than after 24 h of induction whereas a decrease in the amount of fusion proteins was not observed in western blot analysis (see also Figure 7). The difference in binding between ARP-R9 (in both ARP-R9 transformants) and R9-ARP (in AWC R9-ARP#14) is statistically significant for medium samples taken 24 h after induction, in contrast to 4 h after induction.

ARP was chosen as an effector molecule in the fusion proteins for its peroxidase activity and subsequent bleaching applications (Kjalke *et al.* 1992). Therefore, we determined whether ARP showed peroxidase activity when the fusion protein was bound to RR6. The same ELISA experiment as described previously was repeated, however, now without the incubation with α-ARP and the secondary antibody. Instead, the microtiter plates were immediately developed using ABTS as substrate.

As shown in Figure 5B, R9-ARP shows peroxidase activity in this binding assay. The wild-type strain did not show any peroxidase activity and purified ARP was unable to bind in the assay (results not shown), which indicates that the activity observed was derived from the bound fusion protein to the antigen RR6. In this assay, no peroxidase activity was observed for ARP-R9, although previously it was shown that some ARP-R9 did bind to the RR6 coated microtiter plate.

Analysis of ARP activity of the fusion proteins with native PAGE and mobility of both fusion proteins under denaturing and reducing conditions

Transformants were initially selected by their ability to form dark green halos in an ABTS assay, suggesting that active peroxidase was secreted by the fungus. However, no ARP activity was detected when ARP-R9 was bound to a RR6-coated microtiter plate. Therefore, we analysed whether the ARP moiety in ARP-R9 is still active when not bound to its antigen RR6. Medium samples from AWC ARP-R9#2 and R9-ARP#14 transformants were separated on a native gel and peroxidase activity was detected by an ABTS overlay.

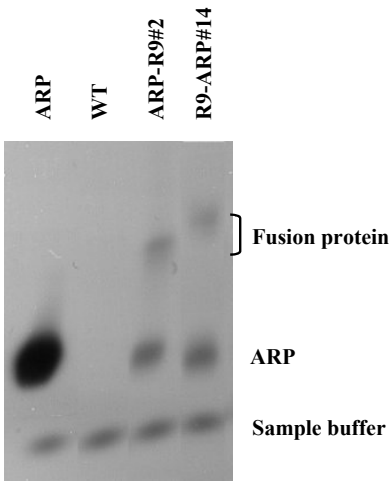

Figure 6. Native PHAST-gel with ABTS overlay showing peroxidase activity of both fusion proteins ARP-R9 and R9-ARP and cleaved-off ARP. Medium samples were taken 24 h after D-xylose induction (see also Figure 7). Purified ARP from Fluka was used as a positive control for ARP activity.

As shown in Figure 6, both fusion proteins showed ARP activity. Furthermore, activity was detected of a cleaved product with a similar molecular weight as ARP. Medium sample of the wild type strain did not show any peroxidase activity, suggesting that the activity observed is a result of the produced fusion proteins. Another observation from the native gel is the difference in running behaviour. To investigate a possible difference in molecular weight, the same medium samples as used for the ELISA experiments were analysed next to each other on western blot. As shown in Figure 7 a clear difference in migration position is observed between ARP-R9 and R9-ARP (compare lanes 1 and 2). Some cleaved-off ARP is seen now for AWC ARP-R9#2, probably resulting from the larger amount of protein loaded onto the SDS-PAGE gel.

To analyse the possibility that R9-ARP had a slower mobility on SDS-PAGE due to differences in glycosylation between the fusion proteins (*e.g.* ARP contains one N-glycosylation site; Kjalke *et al.* 1992), medium samples of both fusion proteins were incubated with EndoH to remove their high mannose oligosaccharides. Digestion with EndoH altered the mobility of both proteins to the same degree (results not shown), indicating that the difference in molecular weight observed between the fusion proteins is not due to a difference in N-glycosylation.

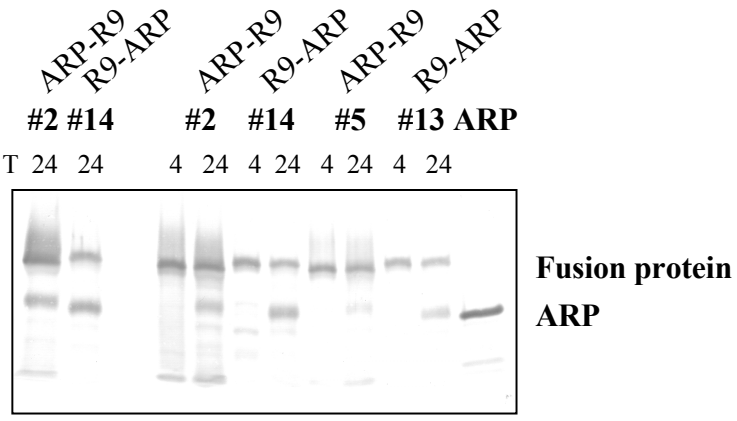

Figure 7. Western blot analysis of medium samples of both multi-copy transformants (ARP-R9#2 and R9-ARP#14) and single-copy transformants (ARP-R9#5 and ARP-R9#13), taken 4 and 24 h after transfer of mycelium to induction medium. The blot was incubated with α-ARP and the position of the fusion proteins and cleaved off ARP is indicated.

Degradation of the fusion proteins

Previously, we found that wild type *A. awamori* secretes up to 75 mg/L EXLA in the culture medium (Joosten *et al.* 2005a). That is 2-8 fold more than the production of ARP-R9 or R9-ARP (without taking the difference in molecular weight into account). A possible reason for this low production is proteolytic degradation, either extra- and/or intracellularly. Recently we found that the level of V_{HH} R2 production increases five-fold after addition of BSA to the culture medium (Joosten *et al.* 2005a). To test whether the yield of fusion protein increases and also the cleavage of ARP from the fusion protein could be reduced, 0.25 % BSA was added to the culture medium. As shown in Figure 8, more fusion protein is detected when medium is supplemented with BSA in AWC ARP-R9 transformants. Furthermore, in AWC ARP-R9 transformants less proteolytic degradation of ARP was observed. In medium samples of AWC R9-ARP transformants the effect of BSA was not so clearly visible as in ARP-R9 medium samples, due to the aberrant running behaviour of medium samples (probably by the excess of BSA in the medium). Furthermore, reduction of proteolytic degradation was not observed in these cultures. These results suggest that proteolytic degradation influences the yields of the fusion protein in the culture medium.

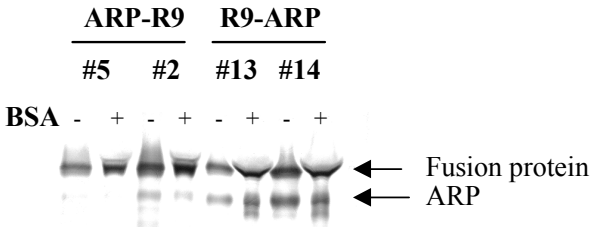

Figure 8. Effect of the addition of BSA to the culture medium on production of fusion proteins. Medium samples of both ARP-R9 and R9-ARP transformants were taken 24 h after transfer of mycelium to induction medium with (+) or without (-) 0.25 % BSA. The blot was incubated with α-ARP and position of the fusion proteins and cleaved-off ARP is indicated.

Intracellular accumulation of fusion protein

Recently, we found that a considerable portion of V_{HH} R2 produced by *A. awamori* was retained within the cell wall and treatment of the mycelium with detergents allowed release of the V_{HH}s (Joosten *et al.* 2005a). To investigate whether fusion protein also adheres to the fungal cell wall, mycelium was washed with detergent (1 % SDS in MM without a carbon source), whereas mycelium was also disrupted and different subsequent extraction procedures were performed to determine whether fusion protein was also localised inside the cell.

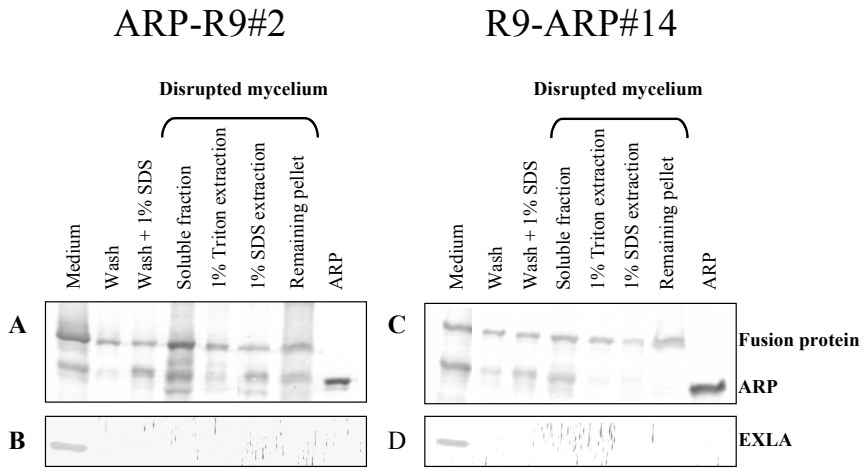

Figure 9. Localisation of fusion protein and EXLA in *A. awamori* transformants AWC ARP-R9#2 (panels A and B) and AWC R9-ARP#14 (panels C and D). Medium, mycelium washes (with or without 1 % SDS) and extracts of disrupted mycelium were loaded on SDS-PAGE. After western blotting, fusion protein was detected on blot with α-ARP (panels A and C) and endogenous secreted EXLA with α-EXLA. See Materials and methods for procedure of mycelium extraction.

As shown in Figure 9, some fusion protein is detected in the washing solution of both AWC ARP-R9#2 and AWC R9-ARP#14. When 1 % SDS was added to the washing solution, more cleaved-off ARP is observed. Since there is almost no difference between washing steps with or without SDS, the fraction of fusion protein found here is probably loosely attached to the cell wall. The total amount of fusion protein found 'attached to the cell wall' is about 25 % and 50 % of what is present in the medium from AWC ARP-R9#2 and AWC R9-ARP#14, respectively. In the extractions of disrupted mycelium (in which extraction volumes were kept equal), a relatively large amount of fusion protein is found intracellular in both type of transformants. Equal amounts compared to the amount secreted in the culture medium of both fusion proteins, are found within the soluble faction and up to 25-50 % in detergents extracted mycelium. In both transformants similar amounts of fusion protein are detected in the final remaining pellet. Furthermore, a protein band running at the same position as ARP is observed in all intracellular fractions of AWC ARP-R9#2 and only in the soluble fraction of AWC R9-ARP#14.

As a control, the endogenous secreted endoxylanase (EXLA) level was determined. EXLA was detected only in the culture medium, suggesting that EXLA is only loosely bound to the cell wall and does not adhere to the cell wall as was shown for both fusion proteins.

Furthermore, no EXLA was detected intracellularly, demonstrating that this protein is efficiently secreted.

CONCLUSIONS AND DISCUSSION

In this model study we analysed the feasibility of production of fusion proteins ("Magic Bullets") by *A. awamori.* In these fusion proteins *A. ramosus* peroxidase (ARP) was fused to the heavy-chain antibody fragment V_{HH} R9. ARP was chosen as effector protein, because of its bleaching capacity (Kjalke *et al*. 1992).

To determine if the orientation of V_{HH} R9 to ARP has an influence on the envisaged use in detergents, the production of the fusion proteins in both orientations was analysed. Therefore, two different expression vectors were constructed (pARP-R9 and pR9-ARP, see Figure 1). From several *A. awamori* transformants the integrated vector copy-number was determined. Both single- and multi-copy transformants were obtained for both expression vectors (Figure 2). Northern blot analysis revealed that fusion genes were efficiently transcribed when compared to the endogenous *exlA* mRNA level (Figure 3).

Multi-copy transformants secreted approximately 30 mg/L ARP-R9 (AWC ARP-R9#2) and 10 mg/L R9-ARP (AWC R9-ARP#14) fusion protein in their culture medium, as shown in Figure 4. Furthermore, both secreted fusion proteins showed peroxidase activity in an ABTS activity test (Figure 6).

The expression of the *exlA* gene, present in one copy in the *A. awamori* genome, results in the secretion of ~75 mg/L EXLA under our cultivation conditions (Joosten *et al.* 2005a). Since *exlA* and fusion transcript levels are almost comparable, it was expected that similar fusion protein levels to that of EXLA protein levels should be present in the culture medium. However, fusion protein levels are clearly lower (2-8 fold).

The amount of R9-ARP in the culture medium was lower than ARP-R9, but a protein band with a lower molecular weight was visible in Figure 4. This lower band was also observed for ARP-R9 in Figures 6-8. Since the resulting protein band showed approximately the same molecular weight as purified ARP on SDS-PAGE, reacted with α-ARP and showed peroxidase activity on a native gel, we concluded that this fragment was ARP cleaved off from R9-ARP. Since the two proteins ARP and V_{HH} R9 are connected via a peptide linker (GTSGS), providing them flexibility and the ability to fold independently, it is assumed that cleavage occurs somewhere in this linker.

One of the major factors affecting the final yield of functional fusion protein production in filamentous fungi is supposed to be the action of proteases. These (secreted) proteases are produced abundantly by most fungal strains (Archer *et al.* 1992, van den Hombergh *et al.* 1997). We recently showed that the addition of 0.25 % BSA to the *Aspergillus* culture medium increased the production level of V_{HH} R2 with a factor 5, resulting in 7.5 mg/L V_{HH} R2 in the culture medium (Joosten *et al.* 2005a). Our results obtained with addition of BSA showed that the final yield of intact fusion protein increased, suggesting that fusion proteins are (partially) cleaved by proteases (see Figure 8). Furthermore, with BSA addition less cleavage of ARP from ARP-R9 was observed. Medium optimisation is thus an attractive starting point to increase fusion protein levels. Recently, it was shown that, when both AWC ARP-R9 and R9-ARP transformants were cultivated in rich medium (CM; Moralejo *et al.* 1999), SCM medium (Ward *et al.* 1990), or with addition of protease-inhibitors (*e.g.* Complete™; Roche), less degradation of fusion protein was observed

(M. Roelofs, unpublished results). Another strategy is the generation of protease-deficient strains, although no information is available yet whether a specific protease is responsible for the degradation and/or cleavage. Moralejo *et al.* (2000 and 2002) found that mutants with a lower proteolytic activity (*pep*A or *pep*B mutants) showed decreased thaumatin degradation in *A. awamori*.

Fusion protein was also detected intracellularly in both transformants (see Figure 9). Most of the protein (equal amounts as those found in the culture medium) was found in the soluble fraction, which indicates that fusion protein is located in the cytoplasm and/or in the ER and/or secretory vesicles. When consecutively the remaining pellet (after extraction without detergent) was extracted with detergents, fusion protein could also be detected in these fractions. This suggests that fusion protein is located also in membrane-surrounded organelles/vesicles or the protein is still adhering to the cell wall components. Intracellular accumulation is often observed when overproducing foreign protein in *S. cerevisiae* (Sagt *et al.* 2002; Thomassen *et al.* 2002). In filamentous fungi intracellular accumulation of heterologous protein is very rarely observed. In general the misfolded proteins are degraded by the vacuole or most probably via ERAD (ER-Associated Degradation; see Conesa *et al.* 2001 for further details on vacuolar targeting and ER quality control in filamentous fungi). Since fusion protein is accumulating inside the cell, it will be interesting to determine whether an Unfolded Protein Response (UPR) is induced (see Chapter 1).

Besides the extracellular degradation of both fusion proteins in the culture medium, also cleaved-off ARP was observed intracellular (Figure 9). Whether this is a consequence of the action of intracellular proteases or a result of the isolation method used, is currently unknown.

A difference in binding to the antigen RR6 between ARP-R9 and R9-ARP was observed in the ELISA binding assay. A possible explanation for this observation could be that the N-terminal orientation of ARP influences binding of the V_{HH} fragment R9 to its antigen RR6 in the binding assay. In support of this hypothesis are crystallographic studies of V_{HH} R9 [Protein Data Bank (PDB): 1SJV], recently published by Spinelli *et al.* (2004). V_{HH}s contain, in contrast to a conventional V_H-V_L pair, only 3 CDRs (complementarity-determining regions) in stead of 6. The function of these CDRs is the recognition and subsequent binding of the antigen. In the V_{HH} R9 structure the N-terminal extension is in close proximity of the three CDRs (Spinelli *et al.* 2004). Possibly, by coupling a protein to the N-terminus of V_{HH} R9 (as in ARP-R9), the binding sites (especially CDR1) are hindered by the much larger ARP molecule. This would then result in a hampered binding capacity of the fusion protein. X-ray studies of the related V_{HH} R2 (PDB: 1QD0; Spinelli *et al*. 2000) revealed that an efficient combining site to RR6 is provided by V_{HH} R2, by using its three CDRs. In V_{HH} R2, especially CDR1 provides the strongest interaction with the azo-dye, more than CDR2 and CDR3. That some 'remaining' binding of ARP-R9 to RR6 was detected in the ELISA is then probably the result of residual binding activity of CDR2 and/or CDR3.

