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Crystallization and preliminary X-ray characterization of a thermostable pectate lyase from *Thermotoga maritima*

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Pectate lyase is an enzyme involved in the degradation of the pectate portion of the primary plant cell wall. A recombinant pectate lyase from *Thermotoga maritima* where three of the four cysteine residues have been mutated (C132I, C156N, C194L) has been crystallized. Crystals of the same morphology and trigonal space group *R*3 with similar unit-cell parameters were obtained under two different conditions. The first, 0.3 *M* $(NH_4)H_2PO_4$ pH 4.2, gave crystals with a maximum size of 0.4 × 0.2 × 0.2 mm in one week that diffracted to a resolution of 1.87 Å and had unit-cell parameters $a = b = 80.6$, $c = 148.8$ Å. The second, 0.1 *M* sodium acetate, 6%(*w*/*v*) PEG 4000 pH 6.5, gave the same size crystals in two weeks that diffracted to a resolution of 2.1 Å and had unit-cell parameters $a = b = 80.0$, $c = 150.1$ Å.

1. Introduction

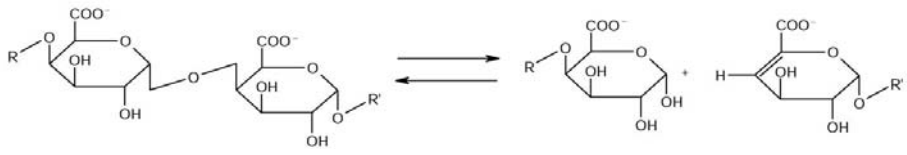
Pectin is a polysaccharide found in the primary cell wall of plants and in the middle lamellae between primary cell walls. Plant pathogens responsible for soft-rot tissue disease, such as *Erwinia* and other plant-invading bacteria, often employ enzymes that degrade pectin as a means of gaining access to vulnerable plant tissue and cell cytoplasm (Herron *et al.*, 2000). The degradation of pectin plays an important role in industrial processes such as the production of fruit juice and wine. The complex structure of pectin requires its degradation to be mediated by several different enzyme types (mainly hydrolases and lyases), each acting on a specific glycosyl or ester bond.

Three major carbohydrate structures are found in pectin; they include homogalacturonan (HGA), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). HGA, often called the smooth region, is a linear polymer of (1→4)-α-linked-D-galacturonic acid residues. Pectate lyase (Pel; EC 4.2.2.2) degrades non-methylated HGA or low-methylated HGA by β-elimination of the α-1,4-glycosidic bond, introducing a double bond in the newly formed non-reducing end of the polysaccharide chain.

Pels are generally characterized by an alkaline pH optimum (pH 8–10), high temperature stability and a requirement for calcium ions. The exact role of the calcium ions (whether they are solely involved in substrate binding, have a role in catalysis or both) has not been completely unravelled. It is of interest to note that lyases degrading pectin do not require calcium ions and display an almost neutral pH optimum (pH 6).

Of the classified carbohydrate-active enzymes (CAZy), the polysaccharide lyases are divided into 12 families based on sequence similarity (Coutinho & Henrissat, 1999). Pels are found in polysaccharide lyase families 1, 2, 3, 9 and 10. There are known crystal structures of Pels in CAZy families 1, 3 and 10. In family 1 they comprise pectate lyase from *Bacillus subtilis* (Pickersgill *et al.*, 1994), PelC and PelE from *Erwinia chrysanthemi* (Lietzke *et al.*, 1996; Yoder & Jurnak, 1995*a*,*b*; Yoder, Lietzke *et al.*, 1993). Also known is the structure of the family 3 Pel from *Bacillus* sp. strain KSM-P15 (Akita *et al.*, 2001).

The enzymes belonging to families 1 and 3 contain an unusual fold of parallel β-strands coiled in a large right-handed helix. This fold was first observed in the structure of Pel C (Yoder, Keen *et al.*, 1993). Since then, several other right-handed parallel β-helix fold-



containing enzymes have been revealed through structural studies: pectin lyases (Mayans *et al.*, 1997; Vitali *et al.*, 1998), several galacturonases (Cho *et al.*, 2001; Petersen *et al.*, 1997; Pickersgill *et al.*, 1998; van Santen *et al.*, 1999), phage p22 coat protein (Steinbacher *et al.*, 1996), virulence factor P.69 pertactin (Emsley *et al.*, 1996) and chondroitinase B (Huang *et al.*, 1999). Parallel right-handed β-helix folds appear to be unique to carbohydrate-binding enzymes and it has been suggested that they are the result of divergent evolution (Jenkins *et al.*, 1998). It should also be noted that examples of left-handed parallel β-helix folds exist, the first of which was UDP *N*-acetylglucosamine acyltransferase (Raetz & Roderick, 1995). Most recently, a unique non-β-helix Pel from family 10 *Pseudomonas fluorescens* ssp. *cellulosa* has been solved (Charnock *et al.*, 2001).

