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Interfacial rheology and stability of air bubbles stabilized by mixtures of hydrophobin and β-casein

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ABSTRACT

Class II hydrophobin (*HFBII*) is a highly surface active molecule and in the context of aeration can be considered to be an air structuring protein conferring exceptional stability to foams for periods far in excess of that obtained with any other commonly used protein. This is of interest to the food industry, since producing shelf stable foams in food formulations is very difficult. Although *HFBII* has proven to be very promising in terms of foam stability when used alone, it is still unknown whether *HFBII* will be able to maintain its functionality when other surface active agents are present, such as in real food systems. The surface rheology: surface shear viscosity (η_S) and surface dilatational elasticity (ε), for *HFBII* and β-casein mixes at various ratios is described in this paper and how this relates to bubble stability. The addition of β-casein up to a certain ratio seems to increase η_S significantly, whilst ε is less affected. This is accompanied by improved stability of air bubbles to coalescence and allows the formation of very small air bubbles that remain extremely stable to disproportionation. Overall, there is the suggestion of some kind of synergy between the two proteins. The exact nature of this interaction is unknown. Measurements of the ζ-potential of the proteins suggest that electrostatic interactions are probably not important at the pH investigated (pH 7). Confocal microscopy of individual bubbles over prolonged periods of time, stabilized by *HFBII* and a fluorescently labeled β-casein, suggests that the enhanced stability is due to highly unusual and complicated interfacial packing phenomena plus local bubble curvature effects that require further investigation.

1. Introduction

Over the past ten years, hydrophobins have attracted a lot of attention especially in the context of aeration as they are considered to be an air-structuring protein, conferring exceptional stability to foams. Hydrophobins are small surface active proteins produced by filamentous fungi, found to cover the hyphal cell walls of fungal aerial structures (Hakanoaa, 2006; Wosten, 2001). Based on their solubility properties, two types of hydrophobins may be distinguished. Class I hydrophobins are highly insoluble and form amyloid-like rodlet membranes that can only be dissolved in certain strong acids. Class II hydrophobins are more water-soluble and can be readily dissolved in SDS or ethanol (Linder, Szilvay, Nakari-Setälä, Soderlund, & Penttilä, 2002; Scholtmeijer, Rink, Hektor, Wösten, & Dilip, 2005). In this study a class II hydrophobin (*HFBII*) was used. This same *HFBII* has recently been used by Cox, Cagnol, Russell, and Izzard (2007), Cox, Aldred, and Russel (2009) to produce liquid foams that do not coarsen and that are stable to coalescence for periods of several months. This period of stability is far in excess of that obtained with any other commonly used proteins. This is of interest to the food industry, since producing shelf stable foams in food formulations is very difficult.

The *HFBII* used in this study was produced by *Trichoderma reesei* and in common with other Class II hydrophobins it is a small globular protein (molecular weight ≈7 kDa) possessing eight conserved cysteine residues that form four intermolecular disulfide bonds. These bonds give *HFBII* an extremely rigid and stable structure (Hakanpaa, Linder, Popov, Schmidt, & Rouvinen, 2006). A hydrophobic patch is also located on the protein surface, estimated to be 12–19% of the total surface area of the protein (Hakanpaa et al., 2004; Kallio, Linder, & Rouvinen, 2007; Linder et al., 2002) and this motif is key in conferring high surface activity to the molecule. Indeed, to date hydrophobins are considered to be the most surface-active proteins known. They are able to spontaneously self-assemble into a robust amphiphilic membrane at a hydrophilic–hydrophobic interface and can change the nature of

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the surface from hydrophobic to hydrophilic or vice versa. The driving force for this self-assembly would seem to be the concealment of the hydrophobic patch. Based on the multimerization model proposed by Hakanpaa, Szilvay, et al. (2006), *HFBII* is found in a dimeric state, concealing 34% of the hydrophobic patch compared to monomers, which is considered to be energetically more favorable. *HFBII* monomers are able to form monolayers at an oil–water (O–W) or air–water (A–W) interface by exposing their hydrophobic patch to the hydrophobic phase, creating an even more stable energetic state, as the entire patch can be concealed (Burtko et al., 2001; Linder et al., 2002; Lumsdon, Green, Stieglits, 2005; Szilvay, Nakari-Setälä & Linder, 2006). Furthermore, unlike other surface active globular proteins, *HFBII* does not seem to unfold significantly once it is adsorbed. The strong adsorption and the close and ordered packing of the molecules result in mechanically strong films that are excellent at resisting bubble shrinkage via disproportionation (Cox et al., 2009; Murray, Dickinson, Lau, Nelson, & Schmidt, 2005). Consequently, hydrophobin seems unique amongst proteins in behaving more like a rigid Janus-type particle (Cox et al., 2007). Linder et al. (2002) and Linder (2009) have provided several good reviews on hydrophobins and their functionality.

Foams and bubbles play a very important role in many food products in terms of their structure and texture. However, foams are renowned for being unstable, even in frozen solidified products such as ice cream. Environmental changes, such as changes in pressure and/or temperature can lead to changes in the foam microstructure that affect both the physical and sensory properties of the product (Heuer, Cox, Singleton, Scott, Barigou & van Ginkel, 2007). The ability to produce liquid stable foams without relying on a gelled or solidified continuous phase is very much desired by food manufacturers since this would allow the development of new aerated products with better functionality, new textures and lower calorie content. One of the ways to create extremely stable bubbles is to adsorb a layer of surface active solids at the surface of the gas bubbles (Dickinson, Ettelaie, Kostakis & Murray, 2004; Du et al., 2003; Horozov, 2008; Hunter, Pugh, Franks, & Jameson, 2008; Murray & Ettelaie, 2004). In practice, finding surface-active particles in the correct size range that are acceptable for consumption is very difficult. Hydrophobins may be able to fulfill these criteria (Murray, Dickinson, & Wang, 2008).

