Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, purification, crystallization and preliminary X-ray analysis of the polysaccharide lyase RB5312 from the marine planctomycete *Rhodopirellula baltica*

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Received 14 November 2007
Accepted 13 February 2008

Polysaccharide lyases belonging to family PL1 act on pectins. These anionic polymers are usually produced by terrestrial plants and therefore pectinolytic enzymes are not frequently observed in marine microorganisms. The protein RB5312 from the marine bacterium *Rhodopirellula baltica* is distantly related to family PL1 pectate lyases, but its exact function is unclear. In this study, the expression and purification of a recombinant form of RB5312 are described. This protein was crystallized using the hanging-drop vapour-diffusion method. The crystals belongs to space group $P2_12_12_1$, with unit-cell parameters $a = 39.05$, $b = 144.05$, $c = 153.97$ Å, $\alpha = \beta = \gamma = 90^\circ$. A complete data set was collected to 1.8 Å resolution from a native crystal.

1. Introduction

The cell walls of marine algae and seagrasses are characterized by an abundance of anionic polysaccharides formed from carboxylic or sulfated sugars (Aquino *et al.*, 2005; Kloareg & Quatrano, 1988). These polymers constitute a crucial carbon source for a number of marine bacteria that secrete specific glycoside hydrolases or polysaccharide lyases (Michel *et al.*, 2006). Among these microorganisms, the Planctomycetes are recognized as one of the key phyla catalyzing important transformations in the global carbon cycle in the sea (Alldredge, 2000; DeLong *et al.*, 1993). Planctomycetes frequently inhabit phytodetrial macroaggregates in marine environments where they mineralize this organic matter, which is mainly composed of polysaccharides (Miskin *et al.*, 1999; Neef *et al.*, 1998; Wang *et al.*, 2002). *Rhodopirellula baltica* is a marine representative of this important bacterial phylum and it was the first to have its genome completely sequenced (Glöckner *et al.*, 2003). Interestingly, its genome revealed an unprecedented large number of sulfatases, accompanied by numerous polysaccharidases. These observations suggest that *R. baltica* is able to degrade a wide range of anionic polysaccharides from marine algae and seagrasses.

Among the various enzymes found in the *R. baltica* genome, two have been annotated as putative pectate lyases: RB5312 (GenBank accession No. CAD74167) and RB5316 (CAD74169) (Glöckner *et al.*, 2003). Both proteins have been assigned to family PL1 of the polysaccharide lyases by the Carbohydrate Active Enzyme website (http://www.cazy.org/; Coutinho & Henrissat, 1999). Pectins are a group of plant polysaccharides that are mainly constituted of D-galacturonic acid (GalA) and three polysaccharide domains are often found: homogalacturonan (HGA), rhamnogalacturonan-I (RGI) and rhamnogalacturonan-II (RGII) (Mohnen, 1999; O'Neill *et al.*, 1990). HGA is a linear homopolymer of (1,4)-α-linked D-galacturonic acid and is deposited in the cell wall of land plants in a form that has 70–80% of GalA residues methyl-esterified at the C-6 carboxyl (Mohnen, 1999; O'Neill *et al.*, 1990). Pectate and pectin lyases cleave the α-(1,4)-linkages in HGA domains by a β-elimination mechanism, releasing an unsaturated C4—C5 bond at the non-reducing end of the cleaved polysaccharide. Pectate lyases (EC 4.2.2.2) are specific for demethylated forms of HGA and require Ca^{2+} for activity (Pilnik & Rombouts, 1981; Scavetta *et al.*, 1999). In contrast, pectin lyases (EC 4.2.2.10) cleave methylated HGA

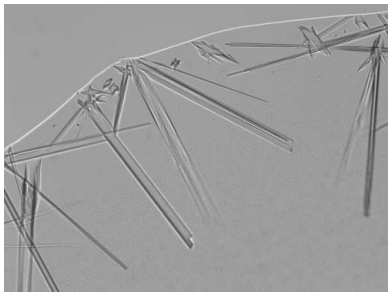

Table 1
Oligonucleotides used for the cloning of *rb5312*.

The primers were designed for a half-hybridization temperature of 345 K.

Forward	GGGGGGAGATCTCAGAAGCCATTGGCCTTTCCGAC
Reverse	CCCCCCGAATTCTTACGGGATGCTGTTGATGTATTGTTC

according to a calcium-independent mechanism (Mayans *et al.*, 1997; Pilnik & Rombouts, 1981).

