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A comparison of the surface activity of the fungal hydrophobin SC3p with those of other proteins

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Abstract

The fungal hydrophobin SC3p, a protein secreted by *Schizophyllum commune*, has become known to form SDS-insoluble layers and to change the physico-chemical properties of an interface. In this study, the surface activity of SC3p was studied by determining the interfacial tensions γ_{lv} and γ_{sl} during adsorption of SC3p at both the liquid–vapour and the solid–liquid interface utilizing the in situ technique axisymmetric drop-shape analysis by profile. To this end, protein solution droplets were put on the solid fluoroethylene–propylene-Teflon. At the liquid–vapour interface, SC3p caused a large decrease of γ_{lv} from 72 to 43 mJ m^{-2} at the concentration of 0.1 mg ml^{-1}. At the solid–liquid interface, γ_{sl} was slightly decreased, whereas the contact angle θ increased, indicating an increase in hydrophobicity of FEP-Teflon, which is unique among the proteins studied so far. Earlier findings indicated a decrease in hydrophobicity of Teflon upon adsorption of SC3p, but this was after a washing and drying step. In order to reconcile these findings with those of the present study, adsorption of SC3p to hydrophobic surfaces is suggested to occur in bilayers. The second layer is supposed to be less strongly adsorbed than the first layer and can be easily removed by washing.

Keywords: Hydrophobin; Interfacial tension; Protein adsorption; *Schizophyllum commune*; Wettability

1. Introduction

Hydrophobins are small secreted fungal proteins rich in hydrophobic amino acids, which have been proposed to be involved in the escape of hyphae from their substrates into the air [1,2] and in the attachment of hyphae to hydrophobic surfaces as in fungus–host interactions [3–5]. Many natural surfaces are hydrophobic, including various plant and insect surfaces and the human skin. On the contrary, the fungal wall is essentially hydrophilic, so that hydrophobin production could be a virulence factor in fungi [3,4].

Wösten et al. [1,2] isolated the hydrophobin SC3p produced by *Schizophyllum commune* in culture medium and described some noteworthy interfacial properties of this protein. The hydrophobin assembled at the wall–air interface of aerial hyphae, therewith covering the surface completely by a highly

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hydrophobic (water contact angles up to 115 ± 10 degrees) rodlet layer. SC3p assemblies are highly insoluble, even in hot sodium dodecylsulphate (SDS), while the monomeric hydrophobin is readily soluble in the aqueous medium. The fact that cold trifluoroacetic acid (TFA) and formic acid are able to dissociate the hydrophobin complexes into monomers, indicates the absence of intermolecular S–S bridges and that assemblage of the monomers is due to strong non-covalent interactions.

Furthermore, SC3p spontaneously assembled from water onto hydrophobic solid surfaces, turning these surfaces hydrophilic after drying [5]. The amount of SC3p adsorbed to solid surfaces decreased with decreasing hydrophobicity of the surfaces. On hydrophobic surfaces more hydrophobin is induced to form SDS-insoluble assemblies than on the hydrophilic surfaces [4]. The surface active character of SC3p is also illustrated by the stabilization of oil emulsions by spontaneous assemblage on the surfaces of the oil droplets [5]. Finally, the amphipathic nature of the assembled protein was suggested by the observation that the hydrophobic rodlet-like side of the membrane showed a different elemental composition as compared to the smooth, hydrophilic side of the membrane [6].

Up till now, the surfactive character of SC3p has not been quantitatively described in terms of its capacity to decrease the interfacial tension of the air–water interface and that of the hydrophobic surface, resulting from its adsorption at these interfaces. Most techniques, such as contact angle measurements on dried adsorbed protein layers, include passages of adsorbed protein layers through liquid–air interfaces, rinsing and drying which probably alter the characteristics of the adsorbed layer by conformational changes and spatial rearrangement of the adsorbed protein molecules. Alternatively, axisymmetric drop-shape analysis by profile (ADSA-P, [7]) is a method by which the contact angle and the surface tension of a liquid droplet are measured simultaneously from the shape of an axisymmetric droplet without distortion of the droplet, so that, in situ, real time studies on interfacial tension changes during protein adsorption can be performed. Recently, interfacial tension changes of albumin, fibrinogen, immunoglobulin G, and human, bovine and porcine insulin solutions were studied by ADSA-P during adsorption from aqueous solution droplets onto both solid–liquid and liquid–vapour interfaces [8,9].

