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Hydrophobins: the protein-amphiphiles of filamentous fungi

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Abstract

Hydrophobins are surface active proteins produced by filamentous fungi. They have a role in fungal growth as structural components and in the interaction of fungi with their environment. They have, for example, been found to be important for aerial growth, and for the attachment of fungi to solid supports. Hydrophobins also render fungal structures, such as spores, hydrophobic. The biophysical properties of the isolated proteins are remarkable, such as strong adhesion, high surface activity and the formation of various self-assembled structures. The first high resolution three dimensional structure of a hydrophobin, HFBII from *Trichoderma reesei*, was recently solved. In this review, the properties of hydrophobins are analyzed in light of these new data. Various application possibilities are also discussed.

Keywords: Hydrophobin; Filamentous fungus; Protein amphiphile; Protein surfactant; Protein self-assembly; Protein adhesion

Contents

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1. Introduction

Hydrophobins are proteins produced by filamentous fungi that have very special properties; they are amphiphiles having hydrophilic and hydrophobic parts and are among the most surface active proteins known. Hydrophobins have consequently raised considerable interest in the scientific community. Their suggested roles in the fungal life are fascinating and the application possibilities are imagination-triggering.

The first hydrophobin genes were found in a search for abundantly expressed genes during the development of *Schizophyllum commune*, without then knowing the identity and nature of the proteins. Based on the deduced protein sequences, Wessels et al. [1] introduced the name hydrophobin for these relatively small fungal proteins of about 10 kDa in size. Apart from containing a large proportion of hydrophobic amino acids, it was noted that one main unifying feature was the presence of eight Cys residues [2], the second and third always being immediate neighbors as also the sixth and the seventh.

Subsequently, it was shown that different hydrophobins are expressed at different stages of fungal life ranging from vegetative hyphae and sporulating cultures to the fruiting bodies (such as mushrooms). Many of the initial observations on hydrophobins were made with mutant strains that were in some way impaired in interacting with their environment. Early examples include the formation of aerial structures, i.e., the ability of fungi to grow towards the air [1], and interaction of pathogenic fungi with the host plants [3]. It is now known that hydrophobins can be found in the medium in liquid fungal cultures, they assemble onto the fungal cell walls, cover fungal spores, and coat the surface and air cavities in fruiting bodies, for example. The properties and biological roles of hydrophobins have been discussed in several recent review articles [4–14].

In fungi, hydrophobins seem to fulfill several different roles. On the one hand they help the fungi to survive in and adapt to the environment, and on the other hand they have various structural roles. It is interesting to consider how these roles are linked to different surface phenomena. At the scale of microbes, surface phenomena become the dominant force, while, e.g., gravity is almost inconsequential. For example, the surface tension of water is a very strong force on this scale, which can easily overcome the gravitational force. The role of hydrophobins seems to be in controlling these surface forces. Thus, the most important functional feature of hydrophobins is their ability to interact with surfaces, coating the surfaces and lowering surface tension. The understanding of the structure–function relations has been hampered by difficulties in obtaining detailed structural data, leading to a dependence on low resolution data and models.

A major step towards understanding the physical and molecular basis of hydrophobin function is the recent determination of the first high resolution 3D-structure of a hydrophobin molecule [15]. The structure is very different from that previously predicted, and gives a new framework for understanding the properties observed for them. In this review, we will examine the properties of hydrophobins in the light of the recent structural data and also discuss the application potential of this class of proteins.

2. Early studies on hydrophobins

The property that initially dominated hydrophobin characterization was the insolubility of the complexes formed by the protein. The high Cys-content of the proteins allowed one to take advantage of radioactive S^{35} labeling of *S. commune* mycelium to trace the protein and to work out how to handle it. The complexes could not be solubilized using hot sodium dodecyl sulphate

(SDS), but formic acid released the protein in a soluble form [16]. Only after an additional treatment with performic acid could the hydrophobin be seen as a soluble protein that was detectable in SDS–PAGE. It was later found that trifluoro acetic acid (TFA) also could solubilize the protein into a SDS–PAGE detectable form. Most of the initial protein characterization was done with the SC3 hydrophobin of *S. commune*, as an effective and very special way of purifying it was developed [17]. It was realized that the protein was initially soluble when it was secreted into the culture medium, and mixing the protein with air caused aggregation to occur. One could, therefore, capture the hydrophobin as a precipitate by agitation. The precipitate was then dissolved in TFA, and after evaporation of TFA, the protein was again soluble in water.

Comparison of amino acid sequences soon also suggested that previously characterized proteins, such as cerato-ulmin (CU), were hydrophobins [18]. CU had been described as a toxin of the fungus *Ophiostoma ulmi*, which causes the Dutch elm disease. The isolated CU protein had shown effects similar as the pathogen itself on trees [19]. However, later experiments showed that CU is a fitness factor for the fungus rather than a toxin against the plant [20]. CU had previously been studied rather extensively, but was not as extreme in its characteristics as the SC3 hydrophobin [21]. Another early example of a hydrophobin is cryparin which was isolated as a highly expressed protein (25% of total mRNA under some conditions) in the chestnut blight-causing fungus *Cryphonectria parasitica* [22,23]. As will be discussed below, characteristic protein aggregates had been noted for cryparin and CU, but these did not require treatments with strong acids to dissolve.

In 1994, Wessels [24] compared the amino acid sequences of the then known nine hydrophobins by looking at the clustering of hydrophobic residues using the method developed by Kyte and Doolittle [25]. The conserved Cys-residues were used to align the sequences. It was found that they could be divided into two groups based on the patterns of clustering of hydrophobic and hydrophilic groups. The two groups were called class I and class II (with SC3 of *S. commune* in I and CU of *O. ulmi* in II). The limited biochemical data available at that time suggested that a difference between these classes might be in the solubility of the aggregates they form. Based on these data, Wessels predicted that class I aggregates would only dissolve in strong acids such as TFA, while class II aggregates could be dissolved using aqueous dilutions of organic solvents. So far this prediction has been remarkably accurate, although the biological significance remains unclear.

Already after a few years of hydrophobin studies, a basic understanding of their function had emerged. Hydrophobins are commonly found as structural proteins located on surfaces of aerial structures [8]. A link between expression of hydrophobins and the ability of the fungus to grow aerial structures was established. In aerial conidia and hyphae, a hydrophobic coating was proposed to have a protecting role both against desiccation and wetting, and aid, e.g., in dispersal of spores [3,16,22,26–28], or water mediated dispersal of conidia [29]. It was also noted that certain hydrophobins seemed to be involved in fungal pathogenesis. Supporting evidence was obtained from physiological studies and with fungal strains which were impaired in hydrophobin expression. It was suggested that hydrophobins could mediate attachment of the fungal infection structures to their targets either as structural components of the appressorium cell wall or by binding to and modifying host surfaces [3,30]. On a general level these initial data gave a picture of the physiological role of hydrophobins that has essentially not changed since.

3. The three dimensional high-resolution structure of HFBII from *Trichoderma reesei*

Given the difficulties in the biophysical characterization of hydrophobin properties and their recalcitrant behavior, the molecular basis of the function of hydrophobins has from the start of the research been elusive. Obtaining high-resolution structural data has been especially difficult. For example, precipitation and aggregation of samples occurs readily, making sample preparation difficult for NMR as well as X-ray analysis. Crystallization of the class II member, HFBII from *T. reesei* did however finally succeed by careful screening of crystallization conditions. Obtaining heavy atom derivates also proved difficult, but it was finally possible to calculate the phases using a bound manganese ion and combining data from different radiation sources [15].

The three dimensional structure of HFBII from *T. reesei* at 1 Å resolution gives us a new basis to understand, at the molecular level, how hydrophobins function [15]. This structure is shown schematically in Fig. 1. Comparison of this structure to others in the databases shows that the hydrophobin fold is unlike that of any other previously analyzed protein, differing for example clearly from other proteins containing several disulfides. The overall shape of the molecule is globular and it has a diameter of about 2 nm. It has a central β structure comprising two β hairpins. In the primary sequence, the first hairpin is located near the N-terminus and the second near the C-terminus. The two hairpins connect and interlock with each other to form one anti-parallel β sheet. This β sheet forms a barrel-like structure. In between the two β hairpins there is one α helix that is formed by residues in the middle part of the primary sequence. In the tertiary structure, this α helix lies outside the central β barrel.

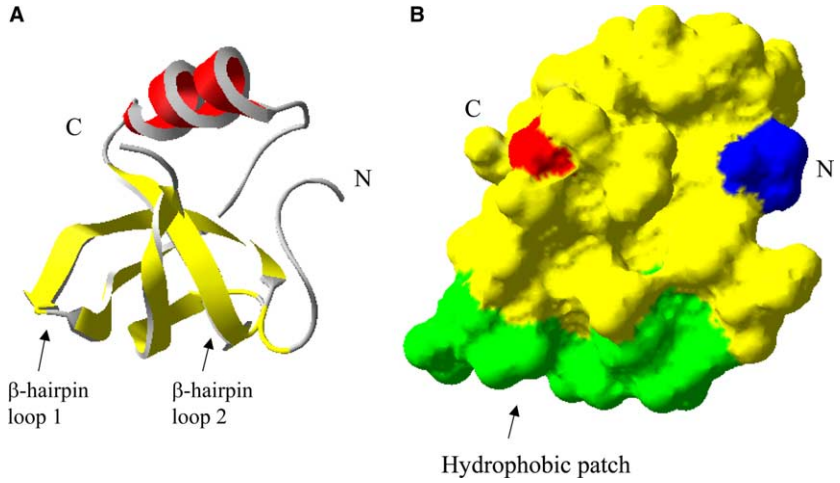


Fig. 1. The structure of the *T. reesei* HFBII hydrophobin shows an amphiphilic molecule with one hydrophilic and one hydrophobic part. (A) Cartoon of the secondary structure elements in HFBII. There is a central β sheet structure that is formed by two β hairpins. The two loops of the hairpins form most of the hydrophobic patch and are indicated. (B) A space-filling model of the structure in the same scale and orientation shows the hydrophobic patch in green, the rest of the surface in yellow, except for the N- and C-termini that are indicated in blue and red, respectively. The coordinates for the structure are available from the protein data bank [94] with the identification number 1R2M.

