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Mini-Review

Hydrophobins: A revolutionary protein with potential uses in the food industry

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Abstract: Hydrophobins are low molecular weight proteins produced by filamentous fungi. These proteins have characteristics of four pairs of cysteine and have surface-active properties due to the presence of hydrophilic-hydrophobic spatial arrangements. This property of hydrophobin makes it capable of stabilizing emulsions and foams which attracts industrial applications. Hydrophobins isolated from GRAS-cleared strains like mushrooms can be used in the food industry as biosurfactants, foaming agents, and stabilizers of air-filled emulsions in food. Due to the requirement for product texture improvements, food industries find hydrophobin as a suitable candidate as it produces foam that is stable for 4 months. Hydrophobins gain more importance when it comes to fat replacement in emulsion-based foods. Air-filled emulsions created using hydrophobins are stable for 45 days and can be used for partial replacement of fat in foods resulting in low-fat products. Hydrophobins play a crucial role in the formation of yeast bio-capsules which are used to complete alcoholic fermentation. These bio-capsules are used in wine production. Hydrophobins are also used to inhibit ice crystal formation in frozen products. Despite being industrially useful molecules, hydrophobins has limitations due to poor yields. This review summarizes the properties of hydrophobins and their existing possibilities in food industry applications.

Keywords: hydrophobins; bio-capsules; food industry; foam; emulsion; nano-emulsion;

1. Introduction

Hydrophobins are proteins with low molecular weight, comprising 100-150 amino acids, and are found in filamentous fungi [1]. These are cysteine-rich proteins with molecular weights ranging from 7 to 15 kDa and can self-assemble into an amphipathic membrane hence, can alter the surface properties that are they can self-assemble and change the surface from hydrophilic to hydrophobic and vice versa, this property of hydrophobins makes its presence essential for the fungal growth and life cycle [2-4]. The expression of hydrophobins depends on different stages of the fungal life cycle and fungal development is mediated by hydrophobins [3, 4]. For instance, hydrophobins play a vital role in producing aerial structures in fungi by lowering the surface tension and letting hyphae breach the water-air interface. It also helps protect the surface of conidia, caps, and fungi spores against wetting by forming layers on them. Hydrophobins also mediate attachment of hyphae to hydrophobic surfaces during pathogenic or symbiotic interactions by self-assembling into an amphipathic layer [3-5].

Hydrophobins are mainly divided into two classes: Class I and Class II, based on the solubility, distribution of cysteine, and arrangement of hydrophilic and hydrophobic amino acid residues in the protein sequence [6]. Class I hydrophobins are stable as they form the rigid rodlet layer. Treatment with strong acids like TFA and formic acid is required to dissociate. Class II hydrophobins do not constitute a rodlet layer and can be dissolved easily using SDS and ethanol [7, 8]. Class I hydrophobins comprise 100-125 amino acids whereas Class II hydrophobins consist of 50-100 amino acids [9]. Research until now shows that class I hydrophobins can be found in Basidiomycetes and Ascomycetes but hydrophobins of class II can only be found in Ascomycetes. According to some evidence, hydrophobins also occur in zygomycetes. However, there is no evidence of the presence of hydrophobins in Chytridiomycetes [10]. First-ever hydrophobin was isolated from the spores of fruiting bodies of *Schizophyllum commune* which belongs to Basidiomycetes [1]. Hydrophobins have been successfully isolated from *Aspergillus fumigatus*, *Neurospora crassa*, *Claviceps fusiformis*, *Claviceps purpurea*, *Trichoderma reesei*, and

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Table 1. Various types and applications of hydrophobins.

