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Crystallization of carbohydrate oxidase from *Microdochium nivale*

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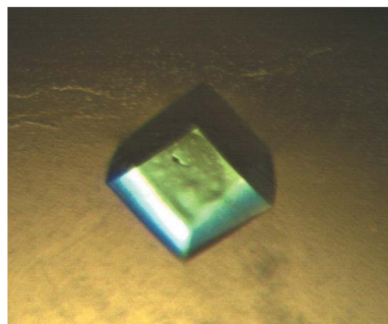
Microdochium nivale carbohydrate oxidase was produced by heterologous recombinant expression in *Aspergillus oryzae*, purified and crystallized. The enzyme crystallizes with varying crystal morphologies depending on the crystallization conditions. Several different crystal forms were obtained using the hanging-drop vapour-diffusion method, two of which were used for diffraction measurements. Hexagon-shaped crystals (form I) diffracted to 2.66 Å resolution, with unit-cell parameters $a = b = 55.7$, $c = 610.4$ Å and apparent space group $P6_222$. Analysis of the data quality showed almost perfect twinning of the crystals. Attempts to solve the structure by molecular replacement did not give satisfactory results. Recently, clusters of rod-shaped crystals (form II) were grown in a solution containing PEG MME 550. These crystals belonged to the monoclinic system $C2$, with unit-cell parameters $a = 132.9$, $b = 56.6$, $c = 86.5$ Å, $\beta = 95.7°$. Data sets were collected to a resolution of 2.4 Å. The structure was solved by the molecular-replacement method. Model refinement is currently in progress.



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1. Introduction

The carbohydrate oxidase from *Microdochium nivale* has been the source of inspiration for a number of publications. The oxidase is a flavoenzyme (Joosten & van Berkel, 2007) containing 475 amino-acid residues and a covalently linked FAD molecule. It has a relative molecular mass of 55 000 Da and an isoelectric point of 9.0 (Xu *et al.*, 2001). The biochemical characteristics of carbohydrate oxidase have also been well described in terms of specificity and kinetic parameters (Xu *et al.*, 2001; Kulys *et al.*, 2001*a*,*b*; Tetianec & Kulys, 2003). The enzyme catalyzes the oxidation of primary alcohols in various monosaccharides or oligosaccharides with an accompanying reduction of molecular oxygen to hydrogen peroxide. The oxidase has a clear preference for disaccharides such as cellobiose and lactose. The conversion of lactose to lactobionic acid has been established as an industrial process and is described in detail by Nordkvist *et al.* (2007). The molecular structure of the oxidase remains unknown. The closest related carbohydrate oxidase with known structure is the gluco-oligosaccharide oxidase from *Acremonium strictum* (Huang *et al.*, 2005), with a sequence identity of 41%. However, there are interesting differences between these two carbohydrate oxidases, for example their substrate specificity. The oxidase from *M. nivale* oxidizes both galactose and xylose, while the oxidase from *A. strictum* fails to utilize these as substrate. A detailed structure of the *M. nivale* oxidase would thus provide additional valuable information about its specificity and mechanism. Indeed, combined structural and biochemical information is undoubtedly the most important basis for the design and development of variant enzymes with new improved properties by protein engineering.

2. Materials and methods

2.1. Protein expression and purification

The carbohydrate oxidase was produced by heterologous recombinant expression in *Aspergillus oryzae* as described by Xu *et al.* (2001). Briefly, the *M. nivale* strain (NN008551) was isolated by K. U.

