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PfaH2: A novel hydrophobin from the ascomycete Paecilomyces farinosus 
PfaH2 – a novel hydrophobin from the ascomycete *Paecilomyces farinosus*

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Running title: PfaH2 hydrophobin from *Paecilomyces farinosus*

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Abstract

The *pfah2* gene coding for a novel hydrophobin PfaH2 from the ascomycete *Paecilomyces farinosus* was identified during sequencing of random clones from a cDNA library. The corresponding protein sequence of PfaH2 deduced from the cDNA comprised 134 amino acids. A 16 aa signal sequence preceded the N-terminus of the mature protein. PfaH2 belonged to the class Ia hydrophobins. The protein was isolated using trifluoroacetic acid extraction and purified via SDS-PAGE and HPLC. The surface activity of the recently described PfaH1 and of PfaH2 was compared by determination of contact angles on glass slides and Teflon tape, and the contact angle of distilled water droplets was measured on glass slides coated with hydrophobin PfaH1 or PfaH2. Surprisingly, both hydrophobins adsorbed to hydrophilic surfaces and changed their physico-chemical properties to a similar quantitative extent, although little amino acid sequence homology was found.

Keywords: ascomycete, contact angle, fungi, hydropathy plot, hydrophobin, *Paecilomyces farinosus*

Abbreviations: CA, contact angle; ESI, electrospray ionization; ITS, internal transcribed spacer; SD, standard deviation; SNL, standard nutrient liquid; TSO, template switching oligo

The authors have no conflict of interest to declare.
1. Introduction

Fungi are producing unique proteins, such as lectins, lignocellulolytic enzymes, protease inhibitors and hydrophobins [1]. Enzymes decomposing lignocellulose, a substrate for future biofuels, may be used for totally different applications, such as the generation of flavour compounds [2]. Likewise, diverse applications are expected for hydrophobins, a class of small, catalytically inactive proteins in aerial structures of asco- and basidiomycetes which mediate the attachment of mycelial hyphae to surfaces [3-5]. According to hydropathy pattern and occurrence they were grouped into classes Ia, Ib and II, with Ia and II occurring in ascomycetes and Ib in basidiomycetes [6]. Intermediate forms are also known [7],[8]. All hydrophobins share a characteristic pattern of eight cysteine residues with cysteine two and three and six and seven being immediate neighbors, respectively [4].

Among the potential applications of hydrophobins reported are, for example, the long-term stabilization of foams [9], of air-filled emulsions [10], or of a water/n-hexane emulsion [11]. Hydrophobins may also be used for biomaterials and medical applications, for example for the improvement of the growth of fibroblasts [12] or for increasing the biocompatibility of hydrophobin covered implants [13]. Hydrophobins have either to be purified from fungal material, or cloned and heterologously expressed. Functional expression of hydrophobins was reported in *Escherichia coli* [14], *Pichia pastoris* [15],[16] and *Trichoderma reesei* [17].

The entomopathogenic ascomycete *Paecilomyces farinosus* (*P. farinosus*, alternative name *Isaria farinosa*) was one of the strains which showed strong water repellent property, when a drop of water was applied to the surface of the mycelium. Recently, a class Ia hydrophobin named PfaH1 was isolated from the mycelium of *P. farinosus* and the full sequence was identified. In this study, using their high constitutive
production, another hydrophobin of \textit{P. farinosus}, PfaH2 (AN CBV17211.1), was purified and its encoding characterized on the sequence level. Additionally, the contact angle for the both purified PfaH1 and PfaH2 on different surfaces was determined to comparatively detect differences of the physico-chemical behavior of both proteins from the same source.