Another possible explanation for the difference in binding to the antigen RR6 between the fusion protein could be that V_{HH} R9 is folded incorrectly in the ARP-R9 fusion protein, resulting for example in a disrupted β-sheet conformation, although this phenomenon not completely explains the residual binding observed in the ELISA.

Although no difference in peroxidase activity between the two fusion proteins was observed on a native gel (Figure 6), bound ARP-R9 did not shown any peroxidase activity in the binding experiment, in contrast to R9-ARP (see Figure 5B). It is possible that upon binding to the RR6-coated microtiter plate (by means of the fused V_{HH} R9 fragment) the

ARP-R9 fusion protein is subjected to a conformational change, which affects the activity of the peroxidase, rendering ARP inaccessible for its peroxidase substrate or hydrogen peroxide.

The levels of fusion protein obtained in the culture medium (10-30 mg/L) are a good starting point for further medium optimisations or strain improvement strategies. For consumer applications large amounts of inexpensive molecules are required, in the g/L range). Since *Aspergillus* fermentations can be performed on large scale, the use of these new molecules in for example detergent industry is within reach (Punt *et al*. 2002). Preliminary fermentation experiments showed that 200 mg/L ARP-R9 can be achieved, without any further improvements (R.J. Gouka, pers. comm.).

From the results we can conclude that the concept of "Magic Bullets" can be considered as a potential method for the improvement of and generation of complete new molecules. The promising levels of bifunctional protein achieved *in A. awamori*, makes the use of fusion proteins in future consumer applications possible.

ACKNOWLEDGEMENTS

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Chapter 6

Expression and production of tomato spot-recognising V_{HH}s (V_{HH}toms) and V_{HH}tom-peroxidase fusion proteins by *A. awamori*

Vivi Joosten, Robin J. Gouka, Theo Goosen, C. Theo Verrips, Cees A. M. J. J. van den Hondel and B. Christien Lokman

ABSTRACT

In this study we analysed the feasibility of the production of tomato-spot recognising antibody fragments (V_{HH}toms) by *Aspergillus awamori* and subsequent fusion of these V_{HH}s to a peroxidase. One of the V_{HH}toms was produced by *A. awamori* in similar quantities as was previously reported for a V_{HH} against the azo-dye RR6. Expression vectors were constructed where V_{HH}toms were genetically fused to either the N- or the C-terminus of the *Arthromyces ramosus* peroxidase (ARP). Both types of fusion proteins were produced by *A. awamori* and both were enzymatically active. Binding experiments showed that V_{HH}tom-ARP fusion proteins were slightly more suited for binding tomato paste than ARP-V_{HH}tom fusion proteins.

INTRODUCTION

Currently, the detergent industry uses several types of hydrolytic enzymes for improved washing performances (Falch 1991). They are being used to remove difficult stains from laundry. An advantage of adding enzymes is that this decreases the use of other chemicals and the energy demand. Redox enzymes are attractive targets for new and improved detergents. One of the enzymes of major interest is the fungal *Arthromyces ramosus* peroxidase (ARP; Akimoto *et al*. 1990; Kjalke *et al*. 1992; Sawai-Hatanaka *et al.* 1995) and recently it was shown that ARP can be produced at high levels (800 mg/L) by the filamentous fungus *Aspergillus awamori* (Lokman *et al.* 2003).

The use of bleaching enzymes in detergents also has some disadvantages, like non-specific bleaching of the spots on laundry, resulting in fading of the textile colour and harmful effects on textile. An option to improve the specific action of bleaching enzymes is their use in "Magic Bullets" (reviewed by Joosten *et al.* 2003). In "Magic Bullets" the enzyme of interest is coupled to a molecule, which specifically recognises the spot on laundry. In this way the enzyme is targeted and enriched at the site were the bleaching activity is required.

Ideal spot-recognising molecules are antibody fragments. Hamers-Casterman *et al.* (1993) discovered a novel class of IgG antibodies in *Camelidae* (camels and llamas), that are devoid of the CH1 domain and lack the light chain completely. Their binding domains consist of only the heavy-chain variable domains, which are called V_{HH}s (Muyldermans *et al.* 1994). V_{HH}s were shown to be very stable under harsh conditions, which makes their use in industrial applications feasible (Ghahroudi *et al.* 1997; van der Linden *et al.* 1999). Furthermore, V_{HH}s could be produced in industrial relevant micro-organisms such as *A. awamori* (Joosten *et al.* 2005a) and *Saccharomyces cerevisiae* (Frenken *et al*. 2000; Thomassen *et al.* 2002).

Recently, a model study was carried out to investigate the feasibility of the production of "Magic Bullets" by *A. awamori* (Joosten *et al.* 2005b; Chapter 5). In this study fusion proteins were generated in which ARP was genetically fused to a model V_{HH} fragment that specifically recognises a small hapten, the azo-dye RR6. To achieve our main goal, the improvement of detergents by targeted bleach, α-RR6-V_{HH}s in the ARP-V_{HH} fusion proteins were replaced by specific spot-recognising V_{HH}s.

In this chapter we first describe the production of tomato-spot recognising V_{HH}s by *A. awamori* (V_{HH}toms) and compare these results with results previously obtained with V_{HH}s against the azo-dye RR6 (Joosten *et al.* 2005a). Second, "Magic Bullets", consisting of V_{HH}toms fused to either the N- or the C-terminus of *A. ramosus* peroxidase (ARP), were constructed and their production levels by *A. awamori* were analysed. Furthermore, binding experiments were carried out to determine the preferred orientation of the V_{HH}tom to ARP (C- or N-terminal coupling) and these results were compared with the results obtained with fusion proteins consisting of ARP and V_{HH}s against RR6.

Our results suggest that by fusion of V_{HH}s that recognise a specific spot on laundry, to enzymes that are able to bleach the stain, a way of specific bleaching can be introduced. For the use of these "Magic Bullets" in the detergent industry large amounts of molecules are needed, which requires a cheap and large-scale production system. *A. awamori* is considered to be a suitable expression system for this purpose.

MATERIALS AND METHODS

Isolation of anti-tomato V_{HH}s

Three genes encoding anti-tomato V_{HH}s (V_{HH}toms) were provided by Unilever Research Vlaardingen (URV), The Netherlands. These V_{HH}toms were obtained by immunising llamas with tomato paste at ID-DLO Lelystad, The Netherlands. After RT-PCR, cDNAs encoding V_{HH}s were subsequently cloned from peripheral blood lymphocytes (as described by Frenken *et al.* 2000). After phage display and subsequent enrichment by biopanning, several binders to tomato paste were isolated. Three V_{HH}toms [V_{HH}1A8 (cIgG2), V_{HH}2E3 (cIgG3) and V_{HH}1H7 (cIgG2)] were selected for further experiments. Their amino acid sequence is depicted in Figure 1, together with the sequences of V_{HH}s against the azo-dye RR6.

Construction of pV_{HH}tom, pV_{HH}tom-ARP and pARP-V_{HH}tom expression vectors

Molecular methods were carried out essentially as described by Sambrook *et al.* (1989). To obtain expression of the genes in *A. awamori*, the genes were placed under control of the strong, inducible *A. awamori* 1,4-β endoxylanase A (*exlA*) promoter (Gouka *et al.* 1996a). Construction of the pV_{HH}tom expression vectors was essentially the same as described for construction of α-RR6 V_{HH} expression vectors (Joosten *et al.* 2005a). The V_{HH}tom genes, present on the yeast expression plasmids pUR1300 (V_{HH}1A8), pUR130x (V_{HH}2E3) and pUR1306 (V_{HH}1H7), were obtained from URV.

In the fusion constructs, the fragments ARP and V_{HH}tom are separated by a "flex-linker" (encoding the amino acid sequence GTSGS) in order to allow proper and independent folding of both proteins. For construction of the pV_{HH}tom-ARP expression vector, a PCR cloning approach was used to introduce 5'- and 3'- ends of the fusion fragments compatible with the general *Aspergillus* expression vector pAW14B12 (van Gemeren *et al.* 1996). Furthermore, in the PCR construct a *Pst*I/*Bst*EII cloning site for V_{HH}s was introduced together with the 5' sequence of the *arp* gene up to the *Xba*I-site (Lokman *et al*. 2003). After subcloning of this PCR fragment in pBluescript SKII (Promega), the V_{HH}tom fragments could be introduced in the *Pst*I/*Bst*EII cloning site, resulting in pBlueV_{HH}tom. The final pV_{HH}tom-ARP expression vector was constructed by ligation of the *Xho*I/*Xba*I fragment from pBlueV_{HH}tom together with pAW14B12 digested with *Xho*I/*Afl*II and the *Xba*I/*Afl*II *arp* fragment from pARP. For construction of the pARP-2E3V_{HH}tom expression vector, an *Eco*RI/*Afl*II fragment from pUR1020 (containing ARP fused to V_{HH}2E3 on a yeast expression vector; URV) was directly ligated into the *Eco*RI/*Afl*II-site of pARP. For pARP-V_{HH}1A8 and pARP-V_{HH}1H7 the V_{HH} fragments first had to be isolated from the yeast expression vectors pUR1300 and pUR1306, respectively, and subsequently exchanged for 2E3 in pUR1020.

Finally, the obtained expression vectors were digested with *Not*I, after which the *Not*I fragment from plasmid pAmdS/pyrG, containing the *A. niger pyrG* gene (coding for orotidine-5'-phosphate decarboxylase; Gouka *et al.* 1995) and the *A. nidulans amdS* gene (coding for acetamidase; Hynes *et al.* 1983) as selection markers, was inserted.

Strains and culture conditions

A. awamori pyrG mutant strain AWC4.20 (Gouka *et al.* 2001), used as a recipient for transformation, is a derivative of *A. awamori* ATCC 11358 (CBS 115.52). *Escherichia coli* strain DH5α (Sambrook *et al.* 1989) was used for propagation of plasmids.

For shake flask cultivations, pre-culture medium consisted of *Aspergillus* minimal medium (MM; Bennett and Lasure, 1991) supplemented with 1 % sucrose as non-inducing

carbon source and 0.1 % yeast extract (Difco). For induction, *Aspergillus* MM was supplemented with 5 % D-xylose as inducing carbon source and 0.1 % yeast extract (Gouka *et al.* 1996). The procedure for transfer of *A. awamori* cultures to induction medium was as previously described (Joosten *et al.* 2005a). Medium and mycelium were separated by filtration over Miracloth and mycelium was frozen in liquid N_2.

Transformation and selection of transformants

For transformation of *A. awamori* AWC4.20 we refer to Joosten *et al.* (2005a). 10 µg of the pV_{HH}tom, pARP-V_{HH}tom or pV_{HH}tom-ARP expression vectors was used in the transformation experiments. Selection of transformants was performed on osmotically stabilised (1.2 M sorbitol) bacteriological agar (Oxoid) plates containing *Aspergillus* MM and 10 mM acetamide or 10 mM acrylamide as a sole nitrogen source, to select for transformants with multiple copies of the expression vectors. Subsequently, transformants containing the peroxidase expression vectors pARP-V_{HH}tom or pV_{HH}tom-ARP were further selected by screening for peroxidase activity, as described by Lokman *et al.* (2003).

Protein analysis methods

After induction of the *exlA* promoter, medium samples of *A. awamori* were taken and subjected to a 10-15 % gradient PHAST gel (Amersham Pharmacia Biotech) and subsequently stained with Coomassie Brilliant Blue (Amersham Pharmacia Biotech).

Western blot analysis was essentially carried out according to Sambrook *et al.* (1989). Nitrocellulose membranes (Protran, Schleicher & Schuell) were blocked with 1 % Protifar (Nutricia) in PBS with 0.05 % Triton X-100 for 60 min. Subsequently, the blots were incubated with polyclonal rabbit anti-ARP (k-69613) or rabbit anti-Llama V_{HH}, diluted 1:2000, obtained from URV. In the second step blots were incubated with goat-anti-rabbit Immunoglobulin G alkaline phosphatase conjugate (Dako, D0487). Blots were developed with nitroblue tetrazoliumchloride (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) as substrate.

For detection of ARP activity on native gels, 10-15 % gradient PHAST gels (Amersham Pharmacia Biotech) were used, according the manufacturers instruction, using native buffer strips. After running, the gels were flooded with an overlay containing 5 mg/ml ABTS in 50 mM Na-phosphate buffer (pH 7.0), 0.015 % H_2O_2 and 1 % agarose.

Binding ELISA

Tomato paste was diluted to a final concentration of 10 % in PBS and homogenised with Ultra-Turrax (30 min; 4 °C). Polysorb microtiter plates (Nalgene Nunc, 475094) were coated overnight at 4 °C with 100 µl diluted tomato paste per well. Washing between the different steps was performed with washing buffer [0.5 % Protifar (Nutricia) in PBS pH 7.4 with 0.05 % Triton X-100 (PBS-T)]. Residual protein-binding sites were blocked with blocking buffer (3 % Protifar in PBS-T) for 1 h at room temperature. Dilutions of medium samples, obtained after culturing of AWC V_{HH}tom-ARP and ARP-V_{HH}tom transformants under inducing conditions, were added to the wells and incubated at room temperature for 1 h. Binding of the V_{HH} moiety of the fusion proteins to tomato paste was detected with rabbit α–ARP (1:2000 in 0.5 % Protifar PBS-T), followed by incubation with alkaline phosphatase-conjugated goat α-rabbit IgG (Dako, D0487) and *p*-nitrophenyl phosphate (pNPP; Sigma) as a substrate. Plates were read at 405 nm in a micro-plate reader (Biorad Laboratories, model 3550-UV).

RESULTS

Production of tomato-spot recognising V_{HH} by *A. awamori*

V_{HH}s were raised in llama against tomato paste and several showed the ability to bind to tomato paste-stained cotton, whereas the V_{HH}s showed no binding to unstained cotton (R.J. Gouka, unpublished results). Three fragments (V_{HH}1A8, V_{HH}2E3 and V_{HH}1H7) were selected for further experiments. Their amino acid sequence (as shown in Figure 1) shows high sequence similarity with anti-RR6 V_{HH} (Frenken *et al.* 2000; Joosten *et al.* 2005a). However, V_{HH}2E3, V_{HH}1A8 and V_{HH}1H7 have all two additional cysteine residues, which might be involved in the formation of an additional disulfide bridge.

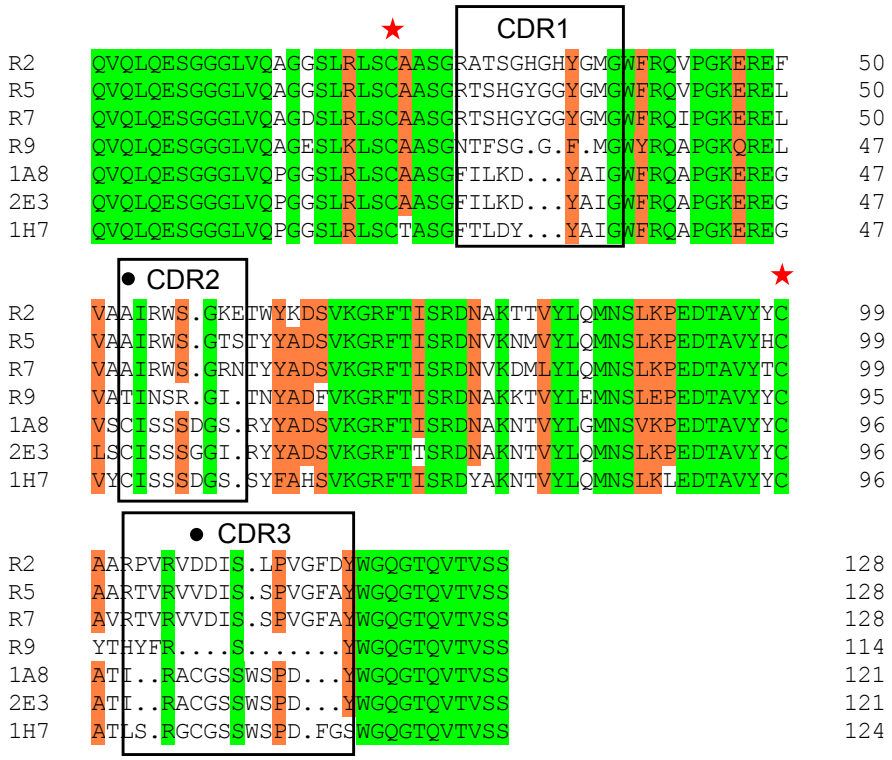

Figure 1. Sequence alignment of the three V_{HH}toms (V_{HH}1A8, V_{HH}2E3 and V_{HH}1H7) analysed in this study and the four anti-RR6 V_{HH}s (V_{HH}s R2, R5, R7 and R9) studied previously (Joosten *et al.* 2005a). Hypervariable regions or complementarity-determining regions (CDRs) are indicated with boxes (Kabat *et al.* 1991), to distinguish them from the more conserved (or framework) regions. The position of conserved cysteine residues in the amino acid sequences is marked with *. In V_{HH}toms, two additional cysteines are present, which are marked with •.

