SURFACE ACTIVITY OF SURFACTIN RECOVERED AND PURIFIED FROM FERMENTATION BROTH USING A TWO-STEP ULTRAFILTRATION (UF) PROCESS

MOHD HAFEZ MOHD ISA, RICHARD A. FRAZIER & PAULA JAUREGI

Department of Food Biosciences University of Reading Whiteknights PO Box 226 Reading RG6 6AP, UK.

Paula Jauregi Department of Food Biosciences University of Reading Whiteknights PO Box 226 Reading RG6 6AP, UK.

ABSTRACT

B. subtilis under certain types of media and fermentation conditions can produce surfactin, a biosurfactant which belongs to the lipopeptide class. Surfactin has exceptional surfactant activity, and exhibits some interesting biological characteristics such as antibacterial activity, antitumoral activity against ascites carcinoma cells, and a hypocholesterolemic activity that inhibits cAMP phosphodiesterase, as well as having anti-HIV properties. A cost effective recovery and purification of surfactin from fermentation broth using a two-step ultrafiltration (UF) process has been developed in order to reduce the cost of surfactin production (Isa et al. 2007; Isa et al. 2008). In this study, competitive adsorption of surfactin and proteins at the air-water interface was studied using surface pressure measurements. Small volumes of bovine serum albumin (BSA) and β-casein solutions were added to the air-water interface on a Langmuir trough and allowed to stabilise before the addition of surfactin to the subphase. Contrasting interfacial behaviour of proteins was observed with β-casein showing faster initial adsorption compared to BSA. On introduction of surfactin both proteins were displaced but a longer time were taken to displace β-casein. Overall the results showed surfactin were highly surface-active by forming a β-sheet structure at the air-water interface after reaching its critical micelle concentration (CMC) and were effective in removing both protein films, which can be explained following the orogenic mechanism. Results showed as well that the two-step UF process is effective to achieve high purity and fully functional surfactin. It is of great importance that the method developed to

recover and purify surfactin or biosurfactants in general is able to produce fully functional end product.

***Keywords**: Bovine serum albumin (BSA); Beta casein (β-casein); surfactin; Critical micelle concentration (CMC).*

INTRODUCTION

Biosurfactants have attracted significant interest in the last few years as compared to chemically synthesized surfactants as better biodegradability and lower toxicity could be expected from microbially produced surface-active substances due to their biogenetic origin (Kosaric 1993). Lipopeptides, one important class of biosurfactants, are produced by microorganisms and are important in scientific and industrial fields such as pharmacy, cosmetics and food additives. About 23 kinds of lipopeptides have been reported over the last two decades, of which 21 were found to be cyclic lipopeptides such as surfactin, lichenysin, iturin and fengysin (Song et al. 2007). One of the most surface-active biosurfactant ever known, surfactin is a cyclic lipopeptide produced by various strains of *Bacillus subtilis*. Surfactin consists of a heptapeptide headgroup with the sequence Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu closed to a lactone ring by a C_{13-15} β-hydroxy fatty acid (Heerklotz & Seelig 2001). It was reported by Ishigami et al. (1995) that the excellent surface-active behavior of surfactin was attributed to the ease of pilling of molecules organized by β-sheet formation after reaching its critical micelle concentration (CMC), and thus can be used as a basis for elucidating its physiological functions.

One of the most important applications of surfactants and proteins at the air-water interface is as foam stabilizers. However, their competitive adsorption at this interface is also of interest to evaluate cleaning potential and the mechanism involved in surfactant displacement and solubilisation of proteins at interfaces. The structures of proteins and surfactants differ greatly and as a result of this so does their behaviour at interfaces. The two species work on a different mechanism to stabilize interfaces, which are incompatible (Gunning et al. 2004). Proteins form a viscoelastic network, sometimes referred to as a two-dimensional gel. The intermolecular interactions of protein molecules provide the strength to maintain its coherence (Gunning et al. 2004). Surfactants, in general form weaker interfacial interactions than proteins, and stabilize droplets and bubbles in the short range through hydrodynamic phenomena known as the Gibbs-Marangoni mechanism (Mackie & Wilde 2005).

