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Crystallization and preliminary X-ray diffraction studies of the catalytic core of acetyl xylan esterase from *Trichoderma reesei*

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Abstract

Acetyl xylan esterase is involved in the biodegradation of hemicellulose. It cleaves *O*-acetyl groups from xylan, which is the most abundant hemicellulose in nature. The catalytic core of acetyl xylan esterase from *T. reesei* has been crystallized and X-ray diffraction data at 2.3 Å collected. The crystal belongs to the triclinic space group *P*1 with unit-cell parameters a = 50.3, b = 62.1, c = 40.0 Å, α = 110.1, β = 113.6 and γ = 97.9°. The asymmetric unit contains two molecules.

1. Introduction

Hemicellulose, which functions as a supporting material in plant cell walls, is a complex mixture of heteropolysaccharides. The most abundant hemicellulose, xylan, is composed of 1,4-linked xylanopyroside units. It may also have various types and amounts of substituents such as *O*-acetyl groups, 4-*O*-methyl glucuronic acid, L-arabinose and phenolic compounds. As a result, several different enzymes are needed for the degradation of xylan. The main chain is hydrolysed by *endo*-1,4-β-xylanases and β-xylosidases, while the side groups are degraded by α-arabinosidases, α-glucuronidases and esterases (Biely, 1985). Since up to 70% of the xylose residues in hardwoods can be acetylated at the C2 and/or C3 positions (Timell, 1967; Lindberg *et al.*, 1973), acetyl xylan esterases are necessary for the total hydrolysis of xylan (Fig. 1). Although there have been numerous enzymological studies of the hydrolysis of xylan, the role of different enzyme components is still partially unclear. To understand the details of xylan degradation, three-dimensional structures of all involved enzymes are required. So far, there is no information about the three-dimensional structure of any acetyl xylan esterase, and only crystallization and preliminary X-ray data of *Penicillium purpurogenum* has been published (Pangborn *et al.*, 1996).

Acetyl xylan esterases are produced by several cellulolytic and hemicellulolytic micro-organisms such as *Fibriobacter succinogenes* (McDermid *et al.*, 1990), *Aspergillus niger* (Kormelink *et al.*, 1993), *Thermoanaerobacterium* sp. (Shao & Wiegel, 1995), *P. purpurogenum* (Egana *et al.*, 1996), *Streptomyces lividans* (Dupont *et al.*, 1996) and *T. reesei* (Sundberg & Poutanen, 1991).

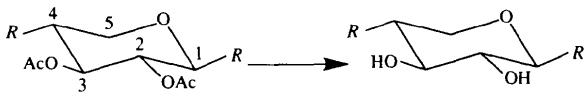

Fig. 1. The reaction catalyzed by acetyl xylan esterase.

Two p*I* forms of acetyl xylan esterase from *T. reesei* (AXEI and AXEII) have been purified and characterized. Recently, a gene, *axe1*, which apparently encodes both isoelectric forms of the acetyl xylan esterase of *T. reesei*, has been isolated and sequenced (Margolles-Clark *et al.*, 1996). The deduced mature protein AXEI is composed of 271 amino-acid residues and contains a catalytic core and a fungal-type C-terminal cellulose-binding domain. Comparison with other acetyl xylan esterases has revealed homology only with the enzyme from *P. purpurogenum*, the partial sequence of which has been published (Egana *et al.*, 1996). However, the catalytic core of AXEI demonstrates some similarity with fungal cutinases and although the overall similarity is very low (about 10%), the catalytic amino-acid residues are conserved assuming the catalytic triad in the enzyme (Margolles-Clark *et al.*, 1996). The studies with partially or fully acetylated methyl xylopyranosides have shown that AXEI is capable of deacetylating substituents of hydroxyl groups both at positions 2 and 3. The rate of hydrolysis was faster if the substrate already had a free hydroxyl group at positions 2 or 3. AXEI is also able to remove the corresponding acetyl groups when acetylated glycopyranosides were used as substrates (Biely *et al.*, 1998). The three-dimensional structure of AXEI would be an important step forward in investigations of the substrate specificity and the reaction mechanism of this poorly known esterase.

2. Materials and methods

2.1. *Crystallization*

AXEI from *T. reesei* was purified as previously described (Sundberg & Poutanen, 1991) and the cellulose-binding domain was removed by papain digestion (Margolles-Clark *et al.*, 1996). While the site of cleavage is not known, it has previously been estimated to be between Leu206 and Ser207. The catalytic core of AXEI was crystallized by the hanging-drop vapour-diffusion technique at room temperature. A 2 µl drop of the protein solution (~10 mg ml^{-1}) was mixed with 2 µl of the buffered precipitation mixture. Crystals grew using 0.8–1.0 *M* potassium/sodium tartrate as the precipitant and 0.1 *M* Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)] as the buffer at pH 8.0–8.4. The addition of 2 µl of 7 mg ml^{-1} of *n*-octyl-β-D-glucoside reduced the formation of twinned crystals.

2.2. *Molecular mass determination*

The molecular mass of the catalytic core of AXEI was determined using a matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometer. Albumin and cytochrome *c* were used for the calibration of the spec-

Table 1. *Data-collection statistics*

d_{min} (Å)	R_{sym} (I)	I/σ	Unique reflections	Completeness (%)	Multiplicity
8.0	4.4	11.7	394	84.4	1.8
6.0	5.7	8.8	507	82.3	1.8
5.0	5.7	9.2	612	83.5	1.8
4.0	5.5	10.4	1488	86.9	1.9
3.5	8.3	7.4	1339	78.3	1.8
3.0	8.5	5.7	2339	77.3	1.7
2.8	9.8	4.6	1310	71.4	1.7
2.5	11.7	3.7	2558	63.5	1.6
2.4	13.3	3.1	1070	57.8	1.5
2.3	13.5	2.9	1134	53.1	1.5
Total	7.9	5.7	12751	70.4	1.7

trometer and sinapic acid was used as the matrix. 0.5 µl of sinapic acid in 90% acetone solution was spread on the target with 0.5 µl of 2% TFA. After drying the sample, 0.5 µl of a 5 mg ml^{-1} solution of AXEI was added. Finally, 0.5 µl of sinapic acid in 70% acetonitrile was also applied to the target after rinsing.