Types of Hydrophobins	Class	Fungal Origin	Application	References
HFBII	II	*Trichoderma reesei*	Stabilizing foams and improving bubble stability against disproportionation in aerated and frozen food products.	[11, 12]
			Stabilizing air-filled emulsions for fat replacement	[12, 13]
			Stabilizing nano-emulsion in nutraceuticals	[14]
HFBI, HFBII, HFBIII	II	*Trichoderma reesei*	Immobilizing yeast and filamentous fungi to form yeast bio-capsules	[15, 16]
SC3	I	*Schizophyllum commune*	Synthesis of water-insoluble drugs for oral regulation	[17]
			Surface modification of Teflon and polymers	[18, 19]
Vmh2	II	*Pleurotus ostreatus*	Immobilization of antibodies	[20]
HFBI	II	*Trichoderma reesei*	Drug nanoparticle coating	[21]
			Surface modification of glass	[22]
			Purification of endoglucanase effectively	[23]

many other Ascomycetes species and from *Pleurotus ostreatus*, *Pleurotus nebrodensis*, *Schizophyllum commune*, *Tircholoma terreum*, *Agaricus bisporus*, *Coprinus cinereus* and other species belonging to Basidiomycetes [1]. Some hydrophobin species are also studied using genomics and bioinformatics which are not yet isolated from their native fungi. For instance, HFBIV and HFBVII of *Trichoderma reesei* are studied using genomics and bioinformatics while HFBI, HFBII, and HFBIII have been isolated and studied [1]. Various types hydrophobins and their applications are presented in table 1, figure 1.

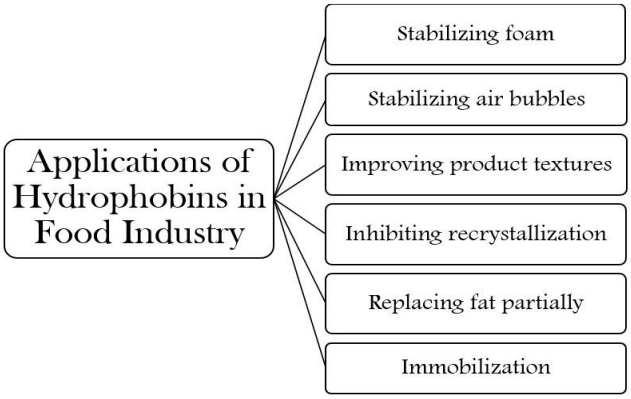

Figure 1. Applications of hydrophobins in food industry.

Due to their amphipathic nature, hydrophobins have many medical applications, industrial applications like surface coating and antifouling agents, and applications in food industries [8, 9]. Hydrophobins can get adsorbed on the hydrophilic surfaces like glass and mica and the hydrophobic surfaces like Teflon and parafilm making it a promising biotechnology tool [5]. Studies have suggested various applications of SC3 hydrophobins such as surface modification of Teflon and polymer, and production of water-soluble drugs for oral administration. HFBI of class II has applications like surface modification of glass, formulation of fluorescent dyes, and purifying endoglucanase efficiently. HFBII hydrophobin of class II can be used effectively as a foaming agent in the food industry in aerated and frozen food products and as an air-filled emulsion to replace fat partially. Studies have suggested that adding hydrophobins can help retain volatile flavor compounds like ocimene in beverages [24]. Hydrophobins with gelled particles produce effective foam which can be used in food products containing gelling polysaccharides like agar, carrageenans, gellan, pectin, or gellable starches [25]. Despite their potentiality, hydrophobins are not widely used in food industries since there is no legislation available; moreover, the limitation of hydrophobin application is also due to the low production. Using hydrophobins in the food industry will require its production and purification on a large scale and be cost-effective [5].

2. Properties of class II hydrophobin

The hydrophobin used predominantly in the food industry is HFBII of class II. These hydrophobins are amphiphilic and do not contain a cylindrical rodlet structure. The inter-cysteine spacings in class II hydrophobins are regular and relatively short and the sequence of amino acids is highly conserved compared to class I hydrophobins [26]. On the air-water interface, class II hydrophobin can form 2D

crystalline films and it was proved by grazing-incidence small-angle X-ray scattering (GISAXS) [27].