Although *HFBII* may be more surface active than other proteins when compared at equal bulk concentrations, real food products may contain a wide range of concentrations of different proteins and other surface active agents. At present, it is unknown whether *HFBII* will be able to maintain its functionality in such systems or if synergistic effects will occur with other proteins. It is therefore of great practical importance to test this point and in this work β-casein (*BC*) was chosen as a second surface active protein for the study of competitive effects with *HFBII*. This is a significant choice, since β-casein is the most surface active milk protein and milk protein powders are widely exploited for their surface active properties in foods and non-food products. The effects of pH and different concentration ratios of *HFBII* to β-casein on the stability of individual bubbles were studied in detail and compared with the corresponding changes in interfacial film rheology.

2. Materials and methods

2.1. Materials and sample preparation

Class II Hydrophobin (*HFBII*) was supplied by Unilever (Colworth, UK), provided in an ammonium acetate buffer solution. It was then freeze-dried and stored in a vacuum oven (Gallenkamp) at 40 °C for 18 h, in order to remove the water and buffer, to enable solutions to be prepared at different pH values. The *HFBII* was then reconstituted in pure water at a concentration of 1.44 wt.% and stored frozen. Before conducting any measurements on *HFBII* samples, 1 min of sonication (via a Kerry sonicator, Kerry Ultrasonics, Hitchin, Herts, UK) at 45 kHz was applied separately to the original 1.44 wt.% solution and also to the diluted samples. This step is necessary to remove any small bubbles and to dissociate any protein aggregates.

β-casein (*BC*) from bovine milk (≥98%, *PAGE*), potassium dihydrogen phosphate ($K_2H_2PO_4$) disodium phosphate (Na_2HPO_4), sodium chloride (NaCl, 7647-14-5), sodium azide (NaN_3), D-gluconic acid δ-lactone (*GDL*) and xanthan gum were supplied by Sigma–Aldrich (Poole, UK). Fluorescently labeled β-casein was obtained by conjugating FITC fluorescent probe to the protein and was provided by Ecole Ltd. (Qingdao, China), with a ratio of 5.5 fluorophores per *BC* molecule. Separate measurements (not shown) of the surface tension of 10^{-2} wt.% FITC-labeled *BC* versus time were not significantly different from the corresponding measurements on non-labeled *BC*, so that the labeling of the *BC* itself was not thought to significantly affect its surface active properties. All solutions used in this study were prepared in phosphate buffered saline using 0.02 mol dm^{-3} KH_2PO_4 + Na_2HPO_4 + 0.05 mol dm^{-3} NaCl buffer with the pH adjusted to pH 7 ± 0.05 via addition of drops of 1 mol dm^{-3} NaOH as necessary. Water from a Milli-Q apparatus (Millipore, Watford, UK), free from surface-active impurities and with a conductivity of less than 10^{-7} S cm^{-1}, was used throughout. To all solutions sodium azide was added (0.02 wt.%) to inhibit microbial growth during measurements. Mixed solutions of β-casein + *HFBII* were prepared with 10^{-4} wt.% *HFBII* and concentrations of β-casein ranging from 10^{-3} wt.% to 10^{-1} wt.%. Each solution was stored overnight at 4 °C before use and each experiment was repeated at least three times.

To investigate the effect of pH on the surface activity of *HFBII*, GDL was used to acidify the solutions. The GDL granules were slowly added to the protein solution and were vigorously stirred for 5 min to allow thorough dissolution and even distribution of the GDL. The sample was then split into two parts: one was used for experimental purposes whilst the pH of the other portion was simultaneously monitored using a pH meter (Jenwau 3310, Essex) in the same laboratory at the same temperature (22 ± 3 °C). At this concentration and temperature, GDL slowly hydrolyzes and lowers the pH uniformly throughout the sample. After 24 h the desired pH was obtained and remained stable. This method was preferable to attempting to dissolve the proteins in buffers of different pH at the start, which led to problems of reproducibility, presumably due to difficult and slow solubilization of the proteins at some pH values.

2.2. Surface shear rheology

The surface shear viscosity was measured using a two-dimensional Couette-type viscometer which has been described in detail previously (Murray, 2002; Borbás, Murray, & Kiss, 2003; Martin, Bos, Stuart, & Van Vliet, 2002) and only a brief explanation will be given here. A biconical disk, hanging from a thin wire of known torsion constant, is positioned with its edge touching the A–W interface of the solution contained in a concentric circular dish. The deflection of the disk is measured by reflection of a 1 mW laser off a mirror on the spindle of the disk onto a scale at a fixed distance from the axis of the spindle. In most of the experiments described here the rheometer was operated in a constant shear-rate mode, although oscillatory measurements of low amplitude and frequency can also be performed (Borbás, Murray, & Kiss, 2003; Jourdain, Christophe Schmitt, Leser, Murray, & Dickinson, 2009). The motion of the beam on the scale was recorded digitally via a CCD camera for subsequent analysis of the disk deflection versus