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# Characterization of a novel pectate lyase, Pel10A, from *Pseudomonas cellulosa*

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Biological recycling of plant material is essential for biosphere maintenance. This perpetual task involves a complex array of enzymes, including extracellular polysaccharide hydrolases and lyases. Whilst much is known about the structure and function of the hydrolases, relatively little is known about the structures and mechanisms of the corresponding lyases. To this end, crystals of the catalytic module of a novel family 10 pectate lyase, Pel10A from *Pseudomonas cellulosa*, were obtained using polyethylene glycol 2000 monomethylether as a precipitant. They belong to space group $P2_1$, with unit-cell parameters $a = 47.7$, $b = 106.1$, $c = 55.4$ Å, $\beta = 92.0^\circ$, and have two molecules in the asymmetric unit. The crystals diffract beyond 1.5 Å using synchrotron radiation.

## 1. Introduction

Polysaccharide lyases (E.C. 4.2.2.*x*) are carbon–oxygen lyases that cleave glycosidic bonds of C5 uronic acid-containing pyranoside substrates. Pectate lyases (E.C. 4.2.2.2), alternatively known as pectate transeliminases, cleave polymeric $\alpha$-1,4-linked galacturonic acid. They play a pivotal role in the degradation of plant cell-wall material and are potent virulence factors of plant pathogenic fungi (Barras *et al.*, 1994). To date, only 12 sequence-based families of polysaccharide lyases have been discovered, in contrast to the 81 families of glycoside hydrolases (Coutinho & Henrissat, 1999). This may reflect their absolute requirement for substrate C5 uronic acid groups. The intricacies of the lyase catalytic mechanism remain elusive, even in the light of biochemical and structural analyses (Scavetta *et al.*, 1999). The enzymes characterized to date have, however, enabled certain general mechanistic features to be identified, such as a dependency on free or methyl-esterified substrate C5 uronic acid groups and divalent cations, typically $Ca^{2+}$. Catalysis results in the elimination of the C4-glycosidic oxygen bond, generating oligosaccharides with 4-deoxy-$\alpha$-D-gluc-4-enuronosyl groups at their non-reducing termini, as illustrated in Fig. 1. The generally accepted mechanism involves proton abstraction from C5 of the +1 subsite sugar residue adjacent to the C6 carboxyl moiety to release the 4,5-unsaturated product *via* a $\beta$-elimination mechanism. It is unclear whether there is negative charge build-up on the glycosidic oxygen in the transition state and hence the requirement for proton donation by an acid catalyst is so far undemonstrated. The lyase and hydrolase mechanisms appear to be of similar efficiency (Brown *et al.*, 2001) and thus it is unclear what evolutionary advantage either of these classes of enzyme offer to their host. Many organisms, such as *Erwinia*, possess both lyases and hydrolases active against the same substrate (Liu *et al.*, 1994). The importance of pectate and pectin degradation in plants is reflected in the dedication of over 146 open reading frames in the *Arabidopsis* genome to this task alone (Henrissat *et al.*, 2001).

Pel10A from *P. cellulosa* is a 68.5 kDa modular enzyme comprising an amino-terminal family 2a carbohydrate-binding module, a central domain of unknown function and a carboxy-terminal 35.8 kDa catalytic module (Brown *et al.*, 2001). Analysis of the primary sequence of the Pel10A catalytic module (hereafter referred to as Pel10Acm) reveals that this enzyme belongs to a recently discovered family of polysaccharide lyases, family 10, which to date comprises just four other members, pectate lyase A from *Azospirillum irakense* (Bekri *et al.*, 1999), pectate

