Interaction and Comparison of a Class I Hydrophobin from *Schizophyllum commune* and Class II Hydrophobins from *Trichoderma reesei*

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Hydrophobins fulfill a wide spectrum of functions in fungal growth and development. These proteins self-assemble at hydrophilic−hydrophobic interfaces into amphipathic membranes. Hydrophobins are divided into two classes based on their hydropathy patterns and solubility. We show here that the properties of the class II hydrophobins HFBI and HFBII of *Trichoderma reesei* differ from those of the class I hydrophobin SC3 of *Schizophyllum commune*. In contrast to SC3, self-assembly of HFBI and HFBII at the water−air interface was neither accompanied by a change in secondary structure nor by a change in ultrastructure. Moreover, maximal lowering of the water surface tension was obtained instantly or took several minutes in the case of HFBII and HFBI, respectively. In contrast, it took several hours in the case of SC3. Oil emulsions prepared with HFBI and SC3 were more stable than those of HFBII, and HFBI and SC3 also interacted more strongly with the hydrophobic Teflon surface making it wettable. Yet, the HFBI coating did not resist treatment with hot detergent, while that of SC3 remained unaffected. Interaction of all the hydrophobins with Teflon was accompanied with a change in the circular dichroism spectra, indicating the formation of an α-helical structure. HFBI and HFBII did not affect self-assembly of the class I hydrophobin SC3 of *S. commune* and vice versa. However, precipitation of SC3 was reduced by the class II hydrophobins, indicating interaction between the assemblies of both classes of hydrophobins.

Introduction

Hydrophobins are small secreted proteins that fulfill a variety of functions in fungal growth and development. They mediate the escape of fungi from the aqueous environment into the air by reducing the water surface tension and coat aerial structures such as spores and fruiting bodies (e.g., mushrooms), making them hydrophobic.[1−3] Moreover, they mediate attachment of hyphae (the filamentous cells of fungi) to hydrophobic surfaces[4,5] and affect the architecture of cell walls.[6] Hydrophobins have the potential to be used as surfactants and emulsifiers in food processing and as surface coatings in medical and technical applications.[7,8]

Hydrophobins have eight conserved cysteine residues, but the amino acid sequences are otherwise diverse.[9] On the basis of their hydropathy patterns and solubility characteristics, Wessels[10] discriminated between class I and class II hydrophobins. Hydrophobins self-assemble at hydrophilic−hydrophobic interfaces (e.g., between water and air, water and oil, or water and a hydrophobic solid like Teflon) into an amphipathic membrane. These membranes turn hydrophilic surfaces hydrophobic, while hydrophobic surfaces can be made hydrophilic.[5,11−15] Moreover, they reduce the water surface tension. With a maximal lowering of the water surface tension from 72 to 24 mJ m^{-2}, the class I hydrophobin SC3 of *Schizophyllum commune* is the most surface active protein known.[3,16]

Membranes of class I members are highly insoluble and can be dissociated with formic acid or trifluoroacetic acid (TFA).[17−19] Circular dichroism (CD) and attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy indicate that class I hydrophobins are rich in β-sheet structure in their soluble state.[16,20] Self-assembly at the water−air proceeds via an intermediate, called the α-helical state, to the stable end form that has increased β-sheet structure.[16,20,21] Initially, this form lacks a clear ultrastructure, but eventually, a mosaic of bundles of fibrils called rodlets can be observed.[14,16,21,22] At the interface between water and hydrophobic solid, hydrophobins are arrested in the intermediate α-helical state. The stable end form can be obtained by treating the interface with diluted detergent at elevated temperatures.[20,21]

Membranes of assembled class II hydrophobins dissociate more easily than those of class I hydrophobins.[23,24,25] The class II hydrophobins HFBI and HFBII of *Trichoderma reesei* adopt ordered structure upon assembly at the water−air interface.[26,27] The three-dimensional crystal structure of HFBII revealed that the hydrophobin is globular and amphiphilic.[28] The hydrophobic part consists mainly of amino acid residues near the loops of two β-hairpins. The hydrophilic part contains one α-helix. The structure of the hydrophobin is stabilized by four disulfide

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bridges, which occur between the cysteine residues as follows: C1−C6, C2−C5, C3−C4, and C7−C8.

S. commune is a filamentous fungus expressing at least four different class I hydrophobin genes.[29] *T. reesei* produces at least three class II hydrophobins: HFBI, HFBII, and HFBIII.[30−32] Apart from the genes encoding these three hydrophobins, several other hydrophobin genes can be found in the recently published genome of *T. reesei*.[33] The *hfb1* gene is expressed on glucose- and sorbitol-containing media but not on lactose and cellulose. In contrast, *hfb2*[30] and *hfb3* (E. Rintala and T. Nakari-Setälä, unpublished results) are expressed on all these carbon sources, but expression on glucose is low, if present at all. HFBI[31] and HFBIII (M. Linder and T. Nakari-Setälä, unpublished results) are associated with the cell walls of vegetative mycelium, whereas HFBII has been isolated from spores.[30] In addition, HFBI and HFBII have been found in the culture medium.[30,31]

Class I hydrophobins occur generally in *Ascomycetes* and *Basidiomycetes* (the two major groups of fungi), while the class II hydrophobins have only been isolated from *Ascomycetes*. Because of the low sequence similarity between class I and class II hydrophobins, it has been speculated that class II hydrophobins have evolved within *Acomycetes* independently of class I.[34] Similarities between these hydrophobin classes were suggested to show the phenomenon of convergent evolution. Interestingly, class I and class II hydrophobins appear to be coexpressed. The class I hydrophobin gene *MPG1* and the class II hydrophobin gene *MHP1* are both expressed during conidiation of the rice pathogen *Magnaporthe grisea*.[35,36] In the corn pathogen *Fusarium verticillioides*, the class I hydrophobins *HYD1* and *HYD2* and the class II hydrophobin *HYD3* are associated with microconidiation[37], while the class II hydrophobin gene *HCf-5* as well as the class I hydrophobin genes *HCf-1*, *HCf-2*, *HCf-3*, and *HCf-4* are upregulated during sporulation of the tomato pathogen *Cladosporium fulvum*.[38,39] These results suggest that class I and class II hydrophobins might be present simultaneously, for instance, on the cell surface or in the growth medium.