Analysis of the surface residues of HFBII reveals a large patch consisting of hydrophobic aliphatic residues. This hydrophobic patch is formed mainly by residues near the loop-regions of the two β hairpins. The surface is relatively flat and comprises about 12% of the total surface area of the protein. About half of the hydrophobic aliphatic residues of HFBII are located at the surface, forming this patch. In contrast, the hydrophobic residues in soluble proteins in general typically form hydrophobic cores that stabilize their folded structures. HFBII can be thought of as being turned inside-out in this respect because it exposes such a large fraction of its hydrophobic residues. To compensate for the destabilizing effect of the exposed hydrophobic residues, the structure is strongly stabilized by disulfide bonds.

The arrangement of the Cys-residues in the primary sequence is shown in Fig. 2A. In this figure, the disulfide bonds between Cys-residues, are shown. In the tertiary structure (Fig. 2B) the disulfides form two continuous units. These units span the entire molecule at two opposite sides of the protein, resulting in an efficient cross-linking of the molecule. This cross-linking results in an efficient stabilization of the protein, and helps to keep a globular shape of the hydrophobin. Both pairs of adjacent Cys-residues form the central parts of these units, explaining why the residues are so highly conserved.

Previous biochemical characterization of CU of *O. ulmi* had suggested that the disulfide bridges may be formed in a symmetrical manner, from the first to the second Cys-residue, from the third to the fourth Cys-residue and so on [31]. Based on this sequential pairing it had been suggested that hydrophobins could have a two-domain structure. Hydropathy plots also suggested a two-domain structure [32]. Because the data on CU and HFBII are contradictory it must be re-evaluated whether there is a generally valid paring. However, as the authors stated when analyzing the data for CU [31], it is clear that there is much uncertainty in the original interpretation.

Because the disulfides are such a central feature of the hydrophobin sequences, their role has been the focus of

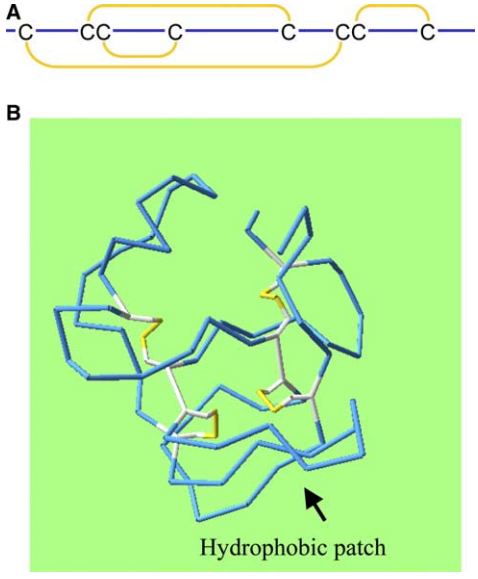


Fig. 2. (A) The order of the eight Cys residues in the hydrophobin primary structures forms a characteristic pattern. All Cys-residues form disulfides and the arrangement found in the *T. reesei* HFBII three dimensional structure is indicated. (B) The disulfides of HFBII are located inside the structure and are clustered into two parts of the structure. Both of the clusters contain four Cys-residues each and form extended covalent linkages that span the entire structure. Sulfur atoms shown in yellow, Cys carbon atoms in white and the rest of the protein backbone in blue.

previous studies. Early work attempted to establish whether the Cys-residues could be involved in forming intermolecular covalent bonds which could explain the extraordinary insolubility observed. However, it was relatively quickly determined that intermolecular disulfide bonds were unlikely and that the Cys residues formed only intramolecular disulfides [33]. Today there does not seem to exist any support for the involvement of intermolecular disulfides. In a more recent study concerning the role of disulfides in SC3 of *S. commune*, it was found that some of the characteristics were maintained even if the Cys-residues were reduced and chemically blocked. On the other hand, the disulfides were found to be essential for the stability of the protein [34].

The high resolution structure of the class II hydrophobin HFBII differs in some respects from previous characterizations of hydrophobins. NMR studies of the class I EAS hydrophobin from *Neurospora crassa* suggested that only the core of the protein is structured, and stabilized by disulfides, but the rest of the protein would be unstructured [35]. However, more recent NMR studies of SC3 of *S. commune* showed that it has a defined structure in solution, indicating that the lack of defined structure in some parts of the protein is not a general feature of class I hydrophobins [36].

The structure of HFBII can thus be summarized as being compact, stable, and amphiphilic. However, as will be discussed in the next chapter, there is some sequence variety between the different classes that allows for structural differences. There are also some clear functional differences between hydrophobins that imply structural differences.

4. Analysis of published hydrophobin sequences

4.1. Sequence alignments

Currently around 70 unique hydrophobin gene sequences are found in the databases. It is expected that the number of genes will further increase through fungal genome and EST sequencing programs. Hydrophobin genes have been found in fungi capable of hyphal growth. These include filamentous fungi and dimorphic yeasts belonging to Ascomycetes and Basidiomycetes. In many cases, more than just one hydrophobin is present in one species. As the number of known hydrophobin sequences has increased it seems to be more and more likely that all filamentous fungi produce hydrophobins. The overall DNA sequence similarity of different hydrophobins is usually low between the two different classes (as described by Wessels [24]) and between species.

Amino acid sequence alignments of deduced hydrophobin sequences found in databases (Fig. 3) were used to construct an unrooted tree (Fig. 4) showing the grouping of hydrophobins between Ascomycetes and Basidiomycetes and the two hydrophobin classes. Of the available unique hydrophobin genes 52 belong to class I and 20 to class II.

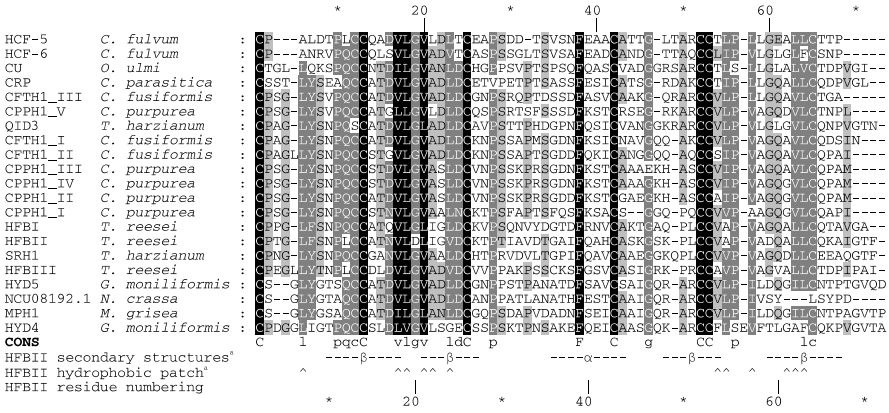
A database search (see Fig. 3) shows that class II hydrophobins have been observed thus far only in Ascomycetes and they form a uniform group in the phylogenetic tree (Fig. 4), whereas class I hydrophobins are observed in both Basidiomycetes and Ascomycetes. The class I sequences cluster into two halves in the phylogenetic tree showing the evolutionary division of hydrophobins from Ascomycetes and Basidiomycetes. For the purpose of comparing sequences we found it convenient to further group the class I hydrophobins into two sub-groups Ia and Ib which represent the hydrophobins of Ascomycetes and Basidiomycetes, respectively. Because of the low sequence similarity between the class I and II hydrophobins, Whiteford and Spanu [11] speculated that class II hydrophobins would have evolved independently of the class I hydrophobins and thus represent a case of convergent evolution. Further structural and functional analysis of hydrophobins of both classes will show what the difference on a molecular level is, and thus such data will likely help to resolve the question of ancestry of the two classes.

In the primary sequence, the most important feature common to all hydrophobins, as discussed previously and seen in the sequence alignments (Fig. 3), is the characteristic pattern of eight Cys-residues. This nearly complete conservation suggests that all hydrophobins irrespective of class would share a common disulfide network and, therefore; we suggest, it is likely that they have a common fold. Besides the conserved Cys-residues and similar hydropathy patterns, the hydrophobins share only a few conserved residues. The overall low sequence conservation may indicate that the Cys-residues are critical for structural reasons while the other residues can vary substantially to give variants with specific properties. Particularly the amino termini of hydrophobin proteins are variable in length and composition.