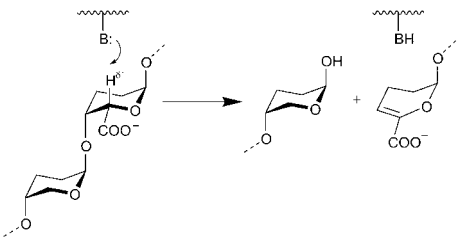


**Figure 1**
Putative mechanism for the elimination of a glycosidic bond by a lyase. This generally accepted $\beta$-elimination mechanism involves proton abstraction from C5 of the +1 subsite sugar residue, leading to the release of a 4,5-unsaturated product.

lyase 15E from alkaliphilic *Bacillus* sp. strain KSM-P15 (Sawada *et al.*, 2000), PelE from *Bacillus alcalophilus* and ORF CC2035 from *Caulobacter crescentus* to which Pel10Acm (residues 325–644), on Fasta3 alignment shares 33.5, 36.6, 33.9 and 20.3% identity, respectively. Enzymes from family 10 exhibit a high pH optimum and are $Ca^{2+}$-dependent. In this paper, we present the production of recombinant Pel10Acm using an *Escherichia coli* expression system, together with its purification, crystallization and preliminary X-ray diffraction analysis.

## 2. Materials and methods

### 2.1. Hyperexpression and purification of recombinant Pel10Acm

Plasmid p4.2.1 (Brown *et al.*, 2001) was transformed into *E. coli* strain BL21(DE3). Bacterial colonies were cultured on 2%(*w*/*v*) LB agar supplemented with 35 µg ml$^{-1}$ kanamycin for 16 h at 310 K. A single colony was used to inoculate a baffled 2 l conical flask containing 1 l LB liquid culture medium supplemented with 35 µg ml$^{-1}$ kanamycin. The bacterial culture was incubated at 310 K with shaking at 160 rev min$^{-1}$ until the optical density at 600 nm reached 0.4, at which point the production of Pel10Acm was induced by the addition of 1 m*M* isopropyl-thio-$\beta$-D-galactoside. High cytoplasmic concentrations of Pel10Acm were tolerated by *E. coli* BL21(DE3) and there were no indications of target proteolysis after 5 h of induction (data not shown) and thus growth was continued until the stationary phase. The bacterial cells were harvested by centrifugation and then sonicated until fully broken in 50 m*M* Na HEPES buffer pH 7.5 at 273 K. Cell debris was removed by centrifugation at 30 000*g* for 45 min and the buffer exchanged for 25 m*M* Na HEPES pH 7.5 containing 1 *M* NaCl and 10 m*M* imadazole using a PD-10 G-25M gel-filtration column (Pharmacia Biotech).

The cell-free extract containing recombinant Pel10Acm was loaded onto a 1.6 × 10 cm chelating Sepharose column (Pharmacia Biotech) which had been charged with five column volumes of 50 m*M* sodium acetate buffer pH 4.5 containing 10 mg ml$^{-1}$ $NiSO_4$ and pre-equilibrated with 10 column volumes of 25 m*M* Na HEPES buffer pH 7.5 containing 1 *M* NaCl and 10 m*M* imidazole. Non-specifically bound proteins were removed and Pel10Acm eventually eluted in a 10–100 m*M* stepped gradient of imidazole. After solvent exchange into 25 m*M* Na HEPES buffer pH 7.5 containing 200 m*M* NaCl, Pel10Acm at 25 mg ml$^{-1}$ was loaded in 1 ml fractions onto a HiLoad 16/60 Superdex 75 gel-filtration column (Pharmacia Biotech). The purified protein was washed with water five times prior to crystallization using a 30 kDa molecular-weight cutoff membrane (Amicon). The Pel10Acm preparation was apparently pure as judged by SDS–PAGE; MALDI-TOF mass spectrometry of a tryptic digest confirmed the identity of the protein and indicated the absence of the N-terminal methionine residue (data not shown).

