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MINI-REVIEW

Applications of hydrophobins: current state and perspectives

Han A. B. Wösten · Karin Scholtmeijer

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Abstract Hydrophobins are proteins exclusively produced by filamentous fungi. They self-assemble at hydrophilic-hydrophobic interfaces into an amphipathic film. This protein film renders hydrophobic surfaces of gas bubbles, liquids, or solid materials wettable, while hydrophilic surfaces can be turned hydrophobic. These properties, among others, make hydrophobins of interest for medical and technical applications. For instance, hydrophobins can be used to disperse hydrophobic materials; to stabilize foam in food products; and to immobilize enzymes, peptides, antibodies, cells, and anorganic molecules on surfaces. At the same time, they may be used to prevent binding of molecules. Furthermore, hydrophobins have therapeutic value as immunomodulators and can been used to produce recombinant proteins.

Keywords Hydrophobins · Self-assembly · Surface modification · Applications

Introduction

Hydrophobins play a key role in the growth and development of the majority of filamentous fungi (Kershaw and Talbot 1998; Wösten and de Vocht 2000; Wösten 2001; Zampieri et al. 2010). By assembly at the medium-air interface, these proteins lower the water surface tension allowing hyphae to breach the medium-air interface (Fig. 1a, b). Self-assembly of hydrophobins at the surface of the cell wall makes aerial hyphae (Fig. 1c), fruiting bodies and the lining of the air cavities in the fruiting bodies (Fig. 1d), and spores (Fig. 1e) hydrophobic and results in attachment of hyphae to hydrophobic surfaces (Fig. 1f) (Wösten 2001). Traditionally, class I and class II hydrophobins were distinguished (Wessels 1994). However, hydrophobins with a cysteine spacing and hydropathy pattern intermediate between these two classes have also been described (Jensen et al. 2010; Seidl-Seiboth et al. 2011; Littlejohn et al. 2011). Hydrophobins self-assemble at hydrophilic-hydrophobic interfaces (Wösten and de Vocht 2000) such as those between water and air, water and oil, and water and hydrophobic solids. Class I hydrophobins assemble into a very stable, amyloid-like membrane that can only be dissociated using trifluoroacetic acid (TFA) and formic acid (Wessels et al. 1991; de Vries et al. 1993). In contrast, class II hydrophobins do not assemble into amyloid-like fibrils and can be dissociated in 60 % ethanol, in 2 % SDS, or by applying pressure (Wösten and de Vocht 2000). Self-assembly of hydrophobins changes a surface from hydrophilic to hydrophobic, while hydrophobic material becomes hydrophilic. The water contact angle of the hydrophobic and hydrophilic side of the assembled hydrophobin ranges between 60° and 120° and between 22° and 60°, respectively (Wösten and de Vocht 2000). In this review, self-assembly of class I and class II hydrophobins is discussed as well as their potential applications.

H. A. B. Wösten (✉)
Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
e-mail: H.A.B.Wosten@uu.nl

K. Scholtmeijer (✉)
Plant Breeding, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands
e-mail: karin.scholtmeijer@wur.nl

Structure of hydrophobins

Hydrophobins are 70–350 amino acids in length (including signal sequence). They all contain eight conserved cysteine residues that form four disulfide bridges, which connect C1–

