

Géza R. Szilvay

Self-assembly of hydrophobin
proteins from the fungus
Trichoderma reesei

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**Self-assembly of hydrophobin
proteins from the fungus
*Trichoderma reesei***

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Keywords hydrophobin, protein self-assembly, protein adhesion, protein multimerization, surface active protein, *Trichoderma reesei*, HFBI, HFBII

Abstract

Hydrophobins are small surface active proteins that are produced by filamentous fungi. The surface activity of hydrophobin proteins leads to the formation of a film at the air-water interface and adsorption to surfaces. The formation of these hydrophobin films and coatings is important in many stages of fungal development. Furthermore, these properties make hydrophobins interesting for potential use in technical applications.

The surfactant-like properties of hydrophobins from *Trichoderma reesei* were studied at the air-water interface, at solid surfaces, and in solution. The hydrophobin HFBI was observed to spontaneously form a cohesive film on a water drop. The film was imaged using atomic force microscopy from both sides, revealing a monomolecular film with a defined molecular structure. The use of hydrophobins as surface immobilization carriers for enzymes was studied using fusion proteins of HFBI or HFBII and an enzyme. Furthermore, site-specifically modified variants of HFBI were shown to retain their ability to self-assemble at interfaces and to be able to bind a second layer of proteins by biomolecular recognition.

In order to understand the function of hydrophobins at interfaces, an understanding of their overall behavior and self-assembly is needed. HFBI and HFBII were shown to associate in solution into dimers and tetramers in a concentration-dependent manner. The association dynamics and protein-protein interactions of HFBI and HFBII were studied using Förster resonance energy transfer and size exclusion chromatography. It was shown that the surface activity of HFBI is not directly dependent on the formation of multimers in solution.

Szilvay, Géza R. Self-assembly of hydrophobin proteins from the fungus *Trichoderma reesei* [*Trichoderma reesei* -homeen hydrofobiini-proteiinien itsejärjestäytyminen]. Espoo 2007. VTT Publications 657. 64 s. + liitt. 43 s.

Avainsanat hydrophobin, protein self-assembly, protein adhesion, protein multimerization, surface active protein, *Trichoderma reesei*, HFBI, HFBII

Tiivistelmä

Hydrofobiinit ovat rihmasienten tuottamia pieniä pinta-aktiivisia proteiineja. Pinta-aktiivisina molekyyleinä hydrofobiini-proteiinit muodostavat kalvon ilman ja veden rajapinnalle sekä kiinnittyvät kiinteille pinnoille. Näiden kalvojen ja pinnoitteiden muodostuminen on tärkeää monissa rihmasienen yksilönkehityksen vaiheissa. Lisäksi nämä ominaisuudet tekevät hydrofobiineista kiinnostavia molekyylejä teknisiin sovelluksiin.

Trichoderma reesei -homeen tuottamien HFBI- ja HFBII-hydrofobiinien pinta-aktiivisuusominaisuuksia tutkittiin ilman ja veden rajapinnalla, kiinteillä pinnoilla sekä liuoksessa. HFBI-hydrofobiinin havaittiin muodostavan yhtenäisen kalvon vesipisaran pinnalle. Kalvon molemmat puolet kuvattiin atomivoimamikroskoopilla ja osoitettiin, että kalvo on muodostunut yhdestä hydrofobiini-molekyylikerroksesta, jolla on hyvin järjestäytynyt molekyylitason rakenne. Entsyymien kiinnittämistä pinnoille hydrofobiinien avulla tutkittiin käyttäen HFBI:n tai HFBII:n ja mallientsyymien fuusioproteiineja. Lisäksi osoitettiin, että kohdennetusti muokattu HFBI-muunnos säilyttää kykynsä itsejärjestäytyä rajapinnoilla ja kykenee sitomaan hydrofobiini-kalvon päälle toisen proteiini-kerroksen käyttäen biomolekulaarista tunnustusta.

Ymmärtääksemme paremmin hydrofobiinien pinta-aktiivisuusominaisuuksia on tarpeen tuntea hydrofobiinien yleisiä ominaisuuksia ja itsejärjestäytymistä. HFBI sekä HFBII yhdistyvät vesiliuoksessa dimeereiksi ja tetrameereiksi, ja reaktio on riippuvainen proteiinien pitoisuudesta. Liuos-multimeerien muodostumisen dynamiikan ja proteiinien välisten vuorovaikutusten tutkimiseen käytettiin Förster-resonanssienergiansiirtomenetelmää sekä geelisuodatuskromatografiaa. Tutkimuksessa osoitettiin, että HFBI:n pinta-aktiivisuus ei ole suoraan riippuvainen liuos-multimeerien muodostumisesta.

Preface

The hydrophobin proteins have fascinated me from ever since I first got the chance to study them. This happened during a research trainee period at VTT Technical Research Centre of Finland in 1999 in the guidance of docent Markus Linder. His enthusiasm about the hydrophobins and their unique properties was very soon transferred also to me. This led to the continuation of my hydrophobin studies as a thesis work at VTT from May 2003 until June 2007. There are numerous people whom I wish to acknowledge for their valuable contributions and to thank them for making this thesis possible.

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Espoo, September 2007

Géza R. Szilvay

List of publications

This thesis is a summary of the following original publications, which are referred to as Appendices I–IV. The articles are included as appendices in the printed version of the thesis.

- I Szilvay, G. R., Paananen, A., Laurikainen, K., Vuorimaa, E., Lemmetyinen, H., Peltonen, J., and Linder, M. B. 2007. Self-assembled hydrophobin protein films at the air-water interface: Structural analysis and molecular engineering. *Biochemistry* 46, 2345–2354.
- II Linder, M., Szilvay, G. R., Nakari-Setälä, T., Söderlund, H., and Penttilä, M. 2002. Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBI from *Trichoderma reesei*. *Protein Science* 11, 2257–2266.
- III Szilvay, G. R., Nakari-Setälä, T., and Linder, M. B. 2006. Behavior of *Trichoderma reesei* hydrophobins in solution: Interactions, dynamics, and multimer formation. *Biochemistry* 45, 8590–8598.
- IV Szilvay, G. R., Kisko, K., Serimaa, R., and Linder, M. B. 2007. The relation between solution association and surface activity of the hydrophobin HFBI from *Trichoderma reesei*. *FEBS Letters* 581, 2721–2726.

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List of abbreviations

AFM	Atomic force microscopy
CD	Circular dichroism
CMC	Critical micellization concentration
FRET	Förster resonance energy transfer
LB	Langmuir-Blodgett
LS	Langmuir-Schaefer
QCM-D	Quartz crystal microbalance with dissipation monitoring
SAXS	Small angle X-ray scattering
SEC	Size exclusion chromatography
SPR	Surface plasmon resonance

1. Introduction

This work describes the unique bio- and physicochemical properties of a group of proteins termed hydrophobins. The fascinating self-assembly of hydrophobin proteins to various structures provides filamentous fungi with a toolbox to adapt to surface forces which they will encounter in their environment (Wösten 2001; Linder *et al.* 2005). These interfaces, i.e. the boundaries between two different phases, can be between air and water, solid and water, or oil and water. The interfacial self-assembling properties also make hydrophobins interesting for materials engineering and as nanoscale building blocks in various devices.

Hydrophobins are small, about 10 kDa sized proteins that are surface active. In other words they adsorb to the interface between air and water and thereby lower the surface tension of water. They have been found in all studied filamentous fungi and several hydrophobin genes have often been identified in a single species. Furthermore, many of the studied hydrophobins have been found to be expressed at high levels.

Hydrophobin proteins form stable films at interfaces and self-assemble readily into different nanoscale structures (Wösten 2001; Linder *et al.* 2005). This thesis concentrates on the hydrophobins HFBI and HFBII from *Trichoderma reesei* (Nakari-Setälä *et al.* 1996; Nakari-Setälä *et al.* 1997) and on their self-assembly. The present work also addresses important questions related to the process that leads to surface activity and self-assembled structures. Based on the results, models for the HFBI self-assembled structures and solution behavior are proposed.

1.1 Hydrophobins in fungal physiology

To appreciate the importance of interfacial forces involved in the environment of microorganisms, one has to consider the remarkably high surface tension of water. The high surface tension is a consequence of water molecules situated on the water surface experiencing an unbalanced interaction between their neighboring molecules; interactions with molecules in the neighboring phase (e.g. air) are less favorable than interactions with bulk phase water molecules. This results in stronger cohesive forces between the molecules at the interface than between the bulk molecules. The enhanced interaction between surface

molecules is observable as surface tension. The high surface tension of water is illustrated by the ability of some small insects, such as water striders, to walk on the water surface. For fungal hyphae, which are several orders of magnitude smaller in size than insects, the interfacial forces are significant. Microorganisms therefore need to produce specific and dedicated molecules in order to adapt to restraints in their living environment caused by interfacial forces. The molecules produced by filamentous fungi for this purpose are hydrophobins.

Hydrophobin proteins have been shown to be involved in many stages of fungal development. Generally, hydrophobins are involved in the adaptation of fungi to their environment by the control of interfacial forces. It has been shown that hydrophobins are very efficient in lowering the surface tension of water (van der Vegt *et al.* 1996; de Vries *et al.* 1999; Wösten *et al.* 1999; Lumsdon *et al.* 2005; Askolin *et al.* 2006). Using a hydrophobin deletion strain of the filamentous fungus *Schizofyllum commune* it was shown that without hydrophobins the fungal hyphae cannot grow out from the water to produce aerial structures (Wösten *et al.* 1999). The hydrophobin SC3 from *S. commune* is highly expressed and can lower the water surface tension from 72 to as low as 24 mN/m (Wösten *et al.* 1999). This reduction in surface tension has been shown to allow the hyphae to escape from the aqueous medium and grow into the air (Figure 1).

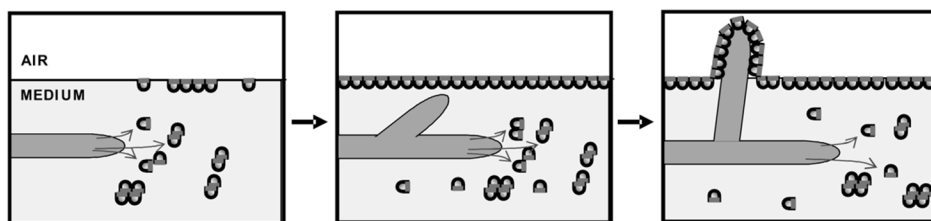


Figure 1. The role of hydrophobins in fungal hyphae growth through the air-water interface as shown by Wösten et al. (1999). As the hyphae grow submerged in aqueous medium they produce hydrophobins into the medium. Hydrophobins adsorb to the air-water interface and lower the water surface tension, thus enabling the hyphae to penetrate the air-water interface and grow into the air. (According to Wösten et al. (1999).)

As the fungal hyphae grow through the air-water interface into the air, the hydrophobins at the interface are believed to coat the emerging hyphae as they penetrate through the interface (Wessels *et al.* 1991b). Some hydrophobins at the

surfaces of aerial hyphae and spores are found to be assembled into a mosaic pattern of rod-like structures, termed rodlets that are about 10 nm in width (Figure 2) (Stringer *et al.* 1991; Bell-Pedersen *et al.* 1992; Lauter *et al.* 1992; Wösten *et al.* 1993). This rodlet coating is very hydrophobic and it is believed to protect the fungal aerial structures from wetting. Hydrophobins have also been observed on the surface of fruiting bodies (Wessels *et al.* 1991a; Lugones *et al.* 1996; de Groot *et al.* 1997). In addition, air channels in fungal aerial structures have been reported to be lined by hydrophobins (Lugones *et al.* 1999; van Wetter *et al.* 2000). This hydrophobic coating has been proposed to protect the air channels from wetting and to ensure successful gas exchange.

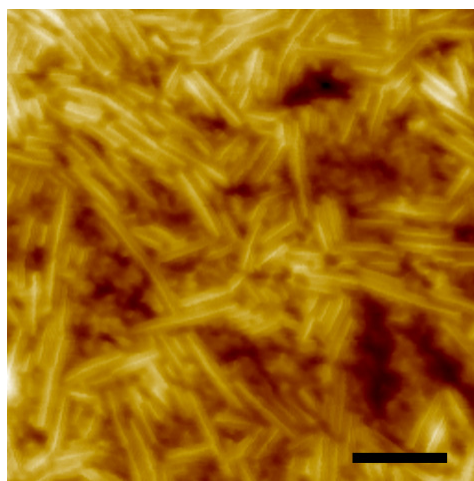


Figure 2. SC3 hydrophobin self-assembles at the air-water interface into rodlets. The assembled structures were dried onto a solid substrate and imaged with atomic force microscopy (AFM). Scale bar is 100 nm. Image by courtesy of Arja Paananen.

Hydrophobins also function as fungal adhesive molecules. Adsorption of fungal structures to surfaces has been shown to be mediated by hydrophobins in many cases. Hydrophobins have been shown for example to be involved in attachment of hyphae to solid substrates (Wösten *et al.* 1994) and adsorption of pathogenic fungi to the surface of a host organism (St. Leger *et al.* 1992; Talbot *et al.* 1993; Talbot *et al.* 1996; Kazmierczak *et al.* 2005).

