Hydrophobins line air channels in fruiting bodies of *Schizophyllum commune* and *Agaricus bisporus*

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The hydrophobin SC4 was isolated from the medium of a dikaryon from *Schizophyllum commune* with disrupted *SC3* genes. Although not glycosylated, its biophysical properties were similar to those of SC3. As the hydrophobins SC3 from *S. commune* and ABH1 and ABH3 from *Agaricus bisporus*, SC4 self-assembled at hydrophilic-hydrophobic interfaces into an SDS insoluble amphipathic film with a typical rodlet structure at its hydrophobic face, and also proved to be a powerful surfactant. Similar rodlet structures were observed in the fruiting body plectenchyma. By immunodetection SC4 could be localized lining air channels within this tissue. A similar localization was found for the ABH1 hydrophobin in fruiting bodies of *A. bisporus*. Probably, these hydrophobin coatings prevent collapse of air channels allowing efficient gas exchange even under wet conditions.

Hydrophobins are small secreted fungal proteins that self-assemble upon exposure to hydrophilic-hydrophobic interfaces with formation of amphipathic membranes which are SDS-insoluble in Class I hydrophobins (Wessels, 1994; 1997). Self-assembly at a water-air interface is accompanied by a large reduction in surface tension (van der Vegt *et al.*, 1996; Wösten & Wessels, 1997). Class I hydrophobins are ubiquitous among filamentous fungi and fulfil a variety of functions all related to the amphipathic nature of the assembled protein membrane. SC3 from *Schizophyllum commune* (Wösten *et al.*, 1994*a*; van Wetter *et al.*, 1996) and ABH3 from *Agaricus bisporus* (Lugones, Wösten & Wessels, 1998) have been related to the formation of hydrophobic aerial hyphae and to attachment of hyphae to hydrophobic surfaces (Wösten, Schuren & Wessels, 1994*b*). The hydrophobins RodA of *Aspergillus nidulans* (Stringer *et al.*, 1991), EAS of *Neurospora crassa* (Bell-Pedersen, Dunlap & Loros, 1992; Lauter, Russo & Yanofsky, 1992); and MPG1 of *Magnaporthe grisea* (Talbot, Ebbole, & Hamer, 1993) have been shown to coat conidiospores with a hydrophobic coating; MPG1 was also shown to be involved in formation of appressoria on hydrophobic surfaces (Talbot *et al.*, 1993; 1996). ABH1 (*A. bisporus*) was found to provide mushrooms with a hydrophobic outer surface (Lugones *et al.*, 1996; de Groot *et al.*, 1996) but was also suggested to be present within the fruiting-body plectenchyma (Lugones *et al.*, 1996).

In contrast, to ABH1, which is only found in fruiting bodies of *A. bisporus* (Lugones *et al.*, 1996; de Groot *et al.*, 1996), SC4, which is found together with SC3 in fruiting bodies of *S. commune* (Ásgeirsdóttir, van Wetter & Wessels, 1995), is secreted by the dikaryotic substrate mycelium into the medium where it is found together with the SC3 hydrophobin (Wessels *et al.*, 1991).

Here we report on the isolation of SC4 and its partial biochemical and biophysical characterization by using a dikaryon in which the *SC3* genes were disrupted. Antibodies raised against SC4 and ABH1 were used to localize the proteins in the fruiting bodies of *S. commune* and *A. bisporus*, respectively. Based on these results we propose a function for these fruiting body hydrophobins.

MATERIALS AND METHODS

Organism and culture conditions

A dikaryotic *Schizophyllum commune* Fr. strain obtained by mating two strains, both isogenized to 4-39 (CBS 341.81) and with the *SC3* gene disrupted by insertion of a phleomycin expression cassette (Wösten *et al.*, 1994*b*; van Wetter *et al.*, 1996), was grown on minimal medium (Dons, de Vries & Wessels, 1979), at 25 °C in the light. For SC4 isolation, 3 ml mycelial macerate was spread at the bottom of a Petri dish and allowed to grow for 3 d at which time a mycelial mat had formed. Forty ml minimal medium were then injected under the mycelial mat which then floated on the medium. The cultures were incubated for 4 additional days with fruiting bodies developing.

