

Sanna Askolin

Characterization of the *Trichoderma reesei* hydrophobins HFBI and HFBII

VTT PUBLICATIONS 601

Characterization of the *Trichoderma reesei* hydrophobins HFBI and HFBII

Sanna Askolin
VTT Biotechnology

Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Department of Chemical Technology, Helsinki University of Technology for public examination and debate in Auditorium TU1 at Helsinki University of Technology (Espoo, Finland) on the 26th of May, 2006, at 12 noon.



ISBN 951-38-6836-2 (soft back ed.)

ISSN 1235-0621 (soft back ed.)

ISBN 951-38-6837-0 (URL: <http://www.vtt.fi/publications/index.jsp>)

ISSN 1455-0849 (URL: <http://www.vtt.fi/publications/index.jsp>)

Copyright © VTT Technical Research Centre of Finland 2006

JULKAISIJA – UTGIVARE – PUBLISHER

VTT, Vuorimiehentie 5, PL 2000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 4374

VTT, Bergsmansvägen 5, PB 2000, 02044 VTT
tel. växel 020 722 111, fax 020 722 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 5, P.O.Box 2000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax + 358 20 722 4374

VTT, Tietotie 2, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 7071

VTT, Datavägen 2, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 7071

VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax +358 20 722 7071

Technical editing Leena Uksskoski

Otamedia Oy, Espoo 2006

Askolin, Sanna. Characterization of the *Trichoderma reesei* hydrophobins HFBI and HFBII [*Trichoderma reesei*in HFBI- ja HFBII-hydrofobiinien karakterisointi]. Espoo 2006. VTT Publications 601. 99 p. + app. 38 p.

Keywords filamentous fungi, *Trichoderma reesei*, surface active proteins, hydrophobins, HFBI, HFBII, purification, characterization, crystallization, microbial surfactant, gene deletion, biological function, foaming

Abstract

Hydrophobins are surface active proteins produced by filamentous fungi. They fulfil a wide variety of functions in fungal growth and development. These proteins for example render fungal aerial structures hydrophobic and affect the attachment of fungi to solid supports. The ability of hydrophobins to modify surface properties by interfacial self-assembly and their high surface activity provide a potential for several applications. In this work, properties and biological roles of the HFBI and HFBII hydrophobins produced by *Trichoderma reesei* were studied. In addition, the hydrophobins were crystallized for structure determination. Moreover, effects of these hydrophobins on the cultivation of *T. reesei* in bioreactors were evaluated.

High production levels of HFBI and HFBII were obtained in *T. reesei* by introducing additional copies of the *hfb1* or *hfb2* genes into the genome. The *T. reesei* HFBI-overproducing strain (VTT D-98692) produced up to 1.4 grams HFBI per liter on glucose-containing culture medium, the highest production level of hydrophobin hitherto reported. The HFBII-overproducing strain (VTT D-99745) and its parent strain secreted 0.24 and 0.03 grams HFBII per liter, respectively, into the culture medium.

HFBI and HFBII were purified from fungal cell walls and liquid culture medium, respectively. The purified hydrophobins were crystallized by vapour diffusion in hanging drops for structural analysis.

Behaviour of the class II hydrophobins HFBI and HFBII of *T. reesei* and the class I hydrophobin SC3 of *Schizophyllum commune* was studied at various interfaces. All the hydrophobins were surface active. HFBI and HFBII reduced

the surface tension of water faster than SC3. Self-assembly of HFBI and HFBII at water-air interfaces induced by mixing with air was not accompanied by a change in the circular dichroism spectra, in contrast to SC3. No clear ultrastructure of the dried class II hydrophobin film was observed in electron microscopy. However, the circular dichroism spectra of HFBI and HFBII changed when they were in contact with Teflon surface, indicating formation of α helix structure, although not to the same extent as with SC3. Both HFBI and SC3 strongly interacted with hydrophobic Teflon surface, rendering it completely wettable. HFBI, HFBII and SC3 all stabilized oil emulsions but HFBI and SC3 appeared to be more effective than HFBII. The presence of HFBI or HFBII affected the solubility of SC3 aggregates, indicating interaction between these hydrophobins. Differences between the behaviour of class I and class II hydrophobins at the water-air interface are proposed to be due to the divergent size and shape of the hydrophobic parts of these proteins.

In order to study the biological roles of HFBI and HFBII, the *hfb1* and *hfb2* genes were deleted in *T. reesei*. The $\Delta hfb1$ strain formed no aerial hyphae in static liquid cultures. Addition of purified HFBI to the medium restored the aerial hyphae formation. The aerial growth was also restored by expressing the gene encoding the SC3 hydrophobin of *S. commune* in the $\Delta hfb1$ strain. Colonies of $\Delta hfb1$ had a wettable and fluffy phenotype when grown on a solid medium. In shaken liquid cultivation, biomass formation of $\Delta hfb1$ was slower compared with the parent strain. Sporulating colonies of $\Delta hfb2$ were wettable and sporulation was only 50% of that of the parent strain. These results indicate that HFBI facilitates aerial growth of *T. reesei*, whereas HFBII is involved in sporulation.

Process technological effects of HFBI and HFBII were studied by cultivating the $\Delta hfb1$, $\Delta hfb2$, HFBI-overproducing and HFBII-overproducing strains in laboratory bioreactors. Vegetative growth properties of the hydrophobin deletion and over-producing strains were similar to those of their parent strains. The strains overproducing the hydrophobins foamed extensively, especially the HFBII-overproducing strain. Foaming of the $\Delta hfb2$ strain (but not $\Delta hfb1$) was lower compared with the parent strain on lactose- and cellulose-containing media. This shows that the main cause of foaming in bioreactor cultivations is the HFBII hydrophobin.

Askolin, Sanna. Characterization of the *Trichoderma reesei* hydrophobins HFBI and HFBII [*Trichoderma reesei*in HFBI- ja HFBII-hydrofobiinien karakterisointi]. Espoo 2006. VTT Publications 601. 99 s. + liitt. 38 s.

Avainsanat microbial surfactant, filamentous fungus, hydrophobicity, production, purification, crystallization, gene deletion, biological function, foaming

Tiivistelmä

Hydrofobiinit ovat rihmamaisten sienten tuottamia pinta-aktiivisia proteiineja. Niillä on useita tehtäviä sienten kasvussa ja kehityksessä. Tässä työssä tutkittiin *Trichoderma reesei*in HFBI- ja HFBII-hydrofobiinien ominaisuuksia ja biologisia rooleja. Lisäksi hydrofobiinit kiteytettiin, ja niiden vaikutusta *T. reesei*in bioreaktorikasvatuksiin arvioitiin.

Korkeat HFBI- ja HFBII-tuottotasot saavutettiin lisäämällä ylimääräisiä kopioita *hfb1*- tai *hfb2*-geenejä *T. reesei*in perimään. HFBI:ä tuotettiin jopa 1,4 grammaa litraa kohden, mikä on korkein tähän mennessä raportoitu hydrofobiinin tuotto-taso. HFBI ja HFBII puhdistettiin ja kiteytettiin rakenneanalyysiä varten.

Luokan II hydrofobiinien HFBI ja HFBII sekä *Schizophyllum commune*n luokan I hydrofobiinin SC3 toimintaa tutkittiin useilla rajapinnoilla. Kaikki hydrofobiinit olivat pinta-aktiivisia. HFBI ja HFBII alensivat veden pintajännityksen nopeammin kuin SC3. HFBI:n ja HFBII:n kerääntyminen vesi- ja ilmafaasiin väliin ei muuttanut niiden CD-spektrejä, toisin kuin SC3:lla. Elektronimikroskopialla ei myöskään havaittu selkeää ultrarakennetta kuivuneilla HFBI- ja HFBII-hydrofobiinikalvoilla. Erojen ehdotetaan johtuvan näiden proteiinien hydrofobisten alueiden erilaisesta koosta ja muodosta. Teflonin lisäys muutti HFBI:n ja HFBII:n CD-spektriä viitaten α -kierrerakenteen muodostumiseen. Sekä HFBI että SC3 tekivät teflonista täysin kastuvan. Kaikki hydrofobiinit stabiloivat öljyemulsioita. HFBI:n tai HFBII:n läsnäolo vaikutti SC3:n liukoisuuteen, mikä viittaa näiden hydrofobiinien väliseen vuorovaikutukseen.

*T. reesei*stä poistettiin *hfb1*- ja *hfb2*-geenit. Staattisissa nestekasvustoissa $\Delta hfb1$ -kanta ei muodostanut ilmarihmoja. Puhdistetun HFBI:n lisääminen elatusaineeseen tai *SC3*-geenin ilmentäminen $\Delta hfb1$ -kannassa palautti ilmarihmojen muodostumisen. $\Delta hfb1$ -pesäkkeillä oli vettyvä ja pörröinen fenotyyppi. Ravistelluissa nestekasvatuksissa $\Delta hfb1$:n biomassan muodostus oli isäntäkantaa hitaampaa. Itiöivät $\Delta hfb2$ -pesäkkeet olivat vettyviä ja itiöinti oli vain 50 % isäntäkannan itiöinnistä. Nämä tulokset osoittavat, että HFBI edesauttaa *T. reesei*n kasvamista ilmaan, kun taas HFBII on osallisena itiöinnissä. HFBI:ä ja HFBII:a ylituottavat kannat vaahtosivat runsaasti bioreaktorikasvatuksissa, erityisesti HFBII:n ylituottokanta. Vaahtoaminen oli $\Delta hfb2$ -kannalla (mutta ei $\Delta hfb1$:lla) isäntäkantaa heikompaa. HFBII:n osoitettiin olevan pääsyy vaahtoamiseen *T. reesei* Rut-C30:n bioreaktorikasvatuksissa.

Preface

This work was carried out at VTT Biotechnology during the years 1998–2003. I thank Professor Juha Ahvenainen, Professor Liisa Viikari and Research Manager Richard Fagerström for providing excellent working facilities at VTT. Professor Matti Leisola at Helsinki University of Technology is gratefully acknowledged for his support during this work.

My warmest thanks are due to my instructors Professor Maija Tenkanen and Research Manager Tiina Nakari-Setälä. I want to thank them for their neverending support, encouragement and valuable comments.

Professor Han Wösten, Dr. Marcel de Vocht and Dr. Karin Scholtmeijer at the University of Groningen in the Netherlands, and Professor Keith Wilson and Dr. Johan Turkenburg at the University of York, UK, are warmly thanked for the pleasant collaboration. Especially, I want to thank Han for his valuable and inspiring comments and for helping me in many ways during my visit in Groningen. I also warmly thank Kalevi Visuri, M.Sc., and Sinikka Uotila, B.Sc., who advised me with the protein crystallization studies in Macrocrystal. I also wish to thank my other co-authors at VTT, Professor Merja Penttilä, Dr. Markus Linder and Michael Bailey, B.Sc., for valuable advice and discussions.

I wish to thank all the personnel at VTT for pleasant and creative atmosphere. My special thanks are due to Riitta Isoniemi and Seija Nordberg for skilful technical assistance and help. Financial support from Neste Oy Foundation, Eemil Aaltonen Foundation, The Foundation of Technology in Finland, The Finnish Foundation for Economic and Technology Sciences – KAUTE and Finnish Konkordia Fund are gratefully acknowledged.

I warmly thank my parents, relatives and friends for their support and encouragement over these years. Finally, I wish to express my dearest thanks to my husband Juha and our sons Ville and Atte for their patience and support, which helped me to finish this work.

List of publications

This work is based on the following publications, which are referred to in the text by their Roman numerals. Additional unpublished data is also presented.

- I Askolin, S., Nakari-Setälä, T. and Tenkanen, M. 2001. Overproduction, purification, and characterization of the *Trichoderma reesei* hydrophobin HFBI. *Appl. Microbiol. Biotechnol.* 57, pp. 124–130.
- II Askolin, S., Turkenburg, J.P., Tenkanen, M., Uotila, S., Wilson, K.S., Penttilä, M. and Visuri, K. 2004. Purification, crystallization and preliminary X-ray diffraction analysis of the *Trichoderma reesei* hydrophobin HFBI. *Acta Cryst. D60*, pp. 1903–1905.
- III Hakanpää, J., Paananen, A., Askolin, S., Nakari-Setälä, T., Parkkinen, T., Penttilä, M., Linder, M.B. and Rouvinen, J. 2004. Atomic resolution structure of the HFBI hydrophobin, a self-assembling amphiphile. *J. Biol. Chem.* 279, pp. 534–539.
- IV Askolin, S., Linder, M., Scholtmeijer, K., Tenkanen, M., Penttilä, M., de Vocht, M.L. and Wösten H.A.B. 2006. Interaction and comparison of a class I hydrophobin from *Schizophyllum commune* and class II hydrophobins from *Trichoderma reesei*. *Biomacromolecules* 7, pp. 1295–1301.
- V Askolin, S., Penttilä, M., Wösten, H.A.B. and Nakari-Setälä, T. 2005. The *Trichoderma reesei* hydrophobin genes *hfb1* and *hfb2* have diverse functions in fungal development. *FEMS Microbiol. Lett.* 253, pp. 281–288.
- VI Bailey, M.J., Askolin, S., Hörhammer, N., Tenkanen, M., Linder, M., Penttilä, M. and Nakari-Setälä, T. 2002. Process technological effects of deletion and amplification of hydrophobins I and II in transformants of *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.* 58, pp. 721–727.

The author of the present thesis had main responsibility for the practical work and writing of publications I, II, IV and V and of VI concerning studies of HFBI. For publication III, she carried out preliminary crystallization of HFBI.

Contents

Abstract.....	3
Tiivistelmä	5
Preface	7
List of publications	8
Contents	9
List of symbols.....	12
1. Introduction.....	14
1.1 Microbial surfactants	14
1.2 Hydrophobins	17
1.2.1 Classification and structural features of hydrophobins	17
1.2.2 Distribution of hydrophobins	19
1.2.3 Protein properties of hydrophobins	25
1.3 The roles of hydrophobins in filamentous fungi.....	29
1.3.1 Formation of aerial hyphae	32
1.3.2 Coating of fungal structures	33
1.3.3 Cell wall maturation.....	35
1.3.4 Pathogenesis and attachment.....	36
1.3.5 Symbiosis	37
1.4 <i>Trichoderma reesei</i> hydrophobins.....	38
1.5 Production of hydrophobins	39
1.6 Purification of hydrophobins	40
1.7 Application potential of hydrophobins	41
1.8 Background and aims of the present study	43
2. Materials and methods	44
2.1 Strains, vectors and culture conditions	44
2.2 DNA techniques	45
2.3 Purification of hydrophobins	46
2.4 Crystallization of HFBI and HFBII	46
2.5 Quantification of HFBI and HFBII	47

2.6	Biochemical characterization of hydrophobins	47
2.7	Biophysical characterization of hydrophobins	48
2.8	Microscopy	48
2.9	Other analyses	49
3.	Results and discussion	50
3.1	Production and purification of HFBI and HFBII (I, II, IV, VI).....	50
3.2	Structural investigation of HFBI and HFBII	52
3.2.1	Crystallization of HFBI and HFBII (II, unpublished results)	52
3.2.2	Three dimensional crystal structure of HFBII (III)	54
3.3	Comparison of the class II hydrophobins HFBI and HFBII with a class I hydrophobin.....	56
3.3.1	Behaviour at water-air interface (IV)	56
3.3.2	Binding to a solid surface (IV).....	59
3.3.3	Emulsification of oils (IV)	61
3.3.4	Stability (I, IV).....	61
3.3.5	Interaction between class I and class II hydrophobins (IV)	62
3.3.6	Hyphothetical models explaining the main differences between class I and class II hydrophobins	63
3.4	Biological roles of HFBI and HFBII (V, VI)	67
3.4.1	Effect of HFBI and HFBII on hydrophobicity of aerial fungal structures (V)	68
3.4.2	Role of HFBI in the formation of aerial hyphae (V).....	69
3.4.3	Effect of HFBI on hyphal growth and morphology (V, VI) ...	70
3.4.4	Effect of HFBI and HFBII on sporulation (V, VI).....	71
3.4.5	Complementation of a phenotype of $\Delta hfb1$ by SC3 (V).....	71
3.5	Process technological effects of HFBI and HFBII in bioreactor cultivations of <i>T. reesei</i> (VI)	72
4.	Conclusions.....	75
	References.....	79

Appendices

Publications I–VI

Appendices II–V of this publication are not included in the PDF version. Please order the printed version to get the complete publication
(<http://www.vtt.fi/inf/pdf>)

List of symbols

aa	amino acid
ADSA-P	axisymmetric drop-shape analysis by profile
AFM	atomic force microscopy
<i>amdS</i>	<i>Aspergillus nidulans</i> gene encoding acetamidase
<i>cbh</i>	<i>Trichoderma reesei</i> gene encoding cellobiohydrolase
C	cysteine
CBH	cellobiohydrolase
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
CMC	critical micelle concentration
EDTA	ethylene diamine tetra-acetic acid
EM	electron microscopy
FEP	fluorinated ethylene propylene (Teflon)
FPU	filter paper units
<i>hfb</i>	<i>T. reesei</i> gene encoding hydrophobin
HFB	<i>T. reesei</i> hydrophobin
<i>hph</i>	<i>Escherichia coli</i> gene encoding hygromycin B phosphotransferase

HPLC	high performance liquid chromatography
IR	infrared
kb	kilobase(s)
LB	Luria Bertani (medium)
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight
M_r	molecular mass
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
<i>pk</i>	gene encoding pyruvate kinase
PTFE	polytetrafluoro ethylene (Teflon)
SAXS	small angle x-ray scattering
SDS	sodium dodecyl sulfate
spp.	species
TFA	trifluoroacetic acid
ThT	Thioflavine T (dye)
3D	three-dimensional

1. Introduction

1.1 Microbial surfactants

Surfactants (“surface active agents”) are amphiphilic molecules, which have both hydrophilic and hydrophobic parts. They adsorb to hydrophilic-hydrophobic interfaces, such as oil-water or air-water, where they form a molecular film. In this way these molecules are capable of reducing the surface tension of a liquid or the interfacial tension between two liquids, and of stabilizing emulsions. Surfactant films can also influence interfacial mass transfer. A wide variety of living organisms produce molecules with surface active properties, from microorganisms and plants (e.g. saponins) to higher organisms including the human body (e.g. bile salts). The main source of microbial surfactants, also called biosurfactants, is bacteria (Healy *et al.*, 1996; Lin, 1996).

Microbial surfactants are a heterogenic group of different chemical structures such as glycolipids, lipopeptides and lipoproteins, phospholipids, neutral lipids, fatty acids and polysaccharide-protein complexes. The hydrophilic parts of biosurfactants generally consist of mono-, di- or polysaccharides, carboxylic acids, amino acids or peptides. The hydrophobic parts are usually saturated, unsaturated or hydroxylated fatty acids with a common chain length of 10–18 carbon atoms. In proteins or peptides, a high proportion of hydrophobic side chains can also form the hydrophobic part. The best known biosurfactants are glycolipids, such as rhamnolipids, trehalolipids and sophorolipids, which are carbohydrates with long-chain fatty acids or hydroxy fatty acids (Lin, 1996; Desai and Banat, 1997; Bognolo, 1999).

The activity of microbial surfactants can be characterized in terms of changes in surface and interfacial tensions, critical micelle concentration (CMC) and stabilisation or destabilisation of emulsions. Many biosurfactants lower the surface tension of water (72 mJ m^{-2}) to approximately 30 mJ m^{-2} (Table 1). Surfactin, a cyclic lipopeptide of *Bacillus subtilis*, reduces water surface tension to 27 mJ m^{-2} and the interfacial tension of water-*n*-hexadecane from 43 to 1 mJ m^{-2} (Cooper *et al.*, 1981; Table 1) and is thus one of the most effective biosurfactants. Addition of surfactant to the solution reduces the surface tension

up to a critical concentration, CMC. At concentrations above the CMC, surfactant molecules associate readily to form structures such as micelles, bilayers and vesicles (Lin, 1996; Desai and Banat, 1997). Emulsions have been characterized as dispersions of microscopic liquid droplets in another liquid continuous phase. Biosurfactants can stabilize emulsions by coating the droplets. One of the most powerful microbial emulsifiers is emulsan, which is a complex of anionic heteropolysaccharide and protein produced by *Acinetobacter calcoaceticus* RAG-1. Emulsan stabilises very effectively hydrocarbons in water by binding tightly to the hydrocarbon droplets (Rosenberg *et al.*, 1979; Zosim *et al.*, 1982; Desai and Banat, 1997).

The properties of many biosurfactants, such as their ability to reduce surface tension, are comparable to those of synthetic surfactants. The CMC values of biosurfactants are approximately from 10 to 40-fold lower than those of synthetic surfactants, which means that they are required in smaller amounts. Some biosurfactants also have good thermal and chemical stabilities. The biodegradability of biosurfactants makes them especially suitable for potential environmental applications such as dispersion of oil spills and bioremediation (Lin, 1996; Desai and Banat, 1997; Bognolo, 1999).

The physiological functions of microbial biosurfactants are not fully understood. Biosurfactants have often been produced by microorganisms grown on water-insoluble substrates (Lin, 1996; Desai and Banat, 1997). It has been suggested that biosurfactants enhance the availability of hydrophobic water-insoluble substrates by emulsification and solubilisation (Lin, 1996; Rosenberg and Ron, 1999; Ron and Rosenberg, 2001). The growth of *Pseudomonas aeruginosa* mutants producing reduced amounts of extracellular rhamnolipid biosurfactant was impaired on *n*-paraffin and hexadecane when compared with the parent strain (Itoh and Suzuki, 1972; Koch *et al.*, 1991). Growth was restored by addition of rhamnolipid to the medium. In addition, biosurfactants may be involved in attachment of cells to interfaces by altering the cell surface hydrophobicity (Lin, 1996; Rosenberg and Ron, 1999). Cell-bound biosurfactants may either increase or decrease the hydrophobicity of the cell surface (Ron and Rosenberg, 2001). Several biosurfactants, mainly lipopeptides and glycolipids, have antimicrobial activity due to solubilization of cell membrane components (Lin, 1996; Ron and Rosenberg, 2001).

Table 1. Properties of microbial surfactants other than hydrophobins. Note that different experimental conditions were used, which may cause uncertainty in comparison of the values.

Surfactant type	Microorganism	Surface tension ^a (mJ m ⁻²)	Concentration (g l ⁻¹)	CMC (g l ⁻¹)	Reference
Glycolipids	<i>Pseudomonas aeruginosa</i>	29	nr	0.11	Abalos <i>et al.</i> , 2001
	<i>Nocardia corynebacterioides</i>	26	0.03	nd	Powalla <i>et al.</i> , 1989
	<i>Serratia rubidaea</i>	26 ^b	0.01	nd	Matsuyama <i>et al.</i> , 1990
Lipopeptides and lipoproteins	<i>Bacillus licheniformis</i>	28	nr	0.012	Yakimov <i>et al.</i> , 1995
	<i>Serratia marcescens</i>	29–34 ^b	0.01	nd	Matsuyama <i>et al.</i> , 1986
	<i>Bacillus subtilis</i>	27	0.025	0.025	Cooper <i>et al.</i> , 1981
	<i>Pseudomonas fluorescens</i>	27 ^b	0.5	0.15 ^b	Neu <i>et al.</i> , 1990
Fatty acids	<i>Nocardia erythropolis</i>	33	nr	nd	MacDonald <i>et al.</i> , 1981
Others	<i>Pseudomonas fluorescens</i>	27	nr	0.01 ^b	Persson <i>et al.</i> , 1988
protein-carbohydrate-complex AP-6					

^aSurface tension of pure water is 72 mJ m⁻²

^bdetermined in buffer or salt solution; CMC critical micelle concentration; nr not reported; nd not determined

1.2 Hydrophobins

Filamentous fungi produce small, moderately hydrophobic proteins called hydrophobins. Due to their surface activity and ability to form amphiphilic protein films at hydrophilic-hydrophobic interfaces (Wessels, 1997; Wösten and Wessels, 1997; Wösten and de Vocht, 2000; Wösten, 2001; Linder *et al.*, 2005), these proteins can be considered as microbial surfactants. The interfacial self-assembly can occur e.g. between water and air, a hydrophilic cell wall and air or water and a hydrophobic solid. Hydrophobins have been localized on the surface of aerial fungal structures such as long branching threads (hypha), specialized spore-producing structures (fruiting bodies) and spores (Wessels, 1997; Wösten, 2001). The name hydrophobin was originally introduced because of the high content of hydrophobic amino acids (Wessels *et al.*, 1991b). Amino acid sequences of hydrophobins contain eight cysteine residues in characteristic pattern, with only a few exceptions. However, the amino acid similarity of hydrophobins is rather low (Wessels, 1994, 1997; Linder *et al.*, 2005). Hydrophobins fulfil a variety of functions in fungal growth and development (Wessels, 1997; Wösten and Wessels, 1997; Wösten, 2001; Whiteford and Spanu, 2002).

During the course of this study, information concerning hydrophobins has increased enormously. This Introduction provides an overview of hydrophobins based on the present literature apart from the publications I–VI. Detailed information concerning the HFBI and HFBII hydrophobins of *Trichoderma reesei* is presented in the Results and Discussion section.

1.2.1 Classification and structural features of hydrophobins

Hydrophobins were divided into two classes, class I and class II, by Wessels (1994). The division was based on the solubility of hydrophobin aggregates and the characteristic distribution of hydrophobic amino acid residues. Both class I and class II hydrophobins form, or self-assemble into, aggregates. Aggregates of the class I hydrophobins (such as SC3 and SC4 of *Schizophyllum commune* and ABH1 of *Agaricus bisporus*) were found to be highly insoluble and to be dissociated by reagents such as formic acid or trifluoroacetic acid (TFA) (Wessels *et al.*, 1991a, b; de Vries *et al.*, 1993). The class II hydrophobin

aggregates (such as those of cerato-ulmin (CU) of *Ophiostoma ulmi* and cryparin (CRP) of *Cryphonectria parasitica*) dissociate more readily and are soluble in e.g. 60% ethanol and 2% sodium dodecyl sulphate (SDS) (Russo *et al.*, 1982; Carpenter *et al.*, 1992; Wessels, 1997; Wösten, 2001).

These two hydrophobin classes can also be distinguished on the basis of the characteristic spacings between their cysteine residues (Wösten and Wessels, 1997) as presented in Figure 1. The number of amino acids between cysteine residues is more variable among class I hydrophobins than among class II hydrophobins. In addition, compared with class II hydrophobins, the spacing between the third and the fourth cysteine residue of class I hydrophobins is relatively long. The cysteine pattern, however, is similar in both hydrophobin classes: the second and the third cysteine residue as well as the sixth and the seventh are located next to each other (Figure 1).

Class I $X_{25-158}\text{-C-X}_{5-9}\text{-C-C-X}_{4-44}\text{-C-X}_{7-23}\text{-C-X}_{5-7}\text{-C-C-X}_{6-18}\text{-C-X}_{2-13}$

Class II $X_{17-165}\text{-C-X}_{7-10}\text{-C-C-X}_{11}\text{-C-X}_{15/16}\text{-C-X}_{6-9}\text{-C-C-X}_{10/11}\text{-C-X}_{3-8}$

Figure 1. Length of the amino acid sequences between eight cysteine residues in class I and class II hydrophobins. X represents any other amino acid than cysteine and the subindex the number of amino acids. The formula was modified from Wösten and Wessels (1997) based on the known amino acid sequences of the hydrophobins listed in Table 2. It applies to each hydrophobin unit of the trihydrophobin CFTH1 and the pentahydrophobin CPPH1 and essentially to the exceptions, QID3 and HFBIII, which contain 7 and 9 cysteine residues, respectively. Signal sequences are included in the formula.

The eight cysteine residues have been reported to form disulphide bridges in the class II hydrophobin CU of *O. ulmi* (Yaguchi *et al.*, 1993). Four disulphide bridges were suggested to be located between neighbouring cysteine residues (between C1 and C2, C3 and C4 etc.). However, the authors acknowledged uncertainty in their results. Nevertheless the structure of hydrophobins has been proposed to be composed of two domains on the bases of these results. In addition to CU, the eight cysteines in the class I hydrophobin SC3 of *S. commune* have also been indicated to be connected by disulphide bridges

(de Vocht *et al.*, 2000). Treatment with reagents that reduce the disulphides (1,4-dithiothreitol) and block free cysteines (iodoacetic acid or iodoacetamide) were shown to initially unfold SC3 according to circular dichroism (CD) and infrared (IR) spectroscopy. It is very likely that the eight cysteines in other hydrophobins are also connected by disulphide bridges.

There is an obvious interest in determining the three dimensional (3D) structures of hydrophobins. *O. ulmi* CU (Yaguchi *et al.*, 1993), *Neurospora crassa* EAS (Mackay *et al.*, 2001) and *S. commune* SC3 (Wang *et al.*, 2004a) hydrophobins have been analysed with nuclear magnetic resonance (NMR) spectroscopy. The results suggest that SC3 is structured in solution, whereas EAS is mostly unstructured apart from a β -sheet core. IR and Raman spectroscopies indicated a high proportion of β sheet structure for CU in solution (Yaguchi *et al.*, 1993). A similar observation was made for the water-soluble SC3 based on IR and CD spectroscopies (de Vocht *et al.*, 1998).

1.2.2 Distribution of hydrophobins

Hydrophobins are found in fungi capable of hyphal growth, including filamentous and dimorphic (i.e. capable of both filamentous and yeast-like growth) fungi of Ascomycetes and Basidiomycetes, the two major groups of fungi (Table 2). The first genes encoding hydrophobins were discovered in *S. commune* (Dons *et al.*, 1984a, b; Mulder and Wessels, 1986; Schuren and Wessels, 1990). Currently, about seventy unique genes encoding hydrophobins are available in databases (Table 2), SC3 of *S. commune* being one of the most studied hydrophobins. In addition, several gene fragments of putative hydrophobins are found. The number of genes will presumably increase further as a result of genome-wide sequencing projects. It appears probably that all filamentous fungi belonging to Ascomycetes and Basidiomycetes produce hydrophobins.

Many fungal species have more than one hydrophobin gene (Table 2). Class I hydrophobins have been found in both Ascomycetes and Basidiomycetes, whereas class II hydrophobins have only been observed in Ascomycetes. Both class I and class II hydrophobins have been shown to occur within a single species. In the tomato pathogen *Cladosporium fulvum* four class I hydrophobins

and two class II hydrophobins have been identified (Spanu, 1997; Segers *et al.*, 1999; Nielsen *et al.*, 2001), whereas in the corn pathogen *Fusarium verticillioides* genes encoding three class I hydrophobins and two class II hydrophobins have been found (Fuchs *et al.*, 2004). The rice pathogen *Magnaporthe grisea* contains at least one class I and one class II hydrophobin encoding gene (Talbot *et al.*, 1993; Kim *et al.*, 2005). A similar situation is expected to occur in many other Ascomycetes.

Nucleotide sequence similarity of hydrophobins between the two classes and between species is usually low (Wessels, 1994, 1997; Linder *et al.*, 2005). Whiteford and Spanu (2002) speculated that class II hydrophobins had probably evolved within Ascomycetes independently of class I hydrophobins, showing the phenomenon of convergent evolution. However, the origin of the two hydrophobin classes remains unclear.

Table 2. *Hydrophobins*.

Organism	Hydrophobin	Number of aa Total ^a Mature protein ^b	M _r ^c (kDa)	Protein isolated	Remarks	Reference or EMBL accession number
CLASS I						
Ascomycetes						
<i>Aspergillus fumigatus</i>	RodA / HYP1	159	16	+		Parta <i>et al.</i> , 1994; Thau <i>et al.</i> , 1994; Paris <i>et al.</i> , 2003
<i>Aspergillus fumigatus</i>	RodB	140	14	+		Paris <i>et al.</i> , 2003
<i>Aspergillus nidulans</i>	DewA	135				Stringer and Timberlake, 1995
<i>Aspergillus nidulans</i>	RodA	157				Stringer <i>et al.</i> , 1991
<i>Aspergillus oryzae</i>	Rola	151		(+) ^d		Takahashi <i>et al.</i> , 2005; AB094496
<i>Aspergillus oryzae</i>	HYPB	145				AB097448
<i>Cladosporium fulvum</i>	HCF-1	105	10	+		Spanu, 1997
<i>Cladosporium fulvum</i>	HCF-2	121				Segers <i>et al.</i> , 1999
<i>Cladosporium fulvum</i>	HCF-3	103				Segers <i>et al.</i> , 1999
<i>Cladosporium fulvum</i>	HCF-4	228				Segers <i>et al.</i> , 1999
<i>Cladosporium herbarum</i>	HCh-1	105		(+) ^d		Weichel <i>et al.</i> , 2003
<i>Fusarium verticillioides</i>	Hyd1	126				Fuchs <i>et al.</i> , 2004; AY155496
<i>Fusarium verticillioides</i>	Hyd2	123				Fuchs <i>et al.</i> , 2004; AY155497
<i>Fusarium verticillioides</i>	Hyd3	96				Fuchs <i>et al.</i> , 2004; AY155498
<i>Magnaporthe grisea</i>	MPG1	112	15	+		Talbot <i>et al.</i> , 1993; 1996
<i>Metarhizium anisopliae</i>	SSGA	96				St Leger <i>et al.</i> , 1992
<i>Neurospora crassa</i>	EAS	108	8.2 ^{ms}	+		Bell-Pedersen <i>et al.</i> , 1992; Lauter <i>et al.</i> , 1992; Templeton <i>et al.</i> , 1995; Mackay <i>et al.</i> , 2001
<i>Paracoccidioides brasiliensis</i>	PbHYD1	100		(+) ^d		Albuquerque <i>et al.</i> , 2004

<i>Paracoccidioides brasiliensis</i>	PbHYD2	133				Albuquerque <i>et al.</i> , 2004
<i>Xanthoria ectaneoides</i>	XEH1	83	10	+		Scherrer <i>et al.</i> , 2000
<i>Xanthoria parietina</i>	XPH1	83	10	+		Scherrer <i>et al.</i> , 2000
Bacidiomycetes						
<i>Agaricus bisporus</i>	ABH1 / HYPA	89	8-9	+		de Groot <i>et al.</i> , 1996; Lugones <i>et al.</i> , 1996
<i>Agaricus bisporus</i>	ABH2 / HYPC	115				de Groot <i>et al.</i> , 1996; Lugones <i>et al.</i> , 1996
<i>Agaricus bisporus</i>	ABH3	119	9.1 ^{ms}	+		Lugones <i>et al.</i> , 1998
<i>Agaricus bisporus</i>	HYPB	119				de Groot <i>et al.</i> , 1999
<i>Agroclype aegerita</i>	Aa-PRI2	123				Santos and Labarère, 1999
<i>Coprinus cinereus</i>	CoH1	113	10	+		Ásgeirsdóttir <i>et al.</i> , 1997
<i>Coprinus cinereus</i>	CoH2	110				Ásgeirsdóttir <i>et al.</i> , 1997
<i>Dictyonema glabratum</i>	DGH1	130	14	+		Trembley <i>et al.</i> , 2002
<i>Dictyonema glabratum</i>	DGH2	115				Trembley <i>et al.</i> , 2002
<i>Dictyonema glabratum</i>	DGH3	118				Trembley <i>et al.</i> , 2002
<i>Flammulina velutipes</i>	FVH1	113				Ando <i>et al.</i> , 2001
<i>Flammulina velutipes</i>	FV-HYD1	121				Yamada <i>et al.</i> , 2005
<i>Lentinula edodes</i>	Le.hyd1	127				Ng <i>et al.</i> , 2000
<i>Lentinula edodes</i>	Le.hyd2	109				Ng <i>et al.</i> , 2000
<i>Paxillus involutus</i>	HYDA	141			9 cysteines	Wright <i>et al.</i> , 2005; AY857495
<i>Pholiota nameko</i>	PNH1	114				Tasaki <i>et al.</i> , 2004
<i>Pholiota nameko</i>	PNH2	110				Tasaki <i>et al.</i> , 2004
<i>Pholiota nameko</i>	PNH3	107				Tasaki <i>et al.</i> , 2004
<i>Pisolithus tinctorius</i>	HYDPT-1	140		(+) ^d		Tagu <i>et al.</i> , 1996, 2001

Table 2. Continued...