### 2.2. Activity determination

The activity of purified Pel10Acm against sodium pectate (Sigma) was determined by following the release of the 4-deoxy-$\alpha$-D-gluc-4-enuronosyl-containing products on a CINTRA 10 UV–visible spectrometer (GBC) at 232 nm, using a 1 cm light-path quartz cuvette. The reaction mixture (0.5 ml) comprised 1 mg ml$^{-1}$ sodium pectate in 50 m*M* CAPS buffer pH 10.3, 0.1 m*M* $CaCl_2$ and 20 µg ml$^{-1}$ enzyme. The reaction components were pre-warmed to and the assay was performed at 310 K and wavelength scans were recorded between 205 and 275 nm after 0, 1, 2, 3 and 4 min incubation, as shown in Fig. 2.

To determine the mode of enzyme action, the products released from pectate by Pel10Acm were analysed by anionic exchange HPLC. The reaction was performed essentially as described above; a 10 µl sample was removed after 16 min and immediately incubated at 373 K for 5 min to inactivate the enzyme. A zero-time sample was produced by first heat inactivating the enzyme, followed by the addition of equivalent amounts of buffer and substrate to the 16 min sample. After dilution by the addition of 490 µl $H_2O$, the samples were loaded onto a Carbopac PA-100 anion-exchange column (Dionex) and eluted as described by Brown *et al.* (2001). The Pel10Acm products were analysed by pulsed amperometric detection as illustrated in Fig. 3.

### 2.3. Crystallization, data collection and processing

Crystals of Pel10Acm were grown by vapour-phase diffusion using the hanging-drop method with screening as described by Brzozowski & Walton (2001). The protein concentration was 30 mg ml$^{-1}$ in Na MES buffer pH 5.2 containing KSCN at a concentration of 200 m*M*. The precipitant was 20%(*w*/*v*) monomethyl polyethyleneglycol 2000 and crystals appeared after 2 d at 291 K. A rayon-fibre loop was used to transfer a single Pel10Acm crystal to a

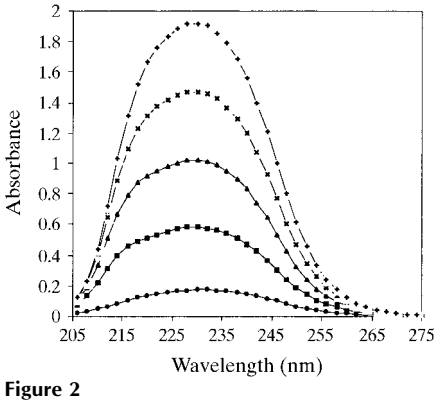


**Figure 2**
Time-course absorption spectra showing the release of 4-deoxy-$\alpha$-D-gluc-4-enuronosyl-containing oligosaccharide products from sodium pectate by Pel10Acm. Wavelength scans were performed using a 1 cm light-path quartz cuvette containing 0.5 ml reaction mixture comprising 50 m*M* CAPS buffer pH 10.3, 0.1 m*M* $CaCl_2$, 1 mg ml$^{-1}$ sodium pectate and 20 µg ml$^{-1}$ Pel10Acm (§2.2). Spectra were recorded after 0 (filled circles), 1 (filled squares), 2 (filled triangles), 3 (×) and 4 (+) minutes of digestion.

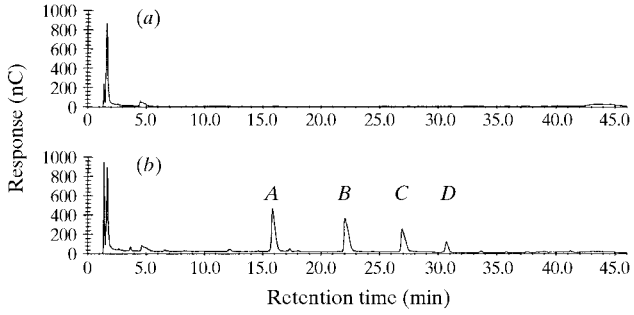


**Figure 3**
Initial release of random oligosaccharides from sodium pectate by Pel10Acm. Pel10Acm (20 µg ml$^{-1}$) was incubated with 1 mg ml$^{-1}$ sodium pectate in 50 m*M* CAPS buffer pH 10.3 and 0.1 m*M* $CaCl_2$ at 310 K (§2.2). At intervals of (*a*) 0 and (*b*) 16 min, aliquots were removed and subjected to HPLC analysis (§2.2). Retention times for the unsaturated digalacturonide (*A*), trigalacturonide (*B*), tetragalacturonide (*C*) and pentagalacturonide (*D*) products are indicated.