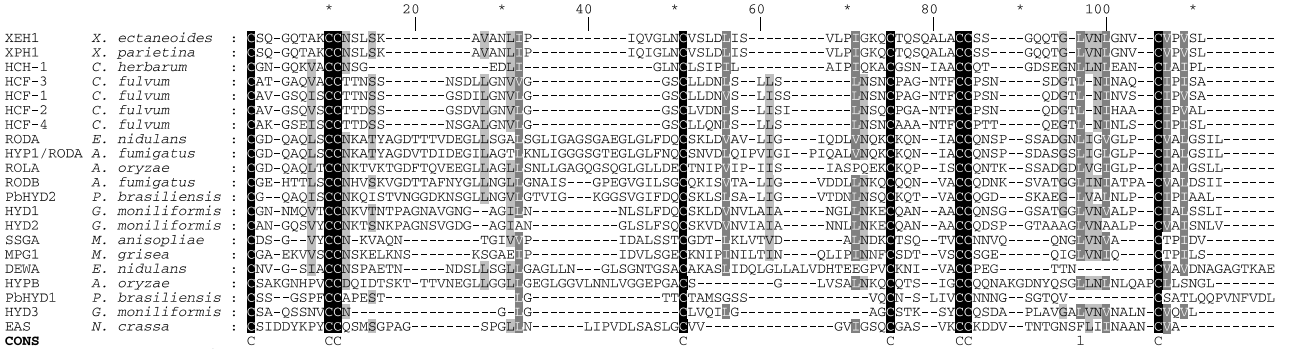
Since the folded structures of hydrophobins are thought to begin only slightly before the first Cys-residue, the amino termini are not included in our sequence alignments (Fig. 3). Nevertheless, the amino terminal sequences may have important roles in the specific functions of individual hydrophobins but presumably have no effect on the general and common properties among hydrophobins. Deletion of the 32 amino acid long glycosylated N-terminus of SC3 from *S. commune* did not alter the self-assembly and surface activity of SC3 [37].

On the other hand, SC3 from *S. commune* has been reported to have 17–22 mannose units linked to Thr-residues in the 32 amino acid part of the N-terminus [38]. Removal of these sugar units resulted in lower

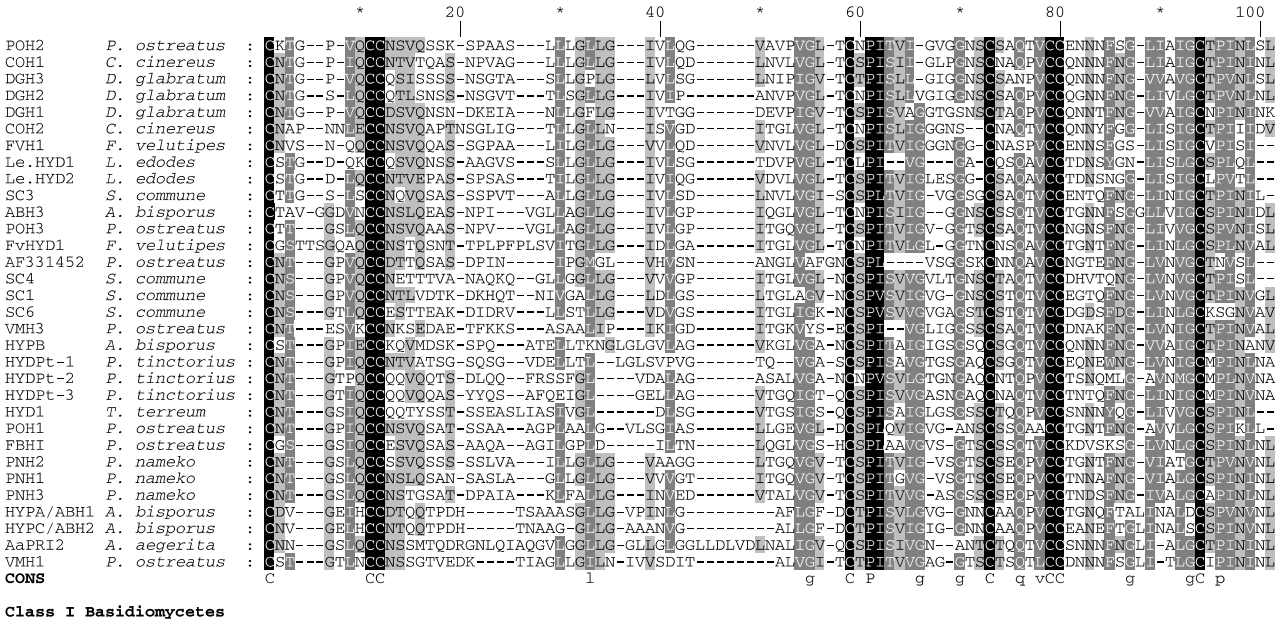
adhesion stability on Teflon surface. It was suggested that the mannose units would help to keep the long N-terminus in a hydrophilic environment. Glycosylation has also been suggested for VMH3 [39] and POH2 [40] from *Pleurotus ostreatus*. It has been shown that HFBI and HFBII from *T. reesei* are not glycosylated [32,41]. Interestingly, CFTH1 from *Claviceps fusiformis* has three homologous hydrophobin domains that are connected by hydrophilic Gly/Asn-rich regions [42]. Despite this unusual primary stucture, the protein showed typical amphiphilic properties like surface activity and surface adsorption, as well as typical localization on aerial hyphae. For the sequence alignments CFTH1 and the pentahydrophobin CPPH1 from *C. purpurea*,



Class II Ascomycetes



Class I Ascomycetes



Class I Basidiomycetes

with five homologous domains and Gly/Asn-rich linker regions, were divided into single domains.

4.2. Conserved features among the primary sequences of class II hydrophobins

Of the different classes Ia, Ib and II, the class II hydrophobins form the most uniform group considering the sequence and loop lengths (sequence identities range from 29% to 95%), which makes it easy to compare how the conserved residues are related to the features of the three dimensional structure of HFBII. The amino acid residues that form the hydrophobic patch in HFBII (comprising residues Val-18, Leu-19, Leu-21, Ile-22 and Val-24 of the first β hairpin loop, Ala-55, Val-57, Ala-61, Leu-62 and Leu-63 of the second β hairpin loop and Leu-7, see Fig. 3) are almost invariably conserved as hydrophobic aliphatic amino acids among class II hydrophobins. Val-54 also contributes to this hydrophobic patch, but in some of the class II proteins a more polar residue is found in position-54. The observed amphiphilicity can thus be ascribed in class II hydrophobins to the presence of a hydrophobic patch on an otherwise hydrophilic surface. If the variations in the patch can give different hydrophobins distinct amphiphilic properties in specific environments remains to be elucidated.

Apart from the Cys-residues and the hydrophobic patch, we observe some other conserved residues. A Phe-residue corresponding to Phe-39 in HFBII is completely conserved among class II hydrophobins. The aromatic ring of Phe-39 in the α-helical region of HFBII is inserted between two Pro-residues (Pro-11 and Pro-50) from the two β hairpin structures into the protein and is likely to stabilize the fold through hydrogen bonds. Pro-11 from the first β strand in HFBII is conserved among class II hydrophobins whereas Pro-50 from the third β strand is in most cases an Ala. There are additionally two well conserved Pro-residues: Pro-56 might have a role in the shape of the β hairpin loop, but the role of Pro-29 is difficult to predict. Preceding the first Cys-pair there is a well conserved Gln although in HFBII it is a Leu-residue that is inside the protein.

4.3. Conserved features among the primary sequences of class I hydrophobins

Class I hydrophobins have more sequence variation and a wider distribution of sequence lengths than the class II hydrophobins (Fig. 3). Especially the class Ia hydrophobins from Ascomycetes show high divergence but distinct subgroups are easily identified. It is interesting that the overall hydrophobicity of the amino acid sequences after the first Cys is higher in most class Ib

