Protein HGFI from the edible mushroom *Grifola frondosa* is a novel 8 kDa class I hydrophobin that forms rodlets in compressed monolayers

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Hydrophobins are a group of low-molecular-mass, cysteine-rich proteins that have unusual biophysical properties. They are highly surface-active and can self-assemble at hydrophobic–hydrophilic interfaces, forming surface layers that are able to reverse the hydrophobicity of surfaces. Here we describe a novel hydrophobin from the edible mushroom *Grifola frondosa*, which was named HGFI and belongs to class I. The hydrophobin gene was identified during sequencing of random clones from a cDNA library, and the corresponding protein was isolated as a hot SDS-insoluble aggregate from the cell wall. The purified HGFI was found to have 83 amino acids. The protein sequence deduced from the cDNA sequence had 107 amino acids, from which a 24 aa signal sequence had been cleaved off in the mature protein. This signal sequence was 5 aa longer than had been predicted on the basis of signal peptide analysis of the cDNA. Rodlet mosaic structures were imaged using atomic force microscopy (AFM) on mica surfaces after drying-down HGFI solutions. Using Langmuir films we were also able to take images of both the hydrophobic and hydrophilic sides of films formed at the air–water interface. No distinct structure was observed in films compressed once, but in films compressed several times rodlet structures could be seen. Most rodlets were aligned in the same direction, indicating that formation of rodlets may be promoted during compression of the monolayer.

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INTRODUCTION

Hydrophobins are a family of small, cysteine-rich secreted proteins that have unusual biophysical properties (Linder *et al.*, 2005). A notable feature is that they assemble on various interfaces (e.g. the interfaces between air and water, oil and water, and a hydrophobic solid and water) and change the properties of the surfaces to which they attach (Wessels, 1994, 1997; Wösten & Wessels, 1997). Hydrophobins have so far only been identified in

basidiomycetes and filamentous ascomycetes, although some evidence indicates that they occur in zygomycetes as well (De Vries *et al.*, 1993).

Hydrophobins play important roles in growth and morphology. For example, they can lower the surface tension of water to enable the growth of hyphae into the air and coat the surfaces of aerial hyphae to conceal the hydrophilic cell wall in the air environment (Talbot, 1997).

Hydrophobin genes are often very highly expressed in fungi (Asgeirsdóttir *et al.*, 1998; Carpenter *et al.*, 1992). They always have one clear distinguishing feature in their primary sequence, namely, eight Cys residues in a characteristic pattern (Schuren & Wessels, 1990). The second and third Cys residues are immediate neighbours, as also are the sixth and seventh. Apart from the conserved Cys residues and similar hydrophobicity patterns, hydrophobins can have very low sequence similarity at both the DNA and protein level. Therefore, it is hard to design universal primers to detect hydrophobin genes by PCR (Wösten, 2001).

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Abbreviations: AFM, atomic force microscopy; FT-ICR, Fourier-transform ion cyclotron resonance; LB, Langmuir–Blodgett; LS, Langmuir–Schaefer; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid.

The GenBank/EMBL/DDBJ accession number for the genomic *hgf1* sequence of *Grifola frondosa* is EF486307.

Hydrophobins can be divided into two groups, called class I and II, based on their hydropathy patterns (Wessels, 1994). The properties of the members of the different classes typically differ, so that aggregates of class I members are much more difficult to dissolve than those of class II members. Often, the strong acid trifluoroacetic acid (TFA) is used to dissolve aggregates of class I hydrophobins.

