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Crystallization and preliminary X-ray diffraction analysis of the glucuronoyl esterase catalytic domain from *Hypocrea jecorina*

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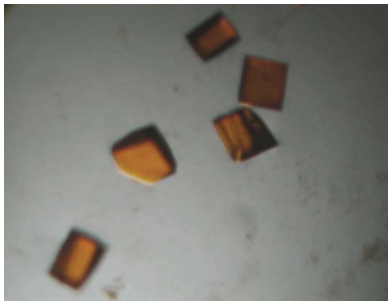


The catalytic domain of the glucuronoyl esterase from *Hypocrea jecorina* (anamorph *Trichoderma reesei*) was overexpresssed, purified and crystallized by the sitting-drop vapor-diffusion method using 1.4 *M* sodium/potassium phosphate pH 6.9. The crystals belonged to space group $P2_12_12_1$ and X-ray diffraction data were collected to 1.9 Å resolution. This is the first enzyme with glucoronoyl esterase activity to be crystallized; its structure will be valuable in lignocellulose-degradation research.

1. Introduction

The continued development of a carbohydrate-based system for the production of fuels and chemicals to replace petroleum will require the recruitment of new renewable materials to add to the current corn starch-based feedstocks. These materials will include agricultural residues such as straw and stovers and new dedicated energy crops such as switchgrass and poplars (Somerville, 2006). In all cases, the vast majority of carbohydrates in these candidate feedstocks are contained in plant cell walls. The major polymers in plant cell walls include cellulose, hemicellulose and lignin, which are intermeshed in a complex organization which provides structure to the plant. This complex structure is extremely recalcitrant to digestion and bioconversion of these materials requires the concerted activity of several polymer-hydrolyzing enzymes. The hydrolysis of the cellulose component has been widely studied and the enzymatic requirement for conversion of this polysaccharide is well understood. However, the total conversion of the plant cell wall also requires digestion and separation of the other component materials. The association of lignin with the hemicellulose (of which xylan is the most abundant type) has long been recognized as a likely limitation on the efficient conversion of cell-wall polysaccharides to monomer sugars (Jeffries, 1990).

Several types of covalent linkages between lignin and xylan in plant cell walls have been described (Jeffries, 1990). One such linkage is an ester bond between hydroxyl groups of lignin moieties and the carboxyl group of the 4-*O*-methyl-D-glucuronic acid (MeGlcA) side groups of glucuronoxylan. An enzyme that is capable of specifically hydrolyzing the alkyl and arylalkyl esters of MeGlcA (see Fig. 1) has been purified from the cellulolytic fungus *Schizophyllum commune* and named glucuronoyl esterase (GE; Špániková & Biely, 2006; Špániková *et al.*, 2007). Based on the partial amino-acid sequence of this enzyme, genes coding for GEs in other fungi have recently been identified (Li *et al.*, 2007). The catalytic domain of GE (Cip2) from *Hypocrea jecorina* is one such enzyme. Sequence analysis revealed that the full-length Cip2 protein contains a carbohydrate-binding module, a linker and a catalytic domain (CD; Foreman *et al.*, 2003) exhibiting GE activity (Li *et al.*, 2007). In this paper, we describe the crystallization and preliminary X-ray diffraction results of the CD of *H. jecorina* GE, the first esterase with this catalytic activity and the first member of the recently established new carbohydrate esterase family 15 (http://www.cazy.org/).

2. Materials and methods

2.1. Overexpression and purification

The CD (corresponding to amino acids 90–460) of *H. jecorina* GE (Cip2; accession No. AY281368; Foreman *et al.*, 2003) was overexpressed in its host, secreted into culture medium (Li *et al.*, unpublished data) and purified to electrophoretic homogeneity by a procedure similar to that described previously for the entire enzyme (Li *et al.*, 2007). Briefly, 7 d old culture supernatant was collected by centrifugation and proteins were precipitated using 70% ammonium sulfate saturation. Precipitated proteins were dissolved in 1.0 *M* ammonium sulfate solution and the soluble proteins were fractionated using Phenyl Sepharose and Q XL Fast Flow columns (Li *et al.*, 2007). GE activity was followed by the thin-layer chromatography method (Špániková & Biely, 2006) and the purity of the enzyme was estimated on SDS–PAGE. Details of how the overexpression cassette was constructed, the identification of transformants secreting the CD and the purification of the protein will be reported elsewhere (Li *et al.*, unpublished results). The protein was shown to be catalytically active against the methyl ester of 4-*O*-methyl-D-glucuronic acid.

