Pectate lyase 10A from *Pseudomonas cellulosa* is a modular enzyme containing a family 2a carbohydrate-binding module

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Pectate lyase 10A (Pel10A) enzyme from *Pseudomonas cellulosa* is composed of 649 residues and has a molecular mass of 68.5 kDa. Sequence analysis revealed that Pel10A contained a signal peptide and two serine-rich linker sequences that separate three modules. Sequence similarity was seen between the 9.2 kDa N-terminal module of Pel10A and family 2a carbohydrate-binding modules (CBMs). This N-terminal module of Pel10A was shown to encode an independently functional module with affinity to crystalline cellulose. A high sequence identity of 66% was seen between the 14.2 kDa central module of Pel10A and the functionally uncharacterized central modules of the xylan-degrading enzymes endoxylanase 10B, arabinofuranosidase 62C and esterase 1D, also from *P. cellulosa*. The 35.8 kDa C-terminal module of Pel10A was shown to have 30 and 36% identities with the family 10 pectate lyases from *Azospirillum irakense* and an alkaliphilic strain of *Bacillus* sp. strain KSM-P15, respectively. This His-tagged C-terminal module of the Pel10A was shown to

encode an independent catalytic module (Pel10Acm). Pel10Acm was shown to cleave pectate and pectin in an endo-fashion and to have optimal activity at pH 10 and in the presence of 2 mM Ca^{2+} . Highest enzyme activity was detected at 62 °C. Pel10Acm was shown to be most active against pectate (i.e. polygalacturonic acid) with progressively less activity against 31, 67 and 89% esterified citrus pectins. These data suggest that Pel10A has a preference for sequences of non-esterified galacturonic acid residues. Significantly, Pel10A and the *P. cellulosa* rhamnogalacturonan lyase 11A, in the accompanying article [McKie, Vincken, Voragen, van den Broek, Stimson and Gilbert (2001) *Biochem. J.* 355, 167–177], are the first CBM-containing pectinases described to date.

Key words: β -elimination, esterified pectin, homogalacturonan, pectinase, rhamnogalacturonan.

INTRODUCTION

Pectic substances contain pectate and pectin, which is the esterified form of pectate. Additionally, both forms are composed of a linear component, homogalacturonan, and a highly branched component, rhamnogalacturonan. In the primary plant cell wall, pectic substances, along with predominantly xylan and mannan, form the matrix in which the highly crystalline microfibrils of cellulose molecules are embedded.

The enzymes that degrade pectic substances, loosely termed pectinases, belong to a larger group of enzymes, defined by P. M. Coutinho and B. Henrissat as carbohydrate-active enzymes (see <http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>). This group of enzymes has been divided into subgroups, three of which, glycoside hydrolases (GHs), polysaccharide lyases (PLs) and carbohydrate esterases (CEs), contain enzymes that degrade pectic substances. Each subgroup is subdivided further into families, the members of which contain structurally related catalytic modules (see <http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>). Furthermore, many carbohydrate-active enzymes are of a modular nature, i.e. they are composed of several modules that can function independently [1]. Thus in addition to a catalytic module, many of these enzymes contain

non-catalytic modules, the most common of which are the carbohydrate-binding modules (CBMs). These CBMs, like the catalytic modules to which they are linked, are grouped into families, the members of which contain structurally related modules [2]. CBMs are thought to potentiate the attachment of the module enzyme, of which they are a part, to specific polysaccharides such as crystalline cellulose, thus facilitating prolonged intimate contact with the plant cell wall [3].

Pectate lyases (examples of which can be found in PL families 1, 2, 3, 9 and 10) catalyse the cleavage of the α -1,4 glycosidic bond, which links adjacent galacturonic acid residues of homogalacturonan. Numerous other enzymes facilitate the degradation of pectic substances. Pectin methyl esterases (CE family 8, CE8), pectin acetyl esterases (CE12) and rhamnogalacturonan acylesterases (CE12) remove the methyl and acetyl groups from pectin. Pectin lyases (PL1), exopolygalacturonate lyases (PL9), polygalacturonases (GH28) and exopolygalacturonases (GH28) degrade homogalacturonan. Rhamnogalacturonan lyases (PL4 and PL11) and rhamnogalacturonases (GH28) degrade rhamnogalacturonan. The branches of rhamnogalacturonan are composed of galactan, arabinan and arabinogalactan and are cleaved by numerous enzymes, including galactanases (GH53) and arabinanases (GH43). PLs and GHs

Abbreviations used: CE, carbohydrate esterase; CBM, carbohydrate-binding module; CDTA, cyclohexane-*trans*-1,2-diaminetetra-acetate; ECP, esterified citrus pectin; GH, glycosyl hydrolase; Pel10A, pectate lyase 10A; Pel10Acm, catalytic module of Pel10A; Pel10Acm2a, family 2a CBM of Pel10A; PGA, polygalacturonic acid; Rgl11A, rhamnogalacturonan lyase 11A; PL, polysaccharide lyase; Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; LB, Luria–Bertani; HPAEC, high-performance anion-exchange chromatography.

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The nucleotide sequence of the *pel10A* gene has been submitted to the GenBank Nucleotide Sequence Database under accession number AF279264.

Figure 1 Molecular architecture of the functionally characterized cellulases, xylanases and pectinases produced by *P. cellulosa*

CBM2, family 2a CBM; CBM10, family 10 CBM; CBM11, family 11 CBM; NodB, deacetylase; FUM1 and FUM2, functionally uncharacterized modules 1 and 2; FN3, fibronectin type 3-like module; Cel9A, endoglucanase 9A; Cel45B, endoglucanase 45B; Cel5C, cellodextrinase 5C; Cel5D, cellodextrinase 5D; Cel5E, endoglucanase 5E; Xyl10A, endoxylanase 10A; Xyl10B, endoxylanase 10B; Xyl62C, arabinofuranosidase 62C; Xyl11D, esterase 1D; Xyl11E, endoxylanase 11E; Xyl10F, endoxylanase 10F; Arb43A, arabinanase 43A; Gal53A, galactanase 53A; Pel10A, pectate lyase 10A; Rgl11A, rhamnogalacturonan lyase 11A.

achieve glycosidic bond cleavage via β -elimination [4], producing oligomers with 4,5-unsaturated residues at the non-reducing end, and general acid catalysis [5,6], producing saturated oligomers, respectively.

Pseudomonas cellulosa is a soil bacterium that produces an extensive repertoire of GHs, e.g. cellulases, xylanases, mannanases, galactanases and arabinanases [7]. Interestingly, all of the cellulases and xylanases from *P. cellulosa* that have been functionally characterized are modular. In contrast, the characterized arabinanase and galactanase enzymes from *P. cellulosa* are composed of a single module (Figure 1).

To ascertain whether all pectinases produced by *P. cellulosa* are composed of single modules and to further characterize the plant-cell-wall-degrading activity of *P. cellulosa*, we screened a *P. cellulosa* library for rhamnogalacturonan-degrading activity. Surprisingly we identified a homogalacturonan-degrading enzyme, pectate lyase 10A (Pel10A).

In this report we show that Pel10A is a modular enzyme containing a functional N-terminal family 2a CBM, a central functionally uncharacterized module, and a functional C-terminal PL family 10 catalytic module. These modules are separated by serine-rich linker sequences. It is worth noting that the attempted expression of entire modular enzymes often results in the production of several protein products. This is due to the sensitivity of the linker sequences of modular enzymes to proteolytic cleavage. Consequently, analysis of modular enzymes usually requires expression of independent modules. Rhamnogalacturonan lyase 11A (Rgl11A), which is described in the

accompanying article [8], is also a modular pectinase from *P. cellulosa*. Rgl11A contains three modules: an N-terminal catalytic module with no similarity to the catalytic module of Pel10A; a central module that exhibited sequence identity to fibronectin-3 modules but not with the central functionally uncharacterized module of Pel10A; and a C-terminal family 2a CBM with 56% identity to the family 2a CBM from Pel10A (Pel10Acbm2a). Pel10A and Rgl11A are the first PL enzymes characterized from *P. cellulosa*, and more importantly, they are the first reported CBM-containing pectinases from any organism.