The presence of putative pectinolytic enzymes in a marine bacterium is surprising since pectins are typical of terrestrial higher plants. Nevertheless, pectin-like polymers have been identified in some marine green algae (Kloareg & Quatrano, 1988) and seagrasses (Ovodov *et al.*, 1975). To evaluate the quality of the functional predictions of Glöckner *et al.* (2003), we have performed our own sequence analyses using *BLAST* searches against the SwissProt database. RB5316 displays 37% and 29% sequence identity to the pectate lyases PelA from *Emericella nidulans* and PelE from *Erwinia chrysanthemi*, respectively (Ho *et al.*, 1995; Lietzke *et al.*, 1994). Therefore, RB5316 is indeed homologous to characterized members of the PL1 family and is likely to be a pectate lyase. In contrast, for the second protein RB5312 only the N-terminal region shares some similarities to characterized proteins: the pectate lyase PelA from *Em. nidulans* (29% sequence identity for Ala22–Ser222, RB5312 numbering) and the noncatalytic pollen allergen AgE from *Ambrosia artemisiifolia* (27% sequence identity for Ala19–Asp160; Rafnar *et al.*, 1991). The C-terminal region of RB5312 (Gly225–Pro455) displays no significant similarity to any characterized protein. At this low level of sequence identity, the exact function of RB5312 is unclear and the prediction that it is a pectate lyase is difficult to substantiate.

Therefore, the focus of our study is to unravel the biochemical function of the marine polysaccharide lyase RB5312. Determination of its crystal structure will also help to establish the role of this divergent member of the PL1 family. We have thus cloned the *rb5312* gene from *R. baltica* into *Escherichia coli* and produced pure recombinant protein. We also report the crystallization of RB5312 and the preliminary X-ray analysis of the crystals.

2. Experimental

2.1. Overexpression and purification of RB5312

According to the program *SignalP* (Bendtsen *et al.*, 2004), RB5312 features an N-terminal signal peptide that is cleaved between residues Ala23 and Gln24. The nucleotide sequence corresponding to the mature RB5312 protein (Gln24–Pro455) was amplified by PCR from *R. baltica* genomic DNA using a set of primers (see Table 1) at a half-hybridization temperature of 345 K. The obtained PCR product was then purified using the Qiagen QIAquick system and digested with *Bgl*II/*Eco*RI (5′/3′ ends) in NEB2 buffer (BioLabs) at 310 K for 3 h. Ligation was performed overnight at 293 K using T4 ligase (Sigma) in a pFO4 vector (derivative of pET15) pre-digested with *Bam*HI/*Eco*RI. This resulted in a gene coding for a recombinant protein (441 residues in total, 48 701 Da) flanked by an N-terminal tag encompassing a methionine followed by six histidine residues and two residues, arginine and serine, corresponding to the *Bgl*II restriction site. The ligation mixture was transformed into *E. coli* DH5α strain. The recombinant plasmid was extracted with the Wizard Plus SV Minipreps kit (Promega) and used to transform *E. coli* BL21 (DE3). The recombinant cells were incubated at 293 K in ZYP-5052 medium (Studier, 2005) with 100 µg ml^{-1} ampicillin until saturation of the culture (final $OD_{600\,nm} \simeq 15$). The cells were harvested by centrifugation (4000*g*, 277 K, 20 min). The cell pellet was resuspended in buffer *A* (50 m*M* NaH_2PO_4 pH 8.0, 500 m*M* NaCl, 50 m*M* imidazole, 5% glycerol). The cells were disrupted using a French press and the lysate was cleared by centrifugation (50 000*g*, 277 K, 30 min). The protein supernatant was applied onto a 10 ml column consisting of IMAC HyperCell resin (Pall Corporation) charged with 100 m*M* $NiSO_4$ and pre-equilibrated with buffer *A*. After a step of washing with buffer *A* (two column volumes), the protein was eluted with a 60 ml linear gradient from buffer *A* to buffer *B* (50 m*M* NaH_2PO_4 pH 8.0, 500 m*M* NaCl, 400 m*M* imidazole, 5% glycerol) at a flow rate of 1 ml min^{-1}. The protein eluted at 160 m*M* imidazole. The protein peak was analyzed by SDS–PAGE (Fig. 1) and the purest fractions were pooled (10 ml final volume). The volume was reduced to 4 ml by ultrafiltration on an Amicon membrane (polyethersulfone, 30 kDa cutoff). The protein sample was injected onto a Superdex 75 HiLoad (GE Healthcare) column pre-equilibated with buffer *C* (50 m*M* HEPES–HCl pH 7.5, 50 m*M* NaCl). Elution was performed with 60 ml buffer *C* at a flow rate of 1 ml min^{-1}. The final protein yield was 2 mg per 500 ml of culture medium. The purified protein was concentrated to 9.7 mg ml^{-1} by ultrafiltration on an Amicon membrane.