2. Materials and methods

2.1. Isolation and purification of PfaH2

Vegetative mycelium of \textit{P. farinosus} from the culture collection of the Institute of Food Chemistry, Leibniz University Hannover, was maintained on the standard nutrient liquid (SNL) medium plates containing 2.0% agar according to [18]. Mycelium was scraped off the plates and hydrophobins were extracted following largely the method of Lugones [19]. The mycelium was frozen at -70 °C and ground under liquid nitrogen. Further isolation and purification of hydrophobins using preparative HPLC followed the method described by [20]. The HPLC system consisted of a Jasco PU-980 HPLC pump (Jasco, Groß-Umstadt, Germany), a Jasco LG 980-02 ternary gradient unit, a two line degasser (Techlab, Braunschweig, Germany), a Jasco MD-910 multi-wavelength detector, all of which controlled from a Jasco LC-NetII/ADC box. Data were collected and analyzed with the Jasco Borwin software package (Version 1.50). The column was a Vydac 214-TP C4 column (50 × 4.6 mm, 5 µm; Grace, Alltech Grom GmbH, Rottenburg, Germany). Elution was obtained using the following gradient: 0-30 min 20 to 60% B, 30-31 min 60 to 80% B, 31 to 32 min 80% B, 32-33 min 80% B to 20% B, 33-35 min 20% B (flow: 1 mL/min; A: H$_2$O with 0.1%
TFA; B: acetonitrile/methanol 95/5 with 0.1% TFA). Elution of protein was monitored at 215 and 240 nm. Protein was collected during elution from the system.

For liquid SNL culture the same SNL medium was used omitting the agar. For a minimal medium glucose, \( L\)-asparagine and yeast extract were omitted. Cultures were grown either as still submerged cultures or on an orbital shaker at 150 rpm at a temperature of 24 °C, until the stationary phase was reached, typically after six days.

2.2. SDS-PAGE analysis of protein solution

For electrophoretic separation, protein containing samples were re-suspended in a sample buffer (200 mM sodium phosphate buffer pH 7.0, 200 mM DTT, 20% glycerol (w/v), 4% SDS (w/v), 25 mg/L bromphenol blue), heated to 95 °C for 10 min and subsequently loaded on the gel. After separation, the gels consisting of a 16% polyacrylamide according to Laemmli [21] were stained either with Coomassie brilliant blue R250 or silver following standard procedures.

2.3. Identification of proteins using MS/MS

For tandem mass spectrometry, protein collected from HPLC separation was freeze-dried. Appropriate fractions were combined after solubilisation with concentrated TFA, the TFA was gently removed with a stream of nitrogen, proteins taken up in SDS sample buffer and electrophoretically separated. Protein bands were excised after staining with Coomassie brilliant blue, washed, reduced and digested with trypsin. The resulting peptides were extracted, desalted and purified according to standard protocols. A Qtof II mass spectrometer (Micromass, UK) equipped with a nanospray
ion source was used for electrospray ionisation (ESI) MS of peptides. For collision-induced dissociation experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell (25–30 eV). The resulting daughter ions were separated by an orthogonal time-of-flight mass analyzer. Peptide mass fingerprints obtained from ESI-Tandem MS analysis were used for cross-species protein identification in public protein primary sequence databases (NCBI).

