The *Pleurotus ostreatus* hydrophobin Vmh2 and its interaction with glucans

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Received on February 9, 2009; revised on December 22, 2009; accepted on January 19, 2010

Hydrophobins are small self-assembling proteins produced by fungi. A class I hydrophobin secreted by the basidiomycete fungus *Pleurotus ostreatus* was purified and identified. The pure protein is not water soluble, whereas complexes formed between the protein and glycans, produced in culture broth containing amylase, are soluble in water. Glycan structure matched to cyclic structures of α-(1-4) linked glucose containing from six to 16 monomers (cyclodextrins). Moreover, it was verified that not only pure cyclodextrins but also a linear oligosaccharide and even the simple glucose monomer are able to solubilize the hydrophobin in water. The aqueous solution of the protein—in the presence of the cyclic glucans—showed propensity to self-assembly, and conformational changes towards beta structure were observed on vortexing the solution. On the other hand, the pure protein dissolved in less polar solvent (60% ethanol) is not prone to self assembly, and no conformational change was observed. When the pure protein was deposited on a hydrophobic surface, it formed a very stable biofilm whose thickness was about 3 nm, whereas the biofilm was not detected on a hydrophilic surface. When the watersoluble protein—in the presence of the cyclic glucans—was used, thicker (up to 10-fold) biofilms were obtained on either hydrophilic or hydrophobic surfaces.

Keywords: biotechnology/cyclodextrins/proteins/self-assembly

Introduction

Hydrophobins are small proteins (about 100 amino acid residues) produced by fungi as soluble forms, self-assembling into an amphipatic membrane when they reach an interface (e.g., medium–air or cell wall–air). Because of their properties, these proteins play a role in the formation of aerial hyphae, spores, in fruiting body and in the attachment of hyphae to hydrophobic surfaces during symbiotic or pathogenic interactions (Wösten 2001). The intriguing properties of these proteins make them of great interest to biotechnologists, as they have potentialities for numerous applications (Hektor and Scholte-meijer 2005). They could be used as coatings to increase biocompatibility of medical implants, to immobilize enzymes on surfaces, in cosmetic industry for hair-care products or in drug delivery.

At the molecular level, hydrophobins have low sequence identity but for the presence of eight cysteine residues forming a conserved four-disulfide bonding pattern (Sunde et al. 2008). Analyses of the known fungal genomes indicate that hydrophobins are encoded as gene families ranging from two to seven members, with the exception of *Coprinus cinereus* genome, which has 23 hydrophobin encoding genes. Hydrophobins have been split into two groups, class I and class II, based on the differences in their hydrophathy patterns, spacing of amino acids between the cysteine residues and properties of the aggregates they form (Linder et al. 2005). Class I hydrophobins generate very insoluble assemblies, which can only be dissolved in strong acids (i.e., 100% trifluoroacetic acid (TFA)). Assemblies of class II can be more easily dissolved in ethanol or sodium dodecyl sulfate. Class II hydrophobins have been only detected in Ascomycetes, while those of class I have been identified in Ascomycetes and Basidiomycetes. One distinguishing feature of class I hydrophobins is the characteristic rodlet structure observed on the hydrophobic side of the amphipathic protein film. The morphology of isolated rodlets is reminiscent of amyloid fibrils, whereas the aggregates of class II hydrophobins lack the rodlet morphology, are non-amyloid and are needle like.

SC3 from *Schyzophyllum commune* is one of the most characterized members of the class I hydrophobin family. SC3 contains approximately 20 mannose residues O-linked through threonine residues in the N-terminal region of the protein, a modification that increases the hydrophilicity of that region of the polypeptide chain (de Vocht et al. 1998). When SC3 has been induced to self-organize at the air/water or Teflon/water interface, a small, transient increase in α-helical secondary structure has been observed, followed by an increase in β-sheet structure (Wang et al. 2002; Wang, Permentier, et al. 2004). More recently, the 3D structure in solution of another class I hydrophobin, EAS from *Neurospora crassa*, has been resolved (Kwan et al. 2006). EAS forms a β-barrel structure interrupted by some disordered regions and displays a complete segregation of charged and hydrophobic residues on its surface. On the
basis of this structure, a model for the polymeric rodlet structure formation has been proposed.