MATERIALS AND METHODS

Materials were purchased from Sigma-Aldrich (Poole, Dorset, U.K.), unless stated otherwise. All methods followed the manufacturer's instructions, where given, or were performed as described by Sambrook et al. [9].

Polymeric substrates

The amount of Ca^{2+} associated with pectic substances varies from preparation to preparation. Thus Ca^{2+} ions were removed from 50 mg·ml⁻¹ solutions of polygalacturonic acid (PGA, sodium salt; Sigma P3850), 31% esterified citrus pectin (ECP; Sigma P9311), 67% ECP (Sigma P9436) and 89% ECP (Sigma P9561), by stirring at room temperature for 5 h with 50 mM cyclohexane-*trans*-1,2-diaminetetra-acetate (CDTA). Precipitates were removed by centrifugation for 15 min at

13000 *g*. Supernatants were dialysed against 18.2 MΩ water. After dialysis the samples were lyophilized and resuspended in 5 mM Tris/HCl buffer, pH 7.0, immediately prior to use.

Primers used

Primers were produced at the Facility for Molecular Biology, University of Newcastle, Newcastle, U.K. P1, 5'-ATG CTT CCG GCT CGT ATG-3'; P3, 5'-TGT CTC TTC AGT CAG GTC CAG-3'; P4, 5'-GCG GGC AGG CTC AGG GTG TAA T-3'; P5, 5'-ATG CCT TAA CGG TAT TGG ATT TTG-3'; P6, 5'-AGC CAC TCA GGG TCG CAT TCC-3'; P7, 5'-AAT CGA GGT TCC ATA GTT AG-3'; P8, 5'-TTG GCT AAT GAA TGT CTG AT-3'; P9, 5'-TGC GAT CAC TGT CAG AGA ATA C-3'; P10, 5'-CCT GGA ATG CGA CCC TGA GTG-3'; P11, 5'-GCC ATC CAG GGT TAG CAT CC-3'; P12, 5'-ACC GCG GTT TCT TCA GT-3'; P13, 5'-CGA GCA GGT TGG CAA GGT AGT G-3'; R1PCR1, 5'-CAT ATG ACA GGC CGG ATG CTA ACC-3'; R1PCR2, 5'-AAG CTT TTA CAG GTA ACC CAC TTT C-3'; R1PCR3, 5'-CAT ATG GCC TGC AGT TAC AAG GTC ACG-3'; and R1PCR4, 5'-GGA TCC TTA GCT GGA GGA AGA ACT CCT-3'.

Media and bacterial strains used

Luria–Bertani (LB) broth, 10 g·l⁻¹ tryptone (Oxoid, Basingstoke, Hants., U.K.)/5 g·l⁻¹ yeast extract (Oxoid)/10 g·l⁻¹ NaCl, pH 7.0; NZY top agar, 5 g·l⁻¹ NaCl/2 g·l⁻¹ MgSO₄·7H₂O/5 g·l⁻¹ yeast extract/10 g·l⁻¹ casein hydrolysate (Oxoid), pH 7.5/0.7% (w/v) agarose. Agar bacteriological (Agar No. 1, Oxoid) was added to 2% (w/v) if required. Ampicillin and kanamycin were used at final concentrations of 100 and 50 µg·ml⁻¹, respectively, if required.

The bacterial strains used were *P. cellulosa* (NCIMB 10462), formerly referred to as *Pseudomonas fluorescens* subsp. *cellulosa*, and *Escherichia coli* XL1-Blue MRF', XL0LR and BL21(DE3) (Stratagene Europe, Amsterdam, The Netherlands).

Expression cloning and sequencing of *pel10A*

Genomic DNA was isolated from a culture of *P. cellulosa*, grown at 25 °C in LB medium containing rifampicin, using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, U.S.A.). This DNA was partially digested with *Sau*3A and the fragments, within the size range 3–10 kb, were gel-purified. These were cloned into *Bam*HI-digested λZAP II vector and packaged using a Gigapack III Gold packaging extract (Stratagene Europe). The genomic library was screened at a density of 500 clones/90 mm Petri dish. AZ-Rhamnogalacturonan (Megazyme, Bray, Ireland) was added to the NZY top agar at a concentration of 1% (w/v). Clear haloes around clones expressing activity against AZ-rhamnogalacturonan were seen against the blue background. One such clone, λR1, was purified, then excised into phagemid using ExAssist helper phage (Stratagene Europe). The phagemid, pR1, was transfected into *E. coli* XL0LR, then propagated on LB agar containing ampicillin and 0.5% (w/v) AZ-rhamnogalacturonan, so as to verify the fidelity of the *in vivo* excision procedure. Phagemid subclone pR1, excised from phage clone λR1, was shown to be active against AZ-rhamnogalacturonan. A *Kpn*I–*Bam*HI fragment from pR1 was subcloned into the low-copy-number plasmid pMTL6000 (a personal gift from N. P. Minton, Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K.) to produce pMM1. This clone was also active against AZ-rhamnogalacturonan.

NucleoSpin plasmid purification kits (Macherey-Nagel GmbH, Düren, Germany) were used to prepare template DNA

of both pMM1 and pR1 for DNA sequencing. A Perkin–Elmer Applied Biosystems 877 Catalyst Workstation (Robot) utilizing Big Dye Terminator chemistry was used to perform the cycle sequencing of the templates with primers P1 and P3–P13. A Perkin–Elmer Applied Biosystems 377 DNA Sequencer was used to determine the sequence of the above products. The gene encoding Pel10A from *P. cellulosa*, *pel10A*, was sequenced in both strands and the sequence analysed using Lasergene software (DNASTAR, Madison, WI, U.S.A.), BLAST, the sequence-similarity search tool of the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast>), Fasta3 and ClustalW, the sequence database searches and interactive analysis tools of the European Bioinformatics Institute (<http://www.ebi.ac.uk>) and SignalP V2.0, the prediction server for signal peptide and cleavage sites in Gram positive, Gram negative and eukaryotic amino acid sequences of the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/SignalP-2.0/>).

pel10A hybridization studies

The segment of *pel10A* encoding the catalytic module of Pel10A (Pel10Acm) was used as the probe for all hybridization techniques. The probe was labelled using the digoxigenin (DIG) DNA Labelling kit (Roche Diagnostics, Lewes, East Sussex, U.K.) and detection achieved using the DIG Luminescent Detection kit (Roche Diagnostics). Southern blotting on to positively charged nylon filters (Roche Diagnostics) were performed as described in *The DIG systems user's guide for filter hybridization* (Roche Diagnostics). *Eco*R1, *Sal*I, *Bam*HI, *Xho*I and *Hind*III digests of *P. cellulosa* genomic DNA were Southern blotted.