Young *Agaricus bisporus* (J. Elange) Imbach (cultivar U1) mushroom primordia measuring 2 mm or 25 mm (buttons) were kindly provided by Mr B. Boer (Mushroom Farm Agarica B. V., Hoogeveen, The Netherlands).

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Isolation of SC4

Culture filtrates were subjected to bubbling with hydrogen gas generated by electrolysis (Lugones *et al*., 1998). The foam was collected and, after lyophilization, material in the foam was treated with trifluoroacetic acid (TFA, de Vries *et al*., 1993) and dissolved in water. After removing insolubles by centrifugation ethanol was added to the supernatant to 60% (v/v). After centrifugation the supernatant was dialysed against water and SC4 was reaggregated on electrolytically released hydrogen bubbles. After TFA treatment the SC4 in the lyophilized material was found to be electrophoretically pure.

Protein analysis

SDS–PAGE, protein staining, Western blotting and immunostaining were performed as described by Lugones *et al*. (1996). MALDI–TOF (Matrix-Assisted Laser Desorption/Ionization Time Of Flight) mass spectroscopy was done by drying 1 µl of a solution of SC4 (1 mg ml^{-1}, TFA treated) in 0·1% aqueous TFA on a target together with 1 µl matrix (20 mg ml^{-1} sinapinic acid in aqueous acetonitrile 40% (v/v) in the presence of 0·1% TFA). The spectra were recorded in a TofSpec E & SE Micromass mass spectrometer.

In vitro *assembly of SC4*

Cleaned polytetrafluoroethylene (PTFE-Teflon) discs (0·5 cm diam., 0·5 mm thick) were incubated with an aqueous SC4 solution (10 µg ml^{-1}) for 16 h. The discs were then extracted with 1% SDS at 100°, to remove monomeric hydrophobin, washed in distilled water and dried. The hydrophobicity of the surface was measured by determining water contact angles (van der Mei, Rosenberg & Busscher, 1991). For exposing the hydrophobic side of the hydrophobin membrane, SC4 was assembled on filter paper (Lugones *et al*., 1996). The paper was dried and extracted with 1% SDS at 100° for 10 min, washed with water and dried for measurement of water contact angles.

Surface tension measurements

Surface tension was determined by Axi-symmetric Drop Shape Analysis by Profile (ADSA-P) as described (Noordmans & Busscher, 1991). 100 µl of freshly prepared aqueous protein solution (100 µg ml^{-1}) was placed on a cleaned fluoroethylene-propylene-Teflon surface (FEP; Norton Fluorplast, Raamsdonksveer, The Netherlands). The droplet profile was digitized with a contour monitor and the data used to calculate the liquid surface tension. All measurements were done at room temperature, at least in duplicate, taking 96 profiles of the water droplet over a period of 8 h. To prevent evaporation, measurements were done with the droplet sitting in a water-vapour-saturated chamber.

Antisera against SC4 and ABH1

Rabbits were injected 5 times at 10 d intervals with 150 µg gel-purified SC4. Purification of the obtained antiserum was necessary and done by incubating the antiserum (diluted 1/100) three times for 60 min with isolated cell walls (Sietsma, Rast & Wessels, 1977) from a *S. commune* monokaryon (4-39) at 1 mg walls (ml antiserum)$^{-1}$. The purified antiserum reacted with SC4 only. Pre-immune serum was unreactive. Preparation and purification of an antiserum against ABH1 have been described (Lugones *et al*., 1996).