Class II hydrophobins are more easily handled because they have less tendency to aggregate. The crystal structures of two class II hydrophobins, HFBI and HFBII of *Thrichoderma reesei*, have been determined, and several other studies on these two proteins have been performed (Hakanpää, Linder, et al. 2006; Hakanpää, Szilvay, et al. 2006). The amino acid sequence of HFBII is 70% similar to HFBI, their 3D structures are similar and they form similar, hexagonally ordered films (Szilvay et al. 2007). The assemblies of HFBI have been recently shown to be more stable than those of HFBII, as the former can tolerate changes in temperature and pH and addition of ethanol better than the latter (Kisko et al. 2008). The differences in their behaviors could reflect different roles in fungal life.

Aside from these studies, very little is known about other hydrophobins, their characteristics and the films they form. It might be mainly due to the difficulties in extracting and handling these self-assembling proteins. A deeper knowledge of the properties and behaviors of other fungal hydrophobins, both in solution or assembled, is needed to generalize results obtained so far. We are contributing to this goal studying the hydrophobins secreted by the basidiomycete fungus *Pleurotus ostreatus*. It is a well-known edible mushroom, and its ligninolytic and bio-degradative abilities have been widely reported (Cohen et al. 2002). Several hydrophobin encoding genes have been identified in *P. ostreatus*, and their expression in different growth stages or culture times has been studied (Peñas et al. 1998; Peñas et al. 2002), whereas very little is known on the encoded proteins, their structures and their potential applications.

In this paper, we analyze at molecular level the hydrophobin secreted by *P. ostreatus* mycelia, its behavior in solution and its interaction with glycans produced by the fungus or other oligo- and monosaccharides.

### Results

**Purification and analyses of the protein**

*P. ostreatus* was grown in static cultures using potato dextrose broth (24 g/L) containing 0.5% yeast extract (PDY) till the mycelia completely covered the liquid surfaces. The amount of the lyophilized material, obtained after air bubbling into the liquid broths and centrifugation, ranged from 5 to 10 mg/L. After the TFA treatment, the dried material was dissolved in water (Hyd-w) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), showing a unique protein band at about 10 kDa (Figure 1A). Since about 0.5–1 mg of proteins per liter of culture broth were determined by bicinchoninic acid (BCA) protein assay, nonprotein components have to be present in the dried material obtained after bubbling. Further treatment of this material with 60% ethanol extracted the hydrophobin in the ethanol solution (Hyd-et), as verified by SDS-PAGE (Figure 1A), leaving the nonprotein component as insoluble precipitate. The presence of glycans in Hyd-w and in the ethanol precipitate was demonstrated by the phenol-sulfuric acid test and by periodic acid staining of the SDS-PAGE (Figure 1B).

After glycan separation by precipitation in 60% ethanol, the protein was only soluble in the presence of ethanol, not in pure water. Therefore these glycans, when present, co-aggregate during air bubbling with hydrophobins, making the protein soluble in water.

However, if the hydrophobin was purified from *P. ostreatus* grown in malt extract (ME) cultural broth (a medium without amylase), the protein was not soluble in water but was soluble in 60% ethanol. In this condition no precipitate was formed.

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*Fig. 1.* SDS-PAGE of Hyd-w (lane 1), Hyd-et (lane 2) and the precipitate obtained from Hyd-et (lane 3). M, Mₙ markers. (A) Half of the gel stained by silver staining, (B) half of the gel stained by periodic acid.