Furthermore, hydrophobins have been reported to be involved in fungi – plant interactions (Viterbo and Chet 2006) and to be localized in mycorrhiza, the

symbiotic structures between fungi and plants (Tagu *et al.* 1996; Tagu *et al.* 2001; Mankel *et al.* 2002). In lichens (symbiotic relationships between fungi and algae or cyanobacteria), hydrophobins have also been observed at the interface between the two symbiotic partners (Honegger 1993; Scherrer *et al.* 2000). It has been proposed that a hydrophobic coating formed by the hydrophobins could protect the gas cavities in the repeated wetting and drying cycles occurring in the lichen's harsh environments (Honegger 1993).

Non-filamentous fungi, such as the yeast *Saccharomyces cerevisiae*, have not been found to contain hydrophobin genes and thus hydrophobins seem to be confined to filamentous fungal growth. Hydrophobins have been found to be produced by filamentous fungi belonging to the phyla of Ascomycetes and Basidiomycetes. Interestingly, bacteria growing in filaments, such as the *Streptomyces*, also produce molecules which they can use to control the properties of interfaces (Wösten and Willey 2000; Ron and Rosenberg 2001). The small peptides streptofactin and SapB (Willey *et al.* 1991; Richter *et al.* 1998; Tillotson *et al.* 1998), and the proteins rodlin (Claessen *et al.* 2002) and chaplin (Claessen *et al.* 2003; Claessen *et al.* 2004) interplay to lower the surface tension, thus enabling aerial growth and the formation of aerial structures with hydrophobic rodlet coatings. The difference between filamentous bacteria and fungi appears to be that bacteria use different kinds of molecules for different tasks. Filamentous fungi, however, use hydrophobins as their multitools for many different surface tasks. A single species expresses different hydrophobins at different developmental stages, so it is evident that specialization has occurred. However, currently our knowledge on the functional differences between different hydrophobins is limited.

In summary, hydrophobins are important in the development of filamentous fungi. They are surface active and thus enable hyphae to grow out from aqueous medium; they form protective coatings, and they mediate attachment of fungal structures to various surfaces. In order to understand the role of hydrophobins in the fungi, hydrophobins have been much studied as isolated proteins. The present work deals with the properties of hydrophobins that are relevant for fungal development, such as surface activity, adsorption and formation of coating films. Furthermore, the solution behavior was studied in order to understand the factors leading to the aforementioned surface properties.

1.2 The amphiphilic structure of hydrophobins

The unifying feature among the amino acid sequences of different hydrophobins is a completely conserved pattern of eight Cys-residues, where the second and third, and the sixth and seventh Cys residues are invariably adjacent. There are no other completely conserved amino acids among hydrophobins. Hydrophobins have been divided into two classes, class I and class II hydrophobins (Wessels 1994) based on the hydropathy patterns of the amino acid sequences (Kyte and Doolittle 1982). This division is also consistent with some of the properties of hydrophobins. Assemblies of class I hydrophobins appear to be more resistant towards solvents and detergents than class II hydrophobins. Furthermore, class I hydrophobins tend to form rodlet structures at interfaces, whereas class II hydrophobins do not. This division into two classes has had a very good predictive power with regard to the hydrophobin properties.

1.2.1 Class II hydrophobins

The hydrophobins HFBI and HFBII from *T. reesei* considered in this study both belong to the class II hydrophobins and their structures have been determined by X-ray crystallography (Hakanpää *et al.* 2004; Hakanpää *et al.* 2006a; Hakanpää *et al.* 2006b). HFBI and HFBII have a very similar fold, which has not been observed in other proteins before. The structure consists of a small antiparallel β -barrel formed by two β -hairpins connected by a stretch of α -helix (Figure 3). The structure is overall very compact and the different parts of the protein are connected by four intermolecular disulfide bridges. The disulfide bond pattern is shown in Figure 3A.

The unique and surprising feature of the HFBII structure is that instead of having its hydrophobic residues inside the protein, about 80% of the hydrophobic side-chains are exposed at the protein surface. This “inside-out” structure is probably stabilized by the aforementioned disulfide bond network. Hydrophobic aliphatic amino acids at the protein surface form a planar hydrophobic area termed the hydrophobic patch (Figure 3D, E). This hydrophobic patch constitutes 12 % of the total surface area of HFBII. The protein surface is otherwise mainly hydrophilic, and thus the surface is segregated into a hydrophobic and a hydrophilic part. This amphiphilic structure governs the properties of both HFBI and HFBII, such as surface activity and surface adsorption. The residues forming the hydrophobic

patch are conserved among class II hydrophobins, suggesting that they all share a similar amphiphilic protein surface (Hakanpää *et al.* 2004; Linder *et al.* 2005). Taken together, the structure can be regarded as a relatively large sized and rigid protein with distinct hydrophilic and hydrophobic regions – a protein amphiphile.

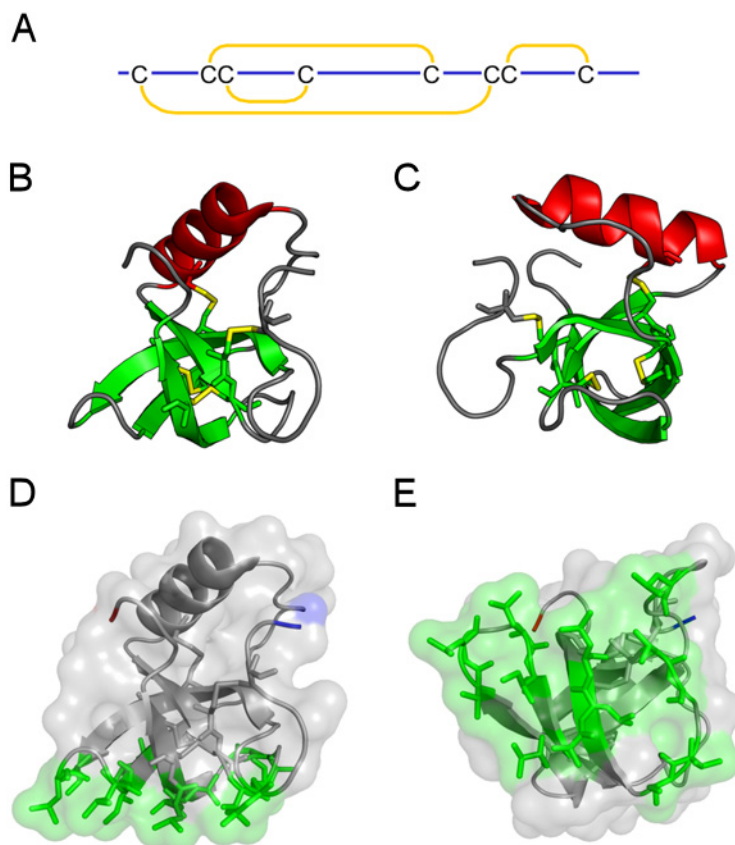


Figure 3. X-ray crystal structure of HFBII (PDB ID 1R2M) (Hakanpää *et al.* 2004). (A) Cartoon of the conserved Cys residue pattern in the hydrophobin amino acid sequences. The disulfide bonding pattern is shown in yellow. (B and C) Cartoon of the protein backbone structure showing the β -barrel formed by two β -hairpins (green) and the connecting α -helix (red). The disulfide bonds of Cys residues are shown in yellow. (D and E) Surface representation of HFBII in which the side chains of the conserved hydrophobic patch are shown and colored green. N- and C- termini are colored in blue and red, respectively. The hydrophobic patch is viewed from the side in panel (D) and from above in (E). The images were produced using PyMol (DeLano 2002).

1.2.2 Class I hydrophobins

The nuclear magnetic resonance (NMR) structure of the class I hydrophobin EAS from *Neurospora crassa* is very similar (Kwan *et al.* 2006) to those of HFBI and HFBII (Figure 4A). Furthermore, the structure of the class I hydrophobin SC3, obtained by molecular dynamics simulation, has a very similar fold (Fan *et al.* 2006). Thus it seems that the observed fold is typical for hydrophobins. The disulfide bond network makes these proteins rigid and no major conformational changes appear to be possible. However, the longer β -hairpin loops in EAS show considerable conformational freedom (Kwan *et al.* 2006) (Figure 4A).

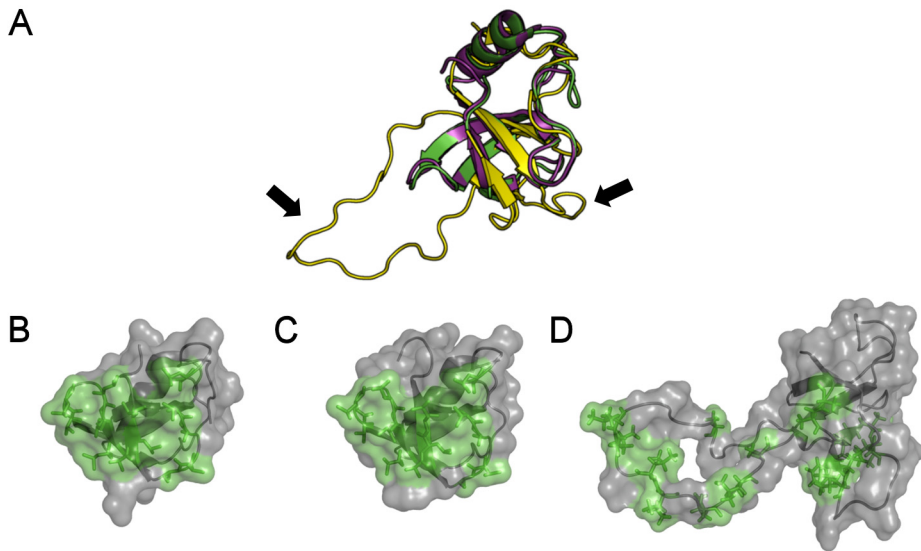


Figure 4. Comparison of the published hydrophobin molecular structures. (A) Cartoon comparing the structures of HFBI (green), HFBII (purple), and EAS (yellow). The alignment of the structures shows the similar core structure among these proteins and the longer and conformationally flexible loops of EAS (arrows). Surface representations of HFBI (B), HFBII (C) and EAS (D). The conserved hydrophobic amino acids of HFBI and HFBII are colored green. The hydrophobic amino acids of EAS in this region are shown in (D). PDB IDs are HFBI: 2FZ6, HFBII: 1R2M, and EAS: 2FMC. The structure alignments and images were produced using PyMol (DeLano 2002).

The amphiphilicity of class I hydrophobins, as seen in the NMR structure of EAS, is not as evident in the structure as the amphiphilicity of class II hydrophobins. Therefore, it might be that the disordered loops of class I hydrophobins have a role in formation of a larger hydrophobic area once the protein encounters an interface.

1.2.3 Hydrophobins in water

The amphiphilic structure of a hydrophobin is important for its function in aqueous solutions. Water molecules interact poorly with hydrophobic molecules and thus hydrophobic molecules are clustered together. This phenomenon, called the hydrophobic effect, depends on the hydrogen bond network of water and the cavity size and shape induced by a hydrophobic solute (Chandler 2005). There is a size dependency of the free energy of solvation for hydrophobic objects in water. According to Chandler (2005) the free energy of solvation *per unit surface area* increases with the size of the hydrophobic object and levels out to a constant value at a diameter of about 1 nm if a spherical object is considered. The hydrophobic patches of HFBI and HFBII are planar and have an area of about 4 nm². It is noteworthy that the size of the patch corresponds approximately to the size at which the solvation free energy per hydrophobic surface area reaches its maximum level. Therefore it is possible that surfactants with continuous planar hydrophobic patches of this size may have unusual properties as surfactants.

Using small angle X-ray scattering (SAXS), the hydrophobins HFBI and HFBII have been shown to associate into dimers and tetramers in solution (Torkkeli *et al.* 2002; Kisko *et al.* 2007a). Furthermore, in the X-ray crystal structures of HFBI and HFBII, the proteins were found to be clustered into dimers or tetramers through their hydrophobic patches (Hakanpää *et al.* 2004; Hakanpää *et al.* 2006b). SC3 has also been shown to exist as monomers, dimers and tetramers in solution (Wang *et al.* 2004a). However, EAS has been proposed to occur only as monomers in solution (Mackay *et al.* 2001). In this study the hydrophobin solution association and the similarity of this association to detergent micelles was investigated.

1.3 Hydrophobin self-assembly

The term molecular self-assembly refers to the autonomous formation of structures or patterns from pre-existing components. This is a reversible process driven only by the properties of the components (Whitesides and Grzybowski 2002). Self-assembly processes can be divided into static processes, in which the system is in equilibrium with its surroundings and into dynamic processes, in which energy is dissipating through the system. Protein self-assembly is very common in biological systems. Examples are the formation of virus capsids, the DNA replication machinery, and disease-related amyloid fibres.