Immunolocalization

Small pieces (about 1 mm^3) of plectenchyma were fixed in 2·5% formaldehyde, 0·5% glutaraldehyde in phosphate buffer (0·2 M, pH 7·2). The samples were degassed and put on ice for 3 h. After washing with water, the samples were dehydrated in an ethanol series, and incubated 16 h in pure Unicryl (Biocell). The samples were then transferred to fresh Unicryl to polymerize for 48 h at −10° under UV light. Ultrathin sections were blocked with 0·5% BSA in PBG-glycine buffer (6·5 mM Na_2HPO_4, 1·5 mM NaH_2PO_4, 2·7 mM KCl, 150 mM NaCl, 20 mM glycine at pH 7·2) for 15 min, incubated for 16 h with purified antiserum (ABH1 1:500, SC4 1:100) in the same blocking buffer, washed three times for 10 min with PBS-glycine buffer and incubated with goat-anti-rabbit serum conjugated to 15 nm gold beads (Amersham, 1:20) in PBS-BSA-glycine buffer for 1 h. After three washes with PBS-glycine (10 min each) and three washes in distilled water (10 min each) the sections were contrasted with uranyl acetate (1% in water) and allowed to dry before examination in a Philips CM 10 electron microscope.

Miscellaneous EM techniques

For visualization of rodlets on the hydrophobic side of assembled SC4, 5 µl of an aqueous solution of SC4 (5 µg ml^{-1}) was allowed to dry on Formvar-coated nickel grids. The surface was shadowed with Pt/C at an angle of 45°. For visualization of rodlets in fruiting bodies, small pieces of tissue (1 mm^3) were put in 4% formaldehyde, degassed and fixed for 3 h. After three washes with water the material was equilibrated with 50% aqueous glycerol, and then frozen in liquid-nitrogen-cooled propane. Freeze-fracturing was done in a freeze-etch unit (Balzers, Liechtenstein). Replicas were made using Pt/C. These were cleaned in $K_2Cr_2O_7$-saturated H_2SO_4 for 90 min and examined in a Philips CM 10 electron microscope.

RESULTS

Isolation of SC4

Dikaryotic mycelium of *Schizophyllum commune* secrets both SC3 and SC4 into the culture medium (Fig. 1, lane 1). With the purification procedure developed for SC3 (Wösten, de Vries & Wessels, 1993), SC3 and SC4 were co-purified from the medium and subsequent separation of the two hydrophobins was unsuccessful. A dikaryon was therefore, constructed with disruptions in the *SC3* genes of both nuclear types. Although normal-looking fruiting bodies were formed, their surfaces and those of aerial hyphae were easily wettable (van Wetter *et al*., 1996) reflecting the absence of SC3. SC4 was isolated from medium of standing cultures of this dikaryon strain (see

Methods) and up to 2 mg l^{-1} could be purified (Fig. 1, lane 2). Shaking cultures, however, secreted much less SC4, probably due to disruption of the binucleate state (Schuurs *et al.*, 1998).

Biochemical and biophysical characterization of SC4

The ability of SC4 to assemble independently from SC3 was evident from the procedure used for its isolation. By treatment of the aggregates with TFA, SC4 was monomerized and became soluble in water. It could be assembled again by vortexing the monomer solution (Fig. 1, lanes 3 and 4).

Mass spectra of SC4 (Fig. 2) revealed four isoforms, which could be fully explained by alternative processing at the *N*-terminus. The isoform with the highest mass (8755) agreed with the amino acid sequence of SC4 (Schuren & Wessels, 1990) when the signal sequence was split after amino acid residue 20, as was previously determined by *N*-terminal sequencing of the mature protein (Wessels *et al.*, 1991). The three other isoforms with lower mol. wt can be explained by cleavage after amino acid residues 24, 25, and 26 (Fig. 2). In contrast to SC3, SC4 thus appears not to be glycosylated. Since SC4 is produced by a heterokaryon, it cannot be excluded that allelic differences are partly responsible for the observed diversity of SC4 forms. The different isoforms of SC4 would not be expected to be resolved on SDS–PAGE. The two different bands seen in Fig. 1 may represent different conformations of the same protein.

The surface activity of SC4 was measured by means of the ADSA-P technique and a lowering of the water-surface tension was found from 72 to 36 mJ m^{-2} (at 100 µg ml^{-1}) which compares to a lowering to 32 mJ m^{-2} for SC3 of *S. commune* (van der Vegt *et al.*, 1996) and to 37 mJ m^{-2} for ABH3 of *Agaricus bisporus* (Lugones *et al.*, 1998).