2.4. RNA isolation, cDNA first strand synthesis, gene cloning and DNA sequencing

200 mg of mycelium were frozen in liquid nitrogen and ground using mortar and pestle. Lysis of cells and total RNA isolation was obtained using NucleoSpin® RNA Plant Kit from Macherey-Nagel (Düren, Germany). The cDNA first strand synthesis was carried out using total RNA and reverse transcriptase and degenerate primers. Degenerate primers were derived from the peptide sequences using the Codon Usage Database (http://www.kazusa.or.jp/codon/) for the *I. farinosa* deposit on the website. After transcription the newly synthesized ssDNA was used as a template for full-length cloning of the corresponding gene. For this the 3'-end of *pfah2* was amplified using the following primer combinations: forward 5’- CAG GGY GTY TCY AAG CCN ACY GG - 3’, reverse 5’-ATT CTA GAG GCC GAG GCG GCC GAC ATG (T)_{30} VN-3’. The PCR conditions were 94 °C 1.5 min, 95 °C 15 s, 59 °C 30 s, 68 °C 45 s, 30 cycles, finally 68 °C for 10 min. To amplify the 5'-end of *pfah2* the template switching oligo (TSO) [22] approach was used. For this custom made DNA oligonucleotide (CMO, 5’-AAG CAG TGG TAT CAA CGC AGA GTA CGC-3’) was ribo-tailed with rG using rGTP and terminal deoxynucleotidyl transferase (TdT) thus creating the TSO-primer (20 U TdT, 1 × TdT buffer, 0,4 mM rGTP, 25 pmol CMO, 40
U ribo-lock; 37 °C for 30 min, then 70 °C for 10 min). First strand synthesis was performed with TSO-primer and 3'-T primer (5'-AAG CAG TGG TAT CAA CGC AGA GTA C(T)₃₀ VN-3'). The concentrations were as follows: 100 ng RNA (purified with standard protocols), 5 µM 3'-T at 70 °C for 10 min, then 1 × RT buffer, 1 mM dNTPs, 8 mM MnCl₂, 50 nM TSO, 200 U reverse transcriptase, 40 U ribo-lock. The reaction proceeded at 42 °C for 60 min, then 50 °C, 10 min, and finally 70 °C, 5 min. The second strand synthesis was conducted after clean-up (PCR clean –up Gel extraction, NucleoSpin® Extract II, Macherey-Nagel) using 5'PCR (5'-AAG CAG TGG TAT CAA CGC AGA GT-3') and 3'-T primer. It consisted of 1 × LP buffer, 200 µM dNTPs, each 4 µM 5'PCR and 3'-T primer, 3% (v/v) DMSO, 1 U LP-mix and 1 µL template cDNA. The PCR reaction was performed using the following program: initiation 94 °C for 2 min, denaturation 94 °C for 20 s, annealing 55 °C for 30 s, elongation 68 °C for 90 s + 1 s/cycle for a total of 30 cycles and final elongation 68 °C for 10 min.

For sequencing the genomic DNA of pfah2 the following primers were used: forward 5'-ATG CGC TTC GCC TTT GCT ATC A - 3' and reverse 5'- CTA GAG AAG GCT GTC GAG AGC G - 3'. Genomic DNA was isolated from P. farinosus mycelium using a Genomic DNA Purification Kit (Fermentas, St. Leon-Rot, Germany). PCR reaction was carried out utilizing the following temperature program: initiation 94 °C for 5 min, denaturation 94 °C for 20 s, annealing 55 °C for 30 s, elongation 68 °C for 30 s for a total of 30 cycles and final elongation 68 °C for 10 min.

2.5. Bioinformatic tools - alignment of sequences
For building of the phylogenetic tree sequences were aligned from the first cysteine to the C-terminus of the protein depicted in the databases (NCBI). The alignment was calculated using the ClustalW algorithm in the ClustalX program version 2.0.12. After clustering using the Bootstrap Neighbor Joining Tree function the tree was visualized using Tree View program version 1.6.6.

Kyte-Doolittle hydropathy pattern [23] was calculated and visualized using the BioEdit software version 7.0.9.0 after alignment of the sequences including the signal sequence using the ClustalW algorithm leaving the gaps in the sequences. Alignment was done using ClustalX program as mentioned above. Sequences were aligned using the profile alignment method and the following sequence as profile 1: -C-CC-C-C-CC-C-. Alignment was done with default parameters, except ‘gap opening’ which was set to 5 instead of 10. The presence and location of signal peptide cleavage sites was determined using SignalP 3.0 tool [24].

2.6. Determination of the protein concentration

The protein concentration was determined using the Bradford reagent (Sigma, Neu-Ulm, Germany). BSA was used as the standard protein. In the concentration range used (0.2 mg/mL – 1.2 mg/mL) the calibration curve was linear with a regression coefficient of $R^2 = 0.992$.

2.7. Contact angle determination with the sessile drop method

The contact angle (CA) was determined using the sessile drop method (SDM) [25] by recording the placement of a drop of liquid on the surface and its subsequent
behavior with a CCD equipped contact angle microscope (OCA 11, DataPhysics, Filderstadt, Germany). CA were evaluated after 30 ms (initial CA) using the software SCA20 (DataPhysics, Filderstadt, Germany). The hydrophobin solutions were used as test liquids on a hydrophilic glass slide, hydrophobic Teflon tape and as a coating of glass slides. The protein concentration in the hydrophobin solutions for the CA determination on glass was 150 µg/mL, on Teflon tape and as coating solution 100 µg/mL. The glass slides were coated with 0.25 µg/cm² protein. For coating, the hydrophobin solutions were dispensed uniformly and dried at room temperature. Per solution six drops were placed, and the CA for each drop was determined as the mean from the CA of the left and the right side. The drop volume was 10 µL, the recording frequency 30 Hz.