cryoprotectant stabilizing solution consisting of the growth buffer supplemented with 25%(*v*/*v*) glycerol. The crystal was then placed into a stream of $N_2$ gas at 120 K. Beamline ID14-4 ($\lambda$ = 0.9366 Å) at the ESRF, Grenoble, equipped with an ADSC-Q4 CCD detector, was used to collect 180° of data, using an oscillation range of 1.0° and an exposure of 1 s per image. Data were processed and reduced using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997).

## 3. Results and discussion

The activity of Pel10Acm against pectate was determined as described in §2.2 using spectrophotometry and anionic exchange HPLC (Dionex), as illustrated in Figs. 2 and 3, respectively. Both the rate of cleavage and the random spectrum of oligosaccharides released were consistent with the findings of Brown *et al.* (2001), confirming that the recombinant Pel10Acm preparation was fully active. Crystals of *P. cellulosa* Pel10Acm belong to space group $P2_1$, with unit-cell parameters $a$ = 47.7, $b$ = 106.1, $c$ = 55.4 Å, $\beta$ = 92.0°. If the asymmetric unit comprises two molecules of Pel10Acm, the resulting $V_M$ and solvent content would be 1.8 Å$^3$ Da$^{-1}$ and 32.7%, respectively, whilst one molecule gives values of 3.7 Å$^3$ Da$^{-1}$ and 66.3% (Matthews, 1968). Data were scaled and reduced using the *HKL* suite of programs (Otwinowski & Minor, 1997). 461 259 observations were merged to give 79 996 unique reflections with an $R_{\rm merge}$ ($\sum_{hkl}\sum_i |I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i I_{hkl}$) of 0.056 (0.131), a mean $I/\sigma(I)$ of 19.4 (10.1), a multiplicity of 3.7 (3.4) and a completeness of 99.7% (100.0%) in the 15–1.55 Å resolution range. Values for the highest resolution shell, 1.58–1.55 Å, are given in parentheses.

In addition to the monoclinic crystal form, Pel10Acm was also cocrystallized with 10 m*M* digalacturonic acid, a product of this enzyme, both in the presence and absence of 25 m*M* $CaCl_2$. Under these conditions, a related but orthorhombic crystal form, space group $P2_12_12$, was obtained, with unit-cell parameters $a$ = 106.3, $b$ = 55.2, $c$ = 47.7 Å. Data were collected at the SRS, Daresbury on station PX9.6 ($\lambda$ = 0.8700 Å). 150 images of 0.7° were collected to a resolution of 1.5 Å. Data were processed as above, resulting in 45 240 unique observations with an $R_{\rm merge}$ of 0.063 (0.311), a multiplicity of 4.2 (4.1) and an $I/\sigma(I)$ of 21.5 (4.5). Values for the highest resolution shell, 1.53–1.50 Å, are given in parentheses. The slightly different packing implied by the occurrence of this orthorhombic crystal form may be the result of conformational changes induced by complex formation.

The structure of Pel10Acm will provide detailed three-dimensional knowledge of this most recently discovered enzyme family. In conjunction with primary sequence analysis, such data will allow the identification of key residues involved in substrate binding and catalysis. This task has so far been unsuccessful owing to the limited number of similar proteins found in this family. Any complex with substrate and cofactor would offer new insights into the mechanism of not only this enzyme but also polysaccharide lyases in general. Toward such an objective, selenomethionine-substituted protein is being prepared for multiple anomalous dispersion analysis.

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