It is the aim of this paper to determine the interfacial tension changes during adsorption of SC3p in situ to a hydrophobic surface, γ_{sl}, and to the water–air interface, γ_{lv}, as a function of time. To this end, a droplet of SC3p solution was placed on a hydrophobic fluoroethylene–propylene-Teflon surface and its shape was observed during a period of up to 2 h, and analysed by ADSA-P. The resulting changes of the contact angle θ, and the interfacial tensions γ_{lv} and γ_{sl} are compared with similar data for the adsorption of a series of other proteins.

2. Materials and methods

2.1. Materials

SC3p is a small protein (14.5 kD) rich in small amino acids like Gly, Leu and Val, and a high content of hydrophobic amino acids [10]. Its hydrophobicity based on amino acid sequence amounts +0.68 according to the Kyte–Doolittle index [11]. The molecule contains 8 cysteine residues for disulfide bridging.

For the purpose of this study, SC3p was purified from the culture medium of *Schizophyllum commune* strain 4-40 (CBS 340.81) and checked for purity with SDS-PAGE as described by Wösten et al. [1]. The protein was dissolved and dissociated in TFA. The TFA was evaporated with nitrogen gas and the protein was dissolved in 10 mM potassium phosphate buffer (10 mM KPi) pH 7.0 at concentrations of 0.1, 0.05 and 0.01 mg ml^{-1}. Furthermore, bovine serum albumin (BSA, Sigma A-4503), human immunoglobulin G (IgG, Sigma I-4506), chicken egg white lysozyme (LSZ, Sigma L-6876), bovine pancreatic ribonuclease A (RNase, Sigma R-5125), bovine milk α-lactalbumin (αLA, a gift from the Netherlands Institute of Dairy Science, NIZO, Ede, The Netherlands), and calcium depleted bovine milk α-lactalbumin (αLA($-Ca^{2+}$), Sigma L-6010) were used as received to prepare single protein solutions in phosphate buffer (10 mM KPi) pH 7.0 in the same concentration range.

Commercial grade fluoroethylene–propylene-

Teflon (FEP-Teflon; Norton Fluorplast, Raamsdonksveer, The Netherlands) was cleaned ultrasonically in ultra pure ethanol (Merck, Germany) to yield water contact angles exceeding 106 degrees.

2.2. Axisymmetric drop-shape analysis by profile and interfacial tension calculations

Generally, droplets of liquids with a low surface tension are more apt to deviate from a perfectly spherical shape than droplets of liquids with a high surface tension. The shape of a curved interface like a liquid droplet on a solid is described by the classical Laplace equation of capillarity

$$\Delta P = \gamma_{lv}\left(\frac{1}{R_1} + \frac{1}{R_2}\right), \qquad (1)$$

where ΔP is the pressure difference across the interface, γ_{lv} is the liquid surface tension, and R_1 and R_2 are the principal radii of the curvature. ADSA-P was developed to calculate the surface tension γ_{lv} and the contact angle θ of an axisymmetric droplet from its shape [7]. An objective function, expressing the difference between a measured droplet profile and a theoretical Laplacian profile, is numerically minimized, while making use of local gravity, density, the three-phase line, the droplet coordinates, and x and y magnification factors as input parameters (see Fig. 1). The exact x and y magnification factors are calculated from profiles of perfectly spherical stainless steel balls with known diameter.

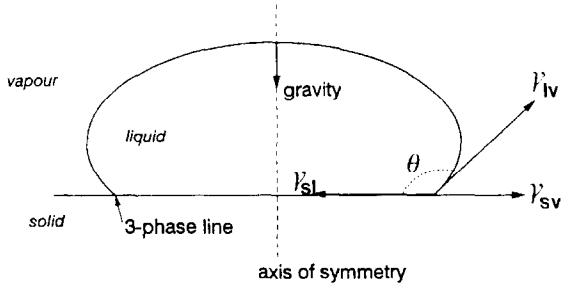

Fig. 1. The shape of an axisymmetric droplet is, amongst others, determined by its volume, the local gravity, the liquid density, and the three interfacial tensions present in the system, γ_{sv}, γ_{sl}, and γ_{lv}. Any changes occurring at the liquid–air or the solid–liquid interface due to e.g. protein adsorption affect the shape of the droplet and the contact angle θ.

