Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary X-ray diffraction analysis of the *Trichoderma reesei* hydrophobin HFBI

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Received 14 June 2003
Accepted 9 August 2004

Hydrophobins are fungal proteins that are capable of altering the hydrophobicity of surfaces by self-assembly at hydrophilic–hydrophobic interfaces. Here, the growth of hydrophobin crystals suitable for X-ray crystallography is reported. The hydrophobin HFBI from *Trichoderma reesei* was crystallized by vapour diffusion in hanging drops in 30% PEG 4000, 0.1 *M* sodium citrate pH 4.3 buffer containing 0.2 *M* ammonium acetate and CYMAL-5 detergent (initial concentration of 2.4 m*M*). HFBI crystals are hexagonal and belong to space group $P6_1$ (or $P6_5$), with unit-cell parameters $a = b = 45.9$, $c = 307.2$ Å. The HFBI used in the crystallization experiments was purified from fungal cell walls.

1. Introduction

Hydrophobins are small (approximately 100 amino acids) moderately hydrophobic proteins secreted by filamentous fungi. They self-assemble into amphiphilic membranes when confronted with hydrophobic–hydrophilic interfaces. This hydrophobin membrane confers hydrophobicity on fungal structures and mediates the attachment of the fungal hyphae to hydrophobic surfaces and the dispersal of spores into the air (Wösten, 2001). Moreover, hydrophobins are some of the most surface-active molecules known (Wösten & de Vocht, 2000). They lower the surface tension of liquids, enabling the escape of fungi from aqueous solution to the air (Wösten *et al.*, 1999). Hydrophobins have several potential applications, including modification of biophysical surface properties such as wettability, protein immobilization and emulsification (Wessels, 1997). Based on their hydropathy patterns and the solubility of the hydrophobin assemblages, hydrophobins are divided into classes I and II (Wessels, 1994).

Hydrophobins contain eight cysteine residues at conserved positions in the sequence, which appear to form disulfide bridges. The locations of four disulfide bridges have been determined for HFBII from *Trichoderma reesei* (Hakanpää *et al.*, 2004). HFBII is the only hydrophobin for which the atomic resolution structure is available. The hydrophobin has a single-domain structure containing one α-helix and four antiparallel β-strands (Hakanpää *et al.*, 2004). An NMR study of the *Neurospora crassa* hydrophobin EAS indicated that the monomers were mostly unstructured apart form a small β-sheet region (Mackay *et al.*, 2001). Changes in the secondary structure of hydrophobins during self-assembly have been observed by circular-dichroism spectroscopy (Wösten & de Vocht, 2000). Self-assembly on a hydrophobic solid induced α-helical structure, while in contrast the percentage of β-sheet in class I hydrophobins increased upon self-assembly at a water–air interface.

The filamentous fungus *T. reesei* produces three class II hydrophobins: HFBI, HFBII and HFBIII (Nakari-Setälä *et al.*, 1996, 1997; Penttilä *et al.*, 2000). In contrast to the spore-wall-specific HFBII, HFBI (7.5 kDa) has been isolated from submerged fungal cell walls of vegetative mycelia in addition to liquid culture medium (Nakari-Setälä *et al.*, 1996, 1997). The amino-acid similarity between HFBI and HFBII is 69% (Nakari-Setälä *et al.*, 1996). Atomic force microscopy studies showed that both the hydrophobins formed highly ordered Langmuir–Blodgett monolayer films (Paananen *et al.*, 2003). HFBI and HFBII formed fibrillar aggregates by shaking (Torkkeli *et al.*, 2002). Contrary to the fibrillar aggregates of HFBI, those of HFBII were stable and their low-resolution structure was able to be studied (Torkkeli *et al.*, 2002). Here, we report for the first time the crystallization and preliminary crystallographic studies of HFBI.