In this study, competitive adsorption of surfactin and proteins at the air-water interface was investigated using two different types of protein. Bovine serum albumin (BSA) is a globular protein and its physiochemical properties are well characterized. It is relatively large globular protein (66.3 kDa) and consists of 607 amino acids with 17 disulfide bonds and one free cysteine group (Kelley & McClements 2003). It has relatively high water solubility because it contains a large number of ionisable amino acids and it is also bind to many types of amphiphilic biological molecules (Kelley & McClements 2003). β-Casein forms about 30% of the casein proteins and is one of the most soluble and most surface active. The amphiphatic nature of β-casein with two distinct hydrophilic (N-terminal domain) and hydrophobic (C-terminal domain) parts allows it to form surfactant-like aggregates or submicelles (Duphas et al. 2005). β-Casein resembles a random coil chain of 209 residues and has a molecular weight of about 24 kDa (Cicuta 2007), with some secondary structure (approximately 9% α-helix, 15% β-sheet) (Mackie & Wilde 2005).

Given the wide range of possible applications of biosurfactants, the aim of this study is to investigate the displacement of protein films by surfactin at the air-water interface, using BSA and β-casein which have different interfacial properties. Comparison of surface pressure of BSA, β-casein, surfactin and the competitive adsorption of surfactin-proteins will add further knowledge on the interfacial properties of surfactin and proteins at the air-water interface.

MATERIALS AND METHODS

BSA and β-casein (96% purity) were obtained from Sigma (Dorset, U.K.) and no further purification was done before use. Surfactin was obtained after recovery and purification from fermentation broth by a two step ultrafiltration (UF) process described in detail elsewhere (Isa et al. 2007; Isa et al. 2008). It was freeze dried prior to use. Solutions of BSA, β-casein and surfactin were made using a phosphate buffer solution of pH 8.5 (ionic strength 0.02 M) using Mili-Q water. All materials and solutions were stored at 4 ^{o}C and taken out of the fridge at least 1 hour prior to use to reach room temperature.

Surface pressure

Surface pressure measurements were carried out by the Wilhelmy plate method using a small PTFE trough (94 x 22 x 5 mm) with a moveable barrier. The micro balance was connected to a computer and data was recorded using the supplied software, Nima516.exe (Nima Technology Ltd., Coventry, England). The Wilhelmy plate method consists of a strip of chromatographic paper in contact with the aqueous subphase and linked to a micro balance (surface pressure

sensor). Protein film was formed by adding 0.1 ml of protein solutions (1 mg/ml) from a pipette directly onto the buffer surface on the trough. The surfactin solution (0.4 ml of 1 mg/ml) was injected using a syringe *via* a small semi-circular opening between the trough and the base of the barrier to allow for addition into the subphase. These amounts gave an approximate 50:1 molar ratio of surfactin to BSA and 18:1 of β-casein to surfactin. The change in surface pressure was then monitored until a plateau was reached.

RESULTS AND DISCUSSION

Adsorption of surfactin at the air-water interface

Figure 1 shows fast and immediate adsorption of surfactin samples after introduction to the subphase of the trough. Final concentration of surfactin was 46 μM and at this concentration surfactin was in the form of micelles (Ishigami et al. 1995). Immediate and fast adsorption of surfactin at air-water interface after introduction from the subphase and the increase in surface pressure continued with a lag period of decreasing surface pressure after the initial adsorption, which lasted for 15 ± 6 minutes before increasing again gradually and reaching final plateau at 39.0 ± 0.47 mN/m after 27 ± 7 minutes. The maximum value of surface pressure of surfactin was in the range of 39.0 - 41.5 mN/m in this study is in agreement with Maget-Dana and Ptak (1992) and Ishigami et al. (1995).

Overall surfactin samples showed fast adsorption at the air-water interface. The lag period phase before reaching the final plateau were probably due to some impurities of residual protein from the recovery and purification process described in Isa et al. (2007) and Isa et al. (2008). Fast adsorption of surfactin at the air-water interface in Figure 1 shows it is highly surface active and this characteristic may be attributable to the ease of pilling of surfactin molecules into a β-sheet formation after reaching CMC (Ishigami et al. 1995).

