An abundant hydrophobin (ABH1) forms hydrophobic rodlet layers in *Agaricus bisporus* fruiting bodies

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The SDS-insoluble protein fraction of *Agaricus bisporus* fruiting bodies was solubilized with trifluoroacetic acid. On SDS-PAGE this fraction was found to contain one abundant protein with an apparent M_r of 16 kDa. The N-terminal amino acid sequence of this protein was determined and RT-PCR used to isolate a cDNA clone which upon sequencing identified the protein as a typical class I hydrophobin (ABH1). The gene (*ABH1*) was isolated and sequenced, and a second hydrophobin gene (*ABH2*) was found about 2.5 kbp downstream of *ABH1*. Purified ABH1 self-assembled at hydrophobic-hydrophilic interfaces, producing the typical rodlet layer known from other hydrophobins. Similar rodlets were observed on the surface of the fruiting body, while immunological localization showed the hydrophobin to be particularly abundant at the outer surface of fruiting bodies, in the veil and in the core tissue of the stipe. Transcripts of *ABH1* were found only in fruiting-body hyphae. The ABH1 hydrophobin is probably solely responsible for the hydrophobicity of the fruiting-body surface but may also line air channels within fruiting bodies.

Keywords: *Agaricus bisporus*, hydrophobin, wall protein, mushroom, fruiting body

INTRODUCTION

Hydrophobins are small, moderately hydrophobic proteins secreted by fungi and characterized by a conserved spacing of eight cysteine residues and a typical hydropathy pattern (Wessels, 1994). Although sequences putatively encoding such proteins have been found in many species, only a few hydrophobins have been characterized. Those that have been studied exhibit interfacial self-assembly, that is, they form amphipathic protein films when confronted with a hydrophobic-hydrophilic interface, such as between air and water. On the basis of the solubility of these protein films two hydrophobin classes were distinguished (Wessels, 1994). Class I hydrophobins, exemplified by the SC3 hydro-

phobin of *Schizophyllum commune* (Schuren & Wessels, 1990), form very stable SDS-insoluble protein films (Wessels *et al.*, 1991; Wösten *et al.*, 1993, 1994a). Class II hydrophobins, exemplified by cerato-ulmin from *Ophiostoma ulmi* (Bowden *et al.*, 1994), form unstable SDS-soluble films (Richards, 1993).

The SC3 hydrophobin of *S. commune* is known to assemble at the surface of aerial hyphae and to cover these with a hydrophobic rodlet layer (Wessels *et al.*, 1991; Wösten *et al.*, 1993, 1994b). Genetic experiments have indicated that the hydrophobic rodlet layers on conidiospores of *Aspergillus nidulans* (Stringer *et al.*, 1991) and *Neurospora crassa* (Bell Pedersen *et al.*, 1992; Lauter *et al.*, 1992) also consist of hydrophobin assemblages. The occurrence of a class I hydrophobin-like protein secreted by the substrate mycelium of the edible mushroom *Agaricus bisporus* has been reported by de Vries *et al.* (1993). Here we report the isolation of a class I hydrophobin from the fruiting bodies of this organism. The gene (*ABH1*) encoding this protein and a closely linked putative hydrophobin gene (*ABH2*) were cloned. ABH1 was found to self-assemble at hydrophilic-hydrophobic interfaces, and to form a hydro-

Abbreviations: TFA, trifluoroacetic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; NBT, nitro blue tetrazolium chloride; RT, reverse transcriptase.

The GenBank accession numbers for the sequences reported in this paper are X92861 and X92860 for *ABH1* and *ABH2*, respectively.

phobic rodlet layer at the surface of the fruiting body and probably at surfaces of air spaces within the plectenchyma.

METHODS

Organism and culture conditions. Young *Agaricus bisporus* (cultivar U1 and a brown variant C9) fruiting body primordia measuring 10 mm in diameter (pinheads) and 25 mm (buttons) were kindly provided by Mr B. Boer (Mushroom Farm Agarica BV, Hoogeveen, The Netherlands). After picking they were immediately processed or frozen in liquid nitrogen and stored at -81°C . Substrate mycelium was obtained by inoculating 25 ml *Schizophyllum commune* minimal medium (Dons *et al.*, 1979) contained in plastic 9 cm Petri dishes with homogenized mycelium of *A. bisporus* cultivar U1 and growing at 24°C for 10 d.