Sumithra (Novo Nordisk A/S, India) and identified by Centraal Bureau voor Schimmelcultures of the Netherlands. The gene encoding the carbohydrate oxidase was cloned into an *A. oryzae* expression vector as a PCR fragment. The construct was transformed into *A. oryzae* protoplasts for expression driven by the TAKA promoter. The transformed strain was grown for oxidase production in 100 ml medium in 500 ml plastic flasks. The medium contained the following (per litre): 1 g $CaCl_2.2H_2O$, 2 g yeast extract, 1 g $MgSO_4$, 2 g citric acid, 5 g KH_2PO_4, 1 g urea, 2 g ammonium sulfate, 20 g maltodextrin and 0.5 ml of a trace-elements solution. The cultures were incubated at 310 K with vigorous aeration (approximately 200 rev min^{-1}) for 4–5 d. The level of oxidase secreted in these shake-flask cultures was typically around 10 mg l^{-1}. Culture broths were harvested by centrifugation and sterile-filtered to remove fungal hyphae. The resulting filtrate was used as the starting material in purification of the oxidase. The enzyme was purified using a two-step ion-exchange protocol including both anion- and cation-exchange chromatography. A 14-fold purification (based on activity per milligram of protein in the starting and final material) and 31% recovery was achieved. The enzyme used for crystallization experiments was in a solution of 10 m*M* Tris–HCl pH 7.5.

2.2. Crystallization

Crystallization experiments were performed at 290 and 298 K using the hanging-drop vapour-diffusion and microbatch methods. Initial screening was carried out with the Hampton Research Crystal Screen (Jancarik & Kim, 1991), Crystal Screen 2 (Cudney *et al.*, 1994) and Index solutions with a protein concentration of 23.5 mg ml^{-1}. The most promising initial crystallization conditions were optimized through variation of precipitant concentration, additives and the volume ratio of the drops.

Regular crystals of dimensions up to 120 µm grew using optimized Crystal Screen solutions Nos. 15 [0.2 *M* ammonium sulfate, 0.1 *M* sodium MES pH 6.5, 20.5%(*w*/*v*) PEG 8000] and 17 [0.08 *M* lithium sulfate monohydrate, 0.04 *M* Tris–HCl pH 8.5, 12%(*w*/*v*) PEG 4000] and with a 2:1 ratio of protein to reservoir drop volume (Fig. 1). However, these crystals degraded immediately upon opening the crystallization well. The use of 5%(*v*/*v*) glycerol as an additive and protection of the experiment under paraffin oil in a microbatch plate [0.1 *M* MES pH 6.5, 0.2 *M* ammonium sulfate, 28%(*w*/*v*) PEG 8000, 5%(*v*/*v*) glycerol, 2:1 ratio of protein to reservoir drop volume] led to even larger crystals (Fig. 2). In this case, the crystals again degraded rapidly when removed from the mother liquor. The addition of fresh reducing agent (dithiothreitol; DTT) to the crystals before manipulation was also tested. An optimal procedure for the hanging-drop setup was established consisting of washing the crystallization drops down from the cover slip into the reservoir, adding 100 µl 0.1 *M* DTT and then mounting a crystal from the reservoir into a cryoloop. However, the additional cryoprotection step that was necessary for these crystals always resulted in their damage.

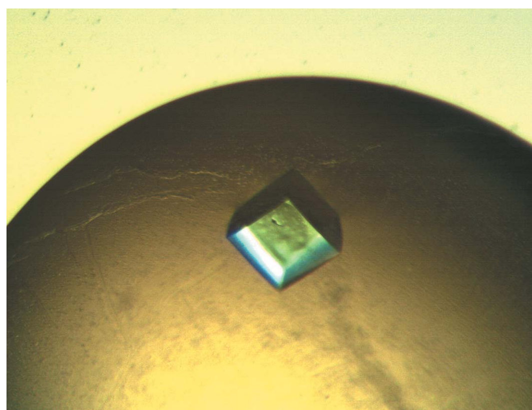

Figure 1
Crystals of carbohydrate oxidase grown by the hanging-drop method in 0.1 *M* MES pH 6.5, 0.2 *M* ammonium sulfate, 20.5%(*w*/*v*) PEG 8000.