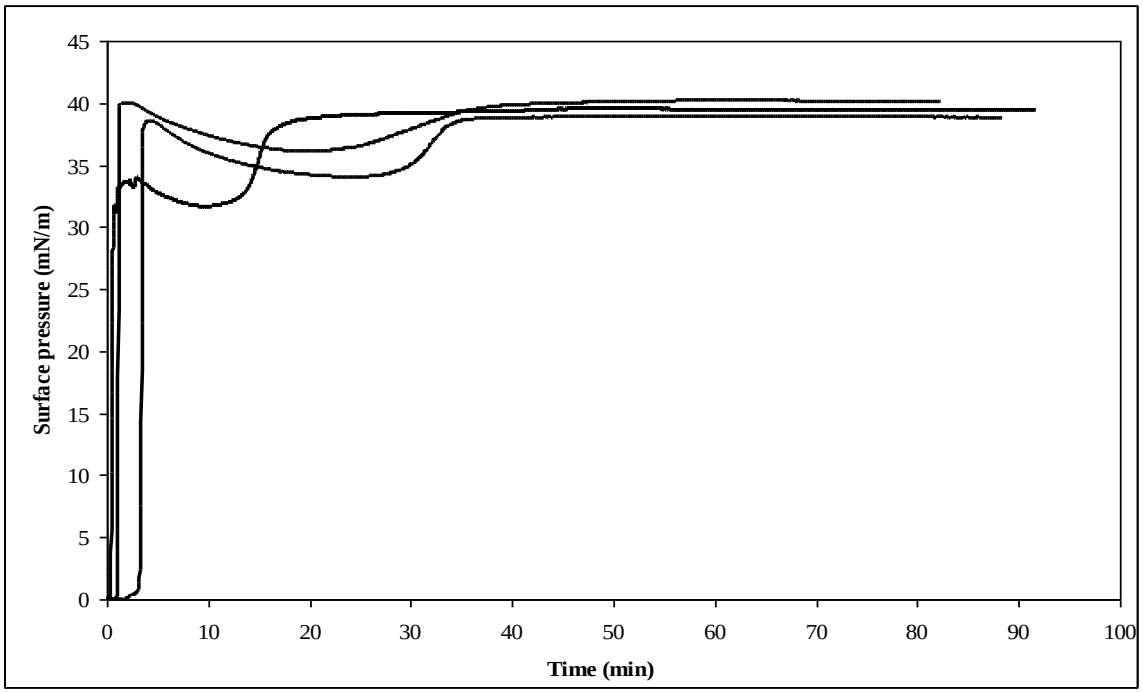

Figure 1 Adsorption of surfactin over time at air-water interface

Adsorption of BSA and β-casein at air-water interface

At the air-water interface, protein molecules try to expose to their hydrophobic parts to the air phase, which leads to an unfolding of the molecules and this process can proceed as long and as far as there is time and space at the interface available (Miller et al. 2000). Figure 2 and Figure 3 show adsorption of two different types of protein at the air-water interface. In Figure 2, surface pressure increased sharply after addition of BSA solutions, and then increased slowly before reaching a final plateau at 14.8 ± 0.8 mN/m after 142 ± 7 minutes. In Figure 3, surface pressure increased sharply after addition of β-casein solutions, and then decreased slowly before increasing again and reaching final plateau at 19.0 ± 0.1 mN/m after 89 ± 3 minutes.

Comparing the adsorption of the two proteins at the air-water interface, β-casein adsorbs much faster with higher surface pressure than BSA. According to Duphas et al. (2005), β-casein is a highly amphiphilic protein and is well known for its emulsifying properties. Furthermore, β-casein is more hydrophobic compared to BSA, which makes it more surface active. According to Mackie and Wilde (2005), there is a good correlation between surface hydrophobicity of a protein molecule and its rate and extent of adsorption to fluid interfaces. After adsorption at the air-water interface, protein may rearrange and unfold. A compact globular protein such as BSA will be partially unfolded at the interface, as this process will be restricted by the highly stabilized

secondary and tertiary structure linked by several disulfide bonds. As for the case of β-casein, considered as a very flexible protein will be unfolded in solution and its neighboring charged side groups will form a network at the air-water interface (Mackie & Wilde 2005; Beaufils et al. 2007).

