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Purification, crystallization and preliminary X-ray analysis of an acetylxylan esterase from *Bacillus pumilus*

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The gene encoding for acetylxylan esterase from *Bacillus pumilus* has been cloned and expressed in *Escherichia coli*. The recombinant protein has been purified to homogeneity and crystallized. The crystals obtained are of regular shape of dimensions 0.05 × 0.05 × 0.05 mm with *R*32 symmetry and diffract to 2.0 Å using synchrotron radiation.

1. Introduction

Xylan is the major constituent of hardwood and represents the second most abundant biopolymer on earth after cellulose. It is present in plant cell walls as a polymer of linear chains of xylose joined by β-1,4-glycosidic linkages. The xylose units in the backbone are modified by different kinds of side-chain substitutions. The most common substituents are arabinose, 4-*O*-methylglucuronic acid and acetyl moieties.

To achieve the complete degradation of such a complex molecule, a set of enzymes is required. The hydrolysis of β-1,4-D-xylan is accomplished by xylanhydrolases (E.C. 3.2.1.8) which break the β-1,4-glycosidic bonds between xylopyranosyl moieties. The products of the hydrolysis are short xylo-olygosaccharides, usually between two and six sugar units. The complete degradation of the polymeric chain to simple sugar moieties is carried out by an additional endo-β-1,3 xylanase (E.C. 3.2.1.32) and β-xylosidases (E.C. 3.2.1.37). The substituent side-chain groups are hydrolysed by α-L-arabinofuranosidases (E.C. 3.2.1.55), α-glucuronidases (E.C. 3.2.1.139) and acetylxylan esterases (E.C. 3.1.1.72).

An acetylxylan esterase from *B. pumilus* (AXE) belonging to the esterase family 7 (Coutinho & Henrissat, 1999) has recently been characterized and overexpressed in *E. coli* (Degrassi *et al.*, 1998, 2000). The protein molecular weight is 40 kDa as determined by SDS–PAGE, while the native molecular weight has been estimated to be 190 kDa by gel-filtration chromatography, thus suggesting either a homotetrameric or pentameric enzyme (Degrassi *et al.*, 1998). The recombinant AXE showed no difference in activity when compared with the native protein, indicating correct expression of the gene in the host strain and correct folding of the enzyme.

In *B. pumilus*, the xylanolytic system has been partially investigated, revealing that a complete xylan-degradation pathway must be present as suggested by the presence of a xylanase and a β-xylosidase, indicating that AXE is one of its components. Furthermore, the production of the enzyme is induced by the presence of xylan and corncob in the growth medium. *B. pumilus* AXE reveals activity towards a broad range of acetylated compounds such as acetylated xylan, xylose tetraacetate, glucose pentaacetate, *p*-nitrophenyl acetate and cephalosporin C (Degrassi *et al.*, 1998).

The high sequence identity with *B. subtilis* cephalosporin C acetylhydrolase (CAH) suggests a possible pharmaceutical application of *B. pumilus* AXE in antibiotic production, *e.g.* in the deacetylation of cephalosporin C (Mitsushima *et al.*, 1995).

To date, no structural information is available for bacterial AXEs, while the crystal structures of AXE II from the fungus *Penicillium purpurogenum* (Ghosh *et al.*, 1999, 2001) and the catalytic core of acetylxylan esterase from the fungus *Trichoderma reesei* (Hakulinen *et al.*, 2000) have recently been determined. Both structures reveal the presence of the catalytic triad Ser-His-Asp, typical of many esterases, lipases and serine proteases, and of a doubly wound α/β sandwich fold with a central parallel β-sheet flanked by two parallel α-helices on each side.

In order to ascertain the role (AXE or CAH) and the catalytic mechanism of *B. pumilus* AXE, we began a study aimed at the X-ray crystal structure determination of this enzyme.