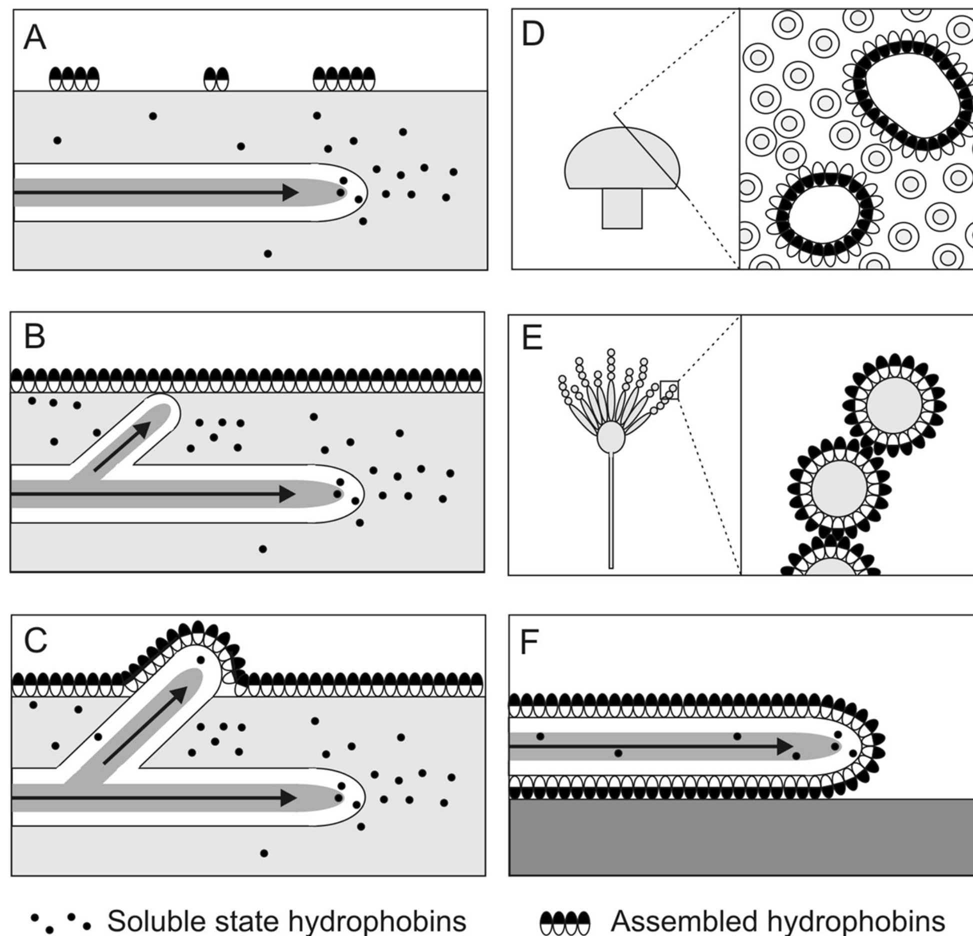

Fig. 1 Model of the biological role of hydrophobins during growth and development of filamentous fungi. Soluble-state hydrophobin is secreted at the tip of the growing, submerged hypha and diffuses into the aqueous environment (**a**). The water surface tension is lowered due to the assembly of hydrophobins into an amphipathic membrane at the medium-air interface (**b**), allowing the hypha to breach the medium-air interface and grow into the air. The hydrophobin secreted by emerging aerial hyphae cannot diffuse into the environment and assembles at the cell wall-air interface (**c**). Aerial cavities in fruiting bodies are lined with an assembled hydrophobin layer preventing water to fill these cavities (**d**). Hydrophobins also play a role in the dispersal of spores by coating them with an amphipathic hydrophobin layer (**e**). Via assembly at the interface between the cell wall and a hydrophobic surface, hydrophobins mediate attachment to the surface, for example, during infection of host tissue (**f**)

C6, C2–C5, C3–C4, and C7–C8 (Hakanpää et al. 2004, 2006a, b; Kershaw et al. 2005; Kwan et al. 2006, 2008; Morris et al. 2012; Ren et al. 2013a, b; Pille et al. 2014). The cysteine residues are important to keep the protein in the soluble state, at least in the case of the class I hydrophobin SC3 from *Schizophyllum commune* (de Vocht et al. 2000). The remaining part of the amino acid sequence is hardly conserved in class I hydrophobins, but class II hydrophobins are more homologous (Ren et al. 2013a).

The 3D structure of the soluble state of the class I hydrophobins EAS (*Neurospora crassa*), DewA (*Aspergillus nidulans*), MPG1 (*Magnaporthe grisea*), and RodA (*Aspergillus fumigatus*) (Kwan et al. 2006; Morris et al. 2012, 2013; Rey et al. 2013; Pille et al. 2014) and the class II hydrophobins HFBI, HFBII (*Trichoderma reesei*), and NC2 (*N. crassa*) (Hakanpää et al. 2004, 2006a, b; Ren et al. 2013b) has been solved. Both types of hydrophobin contain a four-stranded β-barrel core (Fig. 2). Class II hydrophobins are compact and contain two short loops (L1 and L3), while loop 2 (L2) contains α-helical structure (Fig. 2c). Class I hydrophobins show more variation in the length and structure of the loops. For example, L1 and L3 of EAS are unstructured, while L2 contains β-sheet structure (Fig. 2a) (Kwan et al. 2006). In contrast, the N-terminal sequence (not shown) of DewA, RodA, and MPG1 and loops L1 and L2 contain α-helical structure (Fig. 2b) (Ren et al. 2013b; Pille et al. 2014). Charged residues of class I and class II hydrophobins (EAS, DewA, and NC2) were shown to be localized at one side of the surface of the protein, leaving the other side without charge (Kwan et al. 2006; Morris et al. 2013; Ren et al. 2013b). At this side, discrete hydrophobic patches are present in DewA, NC2, EAS, HFBI, and HFBII (ranging in size between 424 and 891 Å^2) giving these molecules an amphipathic nature.