Fig. 3. Sequence alignments from deduced hydrophobin protein sequences found in databases. Clustal W (1.82) [95] was used to align HCF-5 of *Cladosporium fulvum* (accession AJ133703), HCF-6 of *Cladosporium fulvum* (AJ251294), CU of *Ophiostoma ulmi* (U00963), CRP of *Cryphonectria parasitica* (L09559), CFTH1_I-III of *Claviceps fusiformis* (AJ133774), CPPH1_I-V of *Claviceps purpurea* (AJ418045), QID3 of *Trichoderma harzianum* (X71913), HFBI of *Trichoderma reesei* (Z68124), HFBII of *Trichoderma reesei* (Y11894), SRH1 of *Trichoderma harzianum* (Y11841), HFBIII of *Trichoderma reesei* (Nakari-Setälä, T., unpublished data), HYD5 of *Gibberella moniliformis* (AY158024), NCU08192.1 of *Neurospora crassa* (AABX01000408), MHP1 of *Magnaporthe grisea* (AF126872), HYD4 of *Gibberella moniliformis* (AY155499), XEH1 of *Xanthoria ectaneoides* (AJ250793), XPH1 of *Xanthoria parietina* (AJ250794), HCH-1 of *Cladosporium herbarum* (AJ496190), HCF-3 of *Cladosporium fulvum* (AJ566186), HCF-1 of *Cladosporium fulvum* (X98578), HCF-2 of *Cladosporium fulvum* (AJ133700), HCF-4 of *Cladosporium fulvum* (AJ566187), RODA of *Emericella nidulans* (M61113), HYP1/RODA of *Aspergillus fumigatus* (L25258, U06121), ROLA of *Aspergillus oryzae* (AB094496), RODB of *Aspergillus fumigatus* (AY057385), PbHYD2 of *Paracoccidioides brasiliensis* (AY427793), HYD1 of *Gibberella moniliformis* (AY155496), HYD2 of *Gibberella moniliformis* (AY155497), SSGA of *Metarhizium anisopliae* (M85281), MPG1 of *Magnaporthe grisea* (L20685), DEWA of *Emericella nidulans* (U07935), HYPB of *Aspergillus fumigatus* (AB097448), PbHYD1 of *Paracoccidioides brasiliensis* (AF526275), HYD3 of *Gibberella moniliformis* (AY155498), EAS of *Neurospora crassa* (AAB24462, X67339), POH2 of *Pleurotus ostreatus* (Y14657), COH1 of *Coprinus cinereus* (Y10627), DGH3 of *Dictyonema glabratum* (AJ320546), DGH2 of *Dictyonema glabratum* (AJ320545), DGH1 of *Dictyonema glabratum* (AJ320544), COH2 of *Coprinus cinereus* (Y10628), FVH1 of *Flammulina velutipes* (AB026720), Le.HYD1 of *Lentinula edodes* (AF217807), Le.HYD2 of *Lentinula edodes* (AF217808), SC3 of *Schizophyllum commune* (M32329), ABH3 of *Agaricus bisporus* (Y14602), POH3 of *Pleurotus ostreatus* (Y16881), FvHYD1 of *Flammulina velutipes* (AB126686), AF331452 of *Pleurotus ostreatus* (AF331452), SC4 of *Schizophyllum commune* (M32330), SC1 of *Schizophyllum commune* (X00788), SC6 of *Schizophyllum commune* (AJ007504), VMH3 of *Pleurotus ostreatus* (AJ238148), HYPB of *Agaricus bisporus* (Y15940), HYDPt-1 of *Pisolithus tinctorius* (U29605), HYDPt-2 of *Pisolithus tinctorius* (U29606), HYDPt-3 of *Pisolithus tinctorius* (AF097516), HYD1 of *Tricholoma terreum* (AY048578), POH1 of *Pleurotus ostreatus* (Y14656), FBHI of *Pleurotus ostreatus* (AJ004883), PNH2 of *Pholiota nameko* (AB079129), PNH1 of *Pholiota nameko* (AB079128), PNH3 of *Pholiota nameko* (AB079130), HYPA/ABH1 of *Agaricus bisporus* (X89242, X92861), HYPC/ABH2 of *Agaricus bisporus* (X90818, X92860), AaPRI2 of *Agrocybe aegerita* (AF081493), VMH1 of *Pleurotus ostreatus* (AJ238147) using the Gonnet matrix series [96]. The protein databases were searched for annotated hydrophobin sequences showing the typical Cys patterning and omitting protein fragments or sequence variants. Only sequences after the first Cys were used in the alignments due to high variations in the amino termini. The Cys residues were aligned to show the typical Cys-pattern. The trihydrophobin (CFTH1) of *Claviceps fusiformis* and the pentahydrophobin (CPPH1) of *Claviceps purpurea* have multiple homologous hydrophobin domains linked with Gly/Asn-rich regions. For the alignments these protein sequences were divided into single domains and marked with roman numerals according to their order starting from the amino terminus. Note that the shown HFBII sequence begins at position three. [a]Secondary structure elements and the residues forming the hydrophobic patch are obtained from the HFBII crystal structure [15]. [b]EAS secondary structure elements are obtained from an NMR-study [35]. In the consensus sequences the residues that are fully conserved are shown in upper case and residues that are conserved over 80% are in lower case. For the residue shadings similarity groups based on the Blosum62 distance matrix [97] were used. Residue similarities of 100%, 80% and 60% are shown with a black, grey and light grey background, respectively.

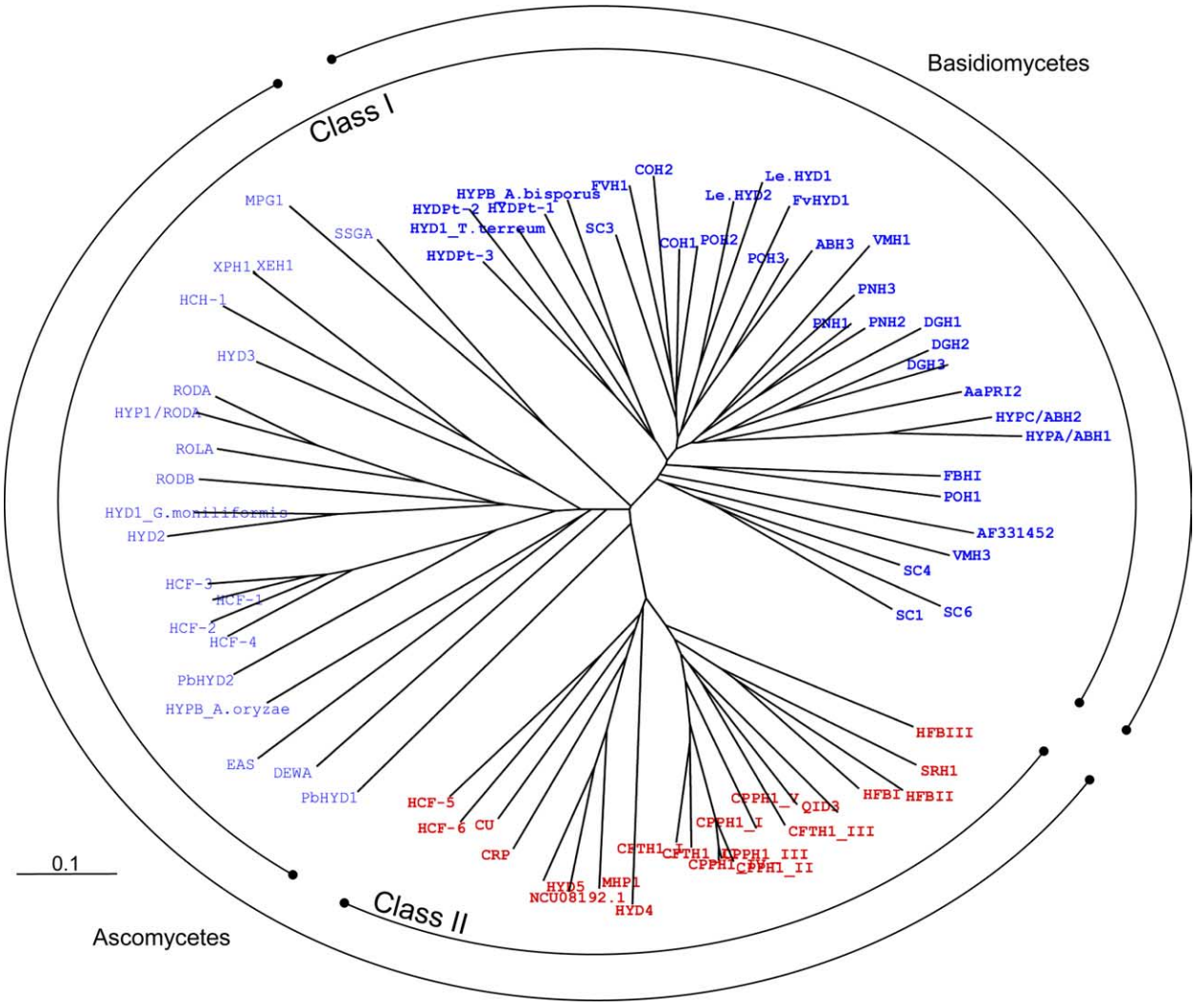


Fig. 4. An unrooted phylogenetic tree of the deduced hydrophobin protein sequences deposited in databases. The phylogenetic relationships of the protein sequences (omitting the sequences preceding the first cysteines) were calculated with Clustal W and an unrooted tree was constructed with PhyloDraw [98]. For sequence nomenclature and database accession numbers see legend in Fig. 3. Class I hydrophobins are shown in blue color with the basidiomycetes in bold face and class II proteins with red color. The scale bar indicates the distance for 0.1 amino acid substitutions per position.

proteins than in most class Ia and II. Of the analyzed class Ib proteins over 60% have a grand average of hydropathy (GRAVY) [25] of 0.5 or more (at the scale of −4.5 to 4.5), whereas less than 20% of the members of classes Ia and II have such high overall hydrophobicity.

For the discussion on possible functional consequences of differences in sequence, we make the assumption that the overall fold of class I and II hydrophobins is similar. It is useful to compare sequences in this way, but there is currently no direct structural evidence that would prove or disprove this assumption.

In the protein sequences, the regions corresponding to the β hairpin loop regions in HFBII (residues between the 3rd and 4th Cys-residue and between 7th and 8th Cys-residue) are interesting because they contain the residues that form the hydrophobic patch in HFBII. Most of the class I hydrophobins have a longer putative first β hairpin loop than class II hydrophobins and incorporate more hydrophobic residues. If exposed on the surface they may form a hydrophobic patch similar to HFBII, only larger. In the class Ib hydrophobins there are 12 conserved hydrophobic aliphatic residues in the putative β hairpin loop regions while there are 11 residues in the hydrophobic patch in HFBII. The sequence variation in the class Ia hydrophobins is high and there are only 6 conserved hydrophobic aliphatic residues in the putative loop regions. This low number may be due to difficulties in aligning these divergent sequences since most sequences have more hydrophobic aliphatic residues that are apparently not conserved. It is interesting to note that HYD3 of *Gibberella moniliformis* from group Ia has the shortest putative first β hairpin loop with only one hydrophobic aliphatic residue. In contrast 11

hydrophobic residues are found in the second β hairpin loop, thus maintaining an overall number of hydrophobic aliphatic amino acids in the loop regions similar to most hydrophobins. Unlike class II hydrophobins there is no conserved phenylalanine in the putative α-helical region of class I hydrophobins. In the class Ia hydrophobins from Ascomycetes there is however a conserved hydrophobic residue at the same position that may be inserted inside the protein in a similar way to the Phe-39 in HFBII.