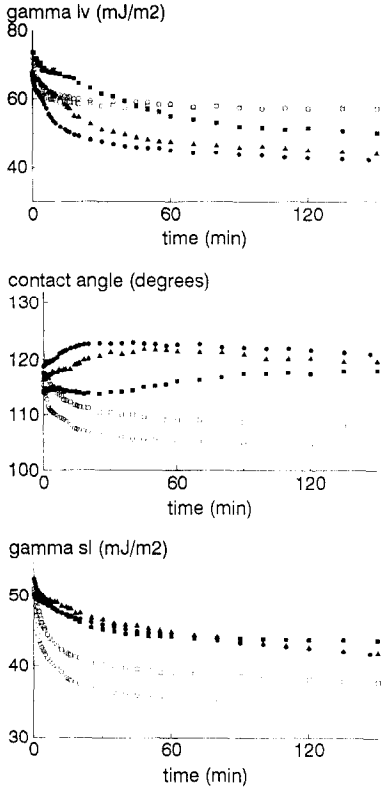

Fig. 2. Measured surface tension $\gamma_{lv}(t)$, contact angle $\theta(t)$, and calculated solid–liquid interfacial tension $\gamma_{sl}(t)$ occurring during adsorption of SC3p from phosphate buffer droplets on FEP-Teflon as determined by ADSA-P as a function of time. SC3p data are averages of duplicate runs which coincide within 4 mJ m^{-2}, 2 degrees, and 3 mJ m^{-2} for γ_{lv}, θ, and γ_{sl} respectively. Results for BSA and αLA are included for comparison. ●: 0.1 mg ml^{-1} SC3p, ■: 0.05 mg ml^{-1} SC3p, ▲: 0.01 mg ml^{-1} SC3p. ○: 0.1 mg ml^{-1} BSA. □: 0.1 mg ml^{-1} αLA.

In this study, ADSA-P was performed as described by Noordmans and Busscher [12]. 100 μl freshly prepared protein solution was put on a cleaned FEP-Teflon surface and the droplet profile was digitized with a contour monitor, especially designed for this purpose, and subsequently used to calculate its contact angle and liquid surface tension. Measurements were done in duplicate as a function of time up to at least 2 h in an enclosed chamber at room temperature. In order to prevent evaporation, a small water reservoir was placed in the chamber to create a saturated vapour.

Subsequently, the contact angle $\theta(t)$ and the liquid surface tension $\gamma_{lv}(t)$ were inserted in the Young

Table 1
Surface tension γ_{lv}, contact angle θ, and solid–liquid interfacial tension γ_{sl} after 2 h of adsorption of various proteins from solution droplets ($c = 0.1$ mg ml^{-1} in 10 mM KPi, pH 7) on FEP-Teflon as measured by ADSA-P [a]

Protein	γ_{lv} (mJ m^{-2})	θ (degrees)	γ_{sl} (mJ m^{-2})
SC3p	43	122	43
Bovine serum albumin	57	104	34
Immunoglobulin G	60	112	42
Lysozyme	54	110	38
Ribonuclease	68	113	46
α-lactalbumin	57	108	38
α-lactalbumin (Ca^{2+}-free)	54	107	36

[a] Data for the proteins other than SC3p are fully presented in Ref. [13].

equation to yield the interfacial tension $\gamma_{sl}(t)$ according to

$$\gamma_{sl}(t) = \gamma_{sv} - \gamma_{lv}(t) \cdot \cos\theta(t), \qquad (2)$$

assuming that the surface tension γ_{sv} of FEP-Teflon amounts to 20 mJ m^{-2} and does not change during the experiment [9].

3. Results

Fig. 2 shows the surface tensions γ_{lv}, the contact angles θ, and the solid–liquid interfacial tensions γ_{sl} as a function of time during adsorption of SC3p from droplets on FEP-Teflon as measured by ADSA-P. For comparison, data for BSA and αLA are given as examples of results found for the other proteins. As a rule, the interfacial tensions γ_{lv} and γ_{sl} are reduced upon adsorption of proteins at an interface. These decreases are both concentration and protein dependent. For SC3p, the maximal interfacial tension changes within 2 h are attained for the highest concentration, i.e., 0.1 mg ml^{-1}.