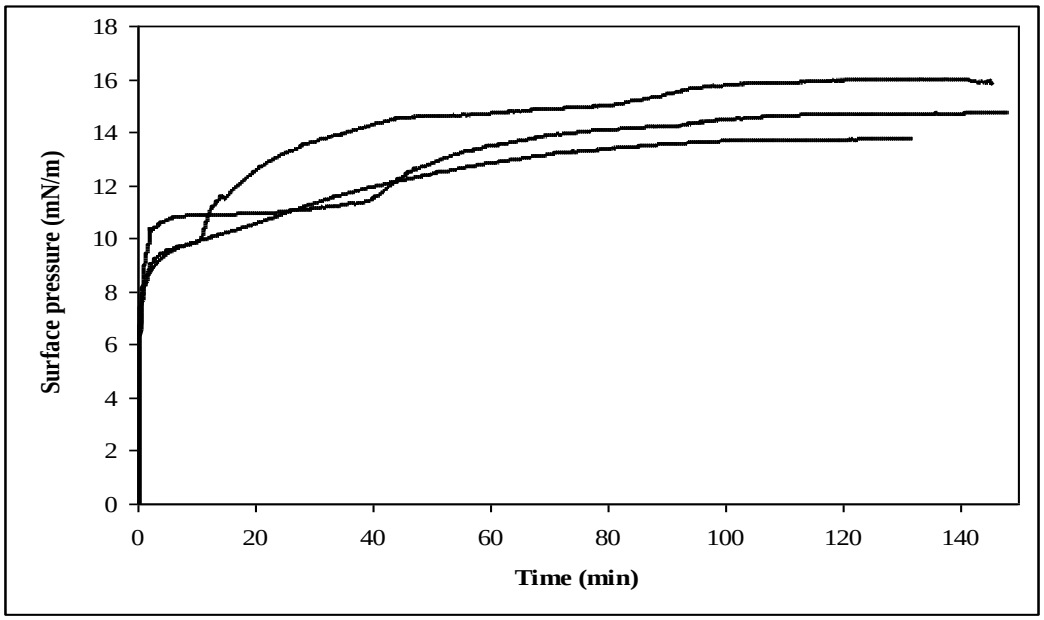

Figure 2 Adsorption of BSA over time at air-water interface.