L1 (between Cys 3 and Cys 4) in class I hydrophobins is larger compared to that of class II hydrophobins (38 and 11 amino acids (aas), respectively) (Ren et al. 2013a). A 12-aa

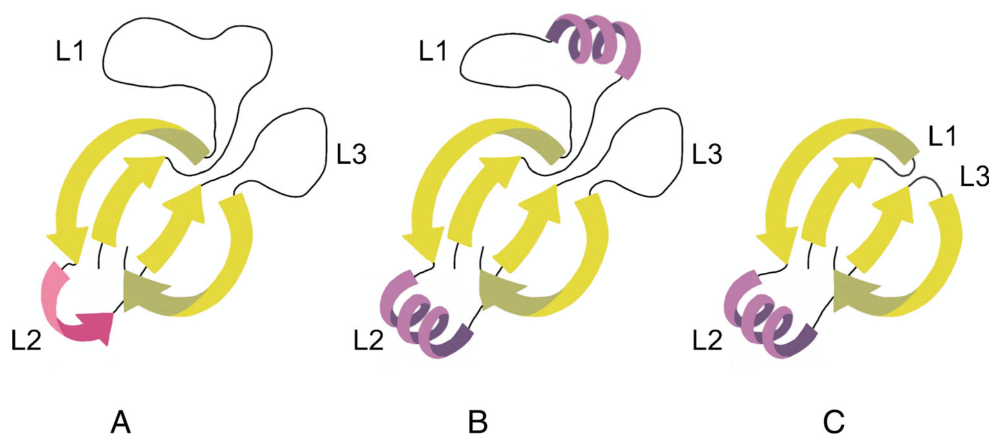

Fig. 2 Schematic representation of the 3D structure of class I (**a**, **b**) and class II (**c**) hydrophobins. Both types of hydrophobins contain a four-stranded β-barrel core (**a**–**c**; in *green*) and either α-helical (**b**, **c**) or β-sheet (**a**) structure in loop 2 (*L2*). In class I hydrophobins (**a**, **b**), loops 1 and 3 (*L1* and *L3*) are relatively large and are unstructured (**a**; EAS) or contain α-helical structure (**b**; DewA, RodA, and MPG1). Class II hydrophobins (**c**; HFBI, HFBII, and NC2) are relatively compact and have a small L1 and L3

peptide of this region in SC3 was predicted to form an amphipathic helix (de Vocht et al. 1998) and was shown to be involved in binding to hydrophobic surfaces (Wang et al. 2004a). L3 (between Cys 7 and Cys 8) of the class I hydrophobin EAS was shown to contain the sequence needed for amyloid formation (Macindoe et al. 2012). The position of this amyloidogenic sequence in class I hydrophobins varies. In DewA it was predicted to be present in L2 (Morris et al. 2013), while in RodA it was predicted to be in L3 like it is in EAS (Pille et al. 2014).

Conformational changes during self-assembly

Monomers or dimers normally represent the water-soluble forms of class I hydrophobins (Mackay et al. 2001; Wang et al. 2002, 2004b; Morris et al. 2013). However, the Vmh2 hydrophobin (*Pleurotus ostreatus*) is only soluble in complexes with glycans (Armenante et al. 2010). These glycans are also needed for assembly (Rea et al. 2012). On the other hand, the water-soluble form of DewA seems to be composed of two stable confomers (Morris et al. 2012, 2013). Recently, it was reported that SC3 spontaneously assembles into amyloid rodlets in water (Zykwinska et al. 2014). However, the SC3 hydrophobin was not treated with TFA prior to analysis, and therefore, partly water-air-assembled SC3 was analyzed. Self-assembly of SC3 at a hydrophilic-hydrophobic interface proceeds via the α-helical and β-sheet 1 state to a stable β-sheet 2 end form (Butko et al. 2001; de Vocht et al. 2002). The α-helical content of SC3 increases upon formation of the α-helical state, while in the β-sheet 1 state, the content of β-sheet structure increases. Both conformations lack a clear ultrastructure when using microscopic techniques. Changes in the secondary structure have not been observed during the transition to the β-sheet 2 state. However, this transition is characterized by the appearance of 10-nm-wide amyloid-like fibrils (also known as rodlets) (Wösten and de Vocht 2000; Butko et al. 2001; Mackay et al. 2001; Kwan et al. 2006). At the water-air interface, the conversion of SC3 from the α-helical state to the β-sheet 1 state occurs within a few minutes (Fig. 2), while transition to the β-sheet 2 state takes several hours. Self-assembly of SC3 is arrested in the α-helical state on a Teflon surface when concentrations of ≤100 μg mL^{-1} are used (de Vocht et al. 1998). This form can be easily removed from the surface using diluted detergent at neutral pH (Fig. 3). However, the combination of diluted detergent and high temperature or low pH (de Vocht et al. 2002; Scholtmeijer et al. 2009) induces the α-helical form to proceed to the β-sheet 2 state (Fig. 3). In addition, this state can be attained by high SC3 concentration (≥300 μg mL^{-1}) and a long incubation time of 16 h. Finally, the conversion to the β-sheet 2 state is promoted by the presence of the cell wall polysaccharide schizophyllan (SPG) allowing conversion to the β-sheet 2 state at concentrations as low as 1 μg mL^{-1} (Scholtmeijer et al. 2009). Maximal lowering of the water surface tension was reached after several hours with SC3 (Askolin et al. 2006), while this occurred instantaneously in the case of ABH1 (*Agaricus bisporus*) (Paslay et al. 2013). SC3 in the β-sheet 2 state cannot be removed from a hydrophobic solid with detergent at any temperature or pH (de Vocht et al. 2002; Scholtmeijer et al. 2009). This coating is therefore highly stable (Fig. 3).