2. Experimental

2.1. Protein production and purification

To produce HFBI, the *T. reesei* HFBI overproducing strain VTT D-98692 (Askolin *et al.*, 2001) was cultivated in feed-batch fermentations on glucose-containing medium essentially as described previously (Askolin *et al.*, 2001). HFBI was purified using two purification methods. The first included mycelial extraction with 1% SDS at pH 9, SDS removal, hydrophobic interaction and ion-exchange chromatography (Askolin *et al.*, 2001), buffer

 DOI: 10.1107/S0907444904019754

exchange to 20 m*M* Tris–HCl buffer pH 8.0 by gel filtration (Bio-Gel P-6DG, Bio-Rad) and concentration by ultrafiltration (YM1 membrane, Amicon). Prior to crystallization, the pH was adjusted to the pH of the crystallization reagent, approximately pH 4.5, by adding an equal volume of 100 m*M* sodium acetate/acetic acid buffer pH 4.5.

For the second purification method of HFBI, the mycelium (1.73 kg wet weight, 13.8% dry weight) was separated by filtration through a 0.25 mm cloth from a working fermentation volume of 25 l (New Brunswick Scientific IF40) after 4 d of cultivation. The mycelium was extracted with 100 m*M* sodium acetate buffer pH 5.0 containing 1% SDS (10 ml of buffer per 1 g dry weight) at room temperature. This lower pH was used to prevent the deamidation of two N-terminal asparagines (Asn2 and Asn4; Askolin *et al.*, 2001). Purification was continued with SDS removal from the mycelial extract by precipitation with 0.4 sample volumes of 2 *M* KCl, centrifugation and ammonium sulfate precipitation in approximately 0.6 *M* ammonium sulfate at 277 K overnight. The precipitate was removed by centrifugation and dissolved in 60% ethanol (400 ml). Part of the solution (50 ml) was buffered to 25 m*M* Tris–HCl pH 8.5 by gel filtration (Bio-Gel P-6DG, Bio-Rad). Proteins eluted from the chromatography column were detected by absorbance at 215 nm. The pH of the purified HFBI was adjusted to pH 6 with 100 m*M* sodium acetate buffer pH 3.8 immediately after the chromatography run. Prior to crystallization, the pH was lowered further to the pH of the crystallization reagent solution. HFBI content and purity were analysed by HPLC essentially as described by Askolin *et al.* (2001). Based on the HPLC analysis, the purified HFBI was homogeneous and intact, indicating that extraction at lower pH prevented N-terminal modifications.

2.2. Crystallization

Crystallization conditions were screened and optimized by vapour diffusion in hanging drops (initial volume of 4.5–10 µl) using purified HFBI. Initial screening was carried out using a standard crystallization screening kit (HR2-110, Hampton Research) at 288 K. The effect of additional detergents on crystallization was studied with a detergent screening kit (HF2-410, Hampton Research).

HFBI purified by the new purification method (see §2.1) was crystallized using microseeding at 277 K. Batches containing HFBI sample and an equal volume of crystallization reagent (total volume of 40 µl) were seeded with previously grown HFBI crystals to induce nucleation.

2.3. Data collection and processing

A cryoprotectant solution was prepared by replacing 25% of the water in the crystallization solution with glycerol. Crystals were soaked briefly in this solution and mounted in a rayon-fibre loop and placed directly into a stream of N_2 gas at 120 K.

A crystal of suitable quality was chosen and used to collect native X-ray diffraction data in the home laboratory at 120 K using a MAR345 imaging-plate detector on a Rigaku RUH3R rotating-anode X-ray generator with a Cu target operating at 50 kV and 100 mA equipped with multilayer focusing X-ray optics (Osmic). The same crystal was subsequently used to collect data on beamline ID14-2 at the ESRF, Grenoble using a MAR Research CCD detector at a wavelength of 0.934 Å. Both data sets were processed and reduced using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997).

