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Expression, purification, crystallization and preliminary X-ray analysis of the κ-carrageenase from *Pseudoalteromonas carrageenovora*

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A recombinant form of His-tagged κ-carrageenase from *Pseudoalteromonas carrageenovora* has been expressed, purified and crystallized. Crystals have been obtained by the vapour-diffusion method using polyethylene glycol (M_r = 4000) as a precipitant. These crystals belong to the space group $P2_12_12_1$, with unit-cell parameters a = 58.2, b = 62.8, c = 77.9 Å, and diffract to 2.2 Å resolution on a rotating-anode X-ray source.

1. Introduction

The red algal polysaccharides known as carrageenans 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 digalactose repeating unit, which varies from one in κ-carrageenan to three in λ-carrageenan. These sulfated galactans exhibit unique rheological properties and are widely used as texturing agents in various industries.

The structural genes of the κ-carrageenase of the marine bacteria *Pseudoalteromonas carrageenovora* and *Cytophaga drobachiensis* strain Dsij have been described in detail (Barbeyron *et al.*, 1994, 1998). These two galactoside hydrolases cleave the internal β(1→4) linkage of κ-carrageenan with a strict substrate specificity. They belong to family 16 of glycoside hydrolases, as do various agarases, β-1,3-glucanases (laminarinases), β-1,3–1,4-glucanases (lichenases) and xyloglucan endo-transglycosylases (XETs), which in plant cell walls catalyze the transfer of segments of β-1,4-xyloglucan molecules to other hemicellulose chains (Henrissat & Bairoch, 1996).

As a member of family 16 of glycoside hydrolases, κ-carrageenases should display a general fold similar to the three-dimensional structures of *Bacillus licheniformis* and *B. macerans* lichenases (Keitel *et al.*, 1993; Hahn *et al.*, 1995). However, in spite of their structural similarities (Barbeyron *et al.*, 1998), κ-carrageenases and lichenases share only 20% overall sequence identity. Even in the conserved catalytic motif κ-carrageenases (as well as laminarinases) depart from lichenases and XETs by the presence of one additional amino acid, an insertion which should affect the active site. A high-resolution three-dimensional structure of a κ-carrageenase is therefore required for defining the amino-acid residues involved in the recognition and cleavage of κ-carrageenans. As a preliminary step towards this goal, we report here the expression, purification and crystallization of the κ-carrageenase from *P. carrageenovora*.

2. Materials and methods

2.1. Expression and purification of *P. carrageenovora* κ-carrageenase

The coding region of the mature κ-carrageenase, *i.e.* without the N-terminal signal peptide and the C-terminal helper, was amplified by the polymerase chain reaction (PCR) with Vent polymerase (New England Biolabs) from the pKA4 plasmid (Barbeyron *et al.*, 1994), using the oligonucleotide primers 5′-GGGCCATGGCATCTATGCAACCTCCC-3′ and 3′-CCACCCGTTATTGTTGGAGCTCG-GGG-5′. The PCR product was then ligated into expression vector pET20b (Novagen), using the *Nco*I and *Xho*I sites of the polylinker. This resulted in a gene encoding κ-carrageenase flanked by a C-terminal hexahistidine tag and a N-terminal PelB signal peptide for targetting into *Escherichia coli* periplasm. The recombinant plasmid, referred to as pETKAc, 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*) *cas*-amino acids, 100 mg ml^{-1} ampicillin and 37 mg ml^{-1} chloramphenicol. Expression of κ-carrageenase was induced with 1 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the culture reached an OD_{600} of 1.0. The culture was maintained for 15 h at 285 K, as higher temperatures resulted in inclusion bodies. Cells were submitted to osmotic shock in order to release the periplasmic proteins,

according to Manoil & Beckwith (1986). Briefly, cells were resuspended in a 0.1 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sucrose and 1 m*M* EDTA, left for 5 min at room temperature, spun down (10000*g*, 10 min) and resuspended in ice-cold water for 5 min. $MgCl_2$ was added to a final concentration of 1 m*M*, the extract was centrifuged (20000*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 60 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 elution was performed with 60 ml of an imidazole linear gradient (60–250 m*M* imidazole) at a flow rate of 1 ml min^{-1}. Fractions were assessed for κ-carrageenase homogeneity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting with a κ-carrageenase-reactive antibody obtained and purified as previouly described (Potin *et al.*, 1995). Enzyme purity was further confirmed by electrospray mass spectrometry, and κ-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} = 82850 M^{-1} cm^{-1} determined according to Pace *et al.* (1995).

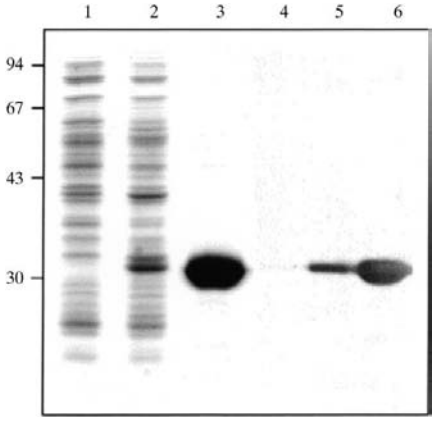

Figure 1
SDS–PAGE and immunoblot analysis of the periplasmic extracts from the pETKAc clone and of the purified κ-carrageenase. Proteins were resolved by SDS–PAGE on a 12.5% polyacrylamide gel, and either detected by Coomassie Blue staining (lanes 1–3) or blotted onto nitrocellulose. The κ-carrageenase was then detected by reaction with the affinity-purified κ-carrageenase-reactive antibody (lanes 4–6). The secondary antibody is an anti-rabbit antibody coupled with alkaline phosphatase. Lanes 1 and 4, extracts from the non-induced pETKAc clone. Lanes 2 and 5, extracts from the IPTG-induced pETKAc clone. Lanes 3 and 6, purified κ-carrageenase after one step of affinity chromatography. The electrophoretic mobilities of low molecular weight markers (Pharmacia) are indicated in kDa.