Here, we studied the properties of the class II hydrophobins HFBI and HFBII of *T. reesei* and compared them to those of the class I hydrophobin SC3 of *S. commune*. Moreover, we addressed whether HFBI and HFBII can interact with SC3.

Experimental Procedures

Proteins and Labeling. SC3 was purified as described[9,14] and labeled with dansyl according to Wang et al.[40] HFBI was extracted from mycelium of *T. reesei* HFBI-overproducing strain VTT D-98692[41] using 1% SDS at pH 5[42] or 9.[41] The protein was subsequently purified as described. HFBII was purified from the culture filtrate of *T. reesei* VTT D-74075 (QM9414)[43] cultivated at 29 °C in a laboratory fermenter (LF.20; Chemap, Switzerland) for 96 h with a working volume of 15 L, agitation of 500 rpm, and aeration of 10 L min^{-1}. The pH was adjusted with NaOH and H_3PO_4 to pH 4.5 ± 0.5. The medium consisted of KH_2PO_4 (4.0 g L^{-1}), $(NH_4)_2SO_4$ (2.8 g L^{-1}), and 15 L of centrifuged extract prepared by heating whey (30 g L^{-1}) and distiller's spent grain (wheat) (30 g L^{-1}) at 115 °C for 30 min. Foam generated by leading air through 1 L of the culture filtrate was collected and dissolved in 60 mL of 20% acetonitrile, containing 0.1% trifuoroacetic acid. The material was freeze-dried and dissolved in 8 mL of acetic acid. The solvent was replaced with water using gel filtration (Econo-Pac 10DG column, 10 mL, Bio-Rad). As a result, 9 mL of HFBII in water (5.2 g L^{-1}) was obtained.

Assembly of Hydrophobins. The assembly of hydrophobins at the water−air interface was studied by rotating 800 μL of hydrophobin solution either in water or in 25 mM Tris/HCl, pH 6.8 in 4 mL glass vials for 1−24 h on a rotary table. The concentrations used (100−200 μg mL^{-1}) are well above the critical concentration for SC3 assembly (i.e., 3 μg mL^{-1})[44] and are close to the concentrations of SC3, HFBI, and HFBII found in culture media.[3,41,45] Samples were examined directly by light microscopy or analyzed by SDS−PAGE after centrifugation for 10 min at 14 000 rpm (Eppendorf centrifuge 5415C, Merck, Germany).

To study emulsifying properties, 10 μL of Sudan black (Merck, Germany)-saturated olive oil or paraffin oil was emulsified in 1 mL of an aqueous hydrophobin solution (100 μg mL^{-1}) by sonication. Samples were placed on a glass slide, covered with a cover glass, and examined under a light microscope.

To assess the assembly of hydrophobin on a hydrophobic solid, 0.5 mm thick Teflon sheets (FEP, Norton Fluoroplast B.V, The Netherlands) were cleaned as described[5] and incubated overnight at room temperature in closed glass vials containing 4 mL of hydrophobin solution. The amount used (100 μg mL^{-1}) represents a 1000-fold excess of SC3 required to coat the Teflon surface.[5] The protein was dissolved in water or in buffer (100 mM sodium citrate buffer, pH 3; 100 mM sodium acetate buffer, pH 5; 100 mM sodium phosphate buffer, pH 7; and 100 mM Tris/HCl buffer, pH 9). After incubation, Teflon pieces were washed 3 times for 10 min with water using a rotary shaker with or without prior extraction for 10 min with 2% SDS at 100 °C. For assembly at the paper−air interface, the lower end of vertical filter paper strips were immersed into hydrophobin solution (100 μg mL^{-1} in 3 mL of 60% ethanol or water) essentially as described by Lugones et al.[11] The solution was allowed to rise up along the strip overnight.

Protein Analysis. Electrophoresis was carried out in 17.5% SDS polyacrylamide gels followed by staining with Coomassie Brilliant Blue. Assembled hydrophobin was dissociated with TFA prior to SDS−PAGE. SDS sample buffer was added after evaporating the solvent by a stream of air. The pH of the samples was adjusted with 25% ammonia, if necessary.

Water Contact Angles and Surface Activity. Wettability of bare and hydrophobin-coated solids was assessed by determining the contact angle of water droplets. Surface tension was determined by axisymmetric drop-shape analysis by profile (ADSA-P) as described by Noordmans and Busscher.[46] A 100 μL drop of aqueous solution of hydrophobin (1−200 μg mL^{-1}) was placed on a cleaned Teflon surface in a water-vapor saturated chamber. Profiles of the drop were digitized with a contour monitor at room temperature during approximately 8 h. The surface tension was calculated from the data based on the principle that the shape of the drop is determined by the counteracting forces of surface tension and gravity.

Circular Dichroism Spectroscopy. The circular dichroism (CD) spectra were measured on an Aviv 62A DS CD spectrometer in the far-UV region under continuous nitrogen flush using a 1 mm quartz cuvette. Protein concentration of the samples in water was 100 μg mL^{-1}. CD spectra of hydrophobins assembled at the water−air interface were obtained by vigorous shaking on a vortex for 2 min. The Teflon−water interface was created by adding 198 nm colloidal Teflon (PFA fluoropolymer resin, type 9940, DuPont, Switzerland) to the aqueous hydrophobin samples. Teflon was filtered through glass wool prior to use and added to the samples giving sufficient surface area for hydrophobin attachment (surface coverage about 10%). The CD spectra of the wavelength scans were recorded using a band and step width of 1 nm using averages of 10 scans. The thermostability of hydrophobins was studied by measuring the ellipticity at 203 nm over the temperature range from 25 to 90 °C, in steps of 5 °C. The CD spectra were also recorded at 90 °C between the wavelengths of 190−250 nm. The reference measurements with water were used to correct the data. Mean residue ellipticity $[\theta]$ (deg cm^2 $dmol^{-1}$) was calculated from the equation $[\theta] = M\theta_{obs}/(10lcn)$ where M is molecular mass, θ_{obs} is observed ellipticity in degrees, l is the cell path length in centimeters, c is the protein concentration in grams per cubic centimeter, and n is the number of amino acid residues. The spectra were not smoothed.