Isolation and purification of ABH1. Frozen pinheads of *A. bisporus* were passed through an X-press. The fragmented material was quickly thawed and suspended in 10 vols hot washing buffer (1%, w/v, SDS, 0.1 M sodium phosphate buffer pH 7.0 at 100°C) and the suspension kept at 100°C for 10 min. After centrifugation the residue was washed six times with 8 vols water and freeze-dried. The dried residue was subsequently extracted with concentrated trifluoroacetic acid (TFA, 0.1 ml mg^{-1}) at 0°C . After removing solids by centrifugation, the TFA was removed from the supernatant with a stream of nitrogen and 60% (v/v) ethanol (0.2 ml per mg dried SDS-insoluble residue) was added to the residue. After standing overnight at room temperature, non-dissolved material was removed by centrifugation and the supernatant was freeze-dried. Before use, the purified ABH1 was treated with TFA as above and dissolved in water.

Gel electrophoresis of proteins. For SDS-PAGE, the TFA-treated material was taken up in sample buffer (50 mM Tris/HCl pH 6.8, 2% SDS, 10%, v/v, glycerol, 5%, v/v, 2-mercaptoethanol, 0.002% bromophenol blue) and the pH adjusted with 5% (v/v) ammonia to 6.8, if required. Electrophoresis was done in 12.5% (w/v) polyacrylamide gels according to Laemmli (1970). Gels were stained with either Coomassie Blue (Neuhof *et al.*, 1988) or silver (Merril *et al.*, 1981). For sequencing, immuno-detection and carbohydrate staining, proteins were transferred to PVDF membranes (Immobilon-P, Millipore) by semi-dry blotting (Multiphor II Electrophoresis System, Pharmacia) at 0.8 mA cm^{-2} for 2 h with electrophoresis buffer containing 20% ethanol.

For sequencing, blots were stained with Coomassie Brilliant Blue R250 in 10% (v/v) acetic acid, 30% (v/v) methanol, and de-stained in the same solvent. Amino-terminal sequencing was carried out on a cut-out band from the gel blot with a pulse liquid sequencer on-line connected to a phenylthiohydantoin analyser (Applied Biosystems).

For immuno-staining, blots were incubated in blocking solution [6.5 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 2.7 mM KCl and 150 mM NaCl, pH 7.2 (1× PBS); 0.1% Triton X-100; 5% (w/v) skim milk powder] for 2 h followed by incubation with ABH1 antiserum (1/2000 in the same buffer) for 1 h and then 1 h with goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Boehringer 605230, 1:6000 in the same buffer). After each incubation, the blots were washed five times with 1× PBS, 0.1% Triton X-100 for 5 min. Blots were then incubated with substrate solution containing 0.4 mM NBT (nitro blue tetrazolium chloride), 0.4 mM BCIP (5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt), 100 mM NaCl, 5 mM MgCl_2 and 100 mM Tris pH 9.5. Proteins in blots were

made visible by the silver staining method for blots described in the Phast System manual (Pharmacia). For detection of carbohydrates, blots were incubated for 1 h at room temperature in 0.5% (w/v) periodic acid, washed once for 5 min in 5% (v/v) acetic acid, and stained with Schiff reagent (Sigma).

Preparation of antiserum against ABH1. After electrophoresis and blotting to PVDF membrane, ABH1 was eluted with TFA from a strip of the membrane. After removing TFA the protein was used for immunization of rabbits. The recovered antiserum reacted specifically with ABH1 and not with other *A. bisporus* proteins, nor with the SC3 or SC4 hydrophobins of *S. commune*. Pre-immune serum was unreactive in all cases. For immunolocalization studies, the antiserum (diluted 1/100) was purified as described by Wösten *et al.* (1994b) by incubating three times for 60 min with a mixture of cell walls from submerged-grown mycelium of *A. bisporus* (1 mg ml^{-1}) and *S. commune* (1 mg ml^{-1}) prepared according to Sietsma *et al.* (1977).

RNA isolation. Frozen fruiting bodies (pinheads) were ground to a powder under liquid nitrogen in a mortar and RNA was extracted by the hot phenol procedure (Wessels *et al.*, 1987).

cDNA clone isolation. First strand synthesis was done in a 50 μl reaction mixture containing 2 μl total-RNA solution, 1 U reverse transcriptase (Superscript-RT, Gibco BRL), 125 μM of each of the four nucleotides, 290 nM oligo-dT (26-mer), 2 mM DTT, and 1 μl RNA-guard (Pharmacia) in 1× RT-buffer (supplied with the enzyme). Incubation was for 1 h at 37°C . After 2 min denaturation at 94°C , an aliquot was taken for PCR (2 μl per 100 μl reaction mixture). Reaction mixtures for PCR contained 0.1 U Supertaq-polymerase (HT Biotechnology) or 1 U Vent-polymerase (New England Biolabs) and buffer supplied by the manufacturers. PCR was performed in 100 μl 1× buffer with 125 μM of each nucleotide, 300 nM oligo-dT (26-mer with an additional *Xba*I restriction site) as reverse primer and 1 μM degenerate or 0.1 μM non-degenerate forward primer. The forward primers contained a *Sac*I site for easy cloning purposes. A moderately stringent annealing profile (1 min, 50°C) was used. Denaturation was at 94°C for 1 min and elongation was at 72°C for 1.5 min.