Rodlet structures are one of the most commonly described assembled structures of class I hydrophobins. When the assembled SC3 hydrophobin was examined by electron or atomic force microscopy, its surface appeared to be made of rodlets spaced about 10 nm apart in a typical mosaic pattern (de Vocht *et al.*, 1998). The formation of class I hydrophobin layers has been found to lead to a rodlet-like mosaic pattern that resembles amyloid fibrils, whereas the aggregates of class II hydrophobins are non-amyloid and needle-like (Hakanpää *et al.*, 2006). Langmuir–Blodgett (LB) films of class II hydrophobins HFB I and HFB II have been studied, and atomic force microscopy (AFM) images of them show highly ordered 2D crystalline structures (Paananen *et al.*, 2003; Szilvay *et al.*, 2007). There are, to our knowledge, no previous reports on Langmuir film structures for class I hydrophobins.

Grifola frondosa (maitake mushroom) is a white rot basidiomycete widely distributed in Asia, North America and Europe. In China, it is considered to be a choice edible mushroom with unique culinary and medicinal qualities (Shen *et al.*, 2002). There has been an increasing biotechnological interest in *G. frondosa* due to its ability to produce potent immuno-stimulatory and anti-tumour-active compounds, such as polysaccharides and proteo-polysaccharides (Nanba, 1993; Cui *et al.*, 2007).

In this work we cloned a hydrophobin gene from *G. frondosa*. A cDNA library was constructed for cloning the highly expressed hydrophobin genes. A class I hydrophobin gene, *hgf1*, was identified on the basis of its hydrophobin-like conserved pattern of Cys residues. An 8 kDa hydrophobin named HGFI corresponding to the *hgf1* cDNA sequence was isolated from the mycelium of a similarly grown culture. Like most hydrophobins, HGFI could self assemble at interfaces, lowering surface tension and changing surface properties. A rodlet layer typical of hydrophobins was observed using AFM when HGFI was dried directly on mica. Highly ordered rodlet layers were also observed using Langmuir films after repeated compression cycles.

METHODS

Strain, media and growth conditions. The *G. frondosa* strain CICC-50075 was used in all experiments. It was routinely grown at 28 °C with shaking at 160 r.p.m. in Erlenmeyer flasks in medium containing 40 g glucose l⁻¹, 2.5 g KH₂PO₄ l⁻¹, 3 g MgSO₄ l⁻¹, 5 g Tryptone l⁻¹ and 30 mg vitamin B₁ l⁻¹ for 5 days. After homogenization, 2 ml culture was transferred to a 9 cm Petri dish and grown in *Schizophyllum commune* minimal medium (Dons *et al.*, 1979) containing 2% agar. After 10 days, the mycelia were collected by scraping. For all cloning work the *Escherichia coli* DH10B strain was used. It was grown at 37 °C in Luria broth.

cDNA library construction. Total RNA of *G. frondosa* was isolated using the TRIzol reagent (BBI) and mRNA was purified from it using the PolyATtract mRNA isolation system (Promega). The cDNA fragments were synthesized with the M-MLV RTase cDNA synthesis kit (Takara). The primer 5'-GAGACTCGAGT^{TTT}-3' (18T), which has an *Xho*I cleavage site (underlined), was used for synthesizing the first strand of the cDNA. *Eco*RI adaptors were linked to cDNA fragments after filling in recessed 3' ends. After phosphorylation, cDNA fragments were cleaved with *Xho*I and connected to the dephosphorylated plasmid pBluescriptII SK(+), which had been digested with *Eco*RI and *Xho*I. The recombinant cDNA was transformed into *E. coli* DH10B.

Identification of a hydrophobin gene in *G. frondosa*.

Transformants in the cDNA library with inserted fragments of 0.5–1.5 kb were sequenced and analysed for hydrophobin-like genes. To identify hydrophobin genes we searched for the characteristic pattern of eight Cys residues. The hydrophobin gene, including introns, was cloned by PCR using *G. frondosa* genomic DNA as template. Primers upstream (5'-TTGCACCTCGCAAACCAC-3') and downstream (5'-CGACAGACACGGCATAATCTC-3') of the cDNA were used. The PCR was performed using Ex Taq DNA polymerase in a total reaction volume of 50 µl. After a denaturing step of 5 min at 94 °C, amplification was carried out for 35 cycles of 1 min at 94 °C, 45 s at 60 °C, and 1 min at 72 °C. The PCR fragment was purified from the gel with a DNA fragment purification kit (Takara) and cloned into a pMD19 T-vector (Takara) for sequencing.