Organism	Hydrophobin	Number of aa Total ^a Mature protein ^b	M _r ^c (kDa)	Protein isolated	Remarks	Reference or EMBL accession number
<i>Pisolithus tinctorius</i>	HYDPT-2	117				Tagu <i>et al.</i> , 1996
<i>Pisolithus tinctorius</i>	HYDPT-3	108				Duplessis <i>et al.</i> , 2001
<i>Pleurotus ostreatus</i>	Fbh1	113	12	+		Peñas <i>et al.</i> , 1998
<i>Pleurotus ostreatus</i>	POH1	113	9	+		Asgeirsdóttir <i>et al.</i> , 1998
<i>Pleurotus ostreatus</i>	POH2	131	20	+	(Glycoprotein)	Asgeirsdóttir <i>et al.</i> , 1998
<i>Pleurotus ostreatus</i>	POH3	113	10	+		Asgeirsdóttir <i>et al.</i> , 1998
<i>Pleurotus ostreatus</i>	Vmh1	116	9	+		Peñas <i>et al.</i> , 2002; AJ420969
<i>Pleurotus ostreatus</i>	Vmh3	108	17	+	(Glycoprotein)	Peñas <i>et al.</i> , 2002; AJ420971
<i>Pleurotus ostreatus</i>	AF331452	110				AF331452
<i>Schizophyllum commune</i>	SC1	109	13.5			Dons <i>et al.</i> , 1984a; Schuren and Wessels, 1990
<i>Schizophyllum commune</i>	SC3	136	13.6– 14.4 ^{ms}	+	Glycoprotein	Schuren and Wessels, 1990; Wessels <i>et al.</i> , 1991a; de Vocht <i>et al.</i> , 1998
<i>Schizophyllum commune</i>	SC4	111	15	+		Schuren and Wessels, 1990; Wessels <i>et al.</i> , 1991a; de Vries <i>et al.</i> , 1993
<i>Schizophyllum commune</i>	SC6	185				Wessels <i>et al.</i> , 1995; AJ007504
<i>Tiricholoma terreum</i>	Hyd1	108	23	+		Mankel <i>et al.</i> , 2002; AY048578
CLASS II						
Ascomycetes						
<i>Cladosporium fulvum</i>	HCF-5	88				Segers <i>et al.</i> , 1999
<i>Cladosporium fulvum</i>	HCF-6	184				Nielsen <i>et al.</i> , 2001
<i>Claviceps fusiformis</i>	CFTH1	394	36,5 ^{ms}	+	Trihydrophobin	de Vries <i>et al.</i> , 1999

<i>Claviceps purpurea</i>	CPPHI	757	85	+	Penta- hydrophobin	Mey <i>et al.</i> , 2003
<i>Cryphonectria parasitica</i>	CRP, cryparin	118	18.6 ^{aaa}	+		Carpenter <i>et al.</i> , 1992; Zhang <i>et al.</i> , 1994
<i>Fusarium verticillioides</i>	Hyd4	100				Fuchs <i>et al.</i> , 2004; AY155499
<i>Fusarium verticillioides</i>	Hyd5	98				Fuchs <i>et al.</i> , 2004; AY158024
<i>Magnaporthe grisea</i>	MPH1, magnaporin	102				Kim <i>et al.</i> , 2001; Kim <i>et al.</i> , 2005
<i>Ophiostoma ulmi</i>	CU, cerato-ulmin	100	7.6 ^{ms}	+		Yaguchi <i>et al.</i> , 1993; Bowden <i>et al.</i> , 1994
<i>Trichoderma harzianum</i>	QID3	143			7 cysteines	Lora <i>et al.</i> , 1994; 1995
<i>Trichoderma harzianum</i>	SRHI	89				Munoz <i>et al.</i> , 1997
<i>Trichoderma reesei</i>	HFB1	97	7.5 ^{ms}	+		Nakari-Setälä <i>et al.</i> , 1996; Linder <i>et al.</i> , 2001
<i>Trichoderma reesei</i>	HFBII	86	7.2 ^{ms}	+		Nakari-Setälä <i>et al.</i> , 1997; Linder <i>et al.</i> , 2001
<i>Trichoderma reesei</i>	HFBIII	103	7.5	+	9 cysteines	Penttilä <i>et al.</i> , 2000; Rintala, 2001
<i>Trichoderma viride</i>	SRHI	89				DQ112069

^aincludes signal sequence for secretion

^bpredicted on the basis of N-terminal amino acid analysis of isolated protein

^cdetermined by SDS-PAGE unless otherwise indicated; aaa molecular mass calculated on the basis of amino acid analysis; ms molecular mass determined by mass spectrometry

^disolated as a recombinant protein

1.2.3 Protein properties of hydrophobins

Hydrophobins are small secreted proteins. The molecular masses of isolated hydrophobins vary from 7 to 85 Da (the pentahydrophobin CPPH1 of *Claviceps purpurea*) (Table 2). The deduced amino-acid sequence of CPPH1 has exceptional primary structure consisting of five class II hydrophobin domains (Mey *et al.*, 2003). Correspondingly, CFTH1 has three three class II hydrophobin domains connected by glycine- and asparagines-rich regions (de Vries *et al.*, 1999). Currently, most of the genes encoding hydrophobins are only known at the nucleotide level and the corresponding proteins have not been isolated and characterized.

The characteristic property of hydrophobins is adsorption to hydrophobic-hydrophilic interfaces, at which they form (or self-assemble into) amphiphilic films (Wessels, 1997; Wösten and Wessels, 1997; Wösten and de Vocht, 2000; Linder *et al.*, 2005). The interface can occur between solid and liquid, liquid and liquid or liquid and vapour. Thus, hydrophobins are very surface active. In early studies, hydrophobins were found to self-assemble into aggregates and form various types of self-assembled structures (Wessels, 1997). Aggregates formed by class II hydrophobins are more soluble than those of class I hydrophobins (Wessels, 1994, 1997), as already described in Section 1.2.1.

The ability to decrease the surface tension of water has been reported for class I hydrophobins ABH3, SC3 and SC4 and class II hydrophobins CFTH1, CRP and HFBII (Table 3), indicating that these proteins migrate into the water-air interface. With maximal reduction of the water surface tension from 72 to 24 mJ m⁻², SC3 is the most surface-active protein known (Wösten *et al.*, 1999; Wösten and de Vocht, 2000).

Hydrophobins are capable of stabilizing oil droplets in water. SC3 has been shown to form a film around oil droplets in water (Wösten *et al.*, 1994c; Wang *et al.*, 2005). In addition, HFBII has been reported to stabilize polyunsaturated fatty acid oil-in-water emulsions (Lumsdon *et al.*, 2005).

Hydrophobin films can make hydrophilic surfaces hydrophobic and *vice versa* (Wösten *et al.*, 1994c; Wösten and Wessels, 1997). The wettability of a solid surface can be estimated from the spreading of water droplets on the solid

surface by measuring their contact angles. A contact angle of 0° represents complete wetting. The hydrophilic (wetable) side of hydrophobin films has been reported to vary from moderate to highly hydrophilic (water contact angles between 22 and 64°) (Table 3). The hydrophobic sides of most hydrophobin films formed on a hydrophilic solid are highly water repellent (Table 3). SC3 has been shown to be glycosylated (de Vocht *et al.*, 1998), and the same has also been proposed for POH2 and Vmh3 of *Pleurotus ostreatus* (Ásgeirsdóttir *et al.*, 1998; Peñas *et al.*, 2002). Deglycosylation of SC3 revealed that glycosylation improved the ability to make Teflon hydrophilic (de Vocht *et al.*, 1998).

The hydrophobic side of class I hydrophobin films has a characteristic pattern of rodlets (Wessels, 1997; Wösten, 2001; Linder *et al.*, 2005). This mosaic of nanometer-sized parallel rods has been observed by electron microscopy (EM) and atomic force microscopy (AFM). Rodlet layers have been reported on aerial structures of fungi, and evidence for the role of hydrophobins in their formation has been obtained by studying hydrophobin deletion and disruption mutants (Wessels, 1997; Wösten, 2001; Linder *et al.*, 2005). For example, deletion of RodA of *Aspergillus nidulans* resulted in spores which lacked the rodlet layer and were less hydrophobic (Stringer *et al.*, 1991). However, other proteins, e.g. chaplins in the bacteria *streptomycetes*, also form rodlet-type-structures (Claessen *et al.*, 2004; Linder *et al.*, 2005). Purified class I hydrophobins have been shown to form rodlet patterned films *in vitro*. When a drop of aqueous class I hydrophobin solution has been allowed to dry down on solid support, rodlets have been observed (Wösten *et al.*, 1993, 1994a; Wessels, 1997; de Vocht *et al.*, 1998, 2000; Lugones *et al.*, 1996; 1998, Mackay *et al.*, 2001). The diameter of these hydrophobin rodlets has been around 10 nm. The length of rodlets is typically hundreds of nanometers, depending on the hydrophobin used and its concentration (Wösten *et al.*, 1994a; Scherrer *et al.*, 2000; Linder *et al.*, 2005). A rodlet layer has also been seen on air vesicles coated with SC3 hydrophobin (Wösten *et al.*, 1993). Interestingly, class I hydrophobin rodlets (de Vocht *et al.*, 2000; Wösten and de Vocht, 2000; Butko *et al.*, 2001; de Vocht, 2001; Mackay *et al.*, 2001; Wang *et al.*, 2005) and amyloid fibrils show similar spectroscopic changes with the dyes Thioflavine T (ThT) and Congo Red. No rodlets have been observed on films formed by the purified class II hydrophobins, such as CRP (Wösten and de Vocht, 2000) and CFTH1 (de Vries *et al.*, 1999).

The class II hydrophobin films have also been shown to be ordered structures. Grazing incidence X-ray diffraction (GIXD) of *T. reesei* HFBII assembled at a water-air interface revealed a highly ordered crystalline structure (Serimaa *et al.*, 2003). AFM of HFBI and HFBII surface films produced using the Langmuir-Blodgett technique showed ordered structures for both hydrophobins, although with some differences (Paananen *et al.*, 2003). Fibril- and rod-shaped microstructures have been reported to appear by shaking an aqueous solution of the class II hydrophobin CU (Takai, 1974; Russo *et al.*, 1982). They have been proposed to be air bubbles of unusual shape stabilized by CU. Similar structures have been reported for shaken aqueous solutions of CRP, HFBI and HFBII (Carpenter *et al.*, 1992; Torkkeli *et al.*, 2002). The needle-like HFBII aggregates have been shown to have a crystalline structure, which was suggested to be composed of packed HFBII tetramers (Torkkeli *et al.*, 2002).

The critical concentration above which the SC3 hydrophobin starts to aggregate has been determined to be 0.004 g l⁻¹ based on increase in fluorescence of the ThT dye in the presence of self-assembled SC3 (de Vocht, 2001). Size-exclusion chromatography of the SC3 solution has indicated that dimers are the dominating form at 1–3 g l⁻¹ (Wang *et al.*, 2004a). HFBI and HFBII have been suggested to exist as tetramers at relatively high concentration (10 g l⁻¹) and probably as dimers and monomers, respectively, at 0.5 g l⁻¹ on the basis of size exclusion chromatography and small angle X-ray scattering (Torkkeli *et al.*, 2002).

IR and CD spectroscopies have indicated that the SC3 hydrophobin is rich in β -sheet structure in solution (de Vocht *et al.*, 1998). Assembly at a water-air interface has been reported to increase the proportion of β -sheet structure (de Vocht *et al.*, 1998, 2002). Formation of α -helix was observed at the interface between water and a hydrophobic solid, which was interpreted as an intermediate state (de Vocht *et al.*, 1998, 2002). Similar observations have been made for the class I hydrophobins SC4 and ABH3 (Wösten and de Vocht, 2000). The stable β -sheet rich end form of SC3 on hydrophobic solid was obtained with diluted detergent at elevated temperature (de Vocht *et al.*, 2002). Formation of α -helix has also been observed in the secondary structure of SC3 at water-air interface before transition to β -sheet rich structure (de Vocht *et al.*, 2002).

Table 3. Properties of hydrophobins. Surface activity of hydrophobins in water and wettability of hydrophobin films as estimated by water contact angles.

Hydrophobin	Surface tension in water ^a (mJ m ⁻²)	Concentration (g l ⁻¹)	Water contact angles (°) of hydrophobin-coated materials Teflon ^b filter paper ^c	Reference
Class I (from Basidiomycetes)				
ABH1	nd		63 ± 8	Lugones <i>et al.</i> , 1996
ABH3	37	0.1	53 ± 1	Lugones <i>et al.</i> , 1998
SC3	24	0.05	48 ± 10	Wösten <i>et al.</i> , 1994c, 1999; Wösten and Wessels, 1997; Wösten and de Vocht, 2000
SC4	36	0.1	48 ± 3	Lugones <i>et al.</i> , 1999
Class II (from Ascomycetes)				
CFTH1	34	0.1	60 ± 5	de Vries <i>et al.</i> , 1999
CRP	32	nr	22 ± 2 ^d	Wösten and de Vocht, 2000
HFBII	45	0.008	64 ± 10	Lumsdon <i>et al.</i> , 2005

^aSurface tension of pure water is 72 mJ m⁻².

^bPolytetrafluoro ethylene (PTFE) Teflon was used unless otherwise indicated. The water contact angle of bare PTFE Teflon is 108 ± 2° (Wösten *et al.*, 1994c).

^cWater contact angle of bare filter paper is 0°.

^dtype of Teflon was not reported; nd, not determined; nr, not reported

1.3 The roles of hydrophobins in filamentous fungi

Hydrophobins have been found to fulfil a variety of functions in fungal growth and development (Table 4). They are involved in e.g. escape of fungal hyphae from aqueous environments, hydrophobicity of fungal structures, fungal attachment, maintenance of open gas channels in fruiting bodies and pathogenesis (Ebbole, 1997; Wessels, 1997; Wösten and Wessels, 1997; Kershaw and Talbot, 1998; Talbot, 1999; Wessels, 1999; Wösten *et al.*, 1999; Wessels, 2000; Tucker and Talbot, 2001; Wösten, 2001; Whiteford and Spanu, 2002; Kazmierczak *et al.*, 2005; Kim *et al.*, 2005). The expression of genes encoding hydrophobins can be very strong. For example, the gene encoding ABH1 (HYPA) produces as high as 60% of the total mRNA in the outer peel tissues of *A. bisporus* mushroom caps (de Groot *et al.*, 1996), whereas the CRP-encoding gene produces 25% of the total mRNA of *C. parasitica* during the rapid growth phase (Zhang *et al.*, 1994).

Table 4. Biological functions of hydrophobins.

Organism	Hydrophobin	Biological function	Mutant phenotype determined	Reference
Ascomycetes				
<i>Aspergillus fumigatus</i>	RodA / HYP1	Conidial wall protein. Affects spore hydrophobicity and conidial killing by macrophages.	+	Thau <i>et al.</i> , 1994; Paris <i>et al.</i> , 2003
<i>Aspergillus nidulans</i>	DewA	Conidial wall protein. Affects spore hydrophobicity.	+	Stringer and Timberlake, 1995
<i>Aspergillus nidulans</i>	RodA	Affects spore hydrophobicity.	+	Stringer <i>et al.</i> , 1991
<i>Cladosporium fulvum</i>	HCf-1	Conidial and hyphal wall protein.	+	Spanu, 1998
		(1) Spore dispersal.		Whiteford and Spanu, 2001
		(2) Affects hydrophobicity of spores and mycelium.		Whiteford <i>et al.</i> , 2004
<i>Cladosporium herbarum</i>	HCh-1	A rare human allergen.		Weichel <i>et al.</i> , 2003
<i>Fusarium verticillioides</i>	Hyd1	Microconidial chain formation.	+	Fuchs <i>et al.</i> , 2004
<i>Fusarium verticillioides</i>	Hyd2	Microconidial chain formation.	+	Fuchs <i>et al.</i> , 2004
<i>Magnaporthe grisea</i>	MPG1	Conidial wall protein.	+	Talbot <i>et al.</i> , 1993, 1996;
		(1) Involved in conidiation,		Beckermann and Ebbole, 1996
		(2) formation of infective structures, appressoria, and		
		(3) attachment of appressoria.		
		(4) Affects hydrophobicity of conidia.		
<i>Neurospora crassa</i>	EAS	Conidial wall protein. Affects spore hydrophobicity.	+	Bell-Pedersen <i>et al.</i> , 1992;
				Lauter <i>et al.</i> , 1992
Basidiomycetes				
<i>Agaricus bisporus</i>	ABH1 / HYP A	Unknown. Probably maintenance of gas channels in fruiting bodies. Localized lining of these structures.		Lugones <i>et al.</i> , 1999

Organism	Hydrophobin	Biological function	Mutant phenotype determined	Reference
<i>Pisolithus tinctorius</i>	HYDPt-1	Unknown. Possibly formation of ectomycorrhiza. Gene expressed during its formation. Localized on hyphal surface in contact with plant roots.		Tagu <i>et al.</i> , 1996, 2001; Martin <i>et al.</i> , 1999
<i>Schizophyllum commune</i>	SC3	Hyphal wall protein. (1) Aids formation of aerial hyphae (2) Involved in attachment to hydrophobic surfaces. (3) Affects hyphal wall composition and (4) hyphal hydrophobicity.	+	Wösten <i>et al.</i> , 1994c; van Wetter <i>et al.</i> , 1996; 2000b; Wösten <i>et al.</i> , 1999
<i>Schizophyllum commune</i>	SC4	Lines gas channels in fruiting bodies. Prevents the channels from filling with water.	+	Lugones <i>et al.</i> , 1999; van Wetter <i>et al.</i> , 2000a
CLASS II				
Ascomycetes				
<i>Cryphonectria parasitica</i>	CRP	Fruiting body and hyphal wall protein. (1) Eruption of fruiting bodies through the bark of the host tree. (2) Affects hydrophobicity of hyphae.	+	Carpenter <i>et al.</i> , 1992; McCabe and van Alfen, 1999; Kazmierczak <i>et al.</i> , 2005
<i>Magnaporthe grisea</i>	MPH1	(1) Development and viability of conidia. (2) Affects formation of infective structures, appressoria.	+	Kim <i>et al.</i> , 2005
<i>Ophiostoma ulmi</i>	CU	Cell wall protein. Localized on aerial hyphae and sporulating structures. (1) Proposed toxin in plant pathogenesis. (2) Aids formation of aerial hyphae. (3) Affects hydrophobicity of hyphae.	+	Takai and Hiratsuka, 1980, 1984; Bowden <i>et al.</i> , 1996; Svircev <i>et al.</i> , 1988

1.3.1 Formation of aerial hyphae

Fungi that grow submerged in a wet environment must penetrate the water-air interface in order to grow into the air. Then, the so-called aerial hyphae may further differentiate to spore-forming structures, which release spores to the air.

The SC3 hydrophobin has been shown to aid fungal aerial growth. Disruption of the *SC3* gene in *S. commune* (yielding the Δ SC3 strain) (Wösten *et al.*, 1994c; van Wetter *et al.*, 1996) resulted in the emergence of only a few aerial hyphae (van Wetter *et al.*, 1996; Wösten *et al.*, 1999). Moreover, surface tension of the Δ SC3 medium did not decrease as much as that of the wild type medium (from 72 to 30 mJ m⁻² after four days of growth) (Wösten *et al.*, 1999). Addition of purified SC3 to the medium of the Δ SC3 strain decreased the surface tension to the normal level and restored formation of abundant aerial hyphae (Wösten *et al.*, 1999).

The proposed model for formation of aerial hyphae (Wösten *et al.*, 1994a; Wessels, 1997; Wösten *et al.*, 1999; Wösten, 2001) can be described in three stages (Figure 2). First, the hyphae grow in liquid culture medium (i.e. submerged) and secrete SC3 (Wösten *et al.*, 1994a). Secondly, the hydrophobin molecules self-assemble as an amphiphilic film at the medium-air interface, accompanied by a drop in surface tension. This enables hyphae to escape from the liquid phase and grow into the air. Thirdly, SC3 secreted by the emerging aerial hyphae self-assembles on the hyphal surface as a hydrophobic rodlet-pattern layer (Figure 2; Wösten *et al.*, 1993, 1994a; van Wetter *et al.*, 2000a). This facilitates the aerial growth by preventing aerial hyphae from wetting.

Similarly to SC3, the class II hydrophobin CU has been shown to affect the formation of aerial hyphae. Disruption of the gene encoding CU in *O. novo-ulmi* resulted in only a few aerial hyphae that were easily wettable (Bowden *et al.*, 1996). Moreover, aerial hyphae formation and CU production have been observed to correlate in different *Ophiostoma* strains (Brasier *et al.*, 1995; Temple *et al.*, 1997). The ability of the class I hydrophobin ABH3 of *A. bisporus* to restore formation of aerial hyphae of the Δ SC3 strain *in vitro* suggests a similar role for ABH3 (Lugones *et al.*, 1998; Wösten *et al.*, 1999).

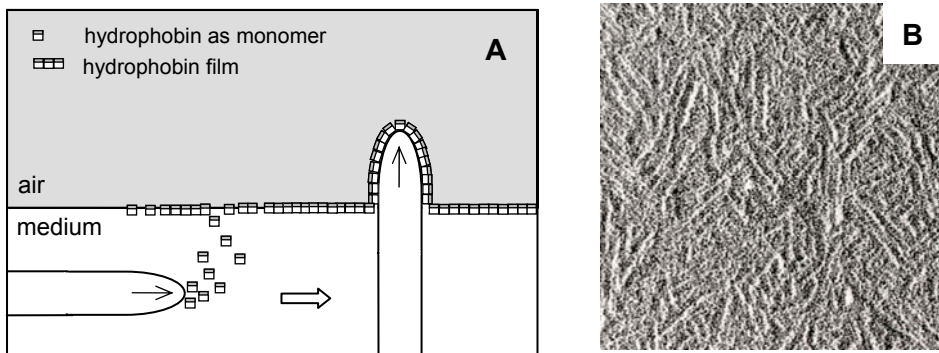


Figure 2. (A) A schematic model for formation of aerial hyphae in *S. commune* presented essentially as described in the literature (Wösten et al., 1994a; Wessels, 1997; Wösten et al., 1999; Wösten, 2001). Hydrophobic and hydrophilic parts of a hydrophobin molecule are indicated as grey and white colours, respectively. The SC3 hydrophobin molecules secreted into the liquid culture medium form a hydrophobin film at the water-medium interface which decrease the surface tension of the liquid, allowing the hyphae to grow into the air. The rodlet-patterned hydrophobin film on the cell wall renders the aerial hyphae hydrophobic. (B) An electron micrograph of the SC3 hydrophobin film. Hydrophobin solution (0.01 g l⁻¹ of SC3) was dried on an electron microscope grid. The diameter of rodlets is approximately 10 nm. Image produced by H.A.B. Wösten and S. Askolin.

1.3.2 Coating of fungal structures

Hydrophobins have been localized and isolated from the surfaces of different fungal structures (Table 4). These include e.g. aerial hyphae, asexual spores, called conidia, and fruiting bodies, which are produced above the soil level. A connection between class I hydrophobins and rodlet layers on fungal surfaces has been demonstrated (Wessels, 1997; Wösten *et al.*, 1997; Wösten, 2001; Linder *et al.*, 2005). For example inactivation of genes encoding the class I hydrophobins RodA of *Aspergillus fumigatus* (Thau *et al.*, 1994; Paris *et al.*, 2003), MPG1 of *M. grisea* (Talbot *et al.*, 1993, 1996) and EAS of *N. crassa* (Bell-Pedersen *et al.*, 1992) has resulted in conidia, which lacked the rodlet layer.

Hydrophobin coating confers hydrophobicity to fungal air-exposed surfaces (Table 4). Deletion or disruption of genes encoding hydrophobins has made the fungal structures (aerial hyphae, aerial conidia and gas channels of fruiting bodies) wettable, resulting in so-called wettable phenotype (Stringer *et al.*, 1991; Stringer and Timberlake, 1995; Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992; Lugones *et al.*, 1996; Talbot *et al.*, 1996; Thau *et al.*, 1994; van Wetter *et al.*, 2000a; Table 4). Thus, the hydrophobin film has a role in preventing aerial fungal structures from soaking up water for example during rain.

The asexual fungal spores, conidia, formed by aerial hyphae of Ascomycetes are generally very hydrophobic, which appears to facilitate their aerial dispersion (Adams, 1995; Wessels, 1997). Several hydrophobins, such as RodA (HYP1) of *A. fumigatus*, EAS of *N. crassa* and HCF-1 of *Cladosporium fulvum*, have been found to be involved in the hydrophobicity of conidia (Bell-Pedersen *et al.*, 1992; Thau *et al.*, 1994; Whiteford and Spanu, 2001; Table 4). Deletion of the genes encoding RodA and EAS has been shown to impair dispersion of conidia through the air (Bell-Pedersen *et al.*, 1992; Thau *et al.*, 1994). Moreover, deletion of the gene encoding HCF-1 has decreased the ability of conidia to be collected by water droplets (Whiteford and Spanu, 2001). Based on these findings, the hydrophobin coating on conidia facilitates conidial spreading.

A putative role for the hydrophobin film on fungal structures is involvement in resistance to different unfavourable environmental conditions. Production of the CU hydrophobin has been shown to correlate with the resistance of dried yeast-like cells of *Ophiostoma* strains, including e.g. the CU-overproducing and CU deletion strain, to storage (Temple *et al.*, 1997). This has suggested that CU may have a role in protecting these fungal cells from desiccation. Hydrophobin coating may also mediate the attachment of spores to hydrophobic surfaces such as insect cuticles, thus aiding their dissemination (Wessels, 1999).

Binding of hydrophobins on fungal cell walls has been proposed to occur when the fungus is confronted with a hydrophilic-hydrophobic interface (Wessels, 1996; Wösten *et al.*, 1994c). This interface can occur between the hydrophilic cell wall and e.g. air or a hydrophobic solid. This is consistent with an immunolocalization study indicating that the SC3 hydrophobin was absent from cell walls of *S. commune* hyphae grown without free contact with air in so-called sandwich culture (Wösten *et al.*, 1994a). However, the class II hydrophobin CRP

has been shown by labelling experiments to bind to the cell walls of *C. parasitica* in liquid phase in the absence of hydrophilic-hydrophobic interface (McCabe and van Alfen, 1999). This binding has been presumed to be due to the lectin-like properties of CRP (Carpenter *et al.*, 1992; McCabe and van Alfen, 1999). Lectins are carbohydrate-binding proteins having one or more sites highly specific to diverse sugar structures (Peumans and van Damme, 1995; Vijayan and Chandra, 1999). Lectin-like activity has also been shown for SC3 and SC4 hydrophobins (van Wetter *et al.*, 2000a).

1.3.3 Cell wall maturation

The main structural components of the cell wall of filamentous Ascomycetes and Basidiomycetes are (1-3)- β -glucan and chitin, which are secreted by the growing hyphae (Wessels, 1992, 1994; Sietsma *et al.*, 1995). Rigidifying of the cell wall occurs gradually as a result of formation of (1-3)/(1-6)- β -glucan-chitin complex (Wessels, 1986; 1994; Sietsma *et al.*, 1995). The SC3 hydrophobin of *S. commune* has been shown to affect the cell wall composition of fungal hyphae (van Wetter *et al.*, 2000b).

The presence of SC3 on surfaces of *S. commune* aerial hyphae has been demonstrated by immunodetection (Wösten *et al.*, 1994a). Disruption of the gene encoding SC3 decreased the amount of the glucan-chitin complex compared with the wild-type (van Wetter *et al.*, 2000b). In addition, the amount of free glucan containing both (1-3)- and (1-6)-linkages (called slime or mucilage) increased (van Wetter *et al.*, 2000b). Thus, SC3 appears to have a role in maturation of the hyphal cell wall.

Implication of a similar role was recently obtained for the CRP hydrophobin of *C. parasitica* (Kazmierczak *et al.*, 2005). Deletion of the gene encoding CRP affected growth of the fungal fruiting bodies through a rigid surface, the bark of a host tree (Kazmierczak *et al.*, 2005). Correspondingly to SC3, CRP may influence maturation and rigidity of the cell wall, which allows eruption of the fruiting bodies through the bark.

1.3.4 Pathogenesis and attachment

Hydrophobins have been linked to diseases caused by pathogenic fungi in several studies. The mechanisms include for example involvement in attachment of fungal infective structures to the host for infection (Talbot *et al.*, 1996; Tucker and Talbot, 2001).

Plant diseases caused by fungal pathogens, such as *M. grisea*, start with adhesion of the fungal spores to the host surface and their germination (Tucker and Talbot, 2001). The germ tube forms a specialised infective structure, the appressorium, which develops hyphae that penetrate and infect the plant cell. Deletion of the gene encoding the class I hydrophobin MPG1 in *M. grisea* caused absence of rodlet layer from conidia, impaired ability to form conidia and appressoria and reduced pathogenicity toward the host plant rice (Talbot *et al.*, 1993, 1996). Disruption of the gene encoding the class II hydrophobin MHP1 of *M. grisea* caused similar phenotypes (Kim *et al.*, 2005). In addition, appressoria and germ tubes of the *MPG1* mutant did not attach to a hydrophobic surface as strongly as those of the wild type strain (Talbot *et al.*, 1996). Thus, MPG1 was proposed to play a role in attachment of germ tubes and appressoria to the host. Whether also MHP1 affects attachment of the infective structure, the appressorium, to the host, like MPG1 (Talbot *et al.*, 1996), has not yet been reported.

A role in attachment has also been suggested for a hydrophobin of the plant-rotting bracket fungus *S. commune*. Growing hyphae of *S. commune* were shown to attach tightly to hydrophobic surface of Teflon (Wösten *et al.*, 1994c). The SC3 hydrophobin secreted by *S. commune* was immunodetected between the hyphae and the Teflon surface (Wösten *et al.*, 1994c). Deletion of the SC3 hydrophobin of *S. commune* was revealed to decrease attachment of the hyphae to Teflon (Wösten *et al.*, 1994c; van Wetter *et al.*, 2000a). These results indicated that SC3 may function in adhesion of *S. commune* hyphae to hydrophobic surfaces such as lignin, the hydrophobic component of wood (Wösten *et al.*, 1994c; van Wetter *et al.*, 2000a).

The role of the CU hydrophobin as a plant toxin has been widely studied. The plant pathogenic fungus *O. ulmi* and its aggressive isolate *O. novo-ulmi* cause a damaging tree disease, Dutch elm disease. Both species produce the CU protein, which was proposed to be the toxin causing the Dutch elm disease already in

1974 (Takai, 1974) and which proved to be a hydrophobin later on. The toxicity of CU was based on the observation that purified CU injected to white elm was able to produce symptoms, such as wilting, similar to those of Dutch elm disease (Takai, 1974; Takai and Hiratsuka, 1984). The symptoms were proposed to be due to blocking of intercellular openings, xylem vessels, by CU-coated air bubbles (Russo *et al.*, 1982). However, deletion of the *cu* gene in *O. novo-ulmi* or overexpression of *cu* in *O. ulmi* did not affect the pathogenicities of the two fungi to elm (Bowden *et al.*, 1996; Temple *et al.*, 1997). On the other hand, expression of *cu* in *O. quericus* made this fungus pathogenic to elm, causing the typical Dutch elm disease symptoms (Del Sorbo *et al.*, 2000). One explanation is that CU is only one of several factors or one of several hydrophobins causing the Dutch elm disease (Wessels, 1994; Bowden *et al.* 1996). Overexpression and deletion of *cu* have demonstrated that CU production correlates with hydrophobicity and adherence of the fungal yeast-like cells to insects, bark beetles (Temple *et al.*, 1997). CU may therefore assist spreading of the fungus from tree to tree (Temple *et al.*, 1997).

A protective role in pathogenesis has been proposed for the hydrophobin coating on the walls of conidia (Paris *et al.*, 2003). *A. fumigatus* is a human pathogen able to cause several respiratory diseases (Bodey and Vartivarian, 1989). Mutant conidia of *A. fumigatus*, in which the gene encoding the RodA hydrophobin has been disrupted, were shown to be sensitive to killing by a specific type of leucocytes (white blood cells), lung alveolar macrophages (Paris *et al.*, 2003). This suggests that RodA affects the resistance of conidia to the host cells (Paris *et al.*, 2003).

1.3.5 Symbiosis

Symbiosis is a close association of two organisms which is beneficial for both partners e.g. for nutrient supply. Hydrophobins have been suggested to be involved in symbiosis in two cases: (1) in allowing gas exchange in lichens, symbiotic associations of fungi and algae or cyanobacteria (Scherrer *et al.*, 2000; Trembley *et al.*, 2002) and (2) in the formation of ectomycorrhiza, a complex structure of fungus and tree roots (Tagu *et al.*, 1996, 2001; Duplessis *et al.*, 2001; Mankel *et al.*, 2002). Supporting data is based on gene expression, protein isolation and localization (Martin *et al.*, 1995, 1999; Tagu *et al.*, 1996; Tagu and Martin, 1996; Scherrer *et al.*, 2000; Duplessis *et al.*, 2001; Mankel *et al.*, 2002; Trembley *et al.*, 2002).

Lichen-forming fungi benefit from the symbiosis by a source of fixed carbon from algae. The fungi are assumed to protect the algae from desiccation. Genes encoding hydrophobins have been cloned, and corresponding proteins have been isolated and localized from both Ascomycete (*Xanthoria* spp.) (Scherrer *et al.*, 2000) and Basidiomycete (*Dictyonema glabratum*) lichen-forming fungi (Trembley *et al.*, 2002). In the gas-filled spaces of *X. parietina* and *D. glabratum* lichens, rodlet layers have been observed (Scherrer *et al.*, 2000; Trembley *et al.*, 2002). A similar rodlet layer has been obtained *in vitro* by drying down the hydrophobins, purified XPH1 of *X. parietina* and partially purified DGH1 of *D. glabratum*, on a solid surface (Scherrer *et al.*, 2000; Trembley *et al.*, 2002). The hydrophobin layer has been suggested to prevent the gas-spaces from filling with water. A similar role has been shown for SC4 hydrophobin lining gas channels in fruiting bodies of *S. commune* (van Wetter *et al.*, 2000a).

By colonizing tree roots and forming ectomycorrhiza, the fungus obtains carbohydrates from the plant, whereas the uptake of water and minerals by the plant is suggested to be improved by the fungus. Genes encoding three hydrophobins of *Pisolithus tinctorius* (HYDPt-1, HYDPt-2 and HYDPt-3) have been found to be highly expressed during early stages of ectomycorrhizal formation when the fungus is colonising the roots of *Eucalyptus globulus* (Tagu *et al.*, 1996; Duplessis *et al.*, 2001). Correspondingly, *T. terreum* was found to express a gene encoding hydrophobin, Hyd-1, when growing in symbiosis with pine roots (*Pinus sylvestris*) (Mankel *et al.*, 2002). One of these hydrophobins, HYDPt-1, has been immunolocalized on cell walls of hyphae in contact with the root surface (Martin *et al.*, 1999; Tagu *et al.*, 2001). Thus, hydrophobins may play a role in the formation of ectomycorrhiza.

1.4 *Trichoderma reesei* hydrophobins

T. reesei is a cellulolytic filamentous fungus, which degrades plant materials in its natural environment, soil. It is widely studied and also an industrially important fungus due to efficient secretion of cellulose- and hemicellulose-degrading enzymes. Three genes encoding class II hydrophobins have been found from *T. reesei* (Nakari *et al.*, 1993; Nakari-Setälä *et al.*, 1996, 1997; Penttilä *et al.*, 2000; Rintala, 2001). The recently published genome of *T. reesei* also reveals other genes encoding hydrophobins (<http://gsphere.lanl.gov/trire1/trire1.home.html>). Annotation

of the genome has not yet been finished and therefore the precise number of the new genes encoding hydrophobins is not known.