Gene cloning and DNA sequencing. A partial *Sau*3A library, in Lambda FIX II, of *A. bisporus* strain Horst^R U3 (Harmsen *et al.*, 1992) was screened using the ABH1 cDNA fragment as probe. Hybridizations were done as described by Schuren *et al.* (1993). Clones of the ABH1 cDNA and genomic subclones were constructed in pUC vectors or derivatives. Nucleotide sequences of the gene and the cDNA were determined by independently sequencing from both strands. Sequencing was performed with the T7 DNA polymerase kit from Pharmacia using the dideoxy chain-termination method (Sanger *et al.*, 1977).

Interfacial self-assembly. For assembly of ABH1 on the hydrophobic surface of polytetrafluoroethylene (PTFE-Teflon), Teflon discs of 0.5 cm diameter, 0.5 mm thick, were first cleaned in chromic acid and then incubated with an aqueous ABH1 solution for 1 h. The discs were subsequently extracted with 1% (w/v) SDS at 100°C , washed in distilled water, dried in air, and the hydrophobicity of the surface was measured by determining water contact angles (van der Mei *et al.*, 1991). Immuno-detection of the hydrophobin on the discs was basically done as indicated for blotted proteins but with the second antibody conjugated to horseradish peroxidase (BRL, 9814SA, 1:3000). Discs were then placed in the wells of a micro-titre plate and 200 μl substrate (*o*-phenylenediamine dihydrochloride, 0.2 mg ml^{-1} , 50 mM phosphate/citrate buffer pH 5.0) was added per well. The reaction was stopped by adding 50 μl 3 M HCl and A_{490} read with an ELISA microplate reader (Bio-Rad, model

450). For assembly of ABH1 on a hydrophilic surface an aqueous solution of the hydrophobin (0.2 mg ml^{-1}) was allowed to ascend in a filter paper strip (Schleicher & Schuell no. 0860, $20 \times 0.6 \text{ cm}$) in open air. After 18 h, allowing for evaporation of about 2 ml water, the paper strip was dried and extracted with 1% SDS at 100°C for 10 min, washed with water and dried for measurement of hydrophobicity. Surface hydrophobicity was estimated by measuring water contact angles.

Immuno-histochemistry. Fruiting body sections ($70 \mu\text{m}$) were cut with a microslicer (Slicetome MW-1000, Meiwa Shoji) under 1% (w/v) PVP (polyvinylpyrrolidone) and placed on glass microscope slides coated with chrome-alum-gelatin. The sections were firmly attached to the slides by exposure to formaldehyde vapours for 30 min and allowed to dry. Sections were placed in blocking solution (1 $\times$ PBS, 0.1% Triton X-100, 1% BSA, 1% PVP, 0.5% normal goat serum, 0.02% NaN_3) for 15 h and then for 15 h in a 1/1200 dilution of purified ABH1 antiserum in blocking solution. After three washes in 1 $\times$ PBS, 0.1% Triton X-100, they were incubated for 5 h in a 1/6000 dilution of goat-anti-rabbit antibodies conjugated to alkaline phosphatase in blocking solution. After six washes of 20 min each in 1 $\times$ PBS, 0.1% Triton X-100, NBT/BCIP was used to detect the enzyme as described for immuno-staining of blots. To differentiate the stain from dark pigments arising from oxidation of phenolic compounds within fruiting bodies, stained sections were treated with a 0.4% solution of sodium hypochlorite for 5 min.

Electron microscopy. For visualization of rodlets on the hydrophobic side of assembled ABH1, $5 \mu\text{l}$ of a solution of ABH1 (approx. $5 \mu\text{g ml}^{-1}$) was allowed to dry on Formvar-coated nickel grids. Surface shadowing with Pt/C was done at an angle of 45°C . For freeze-fracture, tissue pieces of 2 mm^3 were fixed in 4% (v/v) formaldehyde *in vacuo*, equilibrated with 50% aqueous glycerol, and then frozen in liquid-nitrogen-cooled propane. Freeze-fracturing was done in a freeze-etch unit (Balzers). Replicas were made using Pt/C and cleaned in $\text{K}_2\text{Cr}_2\text{O}_7$ -saturated H_2SO_4 for 90 min. Replicas were also made directly from the fruiting body surface. Replicas and shadowed material were examined in a Philips CM 10 electron microscope. Photographs were made on FGP Kodak film.