*Fig. 2.* MALDI-MS spectrum of Hyd-w (linear mode using sinapinic acid as matrix).
Fig. 3. (A) MALDI-MS spectrum (reflectron mode using DHB as matrix) of glycans from Hyd-w, after incubation at 110°C for 2 h at pH 3. Peaks corresponding to cyclic structures \((162n + 23, \text{sodium ion adduct})\) and peaks corresponding to linear structures \((162n + 18 + 23)\) are shown. (B) Comparison of the \(^1\)H NMR spectrum of the \(\alpha-(1\rightarrow4)\) glucan before (bottom) and after (up) the acid hydrolysis. The anomeric signals of the reducing units are labeled (up), and the ring proton signals have been indicated (bottom).
indicating the absence of the glycan component in this sample. This evidence has been confirmed by the lack of any detectable band when the sample was loaded on periodic acid-stained SDS-PAGE.

**Structural analyses**

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) spectra of the samples (Hyd-w and Hyd-et) showed two peaks at 8463 and 8564 m/z (Figure 2), in which the relative intensity of the latter was higher than the former in all the protein purifications.

According to Penas et al. (2002), *P. ostreatus* hydrophobins undergo a proteolytic processing after signal peptide removal. The peak at 8564 m/z can be attributed to Vmh2-1 (TrEMBL accession number Q8WZ12), starting from T25 and with the eight cysteine residues linked in four disulfide bridges, and the peak at 8463 m/z to the same protein after further removal of the N-terminal threonine residue. Analysis of the MALDI-MS spectrum of the tryptic peptides, obtained by in situ hydrolysis, showed the presence of the expected peaks at 2002 m/z (D26-K44) and 2103 m/z (T25-K44), whereas the other expected peak at 6939 m/z (corresponding to the peptide A45-L113) was not detected. The absence of this peak could be due to the difficult extraction of such a large peptide by the polyacrylamide gel.

The identity of the protein and its N-terminal processing was confirmed by liquid chromatography-MS-MS analysis. Fragmentation of the 2103 and 2002 m/z peaks led to the sequences SCSTGSLQG (S29-C37) and CSTGSLQCCSSV (C30-V41) (Supplementary data, Fig. 1), respectively.

The protein was purified from fungal cultures grown at different times (5, 10, 15 or 20 days) and directly analyzed by MALDI-MS. A relative increase of the intensity of the peak corresponding to the form with D26 as N terminus, with respect to that with T25, was observed in older cultures. Furthermore, a peak at 8449 m/z, probably corresponding to the form lacking also D26, was detected in the preparation from 15- and 20-day-old cultures.

The glycan component of Hyd-w was also subjected to chemical and spectroscopic analyses after purification by ethanol precipitation. Carbohydrate analysis by gas-liquid chromatography (GLC)-MS revealed exclusively the presence of 4-substituted D-glucose in pyranose form (4-D-Glc). Chromatography (GLC)-MS revealed exclusively the presence of 4-substituted D-glucose in pyranose form (4-D-Glc). Incubation of the cyclic glucan at 110°C for 2 h at pH 3 led to partial hydrolysis of the cyclic glucans, as shown in the MS spectrum reported in Figure 3A. In agreement, in the 1H NMR spectrum of this sample (Figure 3B), the presence of anomeric reducing signals was detectable.

No precipitate was obtained treating fresh PDY medium with the procedure described for hydrophobin purification. Moreover, the glycan component was absent in hydrophobin samples purified from *P. ostreatus* grown in ME cultural broth, as already mentioned above. Taking into consideration that amylose ((β-(1→4)-Glc), glucan), a component of the PDY cultural broth coming from potato infusion, is absent in the ME culture broth, these results point out that the cyclic glucans are produced by the fungus grown in the presence of amylose.