In contrast to the large decrease in γ_{lv} upon SC3p adsorption, γ_{lv} only decreases slightly for all other proteins studied, including BSA and αLA. Moreover, for all other proteins, this decrease is accompanied by a decrease in contact angle rather than an increase as for SC3p. Table 1 summarizes the observed changes in γ_{lv}, θ, and γ_{sl} after 2 h adsorption of the various proteins employed from solution droplets with 0.1 mg ml^{-1} protein.

4. Discussion

4.1. SC3p at the liquid–vapour interface

In this study, SC3p in a 0.1 mg ml^{-1} phosphate-buffered solution changes the liquid surface tension from 72 to 43 mJ m^{-2}. At this concentration, the

Table 2
Minimal surface tensions γ_{lv} of different types of aqueous surfactant solutions. Surfactant data are obtained from literature

Type of surfactant		γ_{lv} (mJ m^{-2})	Ref.
Fungal protein	SC3p	43 [a]	this study
Proteins	bovine serum albumin	49	[13]
	immunoglobulin G	42	[13]
	lysozyme	40	[13]
	ribonuclease	53	[13]
	α-lactalbumin	37	[13]
	α-lactalbumin ($-Ca^{2+}$)	44	[13]
Biosurfactants by	*Rhodococcus erythropolis*	37	[14]
	Pseudomonas aeruginosa	29	[14]
	Torulopsis bombicola	37	[14]
	Bacillus subtilis	27	[14]
Synthetic surfactants	sodiumdodecylsulphate	37	[14]
	detergent alkylate dodecylbenzene	47	[14]
	dihydroaminefluoride	35	[15]
	oleylaminefluoride	29	[15]

[a] In contrast to the other proteins (5 mg ml^{-1}) the concentration of SC3p was 0.1 mg ml^{-1}.

surface activity of the other proteins at the liquid–vapour interface is clearly less than that of SC3p (see Table 1). The common way, however, to compare surface activities of surfactants, is by their critical micelle concentration (cmc) and minimal surface tension γ_{lv} that can be attained. For proteins, generally no micelles are formed, cmc is badly defined, and the minimal surface tension depends on external factors like pH and ionic strength. Nevertheless, for each protein the interfacial tension changes show a maximum as a function of time and concentration; Table 2 summarizes these approximate minimal values of γ_{lv} for all proteins employed. Table 2 is supplemented with data for synthetic surfactants and some biosurfactants. Although SC3p shows some degree of surface activity at the liquid–vapour interface, most biosurfactants and synthetic surfactants show a larger decrease in interfacial tension γ_{lv} than SC3p [14–16].

It has to be noted, however, that the maximal SC3p concentration investigated is 0.1 mg ml^{-1}, whereas the protein data in Table 2 are mainly valid for concentrations of 5 mg ml^{-1} [13]. Furthermore, the data concern proteins dissolved in phosphate buffer. When SC3p was dissolved in pure water at a concentration of 0.1 mg ml^{-1}, the surface tension γ_{lv} of water decreased from 72 to 32 mJ m^{-2} while the contact angle increased from 112 to 129 degrees. Thus dissolved in water, SC3p is a very powerful surface active protein. It is unclear why SC3p is much more surface active in water than in phosphate buffer. Possibly, the ions present in the medium shield the polar parts at the outer surface of the protein, diminishing the driving force of the adsorption process.

4.2. SC3p at the solid–liquid interface

In this paper, also the solid–liquid interfacial tension of the hydrophobic substratum FEP-Teflon is determined during the adsorption of SC3p. The initial value $\gamma_{sl}(0)$ is hard to determine experimentally, because adsorption of SC3p starts immediately after placing the droplet on the surface, while, due to experimental procedures, the first measurement can only be taken after 2 to 5 seconds. However, γ_{sl} of a solid against water can be calculated from thermodynamic approaches involving measured contact angles and the surface tension value of water [17], and can be taken as a good approximation of $\gamma_{sl}(0)$.

Table 3
γ_{sl} of bare substrata having different wettabilities, calculated from water contact angles θ and a thermodynamic model [18] [a]

Substratum	θ (degrees)	γ_{sl} (mJ m^{-2})
Fluoroethylene–propylene	111	42
Polyethylene	95	32
Poly(methyl methacrylate)	80	23
Tissue culture polystyrene	68	15
Glass	<10	<0.02

[a] Thermodynamic models to calculate interfacial tensions from measured contact angles are not ubiquitously accepted and subject to scientific debate. Therefore, the data given for γ_{sl} should not be considered as absolute values but rather as indications for its variation with substratum hydrophobicity.