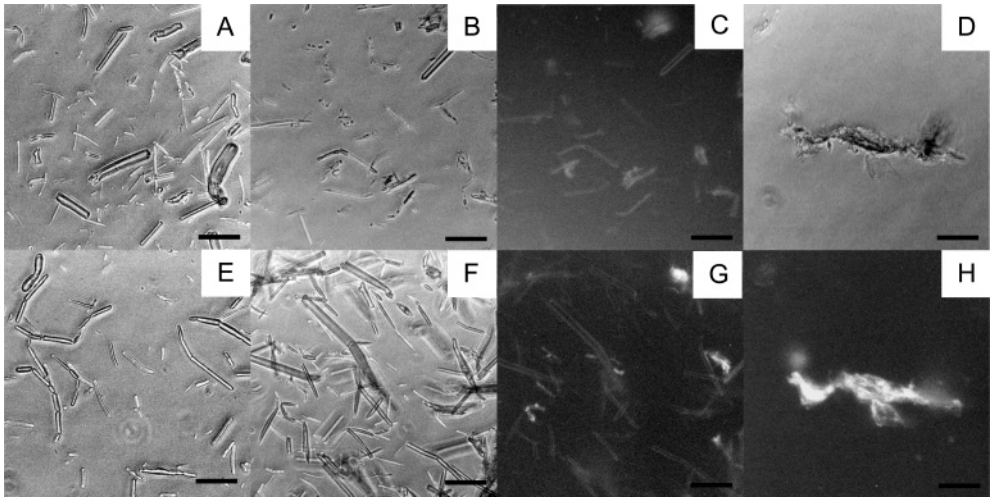

Figure 1. Class I and II hydrophobins after assembly at the water−air interface. Light microscopy of assembled class II hydrophobins HFBI (A) and HFBII (E). Light and fluorescence microscopy of dansyl-labeled class I hydrophobin SC3 in the absence of class II hydrophobins (D and H) and in the presence of HFBI (B and C) and HFBII (F and G), respectively. Bar represents 250 μm.

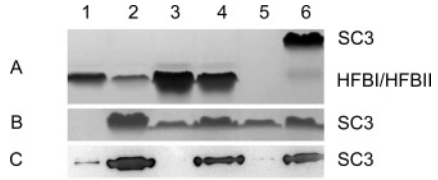

Figure 2. SDS−PAGE analysis of water-soluble and -insoluble fractions after assembly of class I and II hydrophobins at the water−air interface. Samples were centrifuged prior to SDS PAGE analysis. (A) Supernatant (lanes 1, 3, and 5), foam (lanes 2 and 4), and pellet (lane 6) of fractions containing HFBI (lanes 1 and 2), HFBII (lanes 3 and 4), and SC3 (lanes 5 and 6). (B) Supernatant (lanes 1, 3, and 5) and pellet (lanes 2, 4, and 6) fractions containing SC3 in the absence of class II hydrophobins (lanes 1 and 2) or in the presence of HFBI (lanes 3 and 4) or HFBII (lanes 5 and 6). Only SC3 is shown. (C) Same as panel B, but SDS sample buffer was added prior to centrifugation.

Electron Microscopy. A total of 3−5 μL of aqueous hydrophobin solutions (10−50 μg mL^{-1}) were dried on electron microscope grids. Surfaces were shadowed with platinum and carbon at an angle of 45° and examined with a CM10 electron microscope (Philips, The Netherlands).

Results

Hydrophobin Assembly by Mixing with Air. Aqueous solutions (100−200 μg mL^{-1}) of the class II hydrophobins HFBI and HFBII of *T. reesei* and the class I hydrophobin SC3 of *S. commune* were rotated to induce self-assembly at the water−air interface. As a result, HFBI and HFBII solutions obtained a milky appearance. In contrast, the SC3 sample remained transparent but contained particles. Microscopic examination revealed that both class II hydrophobins formed needlelike structures (Figure 1A,E), which seemed to have air trapped inside. In contrast, the class I hydrophobin SC3 formed aggregates as was shown previously.[14] The rotated hydrophobin samples were centrifuged, and pellet and supernatant fractions were analyzed by SDS−PAGE. Centrifugation cleared the opaque solutions of HFBI and HFBII, although some material collected at the surface of the water. Both hydrophobins were mainly found in the supernatant fraction (Figure 2A, lines 1 and 3), while a smaller fraction was found in the foam that collected at the water interface during centrifugation (Figure 2A, lines 2 and 4). After adding water to the foam and centrifugation, no pellet was found, showing that also this material was soluble. All hydrophobin was found in the supernatant fraction when instead of centrifugation, 60% (v/v) ethanol or 2% SDS were added or when the solution was placed at the bench for several hours (data not shown). In contrast, SC3 was found in the pellet fraction after centrifugation (Figure 3A, line 6). SC3 in the pellet could only be dissolved in SDS sample buffer after a TFA treatment.

To see whether class I and II hydrophobins influence each other's assembly, mixtures of the hydrophobins (100 μg mL^{-1} each; i.e., 13, 14, and 9 μM HFBI, HFBII, and SC3, respectively) were rotated as described previously. Light microscopy revealed aggregates, typical for those observed after assembly of SC3, as well as needlelike structures similar to those observed after self-assembly of HFBI and HFBII. These needles were fluorescent when HFBI or HFBII was coassembled with dansyl-labeled SC3 (Figure 1C,G). Therefore, part of the SC3 was present in the assemblages of HFBI and HFBII. SDS−PAGE revealed that a significant part of SC3 (approximately 30−40%) was found in the supernatant fraction when it was mixed with HFBI or HFBII (Figure 2B, lines 3 and 5), while variable amounts of the class II hydrophobins were found in the pellet fraction (not shown). Apparently, class I and II hydrophobins interact with each other. To further analyze this phenomenon, SDS sample buffer was added immediately after mixing (i.e., prior to centrifugation). SC3 was now mainly found in the pellet fraction (Figure 2C, lines 2, 4, and 6), while the class II hydrophobins were soluble. From these data, we conclude that SDS dissociated the class I and II hydrophobins, allowing the class I hydrophobin to precipitate upon centrifugation.

