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Crystallization and preliminary X-ray analysis of *Alicyclobacillus acidocaldarius* endoglucanase CelA

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Crystallization of a family 9 β-1,4-glucanase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* CelA is reported. Thin plates can be obtained by hanging-drop vapour-diffusion crystallization in high concentrations (60%) of MPD. These crystals are unusual in that they do not bind the dye IZIT in the mother liquor and do not appear to dissolve in water after three weeks or in the storage buffer after 2 d. The crystals diffract weakly and the diffraction pattern is compatible with crystal disorder in one direction. After testing several crystals at the ESRF beamlines ID14-1 and ID14-2, a crystal was found which gave ordered diffraction in all directions. A full data set was collected to 3.0 Å resolution, which allowed unambiguous determination of the space group as $P2_12_12$ and the unit-cell parameters as a = 85, b = 129.7, c = 48.6 Å. Initial promising results from molecular-replacement searches are reported.

1. Introduction

Alicyclobacillus acidocaldarius, a thermoacidophilic Gram-positive bacterium, can utilize a variety of polysaccharides, including starch, carboxymethylcellulose and xylan, as its sole carbon and energy sources. Utilization of these polysaccharides implies a well developed battery of polysaccharide-degrading enzymes with diverse specificities and transport systems in order to import the degradation products into the cytoplasm (Schwermann *et al.*, 1994; Matzke *et al.*, 2000).

The gene encoding an *A. acidocaldarius* β-1,4-glucanase (CelA; EC 3.2.1.4) has recently been cloned and overexpressed in *Escherichia coli* and the gene product has been characterized (Eckert *et al.*, 2002). Recombinant CelA (MW = 60 kDa) shows activity on carboxymethylcellulose, lichenan and *p*-nitrophenyl-cellooligosaccharides. The pattern of activity on the latter is quite striking, with high activity on *p*-nitrophenyl-cellobioside and some activity on *p*-nitrophenyl-cellotetraoside, but none on the glucoside and cellotrioside derivatives. This unusual substrate specificity is accompanied by a lack of a signal peptide and irreversible deactivation at the low pHs present extracellularly. This prompted the authors to suggest that this may be an intracellular enzyme involved in imported oligosaccharide degradation, rather than an extracellular polysaccharidase.

The determined CelA sequence allows it to be assigned to family 9 in the glycoside hydrolase classification (Coutinho & Henrissat, 1999; http://afmb.cnrs-mrs.fr/CAZY/). Three structures have so far been reported for this family of glycoside hydrolases: CelD from *Clostridium thermocellum* (subgroup E1; Juy *et al.*, 1992), endoglucanase E4 from *Thermomonospora fusca* (subgroup E2; Sakon *et al.*, 1997) and the homologous enzyme from termite (Khademi *et al.*, 2002). Structures of the enzymes belonging to family 9 display a common catalytic domain with an $(\alpha/\alpha)_6$ twisted α-barrel motif. Sequence analysis has shown that CelA belongs to subgroup E1 and is likely to possess an N-terminal immunoglobulin-like domain like CelD (Eckert *et al.*, 2002).

Here, we report successful crystallization of CelA, data collection and initial molecular-replacement searches, which will hopefully lead to determination of the CelA structure and help in the understanding of its unusual pattern of activity on aryl derivatives.

2. Crystallization

Protein expression and purification was carried out as described in Eckert *et al.* (2002). CelA was cloned in the expression vector pBAD/HisA (Invitrogen) and the recombinant protein was purified by Ni–NTA chromatography. The protein preparation was finally dialyzed in 50 m*M* Tris–HCl pH 7.0.

Initial crystallization screens were carried out using Crystal Screens I and II from Hampton Research with the hanging-drop method in Linbro plates. Drops consisted of

2 µl protein at 3 mg ml^{-1} and 2 µl reservoir. Only clear drops or precipitates were obtained in these initial trials. The precipitates of crystalline appearance were further tested by attempting to dissolve them in excess water, by dyeing them with the commercial IZIT dye (Hampton Research) and by streak-seeding (Stura & Wilson, 1992).