2.2. Crystallization and X-ray diffraction analysis

Crystallization experiments were carried out at 285 K using the hanging-drop vapour-diffusion method. The purified protein was equilibrated by dialysis at the final concentration of 6.5 mg ml^{-1} with 10 m*M* Tris–HCl pH 7.2, 200 m*M* NaCl. Drops were prepared by mixing 2 µl of protein solution with 2 µl of 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).

Crystals were mounted in a thin-walled glass capillary, which was sealed with wax after filling both ends with the reservoir solution. X-ray experiments were carried out using graphite-monochromated Cu *Kα* X-rays from a rotating-anode generator operating at 40 kV and 85 mA. A set of native X-ray diffraction data was collected on a FAST area-detector system (Enraf–Nonius) and processed using the *MADNES* software (Messerschmidt & Pflugrath, 1987). The unit-cell parameters were determined using the autoindexing and parameter-refinement procedures of the *MADNES* software. Reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled and merged with the *PROCOR* program. The space group was determined by examining the intensity distribution of the X-ray data.

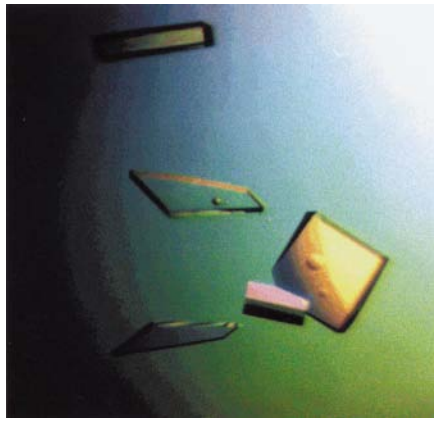

Figure 2
Crystals of κ-carrageenase viewed under polarizing optics. The size of the largest crystal is 0.3 × 0.3 × 0.1 mm.

3. Results and discussion

The structural gene of the mature κ-carrageenase from *P. carrageenovora* was inserted downstream of the T7 polymerase promoter and the ribosomal binding site in the pET20b expression vector (Novagen). Expression of κ-carrageenase from *E. coli* harbouring recombinant plasmid pETKAC resulted in a level of production of about 50 units per millilitre of culture medium, an amount comparable to the highest production level obtained from native *P. carrageenovora* (Potin *et al.*, 1991). This expression yield is well above that achieved using *E. coli* carrying pAT153 and the native κ-carrageenase gene transcribed under its own promoter (Barbeyron *et al.*, 1994). A single affinity-chromatography step was sufficient to recover over 80% of the initial protein quantity as an apparently homogenous preparation (Fig. 1) with a specific activity of about 8000 units mg^{-1}. Interestingly, a similar specific activity was obtained when the protein was expressed together with its C-terminal region (data not shown), validating our previous suggestion (Potin *et al.*, 1995) that this extension is not essential for catalysis nor for proper protein folding. The 32935 ± 3 Da molecular mass of the recombinant κ-carrageenase was determined by electrospray mass spectrometry and is consistent with the 32936 Da theoretical molecular mass expected for the 285 amino-acid construction. These data confirm the enzyme homogeneity and show that the PelB signal peptide was efficiently cleaved. The high enzyme yields obtained upon extraction by osmotic shock further confirmed its export into *E. coli* periplasmic space.

Using the sparse-matrix sampling kit (Jancarik & Kim, 1991), crystalline precipitates and irregular crystals were obtained when polyethylene glycol was used as precipitating agent. Attempts to improve crystal quality were made using polyethylene glycols with different molecular weights and varying the pH of the reservoir solution. The best results were obtained with 0.1 *M* sodium acetate at pH 4.5, 30% polyethylene glycol (M_w = 4000) and 50 m*M* cadmium chloride. Under these conditions, well shaped crystals grew to typical dimensions of 0.3 × 0.3 × 0.15 mm at 288 K within a week (Fig. 2). These crystals diffracted up to 2.2 Å and were very stable in the X-ray beam. X-ray data collected at 2.4 Å from a native crystal yielded 39526 accepted measurements, resulting in a final merged data set of 13227 unique reflections [99% completeness at 2.4 Å, redundancy 3,

$\langle I/\sigma(I)\rangle$ = 13.8] with an $R_{\rm sym}$ of 5.2%. In the outmost shell (2.6–2.4 Å), the completeness, redundancy and $\langle I/\sigma(I)\rangle$ values were 98%, 2.1 and 8.7, respectively. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 58.2, b = 62.8, c = 77.9 Å, $\alpha = \beta = \gamma = 90^\circ$. Assuming the presence of one molecule of recombinant κ-carrageenase in the asymmetric unit resulted in a calculated molecular volume (V_m) of 2.16 Å^3 Da^{-1} and a corresponding solvent content of 37%, consistent with the range frequently observed for protein crystals (Matthews, 1968).

As expected from the low sequence identity, the molecular-replacement method (Navaza, 1994) using the three-dimensional structure of *B. licheniformis* lichenase (PDB code 1GBG) as a model was unsuccessful. With the aim of solving the phase problem by multiple anomalous dispersion, expression of the recombinant κ-carrageenase in the presence of selenomethionine (Hendrickson *et al.*, 1990) is now in progress.

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