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ORIGINAL PAPER

Expression and purification of a functionally active class I fungal hydrophobin from the entomopathogenic fungus *Beauveria bassiana* in *E. coli*

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Abstract Hydrophobins represent a class of unique fungal proteins that have low molecular mass, are cysteine rich, and can self-assemble into two-dimensional arrays at water/air interfaces. These highly surface-active proteins are able to decrease the surface tension of water, thus allowing fungal structures to penetrate hydrophobic–hydrophilic barriers. Due to their unusual biophysical properties, hydrophobins have been suggested for use in a wide range of biotechnological applications. Here we describe a simple method for producing a functionally active class I hydrophobin derived from the entomopathogenic fungus, *Beauveria bassiana*, in an *E. coli* host. N-terminal modifications were required for proper expression and purification, and the hydrophobin was expressed as a fusion partner to a cleavable N-terminus chitin-binding domain-intein construct. The protein was purified and reconstituted from *E. coli* inclusion bodies. Self-assembly of the recombinant hydrophobin was followed kinetically using a thioflavin T fluorescence binding assay, and contact angle measurements of purified recombinant hydrophobin protein (mHyd2) films on a variety of substrata demonstrated its surface modification ability, which remained stable for at least 4 months. Filament or fibril-like structures were imaged using atomic force and transmission electron microscopy. These data confirmed the functional properties of the purified protein and indicate amino acid flexibility at the N-terminus, which can be exploited for various applications of these proteins.

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Introduction

Hydrophobins are a family of low molecular weight amphipathic proteins unique to the fungal kingdom. Although they share limited sequence homology, hydrophobins are characterized by similar hydropathy patterns, eight conserved cysteine residues with conserved spacing that form four disulfide bridges, and solubility profiles [23, 40, 43]. These secreted proteins polymerize to form stable self-assembled monolayers found on the surfaces of fungal aerial structures or at water/air interfaces. Their major functions include mediating fungal attachment to surfaces, lowering the surface tension of liquid/air interfaces allowing for growth of aerial structures, and forming a characteristic spore coat structure known as the rodlet layer. Many fungi possess several different hydrophobin genes, each of which likely plays a unique role in the growth, development, or morphology of the organism, including in cell-wall architecture [35, 37, 44]. Several hydrophobins have also been implicated in the virulence of certain fungal pathogens [19, 34].

Hydrophobins have been divided into two classes based upon hydropathy plots and solubility characteristics. Class I hydrophobins form self-assembled microfibril-like, two-dimensional protein arrays that are highly insoluble to mild acids, boiling sodium dodecyl sulfate (SDS), and other detergents. These structures require strong acids, such as trifluoroacetic acid (TFA) for dissolution [41, 44]. The class I proteins often constitute the hydrophobins that comprise the fungal spore coat, resulting in the characteristic rodlet layer seen on the cell surface of many

dry-spored filamentous fungi. Much of the early work on structural characteristics of class I hydrophobins was performed using the SC3 protein from the wood-rotting fungus *Schizophyllum commune* [39], and the three-dimensional structure of the monomeric form of the *Neurospora crassa*, rodlet hydrophobin, EAS, has been resolved [20, 32]. Class II hydrophobins form polymers that lack the rodlet appearance and are soluble in milder conditions than class I arrays, including some organic solvents (60% ethanol) and detergents (1% SDS). These proteins are often secreted into liquid cultures and self-assemble at liquid/air interfaces, allowing the reduction of surface tension, which in turn allows for fungal structure to grow into the air. The most well-studied class II proteins are hydrophobins I and II (HFBI and HFBII) from *Trichoderma reesei*, with the crystal structures of both proteins having been determined [12, 13]. Class I hydrophobins can also mediate escape of hyphae into the air, and these proteins are more surface active than class II hydrophobins [36, 42, 43].

Due to their surface-modifying activities and their lack of immunogenicity, hydrophobins have been suggested for use in a number of biotechnological applications that include promotion of tissue-culture cell growth, removal of oil from contaminated water, as a dispersal or emulsifying agents, and even as an additive in the food industry [24]. Hydrophobins are being studied for their ability to alter surface properties of mica, Teflon, and polystyrene and as a means for producing catalytic surfaces for biosensing applications [6, 18, 28]. Overall, the unique biophysical and biochemical properties of hydrophobins, such as self-assembly, stability under normally denaturing conditions, and amphipathicity, make these protein prime candidates for use in tissue and scaffold engineering [14, 30] and more recently as substrata for delivery and increase of the bioavailability of hydrophobic drugs [1]. However, a method for efficient production of functionally active hydrophobins remains elusive, with a need for improved technologies for the expression and study of a wider range of hydrophobins than those already examined. Indeed, to date, only one other hydrophobin has been expressed in a functional form in *Escherichia coli* [20].