Hyper-expression and purification of Pel10Acm and Pel10Acbm2a

The following PCR protocol was used to amplify the segment of *pel10A* encoding Pel10Acm and Pel10Acbm2a: one cycle of 3 min at 94 °C; 30 cycles of 1 min at 94 °C followed by 1 min at 55 °C, 2 min at 72 °C and one cycle of 10 min at 72 °C. The final concentrations or amounts of the components of the PCR reactions were: 100 ng of pR1, 0.5 µM for each primer (R1PCR1 and R1PCR2 for Pel10Acm; R1PCR3 and R1PCR4 for Pel10Acbm2a), 2 mM MgCl₂, 0.2 mM for each dNTP and 1.25 units of *Pfu* Turbo polymerase (Stratagene Europe). The amplified products were cloned into pGEM-T Easy vector (Promega, Southampton, Hants., U.K.), then subcloned into pET28a vector (Novagen, Madison, WI, U.S.A.) on an *Nde*I–*Xho*I fragment in the case of Pel10Acm, and an *Nde*I–*Bam*HI fragment in the case of Pel10Acbm2a, to produce plasmids p4.2.1 and pCBMLA, respectively.

Production of Pel10Acm and Pel10Acbm2a was performed by induction of *E. coli* BL21(DE3) cultures carrying p4.2.1 and pCBMLA, respectively, grown to a *D*₆₀₀ of 0.6–1.0 in LB medium containing kanamycin, with isopropyl β-D-thiogalactopyranoside (Biogene, Kimbolton, Cambs., U.K.) at a final concentration of 1 mM. Cells carrying p4.2.1 were grown at 25 °C and 100 rev./min and harvested 16 h post-induction, sonicated on ice, spun at 13000 *g* at 4 °C for 15 min and Pel10Acm purified from the other components in the supernatant using Talon metal affinity chromatography according to the manufacturer's instructions (Clonetechn, Basingstoke, Hants, U.K.). Imidazole (50 mM) was removed using a PD-10 desalting column (Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.). EDTA was added to 50 mM to remove Ca²⁺ ions from Pel10Acm (10 min incubation on ice) and the sample dialysed against 20 mM Tris/HCl buffer, pH 7.4, at 4 °C. Cells carrying

pCBMLA were grown at room temperature and 50 rev./min and harvested 16 h post-induction, sonicated on ice, spun at 13000 g at 4 °C for 15 min, the pellet solubilized in 8 M urea and Pel10Acbm2a purified from the other components in the solubilized pellet using denaturing TALON metal affinity chromatography according to the manufacturer's instructions (Clontech). Purified Pel10Acbm2a was refolded via dialysis against decreasing concentrations of urea. Pel10Acm and Pel10Acbm2a were inspected for purity via SDS/PAGE [10].

Pel10Acm assays

Pel10Acm reactions were determined spectrophotometrically at 232 nm. Unless otherwise specified, the standard enzyme reactions were performed at 37 °C and consisted of 50 mM 3-(cyclohexylamino)propane-1-sulphonic acid (Caps) buffer, pH 10, 0.1 mM CaCl_2 and 1 mg·ml⁻¹ PGA. Protein concentrations were determined as described by Bradford [11]. Activity was expressed as both enzyme units per mg of protein and in terms of the absolute kinetic parameters, k_{cat} and K_m . The absorption coefficient of unsaturated oligogalacturonides used was 5200 M⁻¹·cm⁻¹ [12]. The effect of Ca^{2+} on Pel10Acm activity was determined using the standard reaction conditions, except that 0.25 mg·ml⁻¹ trigalacturonic acid and a range of CaCl_2 concentrations (0.1, 0.2, 0.3, 0.4, 1.0, 2.0 and 4.0 mM) were used. The effects of other divalent cations on Pel10Acm activity were determined using the standard reaction conditions, except that 0.25 mg·ml⁻¹ trigalacturonic acid was used and CaCl_2 was replaced with 1 mM BaCl_2 , CoCl_2 , CuCl_2 , MgCl_2 , MnCl_2 , NiCl_2 , SrCl_2 or ZnCl_2 .

The optimum pH for Pel10Acm activity against PGA, 31 % ECP, 67 % ECP and 89 % ECP was determined using the standard reaction conditions except that both 0.1 and 1.0 mM CaCl_2 were used and 50 mM Caps buffer, pH 9.5, 10.0, 10.5 and 11.0, and 50 mM Tris/HCl buffer, pH 7.5, 8.0, 8.5, 9.0 and 9.5 were used. Buffer effects were compensated for by determining activity at pH 9.5 for both buffers. The effect of temperature on Pel10Acm activity was determined using the standard reaction conditions except that assays were performed at 27, 37, 47, 57, 62, 67 and 77 °C. The thermostability of Pel10Acm was determined by pre-incubating the catalytic module at 27, 37, 47, 52 or 57 °C for 20 min, and assaying these samples using the standard reaction conditions. K_m , V_{max} and k_{cat} values were determined using the standard reaction conditions calculated except that substrate concentrations of 0.2, 0.125, 0.1, 0.0625 and 0.05 mg·ml⁻¹ PGA, 0.3, 0.25, 0.125, 0.0833 and 0.0625 mg·ml⁻¹ 31 % ECP, 1.25, 1, 0.75, 0.625 and 0.5 mg·ml⁻¹ 67 % ECP and 1, 0.5, 0.45, 0.4 and 0.25 mg·ml⁻¹ 89 % ECP were used.

Pel10Acm reactions for high-performance anion-exchange chromatography (HPAEC) were as the standard reaction conditions except that 4 mg·ml⁻¹ PGA, 31 % ECP, 67 % ECP or 89 % ECP were used. The Pel10Acm reactions for HPAEC were separated on a DX 500 ion-chromatography system equipped with CarboPac PA-100 guard (4 mm × 50 mm) and analytical (4 mm × 250 mm) columns (Dionex, Camberley, Surrey, U.K.). The elution conditions were as described by Fraaije et al. [13] and Bekri et al. [14]. Saturated galacturonic acid, digalacturonic acid and trigalacturonic acid standards were used and their retention times were identical with those in [13,14]. Thus the unsaturated oligogalacturonide products released by Pel10Acm were confidently identified by comparison with the retention times reported by Fraaije et al. [13] and Bekri et al. [14]. Detection was facilitated using integrated amperometry with a standard triple potential waveform ($E_1 = 0.05$ V, $t_1 = 0.00$ s; $E_2 = 0.05$ V, $t_2 = 0.2$ s, begin integration; $E_3 = 0.05$ V, $t_3 = 0.4$ s, end integration;

$E_4 = 0.75$ V, $t_4 = 0.41$ s; $E_5 = 0.75$ V, $t_5 = 0.6$ s; $E_6 = -0.15$ V, $t_6 = 0.61$ s; $E_7 = -0.15$ V, $t_7 = 1.0$ s) and a gold working electrode with a Ag/AgCl reference electrode. Separation and detection were performed at 30 °C. System control, data acquisition and data processing were automated through PeakNet 5.1 software (Dionex).

Pel10Acbm2a binding studies

Refolded Pel10Acbm2a (111 µg; or 111 µg of Pel10Acbm2a denatured with 2 % SDS or 6 M guanidine hydrochloride as a control) was incubated on ice with 3 ml of 5 % (w/v) crystalline cellulose (Avicel PH105; Serva Feinbiochemica GmbH, Heidelberg, Germany) in 50 mM Tris/HCl buffer, pH 7.5, for 1 h at 50 rev./min. The mixture was then vacuum-filtered through a porosity grade 3 sintered glass filter (Pyrex). The Avicel deposited on the filter was resuspended in 5 ml of 50 mM Tris/HCl buffer, pH 7.5, and vacuum-filtered five times. After the last wash the Avicel was removed from the filter and bound enzyme eluted from the Avicel by boiling for 5 min in 10 % SDS. The eluant was analysed using SDS/PAGE.