Hydrophobins form self-assembled structures at the air-water interface. Class I hydrophobins form a mosaic of rod-like structures, called rodlets, which are 5–10 nm in width and several hundred nanometers in length (Figure 2) (Wösten *et al.* 1993). Class II hydrophobins have not been observed to form rodlets. Instead, the class II hydrophobins HFBI, HFBII and HFBIII from *T. reesei* have been shown to form films with a self-assembled hexagonally ordered structure (Figure 5) (Paananen *et al.* 2003; Kisko *et al.* 2005; Kisko *et al.* 2007b). Studies on the possible interfacial self-assembly of other class II hydrophobins have not been reported.

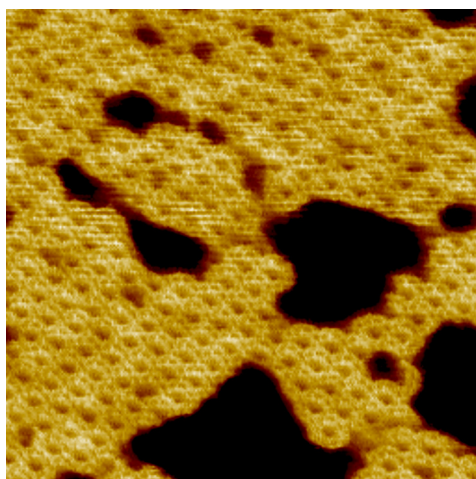


Figure 5. HFBI self-assembles at the air-water interface into a hexagonally ordered monolayer. Imaged with tapping mode AFM in air, image size is 100 nm x 100 nm. (Image courtesy of Arja Paananen.)

The adsorption of class I hydrophobins to the air-water interface and their self-assembly into rodlet structures are associated with changes in the protein structure (de Vocht *et al.* 1998). Most of the studies related to conformational changes at interfaces have concentrated on SC3. When adsorbed to the air-water interface SC3 adopts an intermediate state enriched in α -helical structure. This state then slowly changes to a state that is mainly β -sheet in nature (Wösten and de Vocht 2000; de Vocht *et al.* 2002). This change to the “ β 2-state” (de Vocht *et al.* 2002) is concurrent with the formation of the rodlet structure. At solid surfaces this state is not spontaneously reached and the conformation is retained in the α -helical state. Only by treating this protein layer with hot detergent can the “ β 2-state” be reached on a solid surface. Once this state is formed it is very resistant to solvents and detergents, even to boiling 2 % SDS. It is not yet known where the secondary structure changes are taking place and how the overall structure of SC3 is changed. As the structure is probably stabilized by the four disulfide bonds (as in HFBI and HFBII), one position to look for secondary structure changes would be the long β -hairpin loops that have been observed to be conformationally flexible in the hydrophobin EAS (Figure 4) (Kwan *et al.* 2006).

Conformational changes in class II hydrophobins have been studied only for HFBI (Askolin *et al.* 2006). It was found that unlike SC3, the adsorption of HFBI to hydrophobic Teflon beads is not associated with conformational changes that could be detected using circular dichroism spectroscopy. Furthermore, the surface adsorbed films formed by class II hydrophobins are more easily dissolved than the class I films. However, HFBI in the X-ray crystal structure showed two different conformations in the second β -hairpin loop (Figure 6). In one conformation the loop resembles the conformation observed for HFBII and in the other the loop has moved away from the protein core. Interestingly, this β -hairpin loop has a high conformational freedom in EAS (Kwan *et al.* 2006). This conformational change in HFBI has been proposed to be driven by the formation of the tetramer assembly (Hakanpää *et al.* 2006b). Moreover, this structural flexibility in the hydrophobic patch may enable plasticity for adsorption to different surfaces (Hakanpää *et al.* 2006b).

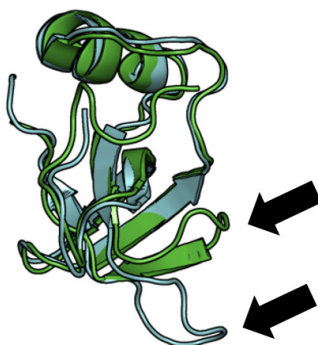


Figure 6. Two conformations observed in the HFBI X-ray crystal structure (PDB ID 2FZ6). Chains A (green) and B (cyan) are aligned to highlight the two conformations of the second β -hairpin loop (arrows). The image was produced with PyMol (DeLano 2002).

In addition to forming films at interfaces, some class II hydrophobins readily form large aggregates in solution. Often these aggregates have needle-like or fibrillar structures (Figure 7) (Torkkeli *et al.* 2002); (Takai 1974). Formation of these aggregates is easily induced by shear forces upon mixing. The assembly mechanism inducing formation of fibril structures is currently not known. Whether fibrils are formed by hydrophobins in bulk solution or by self-assembled hydrophobin structures from the air-water interface that are forced into the solution has remained elusive.

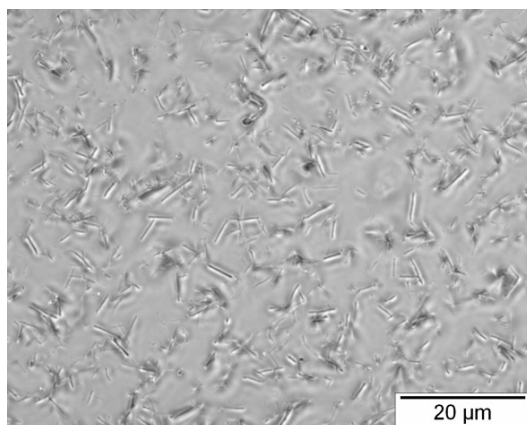


Figure 7. Needle-like fibrils formed by HFBI as observed with a light microscope. Fibril formation of HFBI was induced by extensive shaking of the protein solution. (Modified from publication IV (Appendix IV).)

The self-assembled structures of some class I hydrophobins have been proposed to have amyloid-like features because they interact with dye molecules that bind specifically with amyloid fibrils (de Vocht *et al.* 1998; de Vocht *et al.* 2000; Butko *et al.* 2001; Mackay *et al.* 2001). Among hydrophobins the formation of amyloid structures may be limited to class I hydrophobins, as the fibrils formed by HFBII have been shown not to be amyloid (Torkkeli *et al.* 2002). Amyloid structures are formed by proteins that have adopted a non-native alternative fold which is prone to self-assembly into fibrillar structures. The amyloid fibers are shown to be formed by parallel β -sheets (Sunde *et al.* 1997; Serpell *et al.* 2000; Serpell and Smith 2000; Perutz *et al.* 2002). The emergence of amyloid structures is associated with many unrelated diseases, although it has been proposed that most proteins have an ability to form amyloid structures in suitable conditions (Fändrich and Dobson 2002). Moreover, the formation of amyloid structures is not always associated with disease, as amyloid formation can have functional relevance as observed for example with hydrophobins, bacterial curli protein (Barnhart and Chapman 2006), or yeast prion protein Sup35 (Liebman and Derkatch 1999).

1.4 Application potential of hydrophobins

The effective surface adsorption and surface activity of hydrophobins make them interesting materials for modification of interfaces in various applications. For reviews on the use of hydrophobins in applications see (Scholtmeijer *et al.* 2001; Hektor and Scholtmeijer 2005). Here, the application potential of hydrophobins is briefly summarized.

For many proteins the environment at a solid-liquid or air-water interface is very different to the environment in the biological context of the protein's function. Thus, proteins usually adsorb to interfaces with a low efficiency, in random orientation and, most importantly, upon adsorption they can lose their biological activity (Rusmini *et al.* 2007). This happens either because of denaturation or because unoriented surface adsorption leads to inaccessibility of the active site. By contrast, hydrophobins are efficient in adsorbing to different interfaces and appear to have a specified orientation at the interface (Scholtmeijer *et al.* 2002; Wang *et al.* 2004b; Lumsdon *et al.* 2005).

Native hydrophobins have been used to form protein films and to modify physicochemical properties of surfaces for binding of other molecules in an unspecific manner (Bilewicz *et al.* 2001; Palomo *et al.* 2003; Ikeno *et al.* 2004; Corvis *et al.* 2005; Takahashi *et al.* 2005; Qin *et al.* 2007; Zhao *et al.* 2007). Furthermore, new properties and functionalities can be incorporated to the films in a more controlled manner by means of protein engineering or chemical modification. In such cases, hydrophobins act as general adsorption-mediating modules and the functionalities are chosen according to the specific needs. Published examples include introduction of chemical groups for specific biomolecular binding partners (Kostiainen *et al.* 2006; Kostiainen *et al.* 2007), chelating groups (Corvis *et al.* 2006), nanoparticles (Kurppa *et al.* 2007), binding motifs for cell attachment (Janssen *et al.* 2002; Scholtmeijer *et al.* 2002; Janssen *et al.* 2004), and even immobilization of yeast cells (Nakari-Setälä *et al.* 2002). In this work, the use of modified hydrophobins to introduce enzymatic activity (Appendix II) or protein binding targets (Appendix I) to surfaces was demonstrated. Applications using hydrophobin films could include surface modifications for tissue engineering, construction of sensing surfaces, diagnostic kits, and micro-contact printing.

Hydrophobin solutions have been shown to foam easily (Bailey *et al.* 2002; Sarlin *et al.* 2005; Cox *et al.* 2007). Hydrophobins could thus be used in applications and products where foams are important e.g. in some food products. Furthermore, the properties of the foam could be tailored by using modified hydrophobins. An immunological detection method has been developed to detect fungal contaminants that cause foaming in unwanted cases (Sarlin *et al.* 2005). Furthermore, a hydrophobin deletion strain has been constructed that has been shown to cause less unwanted foaming during fungal fermentations (Bailey *et al.* 2002). HFBI has been shown to effectively separate into a detergent phase in an aqueous two-phase extraction system (Linder *et al.* 2001). Moreover, the use of HFBI as a tag in fusion proteins for efficient protein purification using an aqueous 2-phase extraction system has been demonstrated (Collen *et al.* 2002; Linder *et al.* 2004; Selber *et al.* 2004).

The unique surface adsorption and self-assembly properties of hydrophobins could potentially find use in the field of nanotechnology. Oriented binding, the possibility for site-specific modifications, and the formation of nanoscale self-assembled structures are properties that make hydrophobins good candidates for use in devices where precise control of the molecular building blocks is required.

2. Aims of the present study

This thesis concentrates on the characterization of hydrophobins from *T. reesei* and especially HFBI. The aim of this study was to investigate the self-assembly properties of hydrophobins on surfaces and in solution. The self-assembly in solution into multimers is referred to as multimerization or solution association.

Figure 8 summarizes the objectives of this study. The specific aims were to investigate:

1. Hydrophobin adsorption to interfaces, and the formed self-assembled structures.
2. The use of hydrophobins as adhesion molecules for protein immobilization.
3. Interactions and dynamics of hydrophobin solution multimers and the relation between multimerization and surface activity.

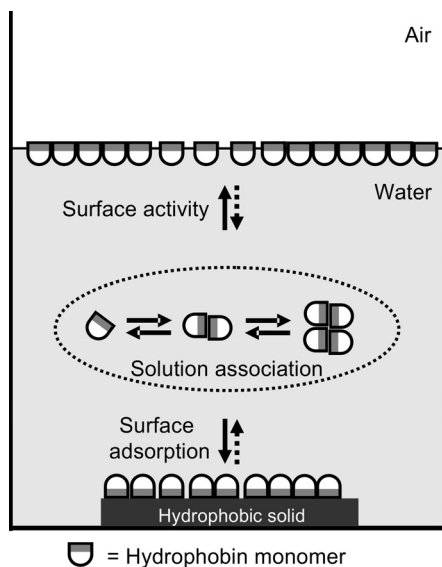


Figure 8. A cartoon representing the function of HFBI and HFBI at interfaces and in solution. The hydrophobins are surface active, adsorb to interfaces, and associate in solution into multimers. The cartoon also represents the study area of this thesis. (Image modified from publication IV (Appendix IV).)

3. Materials and methods

The strains and plasmids used in this study are listed in Table 1. The used methods are described in the Appendices I–IV and are summarized in Table 2.

Table 1. Plasmids and strains used in this study.