**Analyses of the protein–glycan interaction**

A glucan/protein ratio of about 6 (w/w) was determined for Hyd-w samples. Once cyclic glucans were removed by ethanol precipitation, the dried TFA-treated hydrophobin was not soluble either in the cyclic glucans aqueous solution, at the above reported ratio or in PDY broth. A water-soluble protein was re-obtained when the aqueous solution of the cyclic glucans was added to the Hyd-et solution, and the air-bubbling procedure (vortexing, centrifugation, lyophilization and TFA treatment) was performed. On the other hand, when the same procedure was performed using fresh PDY broth, no water-soluble protein was found. The presence of the protein in the aqueous solution was assessed by BCA assay, SDS-PAGE and MALDI-MS spectra. Hence, co-aggregation of the hydrophobin with the fungal cyclic glucans is needed to obtain the hydrophobin in aqueous solution.

In order to investigate the structural requirements of glucans for the hydrophobin solubilization in water, some commercial molecules were tested in comparison with the cyclic glucans produced by the fungus, following the protocol described above. When α, β or γ cyclodextrins were used, the presence of the protein in the aqueous solution was verified in all the samples. Moreover, the protein was solubilized even when maltohexaose, the linear form of the α cyclodextrin, was tested. Afterwards, the ability of glucose to solubilize the hydrophobin was also proved and then analyzed in deeper details. In order to assess the glucose–hydrophobin interaction, a gel filtration chromatography was performed (Supplementary data, Fig. 2) using two different glucose/hydrophobin ratios (6 and 120 w/w). In both cases, the presence of glucose in the protein peak with a ratio of about 5 w/w (corresponding to 200 ÷ 300 mol/mol) was verified. These results suggest that different types of glucans in comparable amounts can interact with the hydrophobin, allowing its water solubility. Among the glucans tested, just the amylose contained in the PDY broth was unable to solubilize the protein.

**Analyses of hydrophobin aggregation**

Since hydrophobins are prone to interfacial self assembly in response to external stimuli like agitation of the solution, we have investigated the hydrophobin behavior in water—in the presence of cyclic glucans—or ethanol solution by analyzing SDS-PAGE, protein concentration, circular dichroism (CD) and fluorescence spectra before and after vortexing.
A remarkable decrease of Hyd-w concentration was observed in the supernatant of vortexed solution. Protein concentration dropped down, as it can be inferred by the intensity of the SDS-PAGE band of the hydrophobin (Figure 4A).

CD spectra of Hyd-w slightly varied from sample to sample (Figure 4B) even when the same dried sample was apparently suspended in the same conditions. In all the samples, however, a significant contribution of random structure was noticed. Conformational changes with a large shift towards β structure were observed after vortexing (Figure 4B).

Significant changes of the secondary structure were also observed between 70°C and 80°C, registering CD spectra of Hyd-w at different temperatures in the range from 30°C to 90°C.

When the pure protein was dissolved in 60% ethanol (Hyd-et), no variation of band intensities on SDS-PAGE was observed after vortexing (Figure 5A). CD spectra of Hyd-et were recorded, showing a substantial contribution of α helix to the secondary structure of the protein (Figure 5B). Interestingly, no change of the spectrum was detectable after vortexing these samples.

We also tried to suspend dried Hyd-et in 20% ethanol. The intensity of the SDS-PAGE band of this sample was much lower than that one obtained in 60% ethanol dissolving the same amount of protein (data not shown), and the corresponding CD spectrum showed similar profile but at lower intensity (Figure 5B). No variation of these parameters was observed after vortexing, as for Hyd-et in 60% ethanol.

Furthermore, when the percentage of ethanol in Hyd-et solutions was decreased, protein bands on SDS-PAGE were observed up to 20% ethanol, while no band was present at 10% ethanol, yet again indicating that the protein aggregates in more polar solution.

The binding of thioflavin-T (ThT) to Hyd-w or Hyd-et was evaluated through the analyses of fluorescence spectra of samples before and after aggregation induced by vortexing. In Figure 6, the remarkable increase of the fluorescence intensity obtained for Hyd-w, not for Hyd-et, is shown thus confirming the formation of amyloid-like aggregates starting from the water solution of the hydrophobin.