Here, we report the preliminary structural studies of an engineered site-directed triple cysteine mutant CAZy family 1 Pel from *Thermotoga maritima*. The enzyme is composed of 340 amino acids, with a total MW of 38 kDa and a pI of 4.9. Optimal conditions for activity of the mutant enzyme are pH 10, a temperature of 373 K and 1.0 m*M* calcium. This is the first structural study of a Pel from a hyperthermophilic bacterium. Comparison of this hyperthermophilic Pel structure with its mesophilic Pel counterparts should provide insight into structure-stabilizing features and may in addition also shed light on the reaction chemistry and substrate-binding specificity of this enzyme.

2. Methods and results

2.1. Cloning, mutation, overexpression, purification and biochemical characterization

The DNA encoding the Pel of *T. maritima* was PCR amplified using genomic DNA of *T. maritima*, DSM3109 (DNA reference: EMBL accession No. AE001722; Protein SPTREMBL accession No. Q9WYR4). The amplified DNA was cloned into the expression plasmid in such a way that the DNA encoding the natural signal peptide of the *T. maritima* Pel was replaced by DNA encoding a signal peptide from a *Bacillus licheniformis* α-amylase already present in the expression plasmid. Thus, when expressed in a *Bacillus* host, the *T. maritima* Pel is recognized as a protein tagged for secretion and is secreted as a mature protein into the supernatant. The expression plasmid carrying the Pel of *T. maritima* was established in a *B. subtilis* 168 RUB200 derivative. After establishment of the plasmid, three codons of the Pel were genetically engineered from cysteine codons to other amino-acid codons (C132I, C156N, C194L). These mutations were made to increase expression levels. The plasmid was then transferred to and overexpressed in *B. lichenformis*. The expressed enzyme was purified by ion-exchange column chromatography followed by size-exclusion column chromatography and assayed for activity. The activity of the mutant enzyme turned out to be comparable to that of the wild-type enzyme. Additional details can be obtained from the World Intellectual Property Organization database No. WO 01/79440 (Bjørnvad *et al.*, 2001).

2.2. Crystallization

All crystallization trials were performed using the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research) sealed with high-vacuum grease (Dow Corning). The protein stock concentration was 15.5 mg ml^{-1} as determined by OD at 280 nm; it was buffered in 0.1 *M* sodium acetate to pH 6.0. Crystallization conditions at room temperature were initially examined using Crystal Screen I (CS I) (Hampton Research). The 4 µl hanging drops consisted of 2 µl of well solution and 2 µl of protein stock solution. In CS I conditions 3 and 37 crystals appeared in one to two weeks. Optimization of these conditions was conducted by varying the concentration of the major precipitant using stock solutions prepared in-house. The final optimized crystallization conditions from CS I 3 (referred to here as condition 1) were 0.3 *M* $(NH_4)H_2PO_4$ (the actual pH of this condition before addition of protein stock is approximately 4.2 according to Bukrinsky & Poulsen, 2001) and from CS I 37 (referred to here as condition 2) were 0.1 *M* sodium acetate 6%(*w*/*v*) PEG 4000 pH 6.5. Interestingly, both conditions result in crystals of the same morphology and size (Fig. 1).

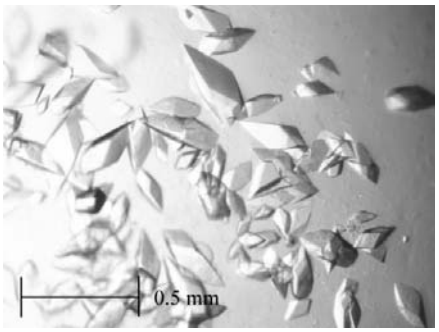


Figure 1
Photographs of hanging drops containing Pel crystals grown in condition 1 (see text) pH 4.2.