Plasmids and strains	Relevant property	Reference
Plasmids		
pMQ121	<i>hfb1</i> gene	(Linder <i>et al.</i> 2001)
pBluescript KS (-)	Cloning vector	Stratagene
pGZ7 (HFBI)	<i>hfb1</i> gene in cloning vector	III
pGZ9AC (NCys-HFBI)	<i>ncys-hfb1</i> gene in cloning vector	III
pGZ12 (NCys-HFBI)	<i>ncys-hfb1</i> expression cassette	III
pGZ10AC (HFBI-CysC)	<i>hfb1-cysc</i> gene in cloning vector	I
pGZ13 (HFBI-CysC)	<i>hfb1-cysc</i> expression cassette	I
pMQ131 (EGIc-HFBI)	<i>eglc-hfb1</i> expression cassette	II, (Penttilä <i>et al.</i> 2000)
pTNS32 (EGIc-HFBII)	<i>eglc-hfb2</i> expression cassette	II
Strain		
XL1-blue <i>Escherichia coli</i>	Cloning strain	(Bullock <i>et al.</i> 1987)
VTT-D-99676 <i>T. reesei</i> Rut-C30 $\Delta hfb2$	<i>hfb2</i> deletion strain	(Bailey <i>et al.</i> 2002; Askolin <i>et al.</i> 2005)
VTT D-061175 (NCys-HFBI), <i>T. reesei</i> Rut-C30 $\Delta hfb2$	NCys-HFBI and (NCys-HFBI) ₂ expression	III, IV
VTT-D-061176 (HFBI-CysC), <i>T. reesei</i> Rut-C30 $\Delta hfb2$	HFBI-CysC and (HFBI-CysC) ₂ expression	I, IV
VTT-D-99702 (EGIc-HFBI), <i>T. reesei</i> QM9414	EGIc-HFBI expression	II
VTT-D-02790 (EGIc-HFBII), <i>T. reesei</i> QM9414	EGIc-HFBII expression	II

Table 2. Methods used in this study.

Method	Described in reference
Recombinant DNA methods	
Gene constructions	I, II, III
<i>T. reesei</i> transformations	I, II, III (Penttilä <i>et al.</i> 1987)
Protein production, purification and chemical conjugation methods	
Production and purification of HFBI	(Paananen <i>et al.</i> 2003)
Production and purification of NCys-HFBI	III
Production and purification of HFBI-CysC	I
Production and purification of (NCys-HFBI) ₂ and (HFBI-CysC) ₂	IV
Production and purification of EGlc-HFBI and EGlc-HFBII	II
Hydrophobin conjugations	III, I
Surface characterization methods	
Surface adsorption measured by enzymatic assay	II
Surface adsorption measured by quartz crystal microbalance	II, I, IV
Surface adsorption measured by surface plasmon resonance	II
Surface tension measurements	I, IV
Langmuir film preparations	I, IV
Hydrophobin air-water interface film deposition to solid surfaces	I
Atomic force microscopy	I
Solution association analysis methods	
Förster resonance energy transfer between hydrophobin monomers	III
Size exclusion chromatography	III, IV
Small angle X-ray scattering	IV
Fibril formation	IV

4. Results and discussion

4.1 HFBI self-assembly at the air-water interface (I)

Hydrophobins, due to their amphiphilic structure, adsorb to the air-water interface like surfactants in general. A clear difference compared to the usual behavior of surfactants can be observed in the air-water interface film that is formed by HFBI or HFBII: the film is very cohesive and small wrinkles can be observed when the surface is disturbed (Figure 9A and B). Moreover, when a dilute HFBI aqueous solution drop is placed on a surface at room temperature, a planar area begins to form after about 15 minutes, starting from the apex of the drop (Figure 9C). The emergence of the observed coherent HFBI film shows that the visco-elastic properties of the formed film are very different from those of a common film formed for example by soap molecules. In this work the molecular structure of this film was investigated (Appendix I). Furthermore, engineered proteins were produced to create protein films with a new functionality.

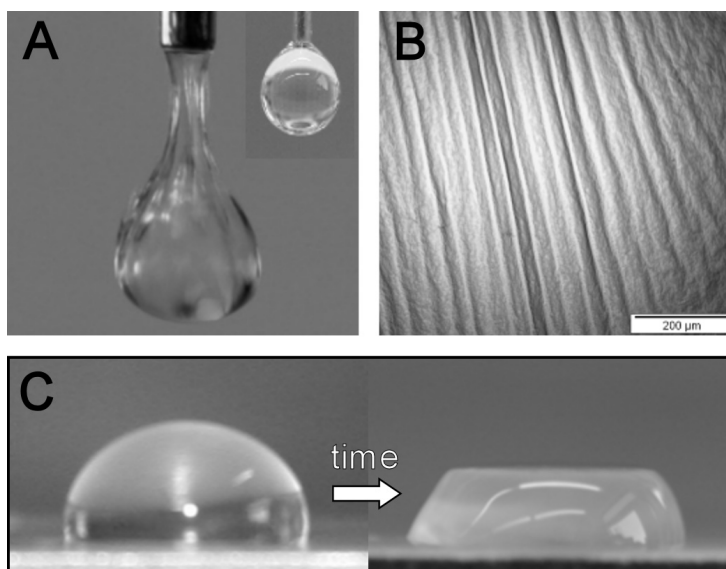


Figure 9. HFBI film formation at the air-water interface. (A) A hanging drop of HFBI solution takes an unusual shape. Pure water shown as inset. (B) Light microscope image of the film showing wrinkles caused by disturbance of the film. (C) After 30 min in ambient environment a planar film is formed on the surface of a drop of 10 µg/ml HFBI solution. (Modified from publication I (Appendix I)).

4.1.1 HFBI air-water interface films

Previously, HFBI and HFBI air-water interface films, prepared using the Langmuir-Blodgett technique (LB) (Figure 10), were shown to consist of highly ordered hexagonal monolayers (Figure 5) (Paananen *et al.* 2003). In the LB method a film formed by surface active molecules is compressed in a Langmuir-trough by moving barriers that reduce the available water surface area (Figure 10). The film is transferred to a solid substrate by lifting an immersed flat substrate through the film so that simultaneous compression deposits the film onto the substrate. The films transferred to solid supports were imaged using AFM. The principles of AFM imaging are summarized in Figure 11.

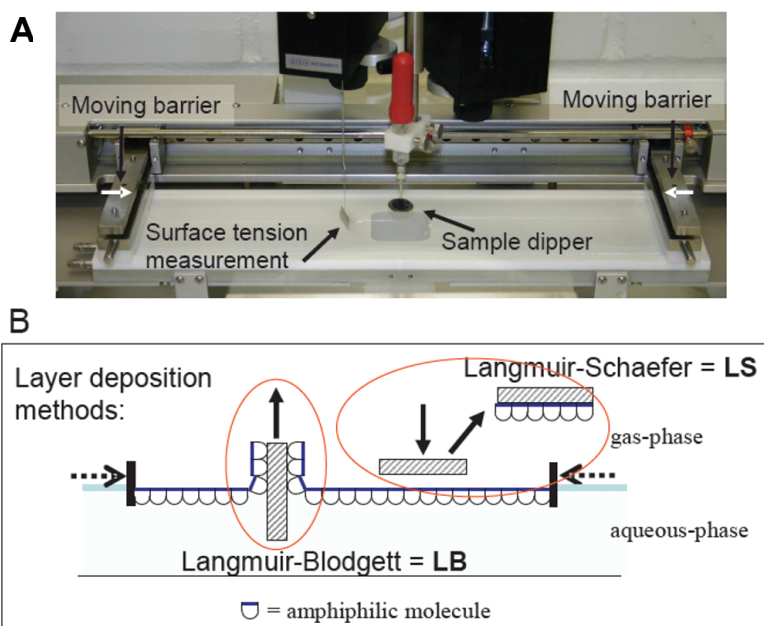


Figure 10. The Langmuir-trough and film deposition methods. (A) The trough is filled with aqueous buffer and the surface tension is measured with a film balance using a Wilhelmy plate. The moving barriers are used to compress the film formed by surface active molecules. The sample dipper is used to deposit the film to solid supports. (B) In the LB-technique, i.e. vertical deposition, a solid substrate is lifted out from the liquid and simultaneously the film is compressed onto the surface. In the Langmuir-Schaefer (LS) technique, i.e. horizontal deposition, a solid substrate is brought into contact with the film whereby the molecules on the surface can adsorb to the surface. Concomitantly the surface is lifted away.

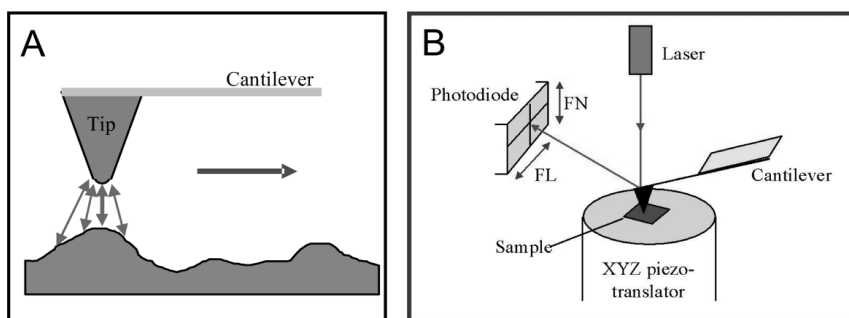


Figure 11. Diagrams of the basic principle of AFM. (A) A sharp tip at the end of a cantilever scans the sample surface line by line and the surface topography is recorded. The tip radius of curvature is typically about 10 nm. (B) Cantilever bending due to height differences on the surface is detected by a laser light that is reflected from the back of the cantilever onto a position sensitive detector (photodiode). The force applied on the surface by the tip (i.e. the cantilever bending) is held constant by adjusting the tip holder – sample distance. This movement is controlled by a feedback loop and simultaneously used to record the topography information. As a scanner (XYZ piezotranslator) scans the surface line by line a topography map of the sample is recorded. Images by courtesy of Arja Paananen.

To study the molecular structure of the HFBI film that is spontaneously formed on a solution drop (Figure 9), we developed a method to pick up this film on a solid substrate for AFM imaging. In this method a hydrophobic, atomically flat graphite substrate was brought into contact with the drop's surface, whereby the film adsorbed to the substrate (Figure 12). The originally hydrophobic substrate surface then became hydrophilic, indicating adsorption of material. We termed this method the drop-surface transfer method. The method is analogous to the Langmuir-Schaefer (LS) method which is used to deposit films from the Langmuir-trough to a solid substrate in a similar manner (Figure 10).

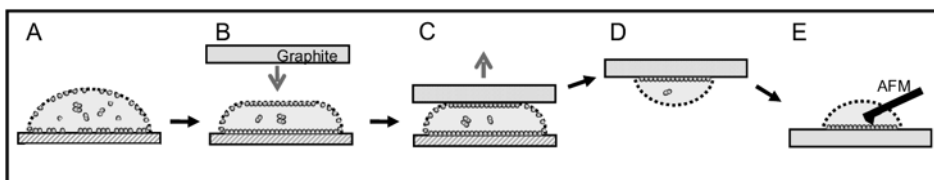


Figure 12. A cartoon of the applied method (termed as the drop-surface transfer) to deposit the hydrophobin film from a solution drop onto a hydrophobic substrate (graphite). (A and B) The surface of a hydrophobin solution drop is gently brought into contact with a hydrophobic graphite substrate. (C and D) The hydrophobin film from the drop's air-water interface adsorbs to the substrate. (E) The deposited film is imaged in buffer with AFM. (Modified from publication I (Appendix I).)

AFM imaging revealed that the deposited HFBI film had a highly ordered hexagonal structure, the same structure as in the previously reported LB samples (Figure 13). Furthermore, the thickness of this film corresponded to the dimensions of an HFBI monomer. The results show that HFBI spontaneously self-assembles into a hexagonally ordered monomolecular film at the surface of a solution drop.

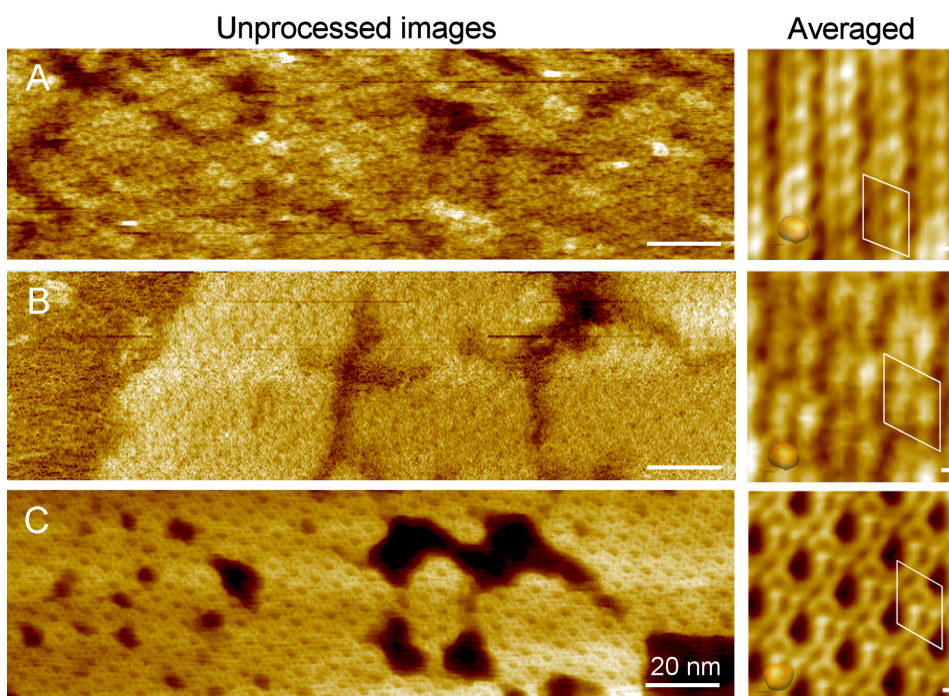


Figure 13. AFM images of HFBI air-water interface films. (A) HFBI film transferred from a solution drop surface using the drop-surface transfer method. (B and C) Langmuir films deposited using the LS (B) and LB (C) methods. Scale bars are 20 nm. Correlation average images are shown in the right column with HFBI monomer and unit cell shown. Scale bars 1 nm. (Modified from publication I (Appendix I).)