To analyse the production of these V_{HH}toms by *A. awamori*, the genes encoding the anti-tomato V_{HH}s were cloned in an *Aspergillus* expression vector, under control of the strong, D-xylose inducible *A. awamori* 1,4-β endoxylanase A (*exlA*) promoter (Gouka *et al.* 1996). Expression vectors were introduced in the *A. awamori* genome by transformation and several putative transformants for each expression vector were obtained. Three transformants per V_{HH}tom construct were further analysed for their V_{HH}tom production in a shake-flask

experiment. In this experiment, transformants were grown under inducing conditions (as described in Materials and methods) and medium samples, taken 24 hours after induction, were subjected to SDS-PAGE followed by western blot analysis (Figure 2).

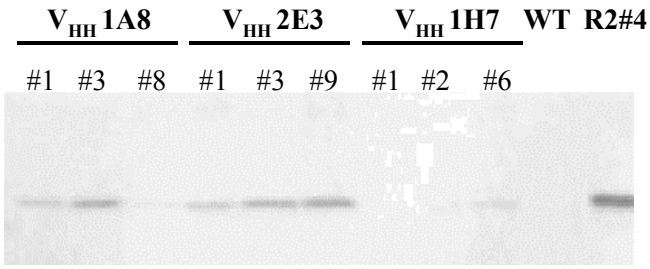

Figure 2. Western blot analysis of medium samples (10 µl) from V_{HH}tom *A. awamori* transformants. AWC R2#4 (Joosten *et al*. 2005a) was used as a positive control. WT is the un-transformed wild type strain AWC. The blot was incubated with α-V_{HH} polyclonal antiserum.

As shown in Figure 2, several transformants secreting V_{HH}toms were obtained. AWC V_{HH}2E3 transformants #3 and #9 secreted the highest amount of V_{HH}tom into the medium, whereas secretion of V_{HH}1H7 by AWC V_{HH}-1H7 transformants was hardly detectable. The production levels for V_{HH}1A8 are between production levels of V_{HH}2E3 and V_{HH}1H7. Although there are only a few differences in amino acid sequences of the framework regions (see also Figure 1), these differences appear to have a significant effect on the final secretion of the different V_{HH}toms. Differences among transformants containing the same V_{HH}tom expression vector could be caused by differences in copy-numbers of the V_{HH}tom expression vector (not determined).

A medium sample of the positive control AWC R2#4, secreting approximately 1.5 mg/L anti-RR6 V_{HH} R2 (Joosten *et al.* 2005a), gave a higher signal than the V_{HH}tom transformants. Moreover, although levels of secreted V_{HH}toms seem to be lower than that of AWC R2#4, it is possible that this is due to the polyclonal antiserum used. The polyclonal antiserum is derived from a rabbit which was immunised with 8 different V_{HH} fragments, including V_{HH} R2, whereas V_{HH}toms were not present. Therefore, quantification is not possible with the method described here, as the affinity of the polyclonal antiserum could be higher for V_{HH} R2 than for the V_{HH}toms.

Production of ARP-V_{HH}tom and V_{HH}tom-ARP fusion proteins

For the construction of the "Magic Bullets" the effector enzyme ARP was fused either at the N- or C-terminus of V_{HH}tom fragments 2E3, 1A8 or 1H7. *A. awamori* was transformed with the different expression vectors and several positive transformants were obtained that were tested further on plates containing *O*-anisidine to detect ARP activity. Extracellular ARP activity resulted in a clear purple halo after several days of growth on agar plates containing MM and *O*-anisidine. Two ARP-positive transformants per construct were analysed further (except for ARP-V_{HH}1H7, since only one ARP-positive transformant was obtained with this expression vector). Transformants were grown under inducing conditions (as described in Materials and methods) and medium samples taken 30 hours after induction were subjected to SDS-PAGE.

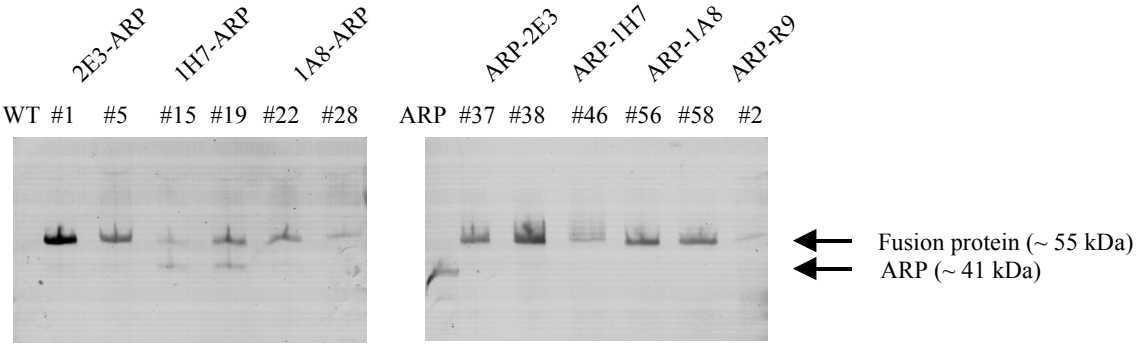

Figure 3. Western blot analysis of medium samples from different AWC V_{HH}tom-ARP and ARP-V_{HH}tom transformants. Samples were taken 30 h after induction on D-xylose. Medium from the wild type (WT) strain AWC was taken as a negative control. The size of the fusion proteins (~55 kDa) and native ARP (~41kDa; Fluka) are indicated. Proteins on western blots were detected with α-ARP.

As shown in Figure 3, all transformants secrete a ~55 kDa protein in their culture medium, which cross-reacts with α-ARP. This band is absent in medium from the wild type strain AWC, which suggests that it corresponds to the V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins. Band intensities differ between the transformants, which may indicate that the fusion proteins are produced at different levels. The difference between transformants with the same construct can indicate differences in copy number of the fusion constructs. Medium from AWC V_{HH}2E3-ARP #1 cross-reacted most strongly with α-ARP. This transformant also showed the strongest colour reaction on *O*-anisidine plates (results not shown), which indicates that this transformant secretes the highest amount of fusion protein, probably due to multi-copy integration of the V_{HH}2E3-ARP expression vector. Furthermore, fusion protein production levels as determined by western blot analysis, reflected mRNA transcript signals detected by northern blot analysis (results not shown). mRNA levels differed between transformants with the same construct, suggesting that no major bottleneck exist on transcriptional level but that differences in fusion protein levels are merely a consequence of the integrated copy number of the expression vector. Northern blot analysis of mRNA isolated from AWC V_{HH}2E3-ARP #1 showed also the strongest hybridisation reaction with a probe based on the expression signals from the *exlA* gene (results not shown).

In medium from AWC ARP-V_{HH}1H7 #46 also a higher band was observed, which may be caused by a difference in glycosylation of the fusion protein. In medium samples from AWC V_{HH}1H7-ARP #15 and #19 and AWC V_{HH}1A8-ARP # 22 also a band is observed which runs at the same position as native ARP. This band probably consists of ARP cleaved off from the V_{HH}tom-ARP fusion protein. Similar to the study with V_{HH} R9, more cleavage of the fusion protein was observed when the V_{HH} fragment was fused at the N-terminus of ARP (Joosten *et al.* 2005b; Chapter 5).

Extracellular fusion protein levels were determined by comparing the amount with serial dilutions of purified ARP (Fluka) on western blot (results not shown). The amount of produced fusion protein was estimated to be around 30 to 40 mg/L for some transformants and even higher (50 mg/L) for AWC V_{HH}2E3-ARP #1.

Activity of the fusion protein

To determine whether the V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins are active, medium samples from the different transformants were separated on a native gel and ARP activity was detected by an ABTS overlay (Figure 4).

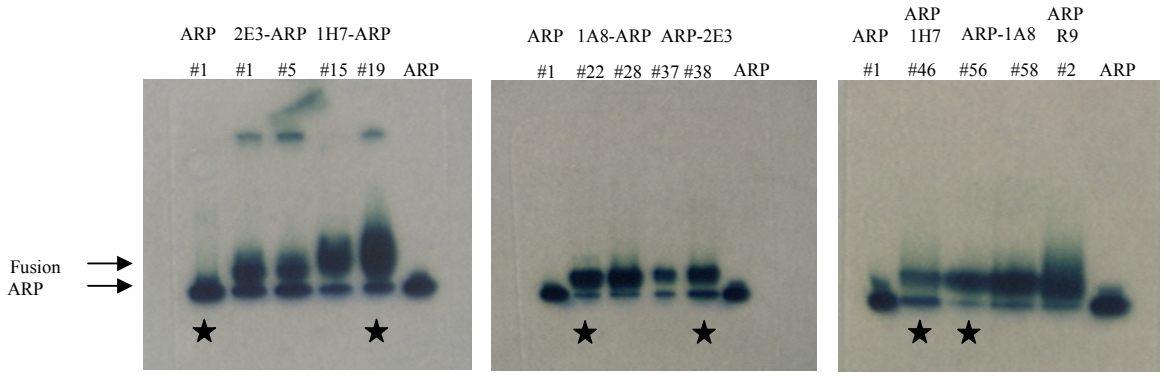

Figure 4. Native 10-15 % gradient PHAST-gel with ABTS overlay showing peroxidase activity of the V_{HH}tom-ARP and ARP-V_{HH}tom fusion protein and cleaved-off ARP. Medium samples (3 µl) were taken 24 h after D-xylose induction. Medium from AWC ARP#1 (Lokman *et al.* 2003) and purified ARP from Fluka were used as a positive control for ARP activity. The positions of the fusion protein and ARP are indicated. Lanes indicated with * correspond to medium samples used in binding experiments (next paragraph).

As shown in Figure 4, all V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins show ARP activity. Furthermore, activity of a cleaved-off product with a similar migration pattern as ARP was detected. ARP activity differed between the transformants and surprisingly, more ARP activity was observed in medium samples of AWC V_{HH}1H7-ARP as compared to AWC V_{HH}2E3-ARP, although on western blot less protein was detected in the medium sample as compared to AWC V_{HH}2E3-ARP. Although cleavage of ARP from the fusion proteins is observed, more activity is detected in the fusion. In a medium sample from the wild type strain AWC no peroxidase activity was observed, which supports the conclusion that the activity observed is the result of the produced V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins (results not shown).

Binding experiments

To determine whether the fusion proteins bind to their antigen present in tomato paste, for each construct one AWC V_{HH}tom-ARP and one AWC ARP-V_{HH}tom transformant was further analysed. A microtiter plate was coated with tomato paste and medium samples of AWC V_{HH}tom-ARP and ARP-V_{HH}tom transformants (V_{HH}2E3-ARP#1; V_{HH}1H7-ARP#19; V_{HH}1A8-ARP#22; ARP-V_{HH}2E3#38; ARP-V_{HH}1H7#46; ARP-V_{HH}1A8#56) were subsequently loaded onto the plate. Bound fusion protein was detected with α-ARP. The results are presented in Figure 5.

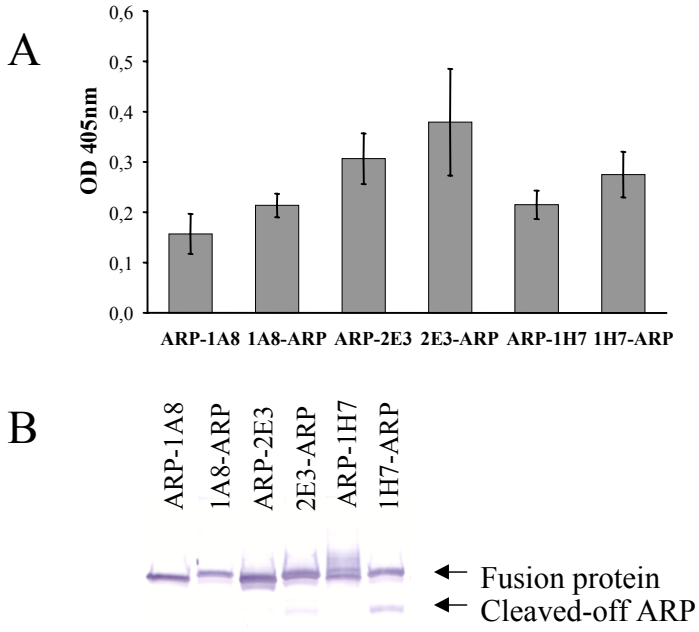

Figure 5. (A) Graphical presentation of the binding of the V_{HH}tom moiety in the V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins to a microtiter plate coated with tomato paste. After applying the medium samples (50µl), the plates were incubated with α-ARP and subsequently with goat-anti rabbit IgG. Detection was performed with pNPP as a substrate. Obtained signals were corrected for background signals with medium samples from the wild type strain AWC. Standard deviations are indicated with error bars. (B) Western blot analysis of the amount of V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins loaded onto the microtiter plate. Detection was performed with α-ARP.

In Figure 5A both orientations of ARP with the same V_{HH}tom are presented next to each other. The results from this experiment suggest that in all cases the N-terminal fusion of the V_{HH}tom to ARP (V_{HH}tom-ARP) showed more binding than the C-terminal fusion (ARP-V_{HH}tom). Furthermore, the fusion protein with V_{HH}-2E3 showed more binding, which probably results from higher amounts of ARP-V_{HH}-2E3 and V_{HH}-2E3-ARP in the culture medium as shown in Figure 5B. Another option is that V_{HH}-2E3 has a higher binding affinity for the tomato paste than the two other V_{HH}toms (V_{HH}-1A8 and V_{HH}-1H7). To be able to compare the binding between the different constructs, the same amount of fusion protein should be loaded onto the ELISA plate. Unfortunately we were unable to load equal amounts.

The results are in agreement with previously obtained results with ARP-R9 and R9-ARP fusion proteins, where the orientation R9-ARP was better suited for binding to the antigen (RR6) than ARP-R9 (Joosten *et al*. 2005b; Chapter 5).

CONCLUSIONS AND DISCUSSION

Recently we reported the production of anti-RR6 V_{HH} by *A. awamori* (Joosten *et al.* 2005a). It was shown that, albeit with low amounts (up to a level of 1.5 mg/L in shake flasks for V_{HH} R2), V_{HH}s were secreted in the extracellular medium and were functional in binding to their

antigen, the azo-dye RR6. Furthermore, a five times improvement was achieved by the addition of BSA to the culture medium, which presumably acts by capturing (partially) the action of extracellular proteases in the culture medium. Since the level of V_{HH} R2 in the extracellular medium was much lower than expected based on both the mRNA level and the capacity of the system [based on the levels obtained with the endogenous 1,4-β endoxylanase A (EXLA)], we hypothesised that production of V_{HH}s was limited at the post-translational level, probably acting intracellularly (Joosten *et al.* 2005a).

To exclude the possibility that the suggested bottleneck was specific for anti-RR6 V_{HH}s, the expression and production of another set of V_{HH}s was investigated. The results in this chapter showed that low amounts of V_{HH}toms were detected in the culture medium, which strengthen the hypothesis that this low level of V_{HH}s observed is a result of a general post-translational bottleneck for V_{HH} production by *A. awamori.* Currently, we are investigating whether an Unfolded protein Response (UPR) and/or ER-Associated Degradation (ERAD) is occurring. Based on the outcome of these investigations new approaches can be used to improve heterologous protein production.

The production of anti-RR6 V_{HH}s in *S. cerevisiae* was over 100 mg/L in shake-flask cultures (Frenken *et al.* 2000). Although at a first glance this production system appears to be more successful than the filamentous fungus *A. awamori*, the level of V_{HH}s that were obtained were reached without any further improvements like medium optimisation and strains improvement techniques. Furthermore, in *S. cerevisiae* considerable amounts of V_{HH}s were detected intracellularly (Thomassen *et al*. 2002). Taken together, for a cost-effective large-scale process for the production of V_{HH}s in both *A. awamori* and *S. cerevisiae*, further improvement is required.

Regarding the production of "Magic Bullets" by *A. awamori*, the results described in this chapter are very promising. V_{HH}tom-ARP and ARP-V_{HH}tom fusion protein levels were comparable with the production of ARP-R9 and R9-ARP fusion proteins (Joosten *et al.* 2005b; Chapter 5), reaching approximately 50 mg/L in the culture medium without any further optimisation techniques. No difference in the levels was observed between the two orientations, although in V_{HH}tom-ARP fusion proteins more cleavage was observed.

Both V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins were shown to possess peroxidase activity under native conditions. However, when both types of fusion proteins were compared in a binding assay, V_{HH}tom-ARP seems to bind slightly better to tomato paste than ARP-V_{HH}tom. Unfortunately, we were unable to determine whether the bound fusion proteins still exhibit peroxidase activity.