Surface Activity. The surface activity of HFBI and HFBII was compared with that of SC3. HFBI, HFBII, and SC3 reduced the water surface tension from 72 mJ m^{-2} to 42, 35, and 27 mJ m^{-2}, respectively, at 100 μg mL^{-1} (Figure 3A). The lowest surface tension of HFBI (37 mJ m^{-2}) and HFBII (28 mJ m^{-2}) was measured at a concentration of 200 and 20 μg mL^{-1}, respectively (Figure 3A). Different times were needed to reach the minimum surface tension with the different hydrophobins (Figure 3B). The final equilibrium value was obtained almost instantly with HFBII at a concentration of 100 μg mL^{-1}, while it took up to several minutes in the case of HFBI. In contrast, equilibrium was observed only after several hours in the case of SC3. This was also observed when SC3 (200 μg mL^{-1}) was

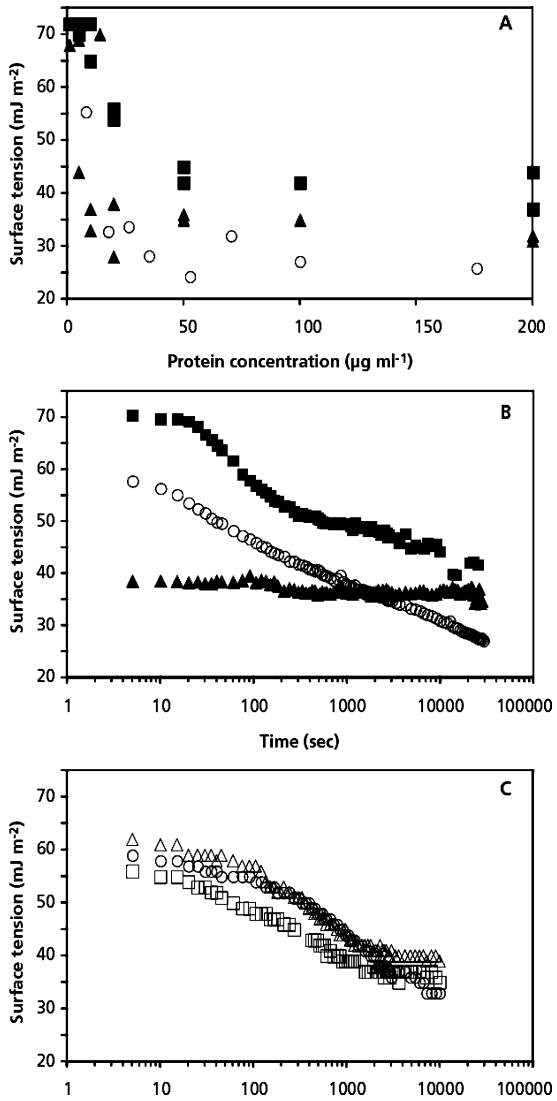

Figure 3. Surface tension of water containing HFBI (■), HFBII (▲), SC3 (○), HFBI and SC3 (□), or HFBII and SC3 (△) as a function of concentration (A) or time (100 μg mL^{-1} of each hydrophobin) (B and C).

mixed 1:1 with either HFBI (200 μg mL^{-1}) or HFBII (200 μg mL^{-1}) (Figure 3C).

Emulsification of Oil with HFBI, HFBII, and SC3. Olive oil and paraffin (1% v/v) were emulsified in an aqueous hydrophobin solution (100 μg mL^{-1}) by sonication. HFBI and SC3 consistently stabilized oil droplets in water more efficiently than HFBII. The HFBII emulsions reproducibly started to clear within an hour, and separation of oil and water was completed after 24 h. In contrast, emulsions made with HFBI did not clear even after 3 days, although a large part of the dyed oil droplets had collected at the top of the solution. Paraffin emulsions stabilized by SC3 were still fine after 3 days, but droplets of olive oil had collected at the top of the water. However, the oil droplets readily dispersed by shaking. The diameter of oil droplets stabilized by HFBI, HFBII, and SC3 were 10 $\pm$ 7, 34 $\pm$ 20, and 8 $\pm$ 5 μm (average of 60 measurements of duplicate experiments $\pm$ standard deviation) for paraffin oil and 7 $\pm$ 6, 44 $\pm$ 35, and 10 $\pm$ 7 μm for olive oil, respectively. As indicated by the standard deviations, sizes varied considerably in all cases.

Binding of HFBI, HFBII, and SC3 to a Solid Surface. Binding to a Hydrophobic Surface. Binding of HFBI and HFBII to Teflon was compared with that of SC3. Teflon pieces that had been incubated overnight in a HFBI or a SC3 solution (100 μg mL^{-1}) and rinsed with water were wettable with water contact angles of 59 $\pm$ 13 and 40 $\pm$ 4°, respectively. In contrast, Teflon incubated with HFBII showed water contact angles similar to those of bare Teflon (109 $\pm$ 2 and 110 $\pm$ 2°, respectively). Wettability slightly decreased after boiling SC3-coated Teflon in 2% SDS (53 $\pm$ 7°). In contrast, HFBI-coated-Teflon became water repellent after this treatment and showed a water contact angle similar to that of bare Teflon (108 $\pm$ 2°). This shows that the HFBI layer resists washes with water but dissociates in the presence of SDS. Coating with HFBI was strongly reduced when buffers (pH 3−9 containing 100 mM salt each) instead of water were used. Coating of SC3 was not affected.

Teflon incubated with a mixture of SC3 and HFBI showed water contact angles of 60 $\pm$ 5 and 72 $\pm$ 15° before and after treating with hot 2% SDS. These values are intermediate between those of incubations with pure SC3 or pure HFBI. Apparently, both hydrophobins were present at the Teflon surface, the latter dissociating upon treatment with SDS. Teflon incubated with SC3 and HFBII showed water contact angles of 58 $\pm$ 12 and 56 $\pm$ 8° before and after treating with hot 2% SDS. The latter value is similar to that obtained with pure SC3, showing that SC3 mainly coats the Teflon. Possibly, HFBII binds to the class I hydrophobin.

Binding to a Hydrophilic Surface. HFBI, HFBII, and SC3 solutions (100 μg mL^{-1} in 60% ethanol) were allowed to rise up along vertical strips of filter paper overnight. SC3 bound over the whole length of the paper strip resulting in water contact angles between 100 and 130°. Thus, SC3 made the surface highly hydrophobic. HFBI and HFBII reduced the hydrophilicity of paper only at the liquid front where the water contact angles were 60−64 and 60−70°, respectively. Similar results were obtained when the class II hydrophobins were dissolved in water instead of 60% ethanol.