Like SC3 (Wösten *et al.*, 1993, 1994*a*), ABH1 (Lugones *et al.*, 1996) and ABH3 (Lugones *et al.*, 1998), SC4 assembled at an air–water interface. The ultrastructure of the hydrophobic side of assembled SC4 was examined. A solution of SC4 when dried and shadowed on Formvar-coated grids showed a mosaic pattern of parallel 10 nm wide rodlets (Fig. 3). These structures very much resembled those already seen in *in vitro* assembled SC3 (Wösten *et al.*, 1993, 1994*a*), ABH1 (Lugones *et al.*, 1996) and ABH3 (Lugones *et al.*, 1998).

To determine the hydrophobicity of the hydrophobic side of an SC4 membrane, paper strips were coated with SC4. As with ABH1 (Lugones *et al.*, 1996), the paper became hydrophobic in an area just beneath the evaporating front. Water contact angles of $115° \pm 3$ were measured after extraction with hot SDS. These values were similar to those found for ABH1 (Lugones *et al.*, 1996).

To determine the hydrophilicity of the hydrophilic face of SC4 membranes, discs of Teflon were immersed in an aqueous solution of SC4 (see Methods). The Teflon discs were then washed with water, dried and contact angles of 1 µl water droplets were measured. Contact angle values dropped from 110° (bare Teflon) to $48° \pm 3$ (coated Teflon). After extraction of the SC4-coated disks with 1% SDS at 100° for 10 min the contact angle values increased to $66° \pm 3$. This compares to values of 63°, and 84° after SDS extraction, found for ABH1 (Lugones *et al.*, 1996).

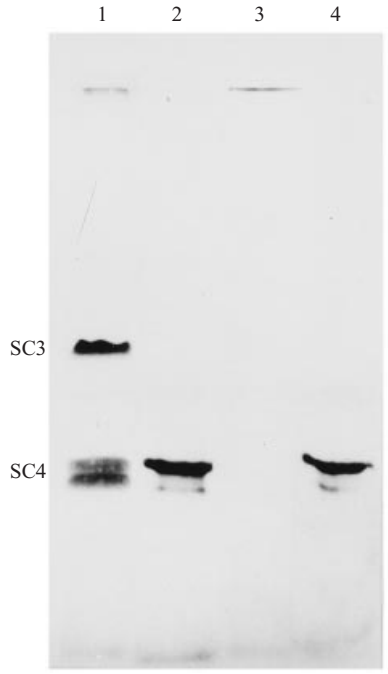


Fig. 1. SDS-PAGE. Lane 1, proteins aggregated from culture medium of wild type dikaryon of *Schizophyllum commune*; lane 2, SC4 aggregated from medium from a dikaryon with a disruption in both *SC3* genes; lane 3, supernatant after vortexing of a SC4 solution; lane 4, pellet after vortexing of the SC4 solution. Proteins in all lanes were treated with TFA prior to electrophoresis. Proteins were stained with silver.