3. Results and discussion

3.1. Identification, isolation and characterization of PfaH2

The extraction of hydrophobins from the mycelium using TFA commenced after the bulk of other proteins was removed using a hot SDS-solution followed by several washing steps with water. The poor solubility in hot SDS solution and the ready solubility in concentrated TFA suggested that PfaH2 belonged to the class I hydrophobins. When applied to SDS-PAGE, a strong signal at 14 kDa, consisting of two poorly resolved proteins occurred. At higher molecular masses a number of weaker signals were visible (Fig. 1a, Lane 1). To completely purify the hydrophobins a sample was applied to preparative HPLC (Fig. 1b), and two separated compounds were collected. The concentrated eluates were submitted to SDS-PAGE, and each gave a single band at around 14 kDa (Fig. 1a, Lanes 2-3). After partial sequencing
using tandem mass spectrometry as described in the methods section, the second peak was designated PfaH2 and gave the unique peptide sequences underlined in Figure 2. According to a BLAST search these sequences matched hydrophobin sequences and were studied in detail (http://www.ncbi.nlm.nih.gov/BLAST/).

3.2. Analysis of the nucleotide sequence and derived amino acid sequence of the \textit{pfah2} gene

Using the mentioned protocol, the complete coding sequence of \textit{pfah2} was determined and the primary structure of the protein was deduced (Fig. 2). The full-length cDNA of the PfaH2 protein had an open reading frame (ORF) of 402 base pairs (bp), coding for a protein with 134 amino acids. The 5’ untranslated region to the poly T tail was 71 bp long. The ORF coding for PfaH2 was on the genomic DNA interrupted by two introns with a length of 53 bp flanked by the characteristic 5’ - AT…AG - 3’ motif [26]. Both are typical class I introns based on the consensus splice site and internal sequence for lariat formation [27].

The 134 amino acids of the polypeptide chain coded for a protein with an estimated molecular weight of 13.69 kDa and an isoelectric point of 6.39. To examine their theoretical ability to be secreted, the SignalP 3.0 tool [24] was applied. A putative cleavage site for a signal peptide was located between amino acids 16 and 17. After cleavage of the putative signal peptide, necessary for directing the protein to the surface of the fungal structures [28], a size of 12.04 kDa remains for the functional protein. In close proximity to the theoretical signal sequence cleavage site a proline residue was found. This proline was also located in a range of the hydrophobin sequences from different \textit{Aspergillus} species [7] and was suggested to be involved in
the correct cleavage of the signal sequence and eventual secretion of the
hydrophobins. Neither N- nor O-glycosylation sites (predicted using NetNGlyc 1.0
and NetOgly 3.1 server, respectively) were detected. Notable, the new sequence
contained no tryptophan residues, which generally are uncommon in hydrophobins
[29]. The amino acid count of PfaH2 (134 aa, Fig. 2) was somewhat larger than
expected (typical range 100 ± 25, [29]), but correlated well with other most
homologous class Ia hydrophobins, such as DewA or RODA (Fig. 3) from A. nidulans
(135 and 157 aa, respectively).

The most characteristic property of hydrophobins is a pattern of eight cysteine
residues \(X_{2-38}C\cdotX_{5-9}C\cdotX_{11-44}C\cdotX_{8-23}C\cdotX_{5-9}C\cdotX_{6-18}C\cdotX_{2-14},\) in which \(X\) indicates
any of the proteinogenic amino acids except cysteine [30]. This pattern, required for
formation the four disulphide bridges necessary to obtain the final tertiary structure
[6],[31], was also found in PfaH2 (Fig. 2), except the sequence preceding the first
cysteine pair which comprised 39 residues: \(X_{39}C\cdotX_{7}C\cdotX_{33}C\cdotX_{18}C\cdotX_{5}C\cdotX_{17}C\cdotX_{7}.\)