The first *T. reesei* gene encoding a hydrophobin, *hfb1*, was found when genes highly expressed on glucose-containing medium were investigated (Nakari *et al.*, 1993; Nakari-Setälä *et al.*, 1996). Heterologous hybridization using *hfb1* as a probe revealed the HFBII hydrophobin encoding gene, *hfb2* (Nakari-Setälä *et al.*, 1997). The HFBIII hydrophobin was first isolated as a protein on lactose-containing medium (M. Tenkanen, unpublished results). The expression of *hfb1* diverges from that of *hfb2* and *hfb3* on different carbon sources. In addition to glucose, *hfb1* is expressed on sorbitol-containing medium, but not on lactose, xylan, cellobiose or cellulose (Nakari-Setälä *et al.*, 1997). Genes encoding HFBII and HFBIII are expressed on cellulose- and lactose-containing media, the carbon sources which also induce e.g. the cellulose- and hemicellulose-hydrolyzing enzymes of *T. reesei*. In addition, *hfb2* and *hfb3* are expressed on sorbitol-containing medium, whereas their expression on glucose is relatively low if detected at all (Nakari-Setälä *et al.*, 1997; Rintala, 2001). Expression of *hfb2* is also induced in xylan- and cellobiose-containing cultures, by carbon and nitrogen depletion and by light and sporulation (Nakari-Setälä *et al.*, 1997). The *hfb3* gene is also expressed in sporulating aerial cultures (Rintala, 2001).

HFB I and HFB II have been isolated from the cell walls of submerged hyphae and aerial spores, respectively, as well as from the culture medium (Nakari-Setälä *et al.*, 1996, 1997). The amino acid similarity between HFB I and HFB II is 69% (Nakari-Setälä *et al.*, 1997). HFB III has been found to be mainly associated with vegetative submerged hyphae (M. Linder and T. Nakari-Setälä, unpublished results).

1.5 Production of hydrophobins

Hydrophobins have hitherto usually been produced and purified in laboratory scale for analysis of their protein properties. However, larger quantities would be required for many potential applications. Production levels of hydrophobins secreted to the culture medium by wild-type fungi are usually rather low. In general, production of hydrophobins has not been widely studied, but some approaches to increase hydrophobin production have been reported.

Production of the class II hydrophobin CU to the liquid culture medium by *O. novo-ulmi* was increased from 0.025 to 0.14 g l⁻¹ by optimization of the medium composition (Takai, 1978; Takai and Richards, 1978). Secretion of CU by *O. ulmi*, in which the native CU production is low, was improved close to the level of *O. novo-ulmi* by introduction of an additional gene encoding CU to the genome, driven by the strong *cu* promoter of *O. novo-ulmi* (Temple *et al.*, 1997).

Several attempts have been made to increase the production level of SC3 including both homologous and heterologous expression systems in fungi (Schuurs *et al.*, 1997; Scholtmeijer, 2000; Scholtmeijer *et al.*, 2001). The most promising results have been obtained from heterologous expression in *T. reesei*, which is capable of secreting large amounts of proteins into the culture medium (Scholtmeijer, 2000; Scholtmeijer *et al.*, 2001). Introduction of the gene encoding SC3 under regulation of the *hfb1* or *hfb2* promoter yielded similar or even higher production of SC3 to the culture medium than secreted by *S. commune* (approximately 0.06 g l⁻¹) (Wösten *et al.*, 1999; Scholtmeijer, 2000). Heterologous expression of the gene encoding the ABH1 hydrophobin of *A. bisporus* in *A. niger* resulted in only low secretion of the protein into the culture medium (Ásgeirsdóttir *et al.*, 1999).

Heterologous hydrophobin production in bacteria, such as for example recombinant fusion proteins of CU (Bolyard and Sticklen, 1992) and *P. ostreatus* POH1 (Peñas *et al.*, 1998) in *E. coli*, has been low (microgram quantities per liter culture). However, more reasonable yields have been obtained by production of recombinant histidine-tagged proteins HCh-1 of *C. herbarum* and RodA (HYP1) of *A. fumigatus* in *E. coli* (9 and 2 milligrams purified protein per liter culture) (Weichel *et al.*, 2003).

1.6 Purification of hydrophobins

The hydrophobins which have hitherto been isolated are presented in Table 2. Purification of hydrophobins has mostly been carried out in laboratory scale for analytical purposes. The amounts of hydrophobins purified have only rarely been reported, probably due to quantification problems.

Purification of class I hydrophobins has usually been based on the low solubility of their aggregates in diluted ethanol or hot diluted SDS. For purification of the SC3 hydrophobin from the cell walls of *S. commune*, impurities were removed using hot 2% SDS followed by release of SC3 with TFA or formic acid (Wessels *et al.*, 1991a, b; de Vries *et al.*, 1993; Wösten *et al.*, 1993). Soluble SC3 has been isolated from culture medium by precipitation, using e.g. trichloroacetic acid or mixing the medium with air, dissolution in TFA and removal of the acid by evaporation (Wösten *et al.*, 1993). Several other class I hydrophobins have been purified essentially according to these methods (e.g. de Groot *et al.*, 1996; Lugones *et al.*, 1996, 1998; Talbot *et al.*, 1996; Ásgeirsdóttir *et al.*, 1997, 1998; Spanu *et al.*, 1997; Peñas *et al.*, 1998; Scherrer *et al.*, 2000; van Wetter *et al.*, 2000a; Trembley *et al.*, 2002; Paris *et al.*, 2003). Chromatographic methods have been used for purification of class I hydrophobins such as EAS of *N. crassa* (Templeton *et al.*, 1995), RodB of *A. fumigatus* (Paris *et al.*, 2003), RoIA of *A. oryzaea* (Takahashi *et al.*, 2005) and SC3 of *S. commune* (Martin *et al.*, 2000; Wang *et al.*, 2002).

Class II hydrophobins, such as CFTH1 of *Claviceps fusiformis*, CRP of *C. parasitica* and HFBI and HFBII of *T. reesei*, have been released from fungal cell walls using diluted solution of SDS or ethanol (Carpenter *et al.*, 1992; Nakari-Setälä *et al.*, 1996, 1997; de Vries *et al.*, 1999; Linder *et al.*, 2001). Other purification methods, including high performance liquid chromatography (HPLC), have been used to further purify these proteins (Carpenter *et al.*, 1992; Nakari-Setälä *et al.*, 1996). For isolation of the soluble class II hydrophobins *O. ulmi* CU and HFBII from culture medium, a weak vacuum was applied or air was led to the medium and the hydrophobin was collected by generating foam (Takai and Richards, 1978; Nakari-Setälä *et al.*, 1997). HFBI and HFBII have also been purified from liquid culture medium in aqueous two-phase systems (Linder *et al.*, 2001).

1.7 Application potential of hydrophobins

The ability of hydrophobins to spontaneously self-assemble at hydrophobic-hydrophilic interfaces gives rise to several potential applications. These include for example immobilising of cells and molecules to surfaces, modification of wettability and biocompatibility of surfaces, use as a fusion partner facilitating

selective protein purification and acting as emulgators (Wessels, 1997; Scholtmeijer *et al.*, 2001; Linder *et al.*, 2005).

Hydrophobins are suitable molecules for immobilization and surface modification, which could be exploited in e.g. biosensors or even in future nanotechnology. Electrodes have been modified by immobilizing redox enzymes on the SC3 hydrophobin-coated electrode surface (Corvis *et al.*, 2005). Small electroactive compounds have been immobilized on electrodes coated with HYDPt-1 of *P. tinctorius* (Bilewicz *et al.*, 2001). HFBI has been used to immobilize *T. reesei* endoglucanase EGI effectively to silanized glass and Teflon as a fusion protein (Linder *et al.*, 2002). Lipases have been immobilized on hydrophilic agarose using mycelium-specific hydrophobins from *P. ostreatus* as an absorbent (Palomo *et al.*, 2003). Binding of *Saccharomyces cerevisiae* cells to hydrophobic silicone-based materials has been increased by targeting HFBI fused with Flo1p flocculin to the cell wall (Nakari-Setälä *et al.*, 2002).

The ability of hydrophobins to modify surface properties makes them attractive candidates to increase the biocompatibility of e.g. medical implants and surgical instruments. For example, binding of human fibroblasts, which give rise to connective tissue, could be encouraged with a hydrophobin film. Coating Teflon with SC4 or genetically engineered SC3 has been shown to improve the growth of fibroblasts on the surface (Janssen *et al.*, 2002, 2004; Scholtmeijer *et al.*, 2002, 2004).

HFBI has been shown to be useful as a purification tag due to its high affinity to nonionic surfactant phases in aqueous two-phase systems (Linder *et al.*, 2001). Proteins, such as endoglucanase EGI, fused with HFBI were separated from aqueous solutions in aqueous two-phase systems even in pilot scale (Penttilä *et al.*, 2000; Collén *et al.*, 2001, 2002; Linder *et al.*, 2004; Selber *et al.*, 2004).

Hydrophobins could be used as an alternative to surfactants in many applications such as emulsifiers or foaming agents in different branches of industry. For example HFBI has been shown to stabilize aqueous dispersion of Kevlar® nanopulp (Lumsdon *et al.*, 2005). Applications in the food industry may be found at least for hydrophobins produced by edible mushrooms. Hydrophobins have also been found to be useful as indicators of beer gushing. Hydrophobins produced by barley-contaminating fungi have been shown to cause gushing

problems in beer, leading to the development of a specific immunoassay (Kleemola *et al.*, 2001; Linder *et al.*, 2005).

1.8 Background and aims of the present study

Hydrophobins are proteins with many remarkable properties, which could be exploited in several applications. These include for example high surface activity and ability to modify surface properties. In the beginning of the present study, the genes encoding the *T. reesei* hydrophobins HFBI and HFBII had already been isolated and their expression studied, but very little was known about the corresponding proteins. The aim of this study was to shed light on the protein properties of these hydrophobins and on their significance for the fungus.

Specific aims of this work were

1. production and purification of HFBI and HFBII
2. crystallization of these hydrophobins for structure determination
3. characterization and comparison of the properties of HFBI and HFBII with those of the SC3 hydrophobin of *S. commune*
4. functional analysis of the genes encoding HFBI and HFBII by gene deletion
5. investigation of the process technological effects of HFBI and HFBII on fermentation of *T. reesei*.

2. Materials and methods

Only a summary of the materials and methods used in this study is presented in this section. More detailed information is given in the publications I–VI.

2.1 Strains, vectors and culture conditions

The *T. reesei* strains used in this study are presented in Table 5. The fungal strains were grown either in liquid media in bioreactors, shake flasks or as static cultivations, or on solid media. Only the Rut-C30-based strains were grown on lactose medium due to poor growth of QM9414 on this carbon source. Culture media and conditions are described in detail in the publications I, II, IV–VI.

E. coli strain DH5 α , used as a bacterial cloning host, was grown in LB (Luria Bertani) medium (Sambrook *et al.*, 1989) containing 100 μg ampicillin ml^{-1} . Plasmids used as cloning vectors are listed in Table 6.

Table 5. Fungal strains used in this study.

<i>Trichoderma reesei</i> strain		Reference
QM9414	(VTT D-74075)	Mandels <i>et al.</i> , 1975
QM9414 $\Delta hfb1$	(VTT D-99724)	V
QM9414 $\Delta hfb2$	(VTT D-99726)	V
QM9414 $\Delta hfb1\Delta hfb2$	(VTT D-99725)	V
QM9414 overproducing HFBI	(VTT D-98692)	I
QM9414 $\Delta hfb1$ producing SC3	(Q139A)	V
QM9414 $\Delta hfb2$ producing SC3	(R26A)	V
Rut-C30	(VTT D-86271)	Montenecourt and Eveleigh, 1979
Rut-C30 $\Delta hfb1$	(B8A)	V
Rut-C30 $\Delta hfb2$	(VTT D-99676)	V
Rut-C30 overproducing HFBII	(VTT D-99745)	VI

Table 6. Plasmid vectors used in this study.

Plasmid	Description	Reference
pEA10	Contains the genomic copy of the <i>hfb1</i> gene	Nakari-Setälä <i>et al.</i> , 1996
pTNS8	Contains the genomic copy of the <i>hfb2</i> gene	Nakari-Setälä <i>et al.</i> , 1997
pTNS24	Contains the deletion construct of the <i>hfb1</i> gene, composed of the acetamide resistance cassette of p3SR2 in between the 2.8 kb and 2.0 kb 5' and 3' flanking sequences of <i>hfb1</i>	V
pTNS27	Contains the deletion construct of the <i>hfb2</i> gene, composed of the hygromycin resistance cassette of pAro21 in between the 1.2 kb and 1.8 kb 5' and 3' flanking sequences of <i>hfb2</i>	V
pS3TR1.1	Contains a DNA construct for expression of <i>SC3</i> from <i>S. commune</i> in <i>T. reesei</i> , composed of the <i>SC3</i> cDNA under the control of the <i>hfb1</i> promoter and terminator sequences	Scholtmeijer, 2000
pS3TR1.2	Contains a DNA construct for expression of <i>SC3</i> from <i>S. commune</i> in <i>T. reesei</i> , composed of the <i>SC3</i> cDNA under the control of the <i>hfb2</i> promoter and <i>hfb1</i> terminator sequences	Scholtmeijer, 2000
pTNS31	Contains a DNA construct for overexpression of the <i>hfb2</i> gene in <i>T. reesei</i> , composed of <i>hfb2</i> under the control of the <i>cbh1</i> promoter and terminator sequences	VI

2.2 DNA techniques

Standard methods were used in DNA modification and transformation of *E. coli* competent cells (Sambrook *et al.*, 1989). *T. reesei* protoplasts were prepared and transformed as described by Penttilä *et al.* (1987). Co-transformation with a selection plasmid pARO21 (Aro *et al.*, 2001) or p3SR2 (Hynes *et al.*, 1983; Tilburn *et al.*, 1983) was performed if the DNA constructs did not contain an appropriate selection marker. Transformants were purified and screened as described in I, V and VI. For DNA analysis, genomic fungal DNA was isolated

using Easy-DNA™ Kit (Invitrogen, The Netherlands). Southern hybridizations were performed according to standard methods (Sambrook *et al.*, 1989) using DNA fragments labelled with [α -³²P]dCTP or digoxigenin (DIG High Prime, Boehringer Mannheim, Germany) as probes.

2.3 Purification of hydrophobins

HFBI was purified from the cell walls of the *T. reesei* strain QM9414 (VTT D-74075) and the HFBI-overproducing strain (VTT D-98692) (I). Two different purification methods were used. The first included extraction of the mycelia with 1% SDS at pH 9.0, removal of dodecyl sulphate as water-insoluble potassium dodecyl sulphate by adding potassium chloride, hydrophobic interaction (Phenyl Sepharose 6 FF, GE Healthcare) and anion exchange (Resource Q, GE Healthcare) chromatography (I). The second purification method for HFBI consisted of extraction of the mycelium with 1% SDS at pH 5, removal of dodecyl sulphate, ammonium sulphate precipitation and gel filtration (Bio-Gel P-6DG, Bio-Rad, USA) (II).

HFBI was purified from culture medium by bubbling, dissolution of the foam in 1% acetic acid (V) or 20% acetonitrile containing 0.1% trifluoroacetic acid (IV), and gel filtration (Econo-Pac 10DG, Bio-Rad, USA). SC3 was purified as described in the literature (Wösten *et al.*, 1993; Wessels, 1997).

2.4 Crystallization of HFBI and HFBI

Hydrophobins were crystallized by the vapour diffusion method in hanging drops (initial volume of 4.5–10 μ l). Initial screening was carried out using a crystallization screening kit (HR2-110, Hampton Research) at 15°C. HFBI was also crystallized by microbatch methods in a volume of 40 μ l. Each batch was seeded with a few HFBI crystals.

2.5 Quantification of HFBI and HFBI

HFBI and HFBI contents were analysed by analytic reverse phase HPLC. Proteins were eluted with an acetonitrile gradient containing 0.1% TFA and pure hydrophobins were used as standards. HFBI was assayed using Resource RPC column (GE Healthcare). The amount of cell-bound HFBI was estimated by extracting HFBI from the mycelium three times with 1% SDS at pH 9 or 6 and analysing the extracts by HPLC (I). HFBI was quantified by a Protein C4 column (Vydac, USA) (VI). Prior to the analysis, HFBI was purified from the culture supernatant using 2% of the non-ionic surfactant Berol 532 (Linder *et al.*, 2001) to remove the closely eluting molecules (VI). HFBI was also quantified on the basis of determination of amino acid content by amino acid analysis.

2.6 Biochemical characterization of hydrophobins

Hydrophobins and their purity were analysed on the basis of molecular mass by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 20% polyacrylamide gels using a Phast System (GE Healthcare) (I) or on 17.5% polyacrylamide gels (IV). Proteins were stained with silver stain (Silver stain kit, Bio-Rad, USA) or Coomassie Brilliant Blue. Possible glycosylation of HFBI was determined on SDS-polyacrylamide gels stained with glycoprotein staining (Zacharius and Zell, 1969). HFBI and SC3 were detected qualitatively by Western analysis, slot blotting and dot blotting using HFBI-specific polyclonal antibodies (Nakari-Setälä *et al.*, 1996) or antiserum raised against SC3 (Wösten *et al.*, 1994a) and further purified by incubating in 1–2 w/v-% acetone powder (Harlow and Lane, 1988) of the *T. reesei* QM9414 strain (IV). Isoelectric focusing and chromatofocusing were performed as described in I.

Stability of HFBI at different pH values was studied using intact HFBI incubated at pH 3, 5, 6, 7 and 9 (I). Protease digestibility of HFBI was determined as described in I. HPLC (Protein C4 column, Vydac, USA) was used for analysing homogeneity and purity of hydrophobin samples (I). The N-terminal amino acids of purified HFBI were sequenced by automated Edman degradation using an Applied Biosystems liquid phase sequencer 477A/120A at the University of Kuopio. Molecular masses of purified HFBI samples were determined with

matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Helin *et al.*, 1999).

2.7 Biophysical characterization of hydrophobins

Surface tension of aqueous hydrophobin solutions was measured by axisymmetric drop-shape analysis by profile (ADSA-P) as described by Noordmans and Busscher (1991). CD spectroscopy was performed using an Aviv 62A DS CD spectrometer to study changes in the secondary structure of hydrophobins (IV).

Assembly of hydrophobins at the water-air interface was studied by rotating hydrophobin solutions for 1–24 h. Samples were examined by light or fluorescent microscopy or analysed by SDS-PAGE after centrifugation. SC3 was labelled with dansyl according to Wang *et al.* (2002). To study emulsifying properties olive or paraffin oil (1 v/v-%) was emulsified in aqueous hydrophobin solution by sonication. Binding of hydrophobins on a hydrophobic solid was assessed by incubating cleaned Teflon sheets (fluorinated ethylene propylene, FEP, Norton Fluoroplast B.V, the Netherlands) in hydrophobin solution overnight at room temperature. Teflon pieces were washed three times for 10 min with water using a rotary shaker with or without prior extraction with 2% SDS for 10 min at 100°C. For assembly at the paper-air interface, hydrophobin solution was allowed to rise up along vertical filter paper strips essentially as described by Lugones *et al.* (1996). Wettability of non-modified or hydrophobin-coated materials, Teflon or filter paper, was determined as the contact angle of water droplets. The methods are described in detail in IV.

2.8 Microscopy

A light microscope and Analysis software imaging tool (Soft Imaging System, Germany) were used for determination of the diameter of fungal hyphae. Prior to microscopy, the culture samples were fixed in 1% formaldehyde and stained with lactophenol blue as described in V. Light microscopy was also used for examining hydrophobin crystals, hydrophobins mixed with air and oil emulsions stabilized by hydrophobins. Fungal colonies were examined using a stereo microscope.

For transmission electron microscopy of fungal hypha, culture samples were fixed, dehydrated, embedded in LX112 Epon, sectioned and stained with uranyl acetate and lead citrate as described in IV and examined in a JEM 1200 EX or a JEM 1200 EX II (Jeol, Japan) electron microscope. Transmission electron microscopy of assembled hydrophobin was performed by drying aqueous hydrophobin solutions on electron microscope grids, surface shadowing with platinum and carbon and examining the grids in a CM10 electron microscope (Philips, the Netherlands) (V).

2.9 Other analyses

Soluble protein concentrations were determined by the method of Lowry *et al.* (1951) after precipitation of proteins with an equal volume of 10% trichloroacetic acid. The precipitation step was omitted when the protein concentration of pure SC3 was determined.

Glucose and lactose concentrations were measured using GOD-Perid (Roche, Germany) and lactose/ β -D-galactose (Boehringer-Mannheim, Germany) analysis kits, respectively, according to the manufacturer's instructions. Overall cellulase activity (filter paper units, FPU), β -1,4-endoglucanase activity (IUPAC 1987) and endo- β -1,4-xylanase activity (Bailey *et al.*, 1992) were assayed using standard methods.

The amount of fungal biomass on liquid glucose- or lactose-containing media was measured gravimetrically (VI). Sporulation efficiency of fungal strains was determined by plating spores on potato dextrose agar (PDA, Difco, USA) plates and incubating the plates in light, followed by collecting and calculating the spores using a haemocytometer (IV).

3. Results and discussion

3.1 Production and purification of HFBI and HFBII (I, II, IV, VI)

High production of hydrophobins is required for many potential applications. In order to increase production of HFBI, two additional copies of the gene encoding HFBI were introduced into the genome of *T. reesei* QM9414 (I). The HFBI-overproducing strain was cultivated in a bioreactor on glucose-based medium, which has previously been shown to induce production of HFBI (Nakari-Setälä *et al.*, 1996). For comparison, the parent strain QM9414 was also cultivated under similar conditions. Most of the HFBI (80-100%) was bound to the fungal cell walls in these submerged cultures of both strains (I, IV). Overproduction of HFBI in *T. reesei* increased the HFBI production almost three times up to 1.4 grams HFBI per liter culture (Table 7), which is the highest production level of hydrophobin hitherto reported.

HFBII was overproduced in *T. reesei* by introducing three extra copies of the gene encoding HFBII into the genome of the efficient cellulase-producing strain Rut-C30 (VI). The additional genes were under the regulation of the strong promoter of the gene encoding cellobiohydrolase I (CBHI). Lactose is known to induce production of both HFBII and cellulose-hydrolyzing enzymes, such as CBHI of *T. reesei* (Nakari-Setälä *et al.*, 1997). Thus, the HFBII-overproducing strain was cultivated on lactose-containing medium in a bioreactor. High HFBII production was obtained, up to 0.24 grams of HFBII per liter culture, compared with 0.03 g l⁻¹ of the parent strain Rut-C30 (Table 7).

Production of other hydrophobins in as high amounts as HFBI (I, VI) and HFBII (VI) has not been published. *S. commune* has been reported to secrete 0.06 g l⁻¹ SC3 and less than 0.01 g l⁻¹ SC4 into the culture medium (Wösten *et al.*, 1999; van Wetter *et al.*, 2000a). Secretion of CU by *O. novo-ulmi* has been estimated to be 0.14 g l⁻¹ (Takai, 1978). The ability to secrete high amounts of proteins makes *T. reesei* a potential host for heterologous production of hydrophobins. Promising results have already been obtained for production of SC3 in *T. reesei* (Scholtmeijer, 2000).

Table 7. Production of HFBI and HFBII by different *T. reesei* strains.

<i>T. reesei</i> strain		Carbon source	HFBI conc. ^a (g l ⁻¹)	HFBII conc. ^b (g l ⁻¹)	Reference
QM9414 overproducing HFBI	VTT D-98692	glucose	1.4	nd	VI
QM9414	VTT D-74075	glucose	0.5	nd	VI
Rut-C30 overproducing HFBII	VTT D-99745	lactose	nd ^c	0.24	VI
Rut-C30	VTT D-86271	lactose	nd ^c	0.03	VI

^a includes both hydrophobin secreted into culture medium and bound to the cell walls of submerged hyphae (cell-bound HFBI was quantified by HPLC based on three sequential extractions of mycelium with 1% SDS in buffer)

^bhydrophobin secreted into the culture medium

^cthe gene encoding HFBI is not expressed in submerged growing cultures on lactose-containing medium (Nakari-Setälä *et al.*, 1997); nd, not determined

In addition to applications, purified proteins are needed for example in protein crystallization and determination of protein properties. For purification of HFBI and HFBII, fungal hyphae from submerged cultures and cell-free culture medium were chosen as starting materials, respectively (I, II, IV). HFBI was purified from the mycelium by extracting proteins with 1% SDS and removing SDS as water-insoluble potassium dodecyl sulphate by precipitation using potassium chloride (I, II). Purification was continued either with hydrophobic interaction chromatography and anion exchange chromatography (I) or ammonium sulphate precipitation (II). HFBII was isolated from the culture medium by bubbling (IV). Accumulation of hydrophobin by generation foam has also been used as a purification method for CU in the early days (Takai and Richards, 1978).

3.2 Structural investigation of HFBI and HFBII

3.2.1 Crystallization of HFBI and HFBII (II, unpublished results)

The 3D structure of a protein provides valuable information needed to understand its biological function. The purified HFBI and HFBII hydrophobins were crystallized for the purposes of structural analysis carried out by x-ray crystallography. The hydrophobins were crystallized in hanging drops by vapour diffusion. Both the HFBI and HFBII crystals obtained were applicable to x-ray crystallography.

HFBI was crystallized as long 3D crystals with a hexagonal sectional area (Figure 1/II). The HFBI crystals were obtained with 30% polyethylene glycol (PEG) 4000, 0.1 M sodium acetate buffer, pH 4.3, containing 0.2 M ammonium acetate and CYMAL-5 detergent (initial concentration 2.4 mM). The X-ray diffraction data was collected at a resolution of 2.5 Å in the European Synchrotron radiation Facility (ESRF) in Grenoble. The HFBI crystals belong to the hexagonal crystal system and space group $P6_1$ (or $P6_5$) with dimensions of the repeating asymmetric unit, unit cell, $a = b = 45.9$ Å, $c = 307.2$ Å (II). Determination of the 3D structure of HFBI is under work.

HFBII was crystallized in several crystal forms using different crystallization reagents. Thus it appeared to crystallize more readily than HFBI. However, most of the crystals were needle-shaped and thus not suitable for x-ray analysis. The size of the needles varied with different reagents, reaching even several hundreds of micrometers in length (Figure 3A). The needle-shaped crystals were often found in the presence of ammonium salts (phosphate, sulphate and acetate) and divalent cations (Ca^{2+} , Mg^{2+} and Zn^{2+}). Small angle x-ray scattering (SAXS) and wide-angle x-ray scattering of needle-shaped crystals of HFBII obtained by shaking in the presence of divalent cations (Cu^{2+}) has revealed that they have a monoclinic structure, which changes to hexagonal when the material has been dried (Torkkeli *et al.*, 2002). Correspondingly, different crystal forms have also been observed by grazing incidence X-ray diffraction analysis of the HFBII film coating water drops (Serimaa *et al.*, 2003). The coating of HFBII on water drops was crystalline, having a monoclinic structure which changed to hexagonal when dried.

Cubic HFBII crystals were obtained for example with 30% PEG 4000, 0.1 M sodium citrate buffer, pH 5.6, containing 0.2 M ammonium acetate. The amount of gelatinous precipitate in the hanging drops was reduced by lowering the PEG concentration. Crystals of good quality were obtained with the crystallization reagent 16% PEG 4000, 0.1 M sodium acetate buffer, pH 5.5, containing 1.3 M ammonium acetate (Figure 3B). The hanging drop was incubated at 15°C for two weeks followed by incubation at 4°C for two months. The drop consisted of 2.5 μl of HFBII (4.8 g l⁻¹ in water) and 2 μl of the crystallization reagent. Incubation at room temperature prior to nucleation decreased the time needed for the crystallization process.

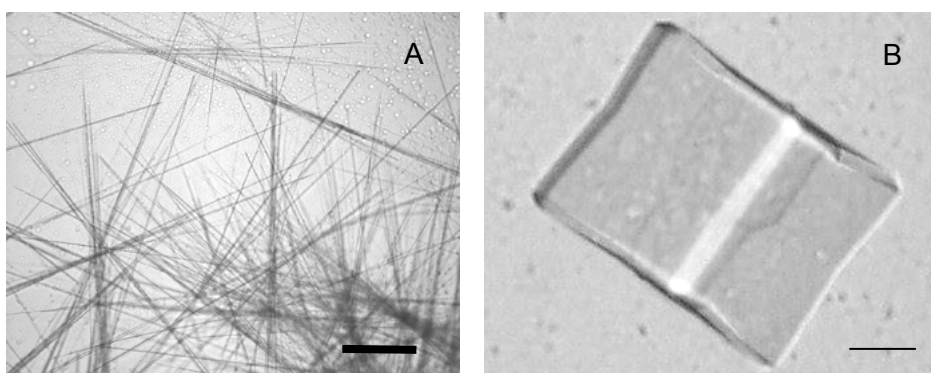


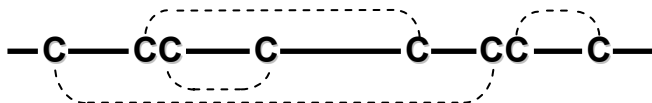
Figure 3. HFBII crystals. A. Crystals were obtained by vapour diffusion in a hanging drop at 15°C with 30% meta-phenylenediamide, 0.1 M sodium citrate buffer, pH 5.6, containing 0.2 M ammonium acetate. The drop consisted of 5 μl aqueous HFBII solution (4.8 g l⁻¹) and 4 μl of the crystallization reagent. B. The crystal was grown with optimized crystallization reagent 16% PEG 4000, 0.1 M sodium acetate buffer, pH 5.5, containing 1.3 M ammonium acetate. See text for details. Thick bar represents 200 μm (A) and thin bar 50 μm (B).

The HFBII crystals (Figure 3B) were analyzed at the University of Joensuu. However, the diffraction results obtained indicated that they had suffered from the transport. The crystallization of HFBII was therefore continued at the University of Joensuu by Hakanpää *et al.* (2004), where crystals rectangular in shape were grown. These HFBII crystals were monoclinic, belonging to the space group C2 with unit cell parameters $a = 78.7 \text{ \AA}$, $b = 46.3 \text{ \AA}$, $c = 34.6 \text{ \AA}$, $\alpha = \gamma = 90.0^\circ$, $\beta = 112.2^\circ$ and diffracted to the resolution of 1.0 \AA with a

synchrotron-radiation source (Hakanpää *et al.*, 2004). They were used for structure determination of HFBII (III).

3.2.2 Three dimensional crystal structure of HFBII (III)

The first 3D atomic-resolution structure of a hydrophobin, HFBII of *T. reesei*, was resolved by x-ray crystallography at the University of Joensuu (Figure 2/III). No high resolution structure of a hydrophobin has previously been reported, possibly due to problems caused by the high tendency of hydrophobins to aggregate. The 3D structure of the class II hydrophobin HFBII is globular and amphiphilic, having a flat hydrophobic area in an otherwise hydrophilic surface. The dimensions of the single domain protein are approximately $24 \times 27 \times 30$ Å. The structure contains four antiparallel β strands and one α helix. The hydrophobic part consists mainly of amino acid residues near the loops of two β hairpins. In the primary structure, the amino acids on the hydrophobic area are situated between the following cysteine residues: C3-C4 and C7-C8 (Figure 4). The structure of the hydrophobin is stabilized by four disulphide bridges, which occur between the cysteine residues as shown in Figure 4.



*Figure 4. Characteristic pattern of eight cysteine residues in hydrophobins. The arrangement of disulphide bonds found in the three dimensional structure of *T. reesei* HFBII is indicated with dashed lines. The flat hydrophobic area on the protein surface is composed of amino acids located between the 3rd and 4th as well as the 7th and 8th cysteine residues.*

In aqueous environment, hydrophobic regions of proteins tend to cluster together to form a water-free environment. This was also observed in the crystal structure of HFBII (Figure 6A/III). The asymmetric unit contained two HFBII molecules, a dimer. The hydrophobic areas of the two molecules were aligned towards each other so that approximately half of the hydrophobic part was still exposed to the aqueous environment. Oligomerization of hydrophobins in aqueous solutions,

such as HFBII and the class I hydrophobin SC3, has been studied by size-exclusion chromatography (Torkkeli *et al.*, 2002; Wang *et al.*, 2004a). The results have indicated that HFBII occurs as tetramers at high hydrophobin concentrations (Torkkeli *et al.*, 2002), whereas dimers were the dominating form of SC3 studied at 1-3 g l⁻¹ (Wang *et al.*, 2004a). Orientation of the hydrophobic areas towards each other in the HFBII dimer indicates that the molecules would prefer a situation in which the whole hydrophobic part would be buried in a water-free environment. This situation is obtained at the hydrophobic-hydrophilic interface, such as water-air.

In addition to the well-conserved spacings between cysteine residues (Figure 1; Wessels, 1994; Wösten and Wessels, 1997), the amino acids forming the hydrophobic surface of HFBII have been found to be highly conserved as hydrophobic amino acids among class II hydrophobins (Linder *et al.*, 2005). Moreover, the class II hydrophobins form a more uniform group with respect to variation in amino acid sequences than class I hydrophobins (Linder *et al.*, 2005). These facts indicate that the class II hydrophobins are structurally related. Therefore, the proposal of disulphide bridges in the class II hydrophobin CU is probably incorrect (Yaguchi *et al.*, 1993). Whether class I hydrophobins also have a similar structure has not yet been established. However, proportions of structural elements in the secondary structure of the class I hydrophobin SC3 (23% α helix, 41% β sheet, 16% β turn and 20% random coil) estimated using IR spectroscopy (de Vocht *et al.*, 1998) are surprisingly similar to those observed in the 3D structure of HFBII (20% α helix, 39% β sheet, 0% β turn and 41% random coil) (J. Rouvinen, personal communication). These observations urge on structural determination of SC3.

The structural information provides a basis for modifying these proteins and broadens the usefulness of the proteins in potential medical and technical applications or even in nanotechnology.

3.3 Comparison of the class II hydrophobins HFBI and HFBII with a class I hydrophobin

Class I and class II hydrophobins have only little sequence similarity apart from the eight cysteine residues (Linder *et al.*, 2005). Moreover, their solubility characteristics are different (Wessels, 1994). Here, the behaviours of the class II hydrophobins HFBI and HFBII of *T. reesei* and the class I hydrophobin SC3 of *S. commune* were compared with each other at various interfaces.

3.3.1 Behaviour at water-air interface (IV)

Amphiphilic molecules reduce the water surface tension by migrating to the water-air interface. The class II hydrophobins HFBI and HFBII were found to be surface active and capable of reducing the surface tension of water, similarly to the class I hydrophobin SC3 (Table 8; Figure 3A/IV). The lowest surface tensions measured for HFBI (37 mJ m^{-2}) and HFBII (28 mJ m^{-2}) (Table 8) are in the range that has been reported for other hydrophobins, including SC3, ($24\text{--}37 \text{ mJ m}^{-2}$) (Table 3). The ability of hydrophobins to reduce the surface tension of a liquid is comparable to other microbial surfactants (Table 1) and conventional surfactants (Jönsson *et al.*, 1998; Beneventi *et al.*, 2001), and the ability of SC3 is even lower. Since experimental conditions such as pH and ionic strength affect surface activities, determination of the optimal conditions for each hydrophobin would allow more detailed comparison.

Different times were needed to reach the minimum water surface tension with HFBI, HFBII and SC3 (Table 8; Figure 3B/IV). Contrary to the class I hydrophobin SC3, the class II hydrophobins HFBII and HFBI reached the minimal water surface tension almost instantly or within minutes, behaving like conventional surfactants. In addition, HFBII was needed in a lower amount than HFBI or SC3 to reach the minimum water surface tension (Table 8; Figure 3A/IV). On the basis of size-exclusion chromatography of aqueous hydrophobin solutions (Torkkeli *et al.*, 2002; Wang *et al.*, 2004a), HFBII appears to have a lower tendency to oligomerize than HFBI or SC3. This could affect the amount of hydrophobin needed. The mobility of commercial surfactants to interfaces has been shown to depend on the chain length of their hydrophobic part, increase in the chain length causing loss of mobility (Beneventi *et al.*, 2001). Thus, faster

decrease of water surface tension by HFBI and HFBII compared with SC3 might be due to faster mobility to the water-air interface caused by the smaller hydrophobic part, as discussed in Section 3.3.6.

Table 8. Physicochemical properties of HFBI, HFBII and SC3.

Hydrophobin	Surface tension in water ^a (mJ m ⁻²)	Conc. (g l ⁻¹)	Equilibrium time	Changes in CD spectra after mixing with air ^b	Clear ultrastructure on hydrophobin film observed ^c
Class II					
HFBI	37	0.2	several minutes	no	no
HFBII	28	0.02	less than one minute	no	no
Class I					
SC3	24	0.05	several hours	yes (de Vocht <i>et al.</i> , 1998)	yes (Wösten <i>et al.</i> , 1993)

^aSurface tension of pure water is 72 mJ m⁻²

^bAqueous hydrophobin solutions with and without mixing with air were analysed by CD spectroscopy.