2. Experimental and results

2.1. Purification of AXE

The *axe* gene of *B. pumilus* was expressed in *E. coli* as previously reported by Degrassi *et al.* (2000). The protein from the culture medium was purified by standard chromatographic techniques according to the published procedure (Degrassi *et al.*, 1998). The recombinant enzyme was purified from a 4 l culture of *E. coli* DH5α carrying the pBPEB2 plasmid

Table 1
Crystal parameters, data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	ESRF ID14-EH4
Wavelength (Å)	0.976
Detector	Quantum 4 CCD
Space group	*R*32
Unit-cell parameters (hexagonal setting)	
a, *b* (Å)	107.58
c (Å)	152.94
Mosaicity (°)	0.8
Resolution range (Å)	37.0–2.0 (2.1–2.0)
No. of measurements	319784
No. of observed reflections, $I \geq 0$	132755
No. of unique reflections, $I \geq 0$	21596 (1970)
Completeness (%)	91.4 (84.0)
Redundancy	6.1 (3.6)
$\langle I/\sigma(I)\rangle$ of measured data	12.7 (2.7)
R_{sym}† (%)	10.4 (27.7)

† $R_{sym}(I) = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i |I_{hkl,i}|$, where $\langle I_{hkl}\rangle$ is the mean intensity of the multiple $I_{hkl,i}$ observations from symmetry-related reflections.

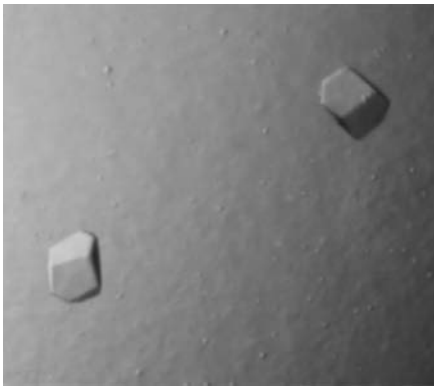

Figure 1
Crystals of *B. pumilus* acetylxylan esterase. Their dimensions are ~0.05 × 0.05 × 0.05 mm.

harbouring the *axe* gene, grown overnight at 310 K on a rotary shaker on LB medium containing 100 µg ml^{-1} ampicillin. The protein solution was brought first to 30% $(NH_4)_2SO_4$ (AS) saturation and the precipitated material was discarded. The supernatant was then brought to 70% AS saturation. The resulting precipitate was resuspended in 1.7 *M* AS, 100 m*M* sodium phosphate buffer pH 7.0 and loaded onto a phenyl Sepharose HP 16/10 column (Pharmacia). The protein was eluted with a linear gradient from 1.7 to 0.0 *M* AS. The active fractions were pooled, dialysed against 20 m*M* bis-tris buffer pH 7.0, loaded onto a Q Sepharose fast-flow 16/10 column (Pharmacia) and eluted with a linear gradient from 0.0 to 1.0 *M* NaCl. Active fractions were pooled, concentrated by ultrafiltration with a YM30 membrane (Amicon Inc., Beverly, Mass.) to 2 ml and loaded onto a Sephacryl HR200 16/60 gel-filtration column (Pharmacia). The protein was eluted at a flow rate of 0.5 ml min^{-1}. Pure fractions were concentrated to 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and used for crystallization experiments.

Table 2
Results of a BLAST search performed on the *B. pumilus* amino-acid sequence.

Organism	Enzyme	Accession code	Sequence length (aa)	Sequence identity (%)
B. pumilus	AXE, E.C. 3.1.1.72	Q9K5F2	320	100
B. subtilis	CAH, E.C. 3.1.1.41	Q59233	325	76
Thermotoga maritima	AXE, E.C. 3.1.1.72	Q9WXT2	325	42
Thermotoga neapolitana	AXE, E.C. 3.1.1.72	O33842	325	41
B. halodurans	CAH, E.C. 3.1.1.41	Q9K7N5	319	38
Thermoanaerobacterium saccharolyticum (JW/SLYS485)	AXE, E.C. 3.1.1.72	S41858	320	36
Streptomyces coelicolor	AXE, E.C. 3.1.1.72	Q9FC27	322	36
B. stearothermophilus	AXE, E.C. 3.1.1.72	Q9JP86	329	36