Structural Changes Upon Mixing with Air and Binding. CD spectroscopy was used to study the secondary structure of water-soluble and assembled HFBI and HFBII. CD spectra of the class II hydrophobins in water (100 μg mL^{-1}) had a maximum close to 190 nm and a minimum close to 200 nm (Figure 4A,B, thick line). Contrary to SC3, no changes in the CD spectra of HFBI and HFBII were observed after vigorous shaking of the solutions (Figure 4A−C, dashed line). Only a slight alteration of intensity was seen, which was due to accumulation of protein at the liquid−air interface. In contrast, when colloidal Teflon was added, the CD spectra of both HFBI and HFBII changed (Figure 4A,B, thin line). Intensity of the signal at 190 nm increased. In contrast, the negative band lost intensity and shifted to 205−207 nm. A weak negative band close to 225 nm appeared, especially in the case of HFBI. These changes indicate formation of α-helical structure.

Protein Stability. CD spectra of HFBI and HFBII in water (100 μg mL^{-1}) were similar at 25 and 90 °C, except for a slight decrease in the intensity of the spectrum at the higher temperature (data not shown). Ellipticity measurements over the temperature range of 25−90 °C at minimum ellipticity wavelength (203 nm) also showed no changes. Similar results have been reported for SC3,[47] showing that both class I and II hydrophobins are stable at high temperature.

Electron Microscopy of Hydrophobin Membranes. Aqueous solutions of hydrophobins (3−5 μL, 10−50 μg mL^{-1}) were dried down on electron microscope grids and surface shadowed. A surface without an apparent ultrastructure was observed in the case of HFBI and HFBII (Figure 5A,B). In contrast, SC3 formed a mosaic of bundles of rodlets (Figure 5C).[14] When SC3

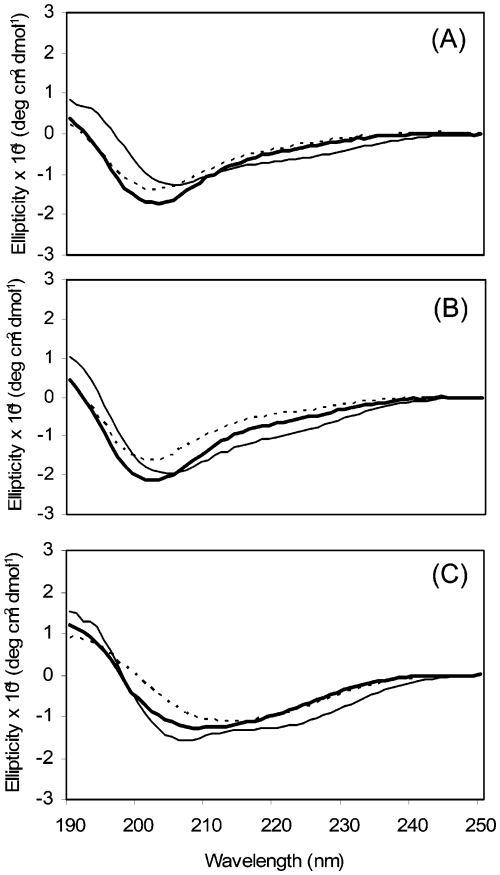

Figure 4. CD spectra of the class II hydrophobins HFBI (A) and HFBII (B) and the class I hydrophobin SC3 (C) in water (thick line), after vigorous shaking (dashed line) and after binding to Teflon (thin line). CD spectra are averages of 10 scans.

was dried down with either HFBI or HFBII (3 μL, 20 μg mL^{-1} of each hydrophobin), regions of rodlets were observed surrounded by hydrophobin without an apparent ultrastructure (Figure 5D,E). We conclude that the class II hydrophobins do not abolish, or at least not completely, the assembly of SC3. They compete for the available interface.

Discussion

Class I hydrophobins have been reported to have similar properties, but differences have been observed in the degree of surface activity and the wettability of the hydrophilic side of the membrane.[1,16] Specificity of lectin activities may differ as well as was shown for the class I hydrophobins SC3 and SC4 of *S. commune*.[48] In this paper, we show that properties of the class II hydrophobins HFBI and HFBII of *T. reesei* differ from those of the class I hydrophobin SC3. Moreover, we present evidence that the class II hydrophobins of *T. reesei* do not coassemble with the class I hydrophobin SC3. Yet, assemblies of these hydrophobins do seem to interact.

Mixing of an aqueous HFBI and HFBII solution with air resulted in needlelike structures that seemed to have air trapped inside. This suggests formation of a hydrophobin membrane around the air bubbles stabilizing their structure. These structures readily disappeared by applying pressure or by adding ethanol, and the proteins were found to be soluble in solution. Similar results were shown for the class II hydrophobins CU and CRP.[23,24,49,50] CD spectra of dissolved HFBI and HFBII were similar to those after assembly at the water−air interface. The needlelike HFBII aggregates have been shown to have a monoclinic crystalline structure, and they are suggested to be composed of packed HFBII tetramers.[51] Surface shadowing revealed that dried assemblages of HFBI and HFBII do not have an apparent ultrastructure. Similar results were obtained with the class II hydrophobins CRP and CFTH1.[16,52] In contrast, class I hydrophobins organize themselves in a highly insoluble membrane consisting of a mosaic of amyloid-like parallel rodlets.[1,14,16,22,53,54] Rodlet formation of SC3 was severely affected when it was dried on a solid support in the presence of HFBI or HFBII. Patches of SC3 rodlets were observed by electron microscopy surrounded by regions without apparent ultrastructure, probably representing assembled class II hydrophobins. These unstructured regions may also contain SC3. After mixing dansyl-labeled SC3 with air in the presence of either HFBI or HFBII, the fluorescently labeled class I hydrophobin was found in aggregates, typical for assembled class I hydrophobin, but also in the typical needles that are observed by light microscopy upon assembly of class II hydrophobins. Despite its presence in the class II assemblages, all SC3 was insoluble in SDS, showing that the class I hydrophobin had self-assembled. Taken together, we conclude that class I and II hydrophobins assemble independently of each other and compete for the available interface. However, class II hydrophobins seem to affect precipitation of class I hydrophobins. Thus, assemblages of these classes of hydrophobins do seem to interact.