The idea of "Magic Bullets" was also explored for another enzyme with industrial bleaching applications: *Acremonium murorum* oxidase (AMO). This enzyme is capable of decolourising plant chromophores (such as anthocyanins). Gouka *et al.* (2001) reported the production of AMO up to 600 mg/L by *A. awamori*. Taking our production levels of ARP (800 mg/L; Lokman *et al.* 2003) and the previously obtained results with the α-RR6 V_{HH}-ARP fusions (Joosten *et al.* 2005b; Chapter 5) into account, the filamentous fungus *A. awamori* can be regarded to be the best candidate for the production of V_{HH}tom-ARP and other V_{HH}-enzyme fusion proteins (with a preference for N-terminal fusion of V_{HH} to the enzyme of interest). Since V_{HH}s can be raised against all kinds of different stains that are difficult to remove from textile by current laundry cleaning procedures, the use of V_{HH}-enzyme fusion proteins to improve detergents comes within reach.

Chapter 7

Production of heme peroxidases by *Aspergillus awamori*

Vivi Joosten, Theo Goosen, Ana V. Conesa, Cees A. M. J. J van den Hondel and B. Christien Lokman

ABSTRACT

This chapter describes studies on the influence of heme supplementation on peroxidase production by *A. awamori*. We demonstrate that the final yield of heterologous *Arthromyces ramosus* peroxidase (ARP) produced by *A. awamori* is positively influenced by the addition of heme to the medium, but this effect depends on the cultivation conditions. In contrast, levels of secreted ARP-V_{HH} fusion proteins and *Phanerochaete chrysosporium* manganese peroxidase (MnP) are not altered by the addition of extracellular heme. This suggests that intracellular heme limitation depends on the amount of heterologous heme-containing protein produced. Our results show that heme can be a limiting factor for the overproduction of heme peroxidases by *A. awamori*.

INTRODUCTION

Peroxidases are heme-containing enzymes that use hydrogen peroxide (H_2O_2) as the electron acceptor to oxidise a broad range of substrates. They are produced by a number of organisms, including bacteria, fungi, plants and animals. Especially fungal peroxidases are of interest for industrial applications such as in wood pulping, paper manufacturing and bleaching applications in textile industries (Michel *et al.* 1991; Paice *et al*. 1995; Sasaki *et al.* 2001; for a review on fungal peroxidases, see Conesa *et al.* 2002; Martinez 2002). Furthermore, the coupling of peroxidases to specific spot-recognising antibody fragments, resulting into a more site-directed action of peroxidases, is of great interest (Joosten *et al*. 2003). However for these applications an inexpensive and large-scale production system is required. Several groups reported the use of filamentous fungi as hosts for the production of peroxidases (Andersen *et al.* 1992; Stewart *et al.* 1996; Larrondo *et al.* 2001). These organisms have the capacity to secrete high amounts of proteins into their culture medium (reviewed by Gouka *et al*.1997; Maras *et al.* 1999; Radzio and Kuck, 1997) and their large-scale fermentation is an established technology (Punt *et al.* 2002).

Some reports showed the successful production of fungal peroxidases by *Aspergillus spp.* (Conesa *et al.* 2000; Lokman *et al.* 2003), but in most cases expression of fungal peroxidases in filamentous fungi has resulted in (very) low production yields (Andersen *et al.* 1992; Stewart *et al.* 1996; Larrondo *et al.* 2001). In general, limitations for heterologous production are believed to be located at the post-transcriptional level. For heme-containing peroxidases it has been shown that the amount of available heme poses such a bottleneck since supplementation of heme in the medium can have a major positive effect on production yields (Andersen *et al.* 1992; Conesa *et al.* 2000; Stewart *et al.* 1996; Elrod *et al.* 1997). In fungal peroxidases, the prosthetic heme group is probably incorporated into the protein during folding in the ER (Conesa 2001; Pinnix *et al.* 1994).

To gain more insight in the influence of available heme during production of peroxidases in filamentous fungi, Conesa (2002) studied the secretion of *Phanerochaete chrysosporium* manganese peroxidase (MnP; EC 1.11.1.13) by *A. niger*. MnP catalyses the oxidation of lignin and phenolic compounds. It was shown that production was limited by the level of heme availability. Recently, we reported a high level of expression of *Arthromyces ramosus* peroxidase (ARP; EC 1.11.1.7) by *A. awamori* (Lokman *et al.* 2003). Based on hemin supplementation experiments, overproduction of ARP, achieving production levels up to 800 mg/L, was shown not to be limited by heme availability. This suggested that *A. awamori* might be the preferred host for overproduction of heme-containing proteins.

In the present study we analysed more thoroughly the potential role of heme limitation on the production of peroxidases by *A. awamori* and in particular on the production of ARP. Different cultivation conditions and different medium additives are evaluated for their influence on the final yield of ARP in the culture medium. Furthermore, *A. awamori* transformants expressing V_{HH}-ARP fusion proteins (Joosten *et al.* 2005b; Chapter 5) or MnP are analysed in a similar fashion.

MATERIALS AND METHODS

Strains and expression vectors

Fungal strains used in this study are listed in Table 1. For generation of *A. awamori* transformants expressing MnP, an *A. awamori pyrG* mutant strain AWC4.20 (Gouka *et al.* 2001) was used as a recipient strain for transformation. The expression vector p*gpd*Mnp1.I-*amdS,* used for transformation, contains the *P. chrysosporium mnp1* gene under the control of the *A. nidulans* constitutive glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter and the *A. nidulans amdS* selection marker (Conesa *et al*. 2002).

Table 1. Fungal strains used in this study.

Species	Strain	Characteristics	Reference
A. niger	MGG029[pAB4-1]	Δ*glaA*, protease deficient strain	Conesa *et al.* 2000
A. niger	MGG029[p*gpd*MnP1.I]#13	Derivative of MGG029 with *P. chrysosporium mnp1* gene expressed under control of *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter.	Conesa *et al.* 2002
A. awamori	AWC 4.20	Recipient strain for transformation	Gouka *et al.* 2001
A. awamori	AWC ARP-R9 #2	Derivative of AWC4.20, containing multiple copies of the *A. ramosus arp* gene genetically linked to α-RR6 V_{HH} R9, under control of the *A. awamori* inducible *exlA* promoter	Joosten *et al.* 2005b
A. awamori	AWC R9-ARP #14	Derivative of AWC4.20, containing multiple copies of α-RR6 V_{HH} R9 gene genetically linked to the *A. ramosus arp* gene, under control of the *A. awamori* inducible *exlA* promoter	Joosten *et al.* 2005b
A. awamori	AWC ARP-R9 #5	Derivative of AWC4.20, containing a single copy of the *A. ramosus arp* gene genetically linked to α-RR6 V_{HH} R9, under control of the *A. awamori* inducible *exlA* promoter	Joosten *et al.* 2005b
A. awamori	AWC R9-ARP #13	Derivative of AWC4.20, containing a single copy of α-RR6 V_{HH} R9 gene genetically linked to the *A. ramosus arp* gene, under control of the *A. awamori* inducible *exlA* promoter	Joosten *et al.* 2005b
A. awamori	AWC ARP #1	Derivative of AWC4.20, containing 5-10 copies of the *A. ramosus arp* gene, under control of the *A. awamori* inducible *exlA* promoter	Lokman *et al.* 2003
A. awamori	AWC ARP #4	Derivative of AWC4.20, containing a single copy of the *A. ramosus arp* gene, under control of the *A. awamori* inducible *exlA* promoter	Lokman *et al.* 2003
A. awamori	AWC MnP #3	Derivative of AWC4.20 with *P. chrysosporium mnp1* gene expressed under control of *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter	This study
A. awamori	AWC MnP #7	Derivative of AWC4.20 with *P. chrysosporium mnp1* gene expressed under control of *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter	This study

Transformation and screening

For *A. awamori* AWC4.20 protoplast production, 1 x 10^6 spores/ml were inoculated in *Aspergillus* minimal medium (MM; Bennett and Lasure, 1991) with 0.5 % yeast extract, 0.2 % casamino acids, 10 mM uridine and 10 mM uracil. Isolation of *A. awamori* protoplasts and subsequent co-transformation of protoplasts was carried out essentially as described by Punt and Van den Hondel (1992), using the expression vector p*gpd*Mnp1.I-*amdS* and pAB4-1 (van Hartingsveldt *et al*. 1987) containing the *A. niger pyrG* (coding for orotidine-5'-phosphate decarboxylase) selection marker. Transformants were selected for uridine prototrophy on osmotically stabilised (1.2 M sorbitol) bacteriological agar (Oxoid) plates containing *Aspergillus* MM. Putative co-transformants containing the MnP expression vector were selected by screening for peroxidase activity, as described in 'Protein analysis methods'.

Culture conditions

For shake flask cultivations of strains containing the expression cassette harbouring the *exlA* promoter, pre-culture medium consisted of *Aspergillus* MM supplemented with 1 % sucrose as non-inducing carbon source and 0.1 % yeast extract (Difco). For induction, MM was supplemented with 5 % D-xylose as inducing carbon source and 0.1 % yeast extract (Gouka *et al.* 1996). 300 ml shake-flasks containing 100 ml pre-culture medium were inoculated with 1 x 10^6 spores/ml and cultivated at 30 °C and 250 rpm. After 16-18 h growth, mycelium was separated from the medium by filtration through Miracloth, washed with physiological salt and transferred to a 300 ml shake-flask containing 50 ml induction medium, with or without the addition of 500 mg/L hemin. Cultures were incubated at 30 °C and 250 rpm and harvested after 24 or 48 h.

Analysis of heme limitation in liquid cultures of MnP-producing transformants was performed under identical conditions as described by Conesa *et al.* (2002), using 500 ml shake-flasks containing 100 ml MM, 0.5 % casamino acids and 5 % maltodextrin (D-2006, Sigma). After inoculation with 1 x 10^6 spores/ml, cultures were grown overnight at 30 ºC, 250 rpm. The mycelium was separated from the medium by filtration through Miracloth, washed with physiological salt and transferred to 50 ml fresh medium with or without the addition of 500 mg/L hemin. Cultures were incubated at 30 °C and 250 rpm and harvested after 24 or 48 h.

Chemicals

Purified *Arthromyces ramosus* ARP (Fluka; 3280 U/mg) was used as standard. Hemin (Ferriprotoporphyrin IX chloride; Fluka) was dissolved at 1.5 g/L in 10 mM NaOH, adjusted to pH 7.5 with HCl and filter-sterilised. The final concentration of hemin in culture medium or plates was 500 mg/L unless stated otherwise. 5'-aminolevulinic acid (ALA; Fluka) was prepared as a 17 g/L stock solution. Bovine Serum Albumin (BSA; Sigma) was prepared as a 3 % stock solution, filter sterilised and added to a final concentration of 0.25 %.

Protein analysis methods

ARP activity was measured by the oxidation of ABTS, as described by Lokman *et al*. (2003). Putative *A. awamori* MnP transformants were assayed for peroxidase activity by inoculating them onto plates containing *Aspergillus* MM supplemented with 1 % glucose and 1.5 % agar. Plates were incubated at 30 ºC for 3 days and then flooded with a thin overlay containing 100 mM Na-succinate (pH 4.5), 2 mM Na-oxalate, 0.015 % H_2O_2, 100 µM $MnSO_4$ and 1 % agarose. Peroxidase-producing transformants developed a green halo.

Western analysis was carried out essentially according to Sambrook *et al.* (1989). Medium samples of *A. awamori* and *A. niger* strains were subjected to Pager™ Gold Precast Gels (BMA) under reducing conditions, followed by blotting onto a nitrocellulose membrane (Protran, Schleicher & Schuell). Blots were blocked with 1 % Protifar (Nutricia) in PBS with 0.05 % Triton X-100 for 60 min. Subsequently, the blots were incubated with polyclonal rabbit anti-ARP (k-69613) obtained from Unilever Research Vlaardingen (URV), a polyclonal antibody against MnPH4 (Dr. D. Cullen, Madison), α-PEPA (Berka *et al.* 1990) or α-EXLA (87011, k-21; obtained from URV). In the second step blots were incubated with goat-anti-rabbit Immunoglobulin G alkaline phosphatase conjugate (Dako, D0487). Blots were developed with nitroblue tetrazoliumchloride (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and amounts of extracellular protein of interest were quantified with Genetools software, version 2.10.03 (Syngene Synoptics Ltd.).

D-Xylose concentration measurement
The remaining concentration of the inducing sugar D-xylose in the culture medium was determined by measuring the production of newly liberated reducing groups using the DNS (2-hydroxy-3,5-dinitrobenzoic acid; Merck) method, as described by Gouka *et al*. (1996).

RESULTS

Influence of the culture volume and hemin addition on ARP activity and ARP protein production by *A. awamori* ARP transformants
Although Lokman *et al.* (2003) found no effect of hemin supplementation on the level of secreted ARP and therefore hypothesised that *A. awamori* would be the preferred host for the production of heme-containing peroxidase, our initial experiments which employed different cultivation conditions indicated otherwise (V. Joosten, unpublished results). Therefore, we decided to analyse the production of ARP in more detail.

ARP transformants AWC ARP #1 (an *A. awamori* transformant containing multiple copies of the *arp* gene) and AWC ARP #4 (containing a single copy *arp* gene), both under control of the inducible *A. awamori exlA* promoter, were cultivated under standard inducing conditions (Joosten *et al.* 2005a). These conditions are different from those used by Lokman *et al.* (2003), where smaller induction volumes and shake-flasks were used. In brief, during our standard induction procedure transformants were pre-cultured in 300 ml shake-flasks containing 100 ml sucrose medium. After o/n growth, mycelium was transferred to 50 ml induction medium containing 5 % D-xylose (denoted as 100 ml $\rightarrow$ 50 ml). As a consequence mycelium is two times concentrated in induction medium.

Figure 1A shows the ARP activity in the culture medium, obtained from the two ARP transformants grown under standard induction conditions. In addition, mycelium of the multicopy transformant AWC ARP#1 was also transferred to 100 ml induction medium, instead of 50 ml (denoted as 100 ml $\rightarrow$ 100 ml). When AWC ARP#1 was grown under standard induction conditions (100 ml $\rightarrow$ 50 ml), the average ARP activity reached a level of 1.6 x 10^7 U/L (corresponding to 0.5 g/L ARP from Fluka) 24 h after induction. A slight decrease of ARP activity over time is suggested (p = 0.08), which may be a consequence of the action of proteases in the concentrated culture. Since at 48 h D-xylose has been almost completely consumed in these cultures (Table 2), the expression of proteases may possibly be induced. When the same transformant was induced without concentration step (100 ml $\rightarrow$ 100 ml), ARP activity reached a level of approximately 1.0 x 10^7 U/L (corresponding to 0.3 g/L purified ARP) after 24 h. This level still increased in time up to 1.4 x 10^7 U/L after 48 h. As shown in Table 2, a significant amount of D-xylose was still present in the medium.

When induction medium was supplemented with hemin, ARP activity increased considerably in the concentrated (100 ml$\rightarrow$ 50 ml) cultures, reaching more than 3.3 x 10^7 U/L (corresponding to more than 1 g/L) after 48 h (p = 0.01). In the non-concentrated culture (100 ml $\rightarrow$ 100 ml), no hemin effect was observed after 24 h, but ARP activity increased more than twofold after 48 h, reaching a level of 4.0 x 10^7 U/L (p = 0.03). Only marginal effects were observed for the single copy transformant AWC ARP#4 upon heme supplementation.