RESULTS

AZ-Rhamnogalacturonan-active enzymes from *P. cellulosa*

AZ-Rhamnogalacturonan-active clones were identified from the *P. cellulosa* genomic library at a frequency of 1/250. One such clone, λR1, was subsequently shown to carry the gene (*pel10A*) encoding Pel10A. This clone was excised into phagemid to produce pR1, which was also active against AZ-rhamnogalacturonan. Southern-blot analysis of *P. cellulosa* genomic DNA probed with a DNA fragment from pR1, which encodes the catalytic module of Pel10A (Pel10Acm), revealed that only one copy of *pel10A* was present in the *P. cellulosa* genome (results not shown).

Nucleotide sequence of *pel10A* and the predicted amino acid sequence of its encoded product Pel10A

Analysis of the nucleotide sequence of *pel10A* (Figure 2) revealed a single open reading frame of 1950 bp encoding a polypeptide of 649 residues with a molecular mass of 68.5 kDa. The open reading frame contained 56 % G+C nucleotides and a codon utilization similar to other enzymes active against plant cell-wall polysaccharides produced by *P. cellulosa* [15]. The proposed translational start codon of *pel10A* was preceded (6 bp) by the sequence 5'-AAGGA-3', which is similar to the ribosome-binding sequence of Gram-negative bacteria [16]. Inspection of the predicted amino acid sequence of Pel10A revealed a typical Gram-negative signal peptide of 31 residues in length and three modules separated by two serine-rich linker sequences (Figure 2). The 9.2 kDa N-terminal module of Pel10A has 56 % identity with the family 2a CBM present in *P. cellulosa* Rgl11A, described in the accompanying paper [8], as well as sharing extensive homology with family 2a CBMs present in several *P. cellulosa* cellulases and xylanases (Figure 3 and Table 1). The 14.2 kDa central module of Pel10A has extensive similarity with the functionally uncharacterized modules of the xylan-degrading enzymes, endoxylanase 10B [17], arabinofuranosidase 62C [17] and esterase 1D [18], from *P. cellulosa* (Figure 4), but has no similarity to any of the Rgl11A modules. The 35.8 kDa C-terminal module of Pel10A shows similarity to pectate lyase A from *Azospirillum irakense* [14] and pectate lyase 15E from the alkaliphilic *Bacillus* sp. strain KSM-P15 [19] (Figure 5). Both the *A. irakense* and the *Bacillus* enzymes are members of family