One precipitate, grown using condition 35 of Crystal Screen II [70% 2-methyl-2,4-pentanediol (MPD), 0.1 *M* HEPES pH 7.5], was used for streak-seeding into a fresh drop mixed using the same reservoir. Needles were produced after 4 d. Intensive efforts were subsequently made in order to optimize crystal quality by varying the pH, MPD concentration, temperature and protein concentration. During optimization, it became clear that crystals could grow without the need for microseeding if a higher initial protein concentration was used (7.6 mg ml^{-1}). Fig. 1(*a*) shows a picture of some of the best crystals grown at room temperature using 57% MPD, 0.1 *M* HEPES pH 7.3 in the reservoir and protein solution with a concentration of 6 mg ml^{-1}. The crystals grow as clusters of needles and/or wafer-thin long plates, which could be separated. The crystals can be up to 0.4 mm long and 0.05 mm wide, but are very thin in the third dimension.

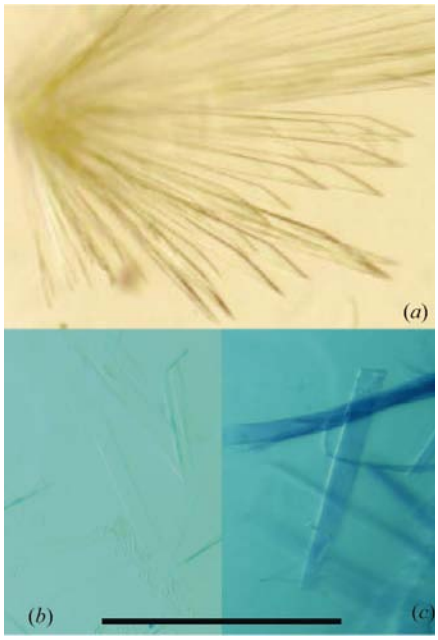

Figure 1
Crystals of CelA: (*a*) best crystals obtained using 57% MPD and 0.1 *M* HEPES pH 7.3 as precipitant; (*b*) colourless crystals 2 d after addition of IZIT to the mother liquor; (*c*) coloured crystals 2 d after addition of IZIT in water. The black bar corresponds to approximately 0.5 mm.

In order to control nucleation various techniques were attempted, such as addition of oil to the reservoir in order to slow equilibration (Chayen, 1997), micro- and macroseeding (Stura & Wilson, 1992) and crystallization in a gel (Robert *et al.*, 1992), but none produced the desired improvement in crystal quality.

Among the additives from the Hampton Research Additive Screen kits, dithiothreitol appeared to increase the speed at which crystals formed, while spermine and cystein altered the morphology, but without considerably improving it. Addition of cellobiose to the crystallization mixture also failed to improve crystal quality; in fact, it seemed to prevent crystal growth.

3. Unexpected results encountered during crystallization

During the initial screening of precipitates, the conditions that eventually led to diffracting crystals of CelA had been evaluated as very unpromising. First of all, the precipitate did not dissolve in water or buffer after 1–2 d, a common test for microcrystalline precipitate. The crystals obtained by microseeding did not dissolve either in water after several weeks or buffer after 2 d. Secondly, neither the precipitate nor the derived crystals were stained by the commercial dye IZIT, which is supposed to discriminate between protein and small-molecule crystals. This is shown in Fig. 1(*b*).

It was, however, unlikely that the crystals were of salt, given the components of the crystallization mixture. We had previously observed that cubic crystals of truncated glycogen-branching enzyme from *E. coli* (Hilden *et al.*, 2000) dyed with IZIT dye lost their colour when transferred to a cryoprotectant solution containing glycerol. We therefore attempted to wash the needles in water and after addition of IZIT dye the colour was rapidly absorbed (Fig. 1*c*). This leads us to conclude that MPD prevents dye absorption by the CelA crystals.

Our results suggest that lack of dye absorption should not be taken as definitive proof that protein crystals have not been obtained. Furthermore, our experiences highlight that a failure to redissolve a precipitate in water or the original buffer within a limited period of time does not necessarily imply that the precipitate is amorphous or that the protein is denatured; it may simply imply that the kinetics of dissolution are exceptionally slow.

Table 1
Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Resolution range (Å)	32.44–3.00 (3.11–3.00)
Completeness (%)	99.4 (95.3)
Unique reflections	68757
Redundancy	6.1 (5.3)
$R_{\rm merge}$	0.154 (0.539)
$I/\sigma(I)$	11.8 (4.0)

4. Data collection

Initial tests of crystals on beamline ID14-1 (ESRF, Grenoble, France) showed diffraction to at least 3.5 Å in one direction, but the reflections were very elongated in the other direction, suggesting anisotropic disorder in the crystals. Data collection was carried out in the mother liquor at 100 K, as MPD at high concentrations is an effective cryo-