2.2. Preliminary pectinolytic activity tests

The pectinolytic activity of RB5312 was tested spectrophotometrically at 235 nm with a UV-2041 PC spectrophotometer (Shimadzu, Japan) by measuring the increase in absorbance caused by the formation of C4–C5 unsaturated sugars. Measurements were taken every 25 s over 10 min at 313 K. The standard assay (1 ml) contained 5 µl purified enzyme at 1 mg ml^{-1}, 995 µl 100 m*M* Tris–HCl pH 8.0 and 1%(*w*/*v*) polygalacturonic acid (PGA) from citrus fruit (Sigma). This activity test was also performed with 995 µl 100 m*M* Tris–HCl pH 8.0, 1%(*w*/*v*) PGA and 5 m*M* EGTA as a control.

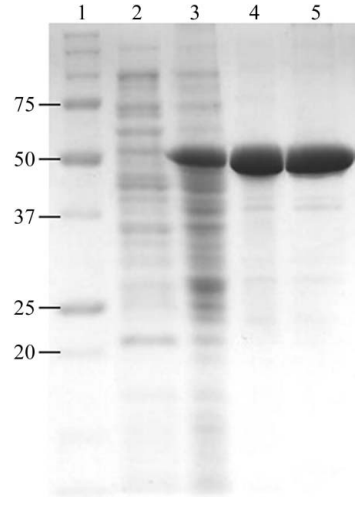

Figure 1
SDS–PAGE gel (12.5%) stained with Coomassie Blue showing the different steps in the heterologous expression and purification of the polysaccharide lyase RB5132. Lane 1, molecular-weight markers (kDa); lane 2, soluble fraction of nontransformed *E. coli* BL21 lysate; lane 3, soluble fraction of *E. coli* lysate with expression; lane 4, fraction with maximum intensity (absorption at 280 nm) after Ni–IMAC column chromatography; lane 5, fraction with maximum intensity after size-exclusion column chromatography.

Table 2
Data-collection statistics of RB5312 crystals.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 39.05, b = 144.05, c = 153.97, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	52.63–1.8 (1.90–1.80)
No. of observations	304224 (14846)
No. of unique reflections	76 671 (7858)
Completeness (%)	93.3 (67.0)
$\langle I/\sigma(I)\rangle$	17.3 (3.9)
Redundancy	4.0 (1.9)
$R_{\rm merge}$† (%)	5.4 (16.7)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

2.3. Protein crystallization

All crystallization experiments were carried out at 292 K. Initial crystallization trials were performed with the Wizard I and II (Emerald BioStructures, Inc.) and JCSG+ Suite (Qiagen) kits; that is, a total of 192 conditions in two 96-well plates from Corning. The trials were set up using a Cartesian crystallization robot and the sitting drops were made up by mixing 300 nl protein solution (9.7 mg ml^{-1} in 50 m*M* HEPES buffer pH 7.5, 50 m*M* NaCl) with 150 nl reservoir solution. Subsequently, the best conditions were optimized in 24-well Linbro plates using the hanging-drop vapour-diffusion method. These drops were prepared on siliconized cover slips by mixing 2 µl protein solution with 1 µl well solution. The drops were equilibrated against 0.5 ml reservoir solution.