The entomopathogenic fungus *Beauveria bassiana* is under intensive study as an alternative to chemical pesticides for the biological control of insect pests. The spore represents the major infectious propagule used in commercial applications, and critical aspects of product formulations attempt to stabilize the tolerance of the spores to abiotic stresses [17, 45]. Spore-coat hydrophobins have been suggested for use in developing more thermotolerant strains of *B. bassiana* [47, 48], and we previously reported the complementary DNA (cDNA) cloning and expression analysis of two class I hydrophobins from this organism [4, 5]. The protein product of one such hydrophobin gene (*hyd2*) was found to be a major component of the rodlet layer on the spore coat of *B. bassiana* aerial conidia [15, 16]. In this report, we present the expression, purification, and characterization of a modified form of the *B. bassiana* Hyd2 protein using the IMPACT-Twin system encoding an N-terminal-modified Ssp DnaB self-cleavable intein tag coupled to a chitin-binding domain. Self-assembly of the purified protein was examined via a thioflavin T (ThT) assembly assay as well as by direct visualization using transmission electron microscopy (TEM) and atomic force microscopy (AFM). These data revealed that the recombinant protein retained the hallmark functional characteristics of hydrophobins.

Materials and methods

Microbial strains and media

E. coli strains TOP10, BL21, and BL21 Rosetta 2 (DE3) (Invitrogen, Carlsbad, CA, USA) were routinely grown in Luria-Bertani (LB) media supplemented with appropriate antibiotics, as determined by the resistance marker of the plasmids. *B. bassiana* ATCC 90517 was grown on potato dextrose agar (PDA). All chemical reagents were obtained from either Fischer Scientific or Sigma-Aldrich unless otherwise noted.

Plasmid constructions

The *B. bassiana hyd2* gene (Genbank accession number EF520285) was first cloned into pCR21-TOPO (Invitrogen) using primers based upon the cDNA sequence of the gene and containing unique 5′ SapI and 3′ PstI sites (underlined) as follows, F (5′-ggtggttgctcttccaacggcggcg ctggccgcggacgcagccacggccgcagt-3′) and R (5′-ggtggtctgc agttatccgaggacggtgat-3′). The signal peptide of the *hyd2* gene as predicted by SignalP was not included in the vector construct. The *hyd2* gene in pCR21 was then subcloned into the pTWIN1 vector (New England Biolabs, Ipswich, MA, USA) using the engineered SapI and PstI sites and in frame with an Ssp DnaB self-cleavable intein tag coupled to a chitin-binding domain as an N-terminal fusion with the *hyd2* gene, yielding pTW-*hyd2*. The integrity of the final clone was verified by sequencing of the insert (UF-ICBR Sequencing CORE).

Protein expression and purification

For expression and purification of the recombinant hydrophobin, pTW-*hyd2* was transformed into Rosetta2 (DE3) Origami cells, which contain the pRARE plasmid that encodes for several rare total RNAs (tRNAs).

