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Crystallization and preliminary X-ray characterization of *Trichoderma reesei* hydrophobin HFBII

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Hydrophobins are small proteins found in filamentous fungi and characterized by their ability to change the character of a surface by spontaneous self-assembly on a hydrophobic–hydrophilic interface. Hydrophobin HFBII from *Trichoderma reesei* was crystallized by the hanging-drop vapour-diffusion method at 293 K. Two crystal forms were obtained: a native form and a form crystallized in the presence of manganese chloride. The native crystals were of high symmetry, cubic $I23$, but only diffracted to 3.25 Å. The crystals grown in the presence of manganese were monoclinic and diffracted to 1.0 Å with a synchrotron-radiation source. The anomalous difference Patterson map calculated from the home laboratory data showed a strong single peak, possibly caused by manganese present in the crystallization solution.

1. Introduction

Hydrophobins are small proteins, about 100 amino-acid residues in size, secreted by filamentous fungi. Characteristically, hydrophobins contain eight cysteine residues in a conserved pattern and share a similar hydropathic pattern. Hydrophobins have several functions in the growth and development of fungi and also in the interactions of fungi with the environment. They are produced fairly abundantly and a fungal species may contain several different hydrophobin genes expressed during different periods and suitable for specific functions (Nakari-Setälä *et al.*, 1997).

A remarkable feature of hydrophobins is their ability to change the character of a surface by spontaneous self-assembly of amphiphilic hydrophobin monomers in the presence of a hydrophobic–hydrophilic interface. In nature, hydrophobins cover the hydrophilic cell walls of fungi, making them hydrophobic, which enables the growth of aerial hyphae and the attachment of hyphae to hydrophobic surfaces (as in pathogenic interactions). Hydrophobins are also very surface active; by reducing the surface tension of water they allow hyphae to penetrate into the air from a liquid media (Wösten *et al.*, 1999). These unique characteristics of hydrophobins make them interesting candidates for numerous applications; for example, in the medical and food industries.

Hydrophobins are divided into two classes according to their hydropathic patterns and the solubility characteristics of the assembled layers (Wessels, 1994). Aggregates formed by class I hydrophobins are highly insoluble (only soluble in trifluoroacetic acid and formic acid), while aggregates of class II hydrophobins are more easily dissociated (*e.g.* in 60% ethanol or 2% SDS). With class II hydrophobins, the spacing of the conserved cysteines, which generally falls into the category $X_{2–38}$-C-$X_{5–9}$-C-C-$X_{11–44}$-C-$X_{8–23}$-C-$X_{5–9}$-C-C-$X_{6–18}$-C-$X_{2–14}$, is also more invariant.

Hydrophobins do not share a high sequence similarity even within their own classes. For example, HFBII, a class II hydrophobin from *Trichoderma reesei*, shares a 46% sequence similarity with another class II hydrophobin, cerato-ulmin from *Ophiostoma ulmi*. However, *T. reesei* also secretes another class II hydrophobin, HFBI, and the sequence similarity between HFBI and HFBII is only 69%. HFBI aggregates in fungal cell walls, whereas HFBII is found in spores (Nakari-Setälä *et al.*, 1996, 1997).

To date, hydrophobins have been isolated from several filamentous fungi and their biological properties are well characterized. However, no three-dimensional structure is available for hydrophobins since crystallization trials have stumbled on the aggregates that are easily formed by the protein. EAS, a class I hydrophobin from *Neurospora crassa*, has been studied by NMR techniques and was found to be largely unstructured in solution, except for a small region stabilized by disulfide bridges (Mackay *et al.*, 2001). Here, we present the crystallization and the preliminary X-ray data of *T. reesei* hydrophobin HFBII. Mature HFBII contains 71 amino-acid residues and has a calculated molecular weight of 7.2 kDa.

Table 1
Measurement statistics.

Values in parentheses correspond to the highest resolution shell.