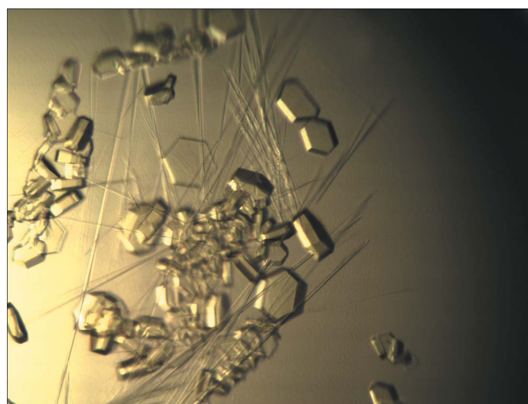

Figure 3
Crystals of carbohydrate oxidase grown by the hanging-drop method in 0.1 *M* HEPES pH 7.0, 17%(*w*/*v*) Jeffamine ED-2001.

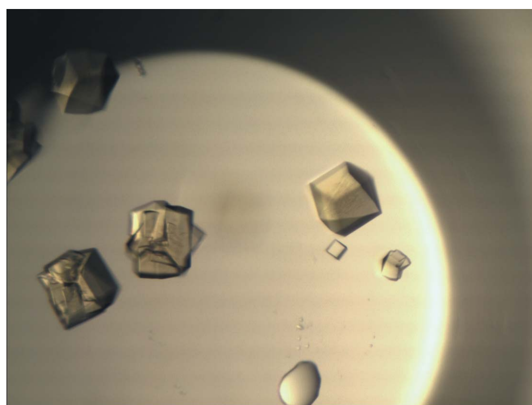

Figure 2
Crystals of carbohydrate oxidase grown by the microbatch method in 0.1 *M* MES pH 6.5, 0.2 *M* ammonium sulfate, 28%(*w*/*v*) PEG 8000, 5%(*v*/*v*) glycerol under paraffin oil. Crystal size is approximately 150 µm.

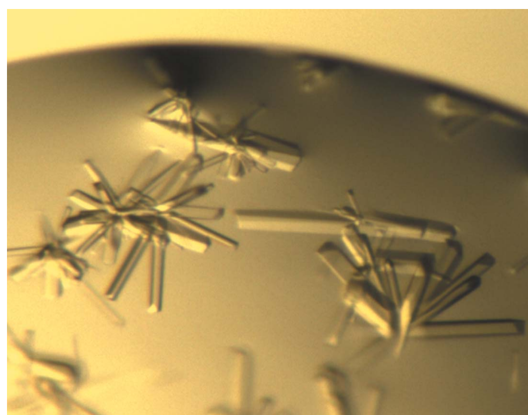

Figure 4
Crystals of carbohydrate oxidase grown by the hanging-drop method in 0.01 *M* $ZnSO_4.7H_2O$, 0.1 *M* MES pH 6.5, 12%(*v*/*v*) PEG MME 550.

Table 1
Data-collection and processing statistics for crystals of carbohydrate oxidase in two crystal forms.

Values in parentheses are for the highest resolution shell.

Crystal form	Hexagon	Rod
Beamline	BM14, MAR 225, Grenoble	X11, MAR 555, Hamburg
Wavelength (Å)	0.8855	0.8148
Space group	Subgroup of $P6_222$	$C2$
Unit-cell parameters (Å, °)	$a = b = 55.7$, $c = 610.4$	$a = 132.9$, $b = 56.6$, $c = 86.5$, $\beta = 95.7$
Resolution (Å)	30–2.66 (2.76–2.66)	30–2.4 (2.55–2.4)
No. of observations	541517	70929 (5833)
No. of unique reflections	10790	24370 (2730)
R_{merge}†	0.083 (0.294)	0.098 (0.362)
$\langle I/\sigma(I)\rangle$	17.7 (2.3)	11.7 (3.4)
Redundancy	5.3 (4.2)	2.9 (2.1)
Completeness (%)	60.2 (12.1)	95 (95)
Data processing	*HKL*-2000	*XDS*

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of a set of equivalent reflections.

Using Index No. 39 [0.1 *M* HEPES pH 7.0, 17%(*w*/*v*) Jeffamine ED-2001, temperature 298 K, 1:2 ratio of protein to reservoir drop volume] as reservoir solution, crystals of hexagonal shape (form I) grew in approximately 4 d (Fig. 3). These crystals had long-term stability and remained stable during cryoprotection. However, either weak or no X-ray diffraction was generally observed. Of a total of 50 tested crystals, only one was found to be suitable for diffraction measurement.