The class Ia EAS hydrophobin was studied by NMR [35] and found to contain three β strands forming β sheet structures. These sheets involve residues just preceding the second and third Cys-residues, between the fifth and sixth Cys residues and a short stretch after the last Cys. If the conserved Cys residues are considered as fixed coordinates all these stretches coincide with the locations of β strands found in the HFBII structure. A corresponding β strand preceding the fourth Cys residue in HFBII was not identified in the EAS NMR structural study.

5. Functional relations in hydrophobins

Hydrophobins are found as multigene families. For instance, eight putative hydrophobins were found in the genome of the white rot fungus *Phanerochaete chrysosporium* (http://genome.jgi-psf.org/). The presence of multiple hydrophobin genes in an organism may be explained by two possibilities. Either they are differentially expressed as a response to different developmental stages or environmental conditions, but are largely able to complement each other, or alternatively they fulfill different functional roles that are reflected in structural differences.

Deletion and complementation studies have been made to resolve these questions. However, experiments in which one or a few hydrophobin genes have been deleted are difficult to interpret when no phenotype is observed. Different hydrophobins can sometimes, but not always, complement each other if they are expressed at the right time and located to the correct place. Complete deletion of hydrophobin genes is uncertain, unless genome data on the total number of hydrophobin genes are available. In one study several hydrophobin genes were tested for complementation in *Magnaporthe grisea* strain where the gene for MPG1 had been deleted [43]. It was found that partial complementation did occur with several hydrophobin genes of the same family, thus indicating a functional relationship.

Even though hydrophobins may be an important way for fungi to deal with surface phenomena in their environment in general, it is seems that individual proteins have properties that are adapted for specific roles. Taking the example of the known three hydrophobins from *T. reesei* it is clear that HFBII is found on the spore walls [44] and is secreted into the medium at high concentrations [32], whereas HFBI [41,45] and HFBIII (M. Linder and T. Nakari-Setälä, unpublished) are largely retained in the mycelium in vegetative cultures. Our recent findings show that different treatments are needed for removal of HFBIII from the mycelium than for HFBI, which can indicate different structural roles for the two proteins. Apart from the probable role of HFBII as a structural component of the conidial wall, our present data do not indicate a clear biological role for each hydrophobin. Deletion strains show phenotypes such as fluffy growth (Δ*hfb1*) or reduced sporulation (Δ*hfb2*) on solid media (S. Askolin, T. Nakari-Setälä, unpublished), while in bioreactor cultivations the Δ*hfb2* mutant showed increased production of spores [45].

Also other studies show that hydrophobins may have different characteristics. The role of SC3 and SC4 of *S. commune* in formation of aerial structures was compared [46]. It was concluded that the two proteins show distinctly different characteristics, although they were able partly to compensate for each other. Paris et al. [47] have shown that the RodBp of *Aspergillus fumigatus* is not able to compenstae for DewAp of *A. nidulans* on conidial surface although the RodB protein was expressed in *A. nidulans*.

6. Self-assembly of hydrophobins

Some of the earliest experimental observations with hydrophobins showed that they form, or self-assemble into, various types of aggregates. In the light of the amphiphilicity observed in the *T. reesei* HFBII three dimensional structure (see Fig. 1) it is now interesting to discuss what could be the molecular basis for the high tendency of hydrophobins to aggregate.

6.1. Surface activity of hydrophobins

One of the first roles suggested for hydrophobins was the lowering of surface tension of water to allow growth of hyphae into the air [8], indicating that hydrophobins are amphiphilic. Amphiphiles are defined as molecules that have distinct hydrophilic and hydrophobic parts. This two-headedness gives amphiphiles properties such as a high tendency to migrate to hydrophobic–hydrophilic interfaces (such as the air–water interface) and the ability to encapsulate and dissolve hydrophobic molecules into aqueous media. As will be discussed below, amphiphilicity can also be a major driving force for self-assembly. One of the most familiar amphiphiles to us is soap, which is used to solubilize dirt. In biology, amphiphiles play enormously important roles and perhaps the most critical one is the formation of

membranes by amphiphilic phospholipids. These amphiphiles and most others that have been studied are of relatively low molecular weight. In contrast, hydrophobins are macromolecular amphiphiles. An interesting question is, what properties we can expect for a macromolecular protein-amphiphile, and what need would fungi have for these types of molecules.

One intrinsic property of amphiphiles is that they reduce the surface tension of water. The physical properties of water are much dependant on the favorable formation of hydrogen bonds between water molecules. The outermost molecules in a drop of water experience a different environment when encountering air or a solid. The water molecules at the interface/surface cannot make favorable contacts, and to minimize the energy, the system will minimize the number of water molecules that need to interact with the surroundings. One consequence is that water drops take a spherical shape when placed on a hydrophobic support, because in a sphere the ratio of surface molecules to bulk molecules is the lowest. Any molecule that can form hydrogen bonds with water on one side and is free of hydrogen bond forming tendencies on the other side will favorably migrate to the interfaces between solid and water or air and water, and thus allow the drop to spread out. This type of molecule is called an amphiphile, and because it migrates to the surface it is surface active.

The spreading of a water drop is a convenient way of measuring surface activity. One can calculate the reduction of surface energy by analyzing the shape of the drop. Broadly speaking, reduction of surface tension is a general property of proteins since they typically contain both hydrophilic and hydrophobic residues. The effect of hydrophobins is, however, much more pronounced than typically observed for a protein. In an early study it was reported that the class I SC3 hydrophobin reduced the surface tension of water from 72 to 43 mJ m^{-2} [48]. However, values as low as 34 were reported later for the same protein [46]. Variations may be explained by variation in experimental parameters such as liquid composition (water, buffer, or culture medium) protein concentration or temperature. CFT1 of *C. fusiformis* had a surface tension of 33.5 mJ m^{-2} at 0.1 mg ml^{-1} [42]. HFBII of *T. reesei* was able to reduce the surface tension of water to 28 mJ m^{-2} at a concentration of 0.02 mg ml^{-1} [15], (S. Askolin, unpublished data). These values are in the range (28–34 mJ m^{-2}) that aqueous solutions of surfactants typically exhibit [49]. The fact that the sample purity, details of analysis, protein concentration, and buffers affect the values obtained leads to problems in comparing the absolute values. Nonetheless, we can draw the conclusion that all hydrophobins seem to lower the surface tension of water by roughly the same degree. There is currently no clear indication that, for example, class I or II hydrophobins would have different abilities to reduce the surface tension of water.

6.2. Solution multimers

The function of amphiphiles is based on the need to hide their hydrophobic parts from the hydrophilic solvent. Low concentrations of surfactant dissolve into water as monomers and coat the surfaces of the container, but above a certain concentration called the critical micellar concentration (CMC), aggregates start to form. Typically, increasing the surfactant concentration will cause a decrease of surface tension until the air/water interface is saturated, and aggregates start to form. In solutions, the shape of the resulting aggregates depends on the structure of the component molecules as summarized by Israelachvili [50]. The size of the hydrophilic head group in relation to the hydrophobic part will determine the packing into either spherical micelles, cylindrical micelles, bilayers, or inverted micelles. Because HFBII of *T. reesei* is rigid, with the hydrophilic and hydrophobic parts forming defined segments, it is not self evident what types of structures the shape control could result in.

Contrary to what might be thought, hydrophobins can be dissolved at quite high concentrations in aqueous solution. For example HFBI and HFBII of *T. reesei* have been dissolved to concentrations of 100 mg ml^{-1} (our unpublished data), SC3 of *S. commune*, and EAS of *N. crassa* to at least 1 mg ml^{-1} [35,36]. Hydrophobins show a behavior in solution that resembles that of typical surfactants, forming different types of aggregates in a concentration-dependent manner. Although the initial assumption was that hydrophobins are monomers in solution, this has been revised. More recent results obtained by analyzing SC3 using size exclusion chromatography show that dimers or tetramers are formed in solution in a concentration-dependent manner [36]. At concentrations lower than 4.5 μg ml^{-1}, fluorescence resonance experiments indicated that monomers were formed. Previously a similar result, observing a loosely associated form of SC3 as its soluble form was demonstrated [51]. For HFBI and HFBII, size exclusion chromatography studies and small angle X-ray scattering showed that both hydrophobins most likely are tetramers at relatively high concentrations (10 mg ml^{-1}). At lower concentrations, dimers may be dominating, but no precise data on the transition was reported [52]. In the crystals used for structure determination of HFBII, [15,53] there were two molecules in the asymmetric unit. The hydrophobic patches were against each other, shielding the patches from the solvent. For the EAS hydrophobin, sedimentation equilibrium experiments identified monomers in solution [35], but the existence of other complexes was not discussed.