C	CGG	CAG	CAC	CGG	ATA	ACT	GAT	TGG	GGG	TGT	GTA	ATG	CGA	GTC	GGC	CTG	GTT	CGC	CTG	CGC	ATC	TGA	CAT	TAA	CAA	TAA	GAA	TGT	-15	
ACT	GAA	GGA	AAA	CCA	ATG	CGA	ACA	CTT	CAT	TTT	TTA	CCT	GTA	TCC	CGT	TTT	TTC	ATG	ACA	TCG	CTC	CTG	GCA	GTA	ACC	GCC	ATC	AGC	72	
					Met	Arg	Thr	Leu	His	Phe	Leu	Pro	Val	Ser	Arg	Phe	Phe	Met	Thr	Ser	Leu	Leu	Ala	Val	Thr	Ala	Ile	Ser	24	
CAG	GTG	GCG	CCT	GCC	TGG	GCC	GCC	TGC	AGT	TAC	AAG	GTC	ACG	AAT	AAT	TGG	GGC	TCG	GGG	TTT	ACC	GCT	GAA	ATT	AAC	GTC	ACA	AAC	159	
Gln	Val	Ala	Pro	Ala	Trp	Ala	Ala	Cys	Ser	Tyr	Lys	Val	Thr	Asn	Asn	Trp	Gly	Ser	Gly	Phe	Thr	Ala	Glu	Ile	Asn	Val	Thr	Asn	53	
GAT	ACC	GGC	CAG	GCC	GTG	AGC	AAC	TGG	TCG	GTG	TCC	TGG	CAG	GAA	AGT	GGT	GCA	ACC	GTG	ACC	AAT	GCC	TGG	AAT	GCG	ACC	CTG	AGT	246	
Asp	Thr	Gly	Gln	Ala	Val	Ser	Asn	Trp	Ser	Val	Ser	Trp	Gln	Glu	Ser	Gly	Ala	Thr	Val	Thr	Asn	Ala	Trp	Asn	Ala	Thr	Leu	Ser	82	
GGC	TCC	AAT	CCT	TAC	ACT	GCT	GCT	TCC	CTG	GGG	TGG	AAT	GGC	ACC	CTG	GCG	CCG	GGC	GCC	TCC	GCC	AGC	TTT	GGC	Phe	CAG	GCC	AAT	333	
Gly	Ser	Asn	Pro	Tyr	Thr	Ala	Ala	Ser	Leu	Gly	Trp	Asn	Gly	Thr	Leu	Ala	Pro	Gly	Gly	Ser	Ala	Ser	Phe	Gly	Thr	Gln	Ala	Asn	111	
GGC	AGC	GCA	GGT	GCT	CCC	GGG	GTG	AAC	GGC	ACC	CTG	TGC	GGA	ACC	GCC	ACC	TCA	TCG	GTG	AGC	AGT	GTG	TCC	AGT	ACA	CCG	GTA	AGC	420	
Gly	Ser	Ala	Gly	Ala	Gly	Gly	Val	Asn	Gly	Thr	Leu	Cys	Gly	Thr	Ala	Thr	Ser	Ser	Val	Ser	Ser	Val	Ser	Ser	Thr	Pro	Val	Ser	140	
AGT	TCG	TCC	AGT	GTC	AGG	AGT	TCT	TCC	TCC	AGC	AGT	TCG	GTA	CAG	AGC	TCC	CAG	GCG	CTC	TCA	ACC	TTG	ACC	CTC	CAG	GAA	GTG	CAG	507	
Ser	Ser	Ser	Ser	Val	Arg	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Val	Gln	Ser	Ser	Gln	Ala	Leu	Ser	Thr	Leu	Thr	Leu	Gln	Glu	Val	Gln	169
GCC	GGC	TTT	TGC	CGC	GTG	GAT	GGT	ATT	GCC	AAT	GAA	TCC	ACC	AAC	GGC	GGA	TTT	ACC	GGT	ACC	GGT	TAC	GCC	AAT	ACC	GAT	AAC	GCG	594	
Ala	Gly	Phe	Cys	Arg	Val	Asp	Gly	Ile	Ala	Asn	Glu	Ser	Thr	Asn	Gly	Gly	Phe	Thr	Gly	Thr	Gly	Tyr	Ala	Asn	Thr	Asp	Asn	Ala	198	
CTG	GGT	GCT	GCC	ATT	GTG	TGG	GCA	GTT	GCG	GCC	AGT	ACC	AGC	AGC	CGC	TAC	AAG	CTC	ACA	GTG	CGC	TTT	GCC	AAT	GGT	GGT	TCA	GCC	681	
Leu	Gly	Ala	Ala	Ile	Val	Trp	Ala	Val	Ala	Ala	Ser	Thr	Ser	Ser	Arg	Tyr	Lys	Leu	Thr	Val	Arg	Phe	Ala	Asn	Gly	Gly	Ser	Ala	227	
AGT	CGC	GAT	GGT	TCA	TTG	CTG	ATT	AAC	GGT	GGC	AGC	AAC	GGT	AAT	TAC	ACC	CTG	AGC	CTG	CCC	GCT	ACC	GGC	GCC	TGG	ACG	ACC	TGG	768	
Ser	Arg	Asp	Gly	Ser	Leu	Ile	Asn	Gly	Ser	Gly	Ser	Asn	Gly	Asn	Tyr	Thr	Leu	Ser	Leu	Pro	Ala	Thr	Gly	Gly	Ala	Trp	Thr	Trp	256	
CAA	ACG	GTG	ACG	GTT	GAG	GTA	GAC	CTG	GTA	CAG	GGC	AAC	AAC	CTG	GTG	CAA	TTG	ACC	TCG	CGC	ACT	GCC	GAT	GGC	CTG	GCC	AAT	ATT	855	
Gln	Thr	Val	Thr	Val	Glu	Val	Asp	Leu	Val	Gln	Gly	Asn	Asn	Leu	Val	Gln	Leu	Thr	Ser	Arg	Thr	Ala	Asp	Gly	Leu	Ala	Asn	Ile	285	
GAC	TAC	CTG	CGA	GTA	GAG	GGG	GCG	TCA	ACC	ACC	GCC	GGT	TCC	TGC	AGC	GGC	AAT	ACC	GGT	AGC	AGT	GTC	TCT	TCA	GTC	AGG	TCC	AGC	942	
Asp	Tyr	Leu	Arg	Val	Glu	Gly	Ala	Ser	Thr	Thr	Ala	Gly	Ser	Cys	Ser	Gly	Ser	Thr	Gly	Ser	Ser	Val	Ser	Ser	Val	Arg	Ser	Ser	314	
TCC	AGT	GTG	GCC	AGC	AGC	TCG	TCC	TCC	AGC	CAG	GCG	ACA	GGC	CGG	ATG	CTA	ACC	CTG	GAT	GGC	AAC	CCG	GCA	GCC	AAT	TGG	TTA	AAC	1029	
Ser	Ser	Val	Ala	Ser	Ser	Ser	Ser	Ser	Ser	Gln	Ala	Thr	Gly	Arg	Met	Leu	Thr	Leu	Asp	Gly	Asn	Pro	Ala	Ala	Asn	Trp	Leu	Asn	343	
AAT	GCG	CGC	ACA	AAA	TGG	TCT	GCC	AGC	CGT	GCG	GAT	GTG	GTG	TTG	TCT	TAC	CAG	CAA	AAT	AAC	GGC	GGC	TGG	CCG	AAG	AAC	CTG	GAT	1116	
Asn	Ala	Arg	Thr	Lys	Trp	Ser	Ala	Ser	Arg	Ala	Val	Val	Leu	Ser	Tyr	Gln	Gln	Asn	Asn	Gly	Gly	Trp	Pro	Lys	Asn	Leu	Asp	372		
TAC	AAC	AGT	GTC	GGC	AAT	GGC	GGT	GGC	GGC	AAT	GAG	AGC	GGC	ACT	ATC	GAC	AAT	GGG	GCA	ACC	ATT	ACT	GAA	ATG	GTA	TTC	CTC	GCC	1203	
Tyr	Asn	Ser	Val	Gly	Asn	Gly	Gly	Gly	Gly	Asn	Glu	Ser	Gly	Thr	Ile	Asp	Asn	Gly	Ala	Thr	Ile	Thr	Glu	Met	Val	Phe	Leu	Ala	401	
GAG	GTT	TAC	AAA	AGC	GGA	GGC	AAT	ACC	AAA	TAC	CGC	GAT	GCC	GTG	CGC	AAG	GCG	GCT	AAC	TTT	TTG	GTG	AAC	TCA	CAA	TAC	AGC	ACC	1290	
Glu	Val	Tyr	Lys	Ser	Gly	Gly	Asn	Thr	Lys	Tyr	Arg	Asp	Ala	Val	Arg	Lys	Ala	Ala	Asn	Phe	Leu	Val	Asn	Ser	Gln	Tyr	Ser	Thr	430	
GGT	GCC	TTG	CCG	CAG	TTT	TAT	CCG	TTG	AAA	GGT	GGC	TAT	TCT	GAT	CAT	GCC	ACC	TTT	AAC	GAT	AAC	GGT	ATG	GCC	TAT	GCC	TTA	ACG	1377	
Gly	Ala	Leu	Pro	Gln	Phe	Tyr	Pro	Leu	Lys	Gly	Gly	Tyr	Ser	Asp	His	Ala	Thr	Phe	Asn	Asp	Gly	Met	Ala	Tyr	Ala	Leu	Thr	459		
GTA	TTG	GAT	TTT	GCG	GCC	AAC	AAG	CGC	GCG	CCT	TTT	GAT	ACC	GAT	GTA	TTC	TCT	GAC	AGT	GAT	CGC	ACG	CGT	TTC	AAA	ACA	GCG	GTG	1464	
Val	Leu	Asp	Phe	Ala	Ala	Asn	Lys	Arg	Ala	Pro	Phe	Asp	Thr	Asp	Val	Phe	Ser	Asp	Ser	Asp	Arg	Thr	Arg	Phe	Lys	Thr	Ala	Val	488	
ACC	AAA	GGC	ACT	GAC	TAT	ATC	CTC	AAG	GCC	CAG	TGG	AAG	CAG	AAT	GGT	GTA	TTA	ACC	GTT	TGG	TGC	GCA	CAG	CAC	GGT	GCC	CTG	GAT	1551	
Thr	Lys	Gly	Thr	Asp	Tyr	Ile	Leu	Lys	Ala	Gln	Trp	Lys	Gln	Asn	Gly	Val	Leu	Thr	Val	Trp	Cys	Ala	Gln	His	Gly	Ala	Leu	Asp	517	
TAC	CAA	CCG	AAA	AAA	GCT	CGC	GCC	TAT	GAA	CTT	GAG	TCA	TTG	AGT	GGC	AGT	GAA	TCT	GTA	GGC	GTC	CTG	GCA	TTC	CTG	ATG	ACG	CAA	1638	
Tyr	Gln	Pro	Lys	Lys	Ala	Arg	Ala	Tyr	Glu	Leu	Glu	Ser	Leu	Ser	Gly	Ser	Glu	Ser	Val	Gly	Val	Leu	Ala	Phe	Leu	Met	Thr	Gln	546	
CCG	CAA	ACG	GCG	GAG	ATC	GAA	CGC	GCG	GTA	AGA	GCG	GGG	GTT	GCC	TGG	TTC	AAT	AGT	CCG	AGA	ACT	TAC	CTT	GAG	GGG	TAT	ACC	TAC	1725	
Pro	Gln	Thr	Ala	Glu	Ile	Glu	Arg	Ala	Val	Arg	Ala	Gly	Val	Ala	Trp	Phe	Asn	Ser	Pro	Arg	Thr	Tyr	Leu	Glu	Gly	Tyr	Thr	Tyr	575	
GAT	TCA	TCC	CTC	GCG	GCT	ACC	AAT	CCC	ATC	GTA	CCC	AGG	GCA	GGC	AGT	AAA	ATG	TGG	TAT	CGT	TTT	TAC	GAT	TTG	AAC	ACT	AAC	CGC	1812	
Asp	Ser	Ser	Leu	Ala	Ala	Thr	Asn	Pro	Ile	Val	Pro	Arg	Ala	Gly	Ser	Lys	Met	Trp	Tyr	Arg	Phe	Tyr	Asp	Leu	Asn	Thr	Asn	Arg	604	
GGT	TTC	TTC	AGT	GAT	CGT	GAC	GGC	AGC	AAG	TTC	TAT	GAC	ATT	ACC	CAA	ATG	TCC	CTG	GAG	CGT	CGC	ACC	GGC	TAT	AGC	TGG	GGT	GGT	1899	
Gly	Phe	Phe	Ser	Asp	Arg	Asp	Gly	Ser	Lys	Phe	Tyr	Asp	Ile	Thr	Gln	Met	Ser	Leu	Glu	Arg	Arg	Thr	Gly	Tyr	Ser	Trp	Gly	Gly	633	
AAC	TAT	GGA	ACC	TCG	ATT	ATC	AAC	TTC	GCC	CAG	AAA	GTG	GGT	TAC	CTG	TAA	CCT	GGT	GTA	CTT	CCC	TCC	GCA	CTA	ATC	AAA	AGG	CCT	1986	
Asn	Tyr	Gly	Thr	Ser	Ile	Ile	Asn	Phe	Ala	Gln	Lys	Val	Gly	Tyr	Leu	End†	1950											649		
GCA	ATT	GCA	GGC	CTT	TTG	ATT	ATC	TTC	GTA	CAC	ATC	CTA	TGG	TGT	TCA	TAA	ATC	ATT	CCC	GAT	TTA	CCA	GAT	ACC	TTT	TTT	CTG	CTG	2073	
CAA	ATG	GGT	AAT	TAG	CAG	ATT	GGC	CAG	CAG	TTG	ATC	ATC	GT																2114	