By using the drop-surface transfer and the LS methods the water-facing (i.e. the hydrophilic) side of the film faces away from the substrate and can be imaged with AFM. In the LB samples, the air-facing (i.e. the hydrophobic) side is imaged. By employing the different deposition methods in this study, both sides of the HFBI film were imaged and therefore a more detailed view of the HFBI film could be obtained. Furthermore, the films deposited using the drop-surface film or LS method had firmly adsorbed to the substrate and thus allowed AFM imaging in aqueous conditions. The possibility for imaging proteins in an aqueous environment is important, as they most often naturally function in aqueous environments and may change their conformation when dried.

4.1.2 Molecular structure of HFBI air-water interface films

Understanding the molecular structure of the self-assembled hydrophobin structures is important for any potential application involving hydrophobin films. When comparing the hexagonal crystalline structure of the monolayer to the monomer size of HFBI from the X-ray crystal structure, it is evident that the repeating unit forming the hexagonal structure consists of multimeric units. The high resolution of the AFM images from both the air-facing side and the water-facing side enabled comparison of the repeating structures in the AFM images with the dimensions of an HFBI monomer. Based on this comparison a putative model of the molecular structure of the hexagonal film was constructed. The model suggests that three monomers forming a trimer are arranged to form a hexagonal pattern (Figure 14). Here, the repeating unit would be a trimer. The assumption is that the hydrophobic patch is either oriented towards the substrate or towards the viewer.

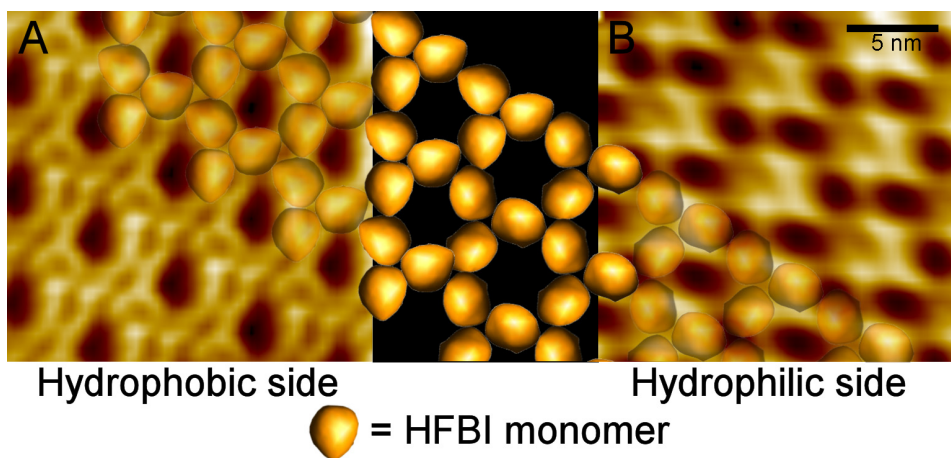


Figure 14. Model of the structure of HFBI film. The film viewed from the hydrophobic side (A) and from the hydrophilic side (B). (A) and (B) are processed AFM images showing the average repeating structures in a single crystalline area. In the middle panel and expanding to both sides is a model of the monomer arrangement in the film. The HFBI monomer is shown as a low resolution surface representation (generated using the UCSF Chimera package (Pettersen et al. 2004)). (Figure modified from publication I (Appendix I).)

The proposed model cannot predict the orientation of the monomers with respect to each other. Nevertheless, it is clear that specific interactions are formed between monomers. These interactions are essential for the visco-elastic properties of the HFBI air-water interface film. However, these intermolecular interactions are not known. The interactions may be hydrogen bonds, ionic bonds, or van der Waals interactions. Hydrophobic interactions are unlikely to be important in the intermolecular interactions since the assumption is that the hydrophobic patch of HFBI is facing the hydrophobic medium (air or solid) and not neighboring monomers. Shape complementarity may be an important factor, as it would increase the number of interaction sites.

HFBI has been shown to associate into dimers and tetramers in aqueous solution but trimers have not been observed (Torkkeli *et al.* 2002; Hakanpää *et al.* 2006b; Kisko *et al.* 2007a). The model of the HFBI film, in which trimeric repeating units form a continuous film, suggests that intermolecular interactions in the film are different from those in solution multimers. Therefore subunit dissociation or rearrangement is required for the HFBI dimers and tetramers in solution to form the proposed trimeric structures.

4.1.3 Avidin immobilization to modified HFBI films

The drop-surface film transfer method enabled fabrication of the hydrophobin hexagonal structure on solid substrate and preservation of this film under aqueous conditions. This made it possible to manipulate the hydrophilic side of the film under aqueous conditions and to monitor molecular interactions, even during simultaneous AFM imaging.

We produced two different HFBI variants having a thiol group at the N- or C-terminus that enables site-specific modification of HFBI using maleimide-thiol coupling chemistry (Figure 15). The variant termed NCys-HFBI has a 13 amino acid addition in the N-terminus and a Cys residue as the second amino acid. The 11 amino acid sequence between the Cys residue and the HFBI sequence is derived from a linker part joining two domains in a *T. reesei* cellulase, the cellobiohydrolase CBHI. This sequence is believed to have a high structural freedom. The variant termed HFBI-CysC has an added Cys residue as the second to last residue.

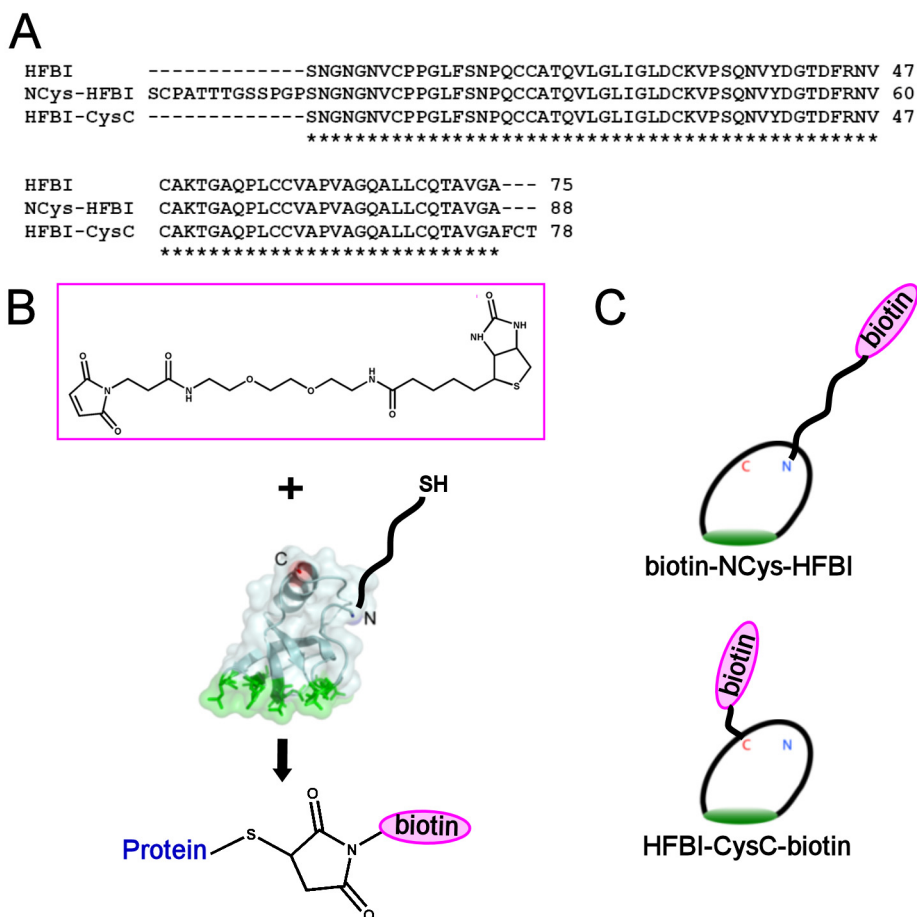


Figure 15. Biotinylation of the HFBI variants. (A) Amino acid sequences of HFBI and the variants NCys-HFBI and HFBI-CysC. (B) Biotin was covalently linked to the introduced thiol group of the protein using maleimide-thiol coupling chemistry. The double bond in the maleimide group reacts with the thiol in the protein and a stable carbon-sulfur bond is formed. (C) Cartoons of biotin-NCys-HFBI and HFBI-CysC-biotin having different linker lengths and positions.

In this work, we used the high affinity biotin – avidin interaction as a probe in the HFBI films (Wilchek *et al.* 2006). The biotin was conjugated to the variant HFBI using maleimide-PEO₂-biotin, resulting in biotin-NCys-HFBI and HFBI-CysC-biotin (Figure 15C).

Both variants were able to form native HFBI-like films and to adsorb to polystyrene. AFM imaging of dried samples showed that both variants formed the same self-assembled structure as native HFBI at the air-water interface (Appendix I, Figure 5 A–C). However, when attempting to image the films in buffer solution, high resolution images could be obtained only of the HFBI-CysC-biotin films. The long flexible linker part of NCys-HFBI apparently interfered with the AFM tip – sample surface interaction by moving under the tip in the aqueous environment. The molecular features of the hexagonally ordered HFBI film are small (around 5 nm) compared to the AFM tip radius that was used (10 nm) and additional structural flexibility appears to have made high resolution imaging unfeasible.

The ability of avidin to bind the biotinylated HFBI films was investigated using AFM and quartz crystal microbalance with dissipation monitoring (QCM-D). In QCM-D the studied surface is on a quartz crystal that oscillates at its resonance frequency. This frequency change is in relation to adsorbed mass. Dissipation (dampening) of the oscillation is dependent on the structure of the adsorbed mass. A rigid layer dampens the oscillation less than a soft layer. The changes in dissipation signal reflect the visco-elastic properties of the adsorbed layer.

Using AFM and QCM-D, avidin was observed to bind well from the hydrophilic, i.e. the water-facing side of the biotinylated HFBI films (Appendix I, Figure 5 D–F and Figure 6). Binding to the native HFBI film was not observed in buffered solutions. When biotin-NCys-HFBI was incubated with avidin in a Langmuir-trough, and thereafter the film was compressed and deposited to solid substrates using the LB and LS sample deposition methods, avidin molecules were visible only on the LS sample, i.e. on the water-facing side.

This one-sided avidin binding of the biotinylated HFBI films indicates that the biotin and hence the N- and C- terminal parts of HFBI are located at the water-facing side of the film. According to the X-ray crystal structure both termini are located in the hydrophilic part of HFBI. Thus, the observed one-sided avidin binding of the HFBI films supports the model in which the hydrophilic side of HFBI is facing water and the hydrophobic patch the air.

The results also demonstrate that the self-assembly properties of HFBI can be used to form layers with nanoscale structures. By using genetically engineered

hydrophobins new functionalities can be introduced to these layers. In this study, the biotin – avidin interaction was used to demonstrate this approach. An avidin layer was formed on top of a modified hydrophobin self-assembled layer by specific molecular interactions. Further layers could potentially be assembled in a layer-by-layer fashion. The results thus exemplify the use of protein engineering to form nanoscale structures with tunable properties.

4.2 HFBI and HFBII surface adsorption and immobilization of fusion partners (II)

Hydrophobins have been shown to attach fungal structures to the surfaces of host organisms and to hydrophobic surfaces such as Teflon (Wösten *et al.* 1994; Talbot *et al.* 1996). In this study, the surface adsorption properties of HFBI and HFBII were studied as partners in fusion proteins and as isolated proteins (Appendix II). The possibility of using hydrophobins for protein immobilization as fusion proteins was also investigated. The adsorption properties to different surfaces were analyzed using surface plasmon resonance (SPR), QCM-D and enzyme activity assay measurements.

SPR is a method in which the adsorbed mass on a surface is determined from the change in refractive index at the surface caused by molecular adsorption. The adsorbed mass cannot be directly calculated from the SPR response but empirically a conversion factor has been determined for estimating the adsorbed mass.

