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Fungal and mushroom hydrophobins: A review

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ABSTRACT: Hydrophobins are surface active proteins that are produced by filamentous fungi including mushrooms. Their ability to self-assemble into an amphipathic membrane at any hydrophilic–hydrophobic interface is most intriguing. These small secreted proteins comprise of eight conserved cysteine residues which form four disulfide bridges and an extraordinary hydrophobic patch. Hydrophobins play critical roles in fungal (and/or mushrooms) growth as structural components and in the interaction of fungi and mushrooms with the environment. The biophysical and biochemical properties of the isolated proteins are remarkable, such as strong adhesion, high surface activity and the formation of various self-assembled structures. With the increasing demands of hydrophobins from fungi and mushroom sources, production and purification in large scale is under challenge. Various applications, ranging from food industries, cosmetics, nanotechnology, biosensors and electrodes, to biomaterials and pharmaceuticals are emerging and a bright future is foreseen.

KEYWORDS: Hydrophobin, Filamentous fungi, Mushroom, Self-assembly, Small proteins

Introduction

Hydrophobins are a large family of small cysteine-rich proteins produced by filamentous fungi with a molecular weight between 7 and 15 kDa (Chaplin and Kennedy, 1994; Scholtmeijer *et al*., 2001). They are extracellular surface active proteins which fulfill a broad spectrum of functions in fungal growth and development (Linder *et al*., 2005; Stübner *et al*., 2010). Hydrophobins are involved in formation of hydrophobic aerial structures such as aerial hyphae, spores and fruiting bodies (e.g. mushrooms or brackets) and mediating attachment of hyphae to hydrophobic surfaces and signaling (Wösten and de Vocht, 2000). These proteins are known for their ability to assemble spontaneously into amphipathic monolayers at hydrophobic–hydrophilic interfaces (Bayry *et al*., 2012). The first hydrophobin genes were discovered in *Schizophyllum commune* in 1991 (Wessels *et al*., 1991). The name hydrophobin was originally used due to their high content of hydrophobic amino acids (Armenante, 2008).

Hydrophobins show very little sequence conservation in general, apart from the presence of 8 cysteine residues implicated in the formation of 4 disulfide bridges (Kwan *et al*., 2006). Based on differences in hydropathy patterns and biophysical properties, they can be divided into two categories: class I and class II (Sarlin *et al*., 2005). Class I hydrophobins are highly insoluble in aqueous solution and can only be dissociated by concentrated strong acids, e.g. trifluoroacetic acid (TFA) and formic acid (Szilvay *et al*., 2007; Linder, 2009). Class I monolayer contains the same highly-ordered core structure known as rodlets, and is positive to Congo red and thioflavin T (Morris *et al*., 2012). Class II hydrophobins are soluble in aqueous dilutions of organic solvents, e.g. ethanol (60%) or hot sodium dodecyl sulfate (2% SDS) (Hektor and Scholtmeijer, 2005; Lumsdon *et al*., 2005). Class II monolayer lacks the rodlet morphology and is less stable (Scholtmeijer *et al*., 2001). Class I hydrophobins have been identified in both ascomycetes and basidiomycetes. Until now, class II hydrophobins have been found in ascomycetes only (Linder, 2009).

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The intriguing properties of hydrophobins provide them with numerous potential applications and draw significant interest to researchers. There are several excellent previous reviews that explore earlier work including hydrophobins structure, functional relations, phylogeny and biological roles, bio-physical and physicochemical characteristics, production and purification, and proposed applications. Here we review some of these recent studies and promote new development involving detecting, isolation, production and applications these valuable proteins.

Structure of hydrophobin

All hydrophobins share 8 conserved cysteine residues and a few other residues (Khalesi *et al*., 2015). However, class I hydrophobins comprise 100–125 amino acids and can be glycosylated while class II hydrophobins are shorter comprising 50–100 amino acid residues (Hektor and Scholtmeijer, 2005).

Hydrophobins are proteins having special spatial arrangements of hydrophobic, hydrophilic and neutral amino acids (Kisko, 2008). There are 4 disulfide bridges formed between cysteine (1–6), (2–5), (3–4) and (7–8). The hydrophobic part including two β-hairpins between cysteine (3–4) and (7–8) is observed in tertiary structure of both class I and II hydrophobins. And a hydrophilic part which includes one α-helix between cysteine (4–5) is present in class II hydrophobins only but not class I hydrophobins (Kallio *et al*., 2007; Linder, 2009).