Isolation and purification of hydrophobin HGFI.

For the isolation of cell wall-bound hydrophobin, aerial hyphae were collected by scraping them from mycelial mats grown in Petri dishes. The mycelium was treated twice with hot 1% SDS (100 mM Tris, pH 7.0) in a boiling water bath for 10 min and washed four times with 10 volumes of water. The residue was freeze-dried and then treated with undiluted TFA in a sonicating water bath for 2 min while the temperature was kept at 0 °C. The mixture was centrifuged at 14 000 g and the supernatant was dried in a stream of nitrogen. The dried material was then dissolved in 60% ethanol, and then further diluted with water to 20% ethanol and lyophilized.

Reverse-phase HPLC (RP-HPLC) was used for further purification of the protein. A Vydac C4 (214TP1010) reversed-phase column (10 × 250 mm) installed on ÄKTA Explorer system (Pharmacia Biotech) was used. Freeze-dried protein was dissolved in 40% acetonitrile containing 0.1% TFA. The sample was eluted using a 20–60% (v/v) acetonitrile gradient containing 0.1% TFA at a flow rate of 4 ml min⁻¹. For analytical HPLC, the same acetonitrile gradient was used with a smaller (4.6 × 50 mm) Vydac C4 (214TP5405) column at a flow rate of 1 ml min⁻¹. The elution was monitored by UV absorption at 215 and 280 nm. An estimate of the protein concentration was obtained by integrating and comparing the 215 nm absorbance with that of samples of known concentrations of *Trichoderma reesei* HFBII protein (Linder *et al.*, 2001). The HFBII concentration was determined by careful weighing of samples of pure dried protein.

Analytical methods. N-terminal amino acid sequences were determined using an ABI Procise 491 (ABI) protein sequencer. A 10 µg freeze-dried sample was loaded on a PVDF membrane and washed with 200 µl 0.1% TFA three times. Then the PVDF membrane was cut up and placed in the reaction cartridge of the ABI Procise 491.

MS was performed using a Bruker 4.7-T APEX-IV Fourier-transform ion cyclotron resonance (FT-ICR) instrument (Bruker Daltonics), interfaced with an external Apollo electrospray ion source. The sample was diluted with acetonitrile/water/acetic acid (49.5:49.5:1.0, by vol.) solution to a final concentration of about 20 pmol µl⁻¹ and directly infused at a flow rate of 1.5 µl min⁻¹. A total of 128 (512-kWord) time-domain transients were co-added and fast

Fig. 1. HGFI sequence and its alignment with some hydrophobins of high similarity. (a) HGFI sequence: *predicted cleavage site for the signal sequence; **start of the mature protein. Cys residues are shown with a grey background. (b) CLUSTAL W (1.83) was used to align hydrophobins with high similarity to HGFI. HGFI was 55 and 53 % identical to Hah2 (GenBank accession no. ABA46362) and Hah1 (accession no. ABA46363) from *H. annosum*, respectively, 53 % identical to *P. nameko* hydrophobin pdi315 (accession no. BAB84547), and 50 % identical to SC3 from *S. commune* (accession no. AAA96324).

Fourier-transformed prior to Gaussian multiplication, magnitude calculation and external frequency-to-*m/z* calibration with respect to the ions of an ES Tuning Mix (Agilent Technologies) calibration mixture.

SDS-PAGE was performed using 16 % Tricine SDS polyacrylamide gels (Schägger & von Jagow, 1987; Lesse *et al.*, 1990).

