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Expression, purification, crystallization and preliminary X-ray analysis of the ι-carrageenase from *Alteromonas fortis*

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This is the first crystallization report of a glycoside hydrolase which belongs to family 82. A recombinant form of His-tagged ι-carrageenase from *Alteromonas fortis* was expressed, purified and crystallized. Crystals were obtained by the vapour-diffusion method using polyethylene glycol (M_W = 6000) as a precipitant. They belong to space group $P2_1$, with unit-cell parameters a = 56.75, b = 91.04, c = 125.01 Å, β = 93.41°. The unit cell contains two molecules in the asymmetric unit related by a non-crystallographic twofold axis. Crystals diffracted to 2.0 Å resolution on a synchrotron beamline.

1. Introduction

Carrageenans are sulfated polysaccharides found in the cell walls of marine red algae. They consist of a linear backbone of D-galactose residues linked by alternating α(1,3) and β(1,4) linkages. A further layer of complexity is the occurrence of a 3,6-anhydro bridge in the α(1,4)-linked galactose residue and the number of ester-sulfate substituents per di-galactose repeating unit, which varies from one in κ-carrageenan to two in ι-carrageenan and three in λ-carrageenan (Kloareg & Quatrano, 1988). These sulfated galactans form thermo- and/or iono-reversible gels structured by double helices and are widely used as texturing agents in various industries.

The enzymatic hydrolysis of these galactans involves specific hydrolases called κ-, ι- and λ-carrageenases. The first known ι-carrageenase was purified from a marine Gram-negative bacterium, *A. fortis* (Greer & Yaphe, 1984). The ι-carrageenase from another marine bacterium, *Zobellia galactanovora*, has been purified and the genes encoding these enzymes have been cloned (Barbeyron *et al.*, 2000). These two proteins are homologous and bear no significant sequence similarities to any other polysaccharidase, including κ-carrageenases. They cleave the internal β(1,4) linkage of ι-carrageenan with overall inversion of the anomeric configuration, whereas κ-carrageenases proceed with retention of configuration (Potin *et al.*, 1995). It was proposed that ι-carrageenases constitute a new structural family of glycoside hydrolases, referred to as family 82 (Barbeyron *et al.*, 2000).

A high-resolution three-dimensional structure of an ι-carrageenase is therefore desirable to reveal the fold of this novel glycoside hydrolase family and to define the amino-acid residues involved in the recognition and cleavage of the double helices of ι-carrageenans. As a preliminary step towards this goal, we report here the expression, purification and crystallization of the ι-carrageenase from *A. fortis*.

2. Materials and methods

2.1. Expression and purification of *A. fortis* ι-carrageenase

The coding region of the mature ι-carrageenase, *i.e.* without the N-terminal signal peptide, was amplified by the polymerase chain reaction (PCR) with Vent polymerase (New England Biolabs) from the pIP2 plasmid (Barbeyron *et al.*, 2000), using the oligonucleotide primers 5′-GGGCCATGGCTGTCT-CCCCCAAGACT-3′ and 3′-CGTCAGCTA-CCCTCGCTGAGCTCGGGG-5′. The PCR product was then ligated into the expression vector pET20b (Novagen), using the *Nco*I and *Xho*I sites of the polylinker. This resulted in a gene encoding ι-carrageenase flanked by a C-terminal hexahistidine tag and a N-terminal PelB signal peptide for targeting the gene product into *Escherichia coli* periplasm. The recombinant plasmid, referred to as pETIAf, was used to transform *E. coli* BL21(DE3) strain harbouring pLysS plasmid.

Recombinant *E. coli* BL21(DE3) cells were incubated at 310 K in M9 medium (Maniatis *et al.*, 1982) complemented with 2%(*w*/*v*) casamino acids, 100 µg ml^{-1} ampicillin and 37 µg ml^{-1} chloramphenicol. The culture was maintained for 15 h at 285 K, as higher temperatures resulted in inclusion bodies. Expression of ι-carrageenase was induced with 1 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the culture reached OD_{600} = 1.0. Cells were submitted to osmotic shock in order to release the periplasmic proteins (Manoil &

Beckwith, 1986). Briefly, cells were resuspended in a 0.1 *M* Tris–HCl buffer (pH 7.2) containing 0.5 *M* sucrose and 1 m*M* EDTA, left for 5 min at room temperature, spun down (10 000*g*, 10 min) and the pellet was resuspended in ice-cold water for 5 min. $MgCl_2$ was added to a final concentration of 1 m*M*, the extract was centrifuged (20 000*g*, 10 min) and the supernatant was supplemented so that the final concentrations were 10 m*M* Tris–HCl pH 7.2, 500 m*M* NaCl and 100 m*M* imidazole (buffer *A*).

