Molecular and biochemical characterization of the thermoactive family 1 pectate lyase from the hyperthermophilic bacterium *Thermotoga maritima*

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The ability of the hyperthermophilic bacterium *Thermotoga maritima* to grow on pectin as a sole carbon source coincides with the secretion of a pectate lyase A (PelA) in the extracellular medium. The *pel*A gene of *T. maritima* was functionally expressed in *Escherichia coli* as the first heterologously produced thermophilic pectinase, and purified to homogeneity. Gel filtration indicated that the native form of PelA is tetrameric. Highest activity (422 units/mg, with a K_m of 0.06 mM) was demonstrated on polygalacturonic acid (PGA), whereas pectins with an increasing degree of methylation were degraded at a decreasing rate. In the tradition of pectate lyases, PelA demonstrated full dependency on Ca^{2+} for stability and activity. The enzyme is highly thermoactive and thermostable, operating optimally at 90 °C and pH 9.0, with a half-life for thermal inactivation of almost 2 h at 95 °C, and an apparent melting temperature of 102.5 °C. Detailed characterization of the product formation with PGA indicated that PelA has a unique eliminative exocleavage pattern liberating unsaturated trigalacturonate as the major product, in contrast with unsaturated digalacturonate for other exopectate lyases known. The unique exo-acting mode of action was supported by progression profiles of PelA on oligogalacturonides (degree of polymerization, 3–8) and the examination of the bond cleavage frequencies.

Key words: pectate lyase, pectin, pectinase, thermostable, *Thermotoga*.

INTRODUCTION

Pectin is an abundant structure component in plant cell walls and functions as a matrix holding cellulose and hemicellulose fibres. It is composed of a main chain of (partly) methyl-esterified α-1 $\rightarrow$ 4-D-polygalacturonate and highly branched rhamnogalacturonan (rhamnose–galacturonate stretches), in which the latter can serve as a binding site for 1 $\rightarrow$ 4-linked side chains like arabinans, galactans or arabinogalactans [1].

Enzymic modification of the pectin polymer is catalysed by a variety of pectinolytic enzymes. They can be classified into (i) esterases that remove the methyl and/or acetyl groups from pectin and (ii) depolymerases that cleave the backbone, either by hydrolysis (hydrolases) or non-hydrolytic β-elimination (lyases) [2]. Almost all bacteria and fungi that are capable of growing on pectin possess pectate lyases that degrade non- or low-methylated pectin (pectate) in either an exo- or endo-active way. They are generally located extracellularly, occasionally membrane-bound and rarely present in the cytoplasm. Pectate lyases belong to the class of polysaccharide lyases, where they are classified in 5 out of 12 families (1, 2, 3, 9 and 10) [P. M. Coutinho and B. Henrissat (1999) http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html]. With the exception of families 2 and 9, all of these have a three-dimensional structure available.

Enzymic pectin degradation is found in several industrial processes, such as the fruit-juice industry, and in textile and paper treatment [3]. To date, attention has been mainly focused on pectinolytic enzymes from mesophilic fungi (e.g. *Aspergillus niger*) and bacteria (e.g. *Erwinia* and *Bacillus* species). The only thermophiles that have been reported to be capable of growing on pectin are *Caldicellulosiruptor* strains [4–6], a few *Clostridia* [7,8] and one archaeon, *Desulfurococcus amylolyticus* [9]. In addition, only a few thermoactive pectate lyases, originating from *Thermoanaerobacter italicus* [10] and *Bacillus* sp. [11], have been biochemically characterized.

Thermotoga maritima, an anaerobic, hyperthermophilic bacterium growing optimally at 80 °C, can metabolize a wide variety of carbohydrates, including polymers such as xylan, starch and cellulose [12–14]. With the completion of the genome sequence of this organism [15], numerous enzymes that play an important role in displaying its heterotrophic potential were listed, including gene products putatively involved in pectin utilization. In the present study, we report on the first heterologously expressed thermoactive pectate lyase A (PelA) from the hyperthermophile *T. maritima*, its biochemical characterization and provide a detailed analysis of its unique exocleaving properties, making PelA the first, extensively characterized, exopectate lyase generating trigalacturonate units.

EXPERIMENTAL

Organisms, growth conditions and plasmids

T. maritima strain MSB8 (DSM 3109) was routinely grown under anaerobic conditions at 80 °C (pH 6.5), in 120 ml bottles, using 30 ml of Marine Medium *Thermotoga* (MMT), containing (per litre): 40 g sea salt (Sigma, Zwijndrecht, The Netherlands),

Abbreviations used: DM, degree of methylation; DP, degree of polymerization; Gal*p*A, galacturonic acid; HPAEC, high-performance anion-exchange chromatography; HPSEC, high-performance size-exclusion chromatography; PAD, pulsed amperometric detection; PelA, pectate lyase A; PGA, polygalacturonic acid; uG_3, unsaturated trigalacturonate.