RESULTS

Isolation and sequencing of ABH1

Extraction of a hot-SDS-insoluble residue of *A. bisporus* fruiting bodies with TFA solubilized proteins which upon PAGE showed three main bands, running at 16, 24 and 33 kDa (Fig. 1, lane 1). By N-terminal amino acid sequencing of the 16 kDa band, the following sequence could be deduced: GKPKASSQCDVGEIHCCDTQQ-TPDHT. The underlined part was used for primer construction. The sequence C-(X_{aa})₆-C-C suggested the presence of a typical hydrophobin.

An antibody raised against the 16 kDa protein reacted with all three bands (Fig. 1, lane 3). Moreover the three bands were found again after re-running the 16 kDa protein (Fig. 1, lane 4). Apparently, the proteins running at 24 and 33 kDa represent multimers or different conformational states of the 16 kDa protein. Oxidation with performic acid produced a shift of the bands to a higher position on the gel (Fig. 1, lanes 1 and 2), as has been observed for other hydrophobins (de Vries *et al.*, 1993). Staining for carbohydrate was negative, indicating

Fig. 1. SDS-PAGE of the SDS-insoluble protein fraction of *A. bisporus* fruiting bodies. Lanes 1 and 2, stained with silver; lanes 3 and 4, immuno-staining with anti-ABH1. Lane 1, TFA treated; lane 2, performic acid treated; lane 3, TFA treated; lane 4, 16 kDa band extracted from gel and run again.

the absence of extensive glycosylation as present in the SC3 hydrophobin of *S. commune* (Ásgeirsdóttir, 1994).

Gene isolation and sequencing

In order to isolate the cDNA encoding the 16 kDa protein, a degenerate primer was synthesized based on the amino acid residues 10–17 of the N-terminal sequence (see above). This sequence was chosen because it allowed the construction of a primer with minimal degeneracy. The primer, 5'-CTCTAGAGCTCGA(T/C)GT(T/C/A/G)-GG(T/C/A/G)GA(A/G)AT(T/C)CA(T/C)TG(T/C)-TG-3', contained an additional *SacI* restriction site (underlined). During PCR with a reverse primer containing oligo-dT₂₆ and an *XhoI* site in addition, elongation was expected to start downstream of the cysteine cluster.

RT-PCR with a total RNA extract of *A. bisporus* pinheads produced a single band approximately 480 bp in length. A 300 bp *SacI*–*XhoI* fragment could be isolated containing an ORF for a protein with a second cysteine cluster in-frame with the amino acid sequence encoded by the forward primer. The deduced amino acid sequence of this cDNA identified a class I hydrophobin, referred to as ABH1 (Fig. 2). The RT-PCR fragment was used to screen a phage library in order to isolate the gene sequence. A 4.1 kbp *HindIII* fragment was found to contain the *ABH1* gene, and a putative second hydrophobin gene (*ABH2*) in addition (Figs 2 and 3). To identify the position of the first intron in *ABH1*, a complete cDNA clone was isolated by performing an RT-PCR using a primer which annealed at the deduced ATG start codon.

ABH2 shows an overall identity of 78% with *ABH1* (80% if only exons are compared). Both genes contain

ABH1

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1 Met Ile Ser Arg Val Leu Val Ala Ala 10 Leu Val Ala Leu Pro Ala Leu Val Thr
1 ATG ATC TCT CGC GTC CTT GTC GCT GCT CTC GTC GCT CTC CCC GCT CTT GTT ACT gtgagtt

                                     20 Ala Thr Pro Ala Pro Gly Lys
42 gat tgcattgcgcc tcgttggtg aaaaatctca ctctcttttg cag GCA ACT CCT GCT CCC GGA AAG

Pro Lys Ala Ser 30 Ser Gln Cys Asp Val Gly Glu Ile His Cys C 40
129 CCT AAA GCC AGC AGT CAG TGC GAC GTC GGT GAA ATC CAT TGC T gtaagtattt cccatgacac

                                     ys Asp Thr Gln Gln Thr Pro Asp His Thr
192 cggcgcactc catatcctca gcgactttct ttttag GC GAC ACT CAG CAG ACT CCC GAC CAC ACC