3.3. Hydropathy and phylogenetic classification of the PfaH2

Difference in hydropathy is one of the criteria for distinguishing class I and II
hydrophobins. Analysis of the hydropathy pattern using the algorithm of Kyte and
Doolittle [23] showed a hydropathy plot similar to other class I hydrophobins. The N-
terminus region predicted to be the signal sequence for secretion was highly
hydrophobic, followed by a hydrophilic region, a hydrophobic core and a hydrophobic
C-terminus interrupted by a hydrophilic area (Fig. 3). The first cysteine doublet was
followed by a stretch of hydrophilic amino acids.
To evaluate the structural and functional relationships between PfaH2 and other hydrophobins found in fungi, a phylogenetic tree was constructed, and PfaH2 was integrated into an alignment of other hydrophobin sequences using ClustalW algorithm (Fig. 4). The created tree showed the affiliation of PfaH2 to class Ia hydrophobins from ascomycetes. Its closest relatives from a phylogenetical standpoint were the class I hydrophobins of HYP1 (A. fumigatus) and RODA (A. nidulans).

On the amino acid level the sequence identity compared to hydrophobin sequences from other ascomycetes ranged from 13 to 36 percent with highest values of 32% for HYP1 hydrophobin (A. fumigatus) and 36% for RODA (A. nidulans). The identity between both proteins from P. farinosus PfaH1 und PfaH2 was only 23%. The result was not surprising, because low identities were exhibited even within the same species: For example, a series of hydrophobins HCf1 to HCf4 from the fungal tomato pathogen C. fulvum were assigned to class I and showed an identity between 46.3 and 69.9%, whereas two other proteins HCf5 and HCf6 belonged to class II possessing an identity among themselves of 52% [32].

3.4. Physicochemical behavior of the hydrophobins PfaH1 and PfaH2

To explore possible fields of applications, some specific biophysical properties of both hydrophobins were determined. The contact angle of hydrophobin drops on hydrophilic (glass slide) and hydrophobic (Teflon tape) surfaces were measured and compared to distilled water (Table 1). To eliminate any possible interferences, both proteins were purified by HPLC as described. A definite increase of the contact angle of the hydrophobin droplets on the glass slide and, less pronounced, a lowering on
the Teflon tape compared to water indicated the amphiphilic properties of both proteins. Despite of low identities on the sequence level, the results of the contact angle definition obtained from both hydrophobins were similar.

Owing to their amphiphilic nature, hydrophobins can be immobilized on different kinds of surfaces including clean glass, Teflon, and polystyrene [20],[33] and are capable to alter the surface properties. The ability of PfaH1 und PfaH2 to change the wetting properties was analyzed with water contact angle measurements. The water contact angle on the glass surface coated with hydrophobins raised apparently indicating a decrease in wettability of glass upon adsorption of PfaH1 und PfaH2 (Table 2).

This change of the surface wettability could increase the biocompatibility, for example for drug delivery applications, or for creating active surfaces with appropriate hydrophobicity for protein immobilization [1],[34]. Coating of hydrophobic particles with the HFBII protein converted them to more hydrophilic ones, and hence improved the particles' cell viability compared to uncoated ones, and enhanced the particles' cellular association [35].

A relation between the amount of adsorbed protein and the resulting film thickness and the wettability of hydrophobin-coated solid surfaces was found [36]. Imperfect formation of hydrophobin films may result in a high variability of surface properties. These findings call for the development of optimal coating methods for different surfaces with both PfaH1 und PfaH2 to better evaluate further applications.
References


Table 1. Contact angle (CA) determination of hydrophobin PfaH1 and PfaH2 on glass slide and Teflon tape. The protein concentration in the hydrophobin solutions on glass was 150 µg/mL, on Teflon tape 100 µg/mL.*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glass slide</th>
<th>SD**</th>
<th>Photo</th>
<th>Teflon tape</th>
<th>SD</th>
<th>Photo</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O dist.</td>
<td>17.4</td>
<td>1.9</td>
<td></td>
<td>121.9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>PfaH1</td>
<td>27.2</td>
<td>3.1</td>
<td></td>
<td>118.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>PfaH2</td>
<td>25.4</td>
<td>3.3</td>
<td></td>
<td>118.1</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