Surface modification with hydrophobins. Protein adhesion to hydrophobic surfaces was studied using pieces of siliconized cover glass (HR3-239, Hampton Research). The siliconized glass sheets were coated with 20 $\mu\text{g ml}^{-1}$ hydrophobin HGFI or HFBI solution and incubated at room temperature for 20 min. After removal of the solution, the sheets were dried in a nitrogen stream and kept at room temperature overnight. The glass was further rinsed with water and contact angle measurements (see below) were used to analyse the surface. PBS, PBS with 0.1 % Tween-20 (PBST), 60 % ethanol or 1 % SDS was used to rinse the siliconized glass surface covered with HGFI to determine the solubility of the adhered protein.

Surface tension and water contact angle measurements. Surface tension and water contact angle measurements were carried out with a KSV Cam200 goniometer (KSV Instruments) and results were calculated using the software provided by the manufacturer. To study protein film formation, a pendant drop of 10 μM protein solution was formed and images were recorded at 66 ms intervals to record the drop shape animation. Protein solution was first pumped through the needle for 7 s forming an 8 μl water drop. After keeping the volume constant for 1 s, the protein solution was pulled back into the needle at constant speed in 4 s.

For surface tension measurements of protein solutions, we used 10 μl drops, for which drop-shape images were recorded and analysed using the same KSV instrument.

Static water contact angles were measured with a 5 μl water droplet on siliconized glass at room temperature. At least three water droplet readings were analysed on different areas of the sample surface.

Drying of protein solution on mica. HGFI was freshly prepared by RP-HPLC, freeze-dried and dissolved in MilliQ water to a concentration of 100 $\mu\text{g ml}^{-1}$. A 10 μl aliquot of the protein solution was deposited on a freshly cleaved mica substrate, and the water was allowed to evaporate overnight in a vacuum desiccator. The samples were stored in a desiccator at room temperature until they were imaged by AFM.

Langmuir films of HGFI. Pressure–area isotherms and Langmuir films of hydrophobins were prepared using a KSV Minimicro Langmuir system (195 $\times$ 51 mm, KSV instruments) using 1 mM sodium acetate buffer, pH 5.0, as the subphase. Analytical-grade chemicals and MilliQ water were used. HFBI pressure–area isotherms were made as controls as described elsewhere (Paananen *et al.*, 2003). Lyophilized HGFI was dissolved in water to a concentration of 27 μM , and 40 μl of the solution was spread on the subphase. Compression of the monolayer was started after the surface pressure had stabilized for 15 min for single compression, or 30 min for multiple compressions. The surface pressure was measured to 0.01 mN m^{-1} using a Wilhelmy paper plate. The monolayers were compressed and expanded at a rate of 250 $\text{mm}^2 \text{min}^{-1}$. Langmuir films were also deposited to solid supports at a deposition pressure of 25 mN m^{-1} . Freshly cleaved mica was used as a substrate for LB films, and freshly cleaved highly oriented pyrolytic graphite (HOPG, ZYA quality) as a substrate for Langmuir–Schaefer (LS) films. In the LB deposition the hydrophilic mica sheet is pulled out through the film, while the barriers compress the film onto the substrate. For an LS deposition the protein film is touched in parallel with an HOPG substrate, whereupon the film adsorbs to the substrate. The samples were dried in a vacuum desiccator.

AFM. The dried droplets and Langmuir films were imaged using a NanoScope IIIa Multimode atomic force microscope ('E' scanner, Veeco). All samples were imaged in dry state using tapping mode and silicon nitride cantilevers with the nominal force constant of 46 N m^{-1} (Ultrasharp, NSC15, Micromasch). Scan rates were approximately 1 Hz using as low a force as possible. The damping ratio (set-point amplitude/free amplitude) was typically about 0.7–0.8. The images were flattened to remove a possible tilt. For image analysis the Scanning Probe Image Processor (SPIP; Image Metrology) was used.