4.2.1 Surface adsorption properties of HFBI and HFBII

Both HFBI and HFBII showed efficient adsorption to hydrophobic surfaces. SPR results showed that a thin film is formed rapidly on a hydrophobic surface (Appendix II, Figure 3). Desorption was observed to be very slow. The surface regeneration using sodium dodecyl sulphate (SDS) apparently altered the surface so that repeated measurements did not give consistent results that could have been used for kinetic calculations. Measurements using QCM-D show that approximately 295 ng/cm² of HFBI (39 pmol/cm²) and HFBII (41 pmol/cm²) is maximally bound on an alkylated quartz surface (Appendix II, Figure 4). The

surface adsorption of HFBI and HFBII takes place in seconds. The hydrophobic patches of both HFBI and HFBII are about 4 nm². A theoretical monolayer packed with molecules occupying 4 nm² each would form a layer of about 40 pmol/cm². Thus the values obtained by QCM correspond well to a monolayer. Furthermore, the dissipation change indicates that the film is relatively rigid, as would be expected for a monolayer.

HFBI and HFBII adsorption to a hydrophilic quartz surface was also observed. However, the adsorption had a significantly lower rate than the adsorption to the hydrophobic surface, indicating different modes of adsorption for these two surfaces (Appendix II, Figure 4). The results show that HFBI and HFBII adsorb preferentially to hydrophobic surfaces probably via their hydrophobic patch, forming a rigid and stable monolayer.

4.2.2 Surface adsorption properties of fusion proteins containing HFBI and HFBII

HFBI and HFBII were joined each as C-terminal fusions to the catalytic domain of endoglucanase I (EGIc), connected by a 33 amino acid linker, resulting in EGIc-HFBI and EGIc-HFBII, respectively. EGI from *T. reesei* is a modular protein with a catalytic domain (EGIc) connected by a linker to a cellulose-binding domain. In the hydrophobin fusions the cellulose-binding domain has been replaced with HFBI or HFBII, thereby changing the protein's binding specificity. EGIc alone was used as a control sample.

The fusion protein EGIc-HFBI behaved as HFBI in SPR and QCM-D measurements, forming a layer of about 30 pmol/cm² on a hydrophobic surface (Appendix II, Figures 3 and 5). Adsorption of the control protein EGIc was negligible. EGIc-HFBII also adsorbed to hydrophobic surfaces. However, after rapid adsorption of EGIc-HFBII, a rapid desorption upon rinsing was observed in SPR. In QCM-D the amount of adsorbed EGIc-HFBII was observed to be only 13 pmol/cm², and thus HFBII fusion protein adsorption was not as effective as the adsorption of the HFBI fusion. Furthermore, a large dissipation change upon adsorption and the rapid desorption rate observed with SPR showed that the adsorption of EGIc-HFBII was weak and transient.

To test whether the hydrophobins could be used to immobilize fusion partners in their active form, the enzyme activities of the fusion proteins were studied. The fusion proteins were introduced to a surface for a specific time, then the surface was washed with excess buffer and finally the activity of bound EGlc towards a synthetic substrate molecule was determined. The enzymatic activity was determined by measuring the fluorescence of released 4-methylumbelliferone from the substrate 4-methylumbelliferyl β -D-cellobioside. As seen from the surface-bound activities, EGlc-HFBI adsorbed to hydrophobic alkylated glass and to a lesser extent to hydrophilic untreated glass (Appendix II, Figure 2). Efficient adsorption was also observed on Teflon and polystyrene (Appendix II, Figure 6). EGlc-HFBII and the control EGlc did not show detectable surface-bound activity.

The results using SPR, QCM-D, and enzyme activity assays show that EGlc-HFBI adsorbed efficiently to hydrophobic surfaces as a monolayer and, importantly, retained the activity of the fusion partner upon adsorption. EGlc-HFBII, however, did not bind efficiently to hydrophobic surfaces. Furthermore, the small amount of adsorbed protein lost its enzymatic activity on adsorption. HFBII appears thus to be less capable of retaining its adsorption properties when fusing with other proteins. HFBI and HFBII are very similar in structure (Hakanpää *et al.* 2004; Hakanpää *et al.* 2006b) but a clear difference is that HFBI has a longer N-terminal part preceding the first Cys-residue. The HFBI N-terminal part may be more flexible and thus allow more versatility in the structures of N-terminal fusions. Furthermore, the solution assemblies formed by EGlc-HFBI and EGlc-HFBII are clearly different, as seen in size exclusion chromatography (SEC). EGlc-HFBI forms very large assemblies that are perhaps octamers or decamers, whereas EGlc-HFBII was found as monomers in SEC. Native HFBI and HFBII form tetramers in solution at 10 mg/ml and dissociate to dimers and monomers when diluted (Torkkeli *et al.* 2002) (Appendix III). The interactions between monomers in the solution assemblies have been shown to be mainly hydrophobic interactions (Appendix III). It seems that these interactions are disrupted in the EGlc-HFBII, possibly because of HFBII folding back towards the EGlc part and thus preventing adsorption via the hydrophobic patch. However, a different linkage between the fusion partners could result in retaining the functionality of both parts.

Protein surface immobilization is extensively used in many areas of biotechnology, including sensors, biomaterials and diagnostic kits. However, very often adsorption of an enzyme to the surface is accompanied by protein denaturation and thus substantial loss in enzymatic activity. In addition, lack of oriented adsorption leads to buried and inaccessible active sites. Furthermore, as most enzymes do not have a high specific adsorption to common surfaces, high protein concentrations are needed in order to obtain reasonable surface coverage. These limitations in enzyme adsorption have been solved by producing sublayers on which modified enzymes can be bound specifically (Rusmini *et al.* 2007). In this way enzyme denaturation is limited and oriented binding can be achieved.

The results of this study show that HFBI can be effectively used to stably immobilize fusion partners in an oriented fashion to hydrophobic surfaces, retaining the biological activity of the fusion partner (Figure 16). The advantages compared to other methods are that adsorption is performed in a single step, using low amounts of protein. Furthermore, the HFBI layer provides the enzyme with a suitable environment. However, for different binding partners, new protein constructs must be made, expressed and purified. A more modular approach has been shown using modified HFBI adsorbed to a surface and then introducing a second layer of molecules that specifically bind to the modified HFBI (Kostiainen *et al.* 2006, Appendix I). This approach could be used to build multilayers of biomolecules in a layer-by-layer fashion. Taken together, the surface adsorption properties of HFBI can be utilized to mediate immobilization of fusion partners or proteins that bind to modified HFBI.

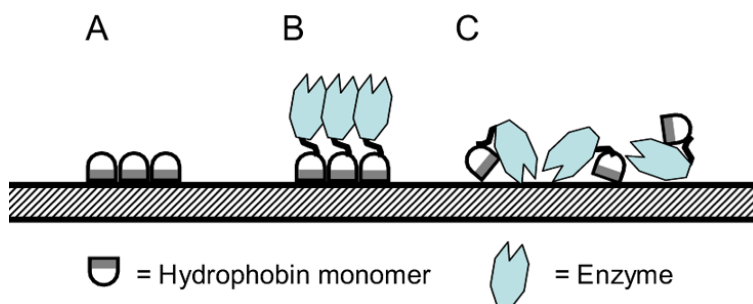


Figure 16. Cartoon of HFBI or HFBIII (A), EGlc-HFBI (B), and EGlc-HFBIII (C) adsorbed to a hydrophobic surface. (B) Adsorption of EGlc-HFBI retains the enzymatic activity of the enzyme. (C) The adsorption of EGlc-HFBIII results in inactive enzyme due to the lack of oriented adsorption.

4.3 HFBI solution behavior (III, IV)

Hydrophobic molecules are energetically unfavorable in solution as they have poor interactions with water. Despite their large hydrophobic patches HFBI and HFBII can be very soluble in water, up to 100 mg/ml (Linder, M.B., unpublished data). The exposure of the hydrophobic patches of hydrophobins towards water is energetically unfavorable and thus the hydrophobic patches are clustered together. It has been previously shown with SAXS and SEC that HFBI and HFBII form tetramers in aqueous solution at about 10 mg/ml (Torkkeli *et al.* 2002). Upon dilution the tetramers dissociate possibly to dimers or monomers. Dimers were also found as asymmetric units in the HFBII X-ray crystal structure (Hakanpää *et al.* 2004) and tetramers in the HFBI structure (Hakanpää *et al.* 2006b). Importantly, the hydrophobic patches of these multimers in the crystals were facing each other.

Common detergent molecules behave in a similar manner and form aggregates such as micelles in aqueous solutions. In micelles the hydrophobic groups of the amphiphilic molecules are clustered inside the micelle and the hydrophilic groups are at the outside, interacting with water. Micellization takes place above a certain detergent concentration that is called the critical micellization concentration (CMC). At the CMC the interfaces are fully occupied by detergent molecules and additional monomers in the bulk phase are not soluble, causing them to cluster into micelles (Figure 17). The CMC is characteristic for a certain detergent and the size and shape of micellar structures depend on the detergent's geometrical shape and the nature of its hydrophilic part.

To learn about the hydrophobin behavior preceding surface adsorption and self-assembly, we examined the solution behavior of HFBI and HFBII (Appendices III and IV) and moreover, the relation between solution behavior and surface properties (Appendix IV).

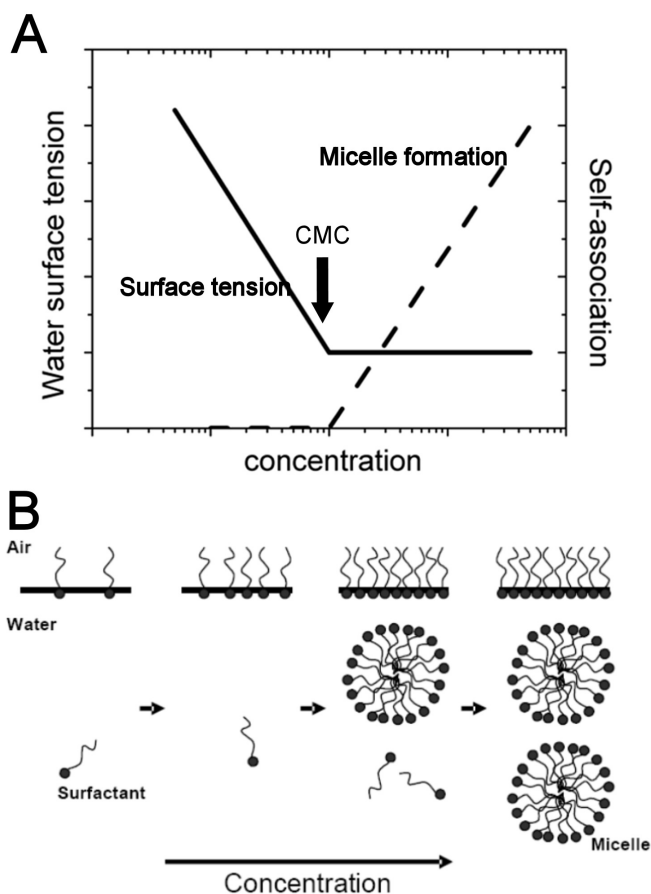


Figure 17. Surfactant micelle formation in aqueous solution. (A) When the surfactant concentration is increased the surface tension decreases (solid line) until a point is reached at which addition of further surfactant molecules does not further reduce the surface tension. This concentration is called the critical micellization concentration (CMC) because addition of further surfactant molecules results in formation of micelles in solution (dashed line). (B) Cartoon of detergents forming micelles above their CMC.

4.3.1 HFBI multimerization

The interactions between hydrophobin monomers were studied using Förster resonance energy transfer (FRET) and SEC (Appendix III). The FRET method (in the literature also referred to as fluorescence resonance energy transfer or

resonance energy transfer) relies on the distance dependent energy transfer between two dye molecules that have a certain spectral overlap (Figure 18A) (Clegg 1992; Lakowicz 1999). We used the genetically engineered HFBI variant NCys-HFBI (Appendix I) to label fluorescent dyes in a site-specific manner (Figure 15). If the hydrophobin monomers and thus the conjugated dyes are close to each other, an excited donor dye will transfer part of its excited state energy to an acceptor dye. The energy transfer efficiency is very strongly dependent on the donor – acceptor distance, thus being sensitive to molecular interactions such as multimeric states of associating molecules.

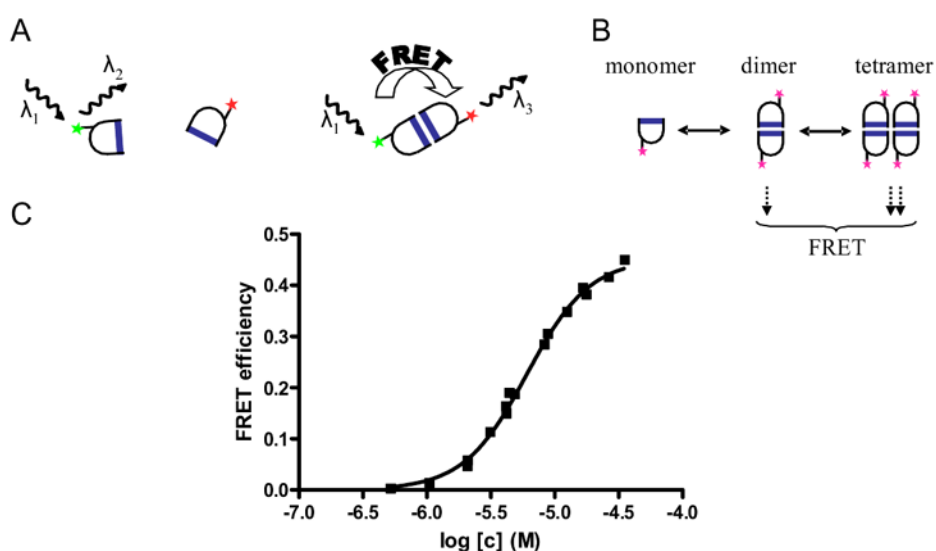


Figure 18. Homomultimerization studied by FRET. (A) FRET occurs only if donor (green) and acceptor (red) dye molecules are in close proximity. (B and C) Formation of dimers and tetramers gives rise to FRET. (C) FRET efficiency and thus multimerization of labeled NCys-HFBI is concentration dependent (Appendix III).