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3.1 g Pipes, 1.0 g yeast extract, 4.0 g tryptone, 2 ml trace elements [16], 0.5 g resazurin, 1 mM $CaCl_2$, 2 mM $MgSO_4$ and 1 mM $Na_2S_2O_3$. Flasks were repeatedly flushed with N_2 and sterilized at 120 °C. Pectin as a substrate with degree of methylation (DM) of 65 and 89 % (Sigma) was prepared separately as a 1 % (w/v) stock and after autoclaving added to a 2 × MMT medium to reach a final concentration of 0.5 % (w/v). Glucose (20 mM) was added separately as filtered solution. *T. maritima* was grown under constant mild shaking (100 rev./min) to reduce inhibition by hydrogen formation. Growth was monitored by measuring the amount of hydrogen produced. To overcome the problem of underestimation when measuring the hydrogen yield at ambient temperature, methane was used as an internal standard as described by Kengen et al. [17].

The bacterial strain used for initial cloning experiments was *E. coli* TG1 [*supE hsd* Δ5] *thi* Δ(*lac-proAB*) F′ (*tra*D35 *proAB*$^+$ *lacI*^{q} *lac*Z ΔM15). *E. coli* BL21(DE3) (*hsdS*) *gal* (λcl*ts* 857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene 1) was used for heterologous expression. The plasmid used for the recombinant work was pET24d from Novagen (Madison, WI, U.S.A.).

Recombinant DNA techniques

Genomic DNA of *T. maritima* was isolated by using an established method described previously [18]. Small-scale plasmid DNA isolation was performed using the QIAGEN purification kit (Qiagen, Hilden, Germany). DNA was digested with restriction endonucleases and ligated with T4 DNA ligase, according to the manufacturer's instructions (Life Technologies, Breda, The Netherlands). DNA fragments were purified from agarose by QiaexII or from a PCR mix by using the PCR purification kit (Qiagen). Chemical transformation of *E. coli* TG1 and BL21(DE3) was performed using established procedures described previously [19].

The gene encoding the pectate lyase (*pel*A, TM0433) was identified in the course of the *T. maritima* genome sequence project [15]. Primers including appropriate restriction sites for cloning purposes (thereby changing serine into an alanine downstream of the initial methionine) were designed to exclude a putative signal sequence of 27 amino acids: BG906 (sense), 5′-GCGC**CCATGG**CTCTCAATGACAAACCTGTGGG (*Nco*I restriction site in boldface). BG887 (anti-sense), 5′-CGGATTGGTTGA**GGATCC**TTACTGAGCC (*Bam*HI restriction site in boldface). A PCR reaction was performed using 300 ng of both oligonucleotides and 100 ng *T. maritima* genomic DNA as a template in a final volume of 50 μl. After an initial denaturation step of 5 min at 95 °C, the DNA thermal cycler (PerkinElmer) was programmed for a PCR with *Pfu* polymerase (1 unit): 1 min at 95 °C, 2 min at 50 °C and 3 min at 68 °C, completed after 35 cycles by a final extension of 7 min at 68 °C. The obtained PCR product was digested with *Nco*I and *Bam*HI and cloned into a pET24d expression vector (Novagen), resulting in pLUW742, which was introduced into *E. coli* TG1 and BL21(DE3).

DNA and amino acid-sequence analysis

Cloned PCR products were sequenced by the dideoxynucleotide chain-termination method [20] with a Li-Cor automatic sequencing system (model 4000L). DNA and protein sequence data were analysed with the DNASTAR package and compared with the GenBank® Database by BLAST [21]. ClustalX was used to create an alignment of the *T. maritima* pectate lyase with homologues from the lyase family. GeneDoc was applied to adjust manually the alignment.