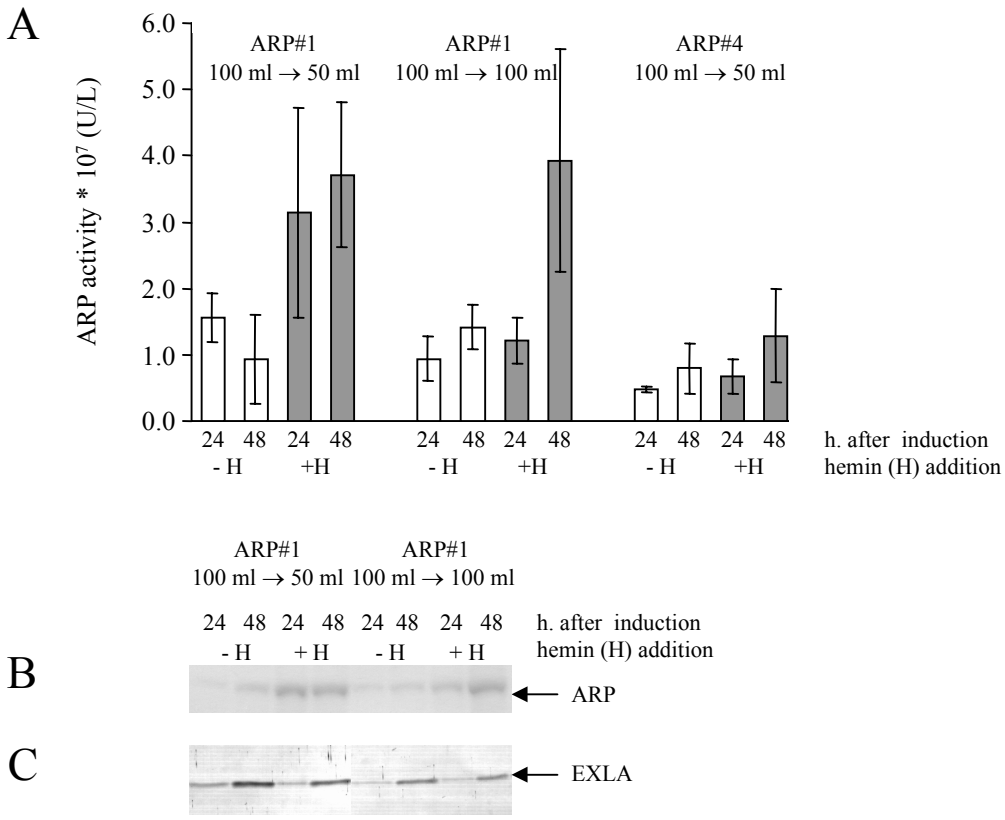

Figure 1A. ARP activity (in 10^7 U/L) in the culture medium of different ARP transformants with (grey bars) or without (white bars) hemin supplementation (500 mg/L) of induction medium. Standard deviations are indicated with error bars. Significance was tested by means of a T-test. Medium samples were taken 24 and 48 h after transfer to induction medium and analysed spectrophotometrically in an ABTS activity assay. In **B** extracellular ARP production levels on a Coomassie-stained SDS-PAGE gel are shown. As a control, endogenous 1,4-β endoxylanase A (EXLA) levels were analysed by western blot analysis with α-EXLA (**C**). In the standard induction procedure (Joosten *et al.* 2005a), transformants AWC ARP #1 (5-10 copies *arp* gene) and AWC ARP #4 (single copy *arp* gene) were pre-cultured in 100 ml sucrose medium. After o/n growth, mycelium was transferred to 50 ml induction medium containing 5 % D-xylose (100 ml → 50 ml). In addition to the 2 times concentration step, AWC ARP#1 was also transferred to 100 ml induction medium (100 ml → 100 ml). Experiments were performed *in duplo*. Transformants were cultivated with (+ H) or without (- H) addition of hemin to the culture medium.

Table 2. Extracellular D-xylose concentration (%) in the culture medium of ARP transformants with or without heme supplementation of the culture medium. Samples were taken 24 or 48 h after transfer to induction medium. D-xylose concentration was measured as described in Materials and methods.

	AWC ARP #1		**AWC ARP #1**		**ARP #4**	
	100 ml → 50 ml		**100 ml → 100 ml**		**100 ml → 50 ml**	
Time (h)	**24**	**48**	**24**	**48**	**24**	**48**
- Hemin	3.2	0.4	3.5	2.3	4.0	0.5
+ Hemin	2.6	0.4	4.0	1.8	2.8	0.5

The ARP activity measured correlated with the amount of ARP protein detected in the culture medium, as shown on the Coomassie-stained SDS-PAGE gel (Figure 1B). This indicates that ARP production levels are increased, and that increased activity is not (merely) a consequence of higher specific activity of the enzyme due to hemin addition. Since hemin addition could influence the overall protein secretion, EXLA levels were determined (Figure 1C). Since *arp* and the *exlA* gene are expressed from the same promoter, both genes should follow the same expression pattern. Samples were analysed on western blot with α-EXLA. As shown in Figure 1C, for both induction conditions the same EXLA secretion is observed with or without hemin addition. Furthermore, the effect of concentration of the mycelium (more EXLA in the concentrated culture due to a higher initial biomass) and induction time (increase of EXLA in time) is clearly visible.

Taken together, these results suggest that hemin addition does increase ARP production levels in the multicopy transformant AWC ARP#1, in contrast to the results obtained by Lokman *et al.* (2003). However, induction conditions do influence the overall ARP yields in the medium as well as the effect of hemin addition.

Effect of different medium additives on ARP activity and ARP protein production by *A. awamori* AWC ARP#1

Although the addition of 500 mg/L hemin to the culture medium seems to be an effective approach for increasing ARP secretion, for large-scale production of ARP supplementation with 500 mg/L hemin is unrealistic due to the high costs of hemin. Furthermore, the effects of such high amounts of hemin are not dramatic, with a 4-fold increase under the most favourable circumstances. Therefore, we investigated whether addition of lower levels of hemin were sufficient to increase secreted ARP levels. Additionally, the effect of addition of casamino acids, the early heme precursor δ-aminolevulinic acid (ALA) and BSA was analysed. Casamino acids accelerate fungal growth and BSA was recently shown to increase V_{HH} production, probably by acting as a substrate for extracellular proteases (Joosten *et al.* 2005a).

A standard induction experiment was carried out (100 ml → 50 ml) with multicopy transformant AWC ARP#1. Both ARP activity (Figure 2A) and ARP production levels (Figure 2B) were determined.

In Figure 2A a clear and significant ($p < 0.001$) increase in ARP activity is shown (~3.3 x 10^7 U/L corresponding to 1.0 g/L purified ARP) when AWC ARP#1 is cultured in heme-supplemented medium (500 mg/L), as compared to non-supplemented medium (~0.8 x 10^7 U/L). Neither ALA alone, nor the combination of ALA with a lower concentration of hemin resulted in a significant increase in ARP activity in the culture medium. Also the addition of BSA did not affect ARP activity levels significantly.

As before, the ARP protein level (Figure 2B) correlates with the measured activity. Also the extracellular levels of EXLA (Figure 2C) were almost similar (even slightly less when 500 mg/L hemin or BSA was added), indicating that the higher levels obtained with hemin addition are not a consequence of a general metabolic effect. The remaining extracellular D-xylose concentration (Table 3) is lower when 500 mg/L hemin or casamino acids were present. For casamino acids this correlates with the higher growth rate.

In conclusion, (1) only a high concentration of hemin increases the secretion of ARP and (2) a barrier must exist for delivering extracellular heme to the right compartment, presumably the Endoplasmic Reticulum (ER).

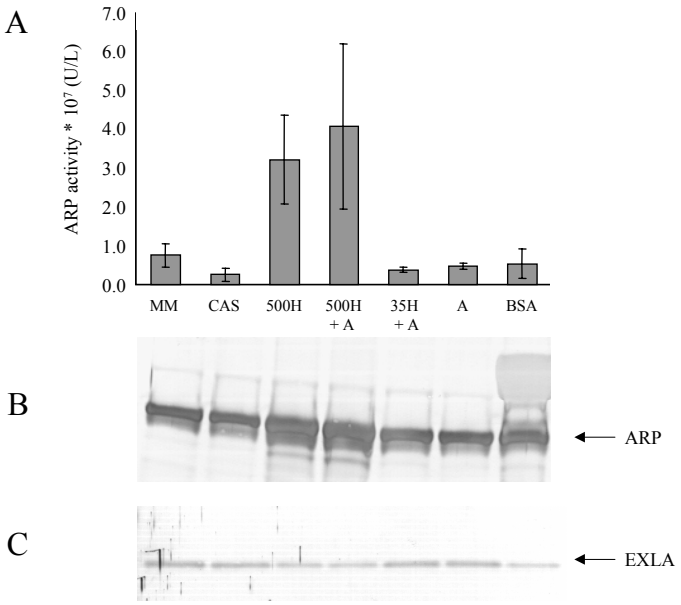

Figure 2. Effect on ARP activity (**A**) (in 10^7 U/L) and ARP protein production (**B**) when culturing AWC ARP#1 with different medium additives. AWC ARP#1 was pre-grown overnight in 100 ml MM with 1 % sucrose. After harvesting, mycelium was transferred to 50 ml MM containing 5 % D-xylose, with or without different additives. MM; Minimal Medium, CAS; 0.5 % casamino acids, 500H; 500 mg/L hemin, 35H; 35 mg/L hemin (~50 µM, as used by Yi *et al.* 2003), A; 17 mg/ml ALA (~100 µM, as used by Yi *et al.* 2003), BSA; 0.25 % BSA. Cultures were harvested 24 h after induction and activity in the medium was analysed spectrophotometrically using ABTS as a substrate (**A**). Purified ARP (Fluka) was used as a reference for activity. Standard deviations are indicated with error bars. Significance was tested by means of a T-test. To determine protein production, medium samples were subjected to western blot analysis, using either α-ARP (**B**) or α-EXLA (**C**) as an internal control.

Table 3. Extracellular D-xylose concentration (%) in the culture medium of AWC ARP#1 when grown with different culture additives, using medium samples taken 24 h after transfer to induction medium. D-xylose concentration was measured as described in Materials and methods.

	MM	**Casamino acids**	**500 mg/L hemin**	**500 mg/L hemin + ALA**	**35 mg/L hemin + ALA**	**ALA**	**BSA**
D-xylose (%)	2.7	1.5	1.8	1.9	2.4	2.4	2.7

Effect of heme supplementation on ARP-V_{HH}/V_{HH}-ARP fusion protein production and activity

Recently we reported the production of ARP fusion proteins, where the ARP-coding region was fused to a DNA segment encoding a llama variable heavy-chain antibody fragment (Joosten *et al.* 2005b). To analyse whether heme addition has a positive effect on fusion protein production levels, both multi-copy and single-copy transformants, producing either ARP-R9 or R9-ARP, were analysed. Cultures were harvested 24 and 48 hours after transfer to induction medium with or without hemin supplementation (500 mg/L), using the standard

induction protocol (100 ml → 50 ml). Medium samples were analysed on western blot (Figure 3) and ARP activity in the medium was analysed in an ABTS assay (Figure 4).

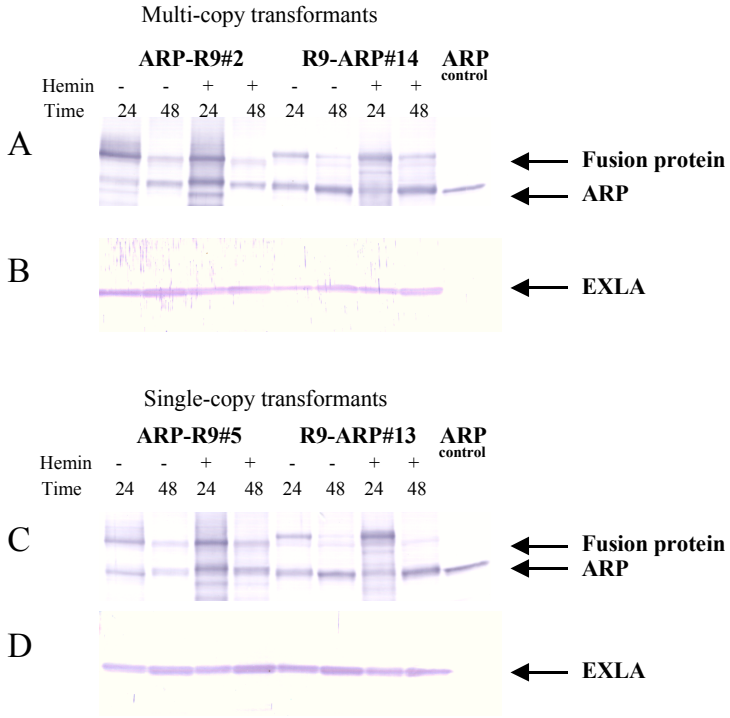

Figure 3. Effect of heme supplementation on *A. awamori* transformants producing ARP-R9 and R9-ARP fusion proteins. Both multi-copy strains (AWC ARP-R9 #2 and AWC R9-ARP #14; Figure 3A, B) and single-copy strains (AWC ARP-R9 #5 and AWC R9-ARP #13; Figure 3C, D) were analysed. Medium samples from heme-supplemented and non-supplemented culture medium were taken both after 24 and 48 h after D-xylose induction and subjected to 8-16 % gradient gels. Western blotting was followed by incubation with a polyclonal antibody against ARP (α-ARP; Figure 3A, C) or α-EXLA (Figure 3B, D) as a control. Purified ARP from *A. ramosus* was used as a control. The position of fusion protein and (cleaved-off) ARP is indicated.

From the western blot analysis (Figure 3) it is clear that in all transformants more fusion protein has been produced at 24 hours than at 48 hours after induction. Furthermore, after 48 hours more proteolysis is observed, which results in higher levels of cleaved-off ARP in the culture medium. D-xylose is almost depleted after 48 hours (results not shown), which might indicate exhaustion of the medium. This consequently could have induced the secretion and/or activation of proteases in the culture medium. Some effect of heme supplementation is visible after 24 hours, but after 48 hours no effect is observed. No difference is observed between multi- and single-copy transformants.

As a control, proteins on western blots were detected with α-EXLA (Figure 3B and D). The amount of extracellular EXLA is higher after 48 hours than after 24 hours of induction, as is expected for an endogenous protein that is relatively stable towards endogenous proteases.

The same medium samples used to analyse the amount of fusion protein secreted in the medium by western blot analysis were also assayed for ARP activity (Figure 4). Hemin addition did not show a significant effect on ARP activity of the ARP-R9 and R9-ARP transformants tested. For both the ARP-R9 and R9-ARP constructs higher activities were observed for the multiple copy transformants in comparison with the single copy transformants (Fig. 4; $p < 0.05$ and $p < 0.1$, respectively). Whereas a decrease in activity was observed for the 48 h samples versus the 24 h samples of the ARP-R9 transformants, the opposite was the case for the R9-ARP transformants.

Overall, the ARP activity levels did not correlate with the results obtained by western blotting experiments (Figure 3). As described in Chapter 5, the amount of fusion protein in the culture medium was to be calculated 30 mg/L for ARP-R9 #2 and 10 mg/L for R9-ARP #14 (24 h after induction in non-supplemented medium). This discrepancy is most likely the result of different specific activities of ARP in the fusion proteins and the additional effect of cleaved-off ARP on the total ARP activity in the culture medium.

Summarising, hemin addition to culture medium of transformants secreting fusion proteins does not result in significantly higher fusion protein secretion yields. In contrast to increased levels of ARP upon hemin addition, as shown in Figure 1 and 2, these results suggest that heme availability is not limiting for the production of these fusion proteins.

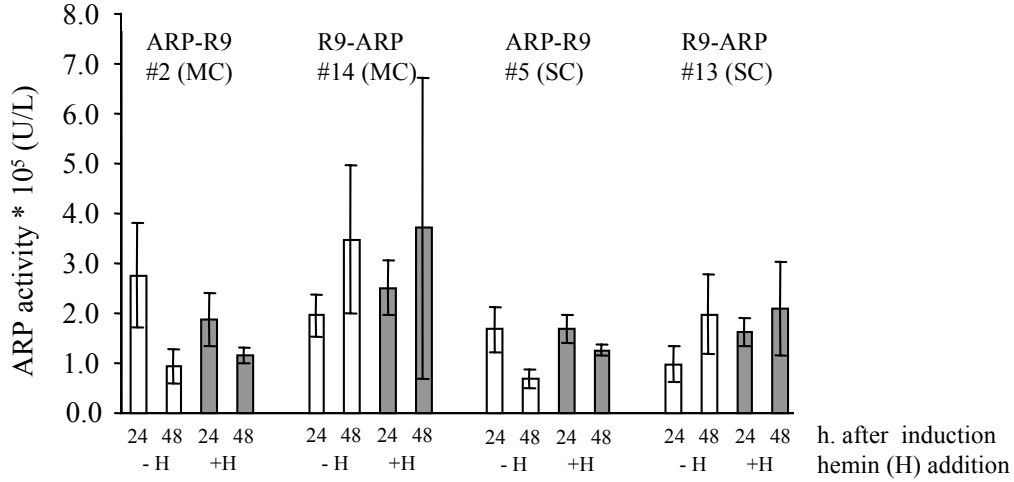

Figure 4. ARP activity (in 10^5 U/L) in medium samples from both AWC ARP-R9 and R9-ARP transformants with (grey bars) or without (white bars) hemin supplementation (500 mg/L). Samples were taken 24 and 48 h after transfer to induction medium and activity analysed spectrophotometrically in an ABTS activity assay. Purified ARP (Fluka) was used as a reference for activity. Standard deviations are indicated with error bars. Significance was tested by means of a T-test. MC; Multi-copy transformant, SC; single-copy transformant.

MnP production by *A. awamori* and the influence of heme

The previous experiments suggested that heme supplementation does not have a general effect on heme–protein production in *A. awamori*, but might have a stimulating effect on production of specific proteins, especially if these are produced at (very) high levels. To substantiate this further, the production of another heme-containing protein, MnP, in *A. awamori* was

analysed. MnP is a manganese peroxidase, for which was shown previously that heme addition could increase the production level in *A. niger* 3.5-fold (Conesa *et al.* 2002).