Isolated HFBII can bind effectively to the surfaces, and while working as a fusion partner it does not cause immobilization, also it does not cause polymerization of the fusion protein in solutions. The class II hydrophobins assemblage and adsorbed surface layers seemed to be dissociated more easily, (for example, by 60% ethanol, 2% sodium dodecyl sulfate that is SDS, or applied pressure). These properties of HFBII make it capable of many practical applications such as biosurfactants, foaming agents, and stabilizers of air-filled emulsions in food, and it can also be used in improving the product texture in food industries [27].

3. Hydrophobins as foaming agents

3.1 Stabilization of foam in milkshakes

Since hydrophobins have a great foam and bubble stabilizing property, much research has been done on this property [28]. HFBII protein of Class II extracted from *Trichoderma reesei* has shown remarkable stability in other food surfactants of simple aerated solutions [29]. Recent work done compares the ability of hydrophobin to stabilize foam in a model system and an example food product: a chocolate milkshake with the formulation that contains different milkshake ingredients such as cocoa powder, fat, protein, and some sugar along with hydrophobin, comparing it with other milkshake having same ingredients except hydrophobin [11].

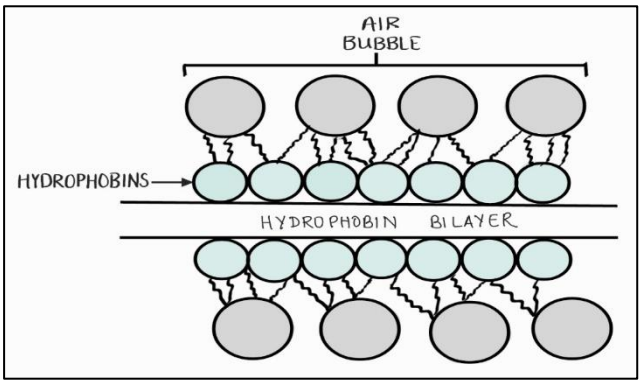

Figure 2. Hydrophobin-air bubble interaction in foam stabilization.

The chocolate milkshake was stabilized using hydrophobin and during this experiment, the shake contained skim milk powder (ca. 35wt% protein content), chocolate powder, xanthan gum, sucrose, HFBII, and water [11]. While making the milkshake it was made sure that it had a good consistency and smooth texture. Two different mixtures of the milkshake, one containing HFBII in an aqueous solution and the other containing water was observed after 3 weeks of aeration and the foam observed had good stability. Also, there was no apparent air phase volume and no visible increase in air bubble size. As soon as it gets absorbed at the surface of the bubble, adding any other ingredient does not appear to affect the surface stabilizing capability of hydrophobin in the food product. In the presence of a thickening agent, it was found that hydrophobin can stabilize the foam to an extent where minimum air phase loss can be observed for over 4 months [11]. Overall, hydrophobin molecules interact and hold the air bubbles or air cells inside the foam, making it stable [28]. This simplified mechanism is shown in figure 2.

3.2 Stabilization of air bubbles using hydrophobin and β-casein

β-casein is a protein that is a surface-active milk protein and is exploited for its surface-active properties in food products. Previous studies suggested that milk proteins like β-casein do not give good stability against the disproportionation of air bubbles. On the other hand, the foam created using HFBII is effectively stable and the disproportionation of air bubbles is stopped compared to foams produced using different proteins like β-casein or β-lactoglobulin [30]. Further studies showed that the stability of air bubbles can be improved by adding hydrophobins with β- Casein as it forms a film around bubbles, hence resisting the bubble shrinkage. The β-casein is proven to increase the surface viscosity of hydrophobins. This was studied by analyzing surface shear rheology, surface dilatational rheology, zeta potential measurements, and Bubble disproportionation of mixtures of β-casein and hydrophobin with varying concentrations of β-casein [31]. It was observed that a mix of β-casein and hydrophobin could create and resist the disproportionation of bubbles for several days. The result of the experiment also suggested that adding β-casein up to a certain level will increase the surface viscosity of hydrophobin. This leads to bettering the bubble stability, decreasing the shrinkage rate, and allowing tiny bubbles to form and survive. This study proves that a combination of hydrophobin and different milk proteins like β-casein can be used to improve the surface properties of food products. Hydrophobins can also be used effectively with other potential milk proteins like sodium caseinate, and β-lactoglobulin to decrease the shrinkage rate of bubbles in food products [32, 33].