RESULTS

cDNA library construction and identification of hydrophobin gene

The cDNA library contained an estimated 2.0×10^5 clones. Transformants with inserted-fragment lengths of 0.5–1.5 kb were screened from the cDNA library and 50 clones were sequenced. Two transformants with the same inserted

Fig. 2. Purification of cell wall-bound hot SDS-insoluble proteins from *G. frondosa*. (a) RP-HPLC of cell wall-bound hot SDS-insoluble proteins. mAU, milli-absorbance units; %B, the percentage of solution B (acetonitrile) in the eluant; Conc, the concentration of acetonitrile according to the right-hand y axis. (b) Tricine SDS-PAGE of the main peak from the RP-HPLC (lane 1) and low-molecular-mass markers (lane 2). The 8 kDa band was identified as HGFI and the 16 kDa band is likely a dimer of this protein.

cDNA fragments contained a hydrophobin-like sequence. A translation of the sequence showed the characteristic pattern of eight Cys residues. We named the gene *hgf1* as an abbreviation of hydrophobin *G. frondosa* 1, and used the name HGFI for the corresponding protein. Sequence comparisons, starting from the first Cys residue, with data in the GenBank database (Altschul *et al.*, 1997; Schäffer *et al.*, 2001) revealed significant degrees of similarity with other hydrophobins identified in *Heterobasidion annosum* and *Pholiota nameko* (Fig. 1). The length of *hgf1* cDNA was 324 bp, which translated into a protein of 107 aa. Signal sequence prediction using SignalP (Nielsen *et al.*, 1997)

suggested a 19 aa signal peptide. The genomic sequence of *hgf1* (NCBI accession no. EF486307) had two introns with lengths of 57 and 72 bp, which were located 246 and 344 bp from the translation initiation codon, respectively.

Isolation of a hydrophobin protein from the outer cell wall layer of *G. frondosa*

A protein later identified as HGFI was extracted from the mycelium of *G. frondosa* as an aggregate that was insoluble in hot SDS. The mycelium used was from a culture at the same growth stage as the one used for cDNA library construction. After removing other proteins with 1% SDS, the mycelium was further extracted with pure TFA by sonication in ice-cold water. TFA was removed with a stream of nitrogen and the residue was dissolved in 60% ethanol and fractionated using RP-HPLC. The chromatogram showed one predominant and several smaller peaks (Fig. 2a). The predominant peak had a very low absorbance at 280 nm, which is expected for a protein such as HGFI that lacks the aromatic residues Tyr and Trp. The fractions corresponding to the peaks were lyophilized and analysed by Tricine SDS-PAGE. In the predominant peak, one protein band with an apparent relative molecular mass of 8 kDa and another faint band of 16 kDa could be seen (Fig. 2b). A likely explanation for the 16 kDa band is that it is a dimeric form of the 8 kDa protein. Proteins in this peak could be observed using silver staining, but only weakly using Coomassie brilliant blue staining.

N-terminal sequencing of the predominant peak gave the sequence QQXTTGQL (X=no positive identification of an amino acid residue could be made). These amino acid residues matched with the translation of the *hgf1* cDNA sequence, indicating that the isolated protein could be HGFI. The third residue, corresponding to the unidentified X in HGFI, was a Cys, which was expected to be difficult to identify if it is in the disulfide form. Also, these data indicated that five amino acid residues more than were predicted for the signal sequence had been cleaved during processing. The FT-ICR mass spectrum of HGFI (Fig. 3) showed that the protein was present mostly as a monomer, and the most abundant isotopic mass was determined to be 8030.75 ± 0.03 Da (averaged over the charge-state distribution). This was 1 Da more than the theoretical mass (8029.76 Da) for HGFI, if it is assumed that the N-terminal glutamine residue exists in its cyclized pyrrolidone carboxylic acid (PCA) form, which is very common for secreted fungal proteins. It is likely that the 1 Da difference is due to a deamidation in HGFI (the corresponding theoretical mass would be 8030.74 Da). Deamidations could be expected in hydrophobins, as shown for *T. reesei* HFBI (Askolin *et al.*, 2001). The measured mass also supported the signal sequence cleavage site suggested by sequencing. In addition to the HGFI monomer, a signal representing a non-covalent protein dimer was also present in the mass spectra. Based on these data we concluded that the isolated protein was HGFI.