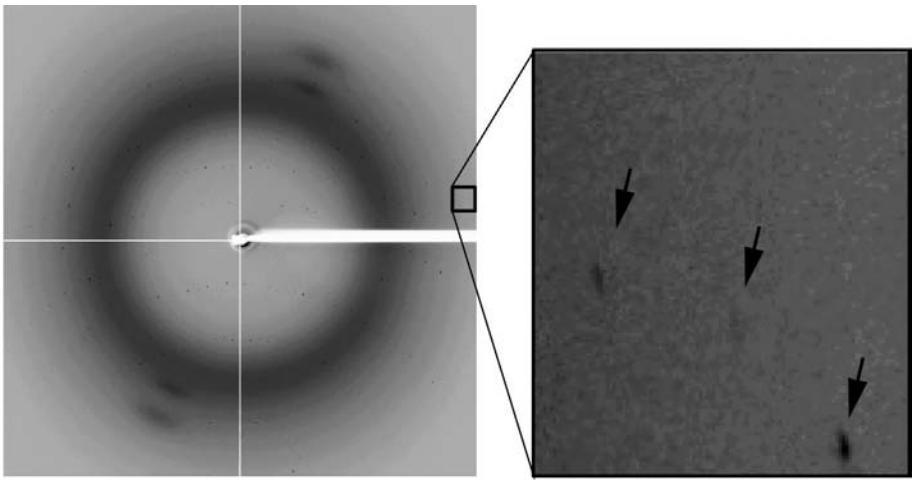

Figure 2
Diffraction pattern of CelA crystal. The insets show that diffraction, although weak, extends to the edges, corresponding to approximately 3.0 Å resolution.

protectant. Since attempts to obtain better crystals were not successful, more than ten crystals were subsequently tested at beamline ID14-2 of the ESRF. Both beamlines operate at a wavelength of 0.93 Å and are equipped with a ADSC Q4 CCD detector. Efforts were taken to choose very thin but single crystals and to mount them in large loops, if possible far from the edges, in order to prevent bending, as suggested by Mayans & Wilmanns (1999).

Only one of the crystals tested (Fig. 1*a*; crystallization conditions in §2) showed a satisfactory spot shape in both directions. Data were collected for this crystal to 3 Å resolution, allowing unambiguous assignment of the space group as $P2_12_12$, with unit-cell parameters $a = 85.0$, $b = 129.7$, $c = 48.6$ Å. The unit-cell parameters are consistent with one molecule in the asymmetric unit and the data were processed with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). Data-collection statistics are shown in Table 1, while a diffraction pattern is shown in Fig. 2. The diffraction is weak overall, resulting in a relatively high $R_{\rm merge}$, but the chosen resolution cutoff is justified by the high redundancy and the reasonable $I/\sigma(I)$ in the last resolution shell.

4.1. Molecular replacement

Molecular-replacement trials were carried out using *EPMR* (Kissinger *et al.*, 1999) with the structure of *C. thermocellum* CelD (PDB code 1clc) as a search model, with and without the N-terminal immunoglobulin-like domain and using default *EPMR* parameters. Using either model, an identical most frequent solution was obtained. The correlation coefficients were 0.280 and 0.264, respectively, for the full and truncated model, while the *R* factors were 0.564 and 0.569, respectively, supporting the view that CelA has a N-terminal immunoglobulin-like domain. This solution seems also promising, as the molecules pack without any serious clashes (Fig. 3). It remains to be seen whether refinement can be succesfully carried out without additional phase information, owing to the limited resolution and homology of the model, which only shares 25 to 30% sequence identity with the target.

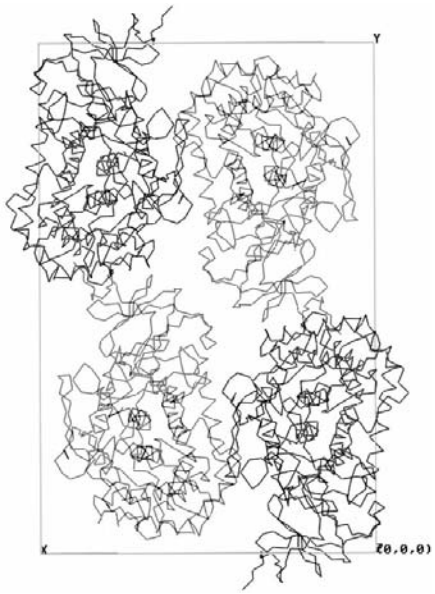

Figure 3
Crystal packing of the 'best' molecular-replacement solution.

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