Culturing conditions and induction of protein expression followed established protocols. Briefly, 1 L of LB supplemented with ampicillin (60 μg/ml) and chloramphenicol (30 μg/ml) was inoculated with an overnight culture (1 ml) of cells grown in the same medium. The culture was grown to an OD_{600} ~0.9 at 37°C with vigorous aeration before protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The culture was then returned to 37°C with shaking for an additional 3 h before the cells were harvested (12,000×*g*, 20 min) and the resultant cell pellet resuspended in 100 ml Lysis buffer [20 mM Tris–hydrochloride (Tris–HCl), 300 mM sodium chloride (NaCl), pH 8.5] and kept at 4°C. Cells were lysed by sonication (conditions) and inclusion bodies harvested by centrifugation (2,000×*g*, 20 min). The resulting pellet was suspended in 100 ml denaturing buffer [7 M guanidine-HCl, 20 mM Tris-HCl, 300 mM NaCl, and 10 mM dithiothreitol (DTT), pH 8.5] for 2–3 h at 4°C. The unfolded protein was then refolded by stepwise dialysis against decreasing concentrations of urea, as follows: (1) 8 M urea, 20 mM Tris-HCl, 300 mM NaCl, 10 mM DTT pH 8.3, (2) 6 M urea, 20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 8.3, (3) 4 M urea, 20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 8.3, (4) 2 M urea, 20 mM Tris-HCl, 300 mM NaCl, 1 mM reduced glutathione, pH 8.3, (5) 20 mM Tris-HCl, 300 mM NaCl, 1 mM reduced glutathione, pH 8.3) [11]. Each step was performed at 4°C for 24 h using ~1 L of dialysis buffer. The refolded Intein-Hyd2 fusion protein was then loaded on a 10-ml chitin bead column (New England Biolabs). The column was then washed with 15 column volumes (~150 ml) of wash buffer (20 mM Tris-HCl, 600 mM NaCl, pH 8.5). For elution of the Hyd2 protein, the pH-mediated intein cleavage was initiated by addition of the cleavage buffer (7 ml, 20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 6.5) into the column, which was left to stand for 24–40 h at room temperature before collection of 1 ml fractions (seven total) off the column. Protein concentration was determined using the Pierce 660 nm Protein Assay (Pierce, Rockford, IL, USA). Aliquots of protein sample were mixed with 4× lithium dodecyl sulfate (LDS) sample buffer plus DTT and run on Bis-Tris Nu-polyacrylamide gel electrophoresis (PAGE) gels with Mes-SDS gel running buffer together with standards (Invitrogen). Protein bands were visualized using Coomassie blue (Bio-Rad).

Isolation of native Hyd2 hydrophobin

The native Hyd2 protein was isolated from *B. bassiana,* as previously described [15, 41]. Briefly, aerial conidia were harvested by flooding 14–21 d PDA plates with sterile distilled water (H_2O). Conidial suspensions were filtered through a layer of Miracloth and the hydrophobins extracted by sonication at 140 W (3-mm-diameter microtip, 50% duty cycle) three times for 7 min using a Sonifier cell disrupter B-30 (Branson Ultrasons, Rungis, France). Unlysed cells and cell debris were removed by centrifugation (10,000×*g*, 10 min) and the supernatant centrifuged for 1 h at 50,000×*g*. The resultant pellet was washed three times with 1% boiling SDS, followed by three washes with distilled H_2O. The final pellet was dissolved in concentrated TFA, which was subsequently removed using a stream of nitrogen. The final pellet was stored at −20°C until use.

Analytical analysis

For protein sequencing analysis, the purified protein was analyzed by lithium dodecyl sulfate (LDS)-PAGE, and the subsequent band was subject to in situ tryptic digestion prior to mass spectrometry (MS) fingerprinting. Capillary reverse-phase (RP)-HPLC separation of protein digests was performed on a 10 cm × 75 μm i.d. PepMap C18 column (LC Packings, Sunnyvale, CA, USA) in combination with a home-built capillary HPLC system operated at a flow rate of 200 nl/min. Inline mass spectrometric analysis of the column eluate was accomplished by a quadrupole ion-trap instrument (LCQ, ThermoFinnigan, San Jose, CA, USA) equipped with a nanoelectrospray source. Fragment ion data generated by data-dependent acquisition via the LCQ were searched against a local sequence database using the SEQUEST (ThermoFinnigan) database search engine. The score for SEQUEST protein identification was considered significant when dCn was ≥0.08 and the cross-correlation score (Xcorr) was >2.2.

Thioflavin T (ThT) assembly assay

The ThT binding assay was performed essentially as described [21]. Briefly, 75–150 μg/ml of either the purified recombinant hydrophobin protein (mHyd2) or the purified native Hyd2 dissolved in buffer (20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 6.5) and 38 μM ThT were added to each well of a 96-well black fluorescence plate (Greiner Biotech). The plate was then sealed using iCycler iQ Optical Quality Sealing Tape (Bio-Rad). Samples were then mixed with high agitation over a 0, 2, 5, 7, and 10-min period by vortexing, after which at each time point the sealant was removed. Fluorescence measurements were taken using a SpectraMAX GeminiXS fluorescence spectrophometer (Germini Devices). The addition of the mHyd2 and ThT was staggered such that all time points ended simultaneously in order to read all samples using one plate. Fluorescence spectra were monitored over a wavelength range of 450–600 nm (with slit widths set at 10 nm

for excitation and emission). Results shown are for the fluorescence maxima at 488 nm and are derived from at least three separate replicates. The experiment was performed with at least two independent batches of purified protein.