50 Ser Ala Ala Ala Ser Gly Leu Leu Gly Val 60 Pro Ile Asn Leu Gly Ala Phe Leu Gly Phe
257 AGC GCC GCC GCG TCT GGT TTG CTT GGT GTT CCC ATC AAC CTT GGT GCT TTC CTC GGT TTC

70 Asp Cys Thr Pro Ile Ser Val Leu Gly Val 80 Gly Gly Asn Asn Cys Ala Ala Gln Pro Val
317 GAC TGT ACC CCC ATT TCC GTC CTT GGC GTC GGT GGC AAC AAC TGT GCT GCT CAG CCT GTC

90 Cys Cys Thr Gly Asn Gln Phe
377 TGC TGC ACA GGA AAT CAA TTC gtaagttaga gcatttgccc tttgacttga ttttaacact atttttt

                                     Thr Ala Leu 100 Ile Asn Ala Leu Asp Cys Ser Pro Val Asn 110 Val Asn Leu ---
445 ctc tcttcag ACC GCA TTG ATT AAC GCT CTT GAC TGC TCT CCT GTC AAT GTC AAC CTC TAG

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ABH2

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1 Met Phe Ser Arg Val Leu Val Ala Ala 10 Leu Val Ala Leu Pro Val Leu Val Ser
1 ATG TTC TCT CGC GTC CTC GTC GCA GCT CTT GTT GCT CTC CCC GTT CTC GTT TCC gtgagtt

                                     20 Ala Ser Pro Thr Pro Gly Gly
42 gac taccctcatt gactgaaatc atttactcac cctctttcgt ag GCA AGT CCC ACT CCT GGC GGA

Tyr Pro Asp Ser 30 Thr Thr Val Val Ser Gln Cys Asn Val Gly 40 Glu Leu His Cys C
128 TAC CCT GAC TCC ACC ACC GTG GTT AGT CAA TGC AAC GTC GGT GAA CTC CAC TGC T gtaa

                                     ys Asn Thr Gln Gln Thr
187 gtctat tccatgacat ctatgtactc catatcctca gtgacttctt ccag GC AAC ACT CAG CAA ACC

50 Pro Asp His Thr Asn Ala Ala Gly Gly Leu 60 Leu Gly Ala Ala Ala Asn Val Gly Ala Leu
254 CCC GAC CAC ACC AAC GCC GCC GGC GGT CTG CTT GGT GCT GCC GCC AAC GTC GGC GCC CTC

70 Leu Gly Phe Asp Cys Thr Pro Ile Ser Val 80 Ile Gly Ile Gly Gly Asn Asn Cys Ala Ala
314 CTC GGT TTC GAC TGT ACC CCC ATT TCC GTC ATT GGC ATC GGT GGC AAC AAC TGT GCT GCT

90 Gln Pro Val Cys Cys Glu Ala Asn Glu Phe
374 CAG CCC GTC TGC TGT GAA GCC AAC GAA TTC gtaagttaga gcttctggtc tttgacttaa aatcta

                                     100 Thr Gly Leu Ile Asn Ala Leu Ser Cys Ser 110 Pro Ile
446 atact attctctctc tctctccct cag ACC GGG TTG ATT AAT GCT CTT AGC TGC TCT CCC ATC

Asn Val Asn Leu ---
504 AAT GTC AAC CTT TAG

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Fig. 2. Nucleotide sequences and deduced amino acid sequences of *ABH1* and *ABH2* of *A. bisporus*. The coding nucleotides are in upper-case letters and introns in lower-case letters. Both nucleotide and amino acid sequences are numbered with reference to the translation start point. Underlined are positions that differ in an allele isolated from the brown variant C9: Asp → Asn, Ala → Gly.

three short introns at the same positions of about 50 base pairs, with splice sites and internal splice signals which conform to the consensus sequences for introns of filamentous fungi (Gurr *et al.*, 1987). In both genes the middle intron splits the third cysteine codon (Fig. 2). The two hydrophobin genes code for proteins with lengths of 112 (*ABH1*) and 115 (*ABH2*) amino acids. After alignment the deduced amino acid sequences show 75 % identity and

7 % similarity in addition. The homology with hydrophobins from other species is much less. For instance the homology between *ABH1*, *ABH2* and the *S. commune* hydrophobins SC3 and SC4 is 34 % identity and 42.5 % and 36 % similarity, respectively, with more homology found in the C-terminal part (Fig. 4). Remarkable is the homology of a box of six amino acids in the N-terminal part of *ABH2* and SC3 (boxed amino acids in Fig. 4).