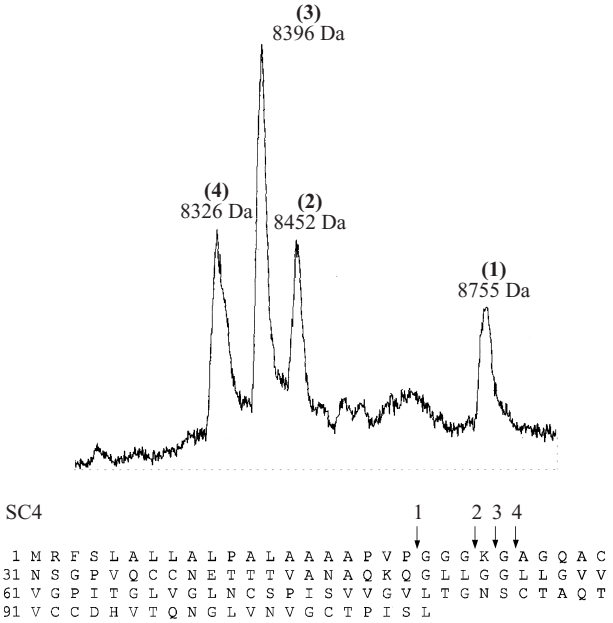
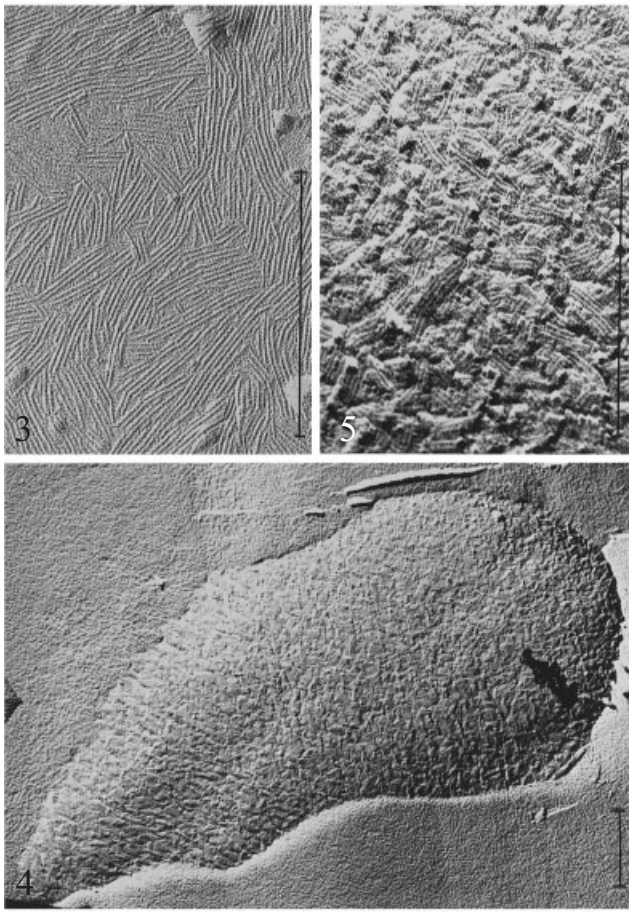


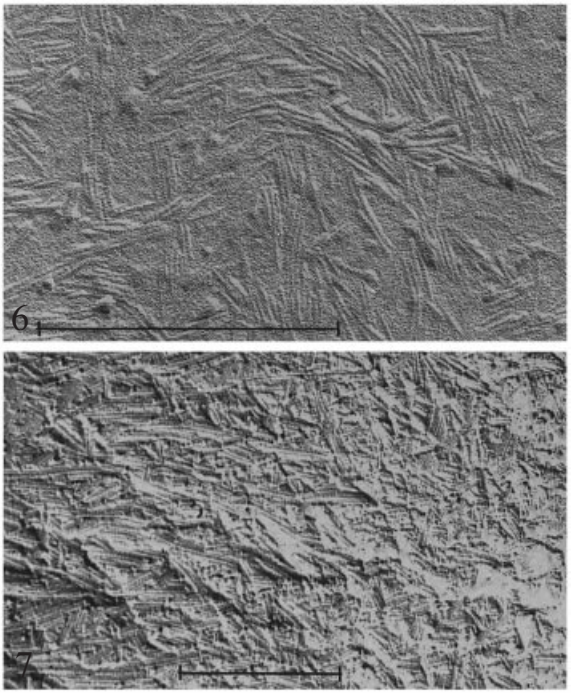
Fig. 2. Upper panel, mass spectrum of pure SC4. Mol. wt for the four isoforms that arise by processing at different positions are indicated. Lower panel, alternative processing sites in SC4 are indicated by arrows.

Presence of rodlets in S. commune *fruiting body plectenchyma*

To examine the internal structure of the plectenchyma, small pieces of *S. commune* fruiting body plectenchyma were freeze-



Figs 3–5. Ultrastructure of assembled SC4. **Fig. 3.** *In vitro* assembled SC4 after shadowing. **Fig. 4.** Replica of the fruit body plectenchyma after freeze fracturing. **Fig. 5.** Higher magnification of **Fig. 4.** Bar = 0·5 μm.