Drop surface transfer

Glass cover slips were prepared by cleaning the slides with detergent and copious rinsing followed by an acid wash (1 M HCl) for 2 h followed by another copious rinsing with deionized water (diH_2O). A 50-µl droplet of mHyd2 (20 µg/ml) was placed onto parafilm, and the droplet was incubated overnight at 80–90% relative humidity to allow self-assembly of the protein at the water/air interface. The hydrophobin monolayer was then transferred to a prepared glass cover slip by bringing it into contact with the hydrophobin droplet [33]. The glass cover slip was then washed 2× with Milli-Q H_2O to remove any unbound protein. The assembled monolayer was then visualized by AFM and TEM.

Contact angle determination

Contact angle measurements were performed using a Ramé-Hart goniometer with DropImage Advanced software, and the data was analyzed using ImageJ 1.42q software. Purified hydrophobin (75–150 µg/ml) were spotted (20–50 µl) onto unmodified glass slides, siliconized glass slides, and Teflon strips. Experiments were performed in triplicate, with at least 15 contact angles measured for each replicate and two independent batches of purified protein. All samples were washed 3× with distilled H_2O and 3× with 1% SDS. Controls included uncoated and BSA (30-µl drop of 125 µg/ml BSA solution)-treated Teflon and siliconized and unmodified glass slides. Glass slides (Fischerbrand microscope cover glass) were siliconized using Sigmacote (Sigma-Aldrich). The receding contact angles (θ_R) are reported.

Atomic force and transmission electron microscopy

Atomic force micrographs were made using a Digital Instruments Multimode SPM Atomic Force Microscope (model #MMAFM-2) placed on a marble stone platform. Images were taken in either tapping or contact mode using an Si_3N_4 probe (Digital Instruments, model #NP-20, spring constant = 0.12 N/m). Images were collected at 512 lines per scan, with a scan rate of 0.96 Hz and tip velocity of 30.6 µm/s Data from the micrographs was analyzed using Nanoscope SPM v4.42, and WsXM v3.0. Transmission electron micrographs were obtained on Formvar-TEM grids by allowing a 30-µl drop of 125 µg/ml mHyd2 solution to evaporate overnight. The Formvar grids were subsequently washed using a drop of water, which was removed by briefly touching the edge of the grid with filter paper. Grids were stained with 2% uranyl acetate for 10 min, and excess stain was removed by wicking with filter paper. Samples were visualized using a Hitachi H7000 TEM.

Results

Expression and purification of mHyd2

Initial attempts to express the *B. bassiana* Hyd2 protein in *E. coli* as a fusion partner with either a C-terminal His, thioredoxin, or V5 epitope tag did not result in the production of significant amounts of the desired protein in the *E. coli* host. Ultimately, a construct based upon the IMPACT (pTWIN1) system in which an N-terminal-modified form of the *hyd*2 gene, lacking its 16 amino acid signal peptide, was cloned in frame with an Ssp-DnaB (chitin-binding domain) intein fusion partner. Attempts to express and purify the native Hyd2 using the pTWIN system were unsuccessful, and therefore, to optimize the pH-mediated cleavage of the intein, the N-terminus of the protein was altered from APHGPSHGPS to GGAGRGRSHGRS. The resultant recombinant product was termed mHyd2. Protein expression was performed using the *E. coli* Rosetta2 (DE3) strain, which was found to yield satisfactory amounts of protein when induced for expression. The Rosetta2 (DE3) expression cells encode for several rare tRNAs, including those for $tRNA^{Ile}_{ata}$, $tRNA^{Leu}_{cta}$, $tRNA^{Gly}_{gga}$, and $tRNA^{Pro}_{ccc}$, that are found 1, 1, 2, and 5 times in the cDNA coding sequence of the hydrophobin gene, respectively. The mHyd2 protein was found essentially within inclusion bodies, and several strategies such as low temperature, reduced expression time, reduced IPTG concentration, 1% glucose, and low-level constitutive expression were unsuccessful in yielding significant amounts of mHyd2 in the soluble fraction. The mHyd2 protein, therefore, was purified from inclusion bodies following a denaturation–renaturation protocol, as described in "Materials and methods". The refolding process was performed using stepwise dialysis of decreasing urea concentrations kept at a basic pH (~8.5) in order to inhibit premature self-cleavage of the intein–chitin binding domain fusion partner, which is promoted at low pH (<6.5). The refolded protein was purified via chitin-binding chromatography and the final product eluted from the column via pH reduction of the eluant. LDS-PAGE analysis revealed the presence of a ~10-kDa protein in the elution fractions (Fig. 1), and the identity of the protein was confirmed by MS peptide fingerprinting. Four peptide fragments, LLAAECSPISVNVLLNQLVPIDNK, LTGPSVLS