Figure 2 Nucleotide sequence of *pel10A* and deduced amino acid sequence of the encoded enzyme Pel10A

The proposed ribosome-binding sequence is underlined. The signal peptide, identified using the SignalP prediction server, is in bold. The sequence similar to the family 2a CBMs present in endoglucanase 9A, endoglucanase 45B, cellobextrinase 5C, endoglucanase 5E, endoxylanase 10A, endoxylanase 10B, arabinofuranosidase 62C, esterase 1D and Rgl11A is boxed with a solid line. Sequence similar to the functionally uncharacterized module in endoxylanase 10B, arabinofuranosidase 62C and esterase 1D is boxed with a broken line. The locations of primers R1PCR1 and R1PCR2, which were used to amplify the gene sequence encoding the catalytic module of Pel10A, are indicated, above their annealing sites, with solid arrows. The locations of primers R1PCR3 and R1PCR4, which were used to amplify the gene sequence encoding the family 2 CBM of Pel10A, are indicated, above their annealing sites, with broken arrows.

10 PLs. The C-terminal module of Pel10A shows no similarity to the catalytic module of Rgl11A.

Results of Pel10Acm assay validation

It is worth noting that most of the Pel10Acm assays were

performed at pH 10 (i.e. the standard reaction conditions). Such a high pH is known to release the esterified groups from esterified pectins [20]. This process is known as saponification and, during the course of a pectate lyase enzymic reaction against esterified pectins, could cause a falsely high reaction rate to be observed, since pectate lyases are thought to cleave bonds between non-

Pel-15E from <i>Bacillus</i>	25	-----LLP--PVSPVSADGDG-----	38
PelA from <i>A. irakense</i>	36	LTFSRVSSLPDTQRAAWQEYLARSEQLSRDKASLAELAPGQPLPPPAEGKGADTMPL	95
Pel10A from <i>P. cellulosa</i>	327	-----TGRMLTLDGNPAANWLNART-----	347
		* . . .	
Pel-15E from <i>Bacillus</i>	39	-----T-TAS--IEQILR-NQRPDGGWR---KDYS--VT---SGEWA-K-----	69
PelA from <i>A. irakense</i>	96	DKPAAWYTSKAARHVADVIVSFQTPAGGWGKNQPRDGALRLPGQHYTGENVAKVKSGSND	155
Pel10A from <i>P. cellulosa</i>	348	-----KWSASRADVVLSYQQNNGGWP---KNLDYNSVGNNGGGNES-----	385
		. : : : * * * : : *	
		↓ ↓ ↓	
Pel-15E from <i>Bacillus</i>	70	--S-----TIDNKATYTEIRRLAAEYTKTR---DSRYSDAAVRGIHFLNMQYAN	114
PelA from <i>A. irakense</i>	156	LDAARDRDWHYVGTIDNDATVTEIRFLAQVVSQLAPEEAAPYRDAALKGIEYLLASQFPN	215
Pel10A from <i>P. cellulosa</i>	356	-----GTIDNGATITTEMVFLAEVYKSGG---NTKYRDAVRKAANFLVNSQYST	430
		* * * * * : * * . . : * : . . : * : . .	
		↓ ↓	
Pel-15E from <i>Bacillus</i>	115	GGWPQIYQGTG-YHRHITYNDNAMINVMMLDDVANRRG--DFAFVNTSLADQSRAAVTR	171
PelA from <i>A. irakense</i>	172	GGWPQVWPLEGGYHDAITYNDALVHVAELLSDIAAGR--GFGFVPPAIRTRALEATNA	273
Pel10A from <i>P. cellulosa</i>	431	GALPQFYPLKGGYS DHATFNDNGMAYALTVLDFANKRAPFDTDVFSDSDRTRFKTAVTK	490
		* . * . : * * * : * : . : * * . . : : * . .	
		↓ ↓ ↓	
Pel-15E from <i>Bacillus</i>	172	GVDCILRTQVVAGGRLTAWGQQHDSVSLAPAGARSYEVPSLTASESTGIVRFLKTRPQTS	231
PelA from <i>A. irakense</i>	274	AIHCIVETQVVDGKRLGWGQQHDLTLRPTSARNFEPAALSSSTESARILLFMEIEAPS	333
Pel10A from <i>P. cellulosa</i>	491	GTDYILKAQWKQNGVLTWVCAQHGDYQPKKARAYELESLSGSESVGLAFLMTQPTA	550
		. . * : . * . * * : . * * : * : * : * : * : . .	
		↓	
Pel-15E from <i>Bacillus</i>	232	-QIRASIQAEEAFQT--VKITGIRVVKTNDDVIVVEDPSVTTPIWARFYEIGTNRPIFV	288
PelA from <i>A. irakense</i>	334	DAVKQAIRGGVAVLNT--SVIRDRAWVKSDQGYQLVTEQGAK-PLWSRFYSLDGNKPVFG	390
Pel10A from <i>P. cellulosa</i>	551	-EIERAVRAGVAFWNSPRTYLEGYTYDSSLAATNPVPRAGS-KMWRFRYDLNTNRGFFS	608
		. : . : . * : : . . : . : : * * * : . * : *	
		↓ ↓ ↓	
Pel-15E from <i>Bacillus</i>	289	GRDGIVKYRLSEIEQERRTGYSWYGNWPASLF-----	320
PelA from <i>A. irakense</i>	391	DRDKTIHDDVMGISQERRTGAWYTTSPQKALSAFTKWEKRS	432
Pel10A from <i>P. cellulosa</i>	609	DRDGSKFYDITQMSLERRTGYSWGGNYGTSIINFAQKVGYL-	649
		. * : . : * * * : * . . :	

Figure 5 Clustal W (1.8) mutiple sequence alignment of the mature pectate lyases from *A. irakense* (PelA) and the alkaliphilic *Bacillus* sp. strain KSM-P15 (Pel-15E), and Pel10Acm from *P. cellulosa*

Percentage identities between the *P. cellulosa* enzyme and the *A. irakense* and *Bacillus* enzymes are 30 and 36%, respectively. ↓, Conserved arginine, aspartic acid and glutamic acid residues; *, identical or conserved residues in all sequences in the alignment; two dots indicate conserved substitutions; one dot indicates semi-conserved substitutions.