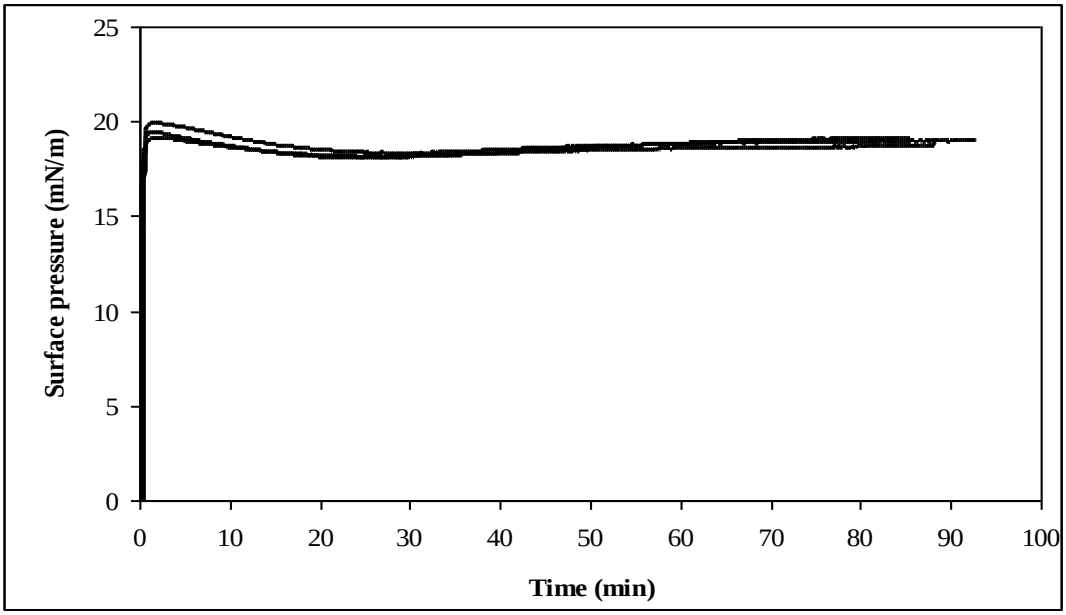

Figure 3 Adsorption of β-casein over time at air-water interface

Competitive adsorption of surfactin and proteins at air-water interface

After the formation of BSA and β-casein films at the air-water interface, surfactin solutions were injected from the subphase of the Langmuir trough. Figure 4 and Figure 5 show the change in surface pressure over time after the formation of a BSA and β-casein films and subsequent addition of surfactin solutions from the subphase. Figure 4 and Figure 5 shows fast and immediate increase of surface pressure after addition surfactin solutions, and reaching final plateau at 40.0 ± 0.5 mN/m and 39.0 ± 0.5 mN/m, respectively. Although both Figure 4 and Figure 5 show surfactin reached approximately similar final surface pressure value, slight differences were observed in the surface pressure increase which involved a lag period of 8 ± 2 minutes and 20 ± 6 minutes, respectively before reaching the final value.

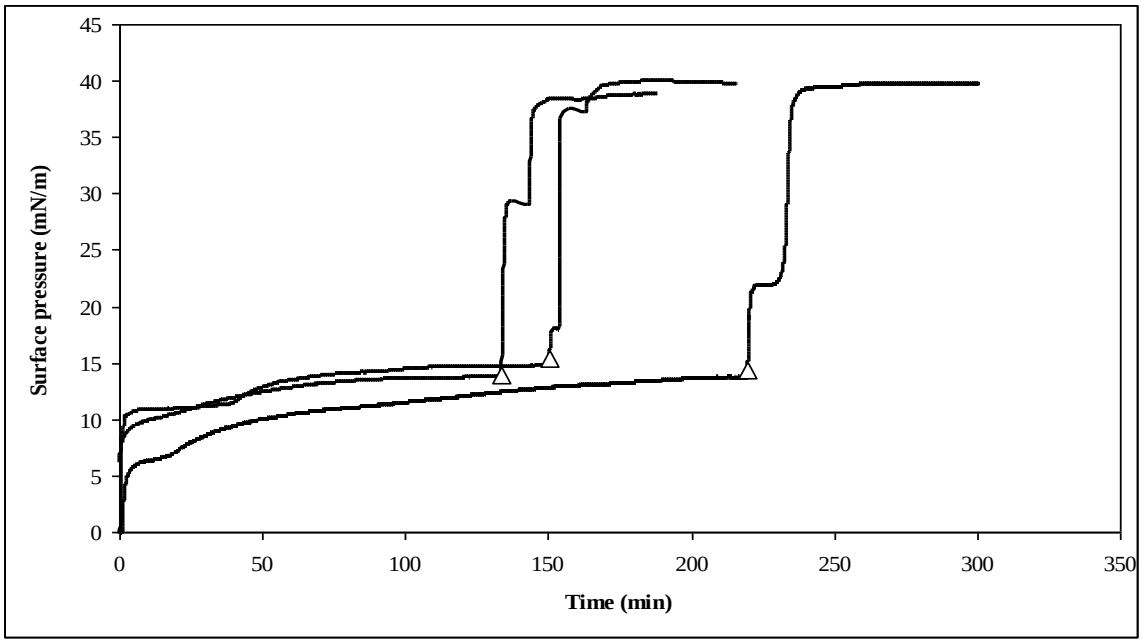

Figure 4 Change in surface pressure over time after addition of BSA and subsequent addition of surfactin solution; open filled triangle indicate when surfactin solution was injected.