The class II hydrophobin NC2 is predominantly present in the monomeric form up to concentrations of 5 mg mL^{-1} (Ren et al. 2013b). HFBI and HFBII only form monomers at very low concentrations (1–2 μg mL^{-1}) and mainly form tetramers at concentrations of 0.5–10 mg mL^{-1} (Torkkeli et al. 2002; Szilvay et al. 2006). The formation of oligomers in solution protects the hydrophobic patch of the monomer from interaction with the hydrophilic surroundings (Hakanpää et al. 2004).

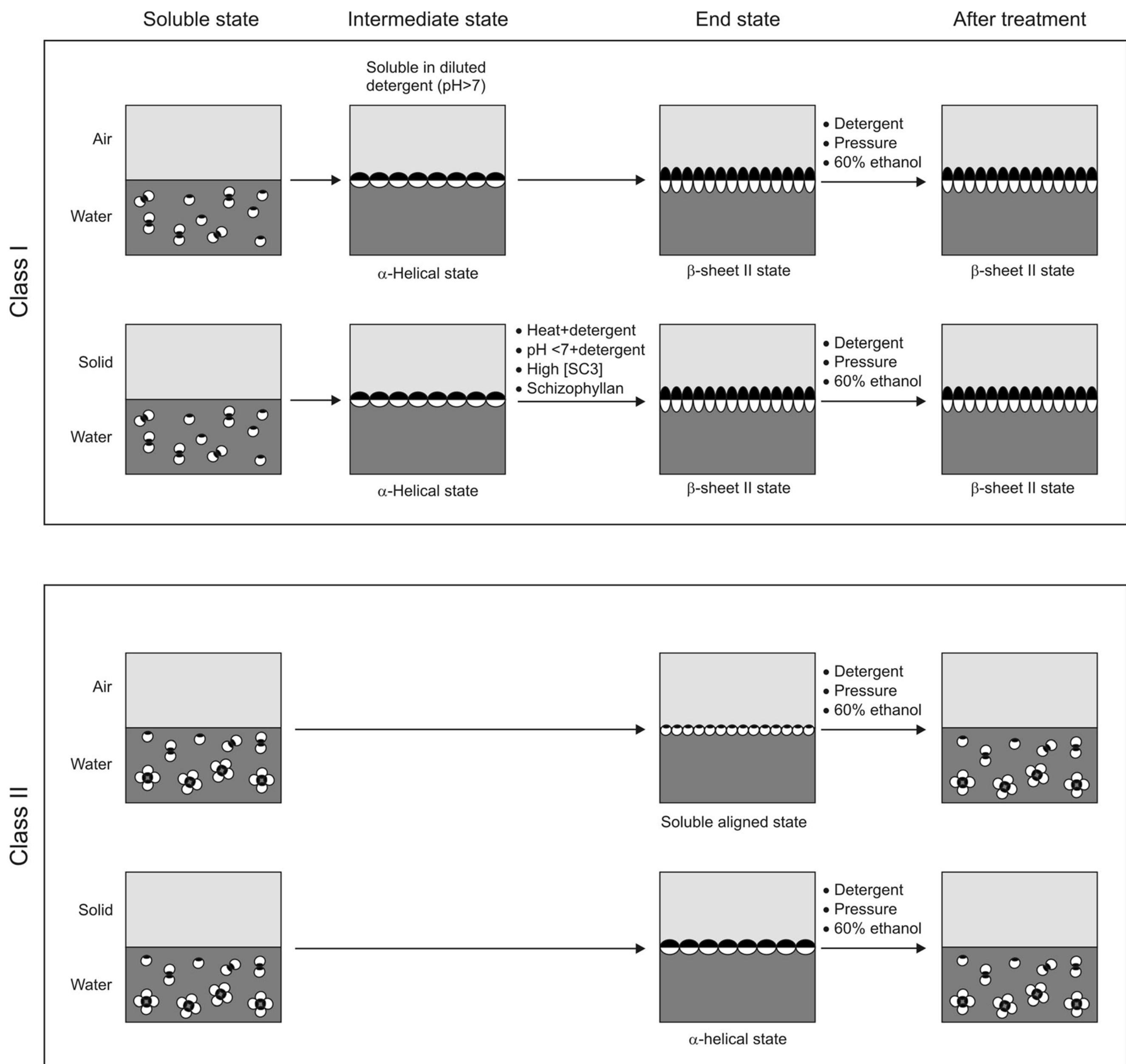

Fig. 3 Assembly of class I and II hydrophobins at hydrophilic-hydrophobic interfaces. Class I hydrophobins (*upper panel*; *SC3*) self-assemble via an α-helical intermediate state and a β-sheet 1 state into the stable β-sheet II end state at water-air interfaces. Upon contact with hydrophobic solids, SC3 may be arrested in the intermediate α-helical configuration. Transition to the stable β-sheet end form is achieved by high protein concentration, the presence of the polysaccharide schizophyllan (SPG), or the combination of heat or low pH and detergents. Class II hydrophobins (*lower panel*) assemble at the water-air interface with a conformation similar to that in the soluble state. In both types of hydrophobins, the hydrophobic patch is directed toward the air and the hydrophilic part to the water (soluble aligned state). At a solid interface, a conformational change into an α-helical form is observed. The end state of class I hydrophobins (*upper panel*) is very stable and cannot be dissociated by pressure, detergent, or 60 % ethanol. In contrast, the end form of class II hydrophobins (lower *panel*) readily dissociates under these conditions

The oligomers were suggested to dissociate at a hydrophilic-hydrophobic interface, resulting in the formation of a monolayer of the class II hydrophobin at the interface. Mutations in HFBI changed the oligomer/monomer ratio (Lienemann et al. 2013). At higher ratios, the adhesiveness of HFBI (mutant) to a solid surface decreased indicating a change in preference for oligomerization over dissociation and binding to a surface. Self-assembly of HFBI and HFBII at the water-air interface is not accompanied by a change in secondary structure or ultrastructure (Askolin et al. 2006; Paananen et al. 2003; Wösten and de Vocht 2000). Maximal lowering of the water surface tension was obtained within minutes (Askolin et al. 2006). This