First, the p*gpd*MnP1.I-*AmdS* expression vector (Conesa *et al.* 2002) was introduced into *A. awamori* AWC4.20 by co-transformation with plasmid pAB4-1 (*pyrG*). Transformants were selected for growth both in the absence of uridine and on acetamide-containing plates. Several transformants were obtained that showed activity of MnP in an ABTS assay (Figure 5). Southern blot analysis confirmed the presence of the expression vector (results not shown). Two transformants (AWC MnP#7 and AWC MnP#3; encircled in Figure 5) were chosen for further experiments.

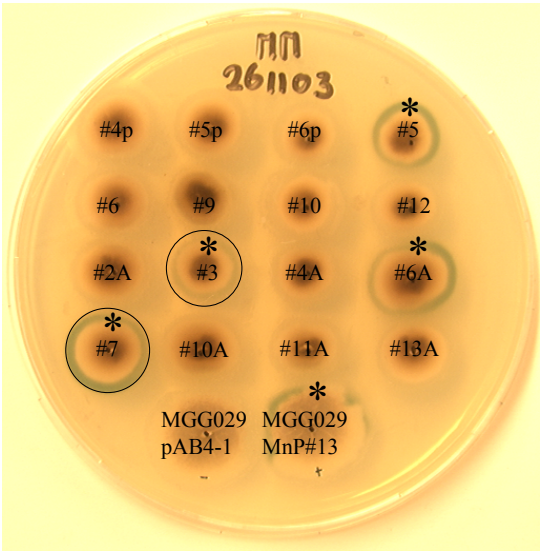

Figure 5. Putative MnP transformants were tested for peroxidase activity, using an ABTS activity test. Positive transformants (indicated with *) show a green halo around the colony. AWC MnP#7 and AWC MnP#3 were chosen for further experiments. *A. niger* MGG029 [p*gpd*MnP1.I]#13 was taken as a positive control and *A. niger* MGG029 transformed with only the co-transforming plasmid pAB4-1 as a negative control (Conesa *et al*. 2002).

To determine MnP production levels of transformants AWC MnP#7 and #3, they were cultivated as described by Conesa *et al.* (2002). The amount of MnP secreted by the two transformants in the culture medium was determined by western blotting, with a known concentration of purified MnP as a standard (results not shown). MnP levels reached approximately 1 mg/L in the culture medium after 24 hours for both transformants. Higher yields were obtained after 48 hours, reaching approximately 5 mg/L. These levels were comparable to the levels obtained with the same expression vector *in A. niger* MGG029 (Conesa *et al.* 2002).

To investigate the effect of heme supplementation on MnP production by *A. awamori*, transformants were grown with and without the addition of 500 mg/L hemin. Cultures were harvested 24 or 48 hours after transfer to fresh MM containing maltodextrin. Medium samples were subjected to western analysis using a polyclonal antibody against MnP (Figure 6).

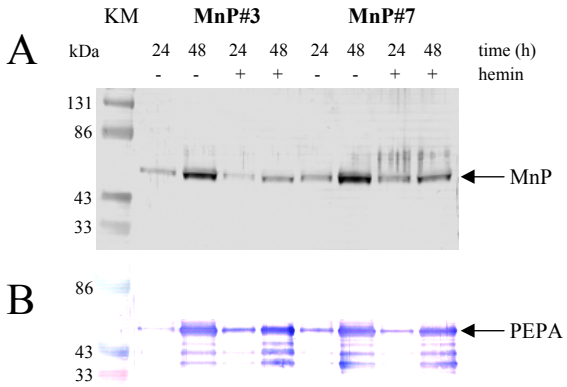

Figure 6. Western blot analysis of extracellular MnP production by AWC MnP#7 and AWC MnP#3 with (+) or without (-) hemin supplementation. Medium samples, taken 24 and 48 h after transfer, were subjected to SDS-PAGE and subsequently analysed by western blotting. Blots were detected with (**A**) α-MnP (raised against MnP isozymes H3-H4, obtained from D. Cullen) and (**B**) α-PEPA (*A. awamori* aspergillopepsin; Berka *et al*. 1990) as a control for the amount of protein loaded onto the gel.

Both transformants show the same production levels and without heme supplementation more MnP is produced after longer incubation periods. Whether at 48 h the maximal production was obtained was not tested. Hemin addition seems to decrease the production of MnP, leading to lower levels compared to production in minimal medium. Production of PEPA (*A. awamori* aspergillopepsin; Berka *et al*. 1990) increased equally in time in both transformants. From this we conclude that MnP production in *A. awamori* under these conditions is not stimulated by hemin supplementation.

CONCLUSIONS AND DISCUSSION

Low heme availability has been suggested to be a limiting factor for the production of heme-containing proteins in different fungal expression hosts (Andersen *et al.* 1992; Conesa 2001; Weber *et al.* 1992). In contrast, some reports showed the successful extracellular production of heme-containing proteins without heme supplementation of the culture medium (Gromada *et al.* 1997; Lokman *et al.* 2003). As was previously shown by Conesa *et al.* (2000) a 7-fold increase of MnP protein production by *A. niger* transformant MGG029 (pMnP1.I)#25 was achieved by supplementation of hemin to the culture medium when the *A. niger glaA* promoter was used to drive the expression of the MnP encoding sequences. When *mnp* was under control of the strong constitutive *A. nidulans gpdA* promoter, a 3.5-fold increase in MnP activity was observed in MGG029 [p*gpd*MnP1.I]#13 when compared to non-supplemented medium (Conesa *et al.* 2002).

In this chapter we analysed possible heme limitation during (over)-production of heme-containing proteins in *A. awamori*. Our first observation was that there is a positive effect of heme supplementation on the production of ARP. This is in contrast with Lokman *et al.* (2003) who reported production of 800 mg/L ARP independently of heme

supplementation of the medium. The maximal ARP activity was obtained 32 h after induction, decreasing to approximately 650 mg/L after 48 h. It was this observation that made the author suggest that *A. awamori* would be the preferred host for the production of fungal peroxidases.

In an attempt to resolve this discrepancy we performed experiments using different cultivation conditions. In our standard induction protocol mycelium is transferred to 300 ml shake-flasks containing 50 ml induction medium. In addition to the standard induction procedure, mycelium was also transferred to 100 ml induction medium. In the experiments described by Lokman *et al.* (2004) much smaller induction volumes (16 ml) and shake-flasks (100 ml) were used, although mycelium was still concentrated twice upon transfer to induction medium (B.C Lokman, pers. comm.). Using larger culture volumes without heme supplementation, we found at 48 h an ARP activity comparable to 400 mg/L ARP (purified ARP from Fluka) under standard induction conditions and 300 mg/L in the non-concentrated culture. This is lower than the 650 mg/L ARP found in unsupplemented medium at t = 48 h as reported by Lokman *et al.* (2003). Surprisingly, ARP activity comparable to an amount of more than 1 g/L ARP was obtained in our experiments with hemin supplementation of the culture medium. This indicates that during production of ARP by *A. awamori* heme limitation is a conditional state that depends on the cultivation conditions.

A possible parameter that might cause the differences observed could be aeration. When using different volumes and smaller shake-flasks, aeration could be sub-optimal. Oxygen limitation leads to a subset of events including the differential expression of a large number of hypoxic genes. Heme is suggested to be involved in the O_2 sensing mechanism and subsequent regulation of transcription of oxygen-responsive genes, as described for *S. cerevisiae* (Kwast *et al.* 1998). Heme would serve as a ligand for transcription factors (Hap1, the Hap2/3/4/5p complex and Rox1; reviewed by Zhang and Hach 1999) and thus the heme concentration would be important. It is proposed that oxygen availability affects the cellular heme levels, and that this, in turn affects the intracellular levels and/or activities of transcription factors (Kwast *et al.* 1998). As an example, the *S. cerevisiae* gene encoding Coproporphyrinogen III oxidase (*HEM13*), which is involved in the biosynthesis of heme (Labbe-Bois and Labbe 1990), is derepressed in the absence of heme and oxygen (Amillet *et al.* 1995; Keng 1992). Therefore, the upregulation of hypoxic genes in *S. cerevisiae* may be part of an adaptive response to make better use of the limiting substrate oxygen (and heme). Although less information is available on heme biogenesis in filamentous fungi, it is likely that a similar pathway is present as in yeast.

The different cultivation volumes and smaller shake-flasks used by Lokman *et al.* (2004) might very well have resulted in oxygen limitation and subsequently the induction of heme biosynthesis. This would explain both the higher ARP production without heme supplementation and the absence of any stimulating effect of the addition of heme. In our standard *A. awamori* shake-flask cultivations, aeration is not expected to be limiting and therefore intracellular heme levels could be lower. Consequently, an extra burden by the expression of a heterologous heme-containing protein could lead to heme limitation.

MnP, ARP and ARP-V_{HH} fusion proteins are heme-containing proteins of interest for industrial large-scale applications, providing that high levels of the protein can be produced in the culture medium at low costs. We analysed their production by *A. awamori* in relation to hemin supplementation and found that in standard shake flask experiments both MnP and ARP-fusion protein production was not increased by hemin addition. Although this seems in contradiction with the results described above, it should be noted that the production levels

reached with MnP (5 mg/L) and fusion proteins (10-30 mg/L) are much lower than those of ARP (200-400 mg/L).

For production of MnP by *A. niger* it has been shown that heme supplementation can significantly increase production levels (Conesa *et al.* 2002). The maximum concentrations of extracellular MnP reached in that study are comparable to what we observed (5 mg/L) and similar levels were also obtained in *A. oryzae*, provided that exogenous hemin was supplied (Stewart *et al*. 1996). Although slightly different cultivation conditions could in part account for the observed differences, it is likely that the genetic distinction between *A. awamori* AWC, *A. niger* MGG029 and *A. oryzae* is responsible for the different heme requirements. This would mean that *A. awamori* is the more suitable host for producing heme-containing proteins after all.

As shown in this chapter 500 mg/L hemin significantly increases ARP protein production up to levels around 1 g/L. However, addition of exogenous heme is undesired in such large-scale production processes, due to the high costs. Furthermore, the effects of even such high amounts of hemin are minor, leading only to a 4 times increase under the most favourable circumstances. Lower hemin concentrations were recently shown to be sufficient for *Caldariomyces fumago* chloroperoxidase (CPO) production by *Aspergillus niger* (Yi *et al.* 2003). These authors also showed that simultaneous addition of the early heme precursor 5-aminolevulinic acid (ALA) and hemin had a positive effect on CPO activity when expressed in *A. niger*. For ARP production in *A. awamori*, lowering the hemin concentration to 35 mg/L abolished the stimulating effect, even in the presence of ALA. Since the amount of hemin present in the medium under these conditions still is a vast excess to the intracellular hemin, it seems that uptake of hemin is problematic. Whether hemin or ALA can diffuse through the fungal cell membrane or the fungus can take up hemin actively (intact or degraded to yield heme) from its environment (see also Conesa 2001) is currently unknown.

Recently we reported the increase of heterologous V_{HH} production upon addition of 0.25 % BSA to the culture medium (Joosten *et al.* 2005a). In contrast, here we show that addition of BSA to the medium does not affect ARP protein or activity levels, suggesting that degradation of ARP by proteases does not have a prominent role under the conditions tested or BSA has no inhibiting effect on proteases responsible for ARP degradation.

The results presented in this chapter indicate that heme availability under certain cultivation conditions can limit the production of peroxidases in *A. awamori.* Since heme supplementation is undesired for large-scale production, alternative approaches have to be considered to deal with this problem. One of these approaches is to increase heme synthesis in the host cell. Although little is known about heme and heme biosynthesis in filamentous fungi, Elrod *et al.* (1997) showed that overexpression of heme biosynthetic enzymes in *A. oryzae* resulted in an increased yield of a heme-containing fungal peroxidase, albeit that these strains still responded to heme supplementation (Elrod *et al*. 2000). If heme incorporation itself is a problem during overproduction of heme-containing proteins, another approach could be the elevation of efficiency of heme incorporation during protein folding in the cell. This action is performed by foldases and molecular chaperones in the ER. For example, Conesa *et al.* (2002) showed that mRNA transcript levels of two ER chaperones (BiP and calnexin) responded upon MnP production and heme addition. When calnexin was over-expressed in *A. niger* MnP production strains, an elevated production and activity was observed.

Overall, from this chapter it is clear that standard shake-flasks cultivations are not well suited for “controlled’ production of heme-containing proteins and heterologous proteins in

general. Conditions can differ from culture to culture and continuously change throughout the cultivation. In chemostat experiments, conditions can be tightly controlled and changed in an unambiguous way. If oxygen depletion does turn on the heme biosynthetic pathway in *A. awamori*, it would be interesting to perform fermentations at different oxygen tensions and evaluate the production of heterologous heme-protein in the culture medium.

ACKNOWLEDGEMENTS

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Chapter 8

Summarising Discussion

INTRODUCTION

In this thesis our main goal was to analyse the potential of the filamentous fungus *Aspergillus awamori* to secrete bifunctional proteins ("Magic Bullets"), consisting of a llama variable heavy-chain antibody fragment (V_{HH}) fused to an effector protein, *Arthromyces ramosus* peroxidase (ARP). The idea behind the research as described in this thesis is the improvement of detergents by developing an improved alternative way of bleaching persistent spots without harming the textile. The strategy chosen was to generate fusion proteins of which the antibody-part should bind to a persistent spot on laundry and the coupled peroxidase could subsequently bleach the spot. By decreasing the content of chemicals in present detergents this strategy will contribute to a more environmentally friendly laundry washing procedure.

V_{HH}s were chosen because of their small size and simple structure, thermostability, solubility and excellent binding capacity (reviewed by Muyldermans 2001). It is expected that their small size and simple structure will enable their production by industrially relevant micro-organisms and also their stability and solubility will make the application in detergents possible. ARP was chosen as the effector protein, since it showed good bleaching capacity (Kjalke *et al.* 1992).

To use bifunctional proteins for large-scale consumer applications such as in detergents, these molecules must be available in large quantities and their production costs should be low. Filamentous fungi, such as *A. awamori* and *A. niger* efficiently secrete proteins and are envisaged as potential host organisms for the production of antibody fragments and bifunctional proteins, as described in **Chapter 2** of this thesis. Furthermore, in **Chapter 2** several additional interesting applications of V_{HH}s and "Magic Bullets" have been described.

PRODUCTION OF V_{HH}s BY *A. AWAMORI*

To study the feasibility of bifunctional protein production by *A. awamori*, first the two separate proteins were expressed and their production by *A. awamori* was analysed. In **Chapter 3** expression and production of model V_{HH}s raised against the azo-dye RR6 was described (Joosten *et al.* 2005a). Expression vectors for α-RR6 V_{HH}s were constructed and transformants containing these vectors were analysed to determine the gene copy-numbers, mRNA levels and secretion of the V_{HH} fragments. Although low amounts of V_{HH}s were detected in the culture medium (in the low mg/L range), these V_{HH}s were shown to be functional, indicating the feasibility to produce them in a filamentous fungal expression system. By comparison of mRNA and proteins levels of 1,4-β endoxylanase and V_{HH}s it became clear that the V_{HH} levels were much too low.

Little information is available on the stability of heterologous mRNA, although it has been shown that codon optimisation can lead to significantly higher expression in filamentous fungi (Gouka *et al.* 1997; Te'o *et al.* 2000). Since preliminary results in fermentation experiments showed that with an ARP-R9 fusion under the control of the *exlA* expression signals, levels of 200 mg/L of the fusion protein could be achieved (B.C. Lokman, unpublished results), codon usage is not regarded as a major problem.

Therefore, experiments were carried out to find possible bottlenecks for V_{HH} production. It was found that secreted V_{HH}s were subjected to (extracellular) degradation, which could be partially prevented by the addition of BSA to the culture medium.

Furthermore, considerable amounts of V_{HH}s were found attached to the fungal cell wall. However, these results only partially explained the low production levels. A possible explanation could be that in *A. awamori* V_{HH} fragments might be subjected to proteolytic degradation in the vacuole or via ERAD (ER-Associated Degradation).

To exclude the possibility that the suggested bottleneck was specific for anti-RR6 V_{HH}s, expression and production of tomato-spot recognising antibody fragments (V_{HH}toms) was investigated (**Chapter 6**). Low amounts of V_{HH}tom were detected in the culture medium, which strengthen the hypothesis that the low level of V_{HH}s observed in the culture medium is a result of a general post-translational bottleneck for V_{HH} production by *A. awamori.*

PRODUCTION OF ARP AND THE INFLUENCE OF HEME SUPPLEMENTATION ON PEROXIDASE PRODUCTION BY *A. AWAMORI*

In **Chapter 4** the results of the production of ARP by *A. awamori* are presented. High amounts of ARP were obtained in the culture medium during shake-flask cultivations, reaching up to 800 mg/L. This level was shown to be independent of heme supplementation of the culture medium. Furthermore, it was hypothesised that *A. awamori* is the preferred production organism for peroxidases (Lokman *et al.* 2003).