The observed NCys-HFBI FRET signal was strongly concentration dependent, showing that multimers are present at above about 20 μ M and monomers below about 1 μ M (Figure 18). Between these concentrations an equilibrium of a mixture of multimers and monomers is present. The subunit interchange (association and dissociation) rate was fast, as FRET was instantly observed (sample preparation took a few minutes) and the signal remained unchanged for hours.

SEC was used to study the nature of the multimers. HFBI was found to form multimers in a concentration dependent manner (Appendix III, Figure 3). At high concentrations the multimers corresponded to tetramers (Torkkeli *et al.* 2002). During the SEC runs, the samples, injected at a volume of 100 μ l, were constantly diluting and finally eluting in a volume of several milliliters. Thus the precise concentrations of the eluted samples were not known. Nevertheless, the protein concentrations used in the FRET experiments were accurately known. When comparing the FRET and SEC multimerization isotherms it is evident that the shapes of these curves are almost identical. The combined data show that the multimerization isotherm of the FRET experiments describes the formation of tetramers from monomers.

All known protein tetramerization pathways proceed through a single pathway of first dimerization and concomitantly dimerization of dimers (Powers and Powers 2003). Tetramerization via successive monomer additions (monomer – dimer – trimer – tetramer) can lead to trapped states in the pathway and thus this pathway is evolutionally not favored. It is very likely that HFBI tetramerization does not differ from other known proteins in this respect and proceeds through dimerization of dimers. This mode of tetramerization always has a cooperative element, as dimerization of dimers depends on the formation of dimers.

Interestingly, the FRET and SEC HFBI multimerization isotherms have sigmoidal characters that would imply a moderately cooperative binding. However, cooperativity may not be the only reason for the sigmoidal curve, as the FRET signal originating from tetramers is presumably larger than from dimers. It is thus unclear whether the observed sigmoidal curve shape originates from cooperative multimerization or as a result of an enhanced FRET signal from the tetramers as compared to the dimers. The conclusion of both interpretations is that the FRET and SEC signals originate from the formation of HFBI dimers and tetramers.

Comparison of the dissociation constants of multimerization (about 6 μ M) (Appendix III) and of surface adsorption (about 0.4 μ M) (Appendix II) shows that HFBI has an order of magnitude higher affinity for interfaces than for forming multimers. Conventional detergents behave in the same way: monomers in solution are energetically unfavorable and they are driven by the hydrophobic effect to adsorb to hydrophobic interfaces. As the concentration is increased and

the interfaces become occupied, detergents self-associate to form micellar structures in which the hydrophobic parts are buried and inaccessible to water. At low concentrations where multimers are not observed, HFBI adsorbs to surfaces and to the air-water interface. At higher concentrations, when the interfaces are already filled, HFBI associates in solution into multimers. The higher preference for interfaces than towards self-association is a common feature among all surfactants (Holmberg *et al.* 2002).

4.3.2 Protein – protein interactions in HFBI multimers

In order to study the nature of the protein – protein interactions in the multimers, we chose an intermediate concentration for FRET studies at which enhancement and inhibition of multimerization can be observed. Ethanol and detergents decreased the FRET, whereas salts along the Hofmeister series enhanced the FRET signal effectively (Appendix III, Table 1). The data indicated that hydrophobic interactions have a major role in the multimerization of HFBI. Furthermore, FRET efficiency was observed to increase with increasing temperature up to about 55 °C and then decrease when heated more (Appendix III, Figure 5). This behavior was reversible upon cooling the samples. As HFBI has been shown to retain its secondary structure up to 90 °C (Askolin *et al.* 2006), the temperature dependence of FRET is interpreted as changes in multimerization state. The hydrophobic effect is assumed to behave equally and to show an increase from low to moderate temperatures and a decrease when heated further (Maibaum *et al.* 2004).

The asymmetric units in HFBI and HFBII crystals are composed of tetramers and dimers, respectively (Hakanpää *et al.* 2004; Hakanpää *et al.* 2006b). In both cases the monomers are in contact through their hydrophobic patches (Figure 19). The multimeric structures observed in the crystals are thus also likely to be valid in solution.

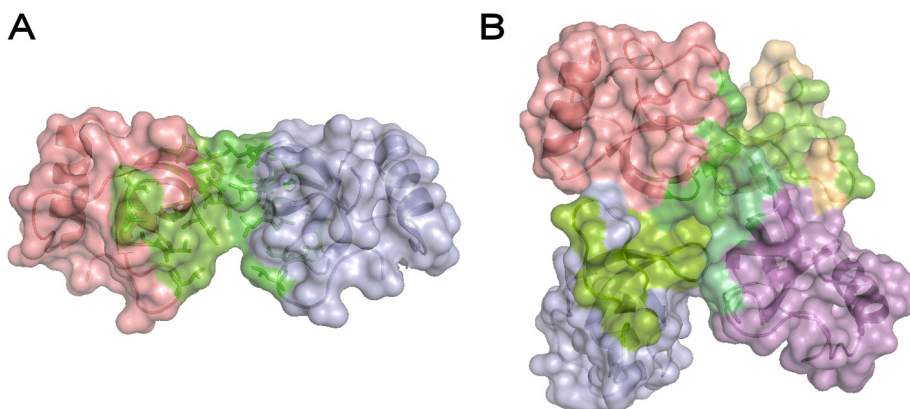


Figure 19. Multimers in the X-ray crystal structures. (A) HFBI dimer (PDB ID 1R2M (Hakanpää et al. 2004)) and (B) HFBI tetramer (PDB ID 2FZ6 (Hakanpää et al. 2006b)) showing the clustered hydrophobic patches (greens) of the interacting subunits. Images were produced using PyMol (DeLano 2002).

The results thus show that HFBI forms surface films and micelle-like multimers in a surfactant-like manner. Both surface adsorption and multimerization are mainly a consequence of hydrophobic interactions. However, in contrast to detergent micelles, HFBI and HFBI form multimers of defined size. They form mainly tetramers, and dimers and monomers upon dilution. In addition to the important hydrophobic interactions, other interactions are also very probably involved.

Formation of detergent micelles does not depend on specific molecular interactions, and mixed micelles containing different detergents are easily formed. To study the specificity of HFBI multimer formation, we investigated whether HFBI and HFBI could interact. SEC shows that HFBI forms multimers in a concentration dependent manner like HFBI. However, the shape of the multimerization isotherm is different from the isotherm of HFBI, showing that HFBI has a lower tendency for multimerization (Appendix III, Figure 3). FRET measurements showed that excess HFBI could reduce FRET but the reduction was not as efficient as with native HFBI (Appendix III, Figure 4). This indicates that HFBI – HFBI interactions are taking place, but that HFBI has a reduced ability to compete for the labeled HFBI. CBHI, a cellulase that is secreted in large amounts by *T. reesei*, was used as a control. Addition of CBHI to the labeled HFBI did not reduce FRET, showing that CBHI does not affect HFBI solution association.

Using SEC, it was shown that at a combined concentration of 135 μM HFBI and HFBII mostly elute separately, although a significant amount of HFBII was eluted together with HFBI. It is likely that at this concentration HFBI and HFBII interact and mixed multimers are formed. The amino acid sequences of HFBI and HFBII are 69 % identical and they share an almost identical hydrophobic patch according to their X-ray structures. The results indicate that HFBI – HFBII chimeric multimers can be formed but that HFBI and HFBII do not preferentially interact. Previously it has been shown that HFBI and SC3 do not form mixed films, also indicating that different hydrophobins do not readily interact at interfaces (Askolin *et al.* 2006).

4.3.3 The role of HFBI solution association in the surfactant properties of HFBI

The effect of hydrophobin solution multimerization on the surface adsorption process has remained unclear. As described in Section 4.3.1, HFBI is surface active at concentrations at which monomers are predominant. Furthermore, adsorption to hydrophobic solid surfaces occurs when monomers are predominant. In other words, the affinity for the air – water interface and hydrophobic surfaces is higher than the affinity for self-association into multimers. Detergent molecules function similarly: at low concentrations monomers are present and they adsorb to interfaces. Detergent monomers in solution start to associate into micelles when the surfaces are fully occupied and the concentration exceeds the CMC (Figure 20A) (Holmberg *et al.* 2002).

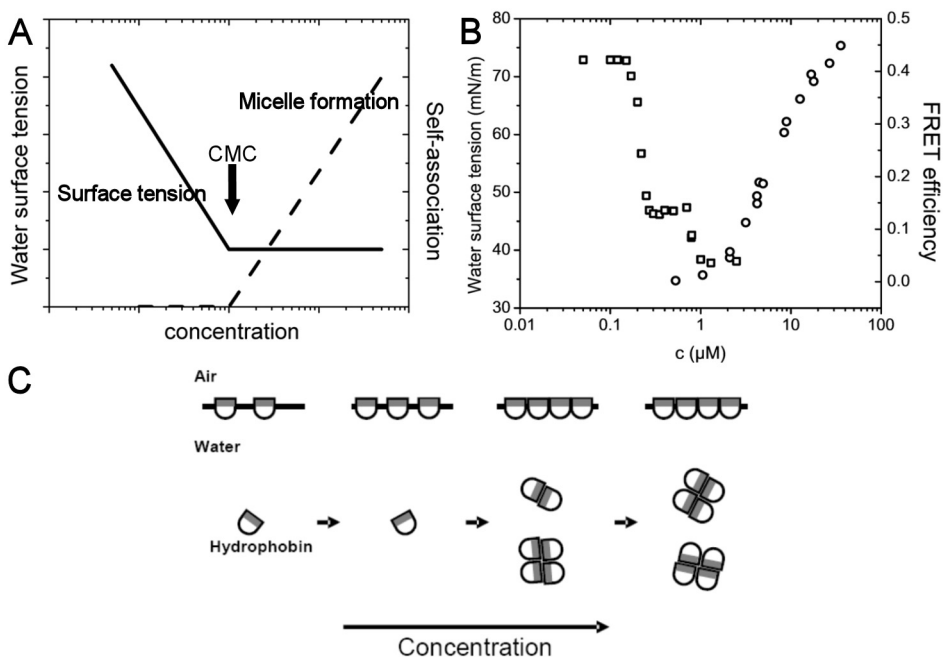


Figure 20. Comparison of detergent (A) and HFBI (B) surfactant behavior. (A) Detergents form micelles above their CMC. (B and C) Upon increasing the HFBI concentration the surface tension (squares) is lowered (Appendix IV) until a point which is called the surface saturation concentration is reached. Addition of further HFBI molecules does not further reduce the surface tension but results in formation of dimers and tetramers in solution (circles) (Appendix III).

In order to study the relationship between the hydrophobin surface and solution properties in more detail we produced two HFBI variants that showed altered solution multimerization properties. The variants NCys-HFBI and HFBI-CysC were purified as disulfide linked dimers that were termed (NCys-HFBI)₂ and (HFBI-CysC)₂, respectively (Figure 21). The variants differ in the length and position of their linker parts. (NCys-HFBI)₂ has a long (24 residue) linker connecting the N-termini and (HFBI-CysC)₂ has a short (4 residue) linker connecting the C-termini.

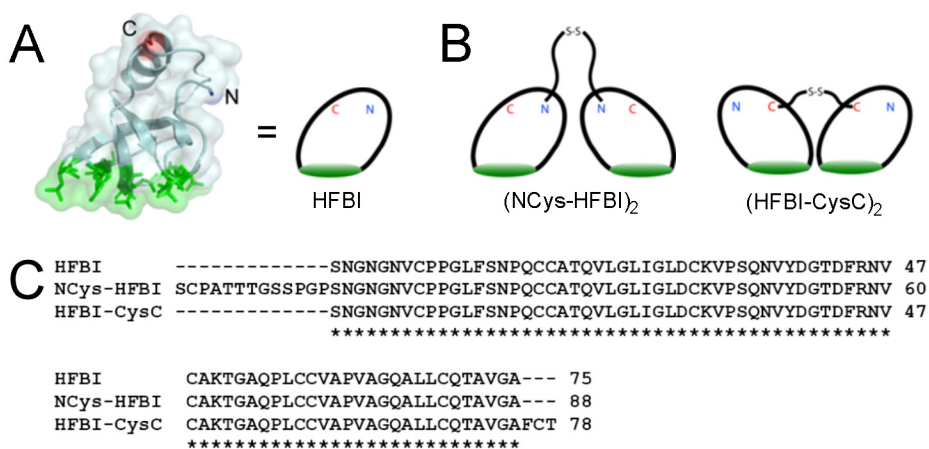


Figure 21. HFBI and dimeric variants used in this study. (A) X-ray crystal structure of HFBI (PDB ID 2FZ6 (Hakanpää et al. 2006b)). The conserved hydrophobic patch is colored green. (B) Cartoons of the dimeric variants $(NCys-HFBI)_2$ and $(HFBI-CysC)_2$. (C) Comparison of amino acid sequences. (Modified from publication IV (Appendix IV).)