* Drop volume 10 µL; recording time 30 ms

** SD, standard deviation
Table 2. Contact angle (CA) determination of distilled water on glass slides with hydrophobin PfaH1 and PfaH2 coating. The protein concentration in the hydrophobin solutions on glass slide as coating was 100 µg/mL.*

<table>
<thead>
<tr>
<th>Coating sample</th>
<th>initial CA [°], H₂O</th>
<th>SD</th>
<th>Photo</th>
</tr>
</thead>
<tbody>
<tr>
<td>dist. water</td>
<td>24.33</td>
<td>5.14</td>
<td><img src="image" alt="Photo" /></td>
</tr>
<tr>
<td>PfaH1</td>
<td>37.18</td>
<td>2.51</td>
<td><img src="image" alt="Photo" /></td>
</tr>
<tr>
<td>PfaH2</td>
<td>33.82</td>
<td>2.24</td>
<td><img src="image" alt="Photo" /></td>
</tr>
</tbody>
</table>

* Drop volume 10 µL; recording time 30 ms

** SD, standard deviation
Figure captions

Figure 1. a) SDS-PAGE of hydrophobin crude extract from mycelium of *P. farinosus* and of purified PfaH1 and PfaH2 hydrophobins. Lane 1, crude extract after TFA treatment; Lane 2, PfaH1 purified with HPLC from mycelium of *P. farinosus*; Lane 3, PfaH2 purified with HPLC from mycelium of *P. farinosus*; Lane 4, low molecular weight marker. b) HPLC chromatogram of the purified hydrophobins.

Figure 2. Nucleotide sequence and deduced amino acid sequence of the *pfah2* gene and PfaH2 protein. The coding sequence appears in the upper line in upper case letters, lower case letters indicate the sequence of the introns 1 and 2, marked with an arrow (↓); depicted below is the deduced amino acid sequence. An arrow (↑) marks the cleavage site of the signal peptide between amino acids 16 and 17 predicted using SignalP 3.0. The sequences obtained by MS are underlined, doubly underlined is the peptide sequence used for primer design. The eight cysteine residues distributed in a characteristic manner are set in bold.

Figure 3. Hydropathy pattern of the PfaH2 protein using the Kyte and Doolittle (1982) algorithm with a window size of nine amino acids plotted against hydrophobins with high sequence identity (HYP1, *A. fumigatus* and RODA, *A. nidulans*), one class II hydrophobin (HFB1, *T. reesei*), and one class Ib hydrophobin (SC3, *S. commune*). Hydrophobic regions are given by a positive index at the respective ordinate, while hydrophilic regions are indicated as negative (above and below horizontal bold line, respectively). The amino acid number is given by the number at the lowest abscissa. An arrow indicates the cleavage site of the signal peptide (calculated with SignalP 3.0). Horizontal lines indicate the position of the eight cysteine residues.
Figure 4. Phylogenetic tree obtained after alignment of hydrophobin protein sequences with PfaH2. The scale bar indicates the distance for 0.1 amino acid substitutions per position. Grouped are class I hydrophobins (Ia) and class II hydrophobins from ascomycetes. Bootstrap values are shown in percentage. The hydrophobins are displayed with their acronym and are as follows: PfaH1 (P. farinosus FR666701), PfaH2 (P. farinosus FR666702), HYP1 (A. fumigatus U06121), RODA (A. nidulans M61113), DewA (A. nidulans UO7935), H Cf1 (C. fulvum X98578), H Cf2 (C. fulvum AJ133700), H Cf3 (C. fulvum AJ133701), H Cf4 (C. fulvum AJ133702), H Cf5 (C. fulvum AJ133703), H Cf6 (C. fulvum AJ251294), CFTH1 (C. fusiformis AJ133774), MPG1 (M. grisea L20685), MPH1 (M. grisea AF126872), SSGA (M. anisopliae M85281), EAS (N. crassa X67339), CU (O. ulmi U00963SRH1 (T. harzianum Y11841), HFB1 (T. reesei Z68124), HFB2 (T. reesei Y11894), XEH1 (X. ectaneoides AJ250793).