Figure 6 Effect of pre-treatment of 89% esterified pectin at different pHs for varying lengths of time on Pel10Acm activity

□, pH 10.5; ■, pH 9.5; ▲, pH 8.5; ●, pH 7.5.

by an increase in CaCl_2 concentration from 0.1 to 1.0 mM (Figure 8). However, Pel10Acm was shown to be relatively less active against PGA at the higher CaCl_2 concentration when

compared with the ECPs, specifically 31 % ECP. The temperature at which the highest Pel10Acm activity could be detected was 62 °C. When subjected to 20 min pre-incubation at various temperatures, Pel10Acm remained 100 % active after pre-incubations at 27, 37 and 47 °C, but was only 62 % active after pre-incubation at 52 °C, and totally inactivated after pre-incubation at 57 °C. Pel10Acm activity was absolutely dependent on Ca^{2+} . Maximum activity was attained at CaCl_2 concentrations of 2.0–4.0 mM (Figure 9). No activity was seen when Ca^{2+} was replaced with Co^{2+} , Cu^{2+} , Ba^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Sr^{2+} or Zn^{2+} . The specificity constant (k_{cat}/K_m) of Pel10Acm decreased with increasing methylation of the substrate (Table 2).

Enzymic mode of action of Pel10Acm activity

Pel10Acm was shown to cleave PGA in an endo-fashion, as revealed by the production of a range of unsaturated oligogalacturonides during the initial stages of a reaction (Figure 10). 31 %, 67 % and 89 % ECPs also cleaved in an endo-fashion, with the retention times of the unsaturated oligogalacturonides released being identical with those released from PGA degradation (results not shown).

Figure 7 12% SDS-polyacrylamide gel of Pel10Acm

Lane 1, low-molecular-mass size standards containing proteins of 66, 45, 36, 29, 24 and 20 kDa; lane 2, Pel10Acm; lane 3, high-molecular-mass size standards containing proteins of 205, 116, 97, 84, 66, 55, 45 and 36 kDa.

Figure 8 Effect of pH on Pel10Acm activity

The pH 10 value against PGA at 1 mM CaCl_2 (i.e. maximum activity) was set to 100%. (A) Activity at 1 mM CaCl_2 ; (B) Activity at 0.1 mM CaCl_2 . ■, PGA; □, 31% ECP; ▲, 67% ECP; ●, 89% ECP.

Figure 9 Effect of Ca^{2+} concentration on Pel10Acm activity

The median replicate at 4 mM Ca^{2+} (i.e. maximum activity) was set to 100%. Error bars represent the S.D. from three replicates.

Table 2 Absolute kinetic parameters for Pel10Acm against polymeric substrates with varying degrees of methylation

Substrate	K_m ($\text{mg} \cdot \text{ml}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{ml} \cdot \text{mg}^{-1}$)
PGA	0.104	259.5	2495
31% ECP	0.131	231.3	1766
67% ECP	1.837	265.0	144
89% ECP	4.141	112.2	27

Binding of Pel10Acm2a to crystalline cellulose

The 14.1 kDa N-terminally His-tagged family 2 CBM of Pel10A (Pel10Acm2a) was shown to bind to Avicel (crystalline cellulose; results not shown). No traces of the module could be detected in the filtrate or washes after binding and the module was fully released from Avicel upon boiling for 5 min with 10% SDS. Denatured Pel10Acm2a did not bind to Avicel (results not shown).

DISCUSSION

P. cellulosa produces a modular pectinase, Pel10A. Pel10A is composed of (i) a signal peptide, (ii) an N-terminally located CBM belonging to CBM family 2a [2], (iii) a central module which shows extensive similarity (66% identity, Figure 4) with the functionally uncharacterized modules present in endoxylanase 10B, arabinofuranosidase 62C and esterase 1D from *P. cellulosa* and (iv) a C-terminally located catalytic module that was characterized as a pectate lyase, and shown to be a member of family 10 of the PL group of carbohydrate-active enzymes (see <http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>). These modules are separated by serine-rich linker regions.

The family 2a CBM of Pel10A from *P. cellulosa* shows significant similarity (between 48 and 54% identity, Figure 3 and Table 1) to the family 2a CBMs of endoxylanase 10A [21], endoxylanase 10B, arabinofuranosidase 62C, esterase 1D, endoglucanase 9A [16], endoglucanase 45B [22], cellodextrinase 5C [15], endoglucanase 5E [23] and Rgl11A [8] from the same organism, and contains the five tryptophan residues shown to be functionally important [24]. The central module of Pel10A, in addition to the extensive similarity with the central functionally

Figure 10 Chromatographs of the products released from PGA upon digestion with Pel10Acm

2U, unsaturated digalacturonide; 3U, unsaturated trigalacturonide; 4U, unsaturated tetragalacturonide; 5U, unsaturated pentagalacturonide; 6U, unsaturated hexagalacturonide.

uncharacterized modules of endoxylanase 10B, arabinofuranosidase 62C and esterase 1D, also shows some similarity (14–15% identity) to the N-terminal segments of mannanases 26A, 26B and 26C from *Piromyces equi* (Table 3) [25,26]. The N-terminal segments of these three mannanases from *P. equi* show 30–33% identity with the N-terminal segment of mannanase 26A from *Clostridium thermocellum* (Table 3), which has been shown to be a CBM with specificity to mannan [27]. The central module of Pel10A has been expressed in *E. coli* and was shown not to bind to a range of soluble and insoluble pectic, cellulosic and hemicellulosic polysaccharides, including mannan (H. F. Xie, personal communication). The catalytic module of Pel10A shows 30 and 36% identities to pectate lyase A from *A. irakense*

Table 3 Percentage identities of the central module of Pel10A and the N-terminal segments of mannanase 26A from *Clostridium thermocellum* and mannanases 26A, 26B and 26C from *Piromyces equi*

Man, mannanase; Ct, *C. thermocellum*; Pe, *P. equi*. Pel10A, residues 163–300; Man26A (Ct), residues 35–165; Man26A (Pe), residues 21–141; Man26B, residues 21–142; Man26C, residues 20–140.

	Identity (%)			
	Pel10A	Man26A (Ct)	Man26A (Pe)	Man26B (Pe)
Pel10A	—	—	—	—
Man26A (Ct)	5	—	—	—
Man26A (Pe)	14	30	—	—
Man26B (Pe)	14	32	85	—
Man26C (Pe)	15	33	66	71

[14] and pectate lyase 15E from alkaliphilic *Bacillus* sp. strain KSM-P15 [19], respectively (Figure 5). Structural and site-directed mutagenesis studies of pectate lyases have indicated the importance of arginine in substrate charge neutralization, and acidic residues as proton donors during catalysis [28,29], and indeed family 10 pectate lyases possess six invariant arginine residues, four invariant aspartic acid residues and four invariant glutamic acid residues (Figure 5).

The recombinant family 2a CBM of Pel10A (Pel10Acbm2a) has been shown to be functionally active and to bind strongly to crystalline cellulose (results not shown). The denatured module did not bind to crystalline cellulose, showing that the His tag does not have any binding affinity and that the family 2a CBM must fold into a three-dimensional structure for binding to occur. Pel10A from *P. cellulosa*, along with the Rgl11A, which is described in the accompanying article [8], are the first reported pectinases to contain CBMs. This begs the question why should Pel10A and Rgl11A, enzymes that attack a polysaccharide thought to be loosely associated with crystalline cellulose, contain a CBM with specificity to cellulose? All other enzymes that are active against pectic substances, including arabinanase 43A [30] and galactanase 53A [31] from *P. cellulosa*, do not contain CBMs. Are pectic substances in the plant cell wall more closely linked to cellulose than is currently assumed? Hence the requirement of a CBM to potentiate the activity of the pectate lyase. Moreover, is it more likely that the backbones of pectic substances, homogalacturonan and rhamnogalacturonan, are more closely associated with cellulose than the arabinan and galactan side chains. Hence the acquisition of a CBM by Pel10A and Rgl11A, but not by arabinanase 43A and galactanase 53A. Alternatively, since most of the plant-cell-wall-degrading enzymes produced by *P. cellulosa* contain CBMs [1], the acquisition of a CBM by Pel10A and Rgl11A may be more to do with the amplification of the CBM-encoding sequences throughout the genome of *P. cellulosa*, which has clearly occurred, rather than any specific advantage conferred on a pectate lyase by a CBM. Such questions about the role of CBMs in enzymes that degrade pectic substances will not be answered until suitable complex substrates are available which are similar to the plant cell wall in structure but, unlike the cell wall, are amenable to enzyme attack.

