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Crystallization and preliminary X-ray study of a family 10 alkali-thermostable xylanase from alkalophilic *Bacillus* sp. strain NG-27

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Xylanases (EC 3.2.1.8) catalyze the hydrolysis of β-1,4-glycosidic linkages within xylan, a major hemicellulose component in the biosphere. The extracellular endoxylanase (XylnA) from the alkalophilic *Bacillus* sp. strain NG-27 belongs to family 10 of the glycoside hydrolases. It is active at 343 K and pH 8.4. Moreover, it has attractive features from the point of view of utilization in the paper pulp, animal feed and baking industries since it is an alkali-thermostable protein. In this study, XylnA was purified from the native host source and crystallized by the hanging-drop vapour-diffusion method. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters a = 174.5, b = 54.7, c = 131.5 Å, β = 131.2°, and diffract to better than 2.2 Å resolution.

1. Introduction

Xylanases (EC 3.2.1.8), the xylan-degrading enzymes, are classified into two distinct families, 10 and 11, of the glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY; Henrissat & Davies, 1997). They hydrolyse xylan, the most abundant hemicellulose in plant cell-wall polysaccharides. The study of xylanases has assumed great importance in view of the industrial applications (Shallom & Shoham, 2003), which include bio-bleaching in the paper industry, which is economical and eco-friendly, the pharmaceutical industry and the clarification of fruit juices. The structures of family 10 have the $(\beta/\alpha)_8$ TIM-barrel fold and have been described for *Streptomyces lividans* (PDB code 1xas; Derewenda *et al.*, 1994), *Cellulomonas fimi* (PDB code 2exo; White *et al.*, 1994), *Pseudomonas fluorescens* (PDB code 1xys; Harris *et al.*, 1994), *Clostridium thermocellum* (PDB code 1xyz; Dominguez *et al.*, 1995), *Thermoascus aurantiacus* (PDB code 1i1w; Natesh *et al.*, 2003), *Penicillium simplicissimum* (PDB code 1bg4; Schmidt *et al.*, 1998), *Streptomyces olivaceoviridis* E-86 (PDB code 1xyf; Fujimoto *et al.*, 2000), *Cellvibrio japonicus* (PDB code 1us2; Pell *et al.*, 2004), *Streptomyces halstedii* (PDB code 1nq6; Canals *et al.*, 2003) and *Geobacillus stearothermophilus* T-6 (PDB code 1hiz; Teplitsky *et al.*, 2004). The structures of the family 11 predominantly consist of β-sheets. Despite their structural differences, they remarkably function in the same manner, with net retention of anomeric configuration (double-displacement mechanism) in enzymatic hydrolysis (Henrissat & Davies, 1997).

We have previously isolated and characterized an extracellular endoxylanase enzyme from the alkalophilic *Bacillus* sp. strain NG-27 (Gupta *et al.*, 2000; Leelavathi *et al.*, 2003). The gene coding for the enzyme has been cloned and sequenced completely (GenBank accession No. AF015445). The open reading frame codes for 353 amino acids with a predicted molecular weight of ~41 kDa. Interestingly, although it is a thermostable protein it does not contain any cysteine residues and disulfide bridges. Even though many thermostable xylanase structures are known to date, there are very few reports of alkalophilic xylanase structures. XylnA is wild-type protein with high degree of thermal stability and a pH optimum of 8.4 and is thus an industrially important enzyme. Therefore, structure solution should provide insights into the molecular basis for the alkali-thermostability of the enzyme.