Fig. 3. Genomic organization of a 4.1 kbp *HindIII* fragment of *A. bisporus* DNA containing *ABH1* and *ABH2*. Restriction analysis, sequencing and hybridization with the *ABH1* cDNA showed the location of the two genes and the direction of transcription (arrows). Restriction sites: H, *HindIII*; X, *XhoI*; E, *EcoRV*; A, *ApaI*; E1, *EcoRI*; S, *SmaI*.

Another *ABH1* allele was found in the brown variant C9, which showed amino acid substitutions at two positions (Fig. 2).

The hydropathy plots of both deduced proteins are similar to those of other known class I hydrophobins (Fig. 5; see Wessels, 1994). *ABH1* and *ABH2* apparently contain hydrophobic signal sequences for secretion. Processing of *ABH1* takes place between a Pro and a Gly, as found in SC3 and SC4 (Schuren & Wessels, 1990; Wessels *et al.*, 1991); the same possibly also occurs in *ABH2*, but since the mature protein was not isolated this remains conjectural.

As in most genes of filamentous fungi (Gurr *et al.*, 1987), codon usage in *ABH1* and *ABH2* is strongly biased. At the third codon position a C or a T is preferred where possible and less than 10% of the codons end with G or A. This resembles the codon usage in other *A. bisporus* genes (van der Vlugt *et al.*, 1993).

Expression of hydrophobins

RT-PCR of RNA using the *ABH1* primer described above and oligo-dT as reverse primer showed a band of approximately 480 bp in the case of fruiting-body RNA

Fig. 5. Hydropathy plot of *ABH1*, *ABH2* and *SC3*. The sequences have been aligned at the first, fourth and fifth cysteine residue. The hydropathy values were calculated following Kyte & Doolittle (1982), using a window size of six amino acids.

only (Fig. 6, lanes 2 and 3). Using a primer specific for a mRNA from the substrate mycelium (see Discussion), a specific fragment of 440 bp was amplified from the

Fig. 4. Alignment of the *ABH1* and *ABH2* amino acid sequences with those of *SC3* and *SC4* from *S. commune* according to the CLUSTAL program of the PC/Genie program package, version 6.60 (Higgins & Sharp, 1988). Shaded amino acids indicate identities. The processing sites in *ABH1*, *SC3* and *SC4* are indicated by an arrow. A homologous box of six amino acids in the N-terminal part of *ABH2* and *SC3* is indicated.

Fig. 6. Agarose gel electrophoresis of RT-PCR products using an ABH1-specific primer (lanes 1–3) and a primer for an mRNA specific for the substrate mycelium (lanes 4–6). Lanes 1 and 4, substrate mycelium cDNA; lanes 2 and 5, fruiting-body cDNA (pinhead stage); lanes 3 and 6, fruiting-body cDNA (button stage).

mycelial RNA only (lanes 4). This shows the integrity of the RNAs from both sources.

In agreement with the PCR result, an *ABH1* cDNA only hybridized to fruiting-body RNA on Northern blots and not with mycelial RNA (results not shown). In addition, no ABH1 could be immunologically detected among proteins secreted by the substrate mycelium (results not shown). Together, these results strongly suggest that ABH1 is only expressed in fruiting bodies of *A. bisporus*. Since only one hydrophobin could be detected in the hot-SDS-insoluble protein fraction (N-terminal sequencing gave no indication of the presence of another hydrophobin), the *ABH2* gene, if coding for a class I hydrophobin, is apparently not, or is very poorly, expressed in fruiting bodies.

Localization of ABH1

Immuno-localization showed that ABH1 is present throughout the fruiting body (Fig. 7). Only the gills were completely unreactive to anti-ABH1. High reactivity was seen in the outer region of pileus and stipe, in the veil and in the core of the stipe. In the inner tissue of pileus and stipe an irregular pattern of weak staining was observed (Fig. 7).

To obtain quantitative information on ABH1 content in different fruiting body parts, a large fruiting body (180 g wet wt) was dissected into various fractions (Table 1). Hot-SDS-extracted residues were prepared as described in

Fig. 7. Immuno-localization of ABH1 in the fruiting body of *A. bisporus*. (a) One-day-old fruiting body (pinhead). (b) Three-day-old fruiting body (button). (c) Four-day-old fruiting body. Note that the roof of the hymenial cavity containing the gills appears free of ABH1; the bottom of the cavity represented by the veil is darkly stained. (d) Transverse section taken through the cap gill tissue of a four-day-old fruiting body. Bars, 0.5 cm.