**Biofilm analyses**

In order to analyze the Vmh2 aggregates, biofilms were formed on hydrophobic (silicon) or hydrophilic (oxidized silicon) surfaces using both Hyd-w or Hyd-et. The biofilm characterization has been performed by variable-angle spectroscopic ellipsometry (VASE) on at least six samples prepared in the same experimental conditions; the optical model for experimental data fitting has been developed in a
previous work (De Stefano et al. 2007). Hyd-et forms a very stable biofilm on silicon whose thickness is 3.1 ± 0.7 nm, whereas Hyd-et did not stably interact with oxidized silicon; thus, the biofilm was not detectable. On the other hand, Hyd-w forms biofilms whose thickness was highly variable, sometimes significantly thicker than when Hyd-et was used on crystalline surface. The Hyd-w biofilms thickness ranged from 3.5 to 30 nm on the silicon surface and from 6 to 38 nm on the oxidized silicon surface.

Discussion

A hydrophobin secreted by the basidiomycete fungus *P. ostreatus* was purified and identified as Vmh2-1. Its encoding gene and cDNA had been previously isolated and sequenced by Pennás et al. (2002). The secreted protein undergoes a proteolytic processing, since the N terminus is either T25 or D26, whereas the expected signal peptide is M1-A21 according to the SignalP prediction program (http://www.cbs.dtu.dk/services/SignalP). Sequence analysis (hydropathy pattern and spacing between the cysteine residues) and robustness of the aggregate (dissolvable in 100% TFA) demonstrate that it belongs to class I hydrophobins.

Although hydrophobins are generally reported to share low sequence identity percentage (Sunde et al. 2008), comparison of the sequence of the often-used example of class I hydrophobin, SC3, with that of Vmh2 reveals 46% of identity on the whole sequence, increasing to 62% if the fragment starting from the first C of the disulfide pattern is considered (Supplementary data, Fig. 3). Actually, the main difference between the two sequences lies in the T stretch upstream of the first C of SC3 that is absent in Vmh2. This T stretch in SC3 is reported to be O-linked to mannose residues, thus further increasing the hydrophilicity of that region and, consequently, of the whole protein (de Vocht et al. 1998). The absence of this region in Vmh2 suggests a higher hydrophobicity of the *P. ostreatus* protein with respect to SC3. As a matter of fact, our results show that pure Vmh2 is not soluble in water. The two more studied class I hydrophobins, SC3 and EAS of *N. crassa*, can be dissolved in water up to 1 mg/mL (Mackay et al. 2001; Wang, Graveland-Bikker, et al. 2004), and class II hydrophobins are reported to be even much more soluble (up to 10 mg/mL) (Kisko et al. 2008). Therefore, to the best of our knowledge, Vmh2 is the most hydrophobic hydrophobin characterized so far.

Analyses of protein samples purified from culture medium containing amylase have shown the presence of glycans in the hydrophobin aggregates. If the protein is dissolved in 60% ethanol, the glycan fraction precipitates, leaving the protein free in this solution. Since the free protein is not soluble in water, the interaction with glycans enables the protein to be water soluble.

The structure of the water-soluble glycans matched to α-(1–4) linked glucose lacking of reducing ends, thus pointing towards cyclic structure containing from six to 16 monomers, namely α, β and γ cyclodextrins and higher homologues. On the basis of our results the cyclic glucans are produced by the fungus when grown in the presence of amylase. Enzymatic activities like 4-α-glucanotransferase (Takaha and Smith 1999) could be responsible for their synthesis. These enzymes are widely distributed in bacteria, yeasts, plants and mammals, and an increasing number of 4-α-glucanotransferases from different sources are known to be able to catalyze cyclization reactions to produce cycloamylose (Yanase et al. 2002).

When some commercial molecules were tested for their ability of restoring the hydrophobin water solubility, like the fungal cyclic glucans do, we ascertained that not only pure α, β and γ cyclodextrins but also the linear molecule maltotetraose and even the simple D-glucose monomer are able to solubilize the hydrophobin Vmh2 in water. Interaction between Vmh2 and glucose has been verified by the finding of a unique peak containing both the molecules eluted by gel filtration chromatography.