Figs 6–7. Ultrastructure of assembled ABH1. **Fig. 6.** *In vitro* assembled ABH1 after shadowing. **Fig. 7.** Replica of the fruit body plectenchyma after freeze fracturing. Bar = 0·5 μm.

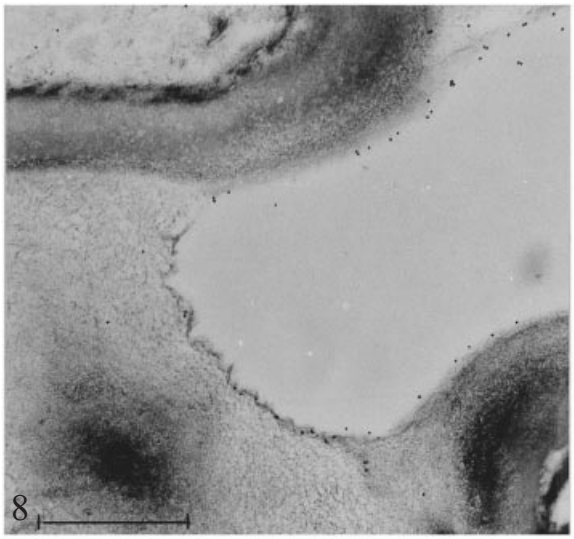


Fig. 8. Immunodetection of SC4 within the plectenchyma of *Schizophyllum commune*. Bar = 0·5 μm.

fractured and shadowed. Surfaces of air cavities were found covered with rodlets (Figs 4, 5) which resembled those of *in vitro* assembled SC4 (Fig. 3). Similar areas of rodlets were observed in the plectenchyma of *A. bisporus* fruiting bodies (Figs 6–7).

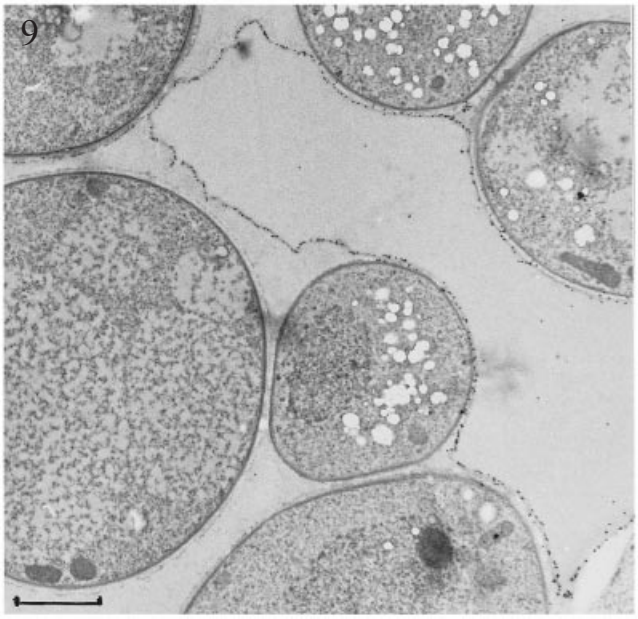
Low temperature SEM (preserving hydrated structures) showed that the plectenchyma of *S. commune* is composed of hyphae embedded in a hydrophilic mucilage that is transversed by air channels (Ásgeirsdóttir *et al.*, 1995). A similar structural organization was found in fruiting bodies of *A. bisporus* (not shown). In none of these cases was it known whether the rodlets were covering all air-exposed surfaces. The hydrophobins were, therefore, localized with antibodies in sectioned material.