6.3. Rodlets

Perhaps the most commonly described form of assembled structure of hydrophobins is the rodlet layer. Imaged by electron microscopy or atomic force microscopy (AFM) nanometer sized rods are observed packed together in a mosaic pattern as shown in Fig. 5. In numerous cases these patterns have been observed in various samples of fungal aerial structures, e.g., on spore surfaces and fungal cell walls. Rodlet layers had, however, been described on fungal structures before any systematic study of hydrophobins. Characterization of these layers had shown many properties similar to those later described for some hydrophobins, such as being highly resistant to attempts to dissolve them by chemicals [54]. The connection between hydrophobins and rodlets was made by studying hydrophobin deletion mutants [26–28]. Air vesicles produced by shaking a solution of hydrophobin also revealed rodlets by freeze-fracturing and imaging by electron microscopy in experiments in vitro [17] and in several subsequent studies [43,47,55]. In a complementation study, where the *mpg1* hydrophobin gene was inactivated in *M. grisea*, the presence of rodlets followed the expression of heterologous hydrophobin genes [43], giving further evidence for the link between hydrophobins and rodlets. There are numerous examples of hydrophobin rodlets identified in fungal structures such as hyphae [56–58], and conidia, [26,59]. However, rodlet–type structures are also formed by other proteins than hydrophobins. For example in the bacteria *Streptomycetes*, proteins called rodlin and chaplins form similar rodlet layers [60].

The rodlets are easily produced in vitro, but have only been observed for hydrophobins of class I. In vitro, rodlets are typically seen when a drop of dilute protein solution is allowed to dry down on a solid support [34,35,38,55]. Because of the way the in vitro samples are prepared, it is likely that the rodlets are formed at the air–water interface, and deposited on the solid support as the liquid evaporates from underneath. The size of the rodlets varies depending on the hydrophobin and how they are produced, with diameters of 5–15 nm and lengths of hundreds of nanometers.

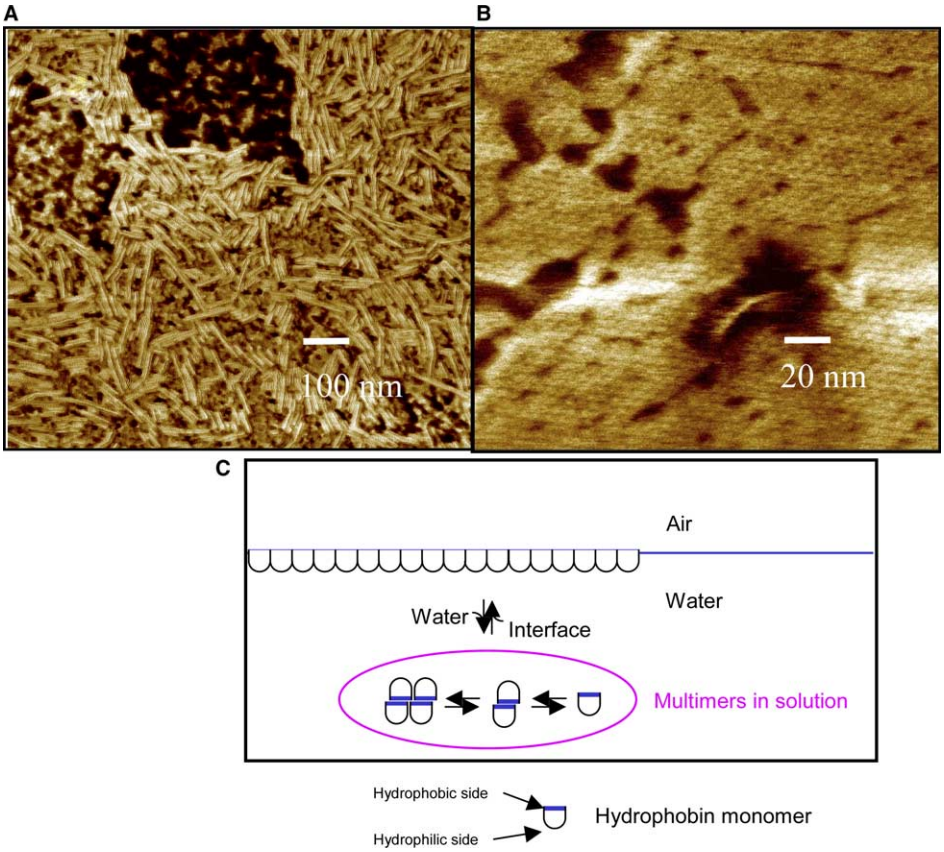


Fig. 5. Supramolecular assemblies of hydrophobins. (A) An atomic force microscopy (AFM) image of a *S. commune* SC3 sample showing rodlets. The sample was prepared by drying down a solution of protein on a sheet of mica. (B) An AFM image of an ordered film of *T. reesei* HFBI on the air/water interface. The film was deposited on a mica support using the Langmuir–Blodgett technique. (C) Possible model for the formation of different complexes of hydrophobins in aqueous solution. The hydrophobin migrates to interfaces and forms aggregates in solution to shield its hydrophobic parts.

An interesting finding relating to the ultrastructure of class I hydrophobin assemblies is that several reports show that the dyes Thioflavin T and Congo Red show the same spectroscopic changes with these hydrophobins as they do with amyloid fibrils [34,35,61,62]. No such changes were noted for the class II hydrophobin HFBII [32]. However, there is currently no direct way of correlating this effect to any specific structural feature of the aggregates although the effect is expected to indicate the presence of stacked β sheets.

6.4. Surface films at the air–water interface

In studies of the air–water interface it was observed that some hydrophobins can form other highly ordered structures of nanometer dimensions on the air water interface. When a drop of a dilute solution of the hydrophobin HFBII was dried down on a flat mica substrate, AFM revealed a highly ordered pattern of objects [15]. The dimensions of these objects were in the range of individual protein molecules packed in a highly regular crystalline form. When working with hydrophobin solutions it is often evident that films form at the air–water interface. These films can be transferred to solid supports for analysis using the Langmuir–Blodgett technique (see Fig. 5). In this case the film is deposited with its hydrophilic side down, because the support is lifted from the aqueous phase. It was shown that the monomolecular films of both HFBI and HFBII are highly crystalline, but distinctly different [52]. This would indicate that surface properties of hydrophobins also involve specific protein–protein interactions.

The presence of surface films, or membranes, on aqueous solution has also been described for the class II hydrophobin cerato-ulmin of *O. ulmi* [19,63]. For the class I SC3 of *S. commune* a technique that involved picking up the surface film from the hydrophobic side and analyzing it by electron microscopy was developed. A surface layer of a drop of solution was simply brought into contact with a solid support. Depending on concentration rodlet patterns started to emerge after prolonged incubations [61]. In this study the air–water interface did not show any ordered structure prior to the formation of rodlets.

6.5. Rods, needles and fibrils

The formation of various aggregates by hydrophobins was noted very early. Takai described the formation of ''rods'' and ''fibrils'' by cerato-ulmin [19] and also described how to use the easy formation of these aggregates to purify the protein [21]. The *T. reesei* hydrophobins HFBI and especially HFBII also readily form these types of long microstructures resembling ''fibrils''. A more detailed analysis revealed that they have a highly crystalline structure [52], making it more appropriate to describe them as crystalline rods or needles. These aggregates are typically formed when solutions of hydrophobin are agitated, but may also be formed spontaneously in standing solutions. These structures have only been observed for class II hydrophobins, but have never been observed associated to fungal structures. It is likely that the rods are a fortuitous consequence of the structural features of the hydrophobins.

6.6. Binding of hydrophobins on to solid surfaces

The binding of hydrophobins on to solid surfaces presents an important aspect of the function of hydrophobins. As the air–water interface is important for aerial growth, the interaction with surfaces can be important for modes of growth that involve attachment of mycelia. This was shown for *S. commune* by studying the attachment to Teflon [64]. Moreover, the surface films on fungal structures can provide protective coatings, by making the surface hydrophobic. Examples that have been described include the caps of mushrooms [65], or conidia and spores [26]. In deletion studies it has often been found that deletion strains are more easily wetted, thus apparently lacking their hydrophobic shield.

In vitro binding of hydrophobins to several different surfaces has been reported. These include biological materials such as insect cuticles and cellulose [20,55], and inorganic materials such as treated or clean glass surfaces, Teflon, or polystyrene [17,38,55,64,66] etc. In one study the involvement of a microbial polysaccharide in the binding of the SC3 hydrophobin to hydrophilic surfaces was demonstrated [67]. In line with this, there is evidence that cryparin has lectin-like activity [22].

The structure of HFBII of *T. reesei*, explains how binding to surfaces could occur. The hydrophobic patch of the protein would face towards hydrophobic surfaces and in the case of binding to hydrophilic surfaces the hydrophobic face would turn outwards. One can argue that lateral interactions would stabilize such layers, locking the individual proteins as pieces of a puzzle, similarly as seen in the Langmuir–Blodgett film of HFBI (Fig. 5B). Currently there are no structural data on the role of putative lateral interactions for stabilization of surface films. However, studying the adsorption of the class Ib hydrophobin HYPA from the mushroom *Agarigus bisporus*, it was noted that thread-like structures were formed on hydrophobic graphite [68]. The study had been performed by placing a hydrophobin solution on the surface and then, after incubation, drawing off surplus liquid. This is in contrast to other published work where rodlets had been noted when drying down drops of protein solution. The study would thus suggest that ordered structures can be formed when hydrophobins bind to a solid surface from solution.