2.2. Crystallization

Protein at a concentration of 25 mg ml^{-1} in 20 m*M* Tris pH 7.5 buffer was used for screening. Initial screening to identify crystallization conditions was carried out using the sitting-drop vapor-diffusion method at 298 K. The commercial crystallization screens Index Screen, SaltRx, Crystal Screen, PEG/Ion Screen (Hampton Research) and Wizard I and II (deCode Genetics) were used. Each experiment consisted of equilibrating a mixture of 0.4 µl protein solution and 0.4 µl screen solution over a reservoir of 0.2 ml screen solution in a 96-well Greiner plate using a Mosquito robot (TTP LabTech). Eight solutions from Index Screen (condition Nos. 18, 41, 45, 59, 66, 74, 79 and 80) and four from Crystal Screen (condition Nos. 10, 15, 31 and 42) produced crystals; however, all the crystals obtained in the initial screening were clusters and not single crystals. Further crystallization screening performed manually with Quik Screen (Hampton Research) by the hanging-drop vapor-diffusion method (1 µl protein solution plus 1 µl screening solution equilibrated over 0.5 ml screening solution) using greased VDX plates (Hampton Research) identified better crystallization conditions (Quik Screen conditions C3, C4, D3 and D4). The crystals typically formed in 2–3 d and grew to full size in a week. The best crystals (0.2 × 0.2 × 0.1 mm) used for data collection (Fig. 2*a*) were obtained using Quik Screen solution C4 (1.4 *M* sodium/potassium phosphate pH 6.9).

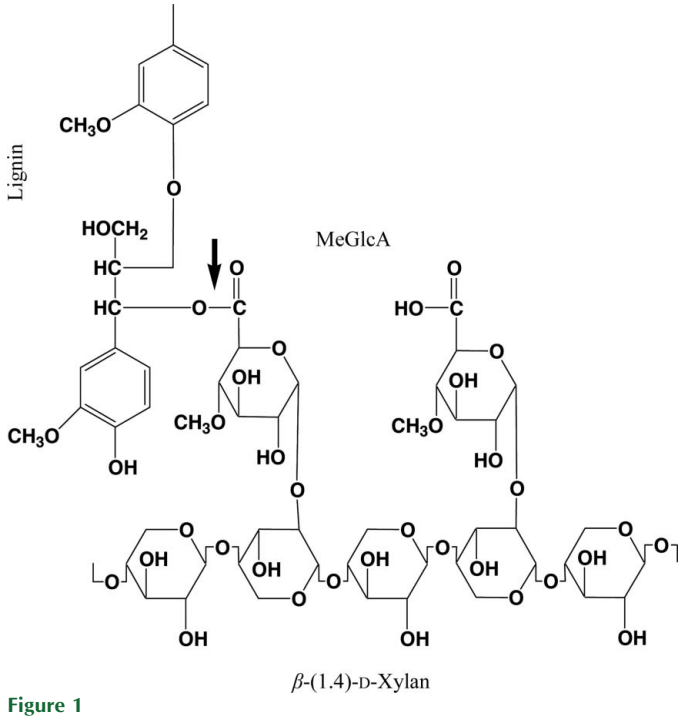
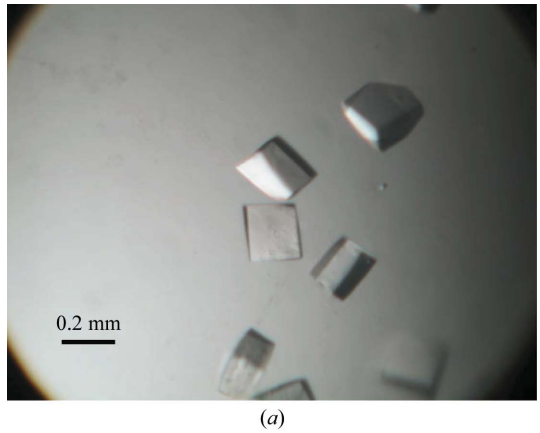


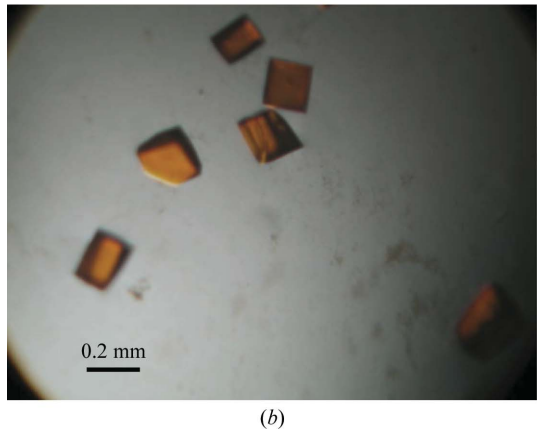
Figure 1
Proposed cross-link structure between lignin and glucuronoxylan in plant cell walls. MeGlcA stands for 4-*O*-methyl-D-glucuronic acid. The arrow indicates the possible ester bond hydrolyzed by GE.