2.3. Data collection and characterization

A 30%(*v*/*v*) glycerol cryosolvent solution was prepared by mixing glycerol with the well solution. The following crystal-mounting procedure was used for crystals from both condition 1 and condition 2. The crystal was looped out of the hanging drop and placed into the cryosolvent for several seconds. Subsequently, the crystal was flash-cooled in a nitrogen-gas stream generated by an Oxford Cryosystems cryocooling device. The diffraction experiment for crystals obtained under condition 1 was performed at EMBL Hamburg Outstation beamline X13 operating with X-rays of wavelength 0.913 Å and equipped with a MAR165 CCD detector at a crystal-to-detector distance of 150 mm. Using an oscillation width of 0.5° per frame for a total φ rotation about the goniometer axis of 180°,

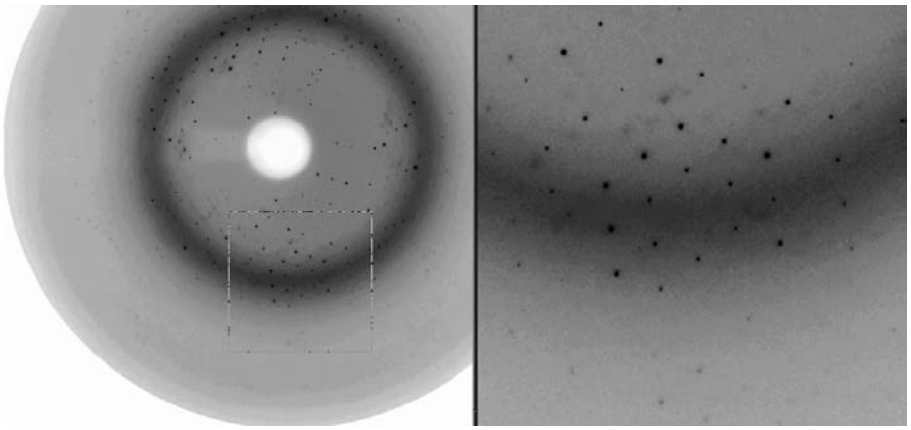


Figure 2
Diffraction pattern of condition 1 crystal.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Crystal	Condition 1	Condition 2
Beamline	EMBL Outstation X13	MAX-lab BL711
Temperature (K)	100	100
Unit-cell parameters (Å)		
$a = b$	80.57	80.03
c	148.83	150.08
Unit-cell volume (Å^3)	966000	961000
Solvent content (%)	57	56
Resolution range (Å)	14.00–1.87 (1.94–1.87)	15.0–2.10 (2.17–2.10)
Wavelength (Å)	0.913	1.023
Total reflections observed	165144	57780
No. of unique reflections	30057	20768
Completeness	100 (99.9)	100 (99.8)
$R_{\rm merge}$†	0.096 (0.589)	0.055 (0.261)
$I/\sigma(I)$	17.8 (2.6)	14.0 (2.1)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity of an individual measurement and $\langle I\rangle$ is the average intensity from multiple observations.

a data set was collected that extended to a resolution of 1.87 Å (Fig. 2). Despite the high resulting $R_{\rm merge}$, we find it justifiable to process the data to this resolution owing to the high redundancy of the data and its acceptable $I/\sigma(I)$ value, supported by χ^2 values close to 1.0 (Table 1).

Data were collected from a single crystal obtained under condition 2 at MAX-lab beamline BL711 with X-rays of wavelength 1.023 Å and using a MAR165 CCD detector. Data to 2.1 Å resolution was obtained using an oscillation of 1.0° per frame and a crystal-to-detector distance of 120 mm for 180° rotation about the goniometer φ axis.

Both data sets were processed using the *HKL* suite version 1.96.1 (Otwinowski & Minor, 1997). Unit-cell parameters and space group were initially assigned using the *DENZO* autoindexing routine. The diffraction pattern only possessed $\bar{3}$ symmetry and absences corresponding to a rhombohedral lattice, which unambiguously assigned the space group as *R*3. Final statistics of the data processing are given in Table 1. The unit-cell parameters correspond to a calculated solvent content of 56% assuming one molecule per asymmetric unit, indicating this to be the likely content.

Following integration and scaling of the data, the mosacity of the condition 2 crystal is higher than that of the condition 1 crystal. This is possibly because of harsher treatment of the condition 2 crystal during the cryotesting procedure. Final statistics for the data can be found in Table 1. Of the available Pel structures, the Pel from *B. subtilis* has the highest sequence identity (35%) to the *T. maritima* enzyme. Use of the *B. subtilis* enzyme as a search model should enable the use of molecular-replacement methods to obtain initial phase information and solve the structure.

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