Fig. 3. FT-ICR mass spectrum of HGFI. The signals representing the observed charge state distribution of multiply protonated proteins $[\text{HGFI} + z\text{H}]^{z+}$ have been designated as $\text{HGF}z+$. The inset shows an isotopically resolved signal for $[\text{HGFI} + 5\text{H}]^{5+}$, with an asterisk indicating the peak that corresponds to the most abundant isotopic mass.

Fig. 4. Surface tension measurements of HGFI in water.

The HGFI solution was very stable, as no change in properties was observed upon storage in MilliQ water at room temperature for at least 1 month. The solubility of HGFI was not as high as that of other class I hydrophobins.

HGFI tended to precipitate above a concentration of $300 \mu\text{g ml}^{-1}$. The protein was completely precipitated by bubbling the solution with air.

Surface modification with HGFI

Water contact angles of dried-down hydrophobins on hydrophobic siliconized glass were used to study the behaviour of HGFI. As a comparison, another hydrophobin, HFBI (class II), was used. Usually, a surface is considered to be hydrophobic if its water contact angle is more than 60° and hydrophilic if it is lower (Lumsdon *et al.*, 2005). The HGFI membrane could be removed with almost all solutions, even with MilliQ water, if the rinsing was done immediately after drying the HGFI solution. If the dried sample was kept overnight at room temperature, the membrane could not be removed with MilliQ water or PBS solution (pH 7.4), but could be dislodged using 60 % ethanol, 1 % SDS or PBST at room temperature, and was readily solubilized using 1 % hot SDS or hot PBST at 80°C . After rinsing with the last two of these solutions, the HGFI-treated siliconized glass surface changed back to

Fig. 5. Hanging drops of $10 \mu\text{M}$ HGFI solution at different time points. An $8 \mu\text{l}$ drop was formed in 7 s (a, b). After maintaining the same volume for 1 s, the hydrophobin solution was drawn back into the needle at constant speed in 4 s (c, d, e and f).

Fig. 6. AFM topography images of rodlets formed by HGFI. A $100 \mu\text{g ml}^{-1}$ solution of HGFI was dried on mica and imaged with AFM as described in Methods. (a) Image size is $1 \times 1 \mu\text{m}$ and height scale is 4.4 nm. (b) A $200 \times 200 \text{ nm}$ image from the centre part of (a). The height scale in (b) is 2.4 nm.

hydrophobic, having water contact angles close to those of the original unmodified surfaces. Both HGFI and HFBI could alter the surface of siliconized glass to hydrophilic. HGFI changed the contact angle from 86.6° to 51.9° . It was found that it was important to rinse the hydrophobin-coated glass with water before measurement. If the hydrophobin-coated glass surface was not rinsed with water prior to measurement, the water contact angle was as low as 8.4° . It is likely that the excess monomers that were dried on the surface were dissolved in the water drop. Being surface-active molecules, these hydrophobin monomers then spread the drop on the glass, resulting in a very low contact angle. All measurements for all surfaces were within 3.0° of the mean values.

Surface activity of HGFI

Lowering surface tension is one of the most important features of hydrophobins. The surface tension of solutions of HGFI at different concentrations was measured, and a concentration–surface tension isotherm was plotted (Fig. 4). Pure (MilliQ) water had a surface tension of 72.0 mJ m^{-2} . A slight increase in surface tension to 74.1 and 72.6 mJ m^{-2} was noted at hydrophobin concentrations of 0.5 and $1 \mu\text{M}$, respectively. Increasing the HGFI concentrations to between 1 and $2 \mu\text{M}$ led to a quick drop in surface tension. At higher concentrations (5 and $10 \mu\text{M}$), the surface tension dropped more slowly to reach a minimum of 44.6 mJ m^{-2} . All surface tension measurement results were within 3.0 mJ m^{-2} of the mean values.