^cA drop of aqueous hydrophobin solution was allowed to dry on an electron microscope grid, surface shadowed and examined by EM.

HFBI and HFBII are not glycosylated or otherwise post-translationally modified, as judged by molecular mass analysis and glycoprotein staining (I; Linder *et al.*, 2001). The surface activity of HFBI and HFBII is thus based solely on amino acid residues. By contrast, SC3 is a glycoprotein, carbohydrate residues of which have been assumed to be mainly located on the hydrophilic side of the protein (Wösten *et al.*, 1994b; de Vocht *et al.*, 1998). Other microbial surfactants generally have fatty acids in their hydrophobic part (Lin, 1996; Desai and Banat, 1997; Bognolo, 1999) and thus differ structurally from the hydrophobins.

In nature, several hydrophobins may be present in the culture medium simultaneously. Interestingly, higher surface activities have been reported for mixtures of anionic and cationic surfactants than for the individual components, probably due to strong interaction between the opposite charges (Li *et al.*, 1996). However, this did not apply to mixtures of the class I hydrophobin SC3 and the class II hydrophobins HFBI or HFBII. The surface activities of these hydrophobin mixtures were between those of the individual hydrophobins (Figure 3B/IV). Moreover, several hours were needed to reach the final equilibrium.

The secondary structures of water-soluble and assembled HFBI and HFBII were studied by CD spectroscopy. CD spectra of the water-soluble HFBI and HFBII were similar to those after assembly at the water-air interface induced by mixing with air (Table 8; Figure 4A, B/IV). In contrast, the CD spectra of aqueous solutions of class I hydrophobins SC3, SC4 and ABH1 have been shown to change after vigorous vortexing, having minima at 215-217 nm indicating β sheet formation (Figure 4C/IV; de Vocht *et al.*, 1998; Wösten and de Vocht, 2000). These observations indicate that the secondary structures of class II hydrophobins do not change when confronting the water-air interface, in contrast to class I hydrophobins. Absence of a conformational change could also explain why the class II hydrophobins HFBI and HFBII reduced the water surface tension faster than the class I hydrophobin SC3.

The HFBI and HFBII films did not have an apparent ultrastructure on the hydrophobic side on the basis of EM (Table 8; Figure 5A, B/IV). The hydrophobin films were prepared *in vitro* by drying down a drop of aqueous hydrophobin solution on solid support and therefore most probably represented the water-air interface of a drop. By contrast, the hydrophobic sides of many class I hydrophobin films, including that of SC3, have been shown to be composed of mosaic of a parallel rodlets (Figure 5C/III; Wösten *et al.*, 1993, 1994a; Wessels, 1997; de Vocht *et al.* 1998, 2000; Lugones *et al.*, 1996, 1998; Mackay *et al.*, 2001). Similar to the films of HFBI and HFBII, those of the class II hydrophobins CRP and CFH1 did not have rodlet structures (de Vries *et al.*, 1999; Wösten and de Vocht, 2000). However, grazing incidence X-ray diffraction analysis (Serimaa *et al.*, 2003) and AFM (III) revealed that the HFBII film at the water-air interface was crystalline and highly ordered. Lack of apparent ultrastructure on the hydrophobic side of the film may be a general feature of class II hydrophobins.

Light microscopy of aqueous HFBI and HFBII solutions mixed with air revealed needle-like structures, which appeared to have air trapped inside (Figure 1A, E/IV). These structures were suggested to be air bubbles stabilized by hydrophobin coating. They were readily dissociated by adding ethanol or applying pressure. Two other class II hydrophobins, CU and CRP, have been reported to behave similarly (Richards and Takai, 1973; Takai and Richards, 1978; Russo *et al.*, 1982; Carpenter *et al.*, 1992; Richards, 1993). By contrast, mixing an aqueous SC3 solution with air aggregated the protein (Figure 1D/IV; Wösten *et al.*, 1993). The higher tendency of the class I hydrophobin SC3 to aggregate may be due to the different size and shape of the hydrophobic part of the protein compared with class II hydrophobins, as discussed in section 3.3.6.

3.3.2 Binding to a solid surface (IV)

Secondary structures of the class II hydrophobins HFBI and HFBII in the presence of an interface between water and a hydrophobic solid were studied by CD spectroscopy. Adding colloidal Teflon to aqueous hydrophobin solutions changed the CD spectra of HFBI and HFBII (Figure 4A, B/IV). The intensity of the signal at 190 nm increased and the negative signal shifted to 205–207 nm and another weak negative signal close to 225 nm appeared, especially in the CD spectra of HFBII. This possibly indicates formation of α helix. Correspondingly, presence of colloidal Teflon has caused changes in the CD spectra of class I hydrophobins SC3, SC4 and ABH3 and class II hydrophobin CRP indicating formation of α helix (Figure 4C/IV; de Vocht *et al.*, 1998; Wösten and de Vocht, 2000).

Binding of HFBI, HFBII and SC3 to hydrophobic (Teflon) and hydrophilic solids (filter paper) was compared. Teflon pieces were incubated in aqueous hydrophobin solutions (0.1 g l^{-1}) overnight and washed with water. HFBI and SC3 rendered Teflon wettable as shown by the water contact angles (Table 9). By contrast, Teflon incubated in an HFBII solution and washed with water was still hydrophobic, with water contact angles similar to those of bare Teflon (Table 9). These results and the observations of Linder *et al.* (2002) indicate that adsorption of HFBII to hydrophobic solids is weaker than that of HFBI.

Table 9. Binding of hydrophobins to hydrophobic (Teflon) or hydrophilic (filter paper) solids.

Hydrophobin	Water contact angles (°) of hydrophobin-coated materials		Changes in CD spectra after adding
	Teflon ^a	Filter paper ^b	Teflon ^c
Class II			
HFBI	59 ± 13	60–70	yes
HFBII	109 ± 2	60–64	yes
Class I			
SC3	40 ± 4	100–130	yes (de Vocht <i>et al.</i> , 1998)

^aPieces of FEP Teflon were incubated in aqueous hydrophobin solution overnight and washed with water three times for 10 min. The water contact angle of bare FEB Teflon is 110 ± 2°.

^bThe water contact angle of bare filter paper is 0°.

^cSecondary structure of hydrophobin with or without addition of colloidal perfluoro alkoxy Teflon to aqueous hydrophobin solution was analyzed by CD spectroscopy.

Solubility of the hydrophobin coating on Teflon differs between class I and class II hydrophobins. The HFBI film responsible for the decrease of surface hydrophobicity was removed with hot diluted SDS. Similar results have been obtained with the class II hydrophobins CRP and CFTH1 (de Vries *et al.*, 1999; Wösten and de Vocht, 2000). The SC3 film on Teflon was more resistant to extraction with hot diluted SDS (water contact angles of 53 ± 7° after the treatment) than the class II hydrophobins, similar to the class I hydrophobins ABH1, ABH3 and SC4 (Lugones *et al.*, 1996, 1998, 1999).

The HFBI and HFBII coatings reduced the hydrophilicity of filter paper, whereas the SC3 coating made the surface highly hydrophobic (Table 9). Based on the capability of HFBI to bind both on hydrophobic and hydrophilic surfaces and being part of hyphal wall structure, HFBI might affect attachment of *T. reesei* to hydrophobic surfaces.

3.3.3 Emulsification of oils (IV)

HFBI and HFBII were found to stabilize oil emulsions, as has been observed for SC3 (Wösten *et al.*, 1994c). HFBI and SC3 appeared to stabilize both olive and paraffin oil-in-water emulsions more effectively than HFBII (Figure 5). Recently, HFBII has been reported to be able to stabilize polyunsaturated fatty acid oil-in-water emulsions (Lumsdon *et al.*, 2005).

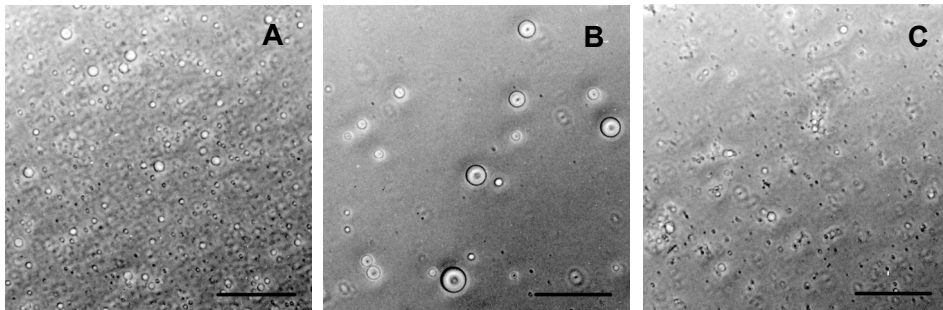


Figure 5. Paraffin oil-in-water (1 v/v-%) emulsions stabilized by the class II hydrophobins HFBI (A) and HFBII (B) and the class I hydrophobin SC3 (C). The bar represents 250 μm .

3.3.4 Stability (I, IV)

As secreted proteins, hydrophobins need to resist diverse unfavourable conditions outside the cell wall. CD spectroscopy of aqueous HFBI and HFBII solutions at high temperatures revealed that these proteins are extremely temperature stable (IV). The CD spectra of HFBI and HFBII were similar both at 25 and 90°C, except for a slight decrease in the intensity at 90°C. This showed that the secondary structures of both hydrophobins remained unchanged even at 90°C, the highest temperature studied. Similar results have been obtained for SC3 (de Vocht *et al.*, 2000). Moreover, no changes in the CD spectra of SC3 were found at pH values between 3 and 12 (de Vocht *et al.*, 2000). The stability of HFBI, HFBII and SC3 most probably derives from the compact structure stabilized by disulphide bridges. This is in agreement with the 3D structure of HFBII (III) and the observed unfolding of SC3 after treatments which reduce disulphide bridges (de Vocht *et al.*, 2000).

Purified HFBI treated at high pH values was found to be heterogeneous on the basis of HPLC chromatograms (I). In addition to intact HFBI, MALDI-TOF mass spectrometry and N-terminal amino acid sequencing revealed a form of HFBI in which the first two N-terminal asparagines (Asn-2 and Asn-4) were deamidated into aspartic acids (Figure 3A/I; Table 1/I). In addition, a similar deamidated form lacking the two first amino acids in the N terminus was found. Cleavage of the peptide bond at asparagine has been assumed to occur through succinimide, an intermediate of the deamidation reaction (Tyler-Cross and Schirch, 1991). Deamidation of proteins and peptides has been reported to depend on environmental conditions, such as pH and ionic strength, and protein structure (Kossiakoff, 1988; Tyler-Cross and Schirch, 1991; Wright, 1991). Indeed, HFBI was shown to remain intact at acidic pH (I). Moreover, the N-terminus of HFBI may locate on the outer surface of the protein, similar to HFBII (III), making the asparagines susceptible to deamidation. Furthermore, the N-terminal asparagines of HFBI are flanked by glycine and serine residues, which have been reported to considerably increase their deamidation rate (Tyler-Cross and Schirch, 1991). Interestingly, chemical modifications, including deamidation, have been used as methods to improve the surface activity and emulsifying properties of proteins (Garti, 1999). Heterogeneity and especially lack of one or more amino acids in the N terminus has also been found to occur in other hydrophobins (Wessels *et al.*, 1991b; Yaguchi *et al.*, 1993; Peñas *et al.*, 1998; de Vocht *et al.*, 1998; de Vries *et al.*, 1999; Mackay *et al.* 2001; Paris *et al.*, 2003).

3.3.5 Interaction between class I and class II hydrophobins (IV)

To study whether the class I and class II hydrophobins interact with each other, the behaviour of hydrophobins in mixtures was analyzed. Interestingly, the presence of a class II hydrophobin (either HFBI or HFBII) was found to affect the solubility of a class I hydrophobin (SC3). A significant fraction of SC3 mixed with air together with HFBI or HFBII in an aqueous solution remained in the supernatant after centrifugation without aggregating (Figure 2B/IV). Fluorescence microscopy of dansyl-labeled SC3 mixed with air in the presence of either HFBI or HFBII showed the fluorescently labelled protein in the needle-like structures (observed for the class II hydrophobins), in addition to aggregates (Figure 1/IV). Electron microscopy of SC3 dried down on surfaces together with

HFBI or HFBII showed regions of rodlet pattern structures, typical for SC3. They were surrounded by regions without apparent ultrastructure, characteristic for HFBI and HFBII (Figure 5/IV). This indicates that these class I and class II hydrophobins assemble independently, competing for the available interface. Moreover, both the class II hydrophobins HFBI and HFBII improved the solubility of the class I hydrophobin SC3.

Both class I and class II hydrophobins have been shown to be expressed at the same developmental stage in a single fungus, e.g. in *C. fulvum* and *M. grisea* during sporulation (Talbot *et al.*, 1993; Segers *et al.*, 1999; Nielsen *et al.*, 2001; Kim *et al.*, 2005). If both class I and class II hydrophobins were secreted simultaneously to liquid growth medium in nature, premature unwanted aggregation of class I hydrophobins might be avoided.

3.3.6 Hypothetical models explaining the main differences between class I and class II hydrophobins

Properties of the class II hydrophobins HFBI and HFBII of *T. reesei* and the class I hydrophobin SC3 of *S. commune* differ from each other in three principal ways. These are solubility of hydrophobin aggregates in e.g. hot diluted SDS (Wösten *et al.*, 1993; IV), ultrastructure of the hydrophobic side of the hydrophobin film (Wösten *et al.*, 1993; IV) and conformational changes after mixing with air (de Vocht *et al.*, 1998; IV). Two hypothetical models are presented to explain these differences.

The first model concerns two characteristics at water-air interfaces reported only for class I hydrophobins, including SC3 and SC4 of *S. commune* and ABH3 of *A. bisporus*. These are the rodlet-patterned ultrastructure on the hydrophobic side of the hydrophobin film (Wösten *et al.*, 1993, 1994a; Wessels, 1997; Lugones *et al.*, 1998) and the changes in the secondary structure after assembly at water-air interfaces observed by CD or IR spectroscopy (de Vocht *et al.*, 1998, 2002; Wösten and de Vocht, 2000). Neither of these properties has been observed for the class II hydrophobins HFBI and HFBII of *T. reesei* (IV). A schematic model presented in Figure 6 proposes that rodlet formation is caused by conformational changes of the hydrophobin molecules together with intermolecular interaction. The reason for the conformational change may be

stabilization of the hydrophobin film by strengthening of the interaction between individual molecules. This model is supported by the observations of de Vocht *et al.* (2002) indicating that changes in secondary structure of SC3 occur prior to rodlet formation.

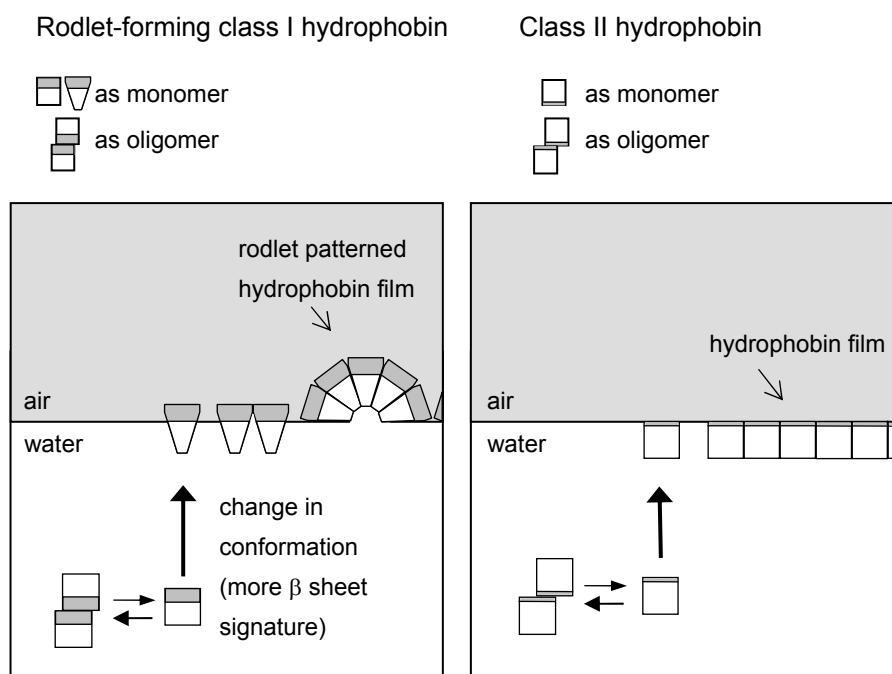


Figure 6. Proposed model of the formation of hydrophobin film at water-air interfaces. Hydrophobic and hydrophilic parts of a hydrophobin molecule are indicated as grey and white colours, respectively. As amphiphilic molecules, hydrophobins tend to hide their hydrophobic parts from the hydrophilic phase, such as water. Conformational change of class I hydrophobins, such as SC3, SC4 and ABH3, indicative of formation of β sheet structure, at the water-air interface is proposed to lead to formation of rodlet-patterned hydrophobin film. Neither conformational change nor rodlet patterns have been observed in the case of class II hydrophobins, such as HFBI and HFBII.

The second model concerns the solubility differences of class I and class II hydrophobins (Wessels, 1994). For example, aggregates of the class I hydrophobin SC3 bound to Teflon were shown to have lower solubility in diluted hot SDS compared to the class II hydrophobin HFBI of *T. reesei* (IV). Hot detergents, such as SDS, have been reported to cause conformational change in the class I hydrophobin SC3, resulting in even stronger binding (de Vocht et al., 2002). Due to the amphiphilic nature of hydrophobin molecules, hydrophobic interaction is here assumed to be the main factor affecting sticking of hydrophobins to each other or to hydrophobic solids. This model proposes that the higher tendency of class I hydrophobins to resist hot detergent when bound to hydrophobic solid is due to the larger hydrophobic area on the protein surface compared with class II hydrophobins, as presented in Figure 7.

This model is supported by the observation that the class I hydrophobin SC3 aggregated more readily by mixing with air than the class II hydrophobins HFBI and HFBII (IV). Moreover, on the basis of EM the class I and class II hydrophobins did not appear to co-assemble (IV), suggesting different sizes and shapes of the hydrophobic surface regions. In addition, the 3D structure of the class II hydrophobin HFBII, the first and hitherto only atomic resolution structure of a hydrophobin (III), supports this model. The hydrophobic part of this amphiphilic molecule is strikingly small (only 12% of the total surface area) (III). The hydrophobic region on the protein surface is composed of most of the hydrophobic amino acids between the 3rd and 4th as well as the 7th and 8th cysteine residues in the amino acid sequence (III). The average total length of amino acids between the two loops composing the hydrophobic area of HFBII is remarkably well conserved both among class I and class II hydrophobins (Table 10). Interestingly, in class I hydrophobins the average of the total length of these two loops is approximately twice that found in class II hydrophobins. The results of Wang *et al.* (2004b) also support this model indicating binding of the loop between the 3rd and 4th cysteine residues of the class I hydrophobin SC3 to hydrophobic solid. Moreover, on the basis of fluorescence resonance energy transfer SC3 molecules have been indicated to be closer to each other after applying hot detergent (Wang *et al.*, 2002).

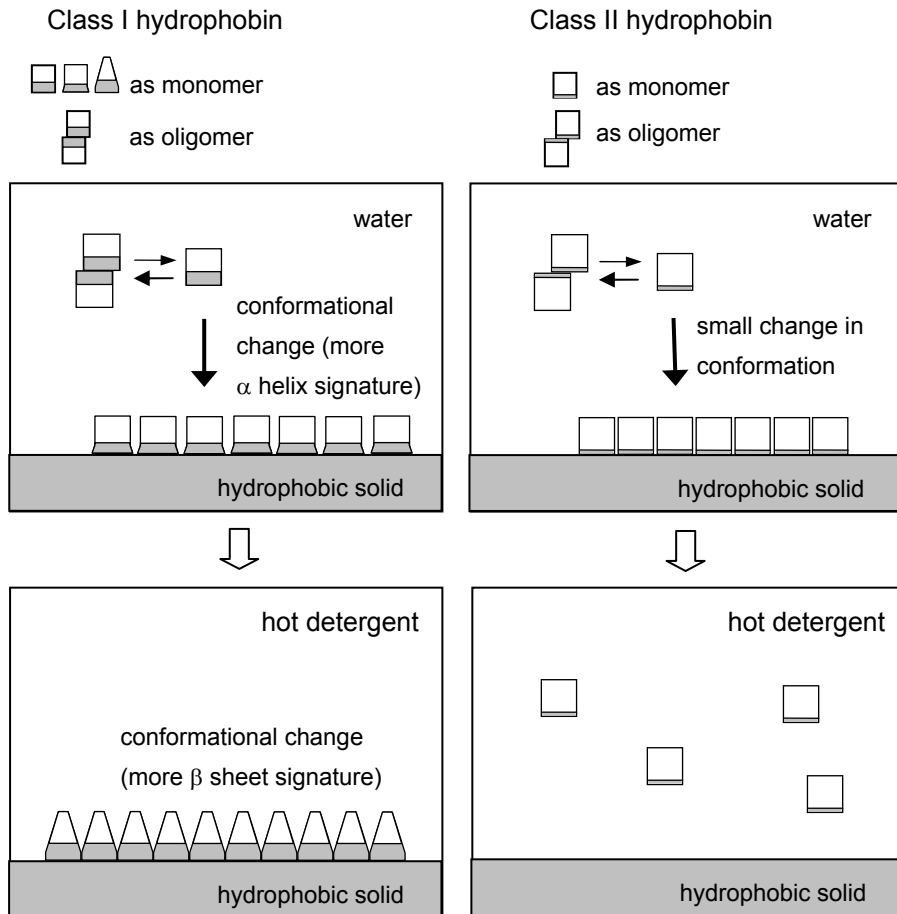


Figure 7. Proposed model of binding of class I and class II hydrophobins, such as SC3, HFBI and HFBI, to a hydrophobic solid in aqueous solution. Hydrophobins, as amphiphilic molecules, tend to bury their hydrophobic parts (indicated with grey colour) into water-free environment by oligomerization or by forming molecular layers. Upon binding to a hydrophobic solid, the hydrophobic part of a hydrophobin molecule faces the hydrophobic solid whereas the hydrophilic part (white) is towards the aqueous solution such as water or growth medium. When hydrophobins are in contact with a hydrophobic solid, such as Teflon, conformational change occurs (de Vocht et al., 1998; IV). Hot detergent (e.g. 2% SDS) solubilizes the class II hydrophobin coating from the hydrophobic solid (IV). By contrast, conformation of the class I hydrophobin changes, resulting in even stronger binding (de Vocht et al., 2002). The solubility difference between class I and class II hydrophobins could be explained by the different size and shape of the hydrophobic parts.

Table 10. Length of the amino acid sequence of hydrophobins between the 3rd and 4th as well as the 7th and 8th cysteine residues. The hydrophobic area on the surface of HFBII is composed of most of the hydrophobic amino acids in these two loops. The number of amino acids has been calculated for all hydrophobins listed in Table 2 apart from the exceptions QID3 (7 cysteine residues) and HYDA and HFBIII (9 cysteine residues).

Hydrophobin class	Number of amino acids		
	between C3 and C4	between C7 and C8	sum of the two loops
I	4-44	6-18	42.0 ± 8.4
II	11	10/11	21.1 ± 0.3

3.4 Biological roles of HFBI and HFBII (V, VI)

In order to determine the biological roles of HFBI and HFBII, *T. reesei* strains were constructed in which either the *hfb1* gene ($\Delta hfb1$) or *hfb2* ($\Delta hfb2$) or both the hydrophobin genes ($\Delta hfb1\Delta hfb2$) were deleted (Table 5). The deletion strains were studied on several carbon sources due to the divergent expression of *hfb1* and *hfb2* (Nakari-Setälä *et al.*, 1997). In addition, the ability of SC3 to substitute for HFBI and HFBII was investigated. Proposed biological roles of HFBI and HFBII are presented in Table 11.

Table 11. Biological functions of the HFBI and HFBII hydrophobins of *T. reesei*.

Hydrophobin	Biological function
HFBI	(1) Aids formation of aerial hyphae. (2) Affects hydrophobicity of hyphae.
HFBII	(1) Involved in sporulation and (2) hydrophobicity of spores and hyphae.

3.4.1 Effect of HFBI and HFBII on hydrophobicity of aerial fungal structures (V)

The QM9414 $\Delta hfb1$, $\Delta hfb2$, $\Delta hfb1\Delta hfb2$ and the parent strain were cultivated on glucose- (the *hfb1* gene expressed and expression of the *hfb2* gene low if detectable) and cellulose-containing solid media (the *hfb2* gene expressed (Nakari-Setälä *et al.*, 1997)). On glucose, the non-sporulating colonies of the $\Delta hfb1$, $\Delta hfb2$ and $\Delta hfb1\Delta hfb2$ were wettable whereas the parent strain remained dry. On cellulose-containing medium, on which the fungi were sporulating, the $\Delta hfb2$ and $\Delta hfb1\Delta hfb2$ colonies had an easily wettable phenotype in contrast to the parent strain and $\Delta hfb1$. These results showed that on glucose medium both HFBI and HFBII had a role in making fungal hyphae hydrophobic. In addition, HFBII affected the hydrophobicity of spores on solid cellulose medium. Thus, HFBI and HFBII appear to prevent these air-exposed fungal structures in soaking up water during wet weather.

Expression of *hfb2* on glucose-containing medium has previously been observed to be induced by depletion of nitrogen or glucose (Nakari-Setälä *et al.*, 1997). Thus, the effect of HFBII on water repellency of aerial structures may be more pronounced in the older parts of colonies. Similar to HFBI and HFBII, disruption of the *cu* and *SC3* hydrophobin genes has resulted in wettable phenotypes of aerial hyphae (Bowden *et al.*, 1996; van Wetter *et al.*, 1996). HFBII is the first class II hydrophobin shown to affect spore wall hydrophobicity. The class I hydrophobins involved in spore wall hydrophobicity include DewA (Stringer and Timberlake, 1995), HCF-1 (Whiteford and Spanu, 2001), RODA (Thau *et al.*, 1994), RodA (Stringer *et al.*, 1991), EAS (Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992) and MPG1 (Talbot *et al.*, 1996).

In addition to making fungal structures water repellent a hydrophobin layer may mediate fungal attachment to hydrophobic surfaces. Expression of HFBI on yeast cell surfaces has been shown to increase binding of the cells to hydrophobic materials (Nakari-Setälä *et al.*, 2002). *T. reesei* degrades plant material by producing cellulolytic enzymes. Addition of a surfactant has been found to increase the enzymatic hydrolysis of the cellulose component of lignocellulose into soluble sugars (Eriksson *et al.*, 2002). Surfactant decreased the adsorption of the enzyme (CBHI) to hydrophobic lignin, probably by binding

to the hydrophobic surface itself. In nature, the hydrophobins of *T. reesei* may have a similar function and thereby enhance the survival of the fungus.

3.4.2 Role of HFBI in the formation of aerial hyphae (V)

After the filamentous fungus has colonised the moist substrate it needs to escape from water and grow into the air. This is facilitated by a drop in water surface tension (Wösten *et al.*, 1999). Both HFBI and HFBII are able to decrease the surface tension, at least when added to pure water (Figure 3/IV). The role of HFBI and HFBII in the formation of aerial hyphae by *T. reesei* was studied in static liquid cultivation. On the *hfb1*-inducing carbon source, glucose, the *hfb1* deletion strains ($\Delta hfb1$ and $\Delta hfb1\Delta hfb2$) did not form any aerial hyphae on the liquid surface, in contrast to the parent strain QM9414 and $\Delta hfb2$. Addition of purified HFBI to the liquid culture medium of the *hfb1* deletion strains restored the formation of aerial hyphae (Figure 5/V). These results showed that HFBI aids formation of aerial hyphae of *T. reesei* on glucose medium. The capability of HFBI to render aerial hyphae hydrophobic may prevent the hyphae from sinking again in moist substrate.

Purified HFBII only partly restored aerial hyphae formation when added to the culture medium of the *hfb1* deletion strains (Figure 5/V). The reason for inability of HFBII to complement HFBI completely remains unclear. It is possible that different compounds in the medium affected the surface activity of HFBII. On lactose medium, which induces expression of the *hfb2* gene, $\Delta hfb1$, $\Delta hfb2$ and the parent strain Rut-C30 all produced aerial hyphae. On this carbon source, emergence of aerial hypha is probably aided by another hydrophobin present in the culture medium. Based on these observations, HFBII has only a minor role in the emergence of aerial hyphae.

The hydrophobins CU of *O. novo-ulmi* and SC3 of *S. commune* have also been shown to affect aerial growth of the corresponding fungi using gene disruption (Bowden *et al.*, 1996; van Wetter *et al.*, 1996; Wösten *et al.*, 1999). In addition to SC3, some other surface active molecules, such as the class I hydrophobins ABH3 and SC4, have been capable of restoring the formation of aerial hyphae when added to the culture medium of the $\Delta SC3$ strain (Wösten *et al.*, 1999).

3.4.3 Effect of HFBI on hyphal growth and morphology (V, VI)

In shaken liquid glucose-containing cultures, the QM9414 $\Delta hfb1$ strain formed less biomass than the parent and $\Delta hfb2$ strain, especially during exponential growth (Figure 3A/V). In addition, the hyphae of $\Delta hfb1$ were thinner, particularly after two days of growth (Figure 4/V). On the basis of electron microscope examination, only the overall diameter of the hyphae was affected, not the thickness of the cell wall (Figure 4B/V). In bioreactor cultivation on glucose medium, the hyphal diameters of the $hfb1$ deletion and HFBI-overproducing strains were similar to that of the parent strain (VI). The differences presumably occurred earlier during the 3-day cultivation of the inoculum. On $hfb1$ repressing carbon source, lactose, no distinct differences between the growth and hyphal diameter of Rut-C30 $\Delta hfb1$, $\Delta hfb2$ and the parent strain were observed. On solid glucose-containing medium, the 3-days old colonies of the QM9414 $hfb1$ deletion strains ($\Delta hfb1$ and $\Delta hfb1\Delta hfb2$) showed fluffy appearance in contrast to the parent and the $\Delta hfb2$ strain (Figure 1/V). This suggested that deletion of the $hfb1$ gene also affected hyphal growth on the solid medium. The amount of biomass was not determined but formation of aerial hyphae was found to be delayed. Based on these results, HFBI affects the appearance of hyphae both in liquid and on solid medium and biomass formation in liquid glucose-containing medium, possibly by influencing cell wall composition as has been shown for SC3 of *S. commune* (van Wetter *et al.*, 2000b).

The effects of deletion of the $hfb1$ gene on biomass formation and hyphal thickness in liquid cultures as well as on colony morphology on solid media were most obvious in the early growth phase of the fungus (after 2–3 days of cultivation), when the production of HFBI had already been initiated in the parent strain (Nakari *et al.*, 1993; Nakari-Setälä *et al.*, 1997; VI). The differences were less obvious, if present at all, after seven days of growth. It is possible that some other hydrophobin of *T. reesei* takes over the functions of HFBI in the late growth phase.

3.4.4 Effect of HFBI and HFBII on sporulation (V, VI)

Both HFBI and HFBII were found to affect sporulation of *T. reesei*. On potato dextrose agar medium, on which QM9414 sporulates extensively, sporulation of both $\Delta hfb1$ and $\Delta hfb2$ was approximately 50% compared with that of the parent strain (Figure 2/V). The effect was even more striking when both the hydrophobin genes were deleted. The influence of HFBI on sporulation is probably indirectly caused by reduced formation of aerial hyphae (see Section 3.4.2), whereas the effect of HFBII is proposed to be more direct.

Reduced sporulation has also been observed in *M. grisea* due to inactivation of either the *MPG1* or *MHP1* hydrophobin gene (Talbot *et al.*, 1993, 1996; Kim *et al.*, 2005). Similar to HFBII, *MPG1* also affects spore hydrophobicity (Talbot *et al.*, 1996). In contrast to the *MPG1* mutant, the viability of the *MHP1* mutant conidia was affected as shown by its decreased ability to germinate (Kim *et al.*, 2005). Whether viability of the spores of the *hfb1* and *hfb2* deletion strains was affected has not been studied. *SC3* also seems to indirectly influence sporulation of *S. commune*. Deletion of *SC3* in dikaryons decreased the number of fruiting bodies, the spore-producing structures, and delayed their formation (van Wetter *et al.*, 2000a).

After depletion of the carbon source, lactose, in liquid bioreactor cultivation of the Rut-C30 $\Delta hfb2$ strain, sporulation unexpectedly increased (VI). Starvation stress together with the absence of HFBII seemed to have an important role in changing the sporulation behaviour of the strain. However, the actual mechanism of how HFBII controls sporulation of these liquid cultures is unclear.

3.4.5 Complementation of a phenotype of $\Delta hfb1$ by *SC3* (V)

In order to investigate the ability of the class I hydrophobin *SC3* of *S. commune* to substitute for the class II hydrophobins HFBI and HFBII, $\Delta hfb1$ and $\Delta hfb2$ strains expressing *SC3* under the *hfb1* or *hfb2* regulatory sequences were constructed (Table 5). Expression of *SC3* in the *hfb1* and *hfb2* deletion strains did not restore biomass formation (Figure 3A/V) or hyphal thickness (Figure 4A/V) in submerged glucose-containing cultures, sporulation on solid medium (Figure 2/V), colony morphology or hydrophobicity of aerial hyphae and spores

in glucose- and cellulose containing cultures, respectively. However, the $\Delta hfb1$ strain expressing SC3 formed aerial hyphae (Figure 5/V), showing that SC3 can partially substitute for HFBI *in vivo*. Concistently with this, when purified SC3 was added to the culture medium of the $\Delta hfb1$ strain aerial hyphae was formed. In contrast, HFBI and HFBII were unable to substitute for SC3 when added to the liquid culture medium of *S. commune*, since they were proteolytically degraded (S. Askolin and H.A.B. Wösten, unpublished results).

Class I hydrophobins have been reported to partially complement for each other (Kershaw *et al.*, 1998; van Wetter *et al.*, 2000a). The ability of SC3 to complement the aerial hyphae-deficient phenotype of $\Delta hfb1$ showed for the first time that the class I and class II hydrophobins can partially replace each other.

3.5 Process technological effects of HFBI and HFBII in bioreactor cultivations of *T. reesei* (VI)

T. reesei is an important producer of industrial enzymes. Its high secretion capacity can also be exploited for heterologous protein production. However, foaming can cause problems during the production processes. The roles of HFBI and HFBII in foaming of the culture broth of *T. reesei* in bioreactors were studied. The *T. reesei* QM9414 and Rut-C30 strains in which the *hfb1* and *hfb2* genes were either deleted or amplified (Table 5) were cultivated on medium containing glucose (the *hfb1* gene expressed), lactose or cellulose (the *hfb2* gene and genes encoding cellulolytic enzymes expressed (Nakari-Setälä *et al.*, 1997)).

Both the HFBI and HFBII hydrophobins caused foaming of the culture broths of *T. reesei*. The strains overproducing HFBI and HFBII foamed extensively (Table 12). Due to severe problems caused by extremely intense foaming on cellulose-containing medium, the HFBII-overproducing strain was cultivated only on lactose. On this medium, consumption of antifoam agent in the cultivation of the parent strain Rut-C30 and the HFBII-overproducing strain were 0.3 and 24 ml l⁻¹, respectively. In agreement with this result, deletion of the *hfb2* gene decreased the antifoam consumption (Table 12). In cultivation of the HFBI-overproducing strain the amount of antifoam agent used was almost double that needed by the parent strain QM9414. The low level of foaming observed in cultivations of both $\Delta hfb1$ and QM9414 (2.7 and 2.3 ml antifoam consumed per liter; 70 and 73 ml

antifoam per milligram dry weight) was possibly due to other molecules such as for example another hydrophobin in the medium. Unlike HFBII, HFBI is mainly bound to the hyphal cell walls.