In order to obtain a proper appreciation of the contact angles and interfacial tensions determined, we have calculated γ_{sl} for substrata of different hydrophobicity against water (Table 3). It can be seen that γ_{sl} is maximal for water against a hydrophobic substratum and that γ_{sl} subsequently decreases with decreasing water contact angles on the substrata. Thus the data in Table 1 suggest that SC3p adsorbed to FEP-Teflon does not decrease but rather increases the FEP-Teflon hydrophobicity, opposite to all other proteins.

4.3. Analysis of the kinetics of the interfacial tension changes

Interfacial tension changes during protein adsorption are due both to adsorption of the proteins in their native state and to prolonged conformational changes of the adsorbed protein. As both parts of the process have their own characteristic time constants, Serrien et al. [19] have proposed to describe the kinetics of these interfacial tension changes as

$$\gamma(t) = \gamma(\infty) + \left(\alpha \exp - \sqrt{\frac{4t}{\pi\tau}} + \beta \right) \exp(-kt), \quad (3)$$

in which $\gamma(\infty)$ is the interfacial tension in the steady state, τ the diffusion relaxation time, and k the rate constant for the conformational changes at the inter-

Table 4
Components α and β of the interfacial tension decreases due to the diffusion-limited adsorption and conformational changes during adsorption of SC3p and other proteins at the liquid–vapour and the solid–liquid interface ($c = 0.1$ mg ml^{-1} in 10 mM KPi, pH 7)

Protein [a]	α (mJ m^{-2})	β (mJ m^{-2})
Liquid–vapour		
SC3p	0	21
bovine serum albumin	14	3
lysozyme	11	6
ribonuclease	0	2
α-lactalbumin	11	3
α-lactalbumin ($-Ca^{2+}$)	9	7
Solid–liquid		
SC3p	0	7
bovine serum albumin	17	4
lysozyme	7	5
ribonuclease	0	3
α-lactalbumin	14	3
α-lactalbumin ($-Ca^{2+}$)	14	7

[a] Data for the proteins other than SC3p are fully presented in Ref. [13].

face. α and β are interfacial tension components such that $\alpha + \beta = \gamma(0) - \gamma(\infty)$. The first term $\exp[-\sqrt{(4t/\pi\tau)}]$ accounts for the decrease of the interfacial tension due to diffusion-limited adsorption, and the second term $\exp(-kt)$ for the decrease due to conformational changes.

Table 4 presents the components α and β for SC3p and the other proteins at the liquid–vapour and the solid–liquid interface. As can be seen, the presence of the protein as indicated by α is predominantly responsible for the interfacial tension changes of most other proteins at the liquid–vapour and the solid–liquid interface, except for RNase; but only for SC3p the conformational part is much greater than the adsorption part. This indicates that SC3p is more apt to extensively change its conformation upon adsorption than the other proteins.

4.4. Hypothesis on the adsorption mechanism of SC3p

The interfacial tension γ_{lv} of SC3p solution droplets on FEP-Teflon greatly decreases from 72 to 43 mJ m^{-2} at 0.1 mg ml^{-1}. Yet, the contact angle increases, opposite to observations for all other proteins, indicating that the FEP-Teflon surface is becoming more hydrophobic. Note that the above holds for measurements done in situ, i.e., without extensive washing and drying of the adsorbed layer.

Previously, Wösten et al. [4,5] found that SC3p, adsorbed overnight to hydrophobic substrata and after extensive washing and drying, made these surfaces hydrophilic. Water contact angles on adsorbed, washed and dried SC3p layers on Teflon were reported to be 48 degrees [5]. After extraction with hot SDS and washing, the contact angle was still 62 degrees, indicative for a hydrophilic surface.