Crystallization of HFBII, class II member, from *Trichoderma reesei* has revealed three dimensional structure at 1 Å resolution with globular shape of 2 nm in diameter (Hakanpää *et al*., 2004). The two β-hairpins connect and interlock with each other to form one anti-parallel β-sheet which further forms a barrel-like structure, called as “the hydrophobic patch”. NMR studies of the class I hydrophobin EAS from *Neurospora crassa* and SC3 of *S. commune* suggested similar disulfide bridging pattern as HFBII, but with more diversity (Wang *et al*., 2004; Kwan *et al*., 2008) The disulfide bridges seem essential for hydrophobins structure, although class I hydrophobins retain their functionality even after reduction and blocking of disulfides (De Vocht *et al*., 2000).

A database search confirmed that class II hydrophobins only exist in ascomycetes with a uniform group in the phylogenetic tree (Linder *et al*., 2005). It was speculated that class II hydrophobins have evolved independently of class I hydrophobins and thus represent a case of convergent evolution (Whiteford and Spanu, 2002). Class I hydrophobins can be divided into two sub-groups Ia and Ib representing hydrophobins of ascomycetes and basidiomycetes, respectively. Class Ia hydrophobins from ascomycetes show high divergence with more sequence variation and a wider distribution of sequence lengths. The overall hydrophobicity of most class Ib hydrophobins is higher than in class Ia and II at a grand average of 60% for hydropathy (GRAVY) (Linder *et al*., 2005).

Properties of hydrophobin

Hydrophobins have been reported as the most surface active proteins discovered thus far (Cooper and Kennedy, 2010). The most intrinsic property of hydrophobins is the self-assembled amphipathic membranes at hydrophobic–hydrophilic surfaces and interfaces (Linder, 2009). These tough, ordered and robust membranes are crystalline and viscoelastic which are important for aerial growth of fungi (Kallio *et al*., 2007). Surface membranes on the air–water interface are monolayers for class II hydrophobins but mono/multilayers for class I (Garbe *et al*., 2009).

The capability to form rodlets at the air–water interface is one of the early observations that were made for class I hydrophobins (Wessels, 1994). Rodlets show interesting analogies with amyloid fibres. Langmuir trough of HGFI from *Grifola frondosa* and hydrophobin from *Pleurotus ostreatus* indicated the rodlets formed at the air–water interface during compression of a surface film through a bilayer intermediate (Yu *et al*., 2008; Houmadi *et al*., 2008).

Another unique property of hydrophobins is their tendency to form very stable foams due to the high surface elasticity of membranes (Wang *et al*., 2005). The foaming tendency may be stronger for class II hydrophobins than for class I. Foams and bubble stability of HFBII was found stable for at least 4 months, and even up to several years in some cases at relatively low concentration of 0.1 wt% (Cox *et al*., 2009).

The foaming ability of hydrophobins also leads to a negative aspect of gushing in carbonated beverages, especially beer. The main gushing component was isolated as Class II hydrophobin of *Fusarium culmorum*

which occurred in *Fusarium* infection of barley (Sarlin *et al*., 2005). The fungi *Fusarium*, *Nigrospra* and *Trichoderma* are the most active producers of hydrophobins observed in carbonated beverages and caused gushing.

The capability of hydrophobins to adhere to various surfaces can be applied as surfactants and emulsifiers (Lumsdon *et al*., 2005). All hydrophobins coat surfaces and so lower the surface tension, but there is a difference in the binding characteristics (Askolin *et al*., 2006). While class I members can be made to adhere very strongly, this is not seen for class II members which dissociate more easily. It's interesting that much lower amount of hydrophobins needed to reach a specific low surface tension compared to other smaller molecular-weight surfactants (Lumsdon *et al*., 2005).

Toxicity and immunological properties

Hydrophobins are not toxic nor cytotoxic or immunogenic for humans upon consumption of mushrooms. It has been suggested (Wösten, 2001) and later confirmed (Aimanianda *et al*., 2009) that by covering fungal aerial structures, hydrophobins shield antigens in the cell wall, thereby protecting the fungal structure against the immune system.

The surface rodlet-layer has a critical role in masking the immunogenicity of airborne fungal spores (Aimanianda *et al*., 2009). By covering the spore surface, the rodlet-layer imparts immunological inertness to the spores and ensures that pathogen-associated molecular patterns (PAMPs) are not recognized by innate and adaptive immune cells, thus preventing the activation of host immune system, inflammation, and tissue damage. Several lines of evidence suggest that the rodlet-layer, which covers the spores of both pathogenic and nonpathogenic fungal species, prevents immune recognition (Bruns *et al*., 2010; Dagenais *et al*., 2010).