2.4. Data collection and X-ray diffraction analysis

X-ray diffraction data were collected from a crystal of recombinant RB5312 at 100 K on beamline ID14-EH2 at the ESRF (Grenoble, France) using an ADSC Quantum 4R CCD detector. All crystals were flash-cooled in a liquid-nitrogen stream. Since the crystallization condition contained 40% 2-methyl-2,4-pentanediol (MPD), it was not necessary to add a cryoprotectant. The wavelength of the synchrotron X-rays was 0.933 Å. The crystal was rotated through 100° with 0.4° oscillation per frame. Further data-collection statistics are given in Table 2. All raw data were processed using the program *MOSFLM* (Leslie, 1992). The resultant data were merged and scaled using the program *SCALA*, which is part of the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). An attempt to solve the structure of RB5312 by molecular replacement was performed using the program *AMoRe* (Navaza, 2001). The model was selected by a sequence search using *BLAST* against the PDB sequence database.

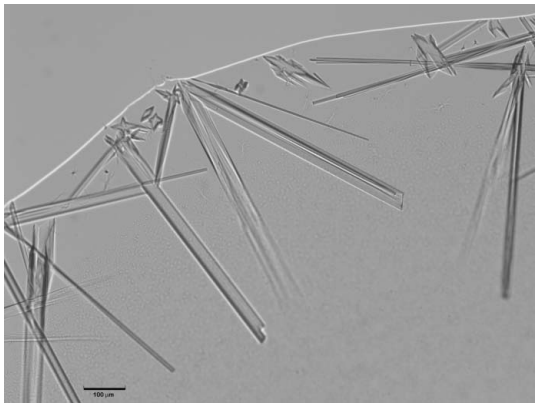

Figure 2
Needle-shaped crystals of polysaccharide lyase RB5132.

3. Results

RB5312 was expressed in *E. coli* without its signal peptide as a soluble protein. This recombinant protein was purified by a combination of IMAC and size-exclusion chromatography in sufficient quantities for crystallization (Fig. 1). A linear increase in $A_{235\,\rm nm}$ was observed when RB5312 was added to a reaction assay containing polygalacturonic acid from citrus fruit. This phenomenon is completely suppressed by the addition of 5 m*M* EGTA. Therefore, RB5312 displays pectinolytic activity and its mechanism is calcium-dependent, suggesting a pectate-lyase specificity. To confirm this substrate specificity, purification of the terminal products of RB5312 is under way. The purified oligosaccharide will be characterized by ^{1}H NMR (Dabin *et al.*, in preparation).

The crystallization screening of RB5312 resulted in the identification of several successful conditions containing MPD and polyethylene glycol (PEG). The optimized conditions for crystal growth are 40% MPD, 4% PEG 8000, 150 m*M* sodium cacodylate pH 6.0 at 292 K. Needle-shaped crystals grew within several days (Fig. 2). Most crystals of the recombinant native protein diffracted to 2.5 Å resolution. However, by screening numerous crystals (∼15) one single crystal (of dimensions 0.3 × 0.05 × 0.05 mm) was found to diffract to 1.8 Å resolution. A complete data set was collected from this crystal and the data-collection quality is reported in Table 2. The space group was determined to be $P2_12_12_1$, with unit-cell parameters a = 39.05, b = 144.05, c = 153.97 Å, $\alpha = \beta = \gamma = 90°$. The asymmetric unit most probably contains two molecules, giving a crystal volume per protein weight ($V_{\rm M}$) of 2.17 Å^3 Da^{-1} and a solvent content of 43% by volume (Matthews, 1968).

The closest sequence match to RB5312 in the PDB is Jun a 1 (PDB code 1pxz), the major cedar pollen allergen from *Juniperus ashei* (Czerwinski *et al.*, 2005). Jun a 1 adopts a β-helix fold and clearly belongs to the PL1 family, but its sequence identity to RB5312 is hardly significant (only 14%). Although the number of insertions and deletions between the two sequences is low, we nevertheless attempted to solve the structure of RB5312 by molecular replacement using the atomic coordinates of 1pxz. However, as expected, these attempts failed. We have therefore decided to produce selenomethionine-labelled protein and the first crystallization trials are under way.

This research was supported by grants to GM from the GIS 'Génomique Marine' and the French Research Ministry (ACI Young Researcher). Additional support was provided by the Region Bretagne (Marine3D program to MC). We are grateful to Drs Rudolph Amann and Frank-Oliver Glöckner for the gift of the *R. baltica* strain and genomic DNA. We thank M. Robert Larocque and Dr Miroslaw Cygler for the gift of the pFO4 vector (BRI, Montreal, Canada). We are indebted to the staff of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) beamline ID14-EH2 for technical support during data collection and processing.

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