suggests that HFBI and HFBII assemble at the water-air interface as a monolayer with a structure similar to that of the water-soluble form (Fig. 3). However, surface adsorption kinetics differ between HFBI and HFBII (Krivosheeva et al. 2013).

Applications

Dispersal of hydrophobic solids, liquids and air

Both class I and class II hydrophobins can be used to disperse hydrophobic solids in water (Wösten et al. 1994; de Vocht et al. 1998; Lumsdon et al. 2005; Askolin et al. 2006). High concentrations of non-ionic surfactants are usually used to disperse Teflon particles in aqueous solutions (e.g., in coatings and lubricants). The stability of this dispersion is affected by its chemical composition and by temperature. Hydrophobins can be used to create Teflon suspensions that are not affected by these conditions (Lumsdon et al. 2005). Dispersions of materials with advantageous electrochemical properties such as highly oriented pyrolytic graphite (HOPG), two-dimensional crystalline graphene, and single- and multi-walled carbon nanotubes (CNT) are of interest for electrochemical applications. However, their aggregation is a major drawback to their use in electrochemical sensors and biosensors. Two-dimensional crystalline graphene sheets and multi-walled carbon nanotubes (MWCNT) were stabilized in aqueous solution by coating with the class II hydrophobin HFBI (Laaksonen et al. 2010; Wang et al. 2010c), while multi- and single-walled CNT, graphene sheets, and HOPG were dispersed using the class I hydrophobins HFGI (*Grifola frondosa*), EAS and HYD3 (*Fusarium verticillioides*) (Wang et al. 2010d; Yang et al. 2013). The hydrophobin modification is a simple, one-step process. Similarly, novel functional fillers and pigments are obtained by making hydrophobin-based suspensions of mineral particles. Calcium carbonate particles were dispersed by using HFBII or HFBI containing a ceramophilic binding site (glutamic-acid-rich protein; HFB I-ZE). Dispersed uniform spheres were obtained with the engineered hydrophobin that were 2-fold smaller when compared to coating with HFBII (Heinonen et al. 2014).