Even though the physical–chemical features of the binding are still unknown, it is evident that carbohydrates do mediate the interaction of the hydrophobin with water. The interaction between carbohydrate and hydrophobic regions of proteins is not surprising since they often bind to proteins by π– stacking interactions. It has been demonstrated that these interactions are facilitated by the formation of hydrogen bonds. However, CH/π stacking interaction, very common in the recognition sites of proteins that recognize carbohydrates, has origin in dispersion forces, which have an impact on the enthalpic term of the free energy (Vandenbussche et al. 2008).

By vortexing the aqueous solution of the Vmh2—in the presence of the fungal cyclic glucans—the protein self-assembles. The conformational changes observed for class I hydrophobins after self-assembling—increase of β sheet structure—have been demonstrated for Vmh2 by vortexing its water solution in the presence of glucans. On the other hand, when the pure protein is dissolved in less polar solvents (60% ethanol), the propensity to self-assemble is greatly reduced, and no conformational change has been observed upon vortexing the solution. Moreover, amyloid-like aggregates are formed by vortexing the hydrophobin water solution, as indicated by ThT binding, whereas no ThT binding was detected by vortexing the ethanol solution of Vmh2.

The behavior of the Vmh2 aggregates has also been investigated by analyzing the biofilms formed by the hydrophobin in different conditions and on different surfaces. We have previously reported that chemically and mechanically stable mono-
and multilayers formed by the *P. ostreatus* secreted hydrophobin (Vmh2 in the Hyd-et form, as described in this paper) (De Stefano et al. 2007, 2008; Houmadi et al. 2008) are obtained by deposition on crystalline silicon (De Stefano et al. 2007) or Porous Silicon (Psi) surfaces (De Stefano et al. 2008). This biofilm changes the wettability of both the silicon surface and the PSI structure. Formation and features of Langmuir Blodgett Vmh2 film have been also investigated and analyzed by atomic force microscopy after transfer onto silicon substrate, revealing the coexistence of a monolayer and rodlets (Houmadi et al. 2008). In this paper, biofilms that had been formed on hydrophobic or on hydrophilic surfaces by depositing the pure protein in ethanol or in water—in the presence of the fungal cyclic glucans—were analyzed by VASE. When the pure protein was deposited on the hydrophobic surface, crystalline silicon, a very stable biofilm was formed, whereas it could not be detected on a hydrophilic surface, the oxidized silicon. On the other hand, biofilms were obtained on both surfaces using Vmh2 dissolved in water. Their thicknesses were always higher but more variable than that obtained from the pure protein on crystalline silicon. Therefore, glucans seem to mediate also hydrophobin binding to hydrophilic surfaces.

Interaction between the most studied class I hydrophobin, SC3 from *S. commune*, and polysaccharides has been studied (Martin et al. 2000; Scholtemeijer et al. 2009). *S. commune* produces and excretes a high molecular weight polysaccharide, schizophyllan (Steiner et al. 1987). Martin et al. (2000) have demonstrated that this polysaccharide stabilizes small hydrophobin oligomers in solution, acting as hydrophilic stabilizer. Moreover, schizophyllan is necessary for SC3 assembly into films on hydrophilic surfaces, whereas the pure hydrophobin can assemble only on a hydrophobic surface.

More recently, Scholtemeijer et al. (2009) have demonstrated that schizophyllan, as well as some other polysaccharides, promotes formation of SC3 amyloid fibrils at the interface between the water and the air or a hydrophobic solid. According to the authors, the small hydrophobin oligomers found in the presence of the glucan could have a higher capacity to assemble at a hydrophobic–hydrophilic interface.