Deletion or amplification of the *hfb1* gene did not affect either the growth or the amount of proteins secreted into the glucose-containing culture medium of *T. reesei* QM9414 in bioreactor cultivations. Correspondingly, growth parameters of the $\Delta hfb2$, HFBII-overproducing strain and parent strain Rut-C30 resembled each other on lactose-containing culture medium. The biomass formation was not measured on the cellulose-containing medium due to interference of insoluble particles with the measurement. Production of cellulases on cellulose medium by the $\Delta hfb2$ strain was similar to that of Rut-C30, whereas on lactose it was slightly poorer (Table 2/VI). Difference might also be due to normal variation in cultures. Overproduction of HFBII under control of the strong *cbh1* promoter caused slight decrease in enzyme production on lactose medium compared with the parent strain Rut-C30, probably due to the presence of several copies of the *cbh1* promoter in the fungal genome. This factor has been shown to limit protein production probably due to insufficient transcription and/or regulation factors (Karhunen *et al.*, 1993; Margolles-Clark *et al.*, 1996).

Similarly to bioreactor cultivations, overfoaming is also a problem in brewing. Consistently with the results of bioreactor cultivations, when HFBII was added to a bottle of beer, the beer gushed and burst out of the bottle during opening (Linder *et al.*, 2005). Hydrophobin preparations of the fungal barley pathogens *Fusarium poae* and *Nigrospora* sp. also caused beer gushing, suggesting that the actual cause of gushing of bottled beer are hydrophobins produced by fungi contaminating barley and malt (Klemola *et al.*, 2001).

Table 12. Antifoam consumption in bioreactor cultivations of the *T. reesei* parent strains and the strains in which either the *hfb1* or *hfb2* gene was deleted or amplified.

<i>T. reesei</i> strain		Carbon source	Antifoam consumption (ml l ⁻¹)
QM9414	(VTT D-74075)	glucose	2.3
QM9414 $\Delta hfb1$	(VTT D-99724)	glucose	2.7
QM9414 overproducing HFB I	(VTT D-98692)	glucose	4.7
Rut-C30	(VTT D-86271)	cellulose	3.7
Rut-C30 $\Delta hfb2$	(VTT D-99676)	cellulose	0.4
Rut-C30 overproducing HFB II	(VTT D-99745)	cellulose	nd ^a
Rut-C30	(VTT D-86271)	lactose	0.3
Rut-C30 $\Delta hfb2$	(VTT D-99676)	lactose	0.0
Rut-C30 overproducing HFB II	(VTT D-99745)	lactose	24.0

^a not determined due to severe problems caused by extreme foaming

The impact of HFB I and HFB II on foaming can be explained by the high surface activity of the hydrophobins (see Section 3.3.1) (IV). In bioreactor cultivation, the effect was most pronounced in the case of HFB II, which is in agreement with its higher surface activity, at least when measured in water, and with the fact that HFB I is mainly cell bound. On the basis of these results, HFB II is the main cause of foaming in bioreactor cultivations of *T. reesei* used for production of cellulolytic enzymes. Thus, the problems caused by foaming during cultivation of *T. reesei* could be overcome by using strains in which the gene encoding the HFB II hydrophobin has been deleted.

4. Conclusions

Hydrophobins are surface active proteins produced by filamentous fungi. The properties of hydrophobins, including strong adhesion, high surface activity and ability to modify materials, give rise to several application possibilities. In this work, biophysical properties of individual hydrophobins were described. In addition, structural information was obtained by crystallizing hydrophobins and their biological roles were investigated. Increased production of hydrophobins was obtained by construction of new production strains. Moreover, hydrophobin was shown to be the main cause of foaming in bioreactor cultivation of the industrially important enzyme producer *T. reesei*.

Biophysical properties of the HFBI and HFBII hydrophobins of *T. reesei* were rather similar (Section 3.3; Table 13). Both hydrophobins were surface active, able to stabilize oil-in-water emulsions and bound to a hydrophilic material, filter paper. Differences were observed in their ability to decrease water surface tension, stabilize oil emulsions and bind to a hydrophobic material, Teflon. CD spectra of both aqueous HFBI and HFBII solutions changed when confronting Teflon, indicating possible formation of α helix.

Comparison of the class II hydrophobins HFBI and HFBII with the class I hydrophobin SC3 of *S. commune* revealed differences and also similarities, despite low sequence similarity. All these hydrophobins were for example surface active and able to stabilize oil emulsions. However, more time was needed to reach the minimum water surface tension using SC3 compared with HFBI and HFBII. In contrast to HFBI and HFBII, CD spectra of SC3 have been shown to change after mixing with air (de Vocht *et al.*, 1998). In addition, the dried SC3 film had a clear ultrastructure observed by EM (Wösten *et al.*, 1993, 1994a), by contrast to HFBI and HFBII films. Moreover, most SC3 bound to Teflon did not dissolve in hot detergent, unlike HFBI. Models were presented proposing that these differences between class I and class II hydrophobins were due to the different size and shape of the hydrophobic region on the protein surface (Figure 6 and 7).

The presence of HFBI and HFBII affected the solubility of SC3 aggregates, indicating an interaction between these hydrophobins. Both class I and class II

hydrophobins have been shown to occur in a single fungus. If both class I and class II hydrophobins are present simultaneously in nature, class II hydrophobins might decrease premature aggregation of class I hydrophobins.

Table 13. Overview of properties of the *T. reesei* hydrophobins studied.

Property	Hydrophobin	
	HFBI	HFBIII
Ability to reduce water surface tension	++ ^a	+++
Change in CD spectra after mixing with air	no	no
Rodlet pattern layer on hydrophobin film at the water-air interface	no	no
Ability to stabilize oil emulsions	+++	+
Change in CD spectra after confronting Teflon	yes	yes
Able to make Teflon wettable after extensive washing with water	yes	no
Ability to reduce wettability of filter paper	++	++
Temperature stability	+++	+++

^a +++ high; ++ intermediate; + low

High hydrophobin production is needed in many potential applications of hydrophobins. High production of HFBI and HFBIII was obtained by introducing additional *hfb1* or *hfb2* genes into the genome of *T. reesei*. The HFBIII-overproducing strain containing three additional *hfb2* genes under the regulation of the strong promoter of the gene encoding cellobiohydrolase I production resulted in up to 0.24 grams of HFBIII per liter of culture. The HFBI-overproducing strain containing two additional copies of the *hfb1* gene under the regulation of its own promoter reached a production level of 1.4 grams HFBI per liter, the highest production of hydrophobin hitherto reported. The production level might be increased even further by optimization of the medium composition and the production process.

HFBI and HFBIII, purified from fungal cell walls and culture medium, respectively, were crystallized for structure determination. The HFBI crystals

were hexagonal and belonged to space group $P6_1$ (or $P6_5$), with unit-cell parameters $a = b = 45.9$, $c = 307.2$ Å. However, the detailed 3D structure of HFBI could not be determined using these crystals. Crystallization of HFBII was continued at the University of Joensuu where the 3D crystal structure was resolved. The 3D structure allows detailed study of the roles of the individual amino acids in the hydrophobic region on the protein surface by targeted amino acid substitution or deletion. Moreover, it enables modification of the protein to broaden its usefulness.

The biological roles of HFBI and HFBII in *T. reesei* were found to be divergent. The results obtained suggested that HFBI has a role in hyphal development and HFBII in sporulation. Deletion of the *hfb1* gene resulted in absence of aerial hyphae in static liquid cultures on glucose-containing medium, showing that HFBI facilitated aerial growth of *T. reesei*. The class I hydrophobin SC3 of *S. commune* complemented the aerial hyphae deficient phenotype of $\Delta hfb1$. This showed for the first time that class I and class II hydrophobins can partially complement each other. Colonies of $\Delta hfb1$ showed a wettable and fluffy phenotype, and in shaken liquid cultures slow biomass formation and reduced hyphal thickness were observed. Absence of HFBII by deletion of the *hfb2* gene correlated with wettability of spores and sporulation efficiency. Functional analysis of other genes encoding hydrophobins of *T. reesei*, in addition to *hfb1* and *hfb2*, would allow more detailed information of the effect of hydrophobins on the survival of the fungus. The complete 3D structures of other hydrophobins, besides HFBII, would be required in order to relate differences in structure to different functions.

Foaming often causes problems in bioreactor cultivations of the industrially important fungus *T. reesei*. Secretion of HFBII into the culture medium was shown to correlate clearly with amount of foam produced by cultivating $\Delta hfb2$, HFBII-overproducing strain and the parent strain. Thus, problems caused both by foaming and by chemical antifoam agents added to the cultures can be overcome by using production strains in which hydrophobin production is inactivated.

The results of this work increase the level of knowledge of the biochemical, biophysical and molecular properties of hydrophobins. Furthermore, they provide information concerning their biological roles, function and interaction as

well as means to improve fungal strains used in industrial protein production. This work will stimulate new studies concerning biological functions of hydrophobins and facilitate the development of future applications.

References

- Abalos, A., Pinazo, A., Infante, M.R., Casals, M., García, F. and Manresa, A. 2001. Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir* 17, pp. 1367–1371.
- Adams, T.H. 1995. Asexual sporulation in higher fungi. In: Grow, N.A.R. and Gadd, G.M. (Eds.) *The Growing Fungi*. London: Chapman & Hall. Pp. 367–382.
- Albuquerque, P., Kyaw, C.M., Saldanha, R.R., Brigido, M.M., Felipe, M.S.S. and Silva-Pereira, I. 2004. Pbhyl1 and Pbhyl2: two mycelium-specific hydrophobin genes from the dimorphic fungus *Paracoccidioides brasiliensis*. *Fungal Genet. Biol.* 41, pp. 510–520.
- Ando, A., Harada, A., Miura, K. and Tamai, Y. 2001. A gene encoding a hydrophobin, *fvh1*, is specifically expressed after the induction of fruiting in the edible mushroom *Flammulina velutipes*. *Curr. Genet.* 39, pp. 190–197.
- Aro, N., Saloheimo, A., Ilmén, M. and Penttilä, M. 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *J. Biol. Chem.* 276, pp. 24309–24314.
- Ásgeirsdóttir, S.A., de Vries, O.M.H. and Wessels, J.G.H. 1998. Identification of three differentially expressed hydrophobins in *Pleurotus ostreatus* (oyster mushroom). *Microbiology* 144, pp. 2961–2969.
- Ásgeirsdóttir, S.A., Halsall, J.R. and Casselton, L.A. 1997. Expression of two closely linked hydrophobin genes of *Coprinus cinereus* is monocaryon-specific and down-regulated by the oid-1 mutation. *Fungal Genet. Biol.* 22, pp. 54–63.
- Ásgeirsdóttir, S.A., Scholtmeijer, K. and Wessels, J.G.H. 1999. A sandwiched-culture technique for evaluation of heterologous protein production in a filamentous fungus. *Appl. Environ. Microbiol.* 65, pp. 2250–2252.

- Bailey, M.J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* 23, pp. 257–270.
- Beckerman, J.L. and Ebbole, D.J. 1996. *MPG1*, a gene encoding a fungal hydrophobin of *Magnaporthe grisea*, is involved in surface recognition. *Mol. Plant Microbe Interact.* 9, pp. 450–456.
- Bell-Pedersen, D., Dunlap, J.C. and Loros, J.J. 1992. The *Neurospora* circadian clock-controlled gene, *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. *Genes Dev.* 6, pp. 2382–2394.
- Beneventi, D., Carre, B. and Gandini, A. 2001. Role of surfactant structure on surface and foaming properties. *Colloids Surfaces A* 189, pp. 65–73.
- Bilewicz, R., Witomski, J., Van der Heyden, A., Tagu, D., Palin, B. and Rogalska, E. 2001. Modification of electrodes with self-assembled hydrophobin layers. *J. Phys. Chem., B* 105, pp. 9772–9777.
- Bodey, G.P. and Vartivarian, S. 1989. Aspergillosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 8, pp. 413–437.
- Bognolo, G. 1999. Biosurfactants as emulsifying agents for hydrocarbons. *Colloids Surf., A* 152, pp. 41–52.
- Bolyard, M.G. and Sticklen, M.B. 1992. Expression of a modified Dutch elm disease toxin in *Escherichia coli*. *Mol. Plant Microbe Interact.* 5, pp. 520–524.
- Bowden, C.G., Hintz, W.E., Jeng, R., Hubbes, M. and Horgen, P.A. 1994. Isolation and characterization of the cerato-ulmin toxin gene of the Dutch elm disease pathogen, *Ophiostoma ulmi*. *Curr. Genet.* 25, pp. 323–329.
- Bowden, C.G., Smalley, E., Guries, R.P., Hubbes, M., Temple, B. and Horgen, P.A. 1996. Lack of association between cerato-ulmin production and virulence in *Ophiostoma novo-ulmi*. *Mol. Plant Microbe Interact.* 9, pp. 556–564.

Brasier, C.M., Kirk, S.A. and Tegli, S. 1995. Naturally occurring non cerato-ulmin producing mutants of *Ophiostoma novo-ulmi* are pathogenic but lack aerial mycelium. Mycol. Res. 99, pp. 436–440.

Butko, P., Buford, J.P., Goodwin, J.S., Stroud, P.A., McCormick, C.L. and Cannon, G.C. 2001. Spectroscopic evidence for amyloid-like interfacial self-assembly of hydrophobin Sc3. Biochem. Biophys. Res. Commun. 280, pp. 212–215.

Carpenter, C.E., Mueller, R.J., Kazmierczak, P., Zhang, L., Villalon, D.K. and van Alfen, N.K. 1992. Effect of virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. Mol. Plant Microbe Interact. 4, pp. 55–61.

Claessen, D., Stokroos, I., Deelstra, H.J., Penninga, N.A., Bormann, C., Salas, J.A., Dijkhuizen, L. and Wösten, H.A.B. 2004. The formation of the rodlet layer of *streptomyces* is the result of the interplay between rodlines and chaplins. Mol. Microbiol. 53, pp. 433–443.

Collén, A., Penttilä, M., Stålbrand, H., Tjerneld, F. and Veide, A. 2001. Extraction of endoglucanase I (Cel7B) fusion proteins from *Trichoderma reesei* culture filtrate in a poly(ethylene glycol)–phosphate aqueous two-phase system. J. Chromatogr., A 943, pp. 55–62.

Collén, A., Persson, J., Linder, M., Nakari-Setälä, T., Penttilä, M., Tjerneld, F. and Sivars, U. 2002. A novel two-step extraction method with detergent/polymer systems for primary recovery of the fusion protein endoglucanase I-hydrophobin I. Biochim. Biophys. Acta. 1569, pp. 139–150.

Cooper, D.G., MacDonald, C.R., Duff, S.J.B. and Kosaric, N. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42, pp. 408–412.

Corvis, Y., Walcarius, A., Rink, R., Mrabet, N.T. and Rogalska, E. 2005. Preparing catalytic surfaces for sensing applications by immobilizing enzymes via hydrophobin layers. Anal. Chem. 77, pp. 1622–1630.

de Groot, P.W.J., Roeven, R.T.P., van Griensven, L.J.L.D., Visser, J. and Schaap, P.J. 1999. Different temporal and spatial expression of two hydrophobin-encoding genes of the edible mushroom *Agaricus bisporus*. *Microbiology* 145, pp. 1105–1113.

de Groot, P.W.J., Schaap, P.J., Sonnenberg, A.S.M., Visser, J. and van Griensven, L.J.L.D. 1996. The *Agaricus bisporus hypA* gene encodes a hydrophobin and specifically accumulates in peel tissue of mushroom caps during fruit body development. *J. Mol. Biol.* 257, pp. 1008–1018.

Del Sorbo, G., Scala, F., Parella, G., Lorito, M., Comparini, C., Ruocco, M. and Scala, A. 2000. Functional expression of the gene *cu*, encoding the phytotoxic hydrophobin cerato-ulmin, enables *Ophiostoma quercus*, a nonpathogen on elm, to cause symptoms of Dutch elm disease. *Mol. Plant Microbe Interact.* 13, pp. 43–53.

Desai, J.D. and Banat, I.M. 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* 61, pp. 47–64.

de Vocht, M.L. 2001. Structural changes that accompany the self-assembly of hydrophobins. Ph.D. Thesis. Groningen: University of Groningen. 97 p.

de Vocht, M.L., Reviakine, I., Ulrich, W.P., Bergsma-Schutter, W., Wösten, H.A.B., Vogel, H., Brisson, A., Wessels, J.G.H. and Robillard, G.T. 2002. Self-assembly of the hydrophobin SC3 proceeds via two structural intermediates. *Protein Sci.* 11, pp. 1199–1205.

de Vocht, M.L., Reviakine, I., Wösten, H.A.B., Brisson, A., Wessels, J.G.H. and Robillard, G.T. 2000. Structural and functional role of the disulfide bridges in the hydrophobin SC3. *J. Biol. Chem.* 275, pp. 28428–28432.

de Vocht, M.L., Scholtmeijer, K., van der Vegte E.W., de Vries O.M.H., Sonveaux, N., Wösten H.A.B., Ruyschaert, J.-M., Hadziioannou, G., Wessels, J.G.H. and Robillard, G.T. 1998. Structural characterization of the hydrophobin SC3, as a monomeric and after self-assembly at hydrophobic/hydrophilic interfaces. *Biophys. J.* 74, pp. 2059–2068.

- de Vries, O.M.H., Fekkes, M.P., Wösten, H.A.B. and Wessels, J.G.H. 1993. Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi. Arch. Microbiol. 159, pp. 330–335.
- de Vries, O.M.H., Moore, S., Arntz, C., Wessels, J.G.H. and Tudzynski, P. 1999. Identification and characterization of a tri-partite hydrophobin from *Claviceps fusiformis*: A novel type of class II hydrophobin. Eur. J. Biochem. 262, pp. 377–385.
- Dons, J.J.M., Mulder, G.H., Rouwendal, G.J.A., Springer, J., Bremer, W. and Wessels, J.G.H. 1984a. Sequence analysis of a split gene involved in fruiting from the fungus *Schizophyllum commune*. EMBO J. 3, pp. 2101–2106.
- Dons, J.J.M., Springer, J., de Vries, S.C. and Wessels, J.G.H. 1984b. Molecular cloning of a gene abundantly expressed during fruit-body initiation in *Schizophyllum commune*. J. Bacteriol. 157, pp. 802–805.
- Duplessis, S., Sorin, C., Voiblet, C., Palin, B., Martin, F. and Tagu, D. 2001. Cloning and expression analysis of a new hydrophobin cDNA from the ectomycorrhizal basidiomycete *Pisolithus*. Curr. Genet. 39, pp. 335–339.
- Ebbole, D.J. 1997. Hydrophobins and fungal infection of plants and animals. Trends Microbiol. 5, pp. 405–408.
- Eriksson, T., Börjesson, J. and Tjerneld, F. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microbial Technol. 31, pp. 353–364.
- Fuchs, U., Czymmek, K.J. and Sweigard, J.A. 2004. Five hydrophobin genes in *Fusarium verticillioides* include two required for microconidial chain formation. Fungal Genet. Biol. 41, pp. 852–864.
- Garti, N. 1999. What can nature offer from an emulsifier point of view: trends and progress? Colloids Surf., B 152, pp. 125–146.
- Hakanpää, J., Parkkinen, T., Hakulinen, N., Linder, M. and Rouvinen, J. 2004. Crystallization and preliminary X-ray characterization of *Trichoderma reesei* hydrophobin HFBII. Acta Cryst. D60, pp. 163–165.

Harlow, E. and Lane, D. 1988. *Antibodies, A Laboratory Manual*. N.Y., Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Helin, J., Caldentey, J., Kalkkinen, N. and Bamford, D.H. 1999. Analysis of the multimeric state of proteins by matrix assisted laser desorption/ionization mass spectrometry after cross-linking with glutaraldehyde. *Rapid Commun. Mass Spectrom.* 13, pp. 185–190.

Healy, M.G., Devine, C.M. and Murphy, R. 1996. Microbial production of biosurfactants. *Resour., Conserv. Recycling* 18, pp. 41–57.

Hynes, M.J., Corrick, C.M. and King, J.A. 1983. Isolation of genomic clones containing the *amdS* gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. *Mol. Cell. Biol.* 3, pp. 1430–1439.

Itoh, S. and Suzuki, T. 1972. Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in *n*-paraffin-utilizing ability. *Agric. Biol. Chem.* 36, pp. 2233–2235.

IUPAC (International Union of Pure and Applied Chemistry) 1987. Measurement of cellulase activities. *Pure Appl. Chem.* 59, pp. 257–268.

Janssen, M.I., van Leeuwen, M.B.M., Scholtmeijer, K., van Kooten, T.G., Dijkhuizen, L., Wösten, H.A.B. 2002. Coating with genetic engineered hydrophobin promotes growth of fibroblasts on a hydrophobic solid. *Biomaterials* 23, pp. 4847–4854.

Janssen, M.I., van Leeuwen, M.B.M., van Kooten, T.G., de Vries, J., Dijkhuizen, L. and Wösten, H.A.B. 2004. Promotion of fibroblast activity by coating with hydrophobins in the β -sheet end state. *Biomaterials* 25, pp. 2731–2739.

Jönsson, B., Lindman, B., Homberg, K. and Kronberg, B. 1998. *Surfactants and polymers in aqueous solution*. Chichester: John Wiley & Sons. Pp. 438.

Karhunen, T., Mäntylä, A., Nevalainen, K.M.H. and Suominen, P.L. 1993. High frequency one-step gene replacement in *Trichoderma reesei*. I: Endoglucanase overproduction. *Mol. Gen. Genet.* 241, pp. 515–522.

- Kazmierczak, P., Kim, D.H., Turina, M. and Van Alfen, N.K. 2005. A hydrophobin of the chestnut blight fungus, *Cryphonectria parasitica*, is required for stromal pustule eruption. *Eukaryot. Cell* 4, pp. 931–936.
- Kershaw, M.J. and Talbot, N.J. 1998. Hydrophobins and repellents: Proteins with fundamental roles in fungal morphogenesis. *Fungal Genet. Biol.* 23, pp. 18–33.
- Kershaw, M.J., Wakley, G. and Talbot, N.J. 1998. Complementation of the Mpg1 mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins. *EMBO J.* 17, pp. 3838–3849.
- Kim, S., Ahn, I.-P. and Lee, Y.-H. 2001. Analysis of genes expressed during rice-*Magnaporthe grisea* interactions. *Mol. Plant Microbe Interact.* 14, pp. 1340–1346.
- Kim, S., Ahn, I.-P., Rho, H.-S. and Lee, Y.-H. 2005. *MHP1*, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. *Mol. Microbiol.* 57, pp. 1224–1237.
- Kleemola, T., Nakari-Setälä, T., Linder, M., Penttilä, M., Kotaviita, E., Olkku, J. and Haikara, A. 2001. Characterisation and detection of the gushing factors produced by fungi. In: Proc. E.B.C. 28th Congr., Budapest. Pp. 1–10. [CD-ROM]. ISBN 90-70143-21-6.
- Koch, A.K., Käppeli, O., Fiechter, A. and Reiser, K. 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J. Bacteriol.* 173, pp. 4212–4219.
- Kossiakoff, A.A. 1988. Tertiary structure is a principal determinant to protein deamidation. *Science* 240, pp. 191–194.
- Lauter, F.R., Russo, V.E.A. and Yanofsky, C. 1992. Developmental and light regulation of eas, the structural gene for the rodlet protein of *Neurospora*. *Genes Dev.* 6, pp. 2373–2381.
- Li, X., Guangxian, Z., Mingxiao, Z. and Wu, G. 1996. The surface activity and solubilization of anionic-cationic surfactants. *Colloids Surfaces, A* 111, pp. 161–166.

Lin, S.-C. 1996. Biosurfactants: recent advances. *J. Chem. Technol. Biotechnol.* 66, pp. 109–120.

Linder, M.B., Qiao, M., Laumen, F., Selber, K., Hyytiä, T., Nakari-Setälä, T. and Penttilä, M.E. 2004. Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. *Biochemistry* 43, pp. 11873–11882.

Linder, M., Selber, K., Nakari-Setälä, T., Qiao, M., Kula, M.-R. and Penttilä, M. 2001. The hydrophobins HFBI and HFBII from *Trichoderma reesei* showing efficient interactions with nonionic surfactants in aqueous two-phase systems. *Biomacromolecules* 2, pp. 511–517.

Linder, M.B., Szilvay, G.R., Nakari-Setälä T. and Penttilä, M.E. 2005. Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microb. Rev.* 29, pp. 877–896.

Linder, M., Szilvay, G.R., Nakari-Setälä, T., Söderlund, H. and Penttilä, M. 2002. Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from *Trichoderma reesei*. *Protein Sci.* 11, pp. 2257–2266.

Lora, J.M., de la Cruz, J., Benitez, T., Llobell, A. and Pintor-Toro, J.A. 1994. A putative catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*. *Mol. Gen. Genet.* 242, pp. 461–466.

Lora, J.M., Pintor-Toro, J.A., Benitez, T. and Romero, L.C. 1995. Qid3 protein links plant bimodular proteins with fungal hydrophobins. *Mol. Microbiol.* 18, pp. 380–382.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, pp. 265–275.

Lugones, L.G., Bosscher, J.S., Scholtmeijer, K., de Vries, O.M.H. and Wessels, J.G.H. 1996. An abundant hydrophobin (ABH1) forms hydrophobic rodlet layers in *Agaricus bisporus* fruiting bodies. *Microbiology* 142, pp. 1321–1329.

- Lugones, L.G., Wösten, H.A.B. and Wessels, J.G.H. 1998. A hydrophobin (ABH3) specifically secreted by vegetatively growing hyphae of *Agaricus bisporus* (common white button mushroom). *Microbiology* 144, pp. 2345–2353.
- Lugones, L.G., Wösten, H.A.B., Birkenkamp, K.U., Sjollema, K.A., Zagers, J. and Wessels, J.G.H. 1999. Hydrophobins line air channels in fruiting bodies of *Schizophyllum commune* and *Agaricus bisporus*. *Mycol. Res.* 103, pp. 635–640.
- Lumsdon, S.O., Green, J. and Stieglitz, B. 2005. Adsorption of hydrophobin proteins at hydrophobic and hydrophilic interfaces. *Colloids Surf., B* 44, pp. 172–178.
- MacDonald, C.R., Cooper, D.G. and Zajic, J.E. 1981. Surface-active lipids from *Nocardia erythropolis* grown on hydrocarbons. *Appl. Environ. Microbiol.* 41, pp. 117–123.
- Mackay, J.P., Matthews, J.M., Winefield, R.D., Mackay, L.G., Haverkamp, R.G. and Templeton, M.D. 2001. The hydrophobin EAS is largely unstructured in solution and functions by forming amyloid-like structures. *Structure* 9, pp. 83–91.
- Mankel, A., Krause, K. and Kothe, E. 2002. Identification of a hydrophobin gene that is developmentally regulated in the ectomycorrhizal fungus *Tricholoma terreum*. *Appl. Environ. Microbiol.* 68, pp. 1408–1413.
- Mandels, M., Sternberg, D. and Andreotti, R.E. 1975. Growth and cellulase production by *Trichoderma*. In: Bailey, M., Enari, T.-M. and Linko, M. (Eds.). *Symposium on Enzymatic Hydrolysis of Cellulose*. 1975. Helsinki: Finnish National Fund for Research and Development (SITRA). Pp. 81–109.
- Margolles-Clark, E., Hayes, C.K., Harman, G.E. and Penttilä, M. 1996. Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. *Appl. Environ. Microbiol.* 62, pp. 2145–2151.
- Martin, F., Laurent, P., de Carvalho, D., Burgess, T., Murphy, P., Nehls, U. and Tagu, D. 1995. Fungal gene expression during ectomycorrhiza formation. *Can. J. Bot.* 73, pp. 541–547.

Martin, F., Laurent, P., De Carvalho, D., Voiblet, C., Balestrini, R., Bonfante, P. and Tagu, D. 1999. Cell wall proteins of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*: Identification, function, and expression in symbiosis. *Fungal Genet. Biol.* 27, pp. 161–174.

Martin, G.G., Cannon, G.C. and McCormick, C.L. 2000. Sc3p hydrophobin organization in aqueous media and assembly onto surfaces as mediated by the associated polysaccharide Schizophyllan. *Biomacromolecules* 1, pp. 49–60.

Matsuyama, T., Kaneda, K., Ishizuka, I., Toida, T. and Yano, I. 1990. Surface-active novel glycolipid and linked 3-hydroxy fatty acids produced by *Serratia rubidaea*. *J. Bacteriol.* 172, pp. 3015–3022.

Matsuyama, T., Murikami, T., Fujita, M., Fujita, S. and Yano, I. 1986. Extracellular vesicle formation and biosurfactant production by *Serratia marcescens*. *J. Gen. Microbiol.* 132, pp. 865–875.

McCabe, P.M. and Van Alfen, N.K. 1999. Secretion of cryparin, a fungal hydrophobin. *Appl. Environ. Microbiol.* 65, pp. 5431–5435.

Mey, G., Correia, T., Oeser, B., Kershaw, M.J., Garre, V., Arntz, C.C., Talbot, N.J. and Tudzynski, P. 2003. Structural and functional analysis of an oligomeric hydrophobin gene from *Claviceps purpurea*. *Mol. Plant Pathol.* 4, pp. 31–41.

Montenecourt, B.S. and Eveleigh, D.E. 1979. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. *Adv. Chem. Ser.* 181, pp. 289–301.

Mulder, G.H. and Wessels, J.G.H. 1986. Molecular cloning of RNAs differentially expressed in monokaryons and dikaryons of *Schizophyllum commune*. *Exp. Mycol.* 10, pp. 214–227.

Muñoz, G., Nakari-Setälä, T., Agosin, E. and Penttilä, M. 1997. Hydrophobin gene *srh1*, expressed during sporulation of the biocontrol agent *Trichoderma harzianum*. *Curr. Genet.* 32, pp. 225–230.

- Nakari, T., Alatalo, E. and Penttilä, M.E. 1993. Isolation of *Trichoderma reesei* genes highly expressed on glucose-containing media: characterization of the *tefl* gene encoding translation elongation factor 1 α . *Gene* 136, pp. 313–318.
- Nakari-Setälä, T., Aro, N., Ilmen, M., Kalkkinen, N. and Penttilä, M. 1997. Differential expression of the vegetative and spore-bound hydrophobins of *Trichoderma reesei*, Cloning and characterization of the *hfb2* gene. *Eur. J. Biochem.* 248, pp. 415–423.
- Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E. and Penttilä, M. 1996. Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. *Eur. J. Biochem.* 235, pp. 248–255.
- Nakari-Setälä, T., Azeredo, J., Henriques, M., Oliveira, R., Teixeira, J., Linder, M. and Penttilä, M. 2002. Expression of a fungal hydrophobin in the *Saccharomyces cerevisiae* cell wall: effect on cell surface properties and immobilization. *Appl. Environ. Microbiol.* 68, pp. 3385–3391.
- Neu, T.R., Härtner, T. and Poralla, K. 1990. Surface active properties of viscosin: a peptidolipid antibiotic. *Appl. Microbiol. Biotechnol.* 32, pp. 518–520.
- Ng, W.L., Ng, T.P. and Kwan, H.S. 2000. Cloning and characterization of two hydrophobin genes differentially expressed during fruiting body development in *Lentinula edodes*. *FEMS Microbiol. Lett.* 185, pp. 139–145.
- Nielsen, P.S., Clark, A.J., Oliver, R.P., Huber, M. and Spanu, P.D. 2001. HCf-6, a novel class II hydrophobin from *Cladosporium fulvum*. *Microbiol. Res.* 156, pp. 59–63.
- Noordmans, J. and Busscher, H.J. 1991. The influence of droplet volume and contact angle on liquid surface tension measurements by axisymmetric drop shape analysis-profile (ADSA-P). *Colloids Surf., A* 58, pp. 239–249.
- Paananen, A., Vuorimaa, E., Torkkeli, M., Penttilä, M., Kauranen, M., Ikkala, O., Lemmetyinen, H., Serimaa, R. and Linder, M. 2003. Structural hierarchy in molecular films of two class II hydrophobins. *Biochemistry* 42, pp. 5253–5258.

Palomo, J.M., Peñas, M.M., Fernández-Lorente, G.M.C., Pisabarro, A.G., Fernández-Lafuente, R., Ramírez, L. and Guisán, J.M. 2003. Solid-phase handling of hydrophobins: immobilized hydrophobins as a new tool to study lipases. *Biomacromolecules* 4, pp. 204–210.

Paris, S., Debeaupuis, J.-P., Crameri, R., Carey, M., Charlès, F., Prévost, M. C., Schmitt, C., Philippe, B. and Latgé, J.P. 2003. Conidial hydrophobins of *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 69, pp. 1581–1588.

Parta, M., Chang, Y., Rulong, S., Pinto-DaSilva, P. and Kwong-Chung, K.J. 1994. *HYPI*, a hydrophobin gene from *Aspergillus fumigatus*, complements the rodlettless phenotype in *Aspergillus nidulans*. *Infect. Immun.* 62, pp. 4389–4395.

Peñas, M.M., Ásgeirsdóttir, S.A., Lasa, I., Cullanez-Macia, F.A., Pisabarro, A.G., Wessels, J.G.H. and Ramirez, L. 1998. Identification, characterization, and in situ detection of a fruit-body-specific hydrophobin of *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 64, pp. 4028–4034.

Peñas, M.M., Rust, B., Larraya, L.M., Ramírez, L. and Pisabarro, A.G. 2002. Differentially regulated, vegetative-mycelium-specific hydrophobins of the edible basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 68, pp. 3891–3898.

Penttilä, M., Nakari-Setälä, T., Fagerström, R., Selber, K., Kula, M.-R., Linder, M. and Tjerneld, F. 2000. Process for partitioning of proteins. *Int. Pat. Appl. PCT/FI00/00249*. 24.3.2000.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E. and Knowles, J. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61, pp. 155–164.

Persson, A., Österberg, E. and Dostalek, M. 1988. Biosurfactant production by *Pseudomonas fluorescens* 378: growth and product characteristics. *Appl. Microbiol. Biotechnol.* 29, pp. 1–4.

Peumans, W.J. and van Damme, E.J. 1995. Lectins as plant defence proteins. *Plant Physiol.* 109, pp. 347–352.

Powalla, M., Lang, S. and Wray, V. 1989. Penta- and disaccharide lipid formation by *Nocardia corynebacteroides* grown on n-alkanes. *Appl. Microbiol. Biotechnol.* 31, pp. 473–479.

Richards, W.C. 1993. Cerato-ulmin: a unique wilt toxin of instrumental significance in the development of Dutch elm disease. In: Sticklen, M.B. and Sberald, J.L. (Eds.). *Dutch Elm Disease Research: Cellular and Molecular Approaches*. New York: Springer Verlag. Pp. 89–151.

Richards, W.C. and Takai, S. 1973. Novel technique for isolating microstructures present in shake cultures of the fungus *Ceratocystis ulmi*. *Appl. Microbiol.* 26, pp. 443–444.

Rintala, E. 2001. *Trichoderma reesei* uudet hydrofobiinit. Master's thesis. Helsinki: University of Helsinki. 56 (+5) p.

Ron, E.Z. and Rosenberg, E. 2001. Natural roles of biosurfactants. *Environ. Microbiol.* 3, pp. 229–236.

Rosenberg, E. and Ron, E.Z. 1999. High- and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.* 52, pp. 154–162.

Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D.L. 1979. Emulsifier of *Arthrobacter* RAG-1: Isolation and emulsifying properties. *Appl. Environ. Microbiol.* 37, pp. 402–408.

Russo, P.S., Blum, F.D., Ipsen, J.D., Miller, W.G. and Abul-Hajj, Y.J. 1982. The surface activity of the phytotoxin cerato-ulmin. *Can. J. Bot.* 60, pp. 1414–1422.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd Edition. N.Y.: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Santos, C. and Labarère. 1999. *Aa-Pri2*, a single-copy gene from *Agrocybe aegerita*, specifically expressed during fruiting initiation, encodes a hydrophobin with a leucine-zipper domain. *Curr. Genet.* 35, pp. 564–570.

Scherrer, S., de Vries, O.M.H., Dudler, R., Wessels, J.G.H. and Honegger, R. 2000. Interfacial self-assembly of fungal hydrophobins of the lichen-forming ascomycetes *Xanthoria parietina* and *X. ectaneoides*. *Fungal Genet. Biol.* 30, pp. 81–93.

Scholtmeijer, K. 2000. Expression and engineering of hydrophobin genes. Ph.D. thesis. Groningen: University of Groningen. 120 p.