	Home laboratory	EMBL Hamburg	EMBL Hamburg
Metal ion	Mn	Mn	—
Unit-cell parameters			
a (Å)	78.80	78.66	72.15
b (Å)	46.16	46.31	72.15
c (Å)	34.66	34.59	72.15
$\alpha = \gamma$ (°)	90.00	90.00	90.00
β (°)	111.81	112.16	90.00
Space group	$C2$	$C2$	$I23$
Source	Cu $K\alpha$	X11	X11
Wavelength (Å)	1.5418	0.8126	0.8122
Resolution range (Å)	99–1.5 (1.55–1.50)	25–1.0 (1.02–1.00)	25–3.25 (3.31–3.25)
No. observations	97414	216723	22261
No. unique reflections	18379 (1740)	61978 (3006)	1047 (48)
Completeness	98.4 (94.1)	99.8 (99.6)	99.6 (100)
R_{sym}	5.2 (16.6)	3.2 (23.5)	4.6 (29.7)
$I/\sigma(I)$	25.0 (7.6)	7.6 (3.2)	13.1 (8.9)

2. Materials and methods

2.1. Protein purification

HFBII was purified from the culture medium of a fermentation of *T. reesei* strain QM9414. To 500 ml of the culture supernatant, 2%(*w*/*w*) surfactant ($C_{11}EO_2$, Berol 532, Akzo-Nobel, Sweden) was added and then allowed to settle in a separation funnel at 293 K. In order to extract the protein back to an aqueous phase, the surfactant phase was collected and mixed with isobutanol. The volume of isobutanol corresponded to five times the original amount of surfactant. The aqueous phase was purified by reversed-phase high-performance liquid chromatography on a Vydac 1 × 20 cm preparative C4 column (Vydac, USA) using a linear gradient of 0–100% 0.1%(*v*/*v*) trifluoroacetic acid in water to 0.1%(*v*/*v*) trifluoroacetic acid in acetonitrile. The pure protein was lyophilized and stored dry.

2.2. Crystallization

HFBII was crystallized using the hanging-drop vapour-diffusion method at 293 K. Initial trials with Hampton Research Crystal Screen I showed that HFBII crystallized easily with various precipitants over a wide pH range. Well ordered crystals grew from a mother liquor containing 25%(*w*/*v*) polyethylene glycol 2000, 0.4 *M* lithium sulfate and 0.1 *M* Tris–HCl pH 8.5. However, the crystals showed only weak diffraction. When additives were screened, it was found that the crystals grown in the presence of manganese chloride diffracted to high resolution. Crystals grew from a mother liquor containing 25%(*w*/*v*) PEG 2000, 0.2 *M* lithium sulfate and 0.1 *M* Na HEPES pH 7.5. $MnCl_2$ was introduced directly into the crystallization drop, where its final concentration was 0.05 *M*.

The lyophilized protein was dissolved in water for crystallization trials to a concentration of 8 mg ml^{-1}; the protein concentration in the crystallization drop was 4 mg ml^{-1}. To improve the quality of crystals grown in the presence of manganese, a streak-seeding method was used and the concentration of polyethylene glycol was reduced to 15%(*w*/*v*) in the mother liquor. The optimal droplet size was 10 µl for drops containing manganese and 4 µl for drops with no manganese. The average dimensions of the crystals were 0.2 × 0.1 × 0.1 mm (drops with manganese) and 0.2 × 0.2 × 0.05 mm (drops with no manganese). Crystals grown in the presence of manganese were rectangular in shape (Fig. 1*a*), whereas the native crystals were of triangular appearance (Fig. 1*b*).

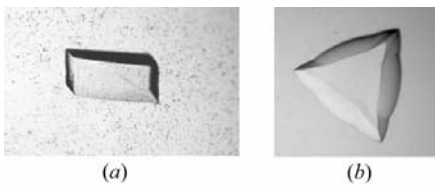

Figure 1
Crystals of *T. reesei* hydrophobin HFBII grown (*a*) in the presence of manganese ions and (*b*) with no manganese present.