The disulfide-linked HFBI variants had a substantially altered solution behavior as compared to the native HFBI. The multimerization equilibrium was shifted strongly towards solution association as seen using SEC (Appendix IV, Figure 3). Dissociation of the multimers formed by disulfide-linked dimers was not observed even after dilution down to the detection limit of the used SEC system. Using SAXS it was shown that the multimers formed by the disulfide-linked dimers were HFBI-like tetramers (Appendix IV, Figure 4). As the dimerization of HFBI is an intermediate step in the route to tetramerization, covalently linking two HFBI units apparently stabilizes this intermediate effectively and thus shifts the equilibrium towards the tetramer state.

Despite the great change in solution association the interfacial properties of the disulfide-linked variant HFBI were only minimally changed. The concentration at which the lowest water surface tension was reached was very similar for the disulfide-linked variants and native HFBI (Appendix IV, Figure 5). In the context of detergents, this concentration value is often referred to as the CMC. In the case of the disulfide-linked variants, however, this does not hold true as the HFBI-like tetramers formed independently of the saturation of interfaces. Although the association equilibrium of the variants was significantly shifted

towards native HFBI-like tetramers, there was only little difference in the surface activity between the variants and the native HFBI. Here, the term surface saturation concentration is used to describe the concentration at which addition of HFBI does not decrease the surface tension further (Figure 20B).

Other surface properties of the disulfide-linked variants, such as Langmuir-film formation and monolayer formation on hydrophobic surfaces, were also almost unaltered (Appendix IV, Figures S1 and S2, Supplemental material). Formation of self-assembled fibrils by shear forces was not altered in the disulfide-linked dimer variants (Appendix IV, Figure S3, Supplemental material). Stabilizing the tetramer state apparently does not prevent hydrophobin fibril formation.

As is typical for proteins, adsorption to the air-water interface is a rather slow process at low concentrations (Appendix IV, Figure 6). The disulfide-linked dimer variants were observed to be slower than native HFBI in lowering the water surface tension. It is probable that at the interface the multimers must rearrange to expose their hydrophobic patches, and that this rearrangement takes longer for the covalently linked variants due to linker-derived restraint. Similarly, the adsorption of detergent micelles to interfaces involves relaxation of the micelle assembly (Toomey *et al.* 2005). (HFBI-CysC)₂ did not lower the surface tension to as low a value as HFBI or (NCys-HFBI)₂, possibly due to the shorter linker in this variant.

In summary, shifting the HFBI solution association equilibrium significantly towards tetramerization did not affect the surface properties such as surface activity, surface adsorption, air – water interface film formation, and self-assembly into fibrils. There is thus no strong relationship between the solution association and the surface properties of HFBI. As the monomers and multimers have both been shown to be surface active it seems that multimer dissociation to monomers is not required for surface activity.

The hydrophobin SC3 from *S. commune* has also been shown to possess an equilibrium between monomers, dimers and tetramers in solution (Wang *et al.* 2004a). It has been suggested that the SC3 multimers could be the building blocks for the self-assembled rodlets observed at the air – water interface. In this work, the self-assembly of HFBI at the air-water interface was not systematically studied as a function of the multimeric state in solution. However,

the bulk hydrophobin concentration may not be important for self-assembly, as the local concentration at the air-water interface experienced by a hydrophobin molecule can be much higher than the concentration in the bulk solution. This high local concentration could effectively promote monomer association and further self-assembly into the observed hexagonally ordered monolayers.

In conclusion, the data show that changing the solution association equilibrium of HFBI does not affect its surface properties. HFBI monomers, as well as dimers and tetramers, are surface active units. As both solution association and surface activity originate from hydrophobic interactions and the amphiphilic structure, it is very likely that monomer rearrangements need to take place at the interface for exposure of the hydrophobic patch. For the formation of self-assembled structures, such as the hexagonally ordered monolayer, the aligned orientation of hydrophobins and the high local concentration experienced by a hydrophobin monomer at the air-water interface may have a greater significance than the preceding multimeric state in solution.

5. Conclusions

The data presented show that the functions of the hydrophobins HFBI and HFBII are governed by their amphiphilic nature. Their functions resemble those of common detergents, such as adsorption to interfaces, formation of monomolecular layers, and association in solution to shield their hydrophobic parts from water. Hydrophobins are thus considered as fungal surfactants. However, there are fundamental differences between hydrophobins and detergents that arise from the unusual molecular structure of hydrophobins. In comparison to detergents, HFBI and HFBII are large and have a defined and rigid structure. In addition, proteins have many different chemical groups at their surface that make specific intermolecular interactions possible.

This unusual amphiphilic structure leads to unique properties at interfaces and in solution. Hydrophobins have been shown to be highly surface active molecules (Wösten *et al.* 1999; Askolin *et al.* 2006, Appendices II and IV). This high surface activity and their tendency to adsorb to various surfaces make them interesting for use in various applications. In Figure 22 the HFBI function at interfaces and in solution is summarized. At the air-water interface HFBI was shown to self-assemble into a highly organized monolayer structure (Figure 22 A, C and D) (Appendix I). Specific intermolecular interactions in the monolayer lead to the self-assembled structure and a cohesive film which can be observed as the formation of a planar film on the surface of a solution drop (as seen in Figure 9). The tendency of HFBI and HFBII towards surface adsorption (Figure 22 B) can be used to bind other molecules effectively and in a specified orientation (Appendices I and II). Furthermore, HFBI and HFBII can form needle-like fibrils in solution (Figure 22 E), although the mechanism inducing the formation of these structures is currently poorly understood.

The amphiphilic nature of hydrophobins also determines their behavior in solution. Similarly to the formation of detergent micelles, HFBI forms multimers in which hydrophobic interactions are significant (Figure 22 A) (Appendix III). However, the HFBI solution association yields multimers with defined structures and appears to involve some degree of specificity (Appendix III). Although solution multimerization appears to be important for the solubility of HFBI, the surface active properties and multimerization are not functionally interconnected

(Appendix IV). Multimer formation is a transient event; multimers disassemble and reassemble at a rapid rate, and furthermore the affinity for multimerization is relatively weak. This ensures that adsorption to interfaces is retained even in the presence of solution multimers (Figure 22 A–C) (Appendices III and IV).

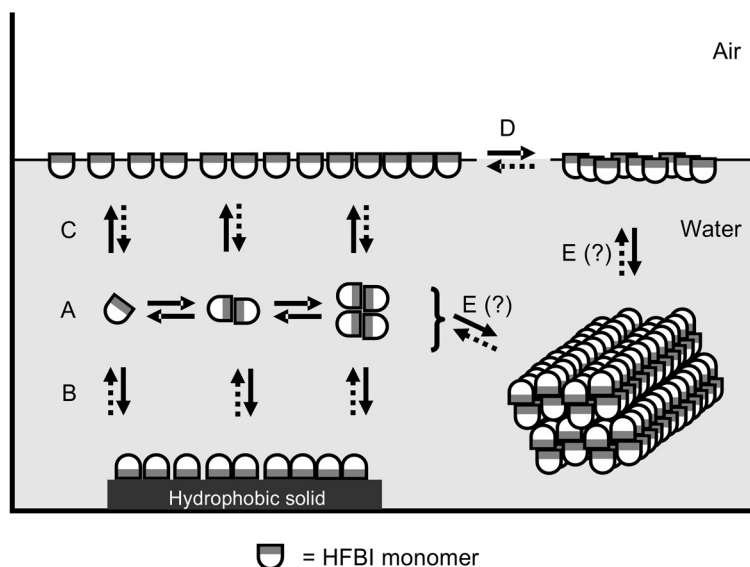


Figure 22. Proposed model for HFBI function in solution and at interfaces. (A) HFBI associates in solution into dimers and tetramers. (B and C) The surface activity is not dependent on the multimeric state of HFBI. It is likely that the adsorption of multimers to the interface is accompanied by a subunit rearrangement. (D) At the air-water interface HFBI self-assembles into a hexagonally ordered monolayer. (E) Shaking a HFBI solution causes the formation of needle-like fibrils. It is currently unclear whether the fibrils are formed by soluble HFBI units or by the structures at the interfaces.

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Author(s) Szilvay, Géza R.		
Title Self-assembly of hydrophobin proteins from the fungus <i>Trichoderma reesei</i>		
Abstract Hydrophobins are small surface active proteins that are produced by filamentous fungi. The surface activity of hydrophobin proteins leads to the formation of a film at the air-water interface and adsorption to surfaces. The formation of these hydrophobin films and coatings is important in many stages of fungal development. Furthermore, these properties make hydrophobins interesting for potential use in technical applications. The surfactant-like properties of hydrophobins from <i>Trichoderma reesei</i> were studied at the air-water interface, at surfaces, and in solution. The hydrophobin HFBI was observed to spontaneously form a cohesive film on a water drop. The film was imaged using atomic force microscopy from both sides, revealing a monomolecular film with a defined molecular structure. The use of hydrophobins as surface immobilization carriers for enzymes was studied using fusion proteins of HFBI or HFBII and an enzyme. Furthermore, site-specifically modified variants of HFBI were shown to retain their ability to self-assemble at interfaces and to be able to bind a second layer of proteins by biomolecular recognition. In order to understand the function of hydrophobins at interfaces, an understanding of their overall behavior and self-assembly is needed. HFBI and HFBII were shown to associate in solution into dimers and tetramers in a concentration dependent manner. The association dynamics and protein-protein interactions of HFBI and HFBII were studied using Förster resonance energy transfer and size exclusion chromatography. It was shown that the surface activity of HFBI is not directly dependent on the formation of multimers in solution.		
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Tekijä(t) Szilvay, Géza R.		
Nimeke <i>Trichoderma reesei</i> -homeen hydrofobiiniproteiinien itsejärjestäytyminen		
Tiivistelmä Hydrofobiinit ovat rihmasienten tuottamia pieniä pinta-aktiivisia proteiineja. Pinta-aktiivisina molekyyleinä hydrofobiiniproteiinit muodostavat kalvon ilman ja veden rajapinnalle sekä kiinnittyvät kiinteille pinnoille. Näiden kalvojen ja pinnoitteiden muodostuminen on tärkeää monissa rihmasienen yksilönkehityksen vaiheissa. Lisäksi nämä ominaisuudet tekevät hydrofobiineista kiinnostavia molekyylejä teknisiin sovelluksiin. <i>Trichoderma reesei</i> -homeen tuottamien HFBI- ja HFBII-hydrofobiinien pinta-aktiivisuusominaisuuksia tutkittiin ilman ja veden rajapinnalla, kiinteillä pinnoilla sekä liuoksessa. HFBI-hydrofobiinin havaittiin muodostavan yhtenäisen kalvon vesipisaran pinnalle. Kalvon molemmat puolet kuvattiin atomivoimamikroskoopilla ja osoitettiin, että kalvo on muodostunut yhdestä hydrofobiinimolekyylikerroksesta, jolla on hyvin järjestäytynyt molekyylitason rakenne. Entsyymien kiinnittämistä pinnoille hydrofobiinien avulla tutkittiin käyttäen HFBI:n tai HFBII:n ja mallientsyymien fuusioproteiineja. Lisäksi osoitettiin, että kohdennetusti muokattu HFBI-muunnos säilyttää kykynsä itsejärjestäytyä rajapinnoilla ja kykenee sitomaan hydrofobiini-kalvon päälle toisen proteiinkerroksen käyttäen biomolekulaarista tunnistusta. Ymmärtääksemme paremmin hydrofobiinien pinta-aktiivisuusominaisuuksia on tarpeen tuntea hydrofobiinien yleisiä ominaisuuksia ja itsejärjestäytymistä. HFBI sekä HFBII yhdistyvät vesiliuoksessa dimeereiksi ja tetrameereiksi, ja reaktio on riippuvainen proteiinien pitoisuudesta. Liuos-multimeerien muodostumisen dynamiikan ja proteiinien välisten vuorovaikutusten tutkimiseen käytettiin Förster-resonanssienergiansiirtomenetelmää sekä geelisuodatuskromatografiaa. Tutkimuksessa osoitettiin, että HFBI:n pinta-aktiivisuus ei ole suoraan riippuvainen liuos-multimeerien muodostumisesta.		
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