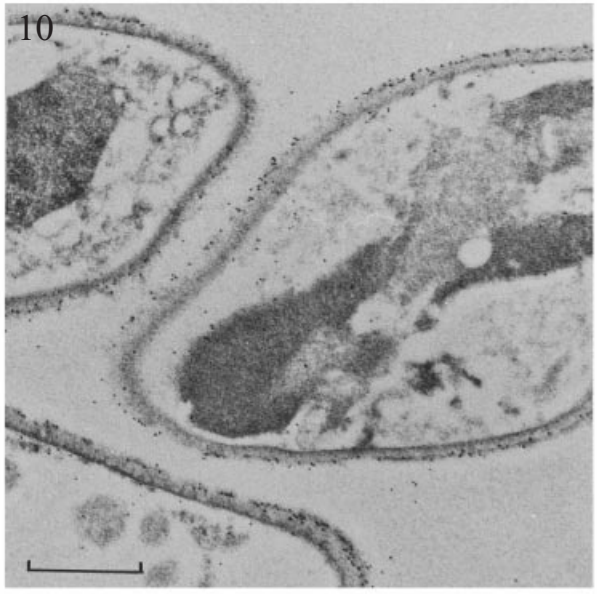
Immunolocalization of SC4 and ABH1

An antiserum against SC4 and visualized with gold-conjugated goat-anti-rabbit antibodies revealed SC4 in the fruiting bodies of *S. commune* to be present only at the surface of extracellular material lining the air spaces (Fig. 8). Similarly, an antiserum raised against ABH1, localized the protein lining the air spaces present in the plectenchyma of *A. bisporus* fruiting bodies (Fig. 9). This location was observed both at the button stage (25 mm) and at the primordium stage (2 mm) of the mushroom. In addition, in the velum and the pellicle of the fruiting bodies where the hyphae are not embedded in mucilage, ABH1 is present at surfaces of hyphal walls that contact the air (Fig. 10). Pre-immune serum was unreactive in all cases (results not shown).

DISCUSSION

The SC4 hydrophobin was purified from the culture medium of a dikaryon of *Schizophyllum commune* containing disruptions in the *SC3* genes of both nuclear types. Although not glycosylated its properties were very similar to those of SC3, the only hydrophobin secreted by the monokaryon. Upon exposure to hydrophobic–hydrophilic interfaces both hydrophobins form an SDS-insoluble amphipathic membrane with





Figs 9–10. Immunodetection of ABH1 within tissues of *Agaricus bisporus*. **Fig. 9.** Plectenchyma. **Fig. 10.** Hyphae of the velum. Bar = 1 μm.

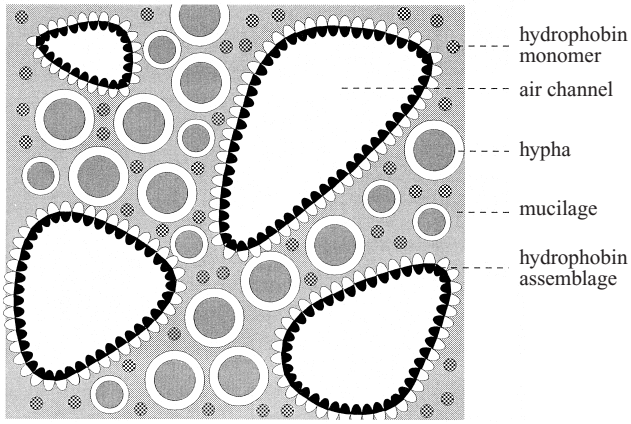


Fig. 11. Model for assembly of hydrophobins within the fruiting body plectenchyma: monomeres secreted by growing hyphae diffuse in the hydrophilic slime that binds hyphae together and assemble at the interface with the air channels.

fascicles of rodlets seen at the hydrophobic side. The ABH1 hydrophobin of *Agaricus bisporus* was previously shown to share these properties (Lugones *et al*., 1996). Assembled (SDS-insoluble) forms of SC4 and ABH1 were found in the fruiting bodies of *S. commune* (Wessels *et al*., 1991) and *A. bisporus* (Lugones *et al*., 1996), respectively. In *S. commune* SC4 was restricted to the inner tissues while the covering hyphae of the fruiting bodies did not express *SC4* but rather *SC3* as did the aerial hyphae formed by the dikaryon (Ásgeirsdóttir *et al*., 1995). In *A. bisporus*, ABH1 is present both in the inner tissues and outer tissues but its concentration is highest in the covering hyphae forming the pellicle and velum (Lugones *et al*., 1996), whereas the aerial hyphae formed by the heterokaryon deposited ABH3 on their surfaces (Lugones *et al*., 1998).

