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Limited coalescence and Ostwald ripening in emulsions stabilized by hydrophobin HFBII and milk proteins

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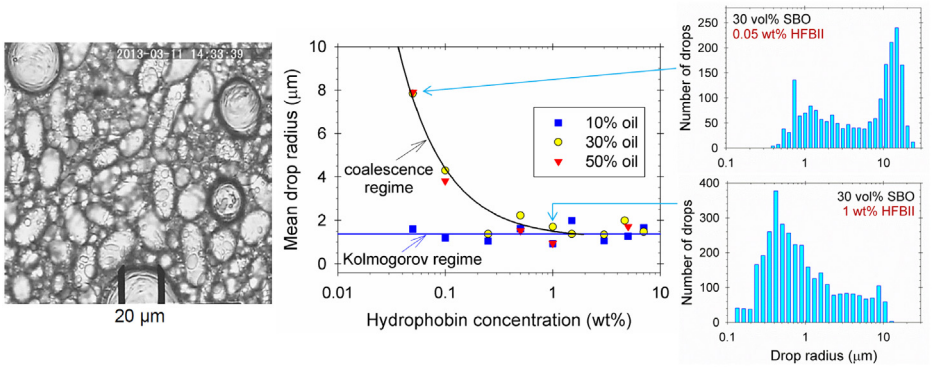
HIGHLIGHTS

- Solidification threshold of HFBII layers at o/w interfaces is determined and interpreted.
- The thinning of o/w/o emulsion films with HFBII ends with the formation of S-bilayer.
- The law of limited coalescence in emulsions with HFBII is quantitatively interpreted.
- Emulsions with HFBII are stable for at least 50 days at rest but unstable upon stirring.
- The dense HFBII adsorption layers encapsulate volatile oils and block Ostwald ripening.

GRAPHICAL ABSTRACT



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ABSTRACT

Hydrophobins are proteins isolated from filamentous fungi, which are excellent foam stabilizers, unlike most of the proteins. In the present study, we demonstrate that hydrophobin HFBII can also serve as excellent emulsion stabilizer. The HFBII adsorption layers at the oil/water interface solidify similarly to those at the air/water interface. The thinning of aqueous films sandwiched between two oil phases ends with the formation of a 6 nm thick protein bilayer, just as in the case of foam films, which results in strong adhesive interactions between the emulsion drops. The drop-size distribution in hydrophobin stabilized oil-in-water emulsions is investigated at various protein concentrations and oil volume fractions. The data analysis indicates that the emulsification occurs in the Kolmogorov regime or in the regime of limited coalescence, depending on the experimental conditions. The emulsions with HFBII are very stable – no changes in the drop-size distributions are observed after storage for 50 days. However, these emulsions are unstable upon stirring, when they are subjected to the action of shear stresses. This instability can be removed by covering the drops with a second adsorption layer from a conventional protein, like β-lactoglobulin. The HFBII surface layer is able to suppress

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the Ostwald ripening in the case when the disperse phase is oil that exhibits a pronounced solubility in water. Hence, the hydrophobin can be used to stabilize microcapsules of fragrances, flavors, colors or preservatives due to its dense adsorption layers that block the transfer of oil molecules.

1. Introduction

Hydrophobins are a class of relatively small proteins (65–100 amino acid residues), which are exclusively produced by filamentous fungi, including some edible mushrooms [1–3]. These proteins possess several remarkable properties. First, the hydrophobin molecules are strongly amphiphilic, like Janus particles, with hydrophobic and hydrophilic patches expressed on their surfaces [1,4]. Second, at air/water and oil/water interfaces they self-assemble into dense adsorption layers (membranes) of high surface dilatational and shear elasticity, which exceeds the elasticity of all other investigated proteins [5–9]. The presence of shear elasticity indicates that the hydrophobin adsorption layers at the air/water interface are not fluid – they solidify soon after their formation [7,10,11]. Third, the hydrophobins are "sticky" proteins [11] – they have been utilized for immobilizing functional molecules at surfaces [12], and for surface modification by appropriate coatings [13].