A series of images of *G. frondosa* hydrophobin HGFI solution pendant drops were taken, which showed an interesting formation of a film at the air–water interface. An $8 \mu\text{l}$ HGFI solution drop was made and then pulled back into the needle at a constant speed. In Fig. 5, six selected images of the hydrophobin solution drop are shown. In Fig. 5(a, b) we can see that at the beginning, the hydrophobin solution drop had a round shape similar to that of a water drop. After that, the hydrophobin solution drop began to shrink (Fig. 5c), but it retained the round drop shape. However, when the drop surface area became small enough, the water drop changed shape as the surface

tension of the solution decreased (Fig. 5d). When the drop volume was further decreased, the shape became irregular and a finely wrinkled film on the drop was evident (Fig. 5e, f). This showed that HGFI readily self assembles into films at the air–water interface. Control experiments with other proteins such as BSA did not show any indication of the formation of similar films.

AFM of rodlet layer and Langmuir films

AFM was used to study the structure of films formed by HGFI at the air–water interface. In a simple experiment a droplet of HGFI solution was dried on a freshly cleaved mica surface. AFM on this surface showed clear rodlet mosaic structures typical of class I hydrophobins (Fig. 6). The rodlets were about 100 – 200 nm in length and extended in all directions. The width of the rodlets was about 5 – 10 nm , although this value may be an overestimation due to the size and shape of the cantilever tip.

Using the Langmuir equipment, pressure–area isotherms of HGFI and HFBI were determined (Fig. 7a). The HFBI isotherm was relatively steep and had a collapse point at 45 mN m^{-1} (near 40 mN m^{-1}). The mean molecular area of HFBI was, as has been reported earlier, $\sim 250 \text{ \AA}^2$ ($\sim 2.5 \text{ nm}^2$) (Paananen *et al.*, 2003). The isotherm of HGFI was not as steep as that of HFBI. As the surface pressure increased to 40 mN m^{-1} , the collapse point was reached and a jagged curve was observed. The calculated mean molecular area was $850 \text{ \AA}^2$ (8.5 nm^2). The HGFI compression curve showed hysteresis and shifted to a lower mean molecular area when multiple compression–expansion cycles were performed. In contrast, HFBI did not show this behaviour (Fig. 7b, c).

Samples compressed only once had no specific morphology (Fig. 8a). However, rodlets could be clearly observed in samples which had undergone multiple compression–expansion cycles. Interestingly, both the hydrophobic (LB films, Fig. 8b) and hydrophilic (LS films, Fig. 8c) sides showed similar rodlet structure. All rodlets extended locally in approximately the same direction. The rodlets were 5 – 10 nm in width and hundreds of nanometres in length.

Fig. 7. Pressure–area isotherms of Langmuir films. (a) HGFI and HFBI compressed once. (b) Three cycles of compression of HGFI. (c) Three cycles of compression of HFBI.

DISCUSSION

A gene corresponding to a hydrophobin was identified in a cDNA library from *G. frondosa*, and named *hgfi*. The translated protein sequence was called HGFI and had the general features of a hydrophobin: (1) it was a small protein (100 ± 25 aa), (2) it had a signal sequence, and (3) it contained eight Cys residues in a characteristic pattern.

Based on sequence similarity and hydropathy plots, the protein was determined to be a class I hydrophobin (Wösten and de Vocht, 2000; Linder *et al.*, 2005).

The corresponding protein, HGFI, was isolated from the fungal mycelia as a hot SDS-insoluble aggregate. Further purification was done using RP-HPLC. The only other class I hydrophobin that has been purified using RP-HPLC is EAS from *Neurospora crassa* (Templeton *et al.*, 1995; Mackay *et al.*, 2001). The often-used example of a class I hydrophobin, SC3, has been purified by size-exclusion chromatography (Wang *et al.*, 2004). Class II hydrophobins such as HFBI and HFBI from *T. reesei* have also been purified by RP-HPLC (Askolin *et al.*, 2001; Bailey *et al.*, 2002).