Purification of PelA

E. coli BL21(DE3) harbouring pLUW742 was grown overnight (37 °C, 150 rev./min) in a 5 ml TYK (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 50 μg/ml kanamycin) preculture, after being isolated as a single colony from the plate (TYK + 1.5 % granulated agar). To inoculate 1 litre of TYK in a baffled 2-litre Erlenmeyer flask, 1 ml of the culture was used. After an overnight growth at 37 °C at 120 rev./min, the culture was centrifuged at 8500 ***g*** for 15 min at 4 °C and the cells were resuspended in 10 ml Tris/HCl (20 mM, pH 8.0). The cell suspension was subjected to sonication (Branson sonifier, 3 × 15″) and cell debris was removed by centrifugation at 16000 ***g*** for 10 min. The resulting supernatant was incubated for 20 min at 80 °C and precipitated proteins were removed by an additional centrifugation step. The heat-stable cell-free extract was loaded on to a Q Sepharose column (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), which was equilibrated with Tris/HCl (20 mM, pH 8.0). Bound proteins were eluted by a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions containing PelA were pooled and concentrated (30 kDa cut-off; Filtron Technology, Northborough, MA, U.S.A.). To determine its native conformation, PelA was run over a gel-filtration column (Superdex 200, Amersham Pharmacia Biotech) and compared with a set of marker proteins, using 20 mM Tris/HCl and 100 mM NaCl (pH 8.0) as elution buffer.

Isolation of extracellular proteins of *T. maritima*

A 30 ml culture of *T. maritima* grown on glucose or pectin was spun down for 10 min at 10000 ***g***. Proteins were recovered from the medium fraction by an 85 % (w/v) ammonium sulphate precipitation [22] and a subsequent centrifugation step (10000 ***g*** for 10 min). The pellet was resuspended in 50 mM Mops buffer (pH 7.7) and ammonium sulphate was removed by washing using Centricon filters (10 kDa cut-off; Millipore, Etten-Leur, The Netherlands).

Gel electrophoresis and zymogram staining

Protein samples were boiled in 5 × sample buffer for 5 min and analysed by SDS/PAGE in 10 % (w/v) gels [23]. After migration, protein bands were stained with Coomassie Brilliant Blue. For zymogram staining, samples were boiled for 2.5 min before applying on to a 10 % SDS/PAGE gel, which contained 0.05 % (w/v) polygalacturonic acid (PGA; the boiling time was reduced to increase the efficiency of refolding into an active enzyme). Denaturing agent SDS was removed by washing gels overnight at 4 °C in 0.1 M Mops buffer (pH 7.7), 1 % (w/v) Triton X-100 and 1 mM dithiothreitol. Subsequently, gels were incubated in 0.1 M Mops buffer (pH 7.0) and 1 mM $CaCl_2$, at 80 °C for 1 h and stained with 0.05 % (w/v) Ruthenium Red (Sigma) for 5 min. Zymograms were washed with water until pectate lyase activity bands appeared.

Enzyme assay

Initial pectate lyase activity was determined spectrophotometrically at 230 nm by measuring the formation of Δ4,5 unsaturated products, using a standard assay adapted from Collmer et al. ($\epsilon_{232} = 4600\ M^{-1} \cdot cm^{-1}$) [24]. Enzyme (10 μl; approx. 0.1–1 μg/assay) was added to a closed 1 ml quartz cuvette containing 990 μl of substrate stock solution [0.25 %

(w/v) PGA, 50 mM Tris/HCl (pH 8.0), 0.6 mM $CaCl_2$]. The subsequent increase in absorbance at 230 nm was monitored as a function of time with a spectrophotometer (Hitachi U-2010). One unit of enzyme forms 1 μmol of Δ4,5 unsaturated product/min. Assays were performed at 80 °C and pH 8.0 unless otherwise stated. The pH of all buffers was equilibrated at the temperatures used. Enzyme thermostability was studied by determining the residual activity after incubation of PelA (0.05 mg/ml) in 50 mM Tris buffer (pH 8.0), with or without 0.6 mM $CaCl_2$, in sealed glass tubes.

Alternatively, assays for HPLC studies were performed at 90 °C in 20 mM Mops buffer and 0.8 mM $CaCl_2$ (pH 7.0), to approach the physiological pH. When using 0.5 % (w/v) PGA (ICN, Cleveland, OH, U.S.A.) or pectin (DM of 30 and 74 %) as a substrate, 1 μg of PelA in 1 ml reaction volume was used. With an appropriate amount of enzyme for each oligogalacturonide, activity assays were performed on saturated 2 mM oligoGal*p*A (oligogalacturonic acid) with a degree of polymerization (DP of 3–8), generated as described previously [25]. The pH of Mops was adjusted for use at 90 °C [26]. Samples (100 μl) were taken at various times and the reaction was stopped by the addition of 10 μl of acetic acid (10 %, v/v) and chilling on ice. Samples were stored at −20 °C until analysed by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC).

HPSEC analysis

HPSEC analysis was conducted on three TSKgel columns (7.8 mm internal diameter × 30 cm/column) in series (G4000 PWXL, G3000 PWXL, G2500 PWXL; Tosohaas, Stuttgart, Germany) in combination with a PWX-guard column (Tosohaas). Elution was performed at 30 °C with 0.2 M sodium nitrate at 0.8 ml/min. The effluent was monitored using a refractive index detector. Calibration was performed using dextrans, pectins and oligoGal*p*A.