HFBI and HFBII were both surface active. Minimal water surface tension of HFBI and HFBII was 37 and 28 mJ m^{-2} at 200 and 20 μg mL^{-1}, respectively. These activities are in the range that has been reported for class I and II hydrophobins.[16] Interestingly, HFBII lowered the water surface tension almost instantly and thus behaved like a conventional surfactant.[55] In contrast, it took up to several minutes to reach the minimal surface tension in case of HFBI. Yet, this was much quicker than SC3, which needs several hours to reach the equilibrium value. These results may explain why HFBII has the most pronounced effect on foaming of the culture medium of *T. reesei*.[56]

SC3 has been shown to form coatings around oil droplets in water.[5] Similar to SC3, HFBI stabilized both olive and paraffin oil-in-water emulsions very effectively. In contrast, the emulsions of HFBII cleared within 24 h. Recently, HFBII has been shown to stabilize polyunsaturated fatty acid (PUFA) oil-in-water emulsions for 24 h.[57] Therefore, the ability of HFBII to stabilize emulsions depends on the oil used. Like the class II hydrophobin CRP,[16] HFBI adsorbed to the surface of Teflon, making this hydrophobic solid wettable. This interaction resists washes with water but not treatment with 2% SDS at 100 °C. CD indicated that HFBII also interacts with the Teflon surface because the α-helix signature was induced both in HFBI and in HFBII upon adding Teflon to the hydrophobin solution. However, the interaction of HFBII with the Teflon surface did not resist washes with water. These data and studies by Linder et al.[58] show that both HFBI and HFBII adsorb to hydrophobic solids but that the interaction of HFBII seems to be weaker than that of HFBI.

It is tempting to speculate that the distinct properties of the class II hydrophobins as well as their behavior when mixed with a class I hydrophobin have biological consequences. It has been reported that both hydrophobin classes are coexpressed during sporulation,[35−39] and this may also occur during other developmental processes. Lowering of the water surface tension is a prerequisite for fungal aerial growth. Both class I and II hydrophobins are expected to fulfill this function. The first aerial hyphae of *S. commune* were formed when the medium surface

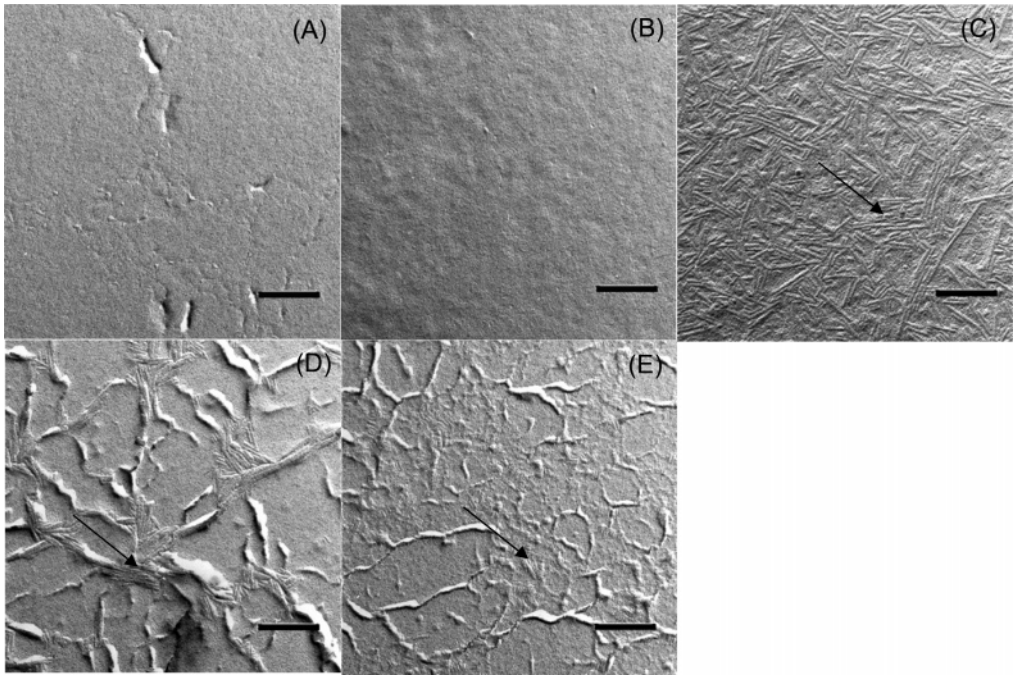

Figure 5. Electron micrographs of the class II hydrophobins HFBI (A) and HFBII (B) and the class I hydrophobin SC3 (C) as well as mixtures of HFBI and SC3 (D) and HFBII and SC3 (E). Hydrophobin solutions (3 μL, 20 μg mL^{-1} of each hydrophobin) were dried down on electron microscope grids. Bar represents 200 nm. Arrows indicate rodlets.

tension was reduced from 72 to 44 mJ m^{-2}. However, most hyphae escaped into the air at 30 mJ m^{-2}.[3] The fact that HFBII is more surface active than HFBI, at least when measured in water, would thus predict that the former is more active in aerial hyphae formation. However, a mutant with a deleted *hfb1* gene did not produce any aerial hyphae when grown on glucose, implying that HFBI does have a role in escape of hyphae.[59] This can be explained by the fact that under these conditions, HFBI accumulates in the culture medium of wild-type strains to a level exceeding 100 μg mL^{-1},[56] while production of HFBII is low if detected at all.[30] This high amount of HFBI would be sufficient to reduce the water surface tension well below 44 mJ m^{-2}. Our results indicate that in the case that equal amounts of a class I and II hydrophobins are secreted, the kinetics of the reduction of the water surface tension is determined by the class I hydrophobin.

Hydrophobins coat fungal structures, once they have escaped into the air. From our data, we can predict that like SC3, HFBI and HFBII would be able to produce such a coating. What happens if both a class I and a class II hydrophobin are secreted by an aerial structure, like in *C. fulvum*, *F. verticillioides*, and *M. grisea* during sporulation?[35−39] Our results indicate that class I and II would compete at the interface. They may interact with each other and form mixed membranes with patches of assembled class I and II hydrophobins. Recently, it was shown that the class I hydrophobin SC3 forms a semipermeable membrane.[60] Whether class II hydrophobins form a similar membrane is not yet known.

Apart from enabling escape into the air and coating aerial structures, hydrophobins mediate attachment to hydrophobic surfaces.[4,5] From the data presented in this paper, it seems unlikely that HFBII has a role in adherence. Whether HFBI has such a role has to be established. At least, the expression of *hfb1* on the cell wall of *Saccharomyces cerevisiae* has been found to increase binding of the yeast on hydrophobic silicone-based surfaces.[61] It would be most interesting to address whether class II hydrophobins can complement each other as effectively as class I hydrophobins do.[51,62] Our findings predict that this would not be the case.