Scholtmeijer, K., Janssen, M.I., Gerssen, B., de Vocht, M.L., van Leeuwen, B.M., van Kooten, T.G., Wösten, H.A.B. and Wessels, J.G.H. 2002. Surface modifications created by using engineered hydrophobins. *Appl. Environ. Microbiol.* 68, pp. 1367–1373.

Scholtmeijer, K., Janssen, M.I., van Leeuwen, M.B.M., van Kooten, T.G., Hektor, H. and Wösten, H.A.B. 2004. The use of hydrophobins to functionalize surfaces. *Bio-Med. Mater. Eng.* 14, pp. 447–454.

Scholtmeijer, K., Wessels, J.G.H. and Wösten, H.A.B. 2001. Fungal hydrophobins in medical and technical applications. *Appl. Microbiol. Biotechnol.* 56, pp. 1–8.

Schuren F.H.J. and Wessels, J.G.H. 1990. Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologies with a gene not regulated by mating-type genes. *Gene* 90, pp. 199–205.

Schuurs, T.A., Schaeffer, E.A.M. and Wessels, J.G.H. 1997. Homology-dependent silencing of the SC3 gene in *Schizophyllum commune*. *Genetics* 147, pp. 589–596.

Segers, G.C., Hamada, W., Oliver, R.P. and Spanu, P.D. 1999. Isolation and characterisation of five different hydrophobin-encoding cDNAs from the fungal tomato pathogen *Cladosporium fulvum*. *Mol. Gen. Genet.* 261, pp. 644–652.

Selber, K., Tjerneld, F., Collén, A., Hyytiä, T., Nakari-Setälä, T., Bailey, M., Fagerström, R., Kan, J., van der Laan, J., Penttilä, M. and Kula, M.-R. 2004. Large-scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. *Process Biochem.* 39, pp. 889–896.

Serimaa, R., Torkkeli, M., Paananen, A., Linder, M., Kisko, K., Knaapila, M., Ikkala, O., Vuorimaa, E., Lemmetyinen, H. and Seeck, O. 2003. Self-assembled structures of hydrophobins HFBI and HFBI. *J. Appl. Cryst.* 36, pp. 499–502.

Sietsma, J.H., Wosten, H.A.B. and Wessels, J.G.H. 1995. Cell wall growth and protein secretion in fungi. *Can. J. Bot.* 73, pp. 388–395.

Spanu, P. 1997. HCF-1, a hydrophobin from the tomato pathogen *Cladosporium fulvum*. *Gene* 193, pp. 89–96.

Spanu, P. 1998. Deletion of *HCF-1*, a hydrophobin gene of *Cladosporium fulvum*, does not affect pathogenicity on tomato. *Physiol. Mol. Plant Pathol.* 52, pp. 323–334.

St. Leger, R.J., Staples, R.C. and Roberts, D.W. 1992. Cloning and regulatory analysis of starvation-stress gene, *ssgA*, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. *Gene* 120, pp. 119–124.

Stringer, M.A., Dean, R.A., Sewall, T.C. and Timberlake, W.E. 1991. *Rodletless*, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes Dev.* 5, pp. 1161–1171.

Stringer, M.A. and Timberlake, W.E. 1995. *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Mol. Microbiol.* 16, pp. 33–44.

Svircev, A.M., Jeng, R.S. and Hubbes, M. 1988. Detection of cerato-ulmin on aggressive isolates of *Ophiostoma ulmi* by immunocytochemistry and scanning electron microscopy. *Phytopathology* 78, pp. 322–327.

Tagu, D. and Martin, F. 1996. Molecular analysis of cell wall proteins expressed during the early steps of ectomycorrhiza development. *New Phytol.* 133, pp. 73–85.

Tagu, D., De Bellis, R., Balestrini, R., De Vries, O.M.H., Piccoli, G., Stocchi, V., Bonfante, P. and Martin, F. 2001. Immunolocalization of hydrophobin HYDPt-1 from the ectomycorrhizal basidiomycete *Pisolithus tinctorius* during colonization of *Eucalyptus globulus* roots. *New Phytol.* 149, pp. 127–135.

Tagu, D., Nasse, B. and Martin, F. 1996. Cloning and characterization of hydrophobins-encoding cDNAs from the ectomycorrhizal Basidiomycete *Pisolithus tinctorius*. *Gene* 168, pp. 93–97.

Takahashi, T., Maeda, H., Yoneda, S., Ohtaki, S., Yamagata, Y., Hasegawa, F., Gomi, K., Nakajima, T. and Abe, K. 2005. The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Mol. Microbiol.* 57, pp. 1780–1796.

Takai, S. 1974. Pathogenicity of cerato-ulmin production in *Ceratocystis ulmi*. *Nature* 252, pp.124–126.

Takai, S. 1978. Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: Cultural factors affecting cerato-ulmin production by the fungus. *Phytopath. Z.* 91, pp. 147–158.

Takai, S. and Hiratsuka, Y. 1980. Accumulation of the material containing the toxin cerato-ulmin on the hyphal surface of *Ceratocystis ulmi*. *Can. J. Bot.* 58, pp. 663–668.

Takai, S. and Hiratsuka, Y. 1984. Scanning electron microscope observations of internal symptoms of white elm following *Ceratocystis ulmi* infection and cerato-ulmin treatment. *Can. J. Bot.* 62, pp. 1365–1371.

Takai, S. and Richards, W.C. 1978. Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: Isolation and some properties of cerato-ulmin from the culture of *C. ulmi*. *Phytopath. Z.* 91, pp. 129–146.

Talbot, N.J. 1999. Fungal biology. Coming up for air and sporulation. *Nature* 398, pp. 295–296

Talbot, N.J., Ebbole, D.J. and Hamer, J.E. 1993. Identification and characterization of MPG1, a gene involved in patogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 5, pp. 1575–1590.

Talbot, N.J., Kershaw, M.J., Wakley, G.E., de Vries, O.M.H., Wessels, J.G.H. and Hamer, J.E. 1996. MPG1 encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell* 8, pp. 985–999.

Tasaki, Y., Ohata, K., Hara, T. and Joh, T. 2004. Three genes specifically expressed during phosphate deficiency in *Pholiota nameko* strain N2 encode hydrophobins. *Curr. Genet.* 45, pp. 19–27.

Temple, B., Horgen, P.A., Bernier, L. and Hintz, W.E. 1997. Cerato-ulmin, a hydrophobin secreted by causal agents of Dutch elm disease, is a parasitic fitness-factor. *Fungal Genet. Biol.* 22, pp. 39–53.

Templeton, M.D., Greenwood, D.R. and Beever, R.E. 1995. Solubilization of *Neurospora crassa* rodlet proteins and identification of the predominant protein as the proteolytically processed eas (ccg-2) gene product. *Exp. Mycol.* 19, pp. 166–169.

Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latge, J.-P. and Paris, S. 1994. Rodletless mutants of *Aspergillus fumigatus*. *Infect. Immun.* 62, pp. 4380–4388.

Tilburn, J., Scazzocchio, C., Taylor, G.T., Zabincky-Zissman, J.H., Lockington, R.A. and Davies, R.W. 1983. Transformation by integration in *Aspergillus nidulans*. *Gene* 51, pp. 43–52.

Torkkeli, M., Serimaa, R., Ikkala, O. and Linder, M. 2002. Aggregation and self-assembly of hydrophobins from *Trichoderma reesei*: low-resolution structural models. *Biophys. J.* 83, pp. 2240–2247.

Trembley, M.L., Ringli, C. and Honegger, R. 2002. Hydrophobins DGH1, DGH2, and DGH3 in the Lichen-Forming Basidiomycete *Dictyonema glabratum*. *Fungal Genet. Biol.* 35, pp. 247–259.

- Tucker, S.L. and Talbot, N.J. 2001. Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu. Rev. Phytopathol.* 39, pp. 385–417.
- Tyler-Cross, R. and Schirch, V. 1991. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. *J. Biol. Chem.* 266, pp. 22549–22556.
- van Wetter, M.-A., Schuren, F.H.J., Schuurs, T.A. ja Wessels, J.G.H. 1996. Targeted mutation of the SC3 hydrophobin gene of *Schizophyllum commune* affects formation of aerial hyphae. *FEMS Microbiol. Lett.* 140, pp. 265–269.
- van Wetter, M.-A., Wösten, H.A.B. and Wessels, J.G.H. 2000a. SC3 and SC4 hydrophobins have distinct roles in formation of aerial structures in dikaryons of *Schizophyllum commune*. *Mol. Microbiol.* 36, pp. 201–210.
- van Wetter, M.-A., Wösten, H.A.B., Sietsma, J.H. and Wessels, J.G.H. 2000b. Hydrophobin gene expression affects hyphal wall composition in *Schizophyllum commune*. *Fungal Genet. Biol.* 31, pp. 99–104.
- Vijayan, M. and Chandra, N. 1999. Lectins. *Curr. Opin. Struct. Biol.* 9, pp. 707–714.
- Wang, X., de Vocht, M.L., Jonge, J.D., Poolman, B. and Robillard, G.T. 2002. Structural changes and molecular interactions of hydrophobin SC3 in solution and on a hydrophobic surface. *Protein Sci.* 11, pp. 1172–1181.
- Wang, X., Graveland-Bikker, J.F., de Kruif, C.G. and Robillard, G.T. 2004a. Oligomerization of hydrophobin SC3 in solution: from soluble state to self-assembly. *Prot. Sci.* 13, pp. 810–821.
- Wang, X., Permentier, H.P., Rink, R., Kruijtzter, J.A.W., Liskamp, R.M.J., Wösten, H.A.B., Poolman, B. and Robillard, G.T. 2004b. Probing the self-assembly and the accompanying structural changes of hydrophobin SC3 on a hydrophobic surface by mass spectrometry. *Biophys. J.* 87, pp. 1919–1928.
- Wang, X., Shi, F., Wösten, H.A.B., Hektor, H., Poolman, B and Robillard, G.T. 2005. The SC3 Hydrophobin Self-Assembles into a Membrane with Distinct Mass Transfer Properties. *Biophys. J.* 88, pp. 3434–3443.

- Weichel, M., Schmid-Grendelmeier, P., Rhyner, C., Achatz, G., Blaser, K. and Cramer, R. 2003. Immunoglobulin E-binding and skin test reactivity to hydrophobin HCh-1 from *Cladosporium herbarum*, the first allergenic cell wall component of fungi. *Clin. Exp. Allergy* 33, pp. 72–77.
- Wessels, J.G.H. 1986. Cell wall synthesis in apical hyphal growth. *Int. Rev. Cytol.* 104, pp. 37–79.
- Wessels, J.G.H. 1992. Protein excretion and morphogenesis in fungi. *Proc. Natl. Sci. Counc., B* 16, pp. 134–143.
- Wessels, J.G.H. 1994. Developmental regulation of fungal cell wall formation. *Annu. Rev. Phytopathol.* 32, pp. 413–437.
- Wessels, J.G.H. 1996. Fungal hydrophobins: proteins that function at an interface. *Trends Plant Sci.* 1, pp. 9–15.
- Wessels, J.G.H. 1997. Hydrophobins: Proteins that change the nature of the fungal surface. *Adv. Microb. Physiol.* 38, pp. 1–45.
- Wessels, J.G.H. 1999. Fungi in their own right. *Fungal Genet. Biol.* 27, pp. 134–145.
- Wessels, J.G.H. 2000. Hydrophobins, unique fungal proteins. *Mycologist* 14, pp. 153–159.
- Wessels, J.G.H., Ásgeirsdóttir, S.A., Birkenkamp, K.U., de Vries, O.M.H., Lugones, L.G., Scheer, J.M.J., Schuren, F.H.J., Schuurs, T.A., van Wetter, M.A. and Wösten, H.A.B. 1995. Genetic regulation of emergent growth in *Schizophyllum commune*. *Can. J. Bot.* 73, pp. S273–S281.
- Wessels, J.G.H., de Vries, O.M.H., Ásgeirsdóttir, S.A. and Schuren F.H.J. 1991a. Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophyllum*. *Plant Cell* 3, pp. 793–799.

- Wessels, J.G.H., de Vries, O.M.H., Ásgeirsdóttir, S.A. and Springer, J. 1991b. The *thn* mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the *Sc3* hydrophobin gene. *J. Gen. Microbiol.* 137, pp. 2439–2445.
- Whiteford, J.R. and Spanu, P.D. 2001. The hydrophobin HcF-1 of *Cladosporium fulvum* is required for efficient water-mediated dispersal of conidia. *Fungal Genet. Biol.* 32, pp. 159–168.
- Whiteford, J.R. and Spanu, P.D. 2002. Hydrophobins and the interactions between fungi and plant. *Mol. Plant Pathol.* 3, pp. 391–400.
- Whiteford, J.R., Lacroix, H., Talbot, N.J. and Spanu, P.D. 2004. Stage-specific cellular localisation of two hydrophobins during plant infection by the pathogenic fungus *Cladosporium fulvum*. *Fungal Genet. Biol.* 41, pp. 624–634.
- Wright, H.T. 1991. Sequence and structure determinants of nonenzymatic deamidation of asparagine and glutamine residues in proteins. *Protein Eng.* 4, pp. 283–294.
- Wright, D.P., Johansson, T., Le Quere, A., Söderström, B. and Tunlid, A. 2005. Spatial patterns of gene expression in the extramatricial mycelium and mycorrhizal root tips formed by the ectomycorrhizal fungus *Paxillus involutus* in association with birch (*Betula pendula*) seedlings in soil microcosms. *New Phytol.* 167, pp. 579–596.
- Wösten, H.A.B. 2001. Hydrophobins: multipurpose proteins. *Annu. Rev. Microbiol.* 55, pp. 625–646.
- Wösten, H.A.B. and de Vocht M.L. 2000. Hydrophobins, the fungal coat unravelled. *Biochim. Biophys. Acta* 1469, pp. 79–86.
- Wösten, H.A.B. and Wessels, J.G.H. 1997. Hydrophobins, from molecular structure to multiple functions in fungal development. *Mycoscience* 38, pp. 363–374.

Wösten, H.A.B., Ásgeirsdóttir, S.A., Krook, J.H., Drenth, J.H.H. and Wessels, J.G.H. 1994a. The fungal hydrophobin Sc3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. *Eur. J. Cell Biol.* 63, pp. 122–129.

Wösten, H.A.B., de Vries, O.M.H. and Wessels, J.G.H. 1993. Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. *Plant Cell* 5, pp. 1567–1574.

Wösten, H.A.B., de Vries, O.M.H., van der Mei, H.C., Busscher, H.J. and Wessels, J.G.H. 1994b. Atomic composition of the hydrophobic and hydrophilic membrane sides of self-assembled SC3p hydrophobin. *J. Bacteriol.* 176, pp. 7085–7086.

Wösten, H.A.B., Schuren, F.H.J. and Wessels, J.G.H. 1994c. Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. *EMBO J.* 13, pp. 5848–5854.

Wösten, H.A.B., van Wetter, M.-A., Lugones, L.G., van der Mei, H.C., Busscher, H.J. and Wessels, J.G.H. 1999. How a fungus escapes the water to grow into the air. *Curr. Biol.* 9, pp. 85–88.

Yaguchi, M., Pusztai-Carey, M., Roy, C., Surewicz, W.K., Carey, P.R., Stevenson, K.J., Richards, W.C. and Takai, S. 1993. In: Sticklen, M.B. and Sherald, J.L. (Eds.). *Dutch Elm Disease Research: Cellular and Molecular Approaches*. New York: Springer. Pp. 152–170.

Yakimov, M.M., Timmis, K.N., Wray, V. and Fredrickson, H.L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Environ. Microbiol.* 61, pp. 1706–1713.

Yamada, M., Sakuraba, S., Shibata, K., Inatomi, S., Okazaki, M. and Shimosaka, M. 2005. Cloning and characterization of a gene coding for a hydrophobin, *Fv-hyd1*, specifically expressed during fruiting body development in the basidiomycete *Flammulina velutipes*. *Appl. Microbiol. Biotechnol.* 67, pp. 240–246.

Zacharius, R.M. and Zell, T.E. 1969. Glycoprotein staining following electrophoresis on acrylamide gel. *Anal. Biochem.* 30, pp. 148–152.

Zhang, L., Villalon, D., Sun, Y., Kazmierczak, P. and van Alfen, N.K. 1994. Virus-associated down-regulation of the gene encoding cryparin, an abundant cell-surface protein from the chestnut blight fungus, *Cryphonectria parasitica*. *Gene* 139, pp. 59–64.

Zosim, Z., Gutnick, D. and Rosenberg, E. 1982. Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. *Biotechnol. Bioeng.* 24, pp. 281–292.

Appendices II–V of this publication are not included in the PDF version. Please order the printed version to get the complete publication (<http://www.vtt.fi/inf/pdf>)

PUBLICATION I

**Overproduction, purification, and
characterization of the *Trichoderma
reesei* hydrophobin HFBI**

In: Appl. Microbiol. Biotechnol. 2001.
Vol. 57, pp. 124–130.

Reprinted with permission from the publisher.

Overproduction, Purification, and Characterization of the *Trichoderma reesei* Hydrophobin HFBI

Sanna Askolin*, Tiina Nakari-Setälä, and Maija Tenkanen
VTT Biotechnology, P.O. Box 1000, 02044 VTT, Finland

Abstract

Many characteristics of fungal hydrophobins such as an ability to change hydrophobicity of different surfaces have potential for several applications. The large scale processes of production and isolation of these proteins susceptible to aggregation and attachment to interfacial surfaces still needs to be studied. We report for the first time of a method for a gram scale production and purification of a hydrophobin, HFBI of *Trichoderma reesei*. High production level of the class II hydrophobin (0.6 g l^{-1}) was obtained by constructing a *T. reesei* HFBI overproducing strain containing three copies of the *hfb1* gene. The strain was cultivated on glucose-containing medium which induces expression of *hfb1*. HFBI hydrophobin was purified from the cell walls of the fungus because most of the HFBI was cell-bound (80%). Purification was carried out with a simple three step method involving extraction of the mycelium with 1% SDS at pH 9.0, followed by KCl precipitation to remove SDS, and hydrophobic interaction chromatography. The yield was 1.8 g HFBI from mycelium (419 g dw), derived from 15 l of culture. HFBI was shown to be rather unstable to N-terminal asparagine deamidation and also in some extent to non-specific proteases while its thermostability was excellent.

* E-mail: Sanna.Askolin@vtt.fi
Phone: +358 20 722 5139
Fax: +358 20 722 2103

Introduction

Hydrophobins are a family of small relatively hydrophobic fungal proteins with interesting properties. These secreted proteins have an ability to convert hydrophobic surfaces to hydrophilic and hydrophilic surfaces to hydrophobic by self-assembly into an amphipathic protein membrane (Wessels 1997). They also belong to the most surface active molecules known (Wösten and de Vocht 2000). The surface activity of hydrophobins is in the level of the commercial synthetic surfactants and other biosurfactants (Lin 1996). In nature, these exceptional characteristics serve different functions. The hydrophobin membrane covers emergent fungal structures making the cell wall hydrophobic which facilitates attachment of hyphae to hydrophobic surfaces, aerial growth of the hyphae, spore dispersal, and proper gas exchange in fungal air channels (Wösten and Wessels 1997; van Wetter et al. 2000). The high surface activity of hydrophobins enables the fungal hyphae to escape from liquid media to the air (Wösten et al. 2000; Wösten and Wessels 1997).

There are several possible applications for hydrophobins including use of hydrophobin membrane in immobilization of cells or proteins to surfaces like in biosensors, changing surface hydrophobicity e.g. in order to increase biocompatibility in tissue engineering, and acting as oil dispersing agents in different branches of industry (Wessels 1997). Biosurfactants have already been found useful in enhancing the degradation of organic pollutants in soil and facilitating oil recovery by mobilization of oil in oil spill (Lin 1996). Hydrophobin coating stabilizing oil droplets could also be useful in e.g. drug delivery (Wessels 1997). Many of the potential applications require large quantities of hydrophobin. So far, the production and purification of hydrophobins has shown to be difficult. Even though hydrophobins have been studied intensively, the research has mainly concentrated in biological aspects of the proteins and not in production.

Hydrophobins are divided into two classes according to the hydropathy patterns and the solubility of the protein assemblages (Wessels 1994). Class I hydrophobins form stable aggregates soluble only in reagents like TFA and formic acid. The assemblages of class II hydrophobins can be dissolved in e.g. 60% ethanol or 2% SDS. We have earlier isolated and characterized two class II hydrophobin genes, *hfb1* and *hfb2*, of the cellulolytic filamentous fungus

Trichoderma reesei and characterized the corresponding proteins. In this paper, we report for the first time of a method for a large scale production and purification of a hydrophobin. The purified hydrophobin HFBI of *T. reesei* is also further characterized by analyzing the stability of the protein.

Materials and methods

Fungal strains. *T. reesei* strains VTT-D-74075 (QM9414) and VTT-D-98692, containing three copies of the *hfb1* gene for overproduction of HFBI on glucose, were used in this study. The strain VTT-D-98692 was constructed by co-transforming QM9414 essentially as described by Penttilä and co-workers (Penttilä et al. 1987) using the plasmids pEA10 (Nakari-Setälä et al. 1993), which contains the genomic copy of the *T. reesei hfb1* gene, and p3SR2 containing the acetamidase gene (Hynes et al. 1983; Tilburn et al. 1983). The Amd⁺ transformants obtained were streaked two times onto acetamide plates (Penttilä et al. 1987). Thereafter, spore suspensions were made from transformants grown on Potato Dextrose agar (Difco). The production of HFBI was tested by slot blotting or Western analysis with HFBI specific antibodies raised against HFBI produced in *E. coli* (Nakari-Setälä et al. 1996) from shake flask or microtiter plate cultivations carried out in buffered *Trichoderma* minimal medium (Nakari-Setälä and Penttilä 1995) supplemented with 4% glucose. The spore suspensions of the HFBI overproducing clones were purified to single spore cultures on selection plates containing acetamide. Overproduction of HFBI was analyzed again from these purified clones as described above and the best transformant (VTT-D-98692) was chosen for the fermenter cultivation.

Southern hybridization. Fungal DNA was isolated using the Easy-DNA Kit of Invitrogen. DNA (2 µg) was cleaved with i) EcoRV, ii) SspI and EcoRI, and iii) SspI and SalI. Both EcoRV and SspI had known recognition sequences within the coding and flanking regions of the *hfb1* sequence whereas the recognition sequences of EcoRI and SalI were unknown. The cleaved DNAs were electrophoresed and transferred onto Magna Charge Nylon filter (Micron Separations Inc.) by capillary blotting. Southern hybridization with *T. reesei hfb1* cDNA labeled with [α -³²P]dCTP was carried out in stringent conditions in 50% formamide at 42°C overnight with 0.7 x 10⁶ cpm of probe per ml of hybridization solution (Sambrook et al. 1989). The washes for the filters were in

2 x SSC – 0.1% sodium dodecyl sulfate (SDS) for 10 min at room temperature, 2 x SSC – 0.1% SDS at 68°C for 1 h and in 0.1 x SSC – 0.1% SDS at 68°C for 130 min. The washed filters were exposed on Kodak X-Omat film.

Production of HFBI hydrophobin. *T. reesei* strains QM9414 and VTT-D-98692 were cultivated in a laboratory fermenter (LF. 20, Chemap AG) with a working volume of 15 l at 29°C, with 400–600 rpm agitation (manual adjustment) and aeration of approximately 10 l min⁻¹. The culture medium consisted of glucose (20.0 g l⁻¹), peptone (4.0 g l⁻¹), yeast extract (1.0 g l⁻¹), KH₂PO₄ (4.0 g l⁻¹), (NH₄)₂SO₄ (2.8 g l⁻¹), MgSO₄·7 H₂O (0.6 g l⁻¹), CaCl₂·2 H₂O (0.8 g l⁻¹), and trace solution (2 ml l⁻¹) (Mandels and Weber 1969). NaOH and H₃PO₄ were used to adjust the pH to 4.5–5.0. The glucose concentration was kept within the limits 10–30 g l⁻¹. The culture medium and biomass were separated by centrifugation (8000 g, 25 min, 6°; Heraeus Sepatech Cryofuge 8000).

Purification of HFBI. The wet mycelium of *T. reesei* VTT-D-98692 (2.15 kg wet weight, 19.5% dw, from 15 l of culture) was extracted three times with 4190 ml of 100 mM Tris/HCl buffer, pH 9.0, containing 1% SDS at room temperature for 1 h with occasional mixing. The solution was chosen according to the preliminary trials in which different pH values, detergents (0.6% Triton X-100, 0.6% Tween 20, and SDS), and SDS concentrations were tested. The diluting effect of the moisture in the mycelium was taken into account in the first extraction by using 1.7x buffer. The mycelium was separated by centrifugation (8000 g, 25 min, 6°C; Heraeus Sepatech Cryofuge 8000). SDS was precipitated from the first extract as water insoluble potassium dodecyl sulfate by adding 0.4 sample volumes of 2 M KCl and discarded after centrifugation. The SDS content was determined as described by Hayashi (Hayashi 1975) using dichloromethane instead of chloroform. Ammonium sulfate concentration and pH of the first extract were adjusted to 0.6 M and pH 7.5, respectively, and the solution (5110 ml) was applied to the column of high substituted Phenyl Sepharose 6 FF (45 x 10 cm, Pharmacia Biotech) equilibrated with 100 mM Tris/HCl, pH 7.5, containing 2 M ammonium sulfate. Most of the HFBI was eluted with water after a linear gradient from the equilibrium buffer to 20 mM Tris/HCl pH 7.5. HFBI-containing fractions were pooled (4590 ml). Part of the pool (3x200 ml) was further purified by anion exchange fast performance liquid chromatography (ResourceTM Q, 6 ml, Pharmacia Biotech) to separate different forms of HFBI after a small scale trial with 20 ml. Proteins were eluted with a linear gradient of

0–0.5 M NaCl in 20 mM Tris/HCl pH 9.0 buffer. The final HFBI preparation was concentrated by ultrafiltration (YM1 membrane, Amicon) and changed to water using gel filtration (Biogel P6-DG, Bio-Rad). The elution of proteins was monitored at a wavelength of 215 nm during all chromatography runs.

Analysis of the HFBI content. HFBI was determined qualitatively by dot blotting or Western analysis using HFBI specific polyclonal antibodies (Nakari-Setälä et al. 1996). Quantitative analysis was performed by reverse phase HPLC (ResourceTM RPC column, 1 ml, Pharmacia Biotech) with a 0–60% acetonitrile (ACN) gradient containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2 ml min⁻¹ using pure HFBI as a standard. The HPLC system included solvent delivery equipment (Waters 6000A), sample injector (Waters 712), a detector (Waters 486), and Millennium 2.15 software. The amount of cell wall-bound HFBI was estimated by extracting the mycelium three times with 100 mM Tris/HCl buffer, pH 9.0 containing 1% SDS, and adding up the HPLC results of the extracts. The purity of the HFBI samples was also monitored using Protein C4 HPLC columns (0.46 x 15 cm, 0.46 x 25 cm, Vydac) with a 20–70% ACN gradient containing 0.1% TFA at a flow rate of 1 ml min⁻¹ and an ÄKTAexplorer HPLC system (Amersham Pharmacia Biotech). Hydrophobin was detected at 215 nm in HPLC runs. The protein content of the standard was determined by amino acid analysis with LKB ALPHA-PLUS amino acid analyzer after hydrolyzing the sample (0.2 ml) with 3 ml of 6 M hydrochloric acid containing norleucine as internal standard at 110°C for 24 h, evaporation to dryness, and dissolution in 1.5 ml 0.2 M sodium citrate, pH 2.2 buffer. The protein concentration obtained from the amino acid analysis was corrected by taking into account the eight cysteines of HFBI, because cysteine, methionine, and tryptophan could not be determined with this method.

Characterization. SDS – polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were performed with a Phast System (Pharmacia-LKB) on a homogeneous 20% polyacrylamide gel and a gel slab with a pH gradient from 3 to 9, respectively, according to the manufacturer's instructions. Proteins were stained with silver stain (Silver Stain Kit, Bio-Rad). When attachment of sugar moieties to the protein was studied, the SDS-polyacrylamide gel was stained with glycoprotein staining (Zacharius and Zell 1969) following electrophoresis. Chromatofocusing of pure HFBI (0.8 mg) was carried out in a column of Polybuffer exchanger PBE-94 (25 ml, Pharmacia) at a flow rate of

1 ml min⁻¹ and monitored at 215 nm. The column was equilibrated with 0.025 M imidazole/HCl pH 7.6. HFBI was eluted from the column with a self-generated pH gradient (7.6–4.0) using Polybuffer 74/HCl pH 4.0 (Pharmacia) at a final dilution of 1 in 8. Molecular masses of the purified HFBI samples were determined with MALDI-TOF mass spectrometry using horse heart myoglobin as a standard (Helin et al. 1999). N-terminal amino acids were sequenced by automated Edman degradation using an Applied Biosystems liquid phase sequencer 477A/120A.

Protease digestibility of the purified HFBI (0.2 mg ml⁻¹ in water) was studied with a method also used for SC3 hydrophobin of *Schizophyllum commune* (O.M.H. de Vries and J.G.H. Wessels, unpublished data; Wösten and Wessels 1997) by using 0.08 mg ml⁻¹ proteinase K (from *Tritirachium album*, Finnzymes), α -chymotrypsin (type VII from bovine pancreas, Sigma), papain (from papaya latex, Sigma), or pepsin (from porcine stomach mucosa, Sigma) at different pH values in the presence of 1% SDS. Contrary to the previous method, digestibility was also analyzed in the absence of SDS. Bovine serum albumin (BSA) was used as a control. Protease stock solutions of 0.5 mg ml⁻¹ proteinase K (pH 8.3), chymotrypsin (pH 8.3), papain (pH 6.7), and pepsin (pH 3.1) were prepared in 50 mM sodium acetate buffer. Proteinase K, chymotrypsin, and papain were activated prior to protease digestion by adding 10 mM CaCl₂, 2 mM CaCl₂, and 5 mM DL-dithiothreitol (DTT), respectively. Papain was preincubated at 35°C for 15 min. Protease digestion was performed by incubating the samples at 31°C for 2 h or 18 h. The Proteinase K, chymotrypsin, and papain reactions were terminated by adding 3 μ M phenylmethylsulfonyl fluoride (PMSF) and the pepsin reaction with 5 μ l ml⁻¹ 25% ammonia. The digestion of HFBI was analyzed by 20% SDS-PAGE followed by silver staining.

The effect of pH on deamidation of HFBI was studied by incubating intact HFBI (25 μ g ml⁻¹) in 100 mM Na₂HPO₄/citric acid pH 3.0; 100 mM sodium acetate, pH 5.0; 100 mM sodium citrate, pH 6.0; sodium phosphate, pH 7.0; and 100 mM Tris/HCl buffer, pH 9.0, for 4h or 26 h at 4°C. The stability of HFBI at pH 9 was also observed daily for a period of 14 days.

Results

Production of HFBI hydrophobin in a glucose-containing medium. In order to obtain high quantities of HFBI hydrophobin, a *T. reesei* strain VTT-D-98692 overproducing HFBI was constructed by introducing multiple copies of the *hfb1* gene into the fungal genome. The expression cassette contained the *hfb1* gene under its own regulatory sequences. Expression of *hfb1* has earlier been shown to be regulated by the carbon source (Nakari-Setälä et al. 1997). High production of HFBI was achieved on glucose-based medium, which induces the expression of the *hfb1* gene. On the basis of Southern hybridization analysis, the HFBI overproducing strain VTT-D-98692 harbors three copies of the *hfb1* gene in its genome (data not shown).

The hydrophobin productivity of the HFBI overproducing strain VTT-D-98692 was compared to the wild type host strain QM9414 by cultivating both strains in a glucose-containing medium in 15-l fed-batch fermentations. The overall production of HFBI initiated after about 24 h and stabilized to a fairly constant level in 80–100 h in the 167 h cultivation of the strain VTT-D-98692 (data not shown). The wild type strain QM9414 was cultivated for 114 h according to a commonly used procedure. Although the length of the two cultivations is divergent we can still estimate the difference in their HFBI production as discussed below. The HFBI production of the overproducing strain, VTT-D-98692, was 0.60 g l^{-1} of which 81% was cell bound. The remainder of the hydrophobin was found in the culture medium. The production level of the overproducing strain was distinctly higher compared to the wild type strain production level 0.17 g l^{-1} . All the hydrophobin produced by the wild type strain QM9414 was bound to the mycelium.

Purification of HFBI. The mycelium of *T. reesei* HFBI overproducing strain VTT-D-98692 was chosen as the starting material of the purification, because most of the HFBI was attached to the fungal cell walls. HFBI was purified from the mycelium in three main steps: 1) Proteins from the mycelium were extracted with 1% SDS, 2) SDS used in the extraction buffer was removed by KCl precipitation, and 3) the purification was continued with hydrophobic interaction chromatography (HIC). The purification procedure is summarized in Figure 1.

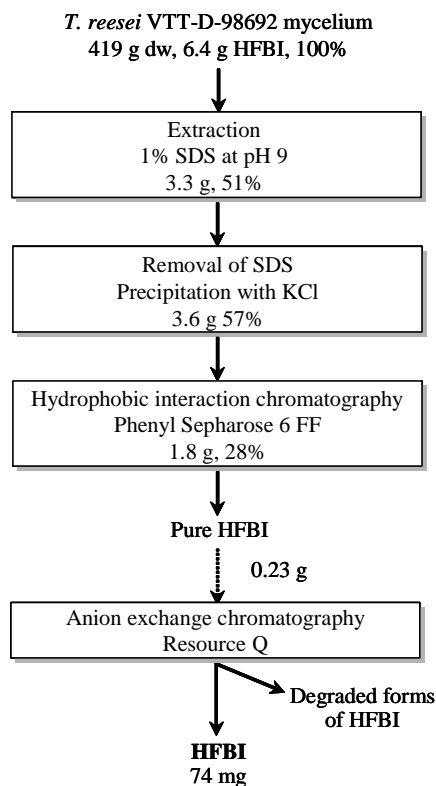


Figure 1. Purification of HFBI hydrophobin. Total amount of HFBI and HFBI yield from starting material are reported for the first three steps. Different forms of HFBI were further separated from part of the purified HFBI. Notice that the amount of HFBI in the mycelium is based on three times extraction with a buffer containing 1% SDS.

The HFBI extraction capacity of SDS increased at alkaline pH, but there was no significant improvement at SDS concentrations above 1%. The two other tested detergents, Triton X-100 and Tween 20, did not reach the extraction efficiency of SDS at the concentration of 0.6% (results not shown). Therefore, the mycelium of *T. reesei* overproducing strain VTT-D-98692 was extracted with 100 mM Tris/HCl buffer, pH 9.0, containing 1% SDS using three sequential extractions. The purification was continued only with the first extract containing most (51%, 1.0 mg ml⁻¹) of the HFBI. The HFBI concentrations of the second and the third extract were 0.4 and 0.1 mg ml⁻¹, respectively. The extracts contained yellow pigment released from the mycelium as reported previously

(Nakari-Setälä et al. 1996). Removal of SDS from the first extract improved the yield of HFBI in the subsequent chromatographic step. The KCl precipitation removed 78% of the SDS without losing any HFBI. Most of the HFBI was eluted with water from the Phenyl Sepharose 6 FF column in HIC. Pooled fractions contained 1.8 g of HFBI and gave a yield of 28% from the total HFBI obtained from the mycelium. Small amounts of the hydrophobin were also eluted with the equilibration buffer and with the lowering salt gradient. According to silver-stained SDS-PAGE gels, the HFBI preparation did not contain any contaminating proteins (Figure 2, lane 1). On the basis of amino acid analysis, the amino acid composition also corresponded to that of HFBI.