Finally, stable crystals (form II) were grown in crystallization condition No. 27 of Crystal Screen 2 optimized to 0.01 *M* $ZnSO_4.7H_2O$, 0.1 *M* MES pH 6.5, 12%(*v*/*v*) PEG MME 550 at a temperature of 298 K. Clusters of rod-shaped crystals (Fig. 4) grew in drops with a 1:2 ratio of protein to precipitant volume. These crystals were suitable for diffraction experiments without the need for cryoprotection.

2.3. X-ray diffraction studies

Crystals grown in PEG 8000 and PEG 4000 were mounted in cryoloops and tested for X-ray diffraction using an in-house rotating-anode Nonius FR591 X-ray source with a MAR 345 image-plate detector. Unfortunately, the resulting diffraction patterns revealed significant damage to the crystal lattice that rendered the crystals unsuitable for structural analysis. Diffraction experiments at room temperature were not considered because of the significant damage that occurred upon opening the crystallization setup.

A crystal of form I was cryoprotected with 30%(*v*/*v*) glycerol and flash-cooled by plunging it into liquid nitrogen. Data collection was performed at 100 K on beamline BM14, ESRF, Grenoble using a MAR Mosaic 225 CCD detector with an oscillation angle of 0.25°. The mini-kappa goniometer geometry was utilized for data collection to minimize the overlap of reflections along the shortest reciprocal-lattice vector (c = 610.4 Å). An optimal κ angle of 25° was found, which led to the best crystal orientation. The data set was processed using *HKL*-2000 (Otwinowski & Minor, 1997). The crystal diffracted to 2.7 Å resolution; however, a rapid intensity fall-off occurred beyond 3.5 Å resolution. The data completeness also decreased beyond this limit owing to the overall anisotropy of the diffraction intensities.

Data from a crystal of form II were collected on beamline X11, EBML Hamburg using a MAR 555 detector. A freshly grown crystal was vitrified in a 100 K liquid-nitrogen stream without any additional cryoprotection. A set of 458 images was collected with an oscillation angle of 0.3° and was processed using the *XDS* program package (Kabsch, 1993). The complete data-collection and processing statistics for both crystal forms are summarized in Table 1.

3. Results

The space group of crystal form I was originally assigned as $P6_222$, with unit-cell parameters $a = b = 55.7$, $c = 610.4$ Å, and checked using the program *POINTLESS* included in the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) with intensities integrated in space group $P1$. According to Matthews coefficient calculations (Matthews, 1974), the unit cell should most likely consist of 12 enzyme molecules, *i.e.* one molecule per asymmetric unit.

According to analysis of the intensity statistics, the sigmoidal shape of the cumulative intensity distribution for acentric data indicated twinning and the fourth moment of I for acentric reflections was around 1.5 (2.0 for ideal untwinned data). The web-based service for analysis of twinning (http://nihserver.mbi.ucla.edu/Twinning) indicated merohedral twinning with a twin factor of 43% (Yeates, 1997). Molecular-replacement trials were performed with *MOLREP* (Vagin & Teplyakov, 1997) and *AMoRe* (Navaza, 1994) using a model with 41% sequence identity (PDB code 1zr6; Huang *et al.*, 2005) in all possible subgroups of $P6_222$ without satisfactory results.

Crystal form II belonged to the monoclinic space group $C2$, with unit-cell parameters $a = 132.9$, $b = 56.6$, $c = 86.5$ Å, $\beta = 95.7°$. Molecular replacement was carried out with the program *MOLREP* using model 1zr6 from the PDB. The search gave a distinct peak correlation coefficient of 0.33 and an R factor of 49.4% without packing conflicts. The electron-density map calculated after rigid-body refinement was clearly interpretable and confirmed the molecular-replacement solution. Model building and refinement are currently under way.

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