2.2. Crystallization

Crystallization trials were performed at 293 K by the hanging-drop method, mixing 2 µl of protein solution with 2 µl of precipitant solution. The ammonium sulfate grid-screening kit from Hampton Research (Laguna Niguel, CA, USA) was used. Very small irregular crystals were observed after few days with 2.4 *M* AS in 100 m*M* HEPES pH 7. Larger and more regular crystals were obtained after optimization in 1.8–2.0 *M* AS in 100 m*M* Tris–HCl pH 8 (Fig. 1).

2.3. Data collection

Diffraction data were collected on a single crystal at 100 K using as cryoprotectant a solution of precipitant in which the salt concentration was increased to 2.4 *M* AS and to which glycerol was added to a final concentration of 20%. The crystal (0.05 × 0.05 × 0.05 mm) was scooped up from the drop with a nylon cryoloop, soaked for about 20 s in the cryosolution and immediately transferred under a cold nitrogen stream at the ID14-EH4 undulator beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Diffraction data were collected using a Quantum 4 CCD detector. X-ray diffraction data were indexed, integrated and subsequently scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), respectively, and the *CCP*4 (Collaborative Computational Project, Number 4, 1994). package was used for the data reduction.

2.4. Results and discussion

A summary of the X-ray diffraction data is given in Table 1. Assuming a molecular weight of 36.012 kDa (as deduced by the amino-acid sequence) and one monomer in the asymmetric unit, the value of the crystal-packing parameter V_M is 2.36 Å^3 Da^{-1}, with a solvent content of 48% (Matthews, 1968). This observation, together with the comparison between the native molecular weight (~190 kDa), suggests that AXE may be arranged in the crystal as a multimer of six identical subunits. A sequence-similarity search showed *B. pumilus* AXE to be homologous to CAH from *B. subtilis* and to *T. maritima* AXE. A comparison of the homologous enzymes from different organisms is given in Table 2. It has been reported that AXE1 from *Thermoanaerobacterium* sp. strain JW/SL-YS485 has a molecular weight determined by SDS–PAGE of 32 kDa with a native MW of 195 kDa (as estimated by gel filtration) or 170 kDa (as determined by native PAGE) and it has also been proposed to be an hexamer of six identical subunit (Shao & Wiegel, 1995). A search of heavy-atom derivatives is currently under way in order to solve the three-dimensional structure of *B. pumilus* AXE.

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addenda and errata

Purification, crystallization and preliminary X-ray analysis of an acetylxylan esterase from *Bacillus pumilus*. Erratum

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This erratum is to apologise for having reported the crystallization and X-ray characterization of *Bacillus pumilus* acetylxylan esterase (AXE) while the protein crystallized was instead an inorganic pyrophosphatase, a contaminant of the expression in *E. coli*. The protein was purified by hydrophobic interaction, ionic exchange and gel filtration, but still contained traces of contaminant proteins. Crystals were obtained in the *R*32 space group perfectly compatible with the homohexameric structure of AXE. The cell parameters were compatible with a reasonable crystal packing as in the model cephalosporin C deacetylase from *Bacillus subtilis* kindly provided before publication by Dr Jim Brannigan *et al*. (PDB code 1ods crystallized in *R*3 and 1odt crystallized in *R*32). Since every attempt to solve the structure by molecular replacement using 1odt as a model failed, a search of the PDB using the cell parameters of the data collected revealed a match with *Escherichia coli* inorganic pyrophosphatase (1ipw). A molecular-replacement solution confirmed that the protein crystallized was indeed *E. coli* inorganic pyrophosphatase present as a contaminant in the protein preparation used for crystallization. This experience should be kept in mind because proteins used for crystallization should be as pure as possible not only to favour the process itself but also to avoid the crystallization of contaminants.

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