However, in **Chapter 7** the production of ARP was analysed under modified cultivation conditions (Joosten *et al.* 2005a) and here it was shown that heme did actually influence the final amount of ARP produced. Since levels higher than 1 g/L were obtained with hemin supplementation, it was hypothesised that oxygen might be limited in the cultivation conditions used in **Chapter 4**.

Furthermore, in **Chapter 7** the production of MnP by *A. awamori* was described. Production levels (~5 mg/L) were comparable to that of previously described for *A. niger* (Conesa *et al.* 2000). These authors showed that heme supplementation of the culture medium could significantly increase MnP production levels (Conesa *et al.* 2002a). When the culture medium of MnP, ARP-V_{HH} and V_{HH}-ARP transformants was subsequently supplemented with hemin, no increase in production of these proteins was observed. Therefore, it was hypothesised in this chapter that a certain heme-protein level has to be reached before the actual overproduction of peroxidases by *A. awamori* results in a limitation of the available heme inside the cell.

PRODUCTION OF "MAGIC BULLETS" BY *A. AWAMORI*

In **Chapter 5** and **Chapter 6** the production of "Magic Bullets" by *A. awamori* is analysed. Therefore, ARP was fused to either the N- or C-terminus of the heavy-chain antibody fragment V_{HH} R9 (**Chapter 5**) or V_{HH}toms (**Chapter 6**). *A. awamori* transformants were obtained which produced up to 30 mg/L fusion protein in the culture medium. These production levels were obtained without any further optimisation of production strains or media components. All fusion proteins showed peroxidase activity in an ABTS activity test.

In **Chapter 5** it was reported that considerable amounts of fusion protein were detected intracellularly, which suggests that the fungus encounters problems in secreting this

kind of proteins. ELISA experiments showed that ARP-R9 has lower affinity for its antigen, the azo-dye RR6, compared to R9-ARP. Furthermore, in contrast to R9-ARP, ARP-R9 bound to its antigen did not show peroxidase activity. These results suggest that C-terminal linkage of ARP to the antibody fragment is the preferred orientation. Based on the recently published crystal structure of V_{HH} R9 (Spinelli *et al.* 2004) it was hypothesised that coupling of ARP to the V_{HH} N-terminal end (*e.g.* ARP-R9) shields the binding sites of V_{HH} R9, resulting in a lower ability of V_{HH} R9 to bind the antigen RR6. When subsequently the binding of V_{HH}tom-ARP and ARP-V_{HH}tom fusion proteins was determined, the same preferred orientation was observed (**Chapter 6**).

IMPROVING THE PRODUCTION OF V_{HH}s, ARP AND FUSION PROTEINS BY *A. AWAMORI*

The V_{HH}s, ARP and bifunctional fusion proteins described in this thesis are of great industrial interest. However, at this moment levels in the culture medium are too low to be able to use them for large-sale applications. For ARP alone there seems to be no major problem to increase the production by *A. awamori*. Improving culture conditions and/or classical strain improvement techniques will possibly lead to grams of ARP per litre of culture medium during chemostat cultivations.

In contrast, during overproduction of V_{HH}s and fusion proteins, several limitations at the post-translational level were observed. Concerning the action of extracellular proteases, it was shown both in **Chapter 3** and **Chapter 5** that the addition of BSA increased the amount of V_{HH} R2 and fusion proteins found in the culture medium. As BSA is thought to act as a substrate for extracellular proteases, which are abundantly secreted by filamentous fungi (Mattern *et al.* 1992; van den Hombergh *et al.* 1997), the degradation of the investigated proteins could be (partially) prevented in this way. Therefore, the generation of protease-deficient strains could be a solution for decreasing extracellular degradation.

A possibility to improve secretion of V_{HH}s and fusion proteins is by fusion of the gene (s) to a well-secreted protein such as glucoamylase (GLA) (Gouka *et al.* 1997; Verdoes *et al.* 1995). Recently, Ward *et al.* (2004) showed that antibodies and Fab' fragments could be efficiently produced by *A. niger*, by using GLA as a carrier protein. When optimising V_{HH} production by *A. awamori*, the use of GLA-fusions can be considered as an effective approach.

Concomitantly, also ARP can be used as carrier protein. Production levels of the V_{HH} R9 fragment were very low in the culture medium (~1 mg/L; **Chapter 3**). When V_{HH} R9 was expressed together with ARP as a fusion protein, resulting in approximately 30 mg/L, ARP seems to act as a carrier protein for V_{HH} R9. Generally, the carrier protein is fused to the N-terminus of the heterologous protein, although in our study it is shown that ARP is able to increase V_{HH} R9 production in both orientations.

It is hypothesised that V_{HH} and fusion protein production is mainly limited at the post-translational level, starting with inefficient translocation and/or incorrect folding, resulting in impaired secretion and/or intracellular degradation. The intracellular signalling pathway responsible for sensing incorrectly folded proteins or an overload of the ER upon high levels of protein expression, is named the Unfolded Protein Response (UPR) and is extensively described in **Chapter 1**. If misfolded proteins are present in the ER (due to ER stress,

incorrect folding etc.) they can accumulate in the ER or are transported to the proteasome by a process called ERAD (ER-Associated Degradation; Brodsky and McCracken 1999). Also transport for destruction in the vacuole is possible (Spear and Ng 2003).

In **Chapter 5** it is shown that ARP-R9 and R9-ARP accumulate intracellularly. It would be interesting to investigate whether these are incorrectly folded fusion proteins, which are eventually targeted to the proteasome or vacuole for destruction. In accordance with this hypothesis, the low levels of fusion protein could be a result of intracellular degradation.

V_{HH}s might be subjected to intracellular degradation (**Chapter 3**). Since no accumulation of V_{HH}s was observed intracellularly and ERAD is suspected to be a fast an efficient degradation process, this could be an indication for proteasomal degradation. However, since some secretion of V_{HH} in the culture medium took place, this could also be an indication for involvement of vacuolar degradation.

Answers to these previous questions can hopefully be provided in the future by for example pulse-chase experiments by tracing the fate of a labelled heterologous protein in the secretion pathway) (van Gemeren *et al.* 1998; Pakula *et al.* 2000), the use of inhibitors of the proteasome (Lee and Goldberg 1996; Tomoda and Omura 2000) and vacuole (Staszczak *et al.* 2000) or the generation of (conditional) mutant fungal strains, deficient in a step in the secretion pathway.

FINAL CONCLUSION AND PROSPECTS

The idea behind the research described in this thesis is the improvement of detergents by means of targeted bleach by the addition of "Magic Bullets". An example of the proposed action of "Magic Bullets" is presented in Figure 1.

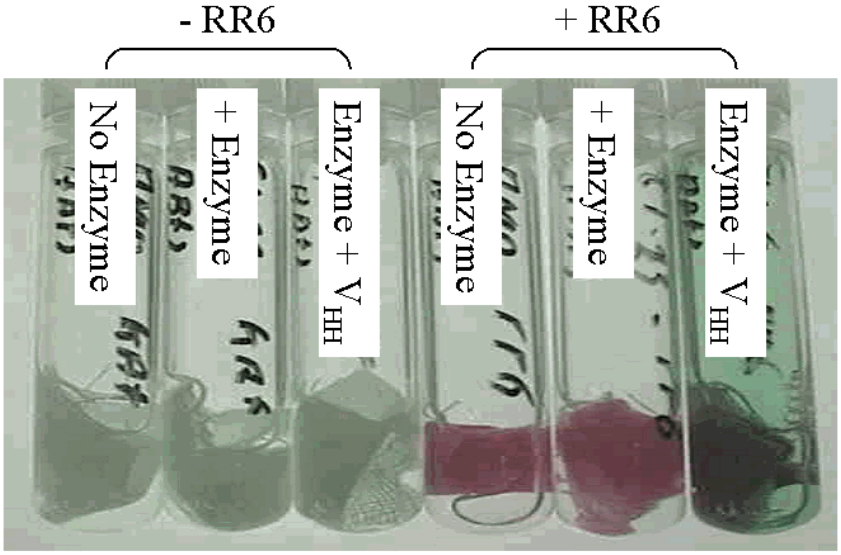

Figure 1. An example of the use of V_{HH}-enzyme fusion proteins ("Magic Bullets") in laundry industry (R.J. Gouka, pers. comm.). Textile swatches were untreated (- RR6) or treated with the azo-dye RR6 (+ RR6). After addition of enzyme or "Magic Bullets" (Enzyme + V_{HH}), the swatches were extensively rinsed. As a control, no enzyme was added. Enzyme activity was determined with an ABTS assay and only detected in tube 6, which shows that the "Magic Bullet" specifically binds to the textile.

In this preliminary experiment (R.J. Gouka, pers. comm.) cotton swatches were treated with the azo-dye RR6 and subsequently "Magic Bullets" were added, consisting of the V_{HH} against RR6 coupled to a bleaching enzyme. After several rounds of rinsing the cotton, the activity of the adhered enzyme was demonstrated with an ABTS peroxidase activity assay. A green coloration was observed, which indicates that still enzyme was present by means of binding of the V_{HH} moiety to the RR6-stained cotton. Furthermore, the present enzyme discoloured the cotton from RR6.

A. awamori is considered as a promising host for the large-scale production of bifunctional proteins, consisting of V_{HH}s fragments fused to an effector molecule. Regarding the initial production levels and the ease of upscaling of the production process, bifunctional proteins can become available in large and cheap amounts for consumer applications. Since V_{HH}s can be raised against all kinds of different stains that are difficult to remove from textile by current laundry cleaning procedures, the use of V_{HH}-enzyme fusion proteins to improve detergents is of major interest. In the near future, "Magic Bullets" produced by filamentous fungi are expected to find their way to consumer applications.

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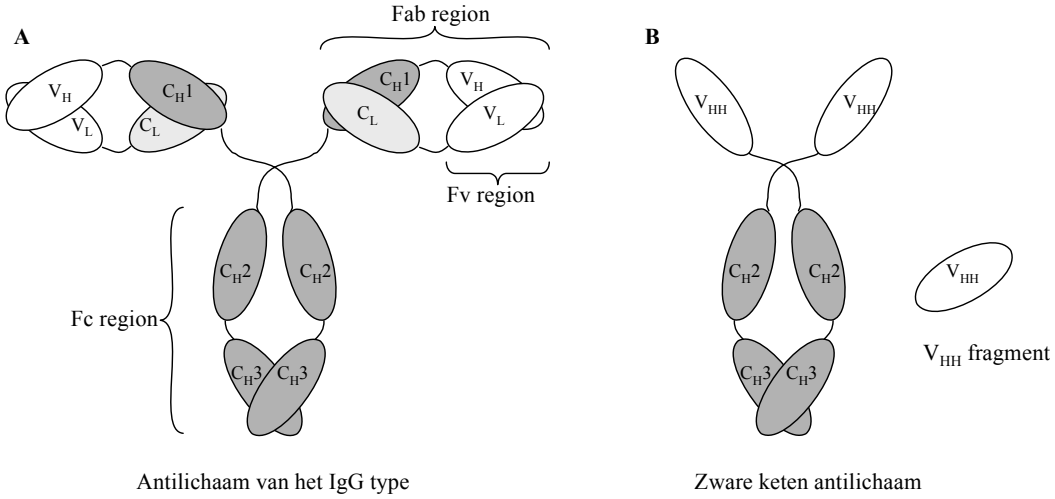
Nederlandse samenvatting

ALGEMENE INTRODUCTIE

Ondanks dat er veel is gedaan aan de verbetering van wasmiddelen, zijn er nog steeds componenten in wasmiddelen aanwezig die schadelijk kunnen zijn voor het milieu. Om bepaalde hardnekkige vlekken te kunnen verwijderen uit wasgoed worden bijvoorbeeld bleekmiddelen gebruikt. Een alternatief voor deze middelen zouden “Magic Bullets” kunnen zijn (in dit proefschrift ook wel fusie eiwitten of bifunctionele eiwitten genoemd). Het idee hierachter is dat bepaalde herkenningsmoleculen worden gebruikt, die de hardnekkige vlekken in het wasgoed gericht herkennen en daaraan binden. Aan het herkenningmolecuul kan vervolgens een enzym worden gekoppeld dat de vlek bleekt. Het gebruik van deze “Magic Bullets” zou verder als voordeel kunnen hebben dat alleen de vlek wordt gebleekt en niet het hele wasgoed, dat kleuren niet doorlopen en dat relatief weinig van deze moleculen nodig zijn om het beoogde doel te bereiken. Ondanks dat het idee achter “Magic Bullets” niet nieuw is (ze worden bijvoorbeeld toegepast in kankertherapie) zijn er enorm veel nieuwe toepassingen te bedenken zoals het gebruik van “Magic Bullets” in tandpasta, deodorant en shampoos.

Herkenningsmoleculen die uitermate geschikt zijn voor de beoogde applicaties zijn antilichamen. Antilichamen zijn eiwitten die heel goed kunnen binden aan hun antigenen (waartegen ze zijn opgewekt). Mensen maken een heleboel verschillende antilichamen die ons beschermen tegen allerlei lichaamsvreemde stoffen en zijn belangrijk in onze afweer. IgG typen antilichamen hebben een heel herkenbare structuur (de zogenaamde Y-vorm; zie Figuur 1A). Ze bestaan uit twee delen: het constante gedeelte (wat in alle antilichamen hetzelfde is) en het variabele domein (wat in elk antilichaam weer verschillend is). Het variabele domein is erg belangrijk want het herkent en bindt aan het antigeen. Daarnaast is er ook nog een verdeling te maken tussen zware en lichte ketens. Zware ketens hebben extra constante domeinen die belangrijk zijn voor het uiteindelijke effect van antilichamen.

In 1993 vond de Belgische professor Hamers dat kamelen, lama’s en dromedarissen een andere soort antilichamen hebben dan andere dieren (zie Figuur 1B), de zogenaamde zware keten antilichamen (heavy-chain antibodies). Deze antilichamen hebben geen lichte ketens en missen ook nog een paar constante zware domeinen. Het domein dat betrokken is bij de binding aan het antigeen wordt V_{HH} genoemd, wat staat voor variabel zware keten antilichaam fragment. Het zijn juist deze domeinen die zo interessant zijn, want het blijkt dat ze erg oplosbaar zijn en erg stabiel bij hoge temperaturen. Daarnaast is aangetoond dat ze bepaalde enzymen kunnen remmen en enzymen kunnen binden op plaatsen die normaal gesproken ontoegankelijk zijn voor ander bindingsmoleculen. Deze V_{HH}s zijn dus erg interessant voor de toepassing in consumentenproducten zoals wasmiddelen, maar ook in medicijnen (bijvoorbeeld als remmers van enzymen of als componenten van kankerremmende medicijnen) en in technische toepassingen zoals bepaalde detectiemethoden.



Figuur 1A. "Normaal" antilichaam van het IgG-type. De constante domeinen (C), die voor alle IgG-type antilichamen hetzelfde zijn, zijn aangeven in grijs. De variabele domeinen (V) in wit. Daarnaast is er ook nog een onderscheid te maken tussen de lichte ketens (L, voor light) en zware ketens (H, voor heavy). **B.** Zware keten antilichaam zoals gevonden bij de familie van de kameelachtigen. De constante domeinen (C), zijn aangegeven in grijs. De V_{HH} fragmenten (wat staat voor variabel zware keten antilichaam fragment) in wit. Deze fragmenten zijn belangrijk voor de binding aan het antigeen.

Als we nu terug gaan naar "Magic Bullets" voor het gebruik in wasmiddelen, dan is er dus een enzym nodig om de vlek te bleken of op te lossen. Een enzym dat interessant is met betrekking tot onze toepassing, is een peroxidase wat uitgescheiden wordt door de schimmel *Arthromyces ramosus* (ARP). Sommige schimmels produceren van nature grote hoeveelheden peroxidases om substraten in hun omgeving af te kunnen breken die vervolgens dienen als voeding.

Om V_{HH}s of "Magic Bullets" op grote schaal te kunnen gebruiken in commerciële producten zijn er grote hoeveelheden nodig. Daarnaast mag de productie niet teveel kosten. Een mogelijke oplossing voor dit probleem is de productie van deze eiwitten door filamenteuze schimmels, zoals *Aspergillus awamori* en *Aspergillus niger*. Deze schimmels, die van nature overal voorkomen, secreteren van zichzelf al heel veel eiwitten in hun omgeving. De moderne biotechnologie maakt het mogelijk om de schimmel deze 'vreemde' eiwitten (ook wel heterologe eiwitten genoemd) te laten produceren en uit te laten scheiden in zijn omgeving.