Conclusion

We have shown that the properties of the class II hydrophobins HFBI and HFBII of *T. reesei* differ from those reported for class I hydrophobins. The membrane formed by the class II hydrophobins at a hydrophilic−hydrophobic interface is less stable than that of class I. Moreover, the assembly of class II hydrophobins at the water−air interface is not accompanied by a change in secondary structure and ultrastructure. The absence of a conformational change may explain why class II hydrophobins reduce the water surface tension more rapidly. Differences were also observed between HFBI and HFBII. HFBII is a stronger surface-active molecule, whereas HFBI more effectively stabilizes oil emulsions and interacts stronger with the hydrophobic solid Teflon. HFBI and HFBII did not affect self-assembly of the class I hydrophobin SC3 of *S. commune* and vice versa. However, assemblies of the class I and class II hydrophobins do seem to interact, resulting in a mixed membrane consisting of patches of assembled class I and II hydrophobins.

The variability of the properties of class II hydrophobins and the finding that mixed hydrophobin membranes can be formed indicate that hydrophobins may fulfill even more functions than have already been described. Variability of properties within the hydrophobin class of proteins is also of interest for use in technical and medical applications.

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References and Notes

(1) Wösten, H. A. B. *Annu. Rev. Microbiol.* **2001**, *55*, 625.
(2) Wösten, H. A. B.; Wessels, J. G. H. *Mycoscience* **1997**, *38*, 363.
(3) Wösten, H. A. B.; van Wetter, M.-A.; Lugones, L. G.; van der Mei, H. C.; Busscher, H. J.; Wessels, J. G. H. *Curr. Biol.* **1999**, *9*, 85.
(4) Temple, B.; Horgen, P. A.; Bernier, L.; Hintz, W. E. *Fungal Genet. Biol.* **1997**, *22*, 39.
(5) Wösten, H. A. B.; Schuren, F. H. J.; Wessels, J. G. H. *EMBO J.* **1994**, *13*, 5848.
(6) van Wetter, M.-A.; Wösten, H. A. B.; Sietsma, J. H.; Wessels, J. G. H. *Fungal Genet. Biol.* **2000**, *31*, 99.
(7) Linder, M.; Szilvay, G.; Nakari-Setälä, T.; Penttilä, M. *FEMS Microbiol. Rev.* **2005**, *29*, 877.
(8) Hektor, H. J.; Scholtmeijer, K. *Curr. Opin. Biotechnol.* **2005**, *16*, 434.
(9) Wessels, J. G. H. *Adv. Microbiol. Physiol.* **1997**, *38*, 1.
(10) Wessels, J. G. H. *Annu. Rev. Phytopathol.* **1994**, *32*, 413.
(11) Lugones, L. G.; Bosscher, J. S.; Scholtmeijer, K.; de Vries, O. M. H.; Wessels, J. G. H. *Microbiology* **1996**, *142*, 1321.
(12) Lugones, L. G.; Wösten, H. A. B.; Birkenkamp, K. U.; Sjollema, K. A.; Zagers, J.; Wessels, J. G. H. *Mycol. Res.* **1999**, *103*, 635.
(13) Lugones, L. G.; Wösten, H. A. B.; Wessels, J. G. H. *Microbiology* **1998**, *144*, 2345.
(14) Wösten, H. A. B.; de Vries, O. M. H.; Wessels, J. G. H. *Plant Cell* **1993**, *5*, 1567.
(15) Wösten, H. A. B.; Ruardy, T. G.; van der Mei, H. C.; Busscher, H. J.; Wessels, J. G. H. *Colloids Surf.* **1995**, *5*, 189.
(16) Wösten, H. A. B.; de Vocht, M. L. *Biochim. Biophys. Acta* **2000**, *1469*, 79.
(17) de Vries, O. M. H.; Fekkes, M. P.; Wösten, H. A. B.; Wessels, J. G. H. *Arch. Microbiol.* **1993**, *159*, 330.
(18) Wessels, J. G. H.; de Vries, O. M. H.; Ásgeirsdóttir, S. A.; Schuren, F. H. J. *Plant Cell* **1991**, *3*, 793.
(19) Wessels, J. G. H.; de Vries, O. M. H.; Ásgeirsdóttir, S. A.; Springer, J. *J. Gen. Microbiol.* **1991**, *137*, 2439.
(20) de Vocht, M. L.; Scholtmeijer, K.; van der Vegte, E. W.; de Vries, O. M. H.; Sonveaux, N.; Wösten, H. A. B.; Ruysschaert, J.-M.; Hadziioannou, G.; Wessels, J. G. H.; Robillard, G. T. *Biophys. J.* **1998**, *74*, 2059.
(21) de Vocht, M. L.; Reviakine, I.; Ulrich, W.-P.; Bergsma-Schutter, W.; Wösten, H. A. B.; Vogel, H.; Brisson, A.; Wessels, J. G. H.; Robillard, G. T. *Protein Sci.* **2002**, *11*, 1199.
(22) Wösten, H. A. B.; Ásgeirsdóttir, S. A.; Krook, J. H.; Drenth, J. H. H.; Wessels, J. G. H. *Eur. J. Cell Biol.* **1994**, *63*, 122.
(23) Carpenter, C. E.; Mueller, R. J.; Kazmierczak, P.; Zhang, L.; Villalon, D. K.; van Alfen, N. K. *Mol. Plant-Microbe Interact.* **1992**, *4*, 55.
(24) Russo, P. S.; Blum, F. D.; Ipsen, J. D.; Miller, W. G.; Abul-Hajj, Y. J. *Can. J. Botany* **1982**, *60*, 1414.
(25) Takai, S.; Richards, W. C. *Phytopath. Z.* **1978**, *91*, 129.
(26) Serimaa, R.; Torkkeli, M.; Paananen, A.; Linder, M.; Kisko, K.; Knaapila, M.; Ikkala, O.; Vuorimaa, E.; Lemmetyinen, H.; Seeck, O. *J. Appl. Crystallogr.* **2003**, *36*, 499.