The optimum activity of the recombinant catalytic module of Pel10A (Pel10Acm) was seen at pH 10 (Figure 8). This compared with approx. pH 9 for PelA from *A. irakense* and pH 10.5 for Pel-15E from the *Bacillus* sp. It is one of the remarkable features

of pectate lyases that their pH optimum is always approx. pH 8.5–10.5, even though pectate lyases are produced by a range of micro-organisms, the majority of which are neutrophilic, e.g. *A. irakense*, *P. cellulosa* and *Erwinia chrysanthemi* [32], and very few of which are alkaliphilic, e.g. *Bacillus* sp. strain KSM-P15. Intriguingly, invasion in potatoes by the pectinolytic plant pathogen *Erwinia carotovora* subsp. *atroseptica* results in a change of pH from 5.0 in fresh tissue to pH 8.5 in the infected tissue [33]. Hence, it is tempting to speculate that pectate lyases have evolved to function optimally at high pH values because their substrate, in nature, is often alkaline in character. Indeed, at high pH values, arginine, a residue that has been implicated in catalysis [29], becomes deprotonated. This is consistent with the high pH optima observed for pectate lyases.

Although Pel10Acm was shown to be more active against PGA at a CaCl_2 concentration of 1 mM, rather than 0.1 mM (Figure 8), it is relatively less active at 1 mM than 0.1 mM when compared with the ECPs, specifically 31% ECP. Although gelling of PGA was not observed at 1 mM CaCl_2 , it was assumed that the relative decrease in activity of Pel10Acm against PGA, when compared with the ECPs, was due to some degree of gelling. Consequently, all assays with polymeric substrates were performed at 0.1 mM CaCl_2 . A Ca^{2+} concentration of between 2.0 and 4.0 mM was shown to be optimal for Pel10Acm activity (Figure 9). Of the other divalent ions tested (Ba^{2+} , Co^{2+} , Cu^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Sr^{2+} and Zn^{2+}) none were shown to substitute for Ca^{2+} at all. In this respect Pel10Acm from *P. cellulosa* was very similar to PelA from *A. irakense*. However, both enzymes differ significantly from Pel-15E from *Bacillus*, which shows significant Ca^{2+} substitution by Sr^{2+} (62% of the activity with Ca^{2+} ; Sr^{2+} substitution was never tested with PelA from *A. irakense*) and Mn^{2+} (36%). Pel-15E also showed slight substitution with Co^{2+} (9%) and Ni^{2+} (7%). Strontium has an atomic radius of 2.15 Å (1 Å = 0.1 nm), which, of the divalent ions tested, is the most similar to that of calcium, i.e. 1.97 Å. This may explain why significant Ca^{2+} substitution by Sr^{2+} is seen with Pel-15E. Perhaps the interactions of Pel10A from *P. cellulosa* and PelA from *A. irakense* with Ca^{2+} are more spatially constrained, hence the absence of substitution by Sr^{2+} .

The divalent-ion experiments with Pel10Acm from *P. cellulosa* were performed with trigalacturonic acid as substrate because PGA often gelled in the presence of some divalent ions at a concentration of 1 mM. However, it was important to use 1 mM concentrations of divalent ions so that any effects can be seen. These experiments, like all the other experiments described herein, were performed with EDTA-treated Pel10Acm (apo-Pel10Acm). Apo-Pel10Acm has absolutely no activity against trigalacturonic acid in the absence of Ca^{2+} and therefore, we believe, is a better enzyme for determining whether other divalent ions can substitute for Ca^{2+} ions, since there are no competing Ca^{2+} ions present in the assay. We have also shown that, in the absence of added Ca^{2+} , apo-Pel10Acm has absolutely no activity against CDTA-treated PGA (results not shown), because again there are no Ca^{2+} ions present. However, apo-Pel10Acm was shown to be active (20% of maximum) against untreated PGA in the absence of added Ca^{2+} . We believe that it is therefore important to use CDTA-treated PGA (and to a lesser extent CDTA-treated ECPs) when performing pectate lyase assays, since it is critical that the activity actually measured is not influenced by unknown amounts of Ca^{2+} associated with the enzyme and/or polymeric substrates. It has been proposed that the role of calcium during catalysis is to neutralize the charge of the uronic acid moieties of the substrate during catalysis [28]. But why is there such an absolute dependence on Ca^{2+} ions for activity? Is it that pectate lyases have evolved a catalytic

mechanism which requires the presence of Ca^{2+} ions because, in Nature, pectins are always found associated closely with Ca^{2+} ?

It can be seen from Table 2 that the specificity constant for Pel10Acm decreases with increasing esterification of the substrate. This suggests that Pel10Acm has a preference for sequences of non-esterified galacturonic acid residues (the so-called 'blocks') in the ECPs. These blocks are less abundant in more highly esterified substrates, hence the reduction in the specificity constant of Pel10Acm with increasing esterification of substrate. Whether this specificity is absolute remains to be determined. The use of differentially esterified oligogalacturonides as substrates will allow us to answer this question.

It is worth noting that Pel10A is active against AZ-rhamnogalacturonan. This is an experimental substrate produced by Megazyme. The fact that Pel10Acm is active against AZ-rhamnogalacturonan suggests that the enzyme also exhibits rhamnogalacturonan-degrading activity. However, this conclusion must be viewed with extreme caution as AZ-rhamnogalacturonan is likely to contain stretches of homogalacturonan in addition to rhamnogalacturonan. Cleavage of these stretches of homogalacturonan may be sufficient to facilitate halo formation, when screening the *P. cellulosa* genomic library with AZ-rhamnogalacturonan.

The $V_{\max}$ values determined for Pel10Acm from *P. cellulosa* (409.2 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ against PGA, 364.6 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ against 31% ECP, 417.8 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ against 67% ECP and 176.9 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ against 89% ECP) are very similar to the $V_{\max}$ values calculated for other pectate lyases [14,32]. Significantly, the $V_{\max}$ values for polygalacturonases [34], which share the same substrate specificity as pectate lyases, but cleave via hydrolysis and not β -elimination, are also similar to the $V_{\max}$ values calculated for Pel10Acm. Moreover, some organisms, e.g. *Erwinia*, produce both pectate lyases and polygalacturonases [35]. Why, since their substrate specificity and activities appear to be identical? Does the answer lie in the different pH profiles of these enzymes? Or is it the fact that the unsaturated products, produced by pectate lyases, are more readily metabolized than the saturated products produced by polygalacturonases, or *vice versa*?

There is still a lot to learn about the enzymic degradation of pectic substances. This substrate, unlike crystalline cellulose, is highly heterogeneous. This may account for the requirement of many enzymes with different specificities and catalytic mechanisms for the complete saccharification of pectic substances. The fact that some of these enzymes have now been shown to contain CBMs is possibly indicative of the complexity of plant cell wall degradation. The facts that (i) one polygalacturonase from *Kluyveromyces fragiles* [36] has, contrary to current opinion, been shown to release esterified galacturonides from pectin, and (ii) Pel10Acm from *P. cellulosa* has been shown to have a preference, if not an absolute requirement, for non-esterified galacturonic acid blocks in ECPs, suggest that the substrate specificity of polygalacturonases and pectate lyases is not as clear cut as once imagined.

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