The bioavailability of water-insoluble drugs can be increased by creating nanoparticles via complex methods such as milling and high-pressure homogenization. The particles are stabilized (preventing aggregation) by addition of, e.g., surfactants (Patravale et al. 2004). Hydrophobins offer a simple and generic alternative to create and stabilize nanoparticles. For instance, hydrophobins can be used in formulations of water-insoluble drugs for oral (Haas Jimoh Akanbi et al. 2010; Valo et al. 2010), topical (Vejnovic et al. 2010a, b), and intravenous (Fang et al. 2014) drug delivery. The oral bioavailability of the hydrophobic drugs cyclosporine A and nifedipine was increased 2- and 6-fold, respectively, when SC3 was added to the drug suspension (Haas Jimoh Akanbi et al. 2010). An example of topical drug delivery using hydrophobins is formulation of the drug terbinafine which enhances the permeation of terbinafine through human nails (Vejnovic et al. 2010b). Instead of directly coating drug nanoparticles, hydrophobins can also be used to modify the surface stability and biocompatibility of already existing drug-loaded particles of porous silicon (Bimbo et al. 2011) and cellulose (Valo et al. 2011, 2013).

Besides hydrophobic solids, hydrophobins are also capable of coating and stabilizing hydrophobic liquids such as oil droplets in water (Wösten et al. 1994; Lumsdon et al. 2005; Reger et al. 2011; Askolin et al. 2006). They were proposed for use in creams and ointments for cosmetic and pharmaceutical purposes and in food applications (Hektor and Scholtmeijer 2005). Combining hydrophobins with emulsion-stabilizing particles such as boehmite (alumina powder with cationic surface) resulted in homogenous emulsions that were stable over months (Reger and Hoffmann 2012).

Improved stability of foam in food products increases the shelf life of these products. Furthermore, physical and sensory properties of products such as ice cream can be improved by using stabilized foam. Finally, aeration of food products that currently do not contain air due to instability problems (e.g., mayonnaise) may become possible allowing the reduction in the amount of fat and/or calories (Cox et al. 2007, 2009; Tchuenbou-Magaia et al. 2009). HFBII stabilizes air bubbles in a superior way when compared to other proteins (Cox et al. 2007, 2009; Blijdenstein et al. 2010). In combination with a thickening agent, HFBII stabilizes foam with little or no air phase loss for up to 2.5 years (Cox et al. 2009). This is significantly better to foams stabilized using common food-aerating or emulsifying agents such as milk proteins or Tween. Partial replacement of fat to reduce the caloric value while maintaining taste and mouth feel can be difficult due to the antifoam activity of the remaining oil. Mixing HFBI-stabilized foam with oil and water resulted in a stable (up to 45 days) oil-air-water emulsion with a 50 % reduction in lipid content compared to an unstable oil-water emulsion with a smaller volume due to the absence of foam (Tchuenbou-Magaia et al. 2009).

Immobilization of molecules and cells

Hydrophobins offer a non-covalent alternative for conventional surface modification methods. The latter are complicated, time consuming, and show unstable hydrophilicity (Sapsford and Ligler 2004). Hydrophobins adsorbed to a substrate can be used to immobilize proteins (Wang et al. 2007, 2010b, d; Qin et al. 2007; Hou et al. 2009; Zhang et al. 2011a, b, c, d). Fusion of dual-chain avidin (dcAvd) to HFBI (HFBI-dcAVd) resulted in immobilization of biotin on a polystyrene surface and thus allows immobilization of a wide variety of biotin-

linked components (Kurppa et al. 2013). Coating of steel sample-loading plates for matrix-assisted laser desorption/ionization (MALDI) with hydrophobin Vmh2 resulted in efficient binding of mixtures of proteins and peptides in the presence of salts and denaturants (Longobardi et al. 2014). Thus, a simple and effective desalting method, suitable for proteomic applications, was obtained. Cutinase was immobilized on RolA (*Aspergillus oryzae*)-coated polybutylenesuccinate-coadipate (PBSA) (Takahashi et al. 2005) and HFB4 and HFB7 (*Trichoderma* spp.)-coated polyethylene terephthalate (PET) (Espino-Rammer et al. 2013). In both cases, the degradation of the PBSA and PET surfaces by cutinase was stimulated. Finally, use of hydrophobins for immobilization of enzymes in biosensors has been described (Corvis et al. 2005; Zhao et al. 2007, 2009). For example, SC3 was used to immobilize glucose oxidase (GOx) and horseradish peroxidase (HRP) on electrodes with activity remaining up to 90 days (Corvis et al. 2005).

Artificial materials can be used to replace or support a variety of body parts including bone, spinal, cardiac, and dental tissues. The non-physiological character of these materials often leads to poor integration into human tissue. Growth of fibroblasts on Teflon served as the first model system to improve biocompatibility via hydrophobins (Scholtmeijer et al. 2002; Janssen et al. 2002, 2004). Class II hydrophobins have also been used to stimulate growth of human embryogenic kidney cells and neural stem cells on solid surfaces via immobilization of collagen or serum proteins (Hou et al. 2008; Li et al. 2009). Attachment of human endothelial cells to a biocompatible surface was obtained via immobilization of anti-CD31 antibodies on HFBI-coated scaffolds (Zhang et al. 2011d). For biomedical applications such as catheters and guide wires, low-friction surfaces are required to reduce tissue injury (Misra et al. 2006). Low-friction surfaces are normally obtained with lubricants such as silicone oil, glycerin, or jelly-type materials. However, weak adhesion to the biomaterial reduces their performance in time. Hydrophobin-coated materials can be used as an alternative since they have ultralow relative friction coefficients. A reduction in the friction coefficient of 50–80 % was obtained when SC3 coatings were compared to bare surfaces (Misra et al. 2006).