In the case of the more hydrophobic protein Vmh2, we have verified that oligomeric or monomeric glucans play a role as hydrophilic stabilizer, similar to that of the polysaccharide schizophyllan, allowing solubility of Vmh2 in water. Starting from these conditions, increase of β-sheet structures and formation of amyloid-like fibrils have been verified. Moreover, biofilms were obtained on hydrophilic surfaces using Vmh2 dissolved in water in the presence of the cyclic glucans. These results point out similarities in the behavior of the two hydrophobins in their relationships with glucans, although we do not know at the moment if in the case of Vmh2 the transition towards the amyloid form is favored by the presence of a polar solvent (water) or of the glucans.

### Materials and methods

**Fungal growth and protein purification**

White-rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306), was maintained through periodic transfer at 4°C on potato dextrose agar (Difco, Detroit, MI) plates in the presence of 0.5% yeast extract (Difco). Mycelia were inoculated (by adding six agar circles of 1 cm diameter) in 2-L flasks containing 500 mL of PDY or 2% ME and grown at 28°C in static cultures.

After 10 days of fungal growth, mycelia were removed by filtration, and hydrophobins released into the medium were aggregated by air bubbling using a Waring blender. Foam was then collected by centrifugation at 4000 × *g*. The precipitate was freeze dried, treated with TFA for 2 h and sonicated for 30 min. After centrifugation at 3200 × *g* for 20 min, the supernatant was dried again in a stream of air and then dissolved in water (Hyd-w) or 60% ethanol (Hyd-et). In the latter case, the solution was kept at 4°C overnight and then centrifuged at 3200 × *g* for 10 min.

Before use, the protein was always disassembled with pure TFA and dried, and then the monomeric protein was dissolved.

Protein concentration of Hyd-w was evaluated by BCA assay (Pierce, Rockford, IL) using bovine serum albumin as standard. Absorbance at 230 nm was measured to find out protein concentration in the presence of substances incompatible with the BCA protein assay. Concentration of Hyd-w evaluated by BCA assay was used as reference.

**Phenol-sulfuric acid test (Chaplin and Kennedy 1994)**

The glycan solution (200 µL) was mixed to 5% phenol (200 µL) and sulfuric acid (1 µL). This mixture was incubated for 10 min at room temperature and 20 min at 37°C, and then absorbance at 485 nm was determined. Standard curve was performed using from 2 to 200 µg of glucose.

**SDS-PAGE**

SDS-PAGE (Laemmli 1970) was performed at 15% polyacrylamide concentration using the Bio-Rad Mini Protean III apparatus (Bio-Rad Laboratories, Hercules, CA). β-Galactosidase (117 kDa), bovine serum albumin (85.0 kDa), ovalbumin (49 kDa), carbonic anhydrase (34.0 kDa), α-lactoglobulin (25.0 kDa) and lysozyme (19 kDa) were used as standards (Fermentas Inc., Glen Burnie, MD). The gels were stained by silver staining or by periodic acid staining (GelCode Glycoprotein Staining Kit, Pierce).

**Mass spectrometry**

MALDI mass spectra were recorded on a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). The analyte solutions were mixed with sinapinic acid (20 mg/mL in 70% acetonitrile, TFA 0.1% *v*/v), 2,5-dihydroxybenzoic acid (DHB) (25 mg/mL in 70% acetonitrile) or α-cyano-4-hydroxycinnamic acid (10 mg/mL in 70% acetonitrile, TFA 0.1%, *v*/v) as matrix, applied to the sample plate and air dried. The spectrometer was used in the linear or reflectron mode. Spectra were calibrated externally.