Hydrophobins are highly surface-active proteins. With a maximal lowering of water surface tension from 72 to 24 mJ m⁻² at 50 µg ml⁻¹, SC3 has been reported to be the most surface-active protein known (Wösten *et al.*, 1999). In this work HGFI lowered the water surface tension to 44.6 mJ m⁻².

It is reported that aggregates formed by class I members cannot be dissolved in hot SDS and in organic solvents such as ethanol, but only in a few solvents such as TFA. Previous comparisons between class I and class II hydrophobins have implicated surface adhesion stability properties as being an important difference between the classes (Askolin *et al.*, 2006). Although aggregates of HGFI are insoluble, it showed adhesion behaviour on siliconized glass similar to that of the class II hydrophobin HFBI. Thus, HGFI could be easily removed with 60 % ethanol, PBST, and 1 % SDS at room temperature or at 80 °C. It is surprising that HGFI formed films that could not resist a similar hot SDS treatment as it was isolated from the cell wall as aggregates insoluble in hot SDS.

A commonly described form of self-assembled structure of hydrophobins is the rodlet layer, which is characteristic of class I hydrophobins but has not been seen in class II members. Rodlet layers were also found on fungal structures in regular patterns even before the identification of hydrophobin proteins (Stringer *et al.*, 1991). Class I hydrophobins on the cell walls generally form rodlets with irregular lengths and stretch in all directions on the surface. The rodlets of HGFI produced in this work were very similar in dimensions to rodlets reported for SC3 (de Vocht *et al.*, 1998). It still remains to be seen whether rodlet patterns can also be found on the surface of *G. frondosa*.

Experiments with Langmuir films showed that the HGFI surface pressure–area curve was not as steep as that of HFBI. The collapse points of HGFI and HFBI were, however, fairly close, at 40 and 45 mN m⁻¹, respectively. Multiple compression–expansion cycling resulted in a clear shift of the mean molecular area of HGFI to lower values. In contrast, the area occupied by HFBI changed much less. The mean molecular area of the HGFI film seemed to decrease after each compression–expansion cycle and after the eighth compression–expansion cycle; the mean

Fig. 8. AFM topography images of HGFI Langmuir films. Due to the way the film is picked up, an LB film shows the hydrophobic side and an LS film shows the hydrophilic side of an amphiphilic film. (a) LB sample from a floating monolayer film compressed once. (b) LB sample from a multiply compressed film. (c) LS sample from a multiply compressed film. Image sizes are $1 \times 1 \mu\text{m}$ and height scales are 1 nm (a), 2 nm (b) and 5 nm (c).

molecular area decreased by 58% to about $360 \text{ \AA}^2$ (3.6 nm^2). LB films of both HFBI and HFBII show polycrystalline structures consisting of 2D oblique single-crystalline domains (Paananen *et al.*, 2003). HGFI forms rodlets in dried droplets and in Langmuir films. Upon multiple compressions rodlet formation was strongly enhanced, resulting in locally aligned rodlets (Fig. 8). The mean molecular area hysteresis can be explained by the tighter packing of monomers as rodlets are formed. The rodlets formed were then not easily dissociated, in contrast to the case of HFBI, in which possible aggregates reversibly dissociate, reforming the surface layer. As a result of the compression cycles the rodlets became more aligned and arranged than typically observed for rodlet layers.

In conclusion, we have discovered and characterized a novel hydrophobin, HGFI, from the edible mushroom *G. frondosa*. As a class I hydrophobin, HGFI can lower the surface tension of water and change the nature of the surfaces to which it adsorbs. Investigation of HGFI Langmuir films showed that rodlets typical of class I hydrophobins are formed at the air–water interface and that compression of the film induces local rearrangements of the rodlets.

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