Production of hydrophobins

Since hydrophobins are available only in milligrams from natural sources, the increasing demands have to be addressed by the large-scale recombinant production (Khalesi *et al*., 2014). Various fungal and bacterial hosts were examined to get functional hydrophobins at a reasonable level. Though hydrophobins are derived from filamentous fungi, *E. coli* was considered for its high and rapid expression. Class I hydrophobin Hyd2 from entomopathogenic fungus *Beauveria bassiana* was successfully expressed in *E. coli* inclusion bodies at 7–10 mg/L (Kirkland and Keyhani, 2010). However, N-terminal modifications of Hyd2 with a fusion partner of chitin-binding domain–intein were required for proper expression and purification. And extra cleavage of intein was needed to obtain proper hydrophobin.

More efficient production of homologous expressed class II hydrophobin HFBI in *T. reesei* was reported at levels up to 600 mg/L, whereas heterogeneous production of class I hydrophobin SC3 from *S. commune* in *T. reesei* was 60 mg/L (Askolin *et al*., 2001; Scholtmeijer *et al*., 2005). Another class I hydrophobin DewA from *Aspergillus nidulans* was also expressed in *T. reesei* using *HFB2* promoter and lactose as carbon source at 33 mg/L (Schmoll *et al*., 2010). Homologous overproduction of SC3 in *S. commune* was hampered by gene silencing and occurrence of THN mutation. When more than one extra copy of SC3 gene is introduced into *S. commune*, silencing of the gene takes place through methylation of the coding DNA, which causes SC3 production to cease (Schuurs *et al*., 1997).

Pichia pastoris is widely employed as an expression system for the production of various hydrophobins. HFBI was heterologously expressed in *P. pastoris* to 120 mg/L using pPIC9 vector under the control of *AOX1* promoter (Niu *et al*., 2012). Both class II hydrophobin FcHyd5p and class I FcHyd3p from *F. culmorum* were heterologously expressed by *P. pastoris* with similar property as their native hydrophobins (Stübner *et al*., 2010; Lutterschmid *et al*., 2011). Class I hydrophobin HGFI from *G. frondosa* was also acquired in *P. pastoris* at 90 mg/L (Wang *et al*., 2010). Class I hydrophobin RodA and RodB from *A. fumigatus* were produced by a fed-batch fermentation at 200–300 mg/L (Pedersen *et al*., 2011). Both rRodA and rRodB converted a glass surface from hydrophilic to hydrophobic similar to native RodA, but only rRodB was able to decrease the hydrophobicity of a Teflon-like surface to the same extent as native RodA.

Although efforts to overcome the limitations in overproduction of class I hydrophobins have not yet produced satisfactory results as class II, attempts to optimize the purification are promising.

Characterization of hydrophobins

Most common characterization methods for hydrophobins are water contact angle (WCA) measurement and atomic force microscopy (AFM) image (Scholtmeijer, 2000). The coating of surfaces of mica sheets and siliconized glass by hydrophobins can result in different WCA with dramatic increment on mica surface but reduction on siliconized glass surface. AFM is effective in distinguishing class I hydrophobins rodlets with class II rods, needles and fibrils.

The emulsifying capacity of hydrophobins can be investigated and compared with the typical food emulsifier (*e.g.,* sodium caseinate) using soy oil emulsion (Niu *et al*., 2012). Class II hydrophobins have higher emulsifying activity and longer stabilizing period of oil droplets than class I hydrophobins. Coating experiments on microtiter plate surface and determined by inorganic dye (*e.g.*, Ponceau S) can also confirm the adhesion effect of hydrophobins (Kottmeier *et al*., 2012). The surface pressure measurement using a film balance can demonstrate the film formation by hydrophobins and subsequent surface pressure increase.

Applications

As hydrophobins have unique and remarkable properties and various biological roles, their application possibilities are also diverse and some even rather surprising. Based on the amphiphilic nature and self-assembly properties, the proposed applications of hydrophobins include biosurfactants, emulsifiers, surface coating and immobilization range from food industries, cosmetics, nanotechnology, biosensors and electrodes, to biomaterials and pharmaceuticals, and even as indicators for beer gushing.