To reconcile these two seemingly contradicting findings, we propose hypothetically that adsorption of SC3p to hydrophobic surfaces occurs in bilayers (see also Fig. 3). The attractive forces originating from a hydrophobic FEP-Teflon surface are strong enough to cause adsorption and subsequent conformational changes of a first adsorbed layer. Due to these conformational changes, the SC3p molecules in this first adsorbed layer have become amphipathic. These attractive forces are strong enough to cause adsorption of a second layer of SC3p, but the attenuation of the attractive forces by the primary adsorbed layer inhibits extensive conformational changes in the second layer. This second layer is probed by the in situ technique ADSA-P and is evidently extremely hydrophobic. Although Fig. 3 depicts the SC3p monomers in the second layer in their native state, they probably have undergone a conformational change to expose a highly hydrophobic surface. This follows from the fact that native SC3p monomers are readily soluble in water and thus must be generally hydrophilic at their surfaces. Also, however, since the second layer is less strongly adsorbed than the first one, it is easily desorbed by removal through a liquid–air interface and washing, exposing the amphipathic, denaturated SC3p and an essentially hydrophilic surface is probed. Note (Fig. 3) that we do not envisage a second adsorbed layer at the liquid–vapour interface because the attractive forces from a low density medium as air are much smaller than those from a relatively high density medium as FEP-Teflon.

Recently, SC3p was also adsorbed to 50-mm long glass plates with a wettability gradient over its length [4]. The amount of adsorbed SC3p gradually in-

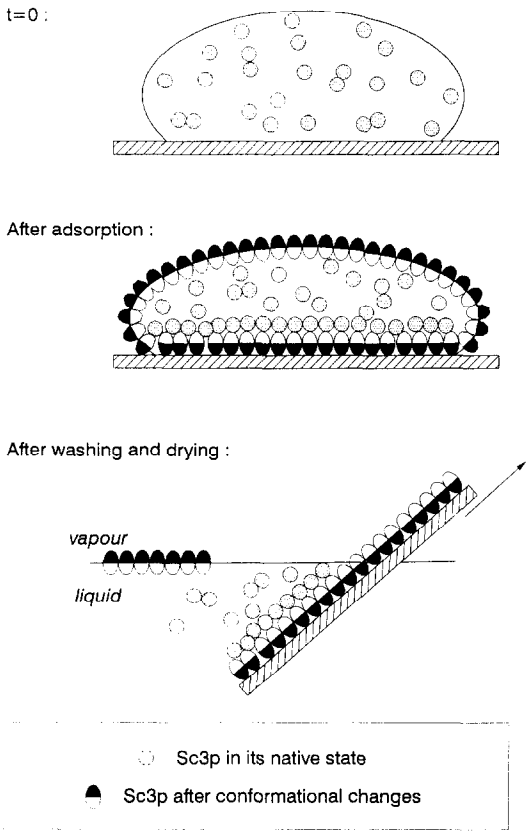

Fig. 3. Proposed model for SC3p adsorption at hydrophobic surfaces. At $t = 0$, dissolved proteins are all in their native state. After adsorption to the liquid–vapour and solid–liquid interfaces, the native protein undergoes a conformational change turning the molecule into an amphipathic molecule with its hydrophilic side (unfilled part) pointing to the aqueous phase and the hydrophobic side (filled part) to FEP-Teflon and air. Only at the solid–liquid interface, the attractive forces originating from a hydrophobic surface are sufficiently strong to induce adsorption of a second layer, but not strong enough to also induce extensive conformational changes of these adsorbed proteins. These secondary adsorbed layers however, are easily removed by washing, leaving an adsorbed, SDS-insoluble monolayer after drying.

creased from the hydrophilic to the hydrophobic end. All adsorbed SC3p could be removed by SDS washing in the region with water contact angles up to 60 degrees, but only a part of the adsorbed SC3p could be removed by SDS washing in the hydrophobic region. This supports our hypothesis that on hydrophobic substrata a bilayer of SC3p is adsorbed with the second layer being adsorbed less strongly that the first layer.

Incidentally it is noted that adsorption of surfactants in bilayers, as described above, has also been observed for ionic surfactants on hydrophilic surfaces. Such bilayers are usually referred to as 'admicelles' [20,21]. Also rodlike structures as found by Wösten et al. for SC3p [1] have been observed for synthetic surfactants [21–23], appearing to be supramolecular micelles.

Summarizing, this study first shows that in situ techniques are required to gain a better insight in protein adsorption phenomena. Washing, passages through liquid–air interfaces and drying may cause artefacts. For the interaction of SC3p with surfaces in the natural environment these artefactual manipulations may be part of the environmental processes occurring and SC3p adsorption under those conditions may be restricted to a monolayer. Under well-controlled in situ conditions, however, SC3p is likely to adsorb in bilayers and upon SC3p adsorption FEP-Teflon becomes even more hydrophobic than in its bare state.

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