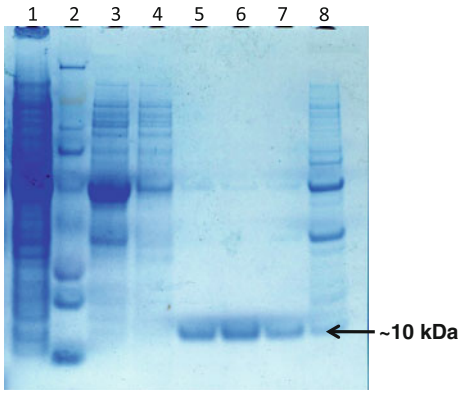

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purification of recombinant hydrophobin protein (mHyd2) expressed in *Escherichia coli*. *Lane* 1, *E. coli* crude extract; *lane* 2 molecular weight standards; *lane* 3, solubilized and refolded protein fraction derived from *E. coli* inclusion bodies (chitin-bead chromatography load); *lane* 4, chitin-bead column flow through and wash; *lanes* 5–7, fractions from column elution in buffer at pH 6.3; *lane* 8, SDS boiled chitin beads (material retained on column)

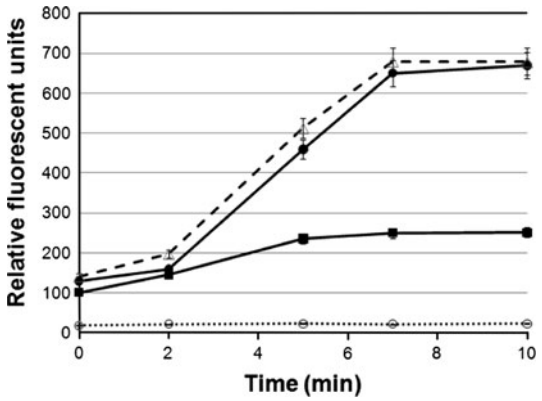

Fig. 2 Time course of protein self-assembly using thioflavin T (ThT) binding as indicator. Solutions containing 0 (*open circle*, *dotted line*), 75 μg/ml (*filled square*), 150 μg/ml (*filled circle*) purified recombinant hydrophobin protein (mHyd2), and (*open triangle*, *dashed line*) 135 μg/ml purified native Hyd2 and 38 μM ThT were mixed vigorously for varying lengths of time and fluorescence intensity at 478–488 nm measured

DLDLR, QQSICCGEQK, and TGDICGNGNTMHCCN DESVTNK were obtained that were exact matches for different segments of the predicted amino acid sequence of the mHyd2 protein. The yield of the purified protein product from the chitin bead column ranged from 200 to 260 μg/ml of elution fraction. The total protein yield was 7–10 mg/L of culture. SDS-PAGE of the purified mHyd2 performed under reducing and nonreducing conditions were consistent with an essentially monomeric nature of the protein, although loss of protein to polymerized forms that failed to enter the gel could not be ruled out.

Self-assembly and surface activity of the mHyd2 protein

Thioflavin T is an aromatic dye that emits an increased fluorescent signal at 485 nm when specifically bound to stacked β-sheet structures, such as those produced during amyloid fiber formation and hydrophobin (self) assembly into two-dimensional protein monolayers [8, 21]. In order to verify that the purified recombinant hydrophobin retained its ability to self assemble, the hydrophobin-ThT solution was vortexed for varying time intervals and fluorescence at 485 was determined. At low-protein concentrations (75 μg/ml), there was a twofold increase in fluorescence over a 5- to 10-min period, whereas at higher protein concentrations (150 μg/ml), there was a sevenfold increase in fluorescence intensity over the same time course (Fig. 2). In both instances, the fluorescent intensity reached a plateau, indicating conversion of the available monomeric form of the protein into the assembled (rodlet) conformation. For comparative purposes, native Hyd2 was purified from *B. bassiana* spores, and the ThT profile using an approximately equivalent concentration of the protein was essentially identical to that observed for mHyd2 (Fig. 2). ThT assays performed in the absence of DTT and with protein dialyzed into buffer without DTT gave essentially the same results.