3. Results and discussion

The first HFBI crystals were obtained with 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6 containing 0.2 *M* ammonium acetate. The crystals were very thin hexagonal plates that grew from spherulites. HFBI crystals larger in all three dimensions were obtained by adding the non-ionic detergent CYMAL-5 (cyclohexyl-pentyl-β-D-maltoside). HFBI crystals suitable for X-ray analysis were obtained after approximately two months with 30% PEG 4000, 0.1 *M* sodium acetate buffer pH 4.3 containing 0.2 *M* ammonium acetate and 1× the critical micelle concentration of CYMAL-5 prior to equilibration at 277 K (or 288 K) (Fig. 1). The drop consisted of 5 µl of HFBI sample (2.6 g l^{-1}, pH adjusted to 4.5), 1 µl of 24 m*M* CYMAL-5 and 4 µl of the optimized crystallization reagent solution. One of the most critical factors in the crystallization was pH. HFBI crystallized within the pH range 4.0–4.5 at 288 K, somewhat different from the calculated isoelectric point of 5.7. HFBI crystals of varying length (0.1–0.6 mm) were obtained using 24–30% PEG 4000 and the other crystallization reagents mentioned above at pH 4.0.

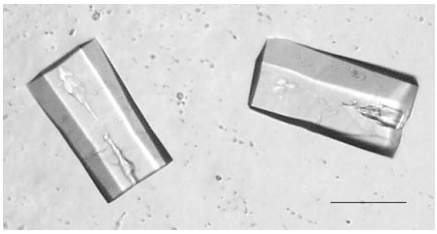

Figure 1
Crystals of HFBI. The crystal dimensions are approximately 190 × 106 µm. The bar represents 100 µm.

Table 1
Diffraction data statistics.

Values in parentheses correspond to reflections in the outer resolution shell.

Space group	$P6_5$ or $P6_1$
Unit-cell parameters (Å)	$a = b = 45.9$, $c = 307.2$
Resolution range (Å)	20–2.5 (2.59–2.5)
Observed reflections	63019 (6255)
Unique reflections	12649 (1251)
Completeness (%)	100 (100)
$R_{\rm merge}$ (%)	8.0 (20)
$\langle I/\sigma(I)\rangle$	21.7 (9.3)

The crystals belong to space group $P6_5$ or $P6_1$, with unit-cell parameters $a = b = 45.9$, $c = 307.2$ Å. Attempts to reduce the data in space groups belonging to Laue group 622 result in an $R_{\rm merge}$ of more than 30% and rejection of almost 15% of the observations, indicating that the crystals contain pseudo-622 point symmetry. Calculation of a self-rotation function using the program *MOLREP* (Vagin & Teplyakov, 1997) does indeed show non-crystallographic twofold axes perpendicular to the sixfold axis, but reveals no other significant features (data not shown). With four molecules in the asymmetric unit, $V_{\rm M}$ is 3.1 Å^3 Da^{-1}, which corresponds to a solvent content of 60% (Matthews, 1968). Alternatively, six molecules in the asymmetric unit would result in a $V_{\rm M}$ of 2.1 Å^3 Da^{-1}, which corresponds to a solvent content of 40%. The crystals diffracted strongly in the home laboratory, which would be in keeping with 4–6 molecules per asymmetric unit, although the latter value is more probable. For the data collected at the ESRF, the resolution was limited to 2.5 Å owing to the long *c* axis (307.1 Å) (Table 1).

Determination of the three-dimensional structure will provide details of surface conformation and the amino-acid residues participating in surface recognition in interfacial self-assembly. It will allow directed amino-acid substitutions to broaden the usefulness of the protein in potential medical and technical applications or even in nanotechnology.

The authors would like to thank Michael Bailey for fermenter cultivations, Riitta Isoniemi for excellent technical assistance in protein purification, the ESRF, Grenoble for

provision of excellent data-collection facilities and the beamline staff for assistance in the use thereof. The financial support of Neste Oy's Foundation (personal grant to SA) and National Technology Agency of Finland (Tekes) is gratefully acknowledged.

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