(27) Paananen, A.; Vuorimaa, E.; Torkkeli, M.; Penttilä, M.; Kauranen, M.; Ikkala, O.; Lemmetyinen, H.; Serimaa, R.; Linder, M. *Biochemistry* **2003**, *42*, 5253.
(28) Hakanpää, J.; Paananen, A.; Askolin, S.; Nakari-Setälä, T.; Parkkinen, T.; Penttilä, M.; Linder, M. B.; Rouvinen, J. *J. Biol. Chem.* **2004**, *279*, 534.
(29) Wessels, J. G. H.; Ásgeirsdóttir, S. A.; Birkenkamp, K. U.; de Vries, O. M. H.; Lugones, L. G.; Scheer, J. M. J.; Schuren, F. H. J.; Schuurs, T. A.; van Wetter, M. A.; Wösten, H. A. B. *Can. J. Botany* **1995**, *73*, 273.
(30) Nakari-Setälä, T.; Aro, N.; Ilmen, M.; Kalkkinen, N.; Penttilä, M. *Eur. J. Biochem.* **1997**, *248*, 415.
(31) Nakari-Setälä, T.; Aro, N.; Kalkkinen, N.; Alatalo, E.; Penttilä, M. *Eur. J. Biochem.* **1996**, *235*, 248.
(32) Penttilä, M.; Nakari-Setälä, T.; Fagerström, R.; Selber, K.; Kula, M.-R.; Linder, M.; Tjerneld, F. International Patent Application PCT/FI00/00249, 24.3., 2000.
(33) http://gsphere.lanl.gov/trire1/trire1.home.html.
(34) Whiteford, J. R.; Spanu, P. D. *Mol. Plant Pathol.* **2002**, *3*, 391.
(35) Talbot, N. J.; Ebbole, D. J.; Hamer, J. E. *Plant Cell* **1993**, *5*, 1575.
(36) Kim, S.; Ahn, I.-P.; Rho, H.-S.; Lee, Y.-H. *Mol. Microbiol.* **2005**. *57*, 1224.
(37) Fuchs, U.; Czymmek, K. J.; Sweigard, J. A. *Fungal Genet. Biol.* **2004**, *41*, 852.
(38) Nielsen, P. S.; Clark, A. J.; Oliver, R. P.; Huber, M.; Spanu, P. D. *Microbiol. Res.* **2001**, *156*, 59.
(39) Segers, G. C.; Hamada, W.; Oliver, R. P.; Spanu, P. D. *Mol. Gen. Genet.* **1999**, *261*, 644.
(40) Wang, X.; de Vocht, M. L.; Jonge, J. D.; Poolman, B.; Robillard, G. T. *Protein Sci.* **2002**, *11*, 1172.
(41) Askolin, S.; Nakari-Setälä, T.; Tenkanen, M. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 124.
(42) Askolin, S.; Turkenburg, J. P.; Tenkanen, M.; Uotila, S.; Wilson, K. S.; Penttilä, M.; Visuri, K. *Acta Crystallogr. D* **2004**, *60*, 1903.
(43) Mandels, M. M.; Sternberg, D.; Andreotti, R. E. In *Symposium on enzymatic hydrolysis of cellulose*; Bailey, M. J., Enari, T.-M., Linko, M., Eds.; Finnish National Fund for Research and Development (SITRA): Helsinki, Finland, 1975; p 81.
(44) de Vocht, M. L. Ph.D. Thesis. University of Groningen, Groningen, The Netherlands, 2001.
(45) Linder, M.; Selber, K.; Nakari-Setälä, T.; Qiao, M.; Kula, M.-R.; Penttilä, M. *Biomacromolecules* **2001**, *2*, 511.
(46) Noordmans, J. Busscher, H. J. *Colloids Surf.* **1991**, *58*, 239.
(47) de Vocht, M. L.; Reviakine, I.; Wösten, H. A. B.; Brisson, A.; Wessels, J. G. H.; Robillard, G. T. *J. Biol. Chem.* **2000**, *275*, 28428.
(48) van Wetter, M.-A.; Wösten, H. A. B.; Wessels, J. G. H. *Mol. Microbiol.* **2000**, *36*, 201.
(49) Richards, W. C. In *Dutch Elm Disease Research: Cellular and Molecular Approaches*; Sticklen, M. B., Sherald, J. L., Eds.; Springer-Verlag: New York, 1993; p 89.
(50) Richards, W. C.; Takai, S. *Appl. Microbiol.* **1973**, *26*, 443.
(51) Torkkeli, M.; Serimaa, R.; Ikkala, O.; Linder, M. *Biophys. J.* **2002**, *83*, 2240.
(52) de Vries, O. M. H.; Moore, S.; Arntz, C.; Wessels, J. G. H.; Tudzynski, P. *Eur. J. Biochem.* **1999**, *262*, 377.
(53) Butko, P.; Buford, J. P.; Goodwin, J. S.; Stroud, P. A.; McCormick, C. L.; Cannon, G. C. *Biophys. Res. Commun.* **2001**, *280*, 212.
(54) Mackay, J. P.; Matthews, J. M.; Winefield, R. D.; Mackay, L. G.; Haverkamp, R. G.; Templeton, M. D. *Structure* **2001**, *9*, 83.
(55) Jenny, K.; Käppeli, O.; Fiechter, A. *Appl. Environ. Microbiol.* **1991**, *36*, 5.
(56) Bailey, M. J.; Askolin, S.; Hörhammer, N.; Tenkanen, M.; Linder, M.; Penttilä, M.; Nakari-Setälä, T. *Appl. Microbiol. Biotechnol.* **2002**, *58*, 721.
(57) Lumsdon, S. O.; Green, J.; Stieglitz, B. *Colloids Surf., B* **2005**, *44*, 172.
(58) Linder, M.; Szilvay, G. R.; Nakari-Setälä, T.; Söderlund, H.; Penttilä, M. *Protein Sci.* **2002**, *11*, 2257.
(59) Askolin, S.; Penttilä, M.; Wösten, H. A. B.; Nakari-Setälä, T. *FEMS Microbiol. Lett.* **2005**, *253*, 281.
(60) Wang, X.; Shi, F.; Wösten, H. A. B.; Hektor, H.; Poolman, B.; Robillard, G. T. *Biophys. J.* **2005**, *88*, 3434.
(61) Nakari-Setälä, T.; Azeredo, J.; Henriques, M.; Oliveira, R.; Teixeira, J.; Linder, M.; Penttilä, M. *Appl. Environ. Microbiol.* **2002**, *68*, 3385.
(62) Kershaw, M. J.; Wakley, G.; Talbot, N. J. *EMBO J.* **1998**, *17*, 3838.
(63) Lin, S.-C. *J. Chem. Technol. Biotechnol.* **1996**, *66*, 109.

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