Fungal hydrophobins, proteins as natural emulsifiers

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ABSTRACT: Hydrophobins are proteins produced by filamentous fungi that exhibit very peculiar properties. Their behaviour in solution resembles that of typical surfactants characterized by self-assembling into an amphiphilic membrane. Actually the hydrophobin self-assembly at the cell-wall-air interface plays a key role in the formation of aerial hyphae and fructifying bodies by establishing a water-repellent layer. The interest in this class of proteins is increasing because of their multiple and attractive potential applications. Just to give an example, hydrophobins can be used to stabilize emulsions, and this property might be extremely useful for pharmaceutical applications and in the food industry. We have purified and characterized the hydrophobin that is secreted in the fungal mycelia and characterized the hydrophobin that is secreted in the fungal mycelia of the edible fungus Pleurotus ostreatus. Higher amounts of the same protein have been obtained from the fungal mycelia and by recombinant expression in E. coli. In this paper the emulsifying properties of the protein are also discussed.

Amphiphiles are molecules characterized by distinct hydrophilic and hydrophobic parts. This characteristic provides them with 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. The most common amphiphiles is soap, which is used to solubilize dirt. In biology, amphiphiles play extremely important roles, and the most crucial one is the formation of membranes by amphiphilic phospholipids. Amphiphiles reduce the surface tension of water: in a drop of water the external molecules encounter an environment, e.g., air or a solid surface, different from that experienced from the internal ones. Since the water molecules at the interface/surface cannot make favourable contacts among them, the system tries to minimize the number of water molecules interacting with the surroundings. The spherical shape of a water drop on a hydrophobic support is a consequence of that, being the lowest ratio of surface molecules to bulk molecules in a sphere. The amphiphiles can favourably interact with water on one side and with air or the hydrophobic surface on the other side, thus they migrate to the interfaces, e.g., solid and water or air and water, and allow the drop to spread out. Since amphiphiles migrate to the surface, they are called surface active molecules and the spreading of a water drop is a convenient way of measuring surface activity.

Biosurfactants are surface-active substances synthesized by living cells. Some proteins can be considered as biosurfactants, although very different from regular detergents from a structural point of view. Proteins are globular, and rather rigid macromolecules, when compared with detergents, that are generally small, elongated, and flexible molecules with long hydrocarbon tails and hydrophilic head groups. Hydrophobins are among the most surface active proteins known. They are produced by filamentous fungi, and endowed with very special properties [1, 2]. They are able to reduce the surface tension from 70 mN m⁻¹ to approximately 30 mN m⁻¹ [3]. Their behaviour as surfactants can be explained from a structural point of view. On one side of their molecular surface, some exposed hydrophobic aliphatic side chains form a flat hydrophobic patch, whilst polar or charged residues are confined to the other side [4, 5]. Hydrophobins are produced as soluble proteins and when they reach an interface (e.g., medium–air or cell wall–air) they self-assemble into an amphiphilic membrane. Several features of fungal development have been attributed to these proteins [6, 7]. For example, the self-assembly of hydrophobins seems to play an essential role in the formation of aerial hyphae and fructifying bodies. Assembly at the medium–air interface results in the reduction of the surface tension, allowing hyphae to break the water-air interface [8]. Self-assembly at the cell wall-air interface results in the coating of aerial hyphae and fructifying bodies with a water-repellent layer [9, 10]. Hydrophobins are also involved in the attachment of hyphae to hydrophobic surfaces [11]; for example, during symbiotic or pathogenic interactions hydrophobins self-assemble into an amphiphilic layer between the fungal wall and the hydrophobic surface of the host [12, 13].

Because of the multiple attractive potential applications of this class of proteins, multinational companies as BASF, Unilever or L’Oreal have increasingly addressed their attention to them, as demonstrated by the conspicuous number of patents. For example hydrophobins can be a good additive to hair care products in the cosmetic industry. They can prolong the residence time of those products that do not firmly adhere and are easily removed upon shampooing (i.e. WO/2003/053383 Cosmetic use of at least a hydrophobin for treating keratinous materials). Hydrophobins can be used to stabilize emulsions in creams and ointments (i.e. WO/2007/006765 Aqueous monomer emulsions containing hydrophobin). This property might also be beneficial for...
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OIL SOLUBLE POWDER

油溶性粉末

Brown 棕 Yellow 绿 Green 绿 Red 红
pharmaceutical applications [WO/2010/060811 Surface active proteins as excipients in solid pharmaceutical formulations] and applications in the food industry [WO/2008/116733 Aerated food products being warm containing soluble and/or insoluble solids and methods for producing them], which both require stable emulsions for certain formulations and ingredients. Recently, Akanabi et al. (14) show that the fungal hydrophbin SC3 can be used to make suspension of water insoluble drugs, resulting in a constant longer lasting drug level in the body. Standardized emulsion testing under realistic laundry conditions demonstrates that tiny amounts of hydrophbin can boost the emulsifying power of an appropriate technical surfactant (15). Before considering the use of hydrophbins as an additive, especially for the food industry, their production levels and price should be affordable. If this is the case, the advantage of hydrophbins is that several of them can be considered food-grade surfactants. Hydrophbins from edible fungi, for example, are already consumed in large amounts by people eating these common mushrooms.