In dit proefschrift wordt het onderzoek beschreven dat is uitgevoerd om te bepalen of *A. awamori* een geschikte gastheer is voor de productie van V_{HH}s en "Magic Bullets". In **Hoofdstuk 2** is een literatuurstudie beschreven en daarin is geprobeerd duidelijk te maken dat filamenteuze schimmels waaronder *A. awamori*, mogelijke kandidaten zijn voor de beoogde productie van V_{HH}s en "Magic Bullets". Vervolgens is dit in praktijk getest, beschreven in de daaropvolgende hoofdstukken.

PRODUCTIE VAN V_{HH}s DOOR *A. AWAMORI*

De mogelijkheid om fusie-eiwitten te laten produceren door *A. awamori* werd onderzocht door eerst de expressie en productie van twee afzonderlijke componenten van de fusie-eiwitten te onderzoeken. In **Hoofdstuk 3** werd de expressie en productie van model V_{HH}s door *A. awamori* geanalyseerd. Deze model-V_{HH}s zijn opgewekt tegen de rode kleurstof RR6. Expressievectoren voor de anti-RR6 V_{HH} genen werden geconstrueerd en de verkregen transformanten werden geanalyseerd op het aantal kopieën van de expressievector, het mRNA niveau en uiteindelijk de uitscheiding van de model-V_{HH}s. Ondanks dat de hoeveelheid uitgescheiden V_{HH}s laag was, waren deze V_{HH}s wel functioneel (d.w.z. dat ze aan de rode kleurstof RR6 konden binden). Dit geeft aan dat het mogelijk is om V_{HH}s door *A. awamori* te laten produceren. Echter, wanneer men mRNA en productieniveaus van een schimmel-eigen eiwit (1,4-β endoxylanase) vergeleek met die van V_{HH}s, bleek dat het productieniveau van V_{HH}s relatief lager was. De conclusie was dat er meer factoren meespelen: *A. awamori* kan deze antilichaam fragmenten niet optimaal produceren en waarschijnlijk worden ze afgebroken door proteases in de schimmel zelf of in het medium waarin de V_{HH}s uitgescheiden worden. Dat afbraak van V_{HH}s in het medium een oorzaak kon zijn voor de lage productie werd aangetoond door het medium te verzadigen met Bovine Serum Albumin (BSA). Door toevoeging van dit "algemene eiwit" aan het medium dat als substraat kan dienen voor de proteases, kon de opbrengst ietwat verhoogd worden. Daarnaast werd ook gevonden dat een redelijke hoeveelheid van de geproduceerde V_{HH}s aan de celwand van de schimmel bleef hangen en dus niet in het medium kon worden waargenomen. De totale hoeveelheid V_{HH}s bleef lager dan verwacht en een mogelijke verklaring is dan ook dat V_{HH}s efficiënt worden afgebroken in de schimmel zelf.

Om de mogelijkheid uit te sluiten dat de lage opbrengst te wijten is aan de specifiek onderzochte V_{HH}s (tegen de rode kleurstof RR6), werd ook een andere set V_{HH} onderzocht (**Hoofdstuk 6**). Deze specifieke tomaatvlek-gerichte V_{HH}s (V_{HH}toms) vertoonde een soortgelijke productie door *A. awamori*, maar vertoonde wel binding met componenten in tomatenpasta. Er werd geconcludeerd dat de productie van V_{HH} is het algemeen problemen oplevert bij de expressie in *A. awamori*.

PRODUCTIE VAN ARP EN DE INVLOED VAN HEEM SUPPLEMENTATIE

Vervolgens werd de productie van het peroxidase ARP door *A. awamori* onderzocht (**Hoofdstuk 4**). In het kweekmedium werden productieniveaus tot 800 mg/L behaald. ARP is een heem peroxidase en heem wordt ingebouwd in het eiwit tijdens de productie van ARP. Het zou kunnen dat door de hoge productie van ARP een tekort aan heem in de schimmel ontstaat. Dit is onderzocht door tijdens de kweekexperimenten heem toe te voegen aan het medium. De productie van ARP kon hierdoor niet verder verhoogd worden en dus is er waarschijnlijk geen limitatie van heem in de cel. Uit de resultaten van dit hoofdstuk werd de conclusie getrokken dat *A. awamori* een zeer geschikte gastheer zou kunnen zijn voor de productie van heem peroxidases.

In **Hoofdstuk 7** werden echter de groeicondities veranderd en hieruit bleek dat toevoegen van heem wel degelijk de productieniveaus van ARP kon verhogen (tot meer dan 1 gram per liter). Een mogelijke verklaring voor dit effect is dat zuurstof een beperkende factor is onder bepaalde groeicondities.

In **Hoofdstuk 7** werd ook de productie van een ander heem peroxidase door *A. awamori* beschreven. Voor dit mangaan peroxidase (MnP) werden dezelfde productieniveaus behaald onder dezelfde condities als recentelijk beschreven voor *A. niger* (~ 5 mg/L). Toevoegen van heem aan het *A. awamori* medium kon dit niveau niet verhogen. Ook deze toevoeging aan transformanten die de fusie-eiwitten uitscheiden, had geen effect op de uiteindelijke hoeveelheid fusie-eiwit in het medium.

PRODUCTIE VAN "MAGIC BULLETS" DOOR *A. AWAMORI*

In **Hoofdstuk 5** en **6** werd de productie van de "Magic Bullets" beschreven. Hiervoor werden zowel anti-RR6 V_{HH} R9 (**Hoofdstuk 5**) als tomaatvlek-herkennende V_{HH}s (V_{HH}toms; **Hoofdstuk 6**) gekoppeld aan ARP. Omdat de beste oriëntatie van het peroxidase ten opzichte van V_{HH}s (ARP-V_{HH} of V_{HH}-ARP) niet bekend was, werden beide mogelijkheden onderzocht.

A. awamori transformanten werden verkregen die 30mg/L fusie eiwit in hun medium uitscheiden. Deze niveaus werden zelfs behaald zonder optimalisatie van het groeimedium of de gastheer zelf. Verder bleek uit een ABTS activiteitstest dat alle geproduceerde fusie-eiwitten peroxidase-activiteit vertoonde.

Uit de experimenten zoals beschreven in **Hoofdstuk 5** bleek dat er een ruime hoeveelheid fusie-eiwit in de schimmel kon worden aangetoond wat er op zou kunnen duiden dat de schimmel moeite heeft met het uitscheiden van dit soort eiwitten. Ook bleek uit ELISA experimenten dat ARP-R9 minder goed kon binden aan de rode kleurstof dan R9-ARP. Daarnaast vertoonde de aan de kleurstof gebonden ARP-R9 geen peroxidase-activiteit meer. Dit suggereert dat men in deze fusie-eiwitten ARP het beste achter het V_{HH} fragment kan plaatsen. Voor de fusie-eiwitten bestaande uit de V_{HH}toms en ARP werd een zelfde voorkeursoriëntatie van V_{HH}s t.o.v. ARP gevonden (**Hoofdstuk 6**). Een verklaring voor de slechtere binding van ARP-R9 aan de kleurstof kan wellicht afgeleid worden uit de kristalstructuur van V_{HH} R9. Als het relatief grote ARP molecuul voor R9 geconstrueerd wordt, zou dit de bindingsplaatsen voor de kleurstof in het V_{HH} R9 molecuul af kunnen schermen.

VERBETERING VAN DE PRODUCTIE VAN V_{HH}S, ARP EN FUSIE-EIWITTEN

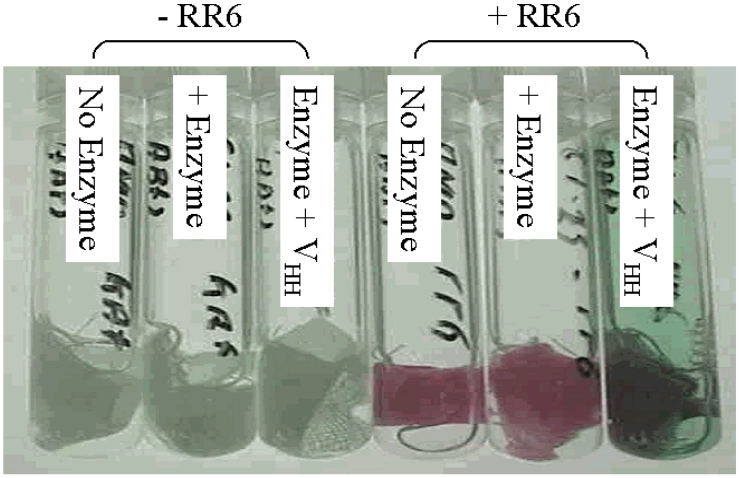
De eiwitten beschreven in dit proefschrift (V_{HH}s, ARP en de "Magic Bullets") zijn van grote industriële waarde. De productieniveaus zijn echter nog niet commercieel aantrekkelijk. Vanuit het oogpunt voor de toepassing in medicijnen, waar vaak kleine hoeveelheden al voldoende zijn en de kosten minder belangrijk zijn, is dit niet zo heel erg belangrijk. Voor het gebruik van "Magic Bullets" in bijvoorbeeld wasmiddelen zal de producent grote hoeveelheden van de fusie-eiwitten nodig hebben, tegen zo laag mogelijke kosten. De consument zal niet bereid zijn om tientallen euro's meer neer te leggen voor een pak waspoeder of deodorant. Dus opschalen van de productie is van groot belang.

Voor het heem peroxidase ARP lijkt deze opschaling niet een heel groot probleem. Omdat er nu al in schudkolven bijna een gram per liter wordt behaald, zullen verbeteringen aan de groeicondities en het gebruik van geoptimaliseerde *Aspergillus* gastheren al snel leiden tot een grote hoeveelheid ARP.

Een verhoogde productie van V_{HH}s en fusie-eiwitten heeft misschien wat meer voeten in de aarde. Er is al aangetoond dat er meerdere niveaus zijn waarop beperkingen tijdens de productie kunnen optreden. In **Hoofdstukken 3** en **5** werd aangetoond dat de hoeveelheid V_{HH} R2 en fusie-eiwitten in het groeimedium wordt beïnvloed door de activiteit van proteases. Filamenteuze schimmels zoals *A. awamori* staan erom bekent dat ze grote hoeveelheden proteases uitscheiden in hun groeimedium. Een afleidingsmanoeuvre, door een protease substraat zoals BSA toe te voegen aan het medium, is te gebruiken in de relatief kleine schudkolf kweken maar niet in grootschalige fermentatie-experimenten. Het is beter om protease-deficiënte gastheren te vinden of zelf te maken, die minder of geen proteases uitscheiden die het eiwit afbreken. Om in de toekomst grootschalig V_{HH}s en fusie-eiwitten te kunnen produceren is het gebruik van deze protease-deficiënte gastheren dus van zeer groot belang. Daarnaast kan het groeimedium geoptimaliseerd worden, zodat de schimmel minder wordt aangezet tot het maken van deze proteases. Beide methoden zijn geschikt om in zeer grote kweekculturen om de productie te verhogen. Meer mogelijkheden om heterologe eiwit productie te verbeteren, zoals de carrier strategie, worden beschreven in **Hoofdstuk 1** van dit proefschrift.

CONCLUSIE EN PERSPECTIEVEN

Het idee achter het onderzoek zoals beschreven in dit proefschrift was de verbetering van wasmiddelen met behulp van meer gerichte bleking van vlekken. Het uiteindelijke gebruik van de fusie-eiwitten in wasmiddelen is niet getest, maar toch is er een goed voorbeeld hoe een V_{HH}, gekoppeld aan een blekings-enzym, de kleurstof op een lapje stof kan bleken (Figuur 2).



Figuur 2. Een voorbeeld van het gebruik van "Magic Bullets" in wasmiddelen (RJ Gouka, pers. comm.). Lapjes stof werden wel (+ RR6) of niet (- RR6) behandeld met de rode kleurstof RR6. Na toevoeging van een fusie-eiwit (V_{HH} + enzym) zijn de lapjes stof zorgvuldig gespoeld om ongebonden fusie-eiwit te verwijderen. Als controle werd ook fusie-eiwit weggelaten of werd alleen het losse enzym toegevoegd. De activiteit van het gebonden fusie-eiwit kon worden aangetoond met behulp van een ABTS assay. In deze assay zet het peroxidase een kleurloos substraat om in een groene kleurstof. Alleen in de laatste buis werd een kleurreactie waargenomen, wat duidt op binding van het fusie-eiwit aan de kleurstof RR6 op het lapje stof.

In dit voorbeeld zijn er lapjes stof behandeld met de rode kleurstof RR6. Vervolgens is er een fusie-eiwit aan toegevoegd en is het lapje stof herhaaldelijk gespoeld. Als het V_{HH} de kleurstof zou binden zou het dus blijven binden, zelfs na meerdere keren spoelen. De binding van het hele fusie-eiwit aan de kleurstof is vervolgens aangetoond met een ABTS activiteitstest. Peroxidase activiteit die na spoelen nog steeds aanwezig is zet een substraat om in een groene kleurstof. Deze groene kleur is zichtbaar in de laatste buis van Figuur 2, wat aangeeft dat er een specifieke binding is van het fusie-eiwit met het RR6-behandelde lapje stof. In deze buis is ook de rode kleur verdwenen op het lapje, dus het peroxidase in het fusie eiwit heeft de rode kleur daarnaast ook nog eens gebleekt.

Concluderend kan gesteld worden dat *A. awamori* een zeer geschikte kandidaat is voor de grootschalige productie van fusie-eiwitten, bestaande uit een bindingsmolecuul zoals een V_{HH} fragment en een effector molecuul zoals het heem peroxidase ARP. Er wordt verwacht dat door het optimaliseren van de groeicondities en de mogelijkheid tot het relatief makkelijk opschalen van de productie, fusie-eiwitten in de toekomst in grote hoeveelheden en voor een redelijke prijs in consumentenproducten te verwerken zijn. De mogelijkheid om V_{HH}s op te wekken tegen een groot aantal vlekken die normaal gesproken moeilijk zijn te verwijderen met behulp van de huidige wasmiddelen, maakt het toepassen van fusie eiwitten in wasmiddelen zeer interessant. Wellicht zullen “Magic Bullets” dan ook in de toekomst toegepast kunnen worden in een breed scala van consumentenproducten.

List of publications

Pettersson, A., J. van der Biezen, V. Joosten, J. Hendriksen, and J. Tommassen. (1999) Sequence variability of the meningococcal lactoferrin-binding protein LbpB. *Gene.* **231**:105-110.

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Curriculum vitae

Vivi Joosten werd op 21 november 1973 geboren te Terheijden.

Na het behalen van haar VWO diploma in 1993 aan het Niels Stensen College te Utrecht, behaalde zij een testimonium natuurkunde aan het James Boswell Instituut. In 1994 begon zij met de studie Biologie aan de Universiteit van Utrecht. Tijdens de studie werden twee afstudeeronderzoeken uitgevoerd. Tijdens het eerste afstudeeronderzoek (9 maanden) bij de vakgroep Moleculaire Microbiologie aan de Universiteit van Utrecht werd onderzoek verricht aan het lactoferrine-bindende eiwit (LbpB) van *Neisseria meningitidis* en de moleculaire karakterisering van het daarvoor coderende *lbpB* gen van diverse *N. menigitidis* serogroep B stammen, begeleid door Dr. Annika Pettersson en Prof. Dr. Wiel Hoekstra. Het tweede afstudeeronderzoek (6 maanden) werd uitgevoerd bij TNO Voeding te Zeist in de toenmalige groep Moleculaire Genetica en Gentechnologie (MGG). In dit onderzoek is Sar1p (een GTPase) van *Aspergillus niger* bestudeerd door middel van een reporterstrategie, onder begeleiding van Dr. Peter Punt. Als afsluiting van haar studie werd een scriptie geschreven met als titel "Spitzenkorper behaviour in filamentous fungi", met als doel om via een literatuurstudie meer inzicht te verkrijgen in de functie van accumulatie van "vesicles" in de top van schimmel hyphen. Scriptie begeleiders waren Prof. Dr. Kees van den Hondel en Prof. Dr. Arie Verkleij.

Na het behalen van het doctoraal examen Biologie in juni 1999, werd in augustus van datzelfde jaar begonnen aan een promotieonderzoek bij TNO Voeding te Zeist, onder begeleiding van Dr. ing. Christien Lokman, Prof. Dr. Kees van den Hondel en Prof. Dr. ir. Theo Verrips. De resultaten van dit onderzoek, dat werd afgerond in september 2003, zijn te lezen in dit proefschrift.

Van oktober tot en met december 2003 was zij als onderzoeker werkzaam op een gezamenlijk project van TNO Voeding te Zeist en TNO Preventie en Gezondheid te Leiden. In dit project werden de expressie en productie van diverse typen collageen in *Aspergillus spp*. onderzocht, onder begeleiding van Dr. Jan Jore.

Per 1 september 2004 is Vivi Joosten werkzaam als postdoc op het Laboratorium voor Biochemie van de Wageningen Universiteit. In dit project doet zij onderzoek naar flavine-afhankelijke sulfhydryl oxidases (SOX).