The periplasmic extract was applied to a 10 ml column of Chelating Fast Flow Sepharose (Pharmacia) charged with $NiSO_4$ and pre-equilibrated with buffer *A*. The column was washed with buffer *A* and then eluted with 60 ml of an imidazole linear gradient (100–300 m*M* imidazole) at a flow rate of 1 ml min^{-1}. Fractions were assessed for ι-carrageenase homogeneity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Enzyme purity was further confirmed by electrospray mass spectrometry. ι-Carrageenase activity was monitored throughout purification by the reducing-sugars method (Kidby & Davidson, 1973). The amounts of purified protein were determined by spectrophotometry at 280 nm using the theoretical molar extinction coefficient (ε_{280} = 58330 M^{-1} cm^{-1}) determined according to Pace *et al.* (1995).

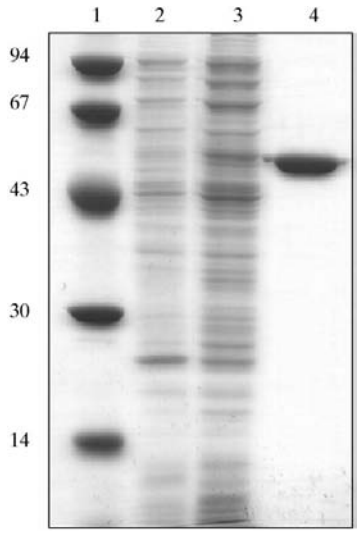

Figure 1
SDS–PAGE of the periplasmic extracts from the pETIAf clone and of the purified ι-carrageenase. Proteins were resolved by SDS–PAGE onto a 12.5% polyacrylamide gel and were detected by Coomassie Blue staining. Lane 2: extracts from the non-induced pETIAf clone. Lane 3: extracts from the IPTG-induced pETIAf clone. Lane 4: purified ι-carrageenase after one step of affinity chromatography. The molecular weight of the markers (Pharmacia), lane 1, are indicated in kDa.

2.2. Crystallization and X-ray diffraction analysis

Crystallization experiments were carried out at 288 K using the hanging-drop vapour-diffusion method. The purified protein was equilibrated by dialysis at a final concentration of 6 mg ml^{-1} with 10 m*M* Tris–HCl pH 7.2, 200 m*M* NaCl. Drops were prepared by mixing 2 µl of the protein solution with 2 µl of the reservoir solution and were allowed to equilibrate against 500 µl of reservoir solution. Crystallization conditions were first investigated using the sparse-matrix sampling kit (Hampton Research). Single crystals were obtained using polyethylene glycol as precipitant.

Crystals were successively soaked for 30 s in crystallization solutions in which the glycerol concentration was increased by steps of 5% to a final concentration of 20%. A single crystal was then mounted on a loop, transferred on the gononiometer head and kept at 100 K in a nitrogen stream. A set of native X-ray diffraction data was collected on a 345 mm MAR image-plate detector (beamline FIP/BM 30, ESRF-Grenoble, France). The unit-cell parameters were determined by the autoindexing and parameter-refinement procedures of *DENZO*. All intensity data were integrated and reduced with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). The space group was determined by examining the intensity distribution of the X-ray data. The scaled and reduced intensity data were converted to amplitudes using *TRUNCATE* (Collaborative Computational Project, Number 4, 1994). The self-rotation function was calculated with *POLARRFN* from the same software package.

3. Results and discussion

The structural gene of the mature ι-carrageenase from *A. fortis* was inserted downstream of the T7 polymerase promoter and of the ribosomal binding site in the pET20b expression vector (Novagen). Expression of ι-carrageenase from *E. coli* harbouring recombinant plasmid pETIAf resulted in a level of production of about 75 U ml^{-1} of culture medium [where one unit (U) is the amount of enzyme which will catalyse the transformation of 1 µmol of substrate per minute], an amount higher than the production level obtained from native *A. fortis* (Barbeyron *et al.*, 2000). A single affinity chromatography step was sufficient to recover over 85% of the initial protein quantity as an apparently homogenous preparation (Fig. 1) with a specific activity of about 5000 U mg^{-1}. For the recombinant ι-carrageenase, a molecular mass of 53316 (3) Da was determined by electrospray mass spectrometry; this value is close to 53319 Da, the expected mass for the 475 amino-acid construction. However, there is a minor population of recombinant ι-carrageenase [53003 (3) Da] which has lost the first methionine introduced by the *Nco*I restriction site. This heterogeneity may be explained by a cleavage mistake when the PelB signal peptide was excized. The high enzyme yields obtained upon extraction by osmotic shock further confirmed ι-carrageenase export into *E. coli* periplasmic space.