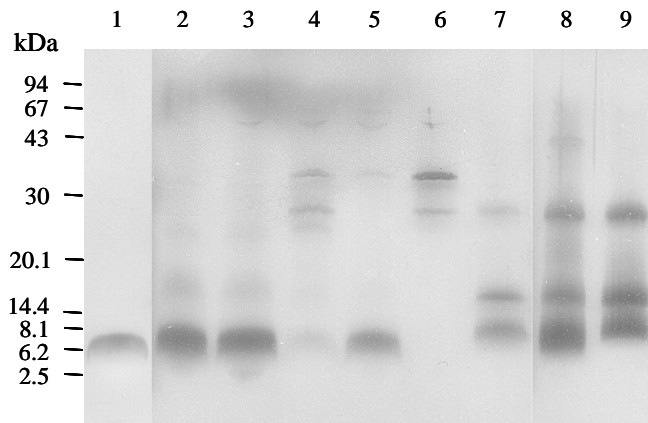


Figure 2. The purity of HFBI on 20% SDS-polyacrylamide gel and protease digestibility of HFBI. HFBI after HIC (0.05 µg) (lane 1). HFBI (0.05 µg) after incubation at 31°C for 18 h in water (lane 2), in 1% SDS (lane 3) or in the presence of the following proteases (0.02 µg) with or without 1% SDS: proteinase K (lane 4), proteinase K with 1% SDS (lane 5), chymotrypsin (lane 7), and chymotrypsin with 1% SDS (lane 8). Protease controls (0.05 µg), proteinase K (lane 6), and chymotrypsin (lane 9) were incubated in water.

The purity of the HIC preparation was further analyzed by a Protein C4 HPLC column (0.46 x 15 cm). The HPLC chromatogram revealed that the preparation was heterogeneous containing three peaks eluting close to each other (Figure 3A). The heterogeneity was preliminary examined with MALDI-TOF mass spectrometry of fractions collected from the HPLC. The preparation turned out to contain several HFBI forms differing in their molecular masses. The

molecular mass of the peak 1 (Figure 3A) seemed to be in accordance with the theoretical molecular mass of HFBI. In order to remove the degraded fractions of the protein, part of the HIC preparation was further purified with an anion exchange column Resource Q (Figure 3B), which separated the peaks from each other. The chromatographic steps removed most of the pigment, but the final HFBI preparation was still slightly yellowish.

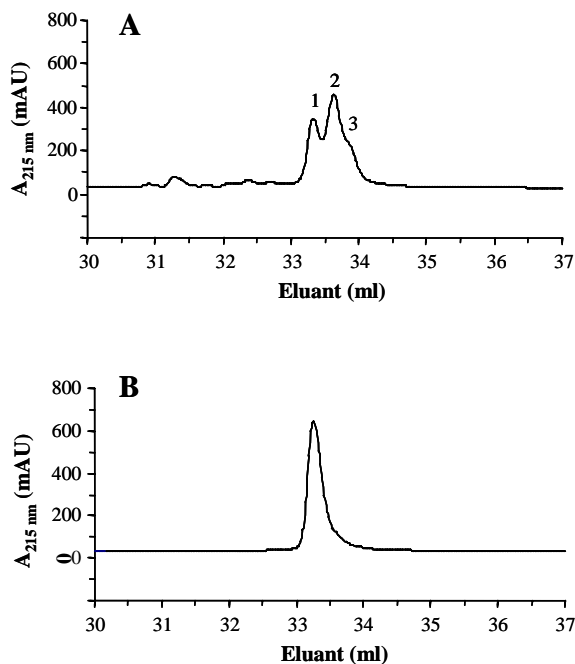


Figure 3. HPLC chromatograms (C4 column, 0.46 x 15 cm) of HFBI preparations. HFBI after HIC (A) and anion exchange chromatography (B). Different forms of HFBI eluted with 33–34 ml.

The different HFBI forms in the three peaks separated by anion exchange chromatography were further studied using MALDI-TOF mass spectrometry and N-terminal amino acid sequencing (Table 1). The molecular mass of the peak 1 (7537 ± 3 Da) is close to the theoretical molecular mass of HFBI (7533 Da). The sequence analysis of the ten amino acids showed, however, that the two first asparagines (Asn-2 and Asn-4) in the N-terminus of the mature protein had deamidated into aspartic acids. The peak 2 contained a similar deamidated form of HFBI which was cleaved after Asn-2 and therefore lacked two amino acids in

the N-terminus. Molecular masses of both the proteins were in agreement with the N-terminal sequences. The deamidation reaction of asparagine to aspartic acid is known to have an intramolecular succinimide intermediate (Wright 1991) the formation of which can lead to peptide bond cleavage at asparagine (Tyler-Cross and Schirch 1991). Therefore, the lack of two N-terminal amino acids was presumably caused by deamidation. The mass decrease of approximately 17 Da and resistance to Edman degradation in amino acid sequencing are indications of a succinimide intermediate formation from asparagine (Bischoff et al. 1993). Both the 7517 and 7521 Da compounds can be assumed to be HFBI having succinimide at position 2 and Ans-4 deamidated to aspartic acid (7517 Da) within the accuracy of the mass spectroscopy (approximately ± 3 Da). The 7320 Da compound may be the corresponding succinimide carrying form for HFBI lacking two N-terminal amino acids. These forms did not give any signal in the N-terminal sequencing. The N-terminal amino acid sequence of peak 3 showed that besides the possible intermediate forms it also contained a form where HFBI was cleaved between Lys-50 and Thr-51, probably by proteolytic degradation. No molecular mass was obtained for this peptide from the mass spectrometry.

Table 1. Different forms of HFBI purified by HIC. The HFBI forms were eluted in three peaks from the C4 HPLC column. Calculation of the theoretical molecular masses is based on the assumption of the four disulfide bonds in HFBI. Underlining indicates the deamidation of asparagine to aspartic acid.

	Molecular mass (Da)		N-terminal amino acid sequence
	Experimental	Theoretical	
HFBI		7533	SNGNGNVCPP
Peak 1	7537 \pm 3	7535	<u>SDGDGNVCPP</u>
Peak 2	7521 \pm 3		
	7335 \pm 3	7332	<u>GDGNVCPPGL</u>
Peak 3	7517 \pm 3		
	7320 \pm 3		
	nd		TGAQLCCVA

Characteristics of the purified HFBI. In order to find a reason for the heterogeneity of the HIC preparation of HFBI both the pH and protease stability of HFBI were studied. Intact HFBI was incubated at different pH values and the sample was analyzed by a Protein C4 HPLC column (0.46 x 25 cm, Vydac) covered with a 45°C heat jacket. After 4 h and 26 h incubation periods the elution profile seemed to be unaltered at pH 3, 5, and 6. Heterogeneity of the sample was discovered at pH 7 and 9 after 4 h. There was an additional peak in the chromatograms, which had a molecular mass reduction of 17 Da compared to the molecular mass of the intact HFBI according to the MALDI-TOF mass spectrometry. The decrease in the molecular mass was possibly caused by formation of a stable aspartyl succinimide intermediate in the deamidation of susceptible asparagine (Bischoff et al 1993). After 14 days approximately 40% of the HFBI had converted to the deamidation form (Figure 4A and B). Short treatments at high pH did not alter the sample significantly (Figure 4A).

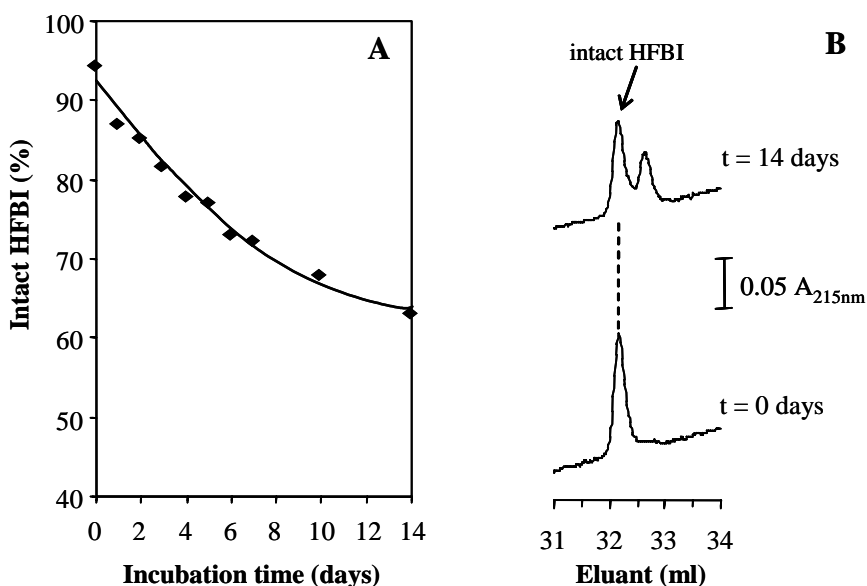


Figure 4. Deamidation of HFBI at pH 9. HFBI was incubated in 100 mM Tris/HCl buffer, pH 9 for 1–14 days at 4°C. The resulting products were analyzed by reverse phase HPLC (C4 column, 0.46 x 25 cm). The portion of intact HFBI as a function of the incubation time (A). HPLC chromatographs of the starting material HFBI in water and HFBI at pH 9 after 14 days of incubation (B).

Resistance of HFBI to a few non-specific proteases was tested and the structure of the protein was observed to be cleaved by proteinase K and α -chymotrypsin after 18 h of incubation (Figure 2) and also to a smaller extent in 2 h. Surprisingly, in the presence of 1% SDS either the proteolysis rate was greatly reduced or the proteolysis did not occur. BSA used as a control was degraded under the same conditions. Papain and pepsin treatments did not degrade HFBI. The degradation of the small HFBI fraction in the HIC preparation between Lys-50 and Thr-51 was presumably caused by proteases produced by *T. reesei*.

On the basis of the determined molecular mass and glycoprotein staining, the purified HFBI was not glycosylated. The calculated pI of HFBI is 5.7. The pI could not be determined either by IEF or chromatofocusing.

Discussion

Hydrophobins have several potential applications, many of which require large quantities of hydrophobin. The potential applications are based on the interesting characteristics rarely witnessed with other proteins: ability to change the wettability of different materials, to attach cells, proteins or other macromolecules to surfaces, and to act as biosurfactants and oil stabilizers facilitating the dispersion of hydrophobic liquids and solids (Wessels 1997). The large scale production and purification of hydrophobins has not earlier been reported. According to Wösten and co-workers (Wösten et al. 1999) *S. commune* secretes up to 0.06 g l^{-1} of class I hydrophobin SC3 to the aqueous medium. This study represents for the first time the production and purification of a hydrophobin in high quantities. The high production of the class II hydrophobin HFBI (0.6 g l^{-1}) was obtained by inserting two additional copies of the *hfb1* gene into the genome of the *T. reesei* wild type strain. Despite the fact that hydrophobins are surface active proteins (Wösten et al. 2000, Wösten and Wessels 1997) HFBI was overproduced successfully without severe problems with foaming, which may be due to most of the hydrophobin being cell bound. Excess HFBI on the mycelium did not significantly interfere with the growth of the fungus. The capacity of the fungal cell walls to bind HFBI may have been saturated when *hfb1* was overexpressed, because some HFBI (20%) was found in the culture medium unlike in the cultivation of the parent strain.

The purification methods of hydrophobins have usually been based on the characteristic solubility of hydrophobin assemblages. Mycelium-bound class I hydrophobins ABH3 of *Agaricus bisporus* (Lugones et al. 1998), MPG1 of *Magnaporthe* (Talbot et al. 1996), POH2 and POH3 of *Pleurotus ostreatus* (Ásgeirsdóttir et al. 1998), and *S. commune* SC3 (de Vries et al. 1993) have been isolated from the cell wall with 100% TFA after removal of impurities and other wall proteins with hot 1–2% SDS. The same method has been used in purification of *Agaricus bisporus* ABH1 (Lugones et al. 1996), *P. ostreatus* POH1 (Ásgeirsdóttir et al. 1998), and *S. commune* SC4 (de Vries et al. 1993) from fruiting bodies. The class II hydrophobin *T. reesei* HFBI has been extracted from the mycelia sequentially with 50 mM sodium citrate pH 6.0, containing 2% SDS, 1% SDS (100°C), and 0.1% TFA / 20% ACN (Nakari-Setälä et al. 1996), *Ophiostoma ulmi* cerato-ulmin with water (Takai and Hiratsuka 1980), and *Cryphonectria parasitica* cryparin with 60% ethanol (Carpenter et al. 1992). Purification of cerato-ulmin and cryparin were continued with ion exchange chromatography and HPLC, respectively.

In this study, HFBI was purified from the cell walls of *T. reesei* overproducing strain by a simple three step method: 1) extraction with 1% SDS at pH 9, 2) removal of SDS by precipitation, and 3) HIC. The high pH value was discovered to improve the extraction capacity possibly by increasing the negative charge of the protein. The whole purification method (extraction-precipitation-chromatography) proved to be an easy way to obtain pure hydrophobin.

The purified HFBI preparation was discovered to be heterogeneous containing different forms of HFBI deamidated in the N-terminal asparagines. Heterogeneity in a protein can affect its properties and thus complicate the application studies. Deamidation of asparagine to aspartic acid is a phenomenon known to occur in proteins and peptides (Wright 1991), and the deamidation rate is suggested to depend on the identity of the nearby amino acid residues, protein conformation, pH, ionic strength, and certain buffers (Kossiakoff 1988, Tyler-Cross and Schirch 1991). Our results suggest that the deamidation in HFBI and thereby also the heterogeneity correlate strongly with pH. By storing and purifying HFBI at pH less than 6–7 the heterogeneity caused by the deamidation could be prevented. The occurrence of the deamidation only in the two N-terminal asparagines can be explained by following glycine residues. The Asn-Gly-sequence has been shown to be particularly susceptible to deamidation

(Tyler-Cross and Schirch 1991). Heterogeneity of the purified HFBI was probably also in minor extent caused by proteases of *T. reesei*. The proteolytic degradation of HFBI might be prevented by e.g. changing the cultivation conditions of *T. reesei* not to support production of proteases, using protease inhibitors, or shortening cultivation and storage times. HFBI was found to be degraded by some proteases in the absence of SDS, but interestingly SDS was found to inhibit the action of proteases on HFBI. It can be speculated that the binding of the amphipathic SDS molecules on the hydrophobin surface hindered the interaction of the hydrophobin with proteases.

Even if HFBI is found to be rather sensitive to deamidation and also in some extent to proteases, HFBI has been proven to be very temperature stable protein. According to the CD measurements the structure of HFBI does not denature even at 90°C (S. Askolin and M. L. de Vocht, unpublished data). The disulfide bridges of HFBI may be instrumental in keeping the protein folded. Disulfide bridges have been detected in cerato-ulmin (Yaguchi et al. 1993), and it is proposed that the eight cysteine residues of other hydrophobins are also connected with disulfide bridges. The secondary structure of SC3 has been determined to share the similar thermal stability with HFBI (de Vocht et al. 2000) and therefore this characteristic may also apply to other hydrophobins.

We have reported here for the first time to be able to produce and purify gram amounts of hydrophobin HFBI, which provides scope for different application experiments in the future. The resistance of HFBI to high temperatures broadens the usefulness of the protein in different applications.

Acknowledgments

The financial support of the National Technology Agency and the Foundation of Neste Oy (S.A., personal grant) is gratefully acknowledged. Seija Nordberg is thanked for skilled technical assistance in the strain construction and Michael Bailey for fermenter cultivations. Markus Linder is thanked for the HFBI sample used in the pH stability determination and for useful discussions about deamidation.

References

- Ásgeirsdóttir, S.A., de Vries, O.M.H., Wessels, J.G.H. (1998) Identification of three differentially expressed hydrophobins in *Pleurotus ostreatus* (oyster mushroom). *Microbiology* 144: 2961–2969.
- Bischoff, R., Lepage, P., Jaquinod, M., Cauet, G., Acker-Klein, M., Clesse, D., Laporte, M., Bayol, A., Van Dorsselaer, A., Roitsch, C. (1993) Sequence-specific deamidation: isolation and biochemical characterization of succinimide intermediates of recombinant hirudin. *Biochemistry* 32: 725–734.
- Carpenter, C.E., Mueller, R.J., Kazmierczak, L.Z., Villalon, D.K., Van Alfen, N.K. (1992) Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. *Mol Plant-Microbe Interact* 4: 55–61.
- de Vocht, M.L., Reviakine, I., Wösten, H.A.B., Brisson, A., Wessels, J.G.H., Robillard, G.T. (2000) Structural and functional role of the disulfide bridges in the hydrophobin SC3. *J Biol Chem* 275: 28428–28432.
- de Vries, O.M.H., Fekkes, M.P., Wösten, H.A.B., Wessels, J.G.H. (1993) Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi. *Arch Microbiol* 159: 330–335.
- Hayashi, K. (1975) A rapid determination of sodium dodecyl sulfate with methylene blue. *Anal Biochem* 67: 503–506.
- Helin, J., Caldentey, J., Kalkkinen, N., Bamford, D.H. (1999) Analysis of the multimeric state of proteins by matrix assisted laser desorption/ionization mass spectrometry after cross-linking with glutaraldehyde. *Rapid Commun Mass Spectrom* 13: 185–190.
- Hynes, M.J., Corrick, C.M., King, J.A. (1983) Isolation of genomic clones containing the *amdS* gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. *Mol Cell Biol* 3: 1430–1439.

Kossiakoff, A.A. (1988) Tertiary structure is a principal determinant to protein deamidation. *Science* 240: 191–194.

Lin, S.-C. (1996) Biosurfactants: recent advances. *J Chem Tech Biotechnol* 66: 109–120.

Lugones, L., Bosscher, J.S., Scholtmeyer, K., de Vries, O.M.H., Wessels, J.G.H. (1996) An abundant hydrophobin (ABH1) forms hydrophobic rodlet layers in *Agaricus bisporus* fruiting bodies. *Microbiology* 142: 1321–1329.

Lugones, L.G., Wösten, H.A.B., Wessels, J.G.H. (1998) A hydrophobin (ABH3) specifically secreted by vegetatively growing hyphae of *Agaricus bisporus* (common white button mushroom). *Microbiology* 144: 2345–2353.

Mandels, M., Weber, J. (1969) The production of cellulases. *Adv Chem Ser* 95: 391–414.

Nakari, T., Alatalo, E., Penttilä, M. (1993) Isolation of *Trichoderma reesei* genes highly expressed on glucose-containing media: characterization of the *tefl* gene encoding translation elongation factor 1 α . *Gene* 136: 313–318.

Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N., Penttilä, M. (1997) Differential expression of the vegetative and spore-bound hydrophobins of *Trichoderma reesei*: cloning and characterization of the *hfb2* gene. *Eur J Biochem* 248: 415–423.

Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E., Penttilä, M. (1996) Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. *Eur J Biochem* 235: 248–255.

Nakari-Setälä, T., Penttilä, M. (1995) Production of *Trichoderma reesei* cellulases on glucose-containing media. *Appl Env Microbiol* 61: 3650–3655.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E., Knowles, J. (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61: 155–164.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Takai, S., Hiratsuka, Y. (1980) Accumulation of the material containing the toxin cerato-ulmin on the hyphal surface of *Ceratocystis ulmi*. *Can J Bot* 58: 663–668.

Talbot, N.J., Kershaw, M.J., Wakley, G.E., de Vries, O.M.H., Wessels, J.G.H., Hamer, J.E. (1996) MPG1 encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell* 8: 985–999.

Tilburn, J., Scazzocchio, C., Taylor, G.G., Zabicky-Zissman, J.H., Lockington, R.A., Davies, R.W. (1983) Transformation by integration in *Aspergillus nidulans*. *Gene* 26: 205–221.

Tyler-Cross, R., Schirch, V. (1991) Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. *J Biol Chem* 266: 22549–22556.

van Wetter, M.-A., Wösten, H.A.B., Wessels, J.G.H. (2000) SC3 and SC4 hydrophobins have distinct roles in formation of aerial structures in dikaryons of *Schizophyllum commune*. *Mol Microbiol* 36: 201–210.

Wessels, JGH (1994) Developmental regulation of fungal cell wall formation. *Ann Rev of Phytopathol* 32: 413–437.

Wessels, J.G.H. (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* 38: 1–45.

Wright, H.T. (1991) Sequence and structure determinants of nonenzymatic deamidation of asparagine and glutamine residues in proteins. *Protein Eng* 4: 283–294.

Wösten, H.A.B, de Vocht, M.L. (2000) Hydrophobins, the fungal coat unravelled. *Biochim Biophys Acta* 1469: 79–86.

Wösten, H.A.B., van Wetter, M.-A., Lugones, L.G., van der Mei, H.C., Busscher, H.J., Wessels, J.G.H. (1999) How a fungus escapes the water to grow into the air. *Curr Biol* 9: 85–88.

Wösten, H.A.B., Wessels, J.G.H. (1997) Hydrophobins, from molecular structure to multiple functions in fungal development. *Mycoscience* 38: 363–374.

Zacharius, R.M., Zell, T.E. (1969) Glycoprotein staining following electrophoresis on acrylamide gel. *Anal Biochem* 30: 148–152.

Yaguchi, M., Pusztai-Carey, M., Roy, C., Surewicz, W.K., Care, P.R., Stevenson, K.J., Richards, W.C., Takai, S. (1993) Amino acid sequence and spectroscopic studies of dutch elm disease toxin, cerato-ulmin. In: Sticklen MB and Sherald JL (eds) *Dutch Elm Disease Research, Cellular and Molecular Approaches*, Springer-Verlag, New York. Pp. 152–170.

PUBLICATION VI

**Process technological effects of
deletion and amplification of
hydrophobins I and II in
transformants of *Trichoderma reesei***

In: Appl. Microbiol. Biotechnol. 2002.

Vol. 58, pp. 721–727.

Reprinted with kind permission of Springer Science
and Business Media.

Process Technological Effects of Deletion and Amplification of Hydrophobins I and II in Transformants of *Trichoderma Reesei*

Michael J Bailey, Sanna Askolin, Nina Hörhammer, Maija Tenkanen*,
Markus Linder, Merja Penttilä and Tiina Nakari-Setälä

VTT, Box 1000, FIN-02044 VTT, Finland

*Present address:

Department of Applied Chemistry and Microbiology, PO Box 27,
FIN-00014 University of Helsinki, Finland

Corresponding author:

Michael Bailey, VTT, Box 1000, FIN-02044 VTT, Finland

e-mail: michael.bailey@vtt.fi, tel: +358-9-4565145, fax: +358-9-4552103

ABSTRACT

Transformants of the *Trichoderma reesei* strains QM9414 and Rut-C30 were constructed in which the genes for the two major hydrophobin proteins, hydrophobins I (HFBI) and II (HFBII), were deleted or amplified by molecular biological techniques. Growth parameters and foam production of the transformant strains were compared with the corresponding properties of the parent strains by cultivation in laboratory bioreactors under conditions of catabolite repression (glucose medium) or induction of cellulolytic enzymes and other secondary metabolites (cellulose and lactose media). All the transformed strains exhibited vegetative growth properties similar to those of their parent. The $\Delta hfb2$ (but not the $\Delta hfb1$) transformant showed reduced tendency to foam, whereas both strains overproducing hydrophobins foamed extensively, particularly in the case of HFBII. Enzyme production on cellulose medium was unaltered in the $\Delta hfb2$ transformant VTT D-99676, but both the $\Delta hfb2$ and HFBII-overproducing transformants exhibited somewhat decreased enzyme

production properties on lactose medium. Production of HFBI by the multi-copy transformant VTT D-98692 was almost 3-fold that of the parent strain QM9414. Overproduction of HFBII by the transformant VTT D-99745, obtained by transformation with three additional copies of the *hfb2* gene under the *cbh1* promoter, was over 5-fold compared to production by the parent strain Rut-C30. The Δ *hfb2* transformant VTT D-99676 produced a greatly increased number of spores on lactose medium compared with the parent strain, whereas the HFBII-overproducing transformant VTT D-99745 produced fewer spores.

INTRODUCTION

Trichoderma reesei, in common with many other fungi, produces extracellular hydrophobins. These low molecular weight proteins have a high cysteine content and high surface activity and amphiphilic properties (Wessels 1997, Wösten and Wessels 1997). Hydrophobin I (HFBI) of *T. reesei* is believed to be located on cell walls, whereas hydrophobin II (HFBII) is present on spore walls (Nakari-Setälä *et al.* 1996, 1997). Mature fungal spores generally have a hydrophobic surface that protects the spores from drying and aids their dispersal. In *T. reesei*, HFBI is produced in the presence of glucose in the growth medium. HFBII, along with extracellular enzymes such as cellulases and xylanases, is produced in enzyme-inducing conditions on complex plant-derived materials or *e.g.* lactose, along with extracellular enzymes such as cellulases and xylanases (Nakari-Setälä *et al.*, 1997). Whereas in liquid cultures HFBI is mainly cell wall-bound in liquid cultures (Askolin *et al.* 2001), HFBII is mainly secreted into the medium as an extracellular protein (Linder *et al.* 2001). Small microgram amounts of hydrophobins of, for example, *Fusarium* have been shown to cause gushing in beer (Kleemola *et al.* 2001). By analogy, foaming in fermentations for the production of extracellular enzymes by *T. reesei* under hydrolase-inducing conditions is probably due largely to production of HFBII.

Although hydrophobins may cause problems in both the brewing and the fermentation industries due to their foam-production properties, they also have interesting physico-chemical properties. They are able to self assemble on solid-liquid or solid-gas interphases, where they form highly stable amphipathic protein membranes (Wessels, 1994). Due to their interesting protein properties, a variety of different applications ranging from protein immobilization and surface

modification to tissue engineering have been suggested for hydrophobins (Wessels 1997, Scholtmeijer *et al.* 2001). Therefore there is interest in producing, on the one hand, fungal strains totally lacking a particular hydrophobin protein and on the other hand, strains overexpressing hydrophobin proteins for harvesting and utilisation in application studies.

In this work, transformants of *T. reesei* strains were produced in which either the *hfb1* or the *hfb2* gene was either deleted or amplified by molecular biological techniques. The transformed strains were cultivated in laboratory fermenters for assessment of the process technological effects of deletion and amplification and for harvesting of hydrophobin proteins for application experiments. The effects of deletion or amplification of the *hfb* genes were evaluated with respect to growth parameters, foaming and, in the case of the *hfb2* gene, production of extracellular enzymes.

MATERIALS AND METHODS

Strains

Trichoderma reesei QM9414 (VTT D-74075) (Mandels *et al.* 1975), its $\Delta hfb1$ transformant VTT D-99724 (Askolin *et al.*, MS in preparation) and the HFBI-overproducing transformant VTT D-98692 (Askolin *et al.* 2001) were used for investigation of the effects of deletion and amplification of the *hfb1* gene in a strain generally accepted as being rather similar to the wild-type isolate QM6a. The strain Rut-C30 (VTT D-86271) (Montenecourt and Eveleigh 1979), its $\Delta hfb2$ transformant VTT D-99676 (Askolin *et al.*, MS in preparation) and the HFBII-overproducing transformant VTT D-99745 were used to examine the effects of deletion and overexpression of HFBII in this efficient cellulase-producing strain.

DNA construction

The *hfb1* and *hfb2* genes were replaced with genes coding for *Aspergillus nidulans* acetamidase (*amdS*) and *Escherichia coli* hygromycin B phosphotransferase (*hph*) in the $\Delta hfb1$ and the $\Delta hfb2$ transformants, respectively. In pTNS24 ($\Delta hfb1$ construct) the *amdS* cassette is flanked by 2.8 and 2.0 kb of *hfb1* 5' and 3' non-

coding sequences, respectively, whereas in pTNS27 ($\Delta hfb2$ construct) the *hph* cassette is flanked with 1.2 kb and 1.75 kb of *hfb2* 5' and 3' flanking sequences (Askolin *et al.*, MS in preparation).

HFBII was overexpressed in strain Rut-C30 using the strong promoter of the cellobiohydrolase gene, *cbh1*. The *hfb2* coding region was amplified with PCR using the primers 5' AGA ACC GCG GAC TGC GCA TCA TGC AGT TCT TCG CCG TC (sense) and 5' TCA TTG GAT CCT TAG AAG GTG CCG ATG GC (antisense) and *phfb2* as a template (Nakari-Setälä *et al.* 1997). The purified PCR fragment was cut with KspI and BamHI and ligated with pMQ121 (Linder *et al.*, 2001), which was similarly digested to replace *hfb1* with *hfb2* in the vector. In the resulting expression vector pTNS31, *hfb2* is under the regulatory control of the *cbh1* promoter and terminator sequences. Prior to fungal transformation, the plasmid pTNS31 was digested with EcoRI and SphI to release the expression cassette.

Fungal transformation

T. reesei Rut-C30 was co-transformed according to Penttilä *et al.* (1987) using 10 µg of digested pTNS31 together with 3 µg of the hygromycin selection plasmid pARO21 (Aro *et al.* 2001). Transformants were streaked three times on selective medium and then transferred to potato dextrose agar for sporulation. Spore suspensions were plated out on selective medium to obtain single spore colonies for further analysis by Southern blotting.

Southern analysis

Southern blotting was used to screen fungal transformants for integration of the DNA constructs into the genome. Fungal DNA was isolated using the Easy-DNA Kit (Invitrogen). DNA of the HFBII-overproducing transformants was cut with XbaI and with NsiI, which have known recognition sequences within the *hfb2* flanking regions and within the *cbh1* promoter and terminator sequences. DNA fragments were size-fractionated by agarose gel electrophoresis and blotted onto nylon membranes using standard methods. Blots were hybridised with [α -³²P]dCTP labelled fragments of the *hfb2* coding region. Hybridisation was performed under stringent conditions according to Sambrook *et al.* (1989) and the blots were then exposed to Kodak XAR-5 X-ray film.

Cultivation methods

The cultivation media used contained, in g l⁻¹: (1) lactose (Valio Ltd, Finland) or glucose (Cerestar, Denmark) 40, peptone 4.0, yeast extract 1.0, KH₂PO₄ 4.0, (NH₄)₂SO₄ 2.8, MgSO₄·7H₂O 0.6, CaCl₂·2H₂O 0.8 (sterilised separately in distilled water), with 2x trace elements (Mandels and Weber 1969), or (2) Solka floc cellulose (James River, Hackensack, N.J.) 40, distiller's spent grain 20, KH₂PO₄ 5.0, (NH₄)₂SO₄ 5.0. Inocula (spore suspensions in 50% glycerol at -80°C) were grown on the same medium with only 20 g l⁻¹ carbon source, buffered with 15 g l⁻¹ KH₂PO₄, in two stages of 1x200 ml (2 days, 200 rpm, 30°C) and 5x200 ml (to 15 or 20 litres) or 3x200 ml (to 10 l) (2.5% v/v transfer, 1 day, 200 rpm, 30°C). The glucose concentration was kept within the limits of 10–25 g l⁻¹ by addition of sterile 50% glucose solution when glucose was used as a carbon source.

The fermenters used were Chemap LF 20 (working volume 15 l) and CF 2000 (10 l) fermenters and in one cultivation of the *Δhfb2* transformant VTT D-99676 a Braun Biostat C30 (20 l). The LF 20 fermenter was not equipped with automatic control of dissolved oxygen (DO). DO control (>20%) in the CF 2000 fermenter was by agitation (500–800 rpm) and in the Braun Biostat by both agitation and enrichment of the incoming air with pure oxygen (0–25%). Oxygen enrichment reduces the need for agitation to maintain the setpoint DO level and therefore the tendency to foaming. Other cultivation conditions were: T=28°C, pH 4–5 (lower limit controlled by addition of NaOH in the cultivations with glucose feeding and by NH₄OH in cultivations in inducing conditions, upper limit by H₃PO₄), aeration 0.5 l l⁻¹ min⁻¹ overall gas flow. Foaming was controlled by automatic addition of Struktol J633 polyoleate antifoam agent (Schill & Seilacher, Germany). All media contained 0.2 ml l⁻¹ antifoam agent (AF) to prevent foaming during sterilisation.

Analyses

Measurement of biomass on glucose and lactose media was performed gravimetrically after filtration of a known volume of culture through a tared Whatman GF/A filter, rinsing with the same volume of distilled water, drying at 103°C for 6 h or overnight and reweighing. Glucose and lactose were assayed using GOD-Perid (Roche, Germany) and lactose/β-D-galactose (Boehringer-

Mannheim, Germany) analysis kits, respectively, following the manufacturer's instructions. The assays for overall cellulase activity (filter paper units, FPU) and β -1,4(1,3)-endoglucanase using hydroxyethyl cellulose (HEC) as substrate (IUPAC 1987) and for endo- β -1,4-xylanase (XYL, Bailey *et al.* 1992) were carried out at pH 5.0 using the standard methods. Soluble protein was assayed by the method of Lowry *et al.* (1951) after precipitation of proteins with an equal volume of 10% trichloroacetic acid.

Hyphal thickness was determined as described by Askolin *et al.* (MS in preparation). The culture samples were fixed in 1% formaldehyde, stained with lactophenol blue (Fluka 61335, Switzerland), and examined with a light microscope and Analysis software imaging tool (Soft Imaging System).

Assay of HFBI and HFBII

HFBI and HFBII were quantified by analytic reversed phase high performance liquid chromatography (RP-HPLC). HFBI was assayed using a Resource RPC column (Pharmacia Biotech, Sweden) with a 0–60% acetonitrile gradient containing 0.1% trifluoroacetic acid and detection of HFBI at 215 nm (Askolin *et al.* 2001). HFBI was extracted from the mycelium three times with 100 mM sodium citrate / citric acid, pH 6.0 containing 1% SDS and the extracts were analysed by HPLC.

Prior to analytical HPLC, HFBII was extracted and purified from the culture supernatant using 2% of the non-ionic surfactant Berol 532 (Linder *et al.* 2001). The extraction was made to avoid problems caused by closely eluting peaks. Berol 532 (20 mg; Akzo-Nobel, Sweden) was added to 10 ml of supernatant and the solution was mixed gently for 1 hour at 21°C on a laboratory shaker. The tubes were centrifuged for 10 minutes at 5000 \times g and the lower phase was removed. One millilitre of 50 mM acetate buffer pH 5.5 and 150 μ l isobutanol were added to the remaining surfactant phase and the centrifugation was repeated. HFBII was quantified from the lower aqueous phase using a 4.6 mm \times 25 cm Vydac protein C4 analytical column (Vydac, Hesperia, Calif.) using a stepwise gradient of 0.1% trifluoroacetic acid in water (A) and in acetonitrile (B). The steps of the gradient were: 0–13 ml 0% B, 13–18 ml 0–20% B, 18–48 ml 20–70% B and 48–51 ml 70–100% B. Detection was by UV at 205 nm, 215 nm and 280 nm. Concentrations were calculated from the integrated peaks using a calibration curve.

RESULTS

Effect of HFBI on growth parameters, protein production and foaming of *T. reesei* QM9414 in fermenter cultivations

To investigate the effect of HFBI on growth parameters, protein production and foaming in the cultivation of *T. reesei*, the host strain QM9414, its $\Delta hfb1$ transformant VTT D-99724 and the HFBI-overproducing transformant VTT D-98692, carrying three copies of the native *hfb1* gene with its own promoter, were cultivated in the 10 l fermenter for 92 h. Since glucose induces the expression of HFBI, glucose medium was used in these fed-batch cultivations. The measured growth parameters of all three strains were very similar. The maximum value of biomass production (Table 1) had been reached by all the strains at 92 h and was within the normal range of variation observed for the host strain QM9414. Mycelial morphology was studied under a light microscope after fixation in 1% formaldehyde and lactophenol blue staining. No differences in morphology or in hyphal thickness were observed between the three strains. According to these results HFBI has no effect on growth or hyphal morphology of *T. reesei* in fermenter cultivations.

Table 1. Consumption of antifoam agent (AF) and maximum production of biomass, total soluble extracellular protein and HFBI by Trichoderma reesei QM9414, its $\Delta hfb1$ transformant VTT D-99724 ($\Delta hfb1$) and the HFBI-overproducing transformant VTT D-98692 ($hfb1+++$) in 92 h laboratory fermenter cultivations on glucose medium. The HFBI concentration includes both soluble and mycelium-bound HFBI. The assay of mycelium-bound HFBI was based on three sequential extractions with a buffer containing 1% SDS. nd, not determined.

Strain	Biomass g l ⁻¹	AF ml l ⁻¹	Soluble protein g l ⁻¹	HFBI g l ⁻¹
QM9414	32.9	2.3	0.2	0.5
QM9414 $\Delta hfb1$	37.0	2.7	0.1	nd
QM9414 $hfb1+++$	37.8	4.7	0.2	1.4

HFBI concentrations were assayed both from mycelial extracts and culture supernatants using HPLC (Askolin *et al.* 2001). Mycelium was extracted three times with 100 mM sodium citrate buffer, pH 6.0, containing 1% SDS. The HFBI-overproducing strain produced 1.4 g l⁻¹ HFBI, which was almost three times higher than the amount produced by the host strain QM9414. Most of the HFBI was cell wall-bound in both strains (Fig. 1). The host strain secreted 0.2 g l⁻¹ total soluble protein into the culture medium, half of which was HFBI. This explains the lower total protein concentration (0.1 g l⁻¹) in the culture medium of the $\Delta hfbl$ transformant (Table 1).