Methods and equal amounts (by dry weight) of the residues were extracted with TFA. TFA extracts were run on SDS-PAGE and the relative amounts of ABH1 estimated by comparing the intensity of the 16 kDa band after staining with Coomassie Blue. The results (Table 1) confirm the qualitative results of immuno-localization. ABH1 turned out to be present in all fractions except the gills; 75% of ABH1 was found in the cap outer tissue, constituting only 10% of the fresh weight of the fruiting body. The inability to detect ABH1 in gill tissue containing immature spores indicates, but does not prove, the absence of ABH1 in these structures.

Interfacial assembly of ABH1

ABH1 was isolated and purified as described in Methods. Discs of Teflon were immersed in an aqueous solution of ABH1 (approx. $20 \mu\text{g ml}^{-1}$) for 16 h. 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° for uncoated to $63 \pm 8^\circ$ for coated Teflon, with lowest values of 51° . ABH1 antiserum

Table 1. Estimation of relative abundance of ABH1 in TFA extracts of hot-SDS-insoluble residues derived from different parts of a large fruiting body

Part of fruiting body	Wet wt (g)	SDS-insoluble residue		
		Dry wt (mg)	Relative amount of ABH1 (16 kDa protein)*	
			per mg	% of total
Outer cap layer	17.9	628	1.00	75
Inner cap	106.8	951	0.05	6
Gills	21.1	288	0.00	0
Outer stipe layer	3.2	92	0.25	3
Inner stipe	30.9	690	0.20	16
Total	179.1	2649	0.32	100

*Relative amounts of ABH1 were estimated by comparing intensities of the 16 kDa protein band after SDS-PAGE and Coomassie Blue staining of serial dilutions of TFA extracts from equal amounts (dry weight) of SDS-insoluble residues. Values are the means of two determinations, which deviated from each other by less than 10%.

reacted with ABH1 assembled on Teflon, showing antigenic epitopes at the hydrophilic side of the coating. After extraction of the ABH1-coated discs with 1% SDS at 100 °C for 10 min no decrease was observed in reaction with the antibody. However the contact angle values increased to $84 \pm 7^\circ$, with lowest values of 72° . For measuring contact angles at the hydrophobic side of an ABH1 film, paper strips were coated with ABH1 as described in Methods. The paper became hydrophobic in an area just beneath the evaporating front, where water contact angles of $113 \pm 4^\circ$ were measured after extraction with hot SDS. The outer surface of the fruiting body cap displayed water contact angles of up to 130° . After extraction of tissue strips with chloroform/methanol (2:1, v/v) at 65 °C (five times, 10 min), surface water contact angles were unchanged. The high hydrophobicity of the fruiting body surface would thus appear to be largely due to the ABH1 hydrophobin.

To investigate the ultrastructure of assembled ABH1, a solution of this protein was allowed to dry on Formvar-coated grids and shadowed. The surface, representing the hydrophobic face, showed a mosaic pattern of parallel rodlets 10 nm wide (Fig. 8a). To examine the presence of rodlets *in vivo*, fragments of outer and inner cap tissue were freeze-fractured and surface replicas viewed in the electron microscope. Patches with rodlets were generally observed in both cases (Fig. 8b, c) but it was not clear whether the rodlets were completely covering hyphal surfaces or only surfaces exposed to air. Replicas made directly from the surface of the outer cap tissue (without breaking) also exhibited rodlets (Fig. 8d). This proves that the ABH1 rodlet layer is the outermost stratum of the fruiting body pileus.

DISCUSSION

Genes encoding class I hydrophobins have mostly been identified by sequencing cDNAs corresponding to mRNAs that are highly expressed at certain stages of fungal development (Wessels, 1994). Only in the case of hydrophobin genes responsible for rodlet layers on conidia within related *Aspergillus* species has sequence similarity been used to isolate the homologous genes (Parta *et al.*, 1994; Thau *et al.*, 1994). On the other hand, genes for the class II hydrophobins cerato-ulmin and cryparin were cloned on the basis of knowledge about amino acid sequences (Bowden *et al.*, 1993; Zhang *et al.*, 1994). Since assemblages formed by class I hydrophobins are insoluble in hot SDS, in contrast to those formed by class II hydrophobins (Wessels, 1994), we decided to clone class I hydrophobin genes of *A. bisporus* starting with the N-terminal sequence of any protein resisting hot-SDS extraction.