Using the sparse-matrix sampling kit (Jancarik & Kim, 1991), crystalline precipitates were obtained with both polyethylene glycol and ammonium sulfate as precipitating agents. Higher quality single crystals were obtained in polyethylene glycol and the presence of calcium ions appeared to be crucial. Best results were obtained with 0.1 *M* sodium cacodylate pH 6.5, 15–17% polyethylene glycol (M_W 6000) and 200 m*M* calcium acetate. In these conditions, well shaped crystals grew to typical dimensions of 0.75 × 0.25 × 0.15 mm at 288 K within a week (Fig. 2). X-ray data collected at 2.0 Å from a native crystal yielded 237 879 reflections, resulting in a final merged data set of 81 869 unique reflections (95.4% completeness at 2.0 Å) with an R_{sym} of 5.0% (21% in the last shell). The overall $I/\sigma(I)$ is 15.0 with a value of 4.8 in the highest resolution bin. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 56.75, b = 91.04, c = 125.01 Å, $\alpha = \gamma = 90$, β = 93.41°. Assuming the presence of two molecules of recombinant ι-carrageenase in the asymmetric unit resulted in a calculated molecular volume (V_m) of 3.0 $Å^3$ Da^{-1}, consistent with the range frequently

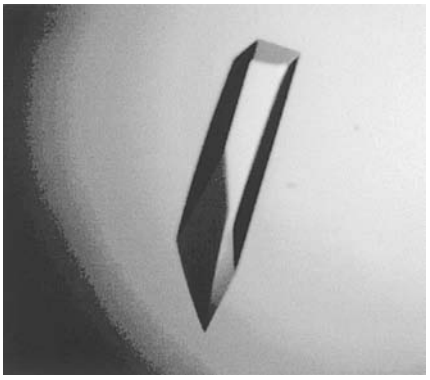

Figure 2
Crystal of ι-carrageenase. Dimensions are 0.8 × 0.2 × 0.2 mm.

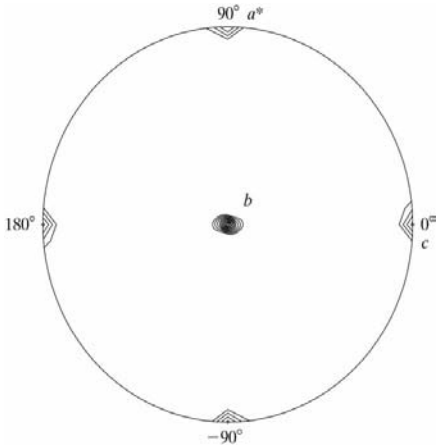

Figure 3
Plot of the $\kappa = 180^\circ$ section of the self-rotation function. The orthogonalization was chosen to place x along c, y along a^* and z along b. The origin peak is along the b axis. Two strong peaks appear at $\omega = 90$, $\varphi = 0.4$ and 90.4°, with a height of 67.1% of the origin. The self-rotation function was calculated with *POLARRFN* (Collaborative Computational Project, Number 4, 1994).

observed for protein crystals (Matthews, 1968). The self-rotation function was calculated for data in the resolution range 20–2 Å and a radius of integration of 15 Å (Fig. 3). Two strong peaks at $\omega = 90$, $\varphi = 0.4$, $\kappa = 180^\circ$ and $\omega = 90$, $\varphi = 90.4$, $\kappa = 180^\circ$ with an intensity height of 67.1% of the origin indicated non-crystallographic twofold axes almost parallel to the c and a^* axes. The combination of the crystallographic and one non-crystallographic rotation axes generated a pseudo-orthorhombic symmetry.

The recombinant ι-carrageenase was also overproduced in the presence of selenomethionine and crystallized using the procedures described above. Structure resolution by the multiple anomalous dispersion method (MAD) is now under way.

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