Interestingly it was found that hydrophobic Teflon surfaces could be coated with the SC3 hydrophobin to

produce a surface even more hydrophobic than Teflon itself [48]. The result was explained by the formation of a double hydrophobin layer, with the hydrophilic parts of the protein forming the middle part of the layer and the hydrophobic parts pointing towards the Teflon and outwards. The formation of such bilayers suggests that the also hydrophilic parts of the hydrophobins can form specific and stable intermolecular interactions [48].

The typical purification procedure that involves extracting a "hot-SDS-insoluble fraction" and then dissolving the residue in TFA, has become a characteristic of class I hydrophobins. The finding in a recent study that SC3 protein bound to Teflon could be removed by 0.1% Tween, which is a nonionic surfactant, at 25 °C was, therefore, very surprising [61]. Moreover, if the surfactant was added at 85 °C, the protein could not be removed. Hot SDS also rendered the protein insoluble. Examination of the protein by methods that are used to study secondary structures of proteins, such as circular dichroism (CD), showed that SC3 acquired an α helix signum when bound to Teflon. The treatment that rendered the protein insoluble caused a change of the CD spectra into one that had a β structure signature. A molecular transition between α-helical and a β sheet state was, therefore, suggested. Unlike on Teflon, SC3 that aggregated at the air–water interface produced stable films that only could be dissolved in TFA. These stable films had the β structure signature [61]. The molecular details of the changes caused by the surfactants in the adsorbed layer are still not known, nor what the molecular basis of the changes that cause differences in the CD spectra. In one computational study on SC3 of *S. commune*, some very interesting possibilities for protein folding at interfaces were suggested [69]. The results implied that folding would occur as the protein encounters interfaces. As will be discussed below it was found that fibroblasts showed different response to films of SC3 in the α or β form [70]. These data also support the finding that changes in the ultrastructure of adsorbed films do occur after an initial binding phase. There is a puzzling contradiction between the data for SC3, which suggest large conformational changes, and the structure of HFBII, which shows a rigid molecule that does not give any suggestions for large changes in structure, although local changes in for example loops could be possible. Possible explanations include that the class I SC3 and the class II HFBII can have very big differences in structure and function by different mechanisms, or that the cores of the structures are similar, but the loop regions in the class I hydrophobins would be significantly larger than in HFBII, which would lead to more easily detectable changes in loops that also could have more significant effects on function.

6.7. Comparison of hydrophobins to other self-assembling molecules

The formation of self-assembled structures by amphiphiles is generally well known. For example, surfactants form various types of micelles, and phospholipids form membranes etc. Micelles and membranes are formed due to competing hydrophobic and hydrophilic interactions. The comparatively large structure of the hydrophobin adds conformational and structural restraints to these interactions, yielding assembled structures that differ in properties from those formed by molecules without these restrains. There is a very active field of materials science that focuses on constructing molecules that organize themselves to form materials that have a defined structure from the macroscopic dimensions down to the positions of the atoms [71–74]. This can be achieved through so called hierarchical self-assembly where molecules combine to form structures that in turn combine and form larger structures and so on in multiple layers until macroscopic dimensions have been achieved [75,76]. A typical example of such a molecule is an amphiphile, preferably a large and shape-persistent one. Terms such as "superamphiphiles" or "giant amphiphiles" have been used to describe these molecules that produce a magnitude of different self-assembling structures, some of which are remarkably similar to those observed for hydrophobins, such as rods, or structured films [77]. Interestingly, films of nanostructured supramolecules can have unusual adhesion or surface properties, including hydrophobicity [78]. In fact, designers of these molecules often look at biological molecules for inspiration and principles to be able to build such materials form the "bottom-up", i.e., by designing the molecules to produce a certain assembled structure [79,80]. We can thus suggest that the formation of various self-assembled structures by hydrophobins is more or less an inevitable consequence of their molecular features as being amphiphilic, large and of defined structure.

7. Application potential of hydrophobins

As described above, hydrophobins have unique and remarkable properties and various biological roles. Thus, it is not unexpected that the application possibilities suggested and demonstrated for hydrophobins are also diverse and some even rather surprising. Due to the amphiphilic nature and self-assembly properties, the proposed applications range from using hydrophobins as surfactants, emulsifiers in food processing, in surface coating and immobilization applications and future nanotechnology, and even as indicators for beer gushing (see also e.g. [7,12]).

Due to the pronounced properties of hydrophobins and the formation of stable mono-molecular layers, some applications may not require large amounts of protein. Hydrophobins are also generally highly expressed. Unfortunately the yields of all types of hydrophobins are, however, still not sufficient for extensive application tests. This limits the progress of finding applications and addressing their economic feasibility. In particular class I hydrophobins are more difficult to obtain and purify, including hot SDS and TFA extractions.

The only hydrophobins that have been produced in good yields until now are the class II *T. reesei* hydrophobins HFBI and HFBII. These can be produced at gram per liter levels with genetically modified *T. reesei* strains [41,45], thus approaching the production levels of industrial bulk enzymes. Also hydrophobin fusion proteins can be produced in good yields [81]. Large-scale purification methods have been developed for these hydrophobins and their fusions, such as aqueous two-phase extraction (ATPS) (see below) and recovery from the foam formed after bubbling air into hydrophobin containing solutions [17,41]. Below are described those application targets for which some experimental data already exists.

7.1. Purification of hydrophobins and hydrophobin fusions in aqueous two-phase systems

Hydrophobins show extreme separation behavior in aqueous two-phase systems (ATPS), which is stronger than that of any other protein known. Both *T. reesei* HFBI and HFBII, but in particular HFBI, separate to the non-ionic surfactant phase when a water solution containing hydrophobin is mixed with technical grade (e.g., Berol ($C_{11}EO_2$), Agrimul ($C_{12-18}EO_4$)) or homogenous non-ionic surfactants with varying length of hydrophilic headgroups [32,81]. ATPS thus provides an excellent technique for selective purification of hydrophobins. ATPS is also used as a method to purify membrane proteins [82].

Due to efficient separation in ATPS, hydrophobins can also be used as fusion partners in selective purification of recombinant proteins of interest. Good production yields, even gram per liter level, of HFBI fusion proteins were obtained in *T. reesei* and the fusion proteins were very efficiently separated into the surfactant phase in ATPS [81,83,84]. In these experiments the endoglucanase EGI of *T. reesei* itself was chosen as a fusion partner since this protein is otherwise difficult to purify from the extracellular protein mixture produced by the fungus. The approach was tested by fusing HFBI to the C-terminus of the complete EGI (about 40 kDa) consisting of a catalytic core and a cellulose binding domain (CBD) separated by a linker, or by fusing EGIcore only to HFBI. Also a fusion of HFBI N-terminally to two CBDs was tested. Fusions can be cleaved from the hydrophobin part by conventional means using, e.g., specific proteases. The two cleaved protein components were subsequently separated in another round of ATPS leading to the recovery of the EGIcore in the water phase. The good separation properties of all these multidomain molecules and the fact that the catalytic activity of the EGI part remained unaltered indicated that domain folding in the hydrophobin fusion proteins occurred correctly and the enzymatic activity was not affected by the partitioning steps. Interestingly, the experiments carried out with homogenous surfactants ($C_{10}EO_2$, $C_{10}EO_3$, $C_{10}EO_4$, $C_{10}EO_5$) suggested that there exists a relationship between the size of the fusion partner, the composition of the surfactant and the partitioning of the fusion protein [81]. A dense hydrophobic surfactant appears to exclude bulky hydrophilic groups.

The heterogenous technical grade surfactant $C_{12-18}EO_4$ worked very well in the separation. In addition, even the whole culture broth including fungal hyphae was used and the fusion protein recovered almost exclusively in the top (surfactant) phase while the other extracellular proteins (endogenous EGI included) and hyphae remained in the bottom (aqueous) phase [85]. Furthermore, ATPS separation of EGIcore-HFBI fusion produced by *T. reesei* has also successfully been carried out at pilot scale [86], showing the industrial potential of the system. Fusion of the protein of interest to HFBI thus provides a cheap, easy and efficient way to purify the protein in ATPS and this approach could be useful for proteins produced in other organisms as well.

7.2. Protein immobilization

The ordered and strong assembly of hydrophobins onto surfaces make them suitable for surface modifications and immobilization purposes. Linder et al. [66] have studied the adhesion properties of the two class II hydrophobins HFBI and HFBII of *T. reesei*, and their fusions with the endoglucanase EGIcore. The HFBI-EGIcore fusion could be efficiently immobilized onto hydrophobic surfaces such as silanized glass and Teflon. The fusions formed a tightly bound, rigid surface layer on the hydrophobic support as analysed by enzymatic activity, surface plasmon resonance (Biacore), and by quartz crystal microbalance. Binding most likely occurred as a monolayer, with calculated amounts of 14 mg m^{-2} of EGIcore-HFBI (in comparison to 2.9–4.4 mg m^{-2} of native hydrophobins). Most of the difference can be ascribed to the much larger size of the fusion protein. The endoglucanase activity was retained upon binding. On the other hand, fusions of EGIcore to HFBII failed to immobilize although HFBII alone bound surfaces efficiently.