2. Experimental procedures

2.1. Purification

The xylanase enzyme was obtained from a strain of the alkalophilic bacteria *Bacillus* sp. NG-27. The enzyme was purified to high homogeneity from the source organism following standard procedures. The bacteria were grown in Luria–Bertani (LB) medium supplemented with 0.5%(*w*/*v*) oat spelt xylan (Sigma) and 1%(*w*/*v*) Na_2CO_3 for 24 h at 300 K. The culture was then centrifuged at 10 000 rev min^{-1} for 20 min. Cell-free supernatant was brought to 80%(*w*/*v*) saturation in ammonium sulfate, followed by centrifugation at 10 000 rev min^{-1} for 30 min. The pellet was resuspended in sterile Milli-Q water and then dialyzed overnight against 10 m*M* Tris–HCl pH 7.0. The dialysate was then incubated at 333 K for 1 h, which precipitated most of the other extracellular proteins, and then centrifuged at 10 000 rev min^{-1} for 20 min. The supernatant was collected and loaded onto a Q-Sepharose (Pharmacia) column equilibrated with buffer *A* (50 m*M* Tris–HCl pH 7.0, 50 m*M* NaCl). The column was then washed with five bed volumes of buffer *A* and subsequently with buffer *B* (50 m*M* Tris–HCl pH 7.0, 200 m*M* NaCl). The protein was eluted with a linear gradient of 200 m*M*–1 *M* NaCl in 50 m*M* Tris–HCl pH 7.0. The xylanase activity was measured in the collected fraction as described elsewhere (Gupta *et al.*, 2000). The fractions containing xylanase activity were pooled and dialyzed overnight against 10 m*M* Tris–HCl pH 7.0. The dialysate was then lyophilized and finally dissolved in sterile Milli-Q water, which was then loaded onto a Hi-Load 16/60 Superdex75 prep-grade (Pharmacia) column equilibrated with buffer *C* (50 m*M* Tris–HCl pH 7.0, 150 m*M* NaCl) with a flow rate of 0.4 ml min^{-1}. The elution profile was detected at 280 nm. The purity of the final fractions was determined by SDS–PAGE using the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (Sigma). Pure samples were finally desalted using a Centricon (10 kDa cutoff, Millipore) and lyophilized. A small amount of lyophilized protein was dissolved in water and checked for enzymatic activity (Gupta *et al.*, 2000). The protein concentration was determined using the CB-X protein-quantification kit (GenoTech).

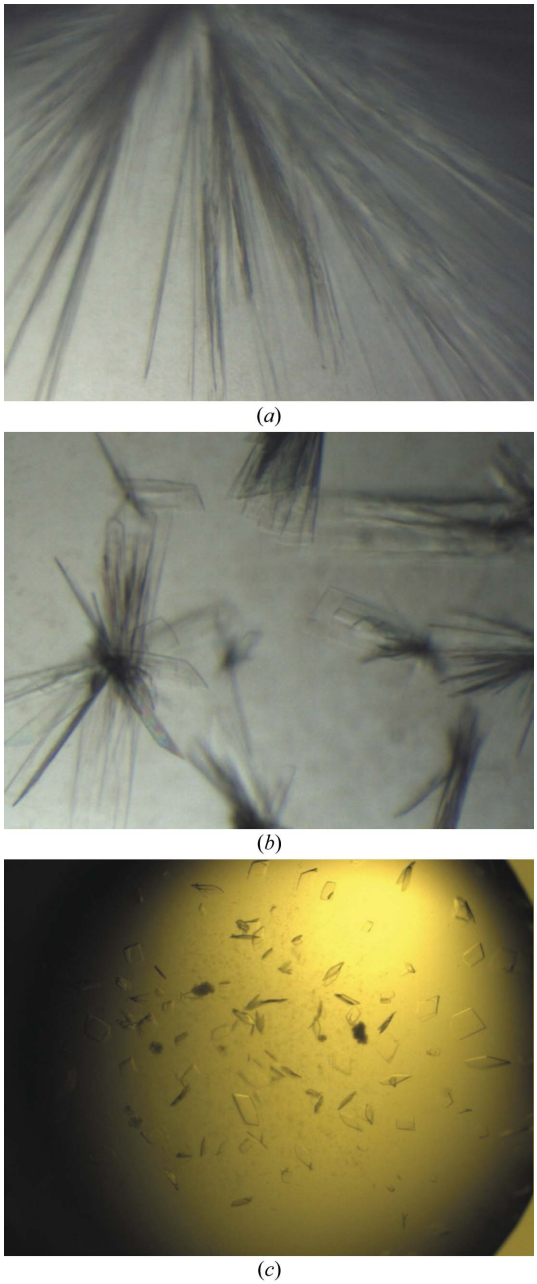

Figure 1
(*a*) Clusters of crystalline needles obtained from the pH 4.6 condition. (*b*) Clusters of plate-like crystals grown using the refined condition. (*c*) Typical diffraction-quality crystals grown at basic pH 8.5 (approximate crystal size is 0.4 × 0.3 × 0.2 mm).

