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 hydrophathy 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 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).
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, Nigrospora 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 (Schnoll 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 FeHyd5p 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 HGBI 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. 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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