In the present study, the class II hydrophobin HFBII was used. The structure of HFBII determined from crystallized samples shows that it is a single-domain protein with dimensions of $24 \times 27 \times 30$ Å [14]. In aqueous solutions, it forms aggregates, which are predominantly tetramers at mg/mL concentrations [15–18]. Because of the adhesive interactions between the HFBII molecules in water, these aggregates can irreversibly grow with time and can reach micrometer sizes [19–21]. The formed large aggregates can be destroyed by ultrasound treatment (sonication). The adsorption of HFBII at air/water and liquid/solid interfaces has been also studied [22–24]. Not only the hydrophobic, but also the hydrophilic parts of HFBII molecules attract each other in aqueous medium, which is evidenced by the strong adhesion between the surfaces of foam films (contact angle >50°) and the spontaneous formation of self-assembled protein bilayers (S-bilayers) of thickness 6 nm [20,25].

Unlike most of the proteins, the hydrophobins are excellent *foam stabilizers* [5,19,26–28]. The Ostwald ripening that is due to diffusion transfer of gas from the smaller to the bigger bubbles [29], can be blocked by the dense and mechanically strong HFBII membranes, which prevent also the bubble coalescence. Detailed literature review and experimental results on hydrophobin stabilized foams can be found in our previous article [30].

The properties of hydrophobins as *emulsifiers* are much less studied in comparison with their properties as foaming agents. Stabilization of emulsion drops (of olive oil in water) by SC3 hydrophobin was first reported by Wösten et al. [31] and it was suggested that oil vesicles covered with hydrophobin membrane could find applications in drug delivery [32,33]. Lumsdon et al. [34] presented data for the stabilization of polyunsaturated fatty acid oil-in-water emulsions by HFBII. Ascolin et al. [35] compared different hydrophobins as emulsifiers and reported that oil-in-water emulsions prepared with HFBI and SC3 were more stable than those with HFBII. Reger et al. [36] investigated emulsions stabilized by two types biotechnically produced water-soluble recombinant hydrophobins and studied the rheology of these emulsions. Further, these authors demonstrated that the combined action of hydrophobins and clay particles produce synergistic effect on emulsification and emulsion stability [37–40]. Cox et al. [41,42] established that hydrophobin HFBII can be used as stabilizer of aerated emulsions for the food industry. Khalesi et al. [43] demonstrated that HFBII membranes can be used for encapsulation and retention of the volatile oil ocimene in the water phase. It was established that the HFBII molecule is stable at the oil/water interface, where it undergoes minimal conformational changes [44]. The surface shear rheology of hydrophobin adsorption layers at oil/water interfaces was also investigated [45].

It should be noted that none of the above studies presents systematic data for the effect of protein concentration and oil volume fraction on the oil-drop distribution and longevity of hydrophobin stabilized emulsion. The present article is the first systematic study of the properties of HFBII as emulsifier. First, we investigate whether the hydrophobin adsorption layers solidify on the oil/water interfaces, as this is observed at the air/water interface. The threshold interfacial tension at solidification is determined (Section 4). Next, in experiments with o/w/o emulsion films we study the interaction of two HFBII adsorption layers across the aqueous phase. One of our goals is to verify whether S-bilayers can be formed also with emulsion films (Section 5). Further, by optical observations we investigate the shape and size distribution of the drops in HFBII stabilized emulsions; calculate the mean drop radii, R_{10} and R_{32}, and study their dependence on the protein concentration, oil volume fraction and emulsion storage time, up to 50 days (Section 6). It turns out, that the emulsions with HFBII are very stable at rest, but they can be easily destabilized upon stirring. This is due to the fact that the solidified structure of adherent oil drops covered by hydrophobin adsorption layers is destroyed by the shear stresses. It is demonstrated that if the emulsion drops are wrapped with a second layer of conventional protein (like β-lactoglobulin), the emulsions become stable upon stirring and centrifugation (Section 7). Finally, we investigated whether the dense hydrophobin adsorption layers can block the Ostwald ripening, which is one of the main destabilizing factors in emulsions where the disperse phase is oil that exhibits pronounced water solubility [46]. Two such oils, limonene and xylene, have been investigated and the stabilizing performance of HFBII was compared with that of other emulsifiers (Section 8).

2. Materials and methods

2.1. Materials

The proteins used in our experiments were as follows:

(1) Hydrophobin HFBII; 70 amino acids; molecular weight M_w = 7.2 kDa; 4 disulfide bonds. The used HFBII sample, provided as a gift by Unilever R&D, was produced via fermentation using *Trichoderma reesei* [20].
(2) β-lactoglobulin (BLG) from bovine milk; 162 amino acids; M_w = 18.3 kDa; 2 disulfide bonds. The used sample was product of Sigma (≥90%, Cat. No. L0130).
(3) The skim milk powder (SMP), received from Unilever, contains 35 wt% protein, mostly caseins and whey proteins; the rest is lactose and some minerals.