2.3. Data collection and processing

Crystals were transferred briefly (15–30 s) to a cryoprotectant solution prior to freezing by direct immersion in liquid nitrogen. The cryoprotectant solution used was as follows: sucrose and NaBr were dissolved in Quik Screen condition C4 to final concentrations of 27%(*w*/*v*) sucrose and 0.5 *M* NaBr for crystal 1 and to 27%(*w*/*v*) sucrose and 1.0 *M* NaBr for crystal 2; 27%(*w*/*v*) sucrose only was dissolved in Quik Screen condition C4 for crystal 3. X-ray diffraction data were collected at the Br *K* absorption edge (crystals 1 and 2) and 8 keV (crystal 3) on the Structural Biology Center 19BM beamline (Advanced Photon Source). The diffraction data were collected on a SBC3-CCD X-ray detector (http://www.sbc.anl.gov/facilities/hardware.html) and processed using the *HKL*-3000 package (Minor *et al.*, 2006).



(*a*)



(*b*)

Figure 2
(*a*) Crystals of the CD of *H. jecorina* GE obtained from QuikScreen condition C4. (*b*) Crystals of the CD of *H. jecorina* GE after exposure to I_2 vapor for 3 h.

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell. See the text for a description of each crystal.

	Crystal 1	Crystal 2	Crystal 3
Unit-cell parameters			
a (Å)	80.3	80.1	80.2
b (Å)	82.0	81.9	81.7
c (Å)	185.9	185.4	186.3
Space group	$P2_12_12$ or $P2_12_12_1$	$P2_12_12$ or $P2_12_12_1$	$P2_12_12_1$
Wavelength (Å)	0.91946	0.91946	1.77120
Resolution (Å)	1.9 (1.97–1.90)	2.0 (2.07–2.00)	2.5 (2.59–2.50)
Total No. of reflections	441563	371350	656752
No. of unique reflections	83127	75481	79160
$R_{\rm merge}$† (%)	8.5 (30.8)	12.9 (33.6)	4.3 (6.8)
Completeness (%)	92 (87)	95 (93)	98 (94)
Redundancy	5 (4)	5 (4)	8 (7)
Mean $I/\sigma(I)$	19 (4)	13 (3.6)	63 (36)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Results and discussion

The crystals of the *H. jecorina* glucuronoyl esterase catalytic domain had orthorhombic Laue symmetry with space group $P2_12_12_1$ and diffracted X-rays to 1.9 Å resolution. The crystallographic parameters and data-collection statistics are shown in Table 1. The exact molecular weight of the protein is not known because of potential glycosylation. The Matthews coefficient $V_{\rm M}$ based on three protein molecules (using the formula weight of 40 kDa) per asymmetric unit is 2.5 $Å^3\,Da^{-1}$, which corresponds to an estimated 52% solvent content (Matthews, 1968). A probability of 78% for three protein molecules per asymmetric unit was suggested by the Matthews probability calculator (Kantardjieff & Rupp, 2003) based on the resolution limit of the crystals.

An attempt was made to determine the structure of the CD using the quick halide-soak method (Dauter *et al.*, 2000) with diffraction data collected at the Br *K* edge using two crystals soaked briefly (15–30 s) in a cryoprotectant solution containing 0.5 *M* NaBr (crystal 1, Table 1) and 1.0 *M* NaBr (crystal 2, Table 1). The presence of anomalous signal to at least 3.0 Å resolution was indicated in both data sets by the χ^2 plots (not shown) of the scaled reflections processed with the 'anomalous' option in *HKL*-3000. However, attempts to locate any bromide ions present in the crystal using the *SHELXD* program in the *HKL*-3000 package were unsuccessful for both data sets in the two possible space groups. It appears that the quick halide soak was not successful in derivatizing the crystals. Cocrystallization efforts that included sodium bromide or potassium iodide in the crystallization mother liquor did not produce any crystals.

We have also tried a recently reported new method that uses the exposure of crystals to I_2 vapor (Miyatake *et al.*, 2006). After the crystals were formed, a 1 µl drop of KI/I_2 solution (prepared by dissolving 25 mg KI and 13 mg I_2 in 0.1 ml water) was placed next to the drop containing crystals in such a way that the two drops were not in direct contact. The crystals turned yellow/brown after exposure to I_2 through vapor diffusion (see Fig. 2*b*). Even though the crystals appeared to be intact optically, after 3 h of exposure to I_2 vapor the crystals did not diffract X-rays, suggesting that the iodine has probably reacted with the protein, significantly affecting the crystal lattice. Therefore, a diffraction data set was collected at 8 keV from a crystal exposed to I_2 vapor for 5 min (crystal 3). Although this data set was not useful in obtaining phase information, it provided the systematic absences to determine the space group as $P2_12_12_1$. Currently, we are pursuing different exposure times of crystals to I_2 vapor and also traditional heavy-atom methods in an effort to determine the structure of the catalytic domain of *H. jecorina* GE, which has enzymatic activity important to biofuels research.

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