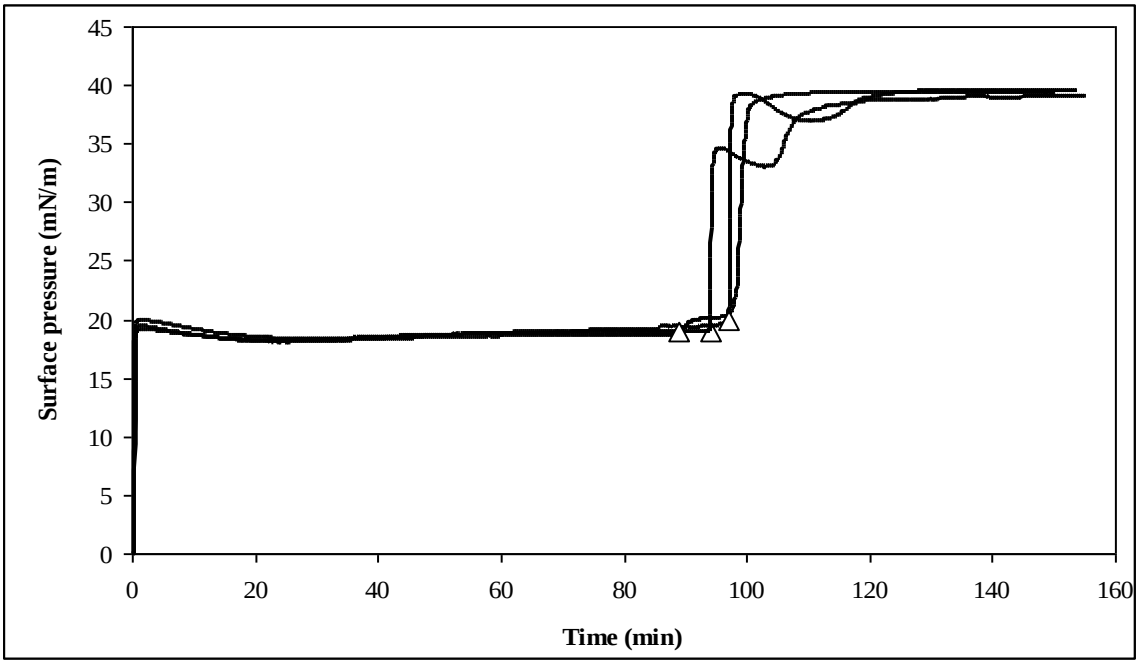

Figure 5 Change in surface pressure over time after addition of β-casein and subsequent addition of surfactin solution; open filled triangle indicate when surfactin solution was injected.

The change in surface pressure increase over time for both BSA and β-casein films after subsequent addition of surfactin solutions from the subphase show high surface activity of surfactin and the ability to displace protein films at air-water interface. Although surfactin was effective in displacing protein films at air-water interface, a lag period were observed before reaching the final plateau in both Figure 4 and Figure 5. These lag period were likely to be as a result of some degree of competitive adsorption by some impurities of residual protein. The residual protein impurities might be surface active and provide slight competition to adsorb at air-water interface.

According to Gunning et al. (2004) and Mackie et al. (1999), the generic mechanism for protein displacement by surfactant at air-water interface follows an orogenic mechanism, which does not occur simply by exchange of individual protein molecules by the more surface active surfactant. Surfactant appears to coadsorb into defects in the protein network, creating surfactant domains acting as nuclei which will then grow and compress the protein network until it eventually fails (Mackie et al. 1999). Failure of the protein network is determined by the mechanical character of the protein network, and that any surfactant-protein binding does not alter the strength of the protein film (Gunning et al. 2004). Despite improved understanding of

protein displacement by surfactant, there is no similar report of protein displacement by biosurfactants in general or surfactin specifically.

CONCLUSIONS

This study of surfactin-protein interactions at air-water interface shows effective displacement of protein films by the more surface-active surfactin. The mechanism of displacement can be explained following the proposed model of orogenic mechanism, although no or very little work has been done on biosurfactants and particularly surfactin to displace proteins films at the air-water interface. Effective displacement of proteins films by surfactin is likely to be due to its very low CMC, attributed by the ease of pilling of surfactin molecules in β-sheet configuration which is a rigid and compact structure. This study shows surfactin can be an innovative alternative to other chemical surfactants for certain types of industrial applications (e.g. emulsifier, foam stabilizer, cleaning agent and food formulation). In addition, it shows surfactin used in this study have had high purity and comparably functional to the standard surfactin, thus confirming the efficiency of the two-step UF process described in Isa et al. (2007) and Isa et al. (2008) to produce high purity of surfactin from fermentation broth.

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