Immunological localization studies reported here showed that SC4 and ABH1 in the inner tissues of the fruiting bodies of *S. commune* and *A. bisporus*, respectively, were exclusively present at the boundaries of the extracellular matrix in which hyphae are embedded and the air channels that traverse the plectenchyma. On the basis of the properties of these hydrophobins as observed *in vitro* we assume that the plectenchyma hyphae secreted the monomers of these hydrophobins which diffused into the extracellular matrix and then assembled at the interface with the air as schematically depicted in Fig. 11. This would provide these air channels with a hydrophobic lining. Since the rise (h) of water in capillaries is proportional to cosθ (θ being the water contact angle) (Netter, 1969) any contact angle above 90° would result in a negative value of h, irrespective of the widths of the capillaries. The measured contact angles for assembled SC4 and ABH1 (115° and 113°, respectively) would then completely prevent water entering the air channels in the fruiting bodies. The hydrophobin coating would then prevent these channels from collapsing and filling with water under wet conditions, thereby remaining efficient conduits for gas exchange within the fruiting body plectenchyma. For instance, in *S. commune*, aerobic conditions are maintained throughout the fruiting body because respiration occurred with an RQ value around unity, much lower than in the supporting substrate mycelium (Wessels, 1965).

ABH1 was localized at the light microscopy level mainly on the outer tissue of the cap (pellicle and velum) (Lugones *et al*., 1996). Quantification of ABH1 extracted from different parts of the fruiting body showed that only 4·6% was present in the plectenchyma. In contrast to the pellicle and velum, the plectenchyma is composed of hyphae embedded in a hydrophilic mucilage. This reduces the surface of cell wall that contacts the air, and could explain the observed differences between fractions in the quantities of ABH1 mg^{-1} cell wall extracted from the different parts of the fruiting body (Lugones *et al*., 1996).

Upon assembly at a water–air interface, SC4 tremendously

lowered the surface tension. Both SC4 of *S. commune* and ABH3 from *A. bisporus* have been shown to substitute for SC3 when added to the culture medium of an *SC3*-disrupted strain of *S. commune* in allowing hyphae to breach the medium-air interface and to grow into the air (Lugones *et al*., 1988, H. A. B. Wösten, unpublished data). In the dikaryon with disrupted *SC3* genes, SC4 must be active in this respect because it is the only hydrophobin secreted into the medium in large amounts. However, such a role cannot be assigned to the ABH1 hydrophobin in *A. bisporus* because *ABH1* was not expressed in the substrate mycelium. Presumably, in *A. bisporus*, this is a role of the ABH3 hydrophobin which is secreted in abundance by both the heterokaryotic and homokaryotic vegetative mycelium of this species but is absent from the fruiting bodies (Lugones *et al*., 1998). In the *S. commune* heterokaryon, the switch between *SC3* and *SC4* expression depended on the proximity of nuclei of different mating type within the hyphae (Schuurs *et al*., 1998). Whether such a mechanism also controls expression of *ABH1* and *ABH3* in *A. bisporus* is unknown.

The question whether SC4 and ABH1 have properties that make them particularly suited for lining the air channels within fruiting bodies with a hydrophobic coat, cannot be answered at the moment. SC4 and ABH1 on the one hand and SC3 and ABH3 on the other hand, share amino acid sequence homologies not present between these two assemblies (Wösten & Wessels, 1997). Possibly, interactions between the hydrophilic wall or extracellular matrix compartments and the hydrophilic side of the assembled hydrophobin membrane play an important role in determining specificity. The functional significance of SC4 in fruiting-body formation is currently investigated by attempting disruption of the *SC4* genes in a dikaryon of *S. commune* and expressing the *SC3* gene under control of the *SC4* promoter.

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