Adequate production levels of hydrophbins would also make possible their use in anti-fouling applications to modify surfaces which are usually significant in size. For example, window panes and cars suffer from fouling, and the growth of unwanted organisms on ships represents a significant problem for the marine shipping industry (16).

Hydrophbins have been split in two groups, class I and class II, based on structural differences and properties of the aggregates they form (17). Class I hydrophbins form highly insoluble aggregates that have the appearance of distinct rodlets and, similarly to amyloid fibrils, are characterized by β-structure. These aggregates can be dissolved in pure trifluoroacetic acid (TFA) whereas class II hydrophbins form polymers that are soluble in some organic solvents and lack the rodlet appearance of class I hydrophbins (1).

Basidiomycete fungi produce only class I hydrophbins, while ascomycetes produce both class I and II proteins. A better understanding of hydrophbin self-assembly process as well as of their behaviour in solution is needed, in order to meet the full exploitation of these proteins. Comparison of the known properties of the few studied proteins with other ones of the same class will advance this knowledge, allowing an outline of the molecular mechanisms underlying these properties. Differences between the proteins belonging to the two diverse classes and among those within the same class, can be fruitfully used to select the molecule that is suitable for a specific application. This will further improve their potentiality. The basidiomycete Pleurotus ostreatus (Figure 1) is a commercially relevant edible mushroom, also used for the bioconversion of wastes and as a source of enzymes for industrial applications. We are studying a Class I hydrophbin (named Vmh2) produced by the mycelium of this fungus. A simple protein purification procedure has been set up; hydrophbins released in the medium are aggregated by aeration, then captured by precipitation. The precipitate is then dissolved in TFA. After TFA evaporation, the protein is solubilised in 60 percent ethanol. This procedure results in a protein production with a yield of about 0.5-1 mg/l of culture broth (18).

Pure Vmh2 is not water soluble, whereas complexes formed between the protein and glucans are soluble in water. It has been demonstrated that glucose is also able to solubilise the hydrophbin in water. The protein in aqueous solution shows high propensity to self-assembly. Conformational changes towards β-structure upon vortexing the solution are observed by circular dicroism spectroscopy. Similar changes occur upon temperature increase: a major change of structure has been detected at about 80°C. On the other hand the pure protein dissolved in less polar solvent (60 percent ethanol) is not prone to self-assembly and no conformational change has been observed (18). The protein is very stable at high temperature and in a broad pH range.

We are seeking for procedures to improve the protein yield. A simple procedure to purify the hydrophbin from the P. ostreatus vegetative mycelia has been set up, obtaining an at least tenfold higher protein yield. Protein expression in easily cultivable and handling hosts can allow higher productivity in shorter time and reduce the costs of production. The versatility and scaling-up possibilities of the recombinant protein production opened up new commercial opportunities for industrial uses of proteins. For these reasons, recombinant expression of the P. ostreatus hydrophbin in E. coli has been achieved and the properties of the recombinant protein compared with those of the extractive one, showing similar behaviours (unpublished data). We are also setting up recombinant expression of the protein in yeast.

Biofilms formed by the extractive protein on different surfaces have been characterized. Chemically and mechanically stable monolayers of the self-assembled protein have

Figure 1. The basidiomycete edible fungus Pleurotus ostreatus.
been obtained. On a hydrophobic surface the pure protein forms a very stable biofilm (19) whose thickness was about 3nm, whereas the biofilm has not been detected on a hydrophilic surface (18, 20). On the other hand, the water-soluble protein -in the presence of glucans- forms thicker (up to ten fold) biofilms either on hydrophilic and hydrophobic surfaces. The hydrophobin modified hydrophobic surface shows a great improvement in wettability. In Figure 2 it is shown that the contact angle of a water drop (90° on the untreated hydrophobic surface) falls down to 25° after hydrophobin deposition, and the surface becomes clearly hydrophilic (20). The hydrophobin solution has also been infiltrated in the nanometric sponge-like matrix of the porous silicon, a material widely used in biomedical and biotechnological applications, increasing its chemical stability and changing its wettability (21).

Moreover, recent results demonstrate the emulsification capacity of the hydrophobin solution with three different oils: olive, peanut, and almond oil. The hydrophobin solutions (0.1 mg/ml) in 20 percent ethanol was vortexed in the presence of each one of the three oils. In Figure 3 the emulsions obtained in the presence or absence of the hydrophobin are shown. These emulsions were stable at least for two days, and even longer (up to 7 days) in the cases of the peanut and almond oils. These data indicate the potentiality of the use of the P. ostreatus hydrophobin as emulsion stabilizer. It is worth noting that this hydrophobin can be considered a food-grade surfactant, since its source, P. ostreatus, is an edible fungus consumed in large amounts.

REFERENCES AND NOTES