2.3. Data collection

Preliminary X-ray data were collected at the home laboratory with a MAR 345dtb image-plate detector using Cu $K\alpha$ radiation (λ = 1.54 Å) from a rotating-anode source equipped with confocal optics from Osmic. Data sets for both crystal forms were collected with the use of synchrotron radiation from the EMBL X11 beamline located at the DORIS storage ring, DESY, Hamburg. The wavelength used was 0.81 Å and the data were collected on an MAR CCD165 detector at 100 K. The crystal-to-detector distance was 250 mm for cubic crystals and 70 and 200 mm for the high- and low-resolution data sets of the monoclinic crystals, respectively. Exposure times were 15 s for cubic crystals and 60 and 1 s for the high- and low-resolution data sets of the monoclinic crystals, respectively. For both crystal forms, mother liquor with the concentration of polyethylene glycol elevated to 30%(*w*/*v*) was used as a cryo-protectant. All data were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Details of the data collected are summarized in Table 1.

3. Results and discussion

The cubic crystals diffracted to about 5 Å in the home laboratory and no data were collected, whereas the monoclinic crystals diffracted to 1.5 Å at 100 K. With synchrotron radiation, the cubic crystals diffracted to 3.25 Å and the monoclinic crystals to 1.0 Å. The space group was determined to be $C2$ for the crystals grown in the presence of manganese, with unit-cell parameters a = 78.66, b = 46.31, c = 34.59 Å, $\alpha = \gamma$ = 90.00, β = 112.16° for the 1.0 Å resolution data set. For cubic crystals, the data were processed in space group $I23$, with unit-cell parameters $a = b = c$ = 72.15 Å, $\alpha = \beta = \gamma$ = 90.00°.

The Matthews coefficient V_M (Matthews, 1968) is estimated to be 1.89 Å^3 Da^{-1} for the monoclinic crystals (two molecules in an asymmetric unit), with a corresponding solvent content of 34.9%. For cubic crystals, V_M is estimated to be 2.03 Å^3 Da^{-1} (one molecule in an asymmetric unit), with a solvent content of 39.3%. These figures match those values usually found for protein crystals quite well.

The anomalous difference Patterson maps were calculated both with the data collected at the home laboratory and the 1.0 Å data collected with synchrotron radiation (resolution range 2.50–22 Å) using the program *XtalView* (McRee, 1992). The map calculated from the home laboratory data showed one clear peak (u = 0.38, v = 0, w = 0.40) and several smaller peaks in the Harker section v = 0. The map calculated from the synchrotron data did not show a corresponding strong peak. Hydrophobin HFBII contains no metal atoms. However, the manganese used in crystallization may bind selectively to the protein. Manganese has an absorption edge of 1.8961 Å, which explains the signal being observed in the home

laboratory data (wavelength 1.5418 Å) and not in the synchrotron data (wavelength 0.8126 Å). The smaller peaks are likely to be produced by anomalous scattering of sulfur from the cysteine residues of hydrophobin. The data collected to atomic resolution may allow structure determination by *ab initio* methods.

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References

Mackay, J. P., Matthews, J. M., Winefield, R. D., Mackay, L. G., Haverkamp, R. G. & Templeton, M. D. (2001). *Structure*, **9**, 83–91.

McRee, D. E. (1992). *J. Mol. Graph.* **10**, 44–47.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N. & Penttilä, M. (1997). *Eur. J. Biochem.* **248**, 415–423.

Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E. & Penttilä, M. (1996). *Eur. J. Biochem.* **235**, 248–255.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Wessels, J. G. H. (1994). *Annu. Rev. Phytopathol.* **32**, 413–437.

Wösten, H. A. B., van Wetter, M.-A., Lugones, L. G., van der Mei, H. C., Busscher, H. J. & Wessels, J. G. H (1999). *Curr. Biol.* **9**, 85–88.