2.2. Crystallization

Crystallization conditions were screened by the hanging-drop vapour-diffusion method using crystallization screening kits (Crystal Screen and Crystal Screen 2) from Hampton Research, USA. An initial protein concentration of 20 mg ml^{-1} (dissolved in water) and a drop size of 4 µl (1:1 ratio of protein and reservoir solution) were used for screening. Clusters of very thin needle-shaped crystals (Fig. 1*a*) grew in a week using a reservoir solution consisting of 0.2 *M* $(NH_4)_2SO_4$, 0.1 *M* sodium acetate pH 4.6 and 25%(*w*/*v*) PEG 4000. A systematic search of other salts in place of ammonium sulfate as well as PEGs was carried out to improve the quality of the crystals. Similar crystals were obtained in a day with divalent cation-containing salts such as $CaCl_2$ or $MgCl_2$. After refinement of the crystallization conditions, XylnA crystals were obtained within 3 d when 2 µl protein solution (10 mg ml^{-1}) were mixed with 3 µl reservoir solution composed of 0.2 *M* $MgCl_2$, 0.1 *M* sodium acetate pH 4.6 and 20%(*w*/*v*) PEG 550 MME. Crystals were plate-shaped and clustered (Fig. 1*b*). We were not able to collect diffraction data from these crystals, presumably owing to the small crystal size.

In the meantime, better crystals were grown using a different crystallization condition with pH 8.5. Plate-like crystals (Fig. 1*c*) were obtained from the condition 0.1 *M* NaCl, 0.2 *M* $MgCl_2$, 0.05 *M* Tris–HCl pH 8.5 and 15%(*w*/*v*) PEG 8000. These results were reproducible and crystals appeared in 4–5 d. They were found to be unstable during room-temperature data collection; diffraction experiments were consequently carried out at 100 K.

2.3. Data collection

A single crystal grown at pH 8.5 was used for complete diffraction data collection. The diffraction intensity data were measured at 100 K using an imaging-plate detector (MAR 345) mounted on a rotating-anode X-ray generator (Cu $K\alpha$ radiation; λ = 1.5418 Å). Crystal freezing included a 3 min soak of the crystal in a cryoprotectant solution consisting of the original crystallization solution and 20%(*v*/*v*) glycerol. The pre-soaked crystal was then submitted to immediate flash-freezing directly within a cold nitrogen-gas stream

Table 1
Crystallographic data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Wavelength (Å)	1.5418
Temperature (K)	100
Space group	*C*2
Unit-cell parameters (Å, °)	a = 174.5, b = 54.7, c = 131.5, β = 131.2
Unit-cell volume (Å^3)	944421
Matthews coefficient (Å^3 Da^{-1})	2.8
Solvent content (%)	56
No. of molecules in AU	2
Resolution (Å)	20.00–2.20 (2.28–2.20)
Observed reflections	282917
Unique reflections	46129 (4100)
Redundancy	6.13
Completeness (%)	96.8 (86.0)
$R_{\rm merge}$† (%)	8.4 (48.6)
$\langle I/\sigma(I)\rangle$	20.3 (3.3)

† $R_{\rm merge} = \sum |I - \langle I\rangle| / \sum I$.

(Oxford Cryosystems). The XylnA crystal diffracted to beyond 2.2 Å. A total of 360 frames were collected with 10 min exposure per frame, 1° oscillation and a crystal-to-detector distance of 200 mm and data were processed using the programs *DENZO* and *SCALEPACK* from the *HKL*2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

Crystals of XylnA were grown by the hanging-drop method and diffraction-quality crystals were obtained from a crystallization condition with pH 8.5. The crystals belonged to the monoclinic space group *C*2, with unit-cell parameters a = 174.5, b = 54.7, c = 131.5 Å, β = 131.2°. Statistics of data collection and processing to 2.2 Å resolution are summarized in Table 1. The Matthews coefficient $V_{\rm M}$ (Matthews, 1968) was calculated to be 2.8 Å^3 Da^{-1}, suggesting the presence of two molecules in the asymmetric unit. This $V_{\rm M}$ value corresponds to a solvent content of approximately 56%.

Preliminary structure solution was obtained by the molecular-replacement method with the program *AMoRe* (Navaza, 1994) using as a search model the crystal structure (PDB code 1hiz) of the extracellular xylanase from *G. stearothermophilus* (Teplitsky *et al.*, 2004). The crystal was shown to contain two XylnA molecules in the asymmetric unit. Model building and refinement of the preliminary structure using the programs *COOT*0.31 (Emsley & Cowtan, 2004) and *CNS*1.1 (Brünger *et al.*, 1998), respectively, are in progress. We are also in the process of studying the crystal structure of the xylanase in complex with xylooligosacharides in order to elucidate the mechanism of substrate recognition and enzymatic activity. The study of this enzyme has been particularly focused on the rational enhancement of its thermostability and alkalostability on the basis of its three-dimensional structure and its detailed catalytic mechanism. It should also help in protein engineering and provide the basis for the design of xylanases with enhanced stability and activity for specific industrial uses.

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