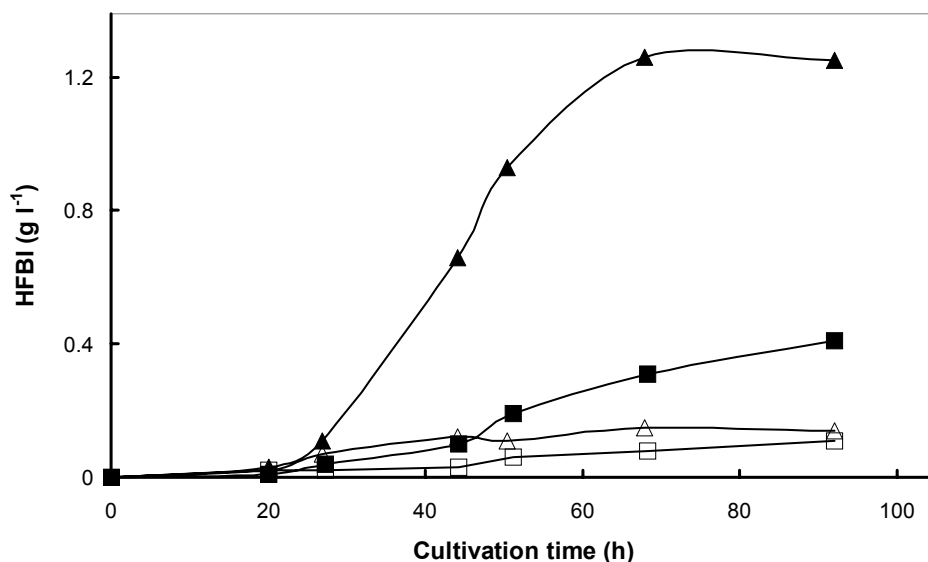


Figure 1. Distribution of HFBI between mycelium and growth medium. HFBI on mycelium (closed symbols) and in growth medium (open symbols) of *Trichoderma reesei* QM9414 (squares) and the HFBI-overproducing strain VTT D-98692 (triangles), respectively. The amount of HFBI on mycelium was estimated by three mycelial extractions with a buffer containing 1% SDS.

Foaming of bioreactor cultures was compared on the basis of consumption of antifoam agent (AF). The medium contained 0.2 ml l⁻¹ AF prior to sterilisation. Addition of AF was automatically controlled during the cultivations. However, the fermentation broth was supplemented manually with 2 ml l⁻¹ AF after 21 h of cultivation in order to minimize the possible effects of varying amounts of AF added automatically at different times. A clear difference in consumption of AF

was observed between the host strain QM9414 and the HFBI-overproducing strain (Table 1). The overproducing strain consumed almost twice the amount of antifoam agent (124 ml per mg dry weight) as that required by the host strain (70 ml per mg dry weight). However, deletion of the *hfb1* had no effect on antifoam consumption (73 ml per mg dry weight) compared to the host strain. These results show that the HFBI protein causes foaming in fermentation cultivations of *T. reesei* when produced in high amounts into the glucose-containing medium. Growth parameters and fungal morphology were not affected by HFBI overproduction.

Effects of deletion and amplification of the *hfb2* gene on growth parameters, sporulation, foaming and protein production

Growth

According to the results of dry weight measurements, carbon source consumption and culture pH, growth of the $\Delta hfb2$ transformant VTT D-99676 on lactose medium in the 15 litre fermenter was similar to that of the parental strain Rut-C30 (Fig. 2). Maximum dry weights reached by Rut-C30 and VTT D-99676 were 16.5 (55 h) and 17.3 (47 h) g l⁻¹, respectively. As a result of manual adjustment of agitation (to 400, 600 or 800 rpm at different cultivation stages), the level of DO in both cultivations was above 20% at all times (results not shown). Growth of the HFBI-overproducing transformant VTT D-99745 on lactose medium in the 10 litre fermenter with DO control (by agitation only) was also similar to that of the control strain Rut-C30. The curves of lactose consumption and culture pH were almost identical to those of the control (Fig. 2). The apparently increased level of maximum biomass (22 g l⁻¹ compared to 16 g l⁻¹ for the control) was almost certainly an artefact caused by the observed incomplete rinsing of the considerable amount of AF present in the culture (see below) in the dry weight analysis. This conclusion is supported by the fact that the apparent increase in biomass between 50 and 72 h occurred after exhaustion of lactose from the medium (Fig 2). Maximum biomass production by the transformant VTT D-99745 in the repeat cultivation with oxygen enrichment and reduced addition of AF was 16.4 g l⁻¹ (result not shown), i.e. very similar to the corresponding value for the control strain Rut-C30.

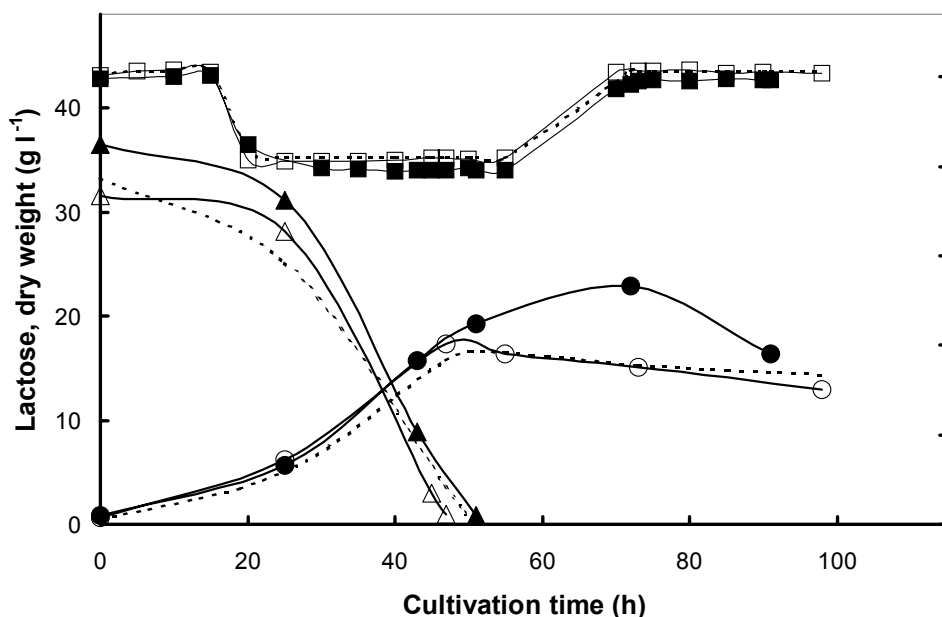


Figure 2. Lactose consumption (triangles), biomass formation (circles) and pH changes (squares) in fermenter cultivations of *Trichoderma reesei* Rut-C30 (dotted lines), its $\Delta hfb2$ transformant VTT D-99676 (open symbols) and its HFBII-overproducing transformant VTT D-99745 (filled symbols) on lactose medium in a laboratory fermenter.

On cellulose medium, estimation of growth was limited to measurement of DO and changes in culture pH. No attempt was made to analyse biomass on the complex lignocellulosic medium that contained solid particles interfering with the measurement. According to the on-line parameters, growth of the $\Delta hfb2$ transformant VTT D-99676 was similar to that of the control strain Rut-C30 (results not shown).

Sporulation

On lactose medium, the culture of the $\Delta hfb2$ transformant VTT D-99676 became intensely green very soon after consumption of the carbon source. The results of microscopy and of agar plating on Plate Count Agar confirmed that this was not due to contamination. Microscopical observation revealed that the reason for the observed green colour was extensive sporulation of the culture. In contrast, production of spores by the control strain Rut-C30 was less intense, although

some spore formation was clearly evident. In logical progression, the culture of the HFBII-overproducing strain VTT D-99745 produced no free spores visible under the microscope. Similar differences in sporulation behaviour compared with the control strain were observed in repeat cultivations of both transformants (results not shown). Further examination of the effects of deletion and amplification of the *hfb2* gene on sporulation was not made in this work.

Foaming

Deletion of the *hfb2* gene had a considerable effect on foaming in the fermenter cultivations. This was particularly true in the case of the cellulose medium: consumption of AF was 3.7 ml l⁻¹ in the case of the control strain compared to only 0.4 ml l⁻¹ for the $\Delta hfb2$ transformant (Table 2). Higher consumption of AF in the cultivation of the control strain was not due to fouling of the foam probe: no over-feeding of antifoam agent occurred at any stage of the cultivation. Foaming was clearly less of a problem on the lactose medium, but the difference between the control and $\Delta hfb2$ strains was evident: whereas the control strain required 0.3 ml l⁻¹ AF, the $\Delta hfb2$ transformant did not consume any AF during the cultivation (Table 2).

Table 2. Maximum production of total soluble extracellular protein, enzyme activities and HFBII and consumption of antifoam agent (AF) by *T. reesei* Rut-C30, its $\Delta hfb2$ transformant VTT D-99676 ($\Delta hfb2$) and its HFBII-overproducing transformant VTT D-99745 ($hfb2+++$) on cellulose (7 day cultivations) and lactose media (4 days). HEC activity against hydroxyethyl cellulose, FPU filter paper units; XYL xylanase, nd not determined. The observed specific production rates of total soluble protein ($\text{g l}^{-1} \text{h}^{-1}$) and HEC activity ($\text{nkcat ml}^{-1} \text{h}^{-1}$) during the linear production phase are also shown.

Strain	Medium	Protein g l^{-1}	$\text{g l}^{-1} \text{h}^{-1}$	HEC nkcat ml^{-1}	$\text{nkcat ml}^{-1} \text{h}^{-1}$	FPU u ml^{-1}	XYL nkcat ml^{-1}	HFBII mg l^{-1}	AF ml l^{-1}
Rut-C30	cellulose	13.9	0.16	1,600	21.6	10.9	1,870	nd	3.7
Rut-C30 $\Delta hfb2$	cellulose	16.4	0.16	1,715	18.7	9.0	2,240	nd	0.4
Rut-C30	lactose	7.6	0.23	890	23.1	4.5	640	30	0.3
Rut-C30 $\Delta hfb2$	lactose	6.5	0.21	690	21.1	4.1	500	0	0.0
Rut-C30 $hfb2+++$	lactose	4.0	0.08	290	5.9	1.3	180	100 ^a	24.0
Rut-C30 $hfb2+++$	lactose ^b	6.8	0.13	580	11.0	2.9	530	240	2.5

^aResult uncertain due to interference by the high content of surface-active antifoam agent

^bRepeat cultivation in a fermenter with DO control by agitation and oxygen enrichment, see text.

Due to the severe problems anticipated in the cultivation of an HFBII-overproducing strain on cellulose medium, the transformant VTT D-99745 was grown only on the lactose medium. Even on this medium, in the fermenter equipped with DO control by agitation (but not by oxygen enrichment, see Materials and Methods), consumption of AF was as high as 24 ml l⁻¹ (Table 2). On the basis of frequent inspection of the condition of the headspace and of the probe, there was never any evidence of unnecessary addition of AF due to fouling of the probe. Evidently, the observed phenomenon was due to overproduction of HFBII.

A repeat cultivation of the strain VTT D-99745 overproducing HFBII was performed in the 20 litre fermenter equipped with DO control by agitation and oxygen enrichment. In this experiment the maximum agitation required for DO control to >20% was limited to 450 rpm (cf. 800 rpm in the previous cultivation) by using oxygen enrichment up to a maximum of 25% of the incoming air. For the greater part of this cultivation, the agitation was below 400 rpm (min 250 rpm). Although the culture evidently had a strong tendency to foam formation, the low agitation sufficed to ensure that addition of AF could be kept within an acceptable range. The total addition of AF to the culture was 2.5 ml l⁻¹ (Table 2), approximately one tenth of that in the first cultivation with this transformant.

Enzyme production

Production of cellulases (soluble protein, HEC) by the *Ahfb2* transformant VTT D-99676 on cellulose medium was similar to that by the control strain Rut-C30 (Fig. 3). The maximum values of all the enzyme activities assayed, along with soluble protein, are displayed in Table 2. The level of xylanase production in the *Ahfb2* transformant was slightly elevated, although the result for FPU (a rather imprecise assay with an accuracy of only ca. ± 20%) was somewhat below that obtained with the control strain Rut-C30. The observed differences in production of enzyme activities and soluble protein by the control and transformant strains probably represented normal between-batch variation rather than any significant differences in enzyme production properties.

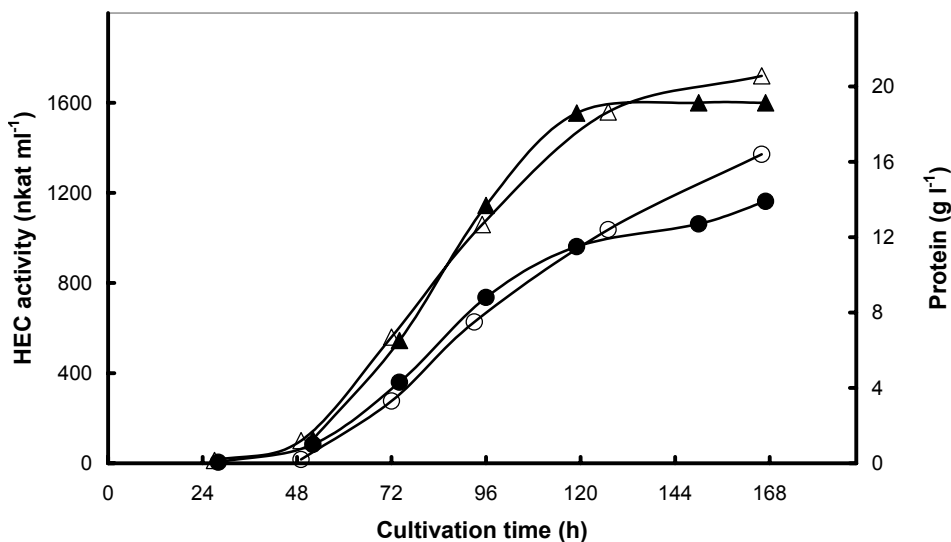


Figure 3. Production of cellulase (HEC, triangles) and total soluble protein (circles) by *Trichoderma reesei* Rut-C30 (open symbols) and its $\Delta hfb2$ transformant (filled symbols) on cellulose-spent grain medium in a laboratory fermenter.

On the lactose medium, enzyme production by the $\Delta hfb2$ transformant was slightly poorer than by the control strain (Table 2). This result was confirmed in repeat cultivations of both strains on the same medium (data not shown). Maximum specific production rates HEC and protein by the transformant strain were similar to those for Rut-C30 (see Table 2), but the length of the linear production phase was shorter (results not shown). Note that whereas the cultivations on cellulose medium were continued for 7 days, a cultivation time of 4 days was sufficient for the attainment of maximum activities on lactose in all cases.

Production of cellulases by the HFBII-overproducing strain VTT D-99745 in the first cultivation on lactose medium was considerably poorer than by the control strain (Table 2). In the repeat cultivation in the fermenter with DO control by oxygen enrichment and lower addition of antifoam agent, the production was clearly improved. However, some depression of enzyme production was still observed (Table 2).

Production of HFBII

Production of HFBII was investigated on the lactose medium. In the first cultivation of the HFBII-overproducing transformant VTT D-99745, with heavy addition of AF (see above), the observed production of about 100 mg l⁻¹ was approximately 3-fold that of the control cultivation (30 mg l⁻¹, see Table 2), despite the lower level of enzyme production. When the cultivation was repeated with lower agitation and reduced addition of AF agent, the production level of HFBII was still higher, at 240 mg l⁻¹. The HFBII-overproducing transformant contains both endogenous *hfb2* and the three extra copies of *hfb2* introduced to the genome under the *cbh1* promoter (data not shown).

DISCUSSION

Hydrophobins are highly surface active proteins of filamentous fungi (Wösten and de Vocht 2000), which were first isolated and characterised in the 1990s (Wessels 1997). Foaming can cause serious problems in fermentation processes, for example, by blocking the air outlet filter and by displacing biomass from the fermenter. Repeated additions of AF decrease the availability of DO, due to the collapse of bubble structures within the culture broth, necessitating increased agitation and thereby increasing the tendency to foam. AF in the culture filtrate may also cause severe problems in the various unit operations of downstream processing, e.g. in membrane filtration. Our results show that in the case of *T. reesei*, the hydrophobins HFBI and especially HFBII play a major role in foaming of the culture broth. However, in this study we showed that foaming can be controlled even in the case of overproduction of hydrophobin protein for harvesting. The extensive foaming of the HFBII-overproducing transformant was effectively reduced by lower agitation achieved with oxygen enrichment, a technique already adaptable to production scale.

Deletion of *hfb2* resulted in a clear decrease in consumption of AF, whereas amplification of the gene caused very high consumption. In the experiment with the highest consumption of AF, massive addition occurred during the phase of enzyme production, after the growth phase (result not shown). This was logical because the excess production of HFBII was governed by the *cbh1* promoter, the homologous product of which normally appears in the culture filtrate after the

phase of maximum growth. The impact of HFBI on foaming was not as pronounced as in the case of HFBI^{II}. This may be due to the fact that most of the HFBI produced was cell wall-bound. In addition, the foam stabilisation characteristics of these two proteins may differ. HFBI was observed to affect foaming only when it was produced in high amounts by the HFBI-overproducing transformant, leading to increased secretion of HFBI into the culture medium. The AF consumption of the $\Delta hfb1$ transformant and the host strain were similar. It is possible that this low level of foaming was in fact caused mainly by factors other than HFBI, and that the effect of *hfb1* deletion was therefore difficult to detect within the limits of the accuracy of the measurement method used.

No major differences were observed in growth parameters, biomass formation or hyphal thickness between the $\Delta hfb1$ transformant, the HFBI-overproducing transformant and the host strain in the controlled fermentation cultivation. The deletion of *hfb1* was previously shown to decrease biomass formation and hyphal thickness in the early growth phase of the fungus in shake flask cultivations (Askolin *et al.*, MS in preparation). This effect was possibly not observed in the fermenter cultivations because the early growth phase had already been superseded during the inoculation cultivations and the growth was effectively controlled in the fermenter, in contrast to shake flasks.

On the basis of the experiments reported in this work, neither deletion nor amplification of the *hfb2* gene had a significant effect on the rate or final level of biomass accumulation on lactose medium in bioreactor cultivation. However, a clear difference was observed in sporulation. Deletion of *hfb2* caused early and massive sporulation, whereas amplification of the gene appeared to result in decreased ability of the fungus to produce free spores in the liquid culture medium. On agar plates, the outward appearance of colonies of all three strains was rather similar, including spore production.

Production of cellulases was apparently unchanged on cellulose medium when the *hfb2* gene was deleted, but a slight decrease in enzyme activities was observed on lactose medium. The maximum rate of enzyme production on lactose medium was approximately the same in the $\Delta hfb2$ transformant VTT D-99676 as in the control strain Rut-C30 (Table 2) but the length of the linear production phase was shorter, probably due to the observed early onset of sporulation. Improved and considerably prolonged production of cellulases by

the *Δhfb2* strain VTT D-99676 in continuous cultivation with process control to prevent the onset of sporulation will be reported in a later work. Interestingly, amplification of the *hfb2* gene also caused a slight decrease in enzyme production on lactose medium, even in the repeat cultivation in which AF consumption was successfully minimised by oxygen enrichment of the incoming air. This may have been due to the depressing effect of overproduction of HFBII protein under the *cbh1* promoter on homologous enzyme production. It has been suggested that several copies of the *cbh1* promoter may be sufficient to titrate out regulatory or transcription factors needed for high enzyme production (Karhunen *et al.*, 1993, Paloheimo *et al.*, 1993, Margolles-Clark *et al.*, 1996). The transformant VTT D-99745 carries three extra copies of the *cbh1* promoter in addition to that contained in the endogenous *cbh1* gene.

Foaming in fermentation processes based on *T. reesei* Rut-C30 was clearly demonstrated to be due mainly to production of HFBII, rather than to the much greater amount of other extracellular proteins (cellulases and hemicellulases) produced by this organism on inducing media. Traditionally, cellulase production has generally been considered to be the prime cause of foaming, rather than the only relatively recently identified hydrophobins. However, despite the strong foaming tendency of HFBII, this protein can be successfully overproduced (by even 8-fold) in a bioreactor equipped with oxygen enrichment to avoid excessive agitation and therefore minimise foaming. The HFBII produced in this work using the overproducing strain VTT D-99745 will be purified for use in application studies.

ACKNOWLEDGEMENTS

The authors thank Tarja Hakkarainen for skillful technical assistance. Financial assistance (to S.A.) from Neste Oy's Foundation is gratefully acknowledged.

REFERENCES

Aro, N., Saloheimo, A., Ilmén, M., Penttilä, M. (2001) ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J Biol Chem 276: 24309–24314.

Askolin, S., Nakari-Setälä, N., Tenkanen, M. (2001) Overproduction, purification, and characterization of the *Trichoderma reesei* hydrophobin HFBI. Appl Microbiol Biotechnol 57: 124–130.

Bailey, M.J., Biely, P., Poutanen, K. (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23: 257–270.

IUPAC (International Union of Pure and Applied Chemistry) (1987) Measurement of cellulase activities. Pure Appl Chem 59: 257–268.

Karhunen, T., Mäntylä, A., Nevalainen, K.M.H., Suominen, P.L. (1993) High frequency one-step gene replacement in *Trichoderma reesei*. I: Endoglucanase overproduction. Mol Gen Genet 241: 515–522.

Kleemola, T., Nakari-Setälä, T., Linder, M., Penttilä, M., Kotaviita, E., Olkku, J., Haikara, A. (2001) Characterisation and detection of the gushing factors produced by fungi. Proceedings of the European Brewing Convention 28th Congress, Budapest 2001, contribution no. 12, 129–138, CD-ROM, ISBN 90-70143-21-6.

Linder, M., Selber, K., Nakari-Setälä, T., Qiao, M., Kula, M.-R., Penttilä, M. (2001) The hydrophobins HFBI and HFBI from *Trichoderma reesei* showing efficient interactions with nonionic surfactants in aqueous two-phase systems. Biomacromolecules 2: 511–517.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Mandels, M., Weber, J. (1969) The production of cellulases. Adv Chem Ser 95: 391–414.

Mandels M.M., Sternberg D., Andreotti R.E. (1975) Growth and cellulase production by *Trichoderma*. In: Bailey M.J., Enari T.-M., Linko M. (eds) Symposium on enzymatic hydrolysis of cellulose. Finnish National Fund for Research and Development (SITRA), Helsinki, Finland, 1975. Pp. 81–109.

Margolles-Clark, E., Hayes, C.K., Harman, G.E., Penttilä, M. (1996) Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. *Appl Environ Microbiol* 62: 2145–2151.

Montenecourt, B.S., Eveleigh, D.E. (1979) Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. *Adv Chem Ser* 181: 289–301.

Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E., Penttilä, M. (1996) Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. *Eur J Biochem* 235:248–255.

Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N., Penttilä, M. (1997) Differential expression of the vegetative and spore-bound hydrophobins of *Trichoderma reesei*: cloning and characterization of the *hfb2* gene. *Eur J Biochem* 248: 415–423.

Paloheimo, M., Miettinen-Oinonen, A., Torkkeli, H., Nevalainen, H., Suominen, P. (1993) Enzyme production by *Trichoderma reesei* using the *cbh1* promoter. In: Suominen, P., Reinikainen, T. (eds) Proceedings of the 2nd TRICEL Symposium on *Trichoderma reesei* cellulases and other hydrolases. Foundation for Biotechnical and Industrial Fermentation Research 8, Espoo, Finland. Pp. 229–238.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E., Knowles J. (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61: 155–164.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular cloning: a Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scholtmeijer, K., Wessels, J.G.H., Wösten, H.A.B. (2001) Fungal hydrophobins in medical and technical applications. *Appl Microbiol Biotechnol* 56:1–8.

Wessels, J.G.H. (1994) Developmental regulation of fungal cell wall formation. *Annu Rev of Phytopathol* 32: 413–437.

Wessels, J.G.H. (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* 38: 1–45.

Wösten, H.A.B., de Vocht, M.L. (2000) Hydrophobins, the fungal coat unravelled. *Biochim Biophys Acta* 1469: 79–86.

Wösten, H.A.B., Wessels, J.G.H. (1997) Hydrophobins, from molecular structure to multiple functions in fungal development. *Mycoscience* 38: 363–374.

Author(s) Askolin, Sanna			
Title Characterization of the <i>Trichoderma reesei</i> hydrophobins HFBI and HFBII			
Abstract Hydrophobins are surface active proteins produced by filamentous fungi. They fulfil a wide variety of functions in fungal growth and development. Properties and biological roles of the <i>Trichoderma reesei</i> hydrophobins HFBI and HFBII were studied in this work. In addition, the hydrophobins were crystallized and their effects on the bioreactor cultivation of <i>T. reesei</i> were evaluated. High production levels of HFBI and HFBII were obtained by introducing additional copies of the <i>hfb1</i> or <i>hfb2</i> genes into the genome of <i>T. reesei</i> . HFBI was produced up to 1.4 grams per liter, the highest production level of hydrophobin hitherto reported. HFBI and HFBII were purified and crystallized for structural analysis. Behaviour of the class II hydrophobins HFBI and HFBII and the class I hydrophobin SC3 of <i>Schizophyllum commune</i> was studied at various interfaces. All the hydrophobins were surface active. HFBI and HFBII reduced the surface tension of water faster than SC3. In contrast to SC3, self-assembly of HFBI and HFBII at water-air interfaces did not change the circular dichroism spectra. In electron microscopy, no clear ultrastructure of the dried class II hydrophobin film was observed, either. Differences are proposed to be due to the divergent size and shape of the hydrophobic parts of these proteins. Addition of Teflon changed the circular dichroism spectra of HFBI and HFBII, indicating formation of α helix structure. Both HFBI and SC3 rendered Teflon completely wettable. All the hydrophobins stabilized oil emulsions. The presence of HFBI or HFBII affected the solubility of SC3, indicating interaction between these hydrophobins. The <i>hfb1</i> and <i>hfb2</i> genes were deleted in <i>T. reesei</i> . The $\Delta hfb1$ strain formed no aerial hyphae in static liquid cultures. Addition of purified HFBI to the medium or expressing the gene encoding SC3 in the $\Delta hfb1$ strain restored the aerial hyphae formation. Colonies of $\Delta hfb1$ had a wettable and fluffy phenotype. In shaken liquid cultivation, biomass formation of $\Delta hfb1$ was slower compared with the parent strain. Sporulating colonies of $\Delta hfb2$ were wettable and sporulation was only 50% of that of the parent strain. These results indicate that HFBI facilitates aerial growth of <i>T. reesei</i> , whereas HFBII is involved in sporulation. The strains overproducing HFBI or HFBII foamed extensively in bioreactor cultivations, especially the HFBII-overproducing strain. Foaming of the $\Delta hfb2$ strain (but not $\Delta hfb1$) was lower compared with the parent. The main cause of foaming in the bioreactor cultivations of <i>T. reesei</i> Rut-C30 was shown to be HFBII			
Keywords filamentous fungi, <i>Trichoderma reesei</i> , surface active proteins, hydrophobins, HFBI, HFBII, purification, characterization, crystallization, microbial surfactant, gene deletion, biological function, foaming			
ISBN 951-38-6836-2 (soft back ed.) 951-38-6837-0 (URL: http://www.vtt.fi/publications/index.jsp)			
Series title and ISSN VTT Publications 1235-0621 (soft back ed.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)			Project number
Date May 2006	Language English, Finnish abstr.	Pages 99 p. + app. 38 p.	Price C
Name of project		Commissioned by	
Contact VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Finland Phone internat. +358 20 722 111 Fax +358 20 722 7071		Sold by VTT P.O.Box 1000, FI-02044 VTT, Finland Phone internat. +358 20 722 4404 Fax +358 20 722 4374	

Tekijä(t) Askolin, Sanna			
Nimeke <i>Trichoderma reesei</i> HFBI- ja HFBII-hydrofobiinien karakterisointi			
Tiivistelmä Hydrofobiinit ovat rihmamaisten sienten tuottamia pinta-aktiivisia proteiineja. Niillä on useita tehtäviä sienten kasvussa ja kehityksessä. Tässä työssä tutkittiin <i>Trichoderma reesei</i> HFBI- ja HFBII-hydrofobiinien ominaisuuksia ja biologisia rooleja. Lisäksi hydrofobiinit kiteytettiin, ja niiden vaikutusta <i>T. reesei</i> bioreaktorikasvatuksiin arvioitiin. Korkeat HFBI- ja HFBII-tuottotasot saavutettiin lisäämällä ylimääräisiä kopioita <i>hfb1</i> - tai <i>hfb2</i> -geenejä <i>T. reesei</i> perimään. HFBI:ä tuotettiin jopa 1,4 grammaa litraa kohden, mikä on korkein tähän mennessä raportoitu hydrofobiinin tuottotaso. HFBI ja HFBII puhdistettiin ja kiteytettiin rakenneanalyysiä varten. Luokan II hydrofobiinien HFBI ja HFBII sekä <i>Schizophyllum commune</i> luokan I hydrofobiinin SC3 toimintaa tutkittiin useilla rajapinnoilla. Kaikki hydrofobiinit olivat pinta-aktiivisia. HFBI ja HFBII alensivat veden pintajännityksen nopeammin kuin SC3. HFBI:n ja HFBII:n kerääntyminen vesi- ja ilmafaasiin väliin ei muuttanut niiden CD-spektrejä, toisin kuin SC3:lla. Elektronimikroskopiolla ei myöskään havaittu selkeää ultrarakennetta kuivuneilla HFBI- ja HFBII-hydrofobiinikalvoilla. Erojen ehdotetaan johtuvan näiden proteiinien hydrofobisten alueiden erilaisesta koosta ja muodosta. Teflonin lisäys muutti HFBI:n ja HFBII:n CD-spektriä viitaten α -kierrekenteen muodostumiseen. Sekä HFBI että SC3 tekivät teflonista täysin kastuvan. Kaikki hydrofobiinit stabiloivat öljyemulsioita. HFBI:n tai HFBII:n läsnäolo vaikutti SC3:n liukoisuuteen, mikä viittaa näiden hydrofobiinien väliseen vuorovaikutukseen. <i>T. reesei</i> stä poistettiin <i>hfb1</i> - ja <i>hfb2</i> -geenit. Staattisissa nestekasvustoissa $\Delta hfb1$ -kanta ei muodostanut ilmarihmoja. Puhdistetun HFBI:n lisääminen elatusaineeseen tai SC3-geenin ilmentäminen $\Delta hfb1$ -kannassa palautti ilmarihmojen muodostumisen. $\Delta hfb1$ -pesäkkeillä oli vettyvä ja pörröinen fenotyyppi. Ravistelluissa nestekasvatuksissa $\Delta hfb1$:n biomassan muodostus oli isäntäkantaa hitaampaa. Itiöivät $\Delta hfb2$ -pesäkkeet olivat vettyviä ja itiöinti oli vain 50 % isäntäkannan itiöinnistä. Nämä tulokset osoittavat, että HFBI edesauttaa <i>T. reesei</i> kasvamista ilmaan, kun taas HFBII on osallisena itiöinnissä. HFBI:ä ja HFBII:a ylituottavat kannat vaahtosivat runsaasti bioreaktorikasvatuksissa, erityisesti HFBII:n ylituottokanta. Vaahtoaminen oli $\Delta hfb2$ -kannalla (mutta ei $\Delta hfb1$:lla) isäntäkantaa heikompaa. HFBII:n osoitettiin olevan pääsyy vaahtoamiseen <i>T. reesei</i> Rut-C30:n bioreaktorikasvatuksissa.			
Avainsanat filamentous fungi, Trichoderma reesei, surface active proteins, hydrophobins, HFBI, HFBII, purification, characterization, crystallization, microbial surfactant, gene deletion, biological function, foaming			
ISBN 951-38-6836-2 (nid.) 951-38-6837-0 (URL: http://www.vtt.fi/publications/index.jsp)			
Avainnimeke ja ISSN VTT Publications 1235-0621 (nid.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)			Projektinnumero
Julkaisu-aika Toukokuu 2006	Kieli Englanti, suom. tiiv.	Sivuja 99 s. + liitt. 38 s.	Hinta C
Projektin nimi		Toimeksiantaja(t)	
Yhteystiedot VTT Tietotie 2, PL 1000, 02044 VTT Puh. vaihde 020 722 111 Faksi 020 722 7071		Myynti VTT PL 1000, 02044 VTT Puh. 020 722 4404 Faksi 020 722 4374	

VTT PUBLICATIONS

- 581 Urala, Nina. Functional foods in Finland. Consumers' views, attitudes and willingness to use. 2005. 79 p. + app. 109 p.
- 582 Human practice in the life cycle of complex systems. Challenges and methods. Edited by Maaria Nuutinen & Juha Luoma. 2005. 147 p.
- 583 Turunen, Erja. Diagnostic tools for HVOF process optimization. 2005. 66 p. + app. 92 p.
- 584 Measures for improving quality and shape stability of sawn softwood timber during drying and under service conditions. Best Practice Manual to improve straightness of sawn timber. Edited by Veikko Tarvainen. 2005. 149 p.
- 585 Hyötyläinen, Raimo. Practical interests in theoretical consideration. Constructive methods in the study of the implementation of information systems. 2005. 159 p.
- 586 Koivisto, Tapio. Developing strategic innovation capability of enterprises. Theoretical and methodological outlines of intervention. 2005. 120 p.
- 587 Ajanko, Sirke, Moilanen, Antero & Juvonen, Juhani. Kierrätyspolttoaineiden laadunvalvonta. 2005. 59 s.
- 588 Ebersberger, Bernd. The Impact of Public R&D Funding. 2005. 199 p. + app. 12 p.
- 589 Kutinlahti, Pirjo. Universities approaching market. Intertwining scientific and entrepreneurial goals. 2005. 187 p. + app. 4 p.
- 590 Jantunen, Erkki. Indirect multisignal monitoring and diagnosis of drill wear. 2005. 80 p. + app. 110 p.
- 591 Rauste, Yrjö. Techniques for wide-area mapping of forest biomass using radar data. 2005. 103 p. + app. 77 p.
- 592 Safety and reliability. Technology theme - Final report. Ed. by Veikko Rouhiainen. 2006. 142 p. + app. 27 p.
- 593 Oedewald, Pia & Reiman, Teemu. Turvallisuuskriittisten organisaatioiden toiminnan erityispiirteet. 2006. 108 s. + liitt. 10 s.
- 594 Lyly, Marika. Added β -glucan as a source of fibre for consumers. 2006. 96 p. + app. 70 p.
- 595 Hänninen, Saara & Rytönen, Jorma. Transportation of liquid bulk chemicals by tankers in the Baltic Sea. 2006. 121 p. + app. 30 p.
- 596 Vähä-Heikkilä, Tauno. MEMS tuning and matching circuits, and millimeter wave on-wafer measurements. 2006. 86 p. + app. 82 p.
- 597 Lallukka, Sami & Raatikainen, Pertti. Passive Optical Networks. Transport concepts. 2006. 123 p.
- 598 Lyyränen, Jussi. Particle formation, deposition, and particle induced corrosion in large-scale medium-speed diesel engines. 2006. 72 p. + app. 123 p.
- 601 Askolin, Sanna. Characterization of the *Trichoderma reesei* hydrophobins HFBI and HFBI. 2006. 99 p. + app. 38 p.
- 602 Rosqvist, Tony, Tuominen, Risto & Sarsama, Janne. Huoltovarmuuden turvaamiseen tähtäävä logistisen järjestelmän riskianalyyssimenetelmä. 2006. 68 s. + liitt. 20 s.

Tätä julkaisua myy

VTT
PL 1000
02044 VTT
Puh. 020 722 4404
Faksi 020 722 4374

Denna publikation säljs av

VTT
PB 1000
02044 VTT
Tel. 020 722 4404
Fax 020 722 4374

This publication is available from

VTT
P.O. Box 1000
FI-02044 VTT, Finland
Phone internat. +358 20 722 4404
Fax +358 20 722 4374