In biotechnological processes aimed at production of metabolites such as ethanol and amino acids, binding of microorganisms to solid surfaces is used to improve the volumetric efficiency, shorten the process time, and achieve more efficient conversion of substrate into product (Nakari-Setälä et al. 2002). The attachment capacity of *Saccharomyces cerevisiae* cells to hydrophobic silicon-based materials was increased 2-fold by displaying HFBI on the yeast surface via the Flo1p flocculin cell wall anchor. Apart from organic molecules and cells, hydrophobins can be used to immobilize anorganic compounds. HYDPt-1 (*Pisolithus tinctorius*) immobilized on electrode surfaces was functionalized with the electroactive compounds ubiquinone (Q10), quinone (Q0), and azobenzene (Bilewicz et al. 2001). Similarly, fullerene molecules were immobilized on SC3-coated electrodes (Corvis et al. 2007). Silicon surfaces and graphene sheets were functionalized with gold nanoparticles coated with mercaptosuccinic acid (MSA) via disulfide formation to cysteines of HFBI derivatives (Laaksonen et al. 2009, 2010). Interest in the fabrication of titanium thin films is increasing because of several attractive applications including microelectronic devices and photonic materials (Santhiya et al. 2010). Creating these thin films from aqueous solutions at low temperature is of great interest due to economic and environmental benefits. The hydrophobin H*Protein B (fusion protein of truncated yaaD, DewA, and His6) was assembled on a silicon substrate followed by deposition of titanium dioxide films at near-ambient conditions (Santhiya et al. 2010). In this example, not only surface modification is achieved but also the surface of interest is created by using hydrophobins.

Antifouling

Adsorption of proteins plays a role during processes such as dental plaque formation or the primary stages of urinary tract infection (Reid 2000). Depending on the hydrophobin used and environmental conditions such as pH and ionic strength, differential binding and/or antifouling properties can be obtained. An adsorbed layer of H*Protein B on octanethiol-coated gold prevented the adsorption of the secondary proteins BSA, casein, and collagen at low-salinity conditions and at pH 8 (von Vacano et al. 2011). Surface analysis showed that the hydrophobin layer stays intact during several hours of exposure to solutions of secondary proteins without any detectable exchange with these proteins. Coating with HFBII resulted in a pronounced change in the degree of plasma protein adsorption to thermally hydrocarbonized porous silicon (THCPSi) nanoparticles (Sarparanta et al. 2012). Identification of the adsorbed proteins revealed that certain opsonins and apolipoproteins are enriched in HFBII-functionalized nanoparticles, whereas the adsorption of abundant plasma components such as serum albumin and fibrinogen was decreased.

Bile stents need to be replaced when microbial film formation results in clogging. Clogging was reduced after coating stents with fusion hydrophobin yaaD-DewA-His6 (H*Protein A) (Weickert et al. 2011). Coating of plastic stents with hydrophobin led to a reduction in the amount of adsorbed material from human bile. DewA and fusion protein derivatives of this hydrophobin with Arg-Gly-Asp (RGD) or laminin globular domain (LG3) binding motif show increased adhesion of human cells (Boeuf et al. 2012). In contrast, fusion of RGD and LG3 to DewA did not increase *Staphylococcus aureus* adhesion to the surfaces. Thus, binding of desired cell types is increased without leading to an increased risk of bacterial infection.

Production and purification of recombinant proteins

Class II hydrophobins, such as HFBI, are efficiently extracted from the bulk protein fraction in aqueous two-phase systems (ATPS) (Linder et al. 2001). Proteins can thus be purified via one-step extraction systems using HFBI as fusion partner. For example, cellulose endoglucanase I (EGI) and the cellulose-binding domains of the cellobiohydrolases CBHI and CBHII were purified using this approach (Linder et al. 2004). A similar approach was used to purify proteins from insect and plant extracts (Lahtinen et al. 2008; Joensuu et al. 2010). Hydrophobins have also been shown to increase the accumulation of recombinant proteins in plants via the formation of novel protein bodies (PBs) (Joensuu et al. 2010). Intracellular PBs were also obtained in *T. reesei* by targeting GFP-HFBI fusion proteins to the ER via the KDEL retention signal (Mustalahti et al. 2013). In some cases, the production is hampered by fusion of the desired protein to HFBI (Kiiskinen et al. 2004; Pereira et al. 2014). Still, the method is of great interest for production of recombinant proteins due to the simple one-step purification method which was scaled up to pilot scale (Reuter et al. 2014).