Water contact angles of dried-down, purified hydrophobin on unmodified glass, siliconized glass, and Teflon were used to examine the surface modification properties of mHyd2. The mHyd2 film produced after 2–24 h was stable and could not be removed with Milli-Q water, PBS (pH 7.4), or 1% SDS at room temperature. The mHyd2 films altered the surface of untreated glass, siliconized glass, and Teflon, in each case making the substrata more hydrophilic, changing the receding contact angles from 44.5° to 15.6°, 71.4° to 11.8°, and 67.9° to 7.7°, respectively (Fig. 3). The film was stable for at least 4 months, retaining its surface modification property (on siliconized glass, the receding contact angle was 10.9 for a 4-month-old film).

Microscopic visualization of mHyd2 films

In order to visually confirm the presence of self-assembled hydrophobin bundles, TEM and AFM was used. Whole-mount negative-stained TEM was performed in which the purified hydrophobin solution was placed on a Formvar grid and allowed to air dry before being processed for imaging. The resulting micrographs showed the presence of small striated filaments or fibrils (Fig. 4). These filaments were approximately 10 nm in diameter and ranged from 50 to 300 nm in length and were essentially disordered. For AFM, samples were prepared using both a drop-surface transfer method as well as by air drying of the mHyd2

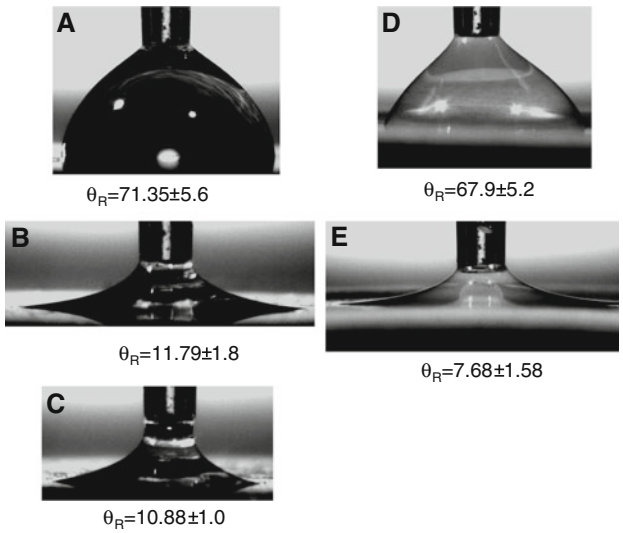

Fig. 3 Water contact angle measurements of purified recombinant hydrophobin protein (mHyd2) films. Water droplet on **a** siliconized glass, **b** mHyd2 film on siliconized glass, **c** mHyd2 on siliconized glass kept at room temperature for 4 months, **d** Teflon, and **e** mHyd2 film on Teflon

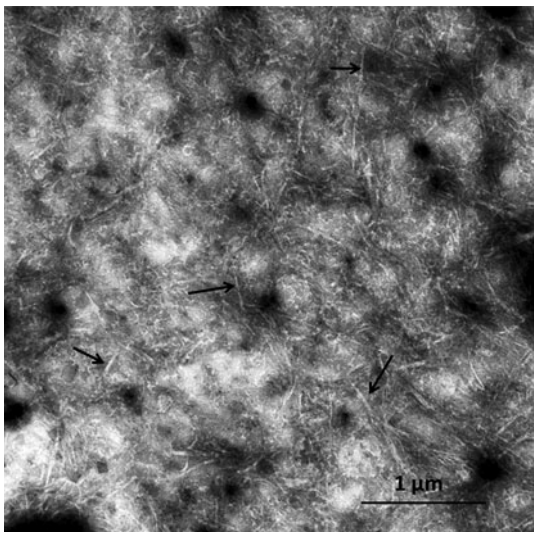

Fig. 4 Transmission electron micrograph (TEM) of self-assembled purified recombinant hydrophobin protein (mHyd2) dried onto a Formvar grid. A 100-µl droplet of 0.18 mg/ml solution of purified mHyd2 was placed on the Formvar grid, and the sample was allowed to air dry (~4 h) before processing. *Arrows* indicate the presence of thin, rodlet-like filaments

solution on glass coverslips, as described in "Materials and methods". These images showed the average height of the monolayer domains to be between 2 and 4 nm thick (Fig. 5). AFM surface analysis revealed the roughness (root mean square, RMS) to be ~1 nm throughout the entire deposited hydrophobin layer, indicating essentially homogenous coverage of the surface with the mHyd2 protein. The patterned hydrophobin film seemed to be compact, with few holes or irregularities.