Palomo et al. [87] did not use hydrophobin fusions for enzyme immobilization but instead used hydrophobins

as such to create protein–protein interaction columns. They first bound the *P. ostreatus* (oyster mushroom) mycelium-bound hydrophobins on hydrophilic glyoxyl-agarose. Thereafter they studied the immobilization of lipases to the column and verified that indeed this immobilization was mediated by the bound hydrophobin. Lipases are active when an amino acid domain (lid) that masks the active site is opened, which concomitantly exposes also hydrophobic patches on the lipase surface. The authors suggested that the mechanism of lipase immobilization on hydrophobins occurs similarly as immobilization to other hydrophobic carriers, i.e., by interfacial activation of the lipase through interaction of the hydrophobic surfaces. The immobilization onto hydrophobins led to high lipase activity and thermal stability.

In addition to immobilization of enzymes, one could envisage the immobilization of a variety of proteins such as antibodies onto columns or other solid supports using hydrophobin fusions. It is expected that hydrophobin fusions allow high concentration and ordered packaging of the protein of interest and furthermore in a more correct orientation and in active form than conventional enzyme immobilization techniques. These types of immobilization applications could range from protein and ligand purification and high-efficiency reaction columns to diagnostics and biosensor applications.

7.3. Surface coating of biomaterials

The strong and stable assembly of class I hydrophobins make them attractive candidate molecules for surface coating of biomaterials such as surgical instruments and medical implants. For instance, the biocompatibility of hydrophobic surfaces could be enhanced by increasing their wettability by applying a hydrophobin layer. Hydrophobin coating could prevent non-specific protein binding in the body and bacterial adhesion. On the other hand, hydrophobin coating could increase the attachment of cells, e.g., fibroblasts, and be useful in tissue engineering [37,70,88].

Janssen and coworkers have carried out extensive studies on the assembly of the *S. commune* class I hydrophobins SC3 and SC4 on Teflon. Different conditions for coating were addressed and it was shown that at high temperatures (80 °C) significantly more hydrophobin could be attached than at room temperature [70]. Uniform assembly in the intermediate α-helical form was obtained but transition to the final β conformation created pores on the surface. Cell growth and morphology was better on the SC4 coated surface. As a measure of cell function it was shown that the β-sheet form of both SC3 and SC4 did not affect mitochondrial activity of the mouse fibroblasts whereas hydrophobin coating in the α-helical form did.

In order to promote growth of fibroblast cells on the hydrophobin coated materials, the SC3 hydrophobin was genetically engineered [37,88]. The N-terminal 25 amino acids of SC3 were replaced by the cell binding domain RGD (Arg–Gly–Asp) of fibronectin. Self-assembly of the engineered hydrophobin molecules was not affected. The hydrophobic side of the layer was as water-resistant as that of native SC3 but the wettability of the hydrophilic side was changed. Addition of the RGD domain to SC3, removal of the N-terminus of SC3, or its replacement with the RGD domain, all promoted growth of mouse fibroblasts on the Teflon surface. It was also verified that SC3 or the truncated SC3 were not cytotoxic to mouse fibroblasts [88] and that the SC3 coatings either in the α-helical or β-sheet conformation were stable when incubated in protein solutions or cell culture medium [70]. SC3 and SC4 coatings also enhanced attachment of human fibroblasts, which do not naturally adhere to Teflon.

7.4. Modification of electrodes

The controlled molecular modification of surfaces is a major challenge in the preparation of function-oriented micro-patterned advanced materials. Bilewicz et al. [89] have studied the assembly of hydrophobins on electrodes and electrode functionalization. They used the class I hydrophobin HYDPt-1 from *Pisolithus tinctorius*, which they bind from aqueous solutions onto hydrophobic electrode materials such as glassy carbon and thin mercury films, or on hydrophilic gold electrodes. The coverage of the hydrophobic surfaces with hydrophobin was higher than that of gold. The hydrophobin-coated electrodes were functionalized with electroactive compounds. It was shown that the hydrophobin layer controlled the access of these compounds from solution to the layer; ubiquinone absorbed to the surface while quinone and azobenzene penetrated into the pores of the hydrophobin layer. The functionalized hydrophobin layers were stable in various conditions such as a wide range of pH, and effectively blocked the oxidation of the electrode substrates and the access of hydrophilic electroactive probes to the electrode surface. These results showed that hydrophobins could act as a hosting matrix and as a material for binding electroactive molecules onto the electrode surface.

7.5. Hydrophobin deletion strains as production hosts

Hydrophobins cause foaming in shaken cultures due to the surface activity. Thus, aerated bioreactor cultures of filamentous fungi are especially expected to suffer from foaming, and antifoam agents need to be added to the cultures. Bailey et al. [45] studied *T. reesei* strains in which the *hfb1* or *hfb2* genes were either inactivated or overexpressed. Overexpression of the hydrophobins led to increased foaming whereas deletion of the *hfb2* gene in particular reduced foaming significantly. Production

strains inactivated in hydrophobin production can have advantages in industrial enzyme production and downstream processing and product formulation.

7.6. Yeast immobilization

The strong immobilization properties may even be utilized in immobilization of cells. The *T. reesei* HFBI was expressed on the surface of yeast by fusing it to the cell wall flocculation protein FLOI of *S. cerevisiae* [90]. The FLOI-HFBI fusion protein was correctly exposed to the yeast cell surface. The recombinant yeast cells separated in ATPS consisting of a non-ionic polyoxyethylene detergent $C_{12–18}EO_5$ more efficiently than the non-transformed host cells. The cell surface characteristics were analyzed using contact angle and ζ potential measurements, which indicated that HFBI expression caused the cells to be more apolar and slightly less negatively charged than the *S.cerevisiae* host strain naturally is. The binding affinity of the hydrophobin producing yeast onto hydrophobic silicone-based materials was twofold increased but no improvement in binding onto hydrophilic carriers could be seen when compared to the parent cells [90]. Hydrophobin-coated production strains could have a benefit in processes where immobilised yeast is used since a higher biomass and more stable attachment to the columns may be obtained.

7.7. Beer gushing

Beer gushing is a phenomenon where beer foams and the contents of a bottle bursts out when a beer bottle is opened. Gushing problems correlate with the quality of barley used in malting and occur in particular in those years when barley has been contaminated with fungal pathogens such as *Fusarium* and *Nigrospora*. The gushing factors were previously described to be small peptides or peptide containing substances, and some were shown to consist of hydrophobic amino acids and be very stable [91]. These characteristics and the surface activity properties of hydrophobins led Kleemola et al. [92] to experiments, which showed that indeed it is the fungal hydrophobins that can be the cause of beer gushing. Hydrophobin preparations from the barley pathogens *F. poae* and *Nigrospora* sp., and the non-pathogenic *T. reesei,* all caused gushing when added into beer bottles in microgram quantities. This led to the successful development of a specific immunoassay for the evaluation of gushing potential of barley and malt [92].

Fig. 6 shows the gushing phenomenon when microgram quantities of *T. reesei* hydrophobin HFBII are added into a beer bottle. Almost the whole liquid content of the bottle can burst out. This type of an experiment is one of the most illustrative, showing in practice the remarkable properties of hydrophobins and their strong surface activity.

7.8. Hydrophobins at oil–water interfaces

In addition to the properties that allow efficient incorporation of hydrophobins into air–water interfaces and stabilization of the air bubbles as described above, hydrophobins also assemble onto oil–water interfaces. Analysis of the *S. commune* SC3, and *T. reesei* HFBI and HFBII showed that these hydrophobins stabilize oil droplets but their effect on the size of the droplets formed and their stability differs (S. Askolin, personal communication). The recent experiments of Wang and colleagues [93] showed that when the class I

Fig. 6. Hydrophobins cause gushing of beer. In this laboratory experiment, 50 μg of *T. reesei* HFBII was added to a 330 ml bottle of beer three days prior to opening. The pictures from left to right were taken a few seconds apart.

hydrophobin SC3 assembles on water-oil interfaces at high concentrations (above 50 μg/ml) a membrane is formed that allows unidirectional passage of small molecules of size up to 10,000 Da from the hydrophobic (e.g., paraffin) side of the layer to the hydrophilic but prevents the passage of molecules ranging from 300 to 10,000 Da from the hydrophilic side. Another interesting phenomenon is that addition of SC3 to a planar lipid bilayer system appears to cause voltage independent pore formation, as measured by the release of calcein [93]. SC3 had an effect on DOPC/DOPE liposomes but did not destabilise DPPC liposomes. It remains to be seen how these hydrophobin properties could be utilized. Proposed application possibilities include the use of hydrophobins as emulgators in food processing, liposome applications and oil refining.

8. Conclusions and future prospects

Filamentous fungi produce hydrophobins to cope with interfacial forces in their surroundings. The properties of hydrophobins are exploited in a number of different situations that often involve interactions with the environment. The structure of the HFBII hydrophobin reveals a large, rigid and amphiphilic molecule. This gives a structural basis for understanding properties such as surface activity, adhesion and formation of supramolecular structures. Hydrophobins can also serve as a model for understanding how such structures are formed. Because surface phenomena often are important in technical applications, there is a large potential for industrial use of hydrophobins. One future line of applications can be in nanotechnology, were the building of self-assembled materials forms an important part. For this goal to be reached we need still a better understanding of structure–function relations on the molecular level, as well as the means to manipulate these.

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