Therapeutic value of hydrophobins

For centuries, medicinal mushrooms have been used in medical therapies in Asia. Various medicinal molecules have been isolated from mushrooms (Wasser 2011). They include polysaccharides (β-D-glucans), polysaccharopeptides, polysaccharide proteins, and proteins. These components, especially polysaccharides, stimulate or inhibit the immune response, and their use in treatment of cancer, immunodeficiency, and autoimmune diseases is being studied. The hydrophobin SC3 also has a strong anti-tumor effect in two tumor mouse models (sarcoma and melanoma) (Haas Jimoh Akanbi et al. 2013). Treatment with SC3 induced a cellular immune response (immunomodulation) and did not cause signs of toxicity. Thus, hydrophobins may be useful as adjuvant in combination with chemotherapy and radiation or may be even used at the same time to formulate hydrophobic anti-cancer drugs. Furthermore, molecules that allow specific targeting to cancer cells could be introduced via genetic engineering or adsorption.

Production and purification of hydrophobins

Large-scale production of hydrophobins is required for industrial application of hydrophobins. A production level of 600 mg L^{-1} HFBI was obtained in *T. reesei* via homologous expression (Askolin et al. 2001) Introduction of extra copies of the *SC3* gene into *S. commune*, however, resulted in silencing of both the endogenous and the introduced copy of SC3 (Schuurs et al. 1997). About 60 mg L^{-1} SC3 was obtained in *T. reesei* and *Aspergillus niger* via heterologous expression, which is similar to the production level in the native organism *S. commune* (Wösten et al. 1999; Scholtmeijer et al. 2005). Production levels of other class I hydrophobins were not significantly higher (Schmoll et al 2010; Wang et al. 2010a; Huanga et al. 2013) and are similar to those obtained with the class II hydrophobin HFBI in *Pichia pastoris* (Niu et al. 2012a, b). In *Escherichia coli*, the production level of class I hydrophobins was 10 to 100-fold lower (Wang et al. 2010e; Kirkland and Keyhani. 2011; Kudo et al. 2011; Morris et al. 2013). Of interest, a fusion of the class I hydrophobin DewA and (a truncated form of) yaaD of *Bacillus subtilis* was produced efficiently in *E. coli*. Although the hydrophobin needed to be isolated from inclusion bodies, this resulted in kilogram scale of purified hydrophobin (Wohlleben et al. 2010).

As mentioned previously, the class II hydrophobins HFBI and HFBII can easily be purified using ATPS (Linder et al. 2001). For some applications, where, e.g., stability is of importance, the use of class I hydrophobins may be preferred. However, for purification of the class I hydrophobin SC3, TFA is needed (Wösten et al. 1993; de Vries et al. 1993). TFA is a corrosive and volatile chemical and is not suitable for large-scale situations. A procedure to allow production and purification of SC3 in its monomeric or soluble form was developed (K. Scholtmeijer, unpublished results). This procedure involves growing *S. commune* in liquid-shaken cultures in the presence of a low amount of polyvinyl alcohol. This detergent prevents assembly of SC3 during cultivation but does not hamper subsequent hydrophobic interaction chromatography (HIC) purification.

Conclusion

As discussed, hydrophobins can be used in various applications. The fact that hydrophobins do seem to be neither toxic (they are ingested by humans upon consumption of mushrooms and fungus-fermented foods) nor cytotoxic or immunogenic (Janssen et al. 2002, 2004) indicates that they can be safely used in medical and food applications. Hydrophobins all show self-assembly at hydrophilic-hydrophobic interfaces, but the properties of their water-soluble forms and assemblages differ. For instance, solubility and stability of class I and class II hydrophobins differ. Moreover, the assembly kinetics and the degree of wettability at both sides of the assemblages vary. Thus, a particular class I or class II hydrophobin can be chosen depending on the requirements of the application. Biophysical and biochemical properties of hydrophobin films can be optimized by genetic engineering (Scholtmeijer et al. 2002; Lahtinen et al. 2008; Joensuu et al. 2010) or by adding

surfactants (Morris et al. 2011; Zhang et al. 2011a, b, c; Tucker et al. 2014), glycans (Scholtmeijer et al. 2009), or other proteins (Danov et al. 2014) during coating. Most hydrophobins cannot yet be produced in gram per liter quantities hampering their use in medical and technical applications. However, BASF succeeded to produce the hydrophobins H*Protein A and H*Protein B in quantities sufficient for large-scale applications. This brings the potential of hydrophobins in applications in daily life nearby.

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