Discussion

Hydrophobins represent a class of proteins with the potential for a diverse range of biotechnological uses. A simple method for expression of functionally recoverable hydrophobin in *E. coli* would greatly facilitate studies as well as applications of these proteins. To date, most hydrophobins have been purified from their host organism, although heterologous expression in yeasts and other fungi has been reported [3, 44]. These methods, however, can be time consuming, difficult to scale up for biotechnological applications, and—in some instances—can yield relatively little protein that may be contaminated with endogenous host hydrophobins. A number of hydrophobins have been expressed in *E. coli*, although it is unclear whether these proteins were functionally active and the expressed proteins were primarily used for antibody production [2, 27]. An exception has been the rodlet layer hydrophobin of *N. crassa*, which is encoded by the *eas* gene and which has been successfully expressed in *E. coli*, and its protein product used in part to facilitate structural studies on this protein [20].

The filamentous entomopathogenic fungus, *B. bassiana*, is a commercially available biopesticide that displays a broad host range against diverse insect species. *Beauveria* spp. are also one of the most widely used whole-cell eukaryotic biocatalysts, and *B. bassiana* represents an emerging model system for examining novel aspects of fungal development and pathogenesis [10, 22, 38]. With regard to practical field applications of this organism, spore stability remains a critical issue regarding formulation and stabilization of the active spores, including manipulating culturing conditions of the fungus to increase the presence of hydrophobin-like proteins to yield spores with improved tolerance to thermal stress [45, 48].

In this report, we demonstrate that the production of a hydrophobin derived from *B. bassiana*, in an *E. coli* host using an intein purification system allows for fast and efficient protein production. The fusion partner is self-cleavable, via adjustment of the protein-containing solution, to a pH that promotes the autocatalytic reaction (pH 6.3), and the use of an intein-based affinity purification system was originally developed as a way of expressing proteins without the addition of any extraneous tags that could limit protein activity [46]. However, to optimize the autocatalytic activity of the intein, a series of amino acid residues at the N-terminus of the Hyd2 protein were altered.

A critical property of hydrophobins is their ability to self-assemble into a monolayer at hydrophobic/hydrophilic interfaces. Reconstituted self-assembly of hydrophobins obtained directly from fungal surfaces or cultures has been demonstrated in many fungi [13, 14, 25, 26, 33, 41]. Hydrophobins exist in three structural states: a monomeric state, which is soluble; an α-helical state; and a β-sheet state when self-assembled at water/air interface [7, 9]. Agitation of hydrophobins in solution promotes self-assembly and is a classic method by which hydrophobins can be purified [21, 41]. Several lines of evidence were used to determine whether the mHyd2 protein retained its activity of self-assembly. Due to its increase upon binding of stacked β-sheet structures, ThT fluorescence has been used to detect amyloid aggregation and hence in the diagnosis of amyloid fibrosis [29]. Hydrophobins have been compared with amyloid fibrils, and this technique has been used to monitor hydrophobin assembly in solution [21, 32, 43]. Using the ThT assay, assembly of the purified mHyd2 protein followed kinetics similar to those seen for the *N. crassa eas* hydrophobin [21] and was essentially identical to native Hyd2 isolated from *B. bassiana* spores. In addition, the surface modification activity of the recombinant protein was confirmed via contact-angle measurements of protein films deposited on untreated, siliconized glass and Teflon. In each case, the mHyd2 protein caused a dramatic decrease in the receding contact angles, altering the surfaces from hydrophobic to hydrophilic. The mHyd2 film was also very stable, retaining essentially full-surface modification activity for a minimum of 4 months, which was the time frame of the experiment, but which likely indicates a far longer stability. The eight cysteine residues found in hydrophobins form critical disulfide linkages that participate in the stability and activity of the protein. A set of experiments was performed attempting to react any free sulfhydryl residues with iodoacetate. Only a slight shift in the migration of mHyd2 in SDS-PAGE gels was noticed for the purified mHyd2 in the presence or absence of DTT, $\pm$8 M urea, and $\pm$iodoacetic acid, suggesting that the cysteine residues were unreactive and that the disulfide bridges in the protein were stable (data not shown). Additional experiments, however, are needed to better examine this issue and to probe the nature and formation of the disulfide bonds in mHyd2.