In situ hydrolysis was carried out on the silver-stained protein bands excised from a 15% polyacrylamide gel run under denaturing conditions (Cleveland et al. 1977). Excised bands were extensively washed with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation in 10 mM dithiothreitol for 45 min at 56°C and carboxamidomethylated by using 55 mM iodoacetamide for 30 min, in the dark at room temperature. The gel particles were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with 15 µg/mL trypsin (Sigma-Aldrich, St.
Quantum coherence was measured in the 1H-detected mode before Fourier transformation. Heteronuclear single resolution enhanced in both dimensions by a shifted sine-bell dimension to give a matrix of 4096 × 2048 points and was zero-filled phase-sensitive correlation spectroscopy experiment was performed using data sets (t1 × t2) of 2048 × 256 points, and 16 scans were acquired. In all homonuclear experiments, the data matrix was zero-filled and the procedure used for hydrophobin purification (vortexing, centrifugation, lyophilization and TFA treatment) was performed. Alternatively, Hyd-et was diluted up to 4% ethanol, and then aqueous solutions (0.6 mg/mL) of PDY broth, or of the cyclic glucans, or of the cyclodextrins (α, β, or γ) or of maltohexaose, or of D-glucose (Sigma-Aldrich) were added. Samples were then vortexed for 10 min, the aggregates lyophilized, TFA treated and solubilized in water.

**Gel filtration chromatography**

One milliliter of a solution containing hydrophobin (0.1 mg) solubilized in the presence of glucose (0.6 or 12 mg) was subjected to size exclusion chromatography on Toyopearl HW-40S (Tosoh Bioscience GmbH, Montgomeryville, PA). The column (1 × 40 cm) was eluted with water at a flow rate of 10 mL/h, and the absorbance of the eluate at 230 and 280 nm was recorded. Protein and sugar contents were determined by BCA assay and by phenol-sulfuric acid test, respectively.

**Circular dichroism and fluorescence spectroscopies**

Far-UV CD spectra were recorded on a Jasco J715 spectropolarimeter equipped with a Peltier thermostatic cell holder (Jasco model PTC-348) in a quartz cell (0.1-cm light path) from 190 to 250 nm. The temperature was kept at 20°C, and the sample compartment was continuously flushed with nitrogen gas. The final spectra were obtained by averaging three scans, using a bandwidth of 1 nm, a step width of 0.5 nm and a 4-s averaging per point. The spectra were then corrected for the background signal using a reference solution without the protein.

CD spectra of the protein were also recorded by varying the temperatures from 30°C to 90°C at a rate of 1°C/min with 10°C step.

Fluorescence spectra were recorded at 25°C with a Perkin-Elmer LS50B fluorescence spectrometer. Slit widths were set at 10 nm in both the excitation and emission monochromators. ThT (Sigma-Aldrich, 100 μM final concentration) was added to Hyd-w and Hyd-et before and after vortexing for 30 min. Samples were excited at 435 nm, and emission was monitored from 460 to 600 nm. The spectra were then corrected by subtracting the ThT spectrum.

**Biofilm on silicon chip**

Silicon samples, single side polished, <100> oriented (chip size: 1 cm × 1 cm), after standard cleaning procedure, were washed in hydrofluoric acid solution for 3 min to remove the native oxide thin layer (1–2 nm) due to silicon oxidation. To obtain oxidized silicon, samples were thermally oxidized in O₂ atmosphere, at 1100°C for 1 h, resulting in an oxide thickness of about 80 nm. A drop (80 μL) of Hyd-et solutions (0.1 mg/mL in 80% ethanol) was spotted on the chip. After 1 h, samples were dried for 10 min on the hot plate (80°C) and then washed by the solvent solution and then by 2% SDS at 100°C for 10 min. Ellipsometric characterization of the hydrophobin biofilm was performed by a variable-angle spectroscopic ellipsometry model (UVISEL, Horiba-Jobin-Yvon). At least six different experiments were performed in each condition.
Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding
This work was supported by the European Commission, Sixth Framework Program (QUORUM contract NMP-2004-032811).

Acknowledgements
The authors gratefully acknowledge Professor Gennaro Marino, Professor Delia Picone and Professor Leila Birolo for the helpful discussions.

Abbreviations
BCA, bicinchoninic acid; CD, circular dichroism; DHB, 2,5-dihydroxybenzoic acid; GLC, gas–liquid chromatography; 1H NMR, proton nuclear magnetic resonance; Hyd-et, hydrofluoroacetic acid; ThT, thioflavin-T; VASE, variable-angle spectroscopic ellipsometry.

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