Hydrophobins have become outstanding candidates for surface active components in food industries for their non-toxicity nor cytotoxicity or immunogenicity especially the ones derived from mushrooms (Murray *et al*., 2009). They can stabilize oil droplets or serve as emulsifiers in food processing, liposome applications and oil refining (Linder *et al*., 2002). Hydrophobins are expected for long terms stabilization of different phases in food products, e.g. to stabilize the dispersed air bubbles in ice cream. They can also modify hydrophilic and hydrophobic surfaces (glass and Teflon, respectively) (Tchuenbou-Magaia *et al*., 2009). SC3 has an influence on DOPC/DOPE liposomes but does not destabilize DPPC liposome.

The amphipathic nature of hydrophobins can also be exploited in separation technologies. Fusion of the goal protein to hydrophobins has been utilized in purification by aqueous two-phase systems (ATPS) (Linder *et al*., 2004). Class II hydrophobins display high separation behavior in ATPS. HFBI was used as a tag to cellulose endoglucanase I (EGI) and EGIcore-HFBI fusion method proved to be a cheap, easy and efficient way to purify EGI (Collen *et al*., 2002). Based on the efficient assembly, hydrophobin fusions can be used for immobilization of enzymes and antibodies. The immobilization of lipase onto *P. ostreatus* hydrophobins led to high lipase activity and thermal stability (Palomo *et al*., 2003). Immobilization of flavor compounds can be another aspect to keep the aroma in food and beverages for longer time.

Hydrophobins coating on biosensors and electrodes can be decrease the hydrophobicity and thus inhibit the denaturation and preserve long-time activity. Multi-wall carbon nanotubes (MWNTs) were coated by HFBI using a novel non-covalent approach (Wang *et al*., 2010). HFBI–MWNTs nanocomposites displayed high sensitivity, wide linear range, low detection limit, and fast response for glucose detection. The *Pisolithus tinctorius* hydrophobin HYDPt-1–coated electrodes were stable in a wide range of pH, and effectively blocked the oxidation of electrode substrates and the access of hydrophilic electroactive probes to electrode surface (Bilewicz *et al*., 2001). Class I hydrophobins from *P. ostreatus* were stable in KOH and thus coated on a silicon to protect it from etching with KOH and passivate optical devices (De Stefano *et al*., 2007).

Hydrophobins are promising in enhancing the biocompatibility of medical implants without eliciting immunogenic reactions (Hektor and Scholtmeijer, 2005). SC3-coated polymers such as polystyrene by spin coating or direct deposition showed a bumpy structure and 70–80% reduction in friction coefficient compared to untreated surface which can be used for personal care and biomedical applications (*e.g.*, catheters) (Misra *et al.*, 2006). The self-assembly property of hydrophobins allows them to be used in formulations of water insoluble drugs for oral administration (Valo *et al*., 2010). The addition of SC3 to hydrophobic drug suspension of cyclosporine A and nifedipine increased their bioavailability two and six folds, respectively

(Haas Jimoh Akanbi *et al*., 2010).

Class II hydrophobins have also been used to stimulate cell growth on solid surfaces (Hou *et al*., 2008). A coating with HFBI was employed to adhere collagen to the hydrophobic surface of PDMS. The layer of HFBI–collagen helps the adhesion and the growth of human embryonic kidney cells while HFBI–serum layer promoted growth of neural stem cells on micro-domains (Li *et al*., 2009).

The assembly of hydrophobins at the interface between hydrophobic and hydrophilic liquids can stabilize emulsions (Vic, 2003). Hydrophobins can be used to stabilize emulsions in creams and ointments. They can prolong the residence time of shampoo by surviving several washes and thus be a good additive to hair care products.

Although the stabilization of foam by hydrophobin can be used in certain positive applications, it may also cause problems in beer brewing and sparkling wines production (Khalesi *et al*., 2012). The presence of too many hydrophobins in beer can provoke 'gushing' of the beverage. Several methods have been developed to trace the gushing factors either in barley or malting (Shokribousjein *et al*., 2011).

Summary and future aspect

The extraordinary properties of hydrophobins offer numerous possibilities for applications in science and technology. Research over the past few years has improved our understanding of the self-assembly process of hydrophobins. However, currently there is no mature product on the market to fulfill proposed applications yet. Thus it's critical to develop production technologies for commercial success of hydrophobins. Molecular engineering of hydrophobins with fusion proteins can give endless variations of functionality. With the importance of surface phenomena in technical applications, the utilization of hydrophobins are tremendous. Also as the building of self-assembled materials, hydrophobins hold bright promise in nanotechnology. Molecular aspects of mushroom technology should contribute to hydrophobin research.

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