3.3 Application of bacterial hydrophobin in ice-cream

Recent studies found that biofilm produced by Bacillus subtilis is highly non-wetting when the water droplet placed on the biofilm of *B. subtilis* had a higher contact angle than the water droplet which was placed on the Teflon film [34]. The production of protein BslA (Biofilm surface layer protein A) was found responsible for the formation of non-wetting biofilm. The potentiality of the protein BslA was observed during interaction with oil/water and air/water interfaces and solid surfaces; it further led the researchers to explore the applications of BslA in applied settings. BslA is already being used in Japanese and Korean fermented food hence, is already found in the food chain. Research shows that BslA can be used in ice cream production as it binds the air, water, and fat together

to create a super smooth consistency. BslA also adheres to fat droplets and air bubbles, making the mixture more stable. It allows the ice creams to remain frozen for a long time during hot weather conditions while inhibiting the growth of ice crystals ensuring the fine and smooth texture of the ice cream [34].

4. Hydrophobins in aerated food products and frozen food products

Aerated food products are made by introducing different gases like air, carbon dioxide, or nitrogen [35]. Two primary considerations arise during the production and storage of aerated products. The first is the capability of foaming agents to produce foam that is foamability and the second one is foam stability which is considered during storage. As aerated products typically fall into four groups- hot foods which include beverages like cappuccino, ambient food like whipped cream, marshmallows, and bakery products, chilled food which includes whipped cream, mousses, and drinks like milkshakes and smoothies, frozen food products like ice-cream, yogurt, milk ice, frozen custard [35]. Although hydrophobins are found in frozen and chilled food products, research claims that the aerated products contain 0.001%wt hydrophobin. It has also been proven that hydrophobin in trace amounts with an effective biopolymer can inhibit bubble coarsening and can stabilize foam in aerated products [35, 36].

To study the properties and effectiveness of the product, aerated frozen products were made using hydrophobin and were compared with products made using other foamable proteins like sodium caseinate and skimmed milk powder [35]. Three different mixes were prepared which contained common ingredients coconut oil, sucrose, and water, and one mix contained sodium caseinate, the other contained skimmed milk powder, and the last contained hydrophobin HFBII [35]. The process of making a mixture containing hydrophobins differs from that of making a mixture containing other proteins. Hydrophobins are constantly added as an aliquot into the solution containing sucrose and cold water whereas the protein is combined with sucrose first and then dispersed into the cold water [35].

The products containing hydrophobins can retain their microstructure in temperature abuse for weeks and show tiny bubble coarsening. In contrast, other milk proteins do not have their microstructure and show severe bubble coarsening. The product containing hydrophobin shows good melting behavior by retaining its shape during melting, resulting in stable foam and ice than the products made using sodium caseinate and skimmed milk [35].

Hydrophobins in frozen products can play an essential role in inhibiting re-crystallization. Frozen products contain crystals of ice that, after a specific duration, start to increase in size, which hinders the quality of the product by affecting its texture, appearance, and taste [36]. Hydrophobins if introduced deliberately in trace amounts like 0.01%wt. can inhibit ice crystal formation and modify ice crystal habits in frozen composition. Further, hydrophobins also impact the melting behavior of frozen products. Introducing hydrophobin in frozen products can improve the texture of the product making it more consistent and smoother and more stable to temperature abuse unlike other milk proteins [35, 36].