A hot-SDS-insoluble complex that could be dissociated with TFA into a single 16 kDa protein was earlier found in the cell walls of vegetatively-growing *A. bisporus* by de Vries *et al.* (1993). In the present paper we describe the isolation and purification of a similarly-sized protein (ABH1) with all the properties of a class I hydrophobin from a hot-SDS-insoluble residue from fruiting bodies of this species. The cDNA sequence isolated on the basis of RT-PCR reveals the expected homology to class I hydrophobin genes cloned from *Schizophyllum commune* and other fungi, particularly with respect to spacings of cysteine residues and hydrophathy plots. In addition, sequencing of the genomic fragment that harbours the *ABH1* gene revealed a highly homologous second hydrophobin gene (*ABH2*) 2.58 kbp downstream of *ABH1*.

No transcript for the *ABH2* gene was found but the *ABH1* mRNA was abundant in the fruiting bodies. Whether *ABH2* is an inactive gene or is transcribed into a low-abundance mRNA requires further investigation. Significantly, the *ABH1* transcript appeared to be absent from the substrate mycelium, indicating that *ABH1* is specifically expressed in fruiting bodies. However, this substrate mycelium does secrete another class I hydrophobin into the culture medium (L. G. Lugones & J. G. H. Wessels, unpublished data). The gene (*ABH3*) for this hydrophobin is currently being cloned and a degenerate primer based on its N-terminal amino acid sequence detects an mRNA that is present in the substrate mycelium but not in the fruiting bodies (Fig. 6).

We have demonstrated that ABH1 assembles *in vitro* into an SDS-insoluble amphipathic protein film when confronted with hydrophobic-hydrophilic interfaces, as earlier shown for the SC3 hydrophobin of *S. commune* (Wösten *et al.*, 1993, 1994a, b). In fact, this is the second class I hydrophobin for which this remarkable property has now been reported. In addition, we have shown that ABH1, like the SC3 hydrophobin of *S. commune* (unpublished), can assemble at the surface of cellulose fibres, coating them with an SDS-insoluble layer that confers non-wettability (water contact angles 110°). We thus

Fig. 8. Ultrastructure of assembled ABH1. (a) *In vitro*-assembled ABH1 after shadowing. (b) Replica of the cap plectenchyma after freeze-fracturing. (c) Replica of the surface of the cap after freeze-fracturing. (d) Replica of the surface of the cap. Bars, 100 nm.

propose that ABH1 secreted by fruiting-body hyphae of *A. bisporus* self-assembles at the wall–air interface, coating these hyphae with an insoluble ABH1 film that exposes its rodlet-decorated hydrophobic side towards the air. This would be similar to the mechanism earlier proposed for formation of the hydrophobic rodlet layer on aerial hyphae of *S. commune* (Wösten *et al.*, 1994b). Indeed, immuno-histochemistry with an ABH1 antiserum and electrophoretic detection of ABH1 in dissected fruiting-body parts showed that ABH1 is most abundant at the surface of the fruiting body, while replicas of the cap surface suggest that the outer surface of the fruiting body is completely covered by a rodlet layer made of ABH1. This layer may be solely responsible for the non-wettability of the surface of the fruiting body. Its main function may be prevention of inflow of water from the outside and protection of the fruiting body against bacterial and fungal parasites. Whether the extremely thin hydrophobin layer (approx. 10 nm) prevents water loss from the fruiting body is doubtful. Because the hydrophobic outer stratum of the fruiting body should not impede metabolic gas exchange too much, we surmise that the hydrophobin layer is also freely permeable to water vapour.

It has been shown that the SC3 hydrophobin probably fulfils a similar function as ABH1 at the surface of fruiting bodies of *S. commune* while the SC3 hydrophobin is excluded from the inner tissue of fruiting bodies where another hydrophobin (SC4) is prevalent (Mulder & Wessels, 1986; Ásgeirsdóttir *et al.*, 1995). The SC4

hydrophobin assemblages seem to line walls of air channels that traverse the fruiting-body plectenchyma, probably conferring hydrophobicity to the channel walls and preventing water penetration (Wessels *et al.*, 1995). Localization of ABH1 shows that this hydrophobin is not confined to the surface but occurs throughout the fruiting body of *A. bisporus*. Within the fruiting body it is most abundant in the centre of the stipe, where hyphae are most loosely packed (Craig *et al.*, 1979), suggesting the presence of ABH1 where air is present. Freeze-fracture replicas show that rodlets occur inside the fruiting bodies (Fig. 8b), probably where air spaces occur within the plectenchyma. Apart from its role at the surface, ABH1 thus may fulfil a function within the plectenchyma of *A. bisporus* fruiting bodies similar to that played by SC4 in *S. commune*.

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