Microscopic visualization of hydrophobin-deposited surfaces has also been used to examine the hydrophobin films. Similar to our results when mHyd2 was dried down onto Formvar-coated TEM grids, negatively stained TEM images of the purified *N. crassa* EAS and *S. commune* SC3 hydrophobins indicated that these proteins assemble into $\sim$10-nm-diameter filaments, with lengths ranging from 35 to >200 nm [32, 41]. Similarly, AFM topography images of the *T. reesei* HFBI and HFBII proteins revealed patterns of ordered assemblies of the hydrophobin molecules similar to the images of the mHyd2 films obtained here

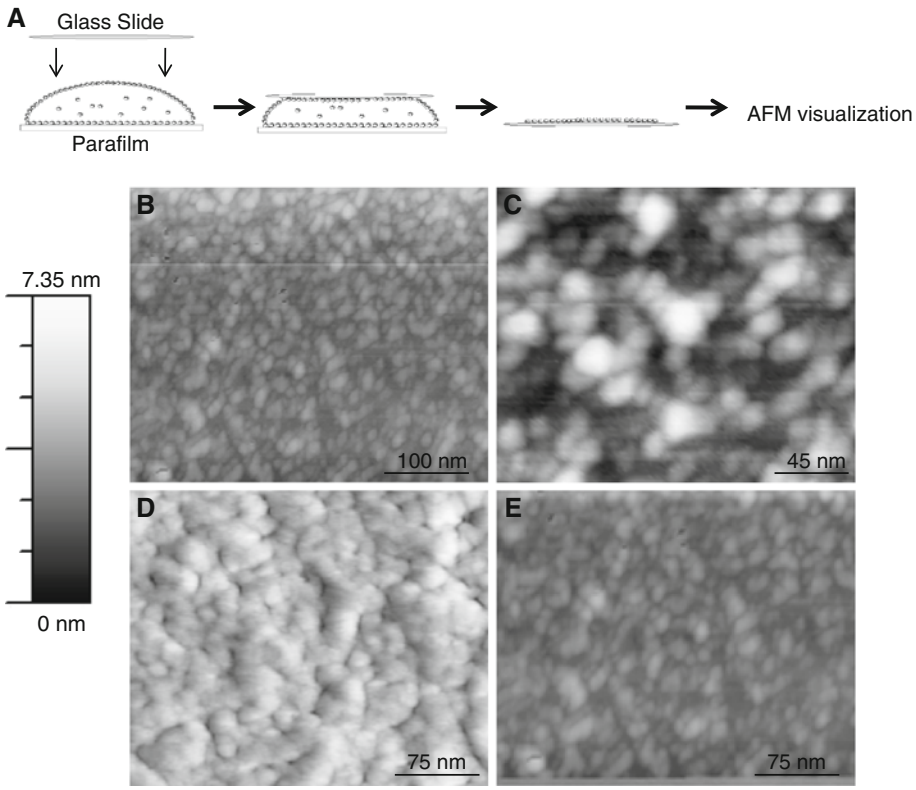

Fig. 5 Purified recombinant hydrophobin protein (mHyd2) was allowed to self-assemble and the resultant monolayer transferred to a glass slide (**a**, drop transfer method). Atomic force microscopy (AFM) topography images of mHyd2 films prepared by the drop transfer method (**b, c**) and by air drying (**d, e**) of a solution of mHyd2 on the glass coverslip

[12, 33]. In conclusion, our results suggest that hydrophobins can be efficiently expressed in a prokaryotic system and that functionally active protein can be recovered following a step-wise refolding strategy. The method should be broadly applicable, thus overcoming production obstacles that have hindered the diversity of hydrophobins examined to date and can be exploited for various biotechnological applications. In addition, our data, similar to what has been reported for the SC3 hydrophobin [31], indicate that the N-terminus has flexibility in amino acid sequence that can be manipulated for construction of various peptide derivatives, thus expanding the bioengineering options of hydrophobins.

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