2.3. *X-ray diffraction*

A native data set was collected on an R-AXIS IIC image-plate area detector mounted on a Rigaku RU200HB rotating anode operated at 180 mA and 50 kV. Graphite-monochromated Cu $K\alpha$ radiation was used. The crystal was first transferred to a cryoprotectant solution containing 40%(v/v) glycerol, 0.5 M potassium/sodium tartrate and 0.1 M Hepes, pH 8.2 for 30 s. After that the crystal was flash-frozen in a cold nitrogen stream using a rayon CryoLoop (Hampton Research) and the data were collected at 120 K. The crystal-to-detector distance was 80 mm and a 180° rotation in steps of 3° oscillation was measured. Each frame was exposed for 60 min. The data set was processed using *Biotex* area-detector processing software (Molecular Structure Corporation, 1996).

3. Results and discussion

Several measurements of the molecular mass of the catalytic core of AXE were performed resulting in a mass of 21 801–21 807 Da (Fig. 2). The enzyme includes a single potential *N*-glycosylation site at Asn63 (Margolles-Clark *et al.*, 1996). If we assume that the catalytic core of AXE includes 208 amino-acid residues, and two β-D-*N*-acetylglucosamine and two β-D-mannose residues, we obtain the mass of 21 806 Da.

Crystals of AXE appeared in the drop after about one week and reached their maximum size after about one month. The crystals initially grew as very thin twinned plates using 1.0 M sodium/potassium tartrate as the precipitant. Addition of *n*-octyl-β-D-glucoside slightly reduced the twinning but the size remained small. Somewhat larger crystals were grown by the macroseeding method, where the original crystals were gently separated and then used as seeds. After a few weeks, transferred crystals attained their final size of about 0.4 × 0.05 × 0.05 mm (Fig. 3).

The crystals diffracted X-rays to at least 2.0 Å resolution and belonged to the triclinic space group $P1$ with unit-cell constants a = 50.3, b = 62.1, c = 40.0 Å, α = 110.1, β = 113.6 and γ = 97.9°. The solvent content was calculated by Matthews formula (Matthews, 1968), using a molecular mass of 21.8 kDa. This gives V_m = 2.21 Å^3 Da^{-1} and a solvent content of 44%, assuming two molecules in an asymmetric unit. An X-ray data set was collected at 2.3 Å resolution. There were 12 751 unique reflections with 21 609 total observations having an R_{merge} of 7.87%. The data set was 70% complete (Table 1). In order to achieve a more complete data set tens of different crystals have been tested. In most cases the crystals were very badly twinned and the remaining ones were not isomorphous, preventing the merging of data. A self-rotation

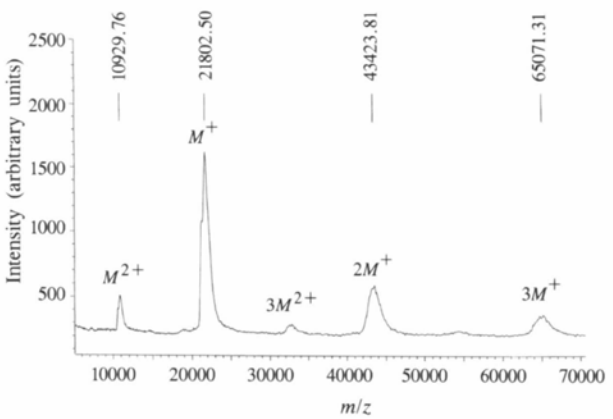

Fig. 2. A MALDI–TOF analysis of the catalytic core of AXEI. The peak of 21802.50 corresponds AXEI core protein.

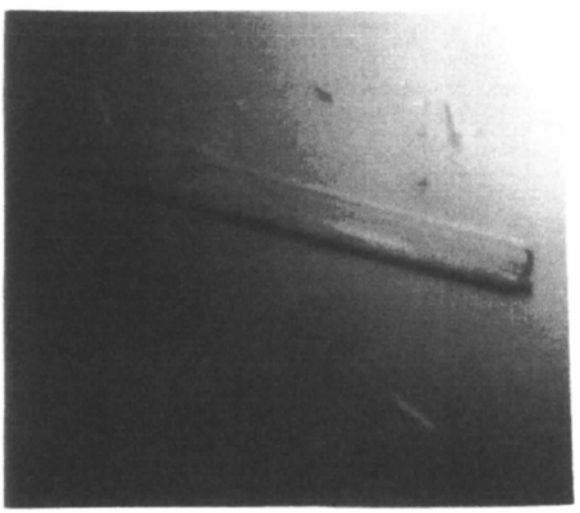

Fig. 3. Crystals of the catalytic core of AXEI. The maximum dimension is *ca* 0.4 mm.

function was calculated using the program *X-PLOR* (Brünger *et al.*, 1987) in order to identify local symmetry in the asymmetric unit. The rotation function (calculated in the range 10–3.5 Å) clearly indicated the presence of a non-crystallographic twofold element at $\psi = 67.5°$ and $\varphi = 65.0°$. The determination of the structure using molecular replacement is currently underway.

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