5. Hydrophobins as emulsions

5.1 Air-filled emulsions for fat replacement

Research has proven that hydrophobins can create air bubbles due to their physical properties, so they are suitable for tri-phasic (air/oil/water) emulsion formation. These air cells created using hydrophobins are approximately 1-100mm in size, and 40% of these air cells fall within the 1-2mm range and are air-filled emulsions [13]. Air-filled emulsions are great for replacing lipid content in food and creating low-fat food products. As full-fat replacement might hamper the taste of the food product, partial fat replacement comes as a solution, and to obtain the low-fat products partial replacement of lipids can be allowed. The remaining fat can be utilized in the flavor delivery. The study was carried out by creating a range of tri-phasic emulsions of air/oil/water by diluting it up to 20% oil-in-water emulsion (oil/water) with the 68% air phase air-filled emulsion. This showed that air cells formed have a rigid surface structure resulting from hydrophobin film, which helps prevent ripening and disproportionation for 45 days. Air-filled emulsions can also survive high shearing in the Silverson High Shear Mixer. All the physical properties of air cells like size, elasticity, and strength allow the air-filled emulsion prepared using hydrophobin to precisely replace oils from emulsified structures [13, 28]. Hydrophobins can also be used in aerated food products that are made using unsaturated fats which contain a considerable amount of liquid fats due to their ability to stabilize aerated emulsions [26].

5.2 Nano-emulsions in nutraceuticals

Several studies have been performed on a natural oil Copaiba found in trees of the genus *Copaifera* spp., because of its health-benefiting properties. In oleoresin, the presence of diterpenes and sesquiterpenes promotes its biological properties like antimicrobial and anti-inflammatory activities and has potential in medical and food applications [14]. The study claimed that a low concentration of copaiba oil has enhanced the spontaneous emulsification process which contributes to forming nanoscale droplets that presumably favor the structuration of the HFBII adsorption layer on the oil-water interface. Using these low concentrations, the hydrophobic attraction to the interface might be selected by creating tiny oil droplets with a larger surface area, following the diffusion of acetone, helping the rapid structuration of adsorption layers. This remarkable property of the structure of HFBII arises due to the exposed hydrophobic region which allows rapid adsorption on the

oily surfaces to form layers of high mechanical strength, even at low protein concentrations. All these studies have shown that HFBII can be potentially used to stabilize the nano-emulsion system as a biopolymer which can be used for delivering bioactive lipids [14].

6. Formation of yeast bio-capsules

The combination of yeast and filamentous fungi adhesive forces creates an immobilization system called yeast bio-capsules. Yeast bio-capsules are used in wine production as they allow the completion of alcoholic fermentation with the help of natural adherent properties of yeast and fungi which allows yeast to be fixed on filamentous fungi. The bio-capsules form biofilm on the wine-air interface and have high immobilization efficiency [15]. Bio-capsules also have other advantages like maintaining integrity after fermentation which makes it possible to reuse them for subsequent fermentation also, they have high immobilization efficiency of up to 84%, and they can prevent loss of cells. Hydrophobins play a crucial role in bio-capsules as they provide hydrophobic interaction between filamentous fungi and yeast. Hence, filamentous fungi which produce high amounts of hydrophobins are beneficial in creating bio-capsules and thus improvising alcoholic fermentation in the production of wine [15, 16].

7. Conclusion and future prospective.

Hydrophobins, the protein secreted by filamentous fungi, have many applications in the food industry due to their amphipathic property, making them capable of producing foam, stabilizing foam, creating emulsions, and stabilizing it as well. Although it is just one step toward applications in the food industry, more research has yet to be done on this protein. So far, the use of hydrophobin in aerated food products and frozen food products to create and stabilize foam, create and stabilize emulsions, inhibiting ice recrystallization has been discovered. Other applications like stabilizing foam in milkshakes have also been proven. Air-filled emulsions created using hydrophobins have the potential to replace fat and create low-fat products which might gain a great demand in the future. Nano-emulsions created using hydrophobins can be used as nutraceuticals, which will have many applications in the medical and food industries. Yeast bio-capsules have been successfully used to complete alcoholic fermentation and the quality of these capsules can be increased by using fungi which produces more hydrophobin. Up till now, class II of hydrophobins have been exploited and have potential uses, especially HFBII is used predominantly, class I hydrophobins are yet to be explored which might have other benefits in medical as well as in the food industry.

Declarations

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