HPAEC analysis

HPAEC analysis at pH 12 was performed as described previously [27]. Δ4,5 unsaturated oligoGal*p*A were selectively detected by UV detector at 235 nm (UV1000; Thermo Separation Products, Burnsville, Minnesota), whereas both saturated and Δ4,5 unsaturated oligoGal*p*A were detected using pulsed amperometric detection (PAD, Electrochemical Detector ED40; Dionex, Sunnyvale, CA, U.S.A.). Pure saturated oligoGal*p*A (DP1–7) and Δ4,5 unsaturated oligoGal*p*A (DP2–7) were used as standards for external calibration of the system. The amount of product formation was quantified by peak integration (Chromquest, San Jose, CA, U.S.A.). The specific activity (nmol of product · min^{-1} · mg $protein^{-1}$) was calculated from the formation of unsaturated oligoGal*p*A in time (initial 4 h). Activity on pectins was corrected for unsaturated oligoGal*p*A, produced by chemical β-elimination.

Differential scanning calorimetry

Thermal unfolding experiments were performed on a MicroCal VP-DSC in the temperature range from 50 to 125 °C at a heating rate of 0.5 °C/min. All experiments were performed in buffer solutions with an ionic strength of 50 mM. The protein (0.3 mg/ml) was dialysed against the buffer solution before the experiment.

RESULTS

Genetic characterization of the *pel*A gene from *T. maritima*

The *pel*A gene (1104 bp encoding 367 amino acids) was annotated in the *T. maritima* genome as pectate lyase (TM0433) [15]. PelA belongs to family 1 of the polysaccharide lyases, a group of carbohydrate-active enzymes that cleave polysaccharide chains by β-elimination [P. M. Coutinho and B. Henrissat (1999) http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html]. A database search [21] indicated highest similarity with a pectate lyase from *Bacillus halodurans* (51 %) [28]. The first 27 amino acids of PelA were predicted to be the signal sequence (Figure 1) [29]. Furthermore, no transmembrane domains could be identified (SMART) [30,31]; all these results together suggest an extracellular localization.

The results of the database search were used for a multiple sequence alignment at amino acid level between pectate and pectin lyases (Figure 1). The invariant residues located in the substrate-binding cleft of pectin and pectate lyases (the active-site related residues Arg^{279} and Asp^{184}; *B. subtilis* BsPel numbering is used throughout the paper) are conserved in all sequences. All three aspartate residues (Asp^{184}, Asp^{223} and Asp^{227}) that were shown to form a complex between pectate lyases and Ca^{2+} [32,33] are present in *T. maritima* PelA (Figure 1). Aromatic residues believed to be present in the substrate-binding cleft and expected to contribute to the affinity for non-charged, highly esterified pectin, are evident in the *A. niger* pectin lyase (PlyA) [32], but could not be identified in PelA (Figure 1).

Cloning, overproduction and purification of PelA

The *pel*A gene without the signal peptide-encoding sequence was amplified by PCR, including a methionine at position −1 of the predicted signal peptidase cleavage site and changing the serine at position +1 to an alanine. The resulting 1026 bp fragment was cloned into a pET24d vector under the control of a T7 promoter, resulting in pLUW742. The gene was functionally overexpressed in *E. coli* BL21(DE3) by overnight incubation at 37 °C. The protein was purified from a cell-free extract by heat incubation, followed by anion-exchange chromatography (Q Sepharose) during which the protein eluted at 0.6 M NaCl. PelA was considered pure after the last purification step, as judged by SDS/PAGE analysis (Figure 2, lane 1).

Growth of *T. maritima* on pectin

T. maritima was grown on glucose for 2 days, at the end of which the cells reached the stationary phase. Besides using these monosaccharides, the organism also showed the capacity to grow well on citrus pectin with a DM of 65 %. The maximal doubling time when grown on pectin was calculated to be 260 min, compared with 82 min when grown on glucose under the same conditions (results not shown). Cultures grown on pectin were tested on PGA for pectate lyase activity. High levels of activity were found in the medium, confirming the extracellular localization of PelA.

Enzyme properties

PelA activity in the medium fraction was confirmed further by zymogram experiments. Zymograms of the recombinant PelA displayed a prominent PGA-degrading activity band at approx. 40 kDa (Figure 2, lane 2). Similar analysis of the medium fraction of pectin-grown *T. maritima* demonstrated an activity band of identical molecular mass (Figure 2, lane 4), whereas no

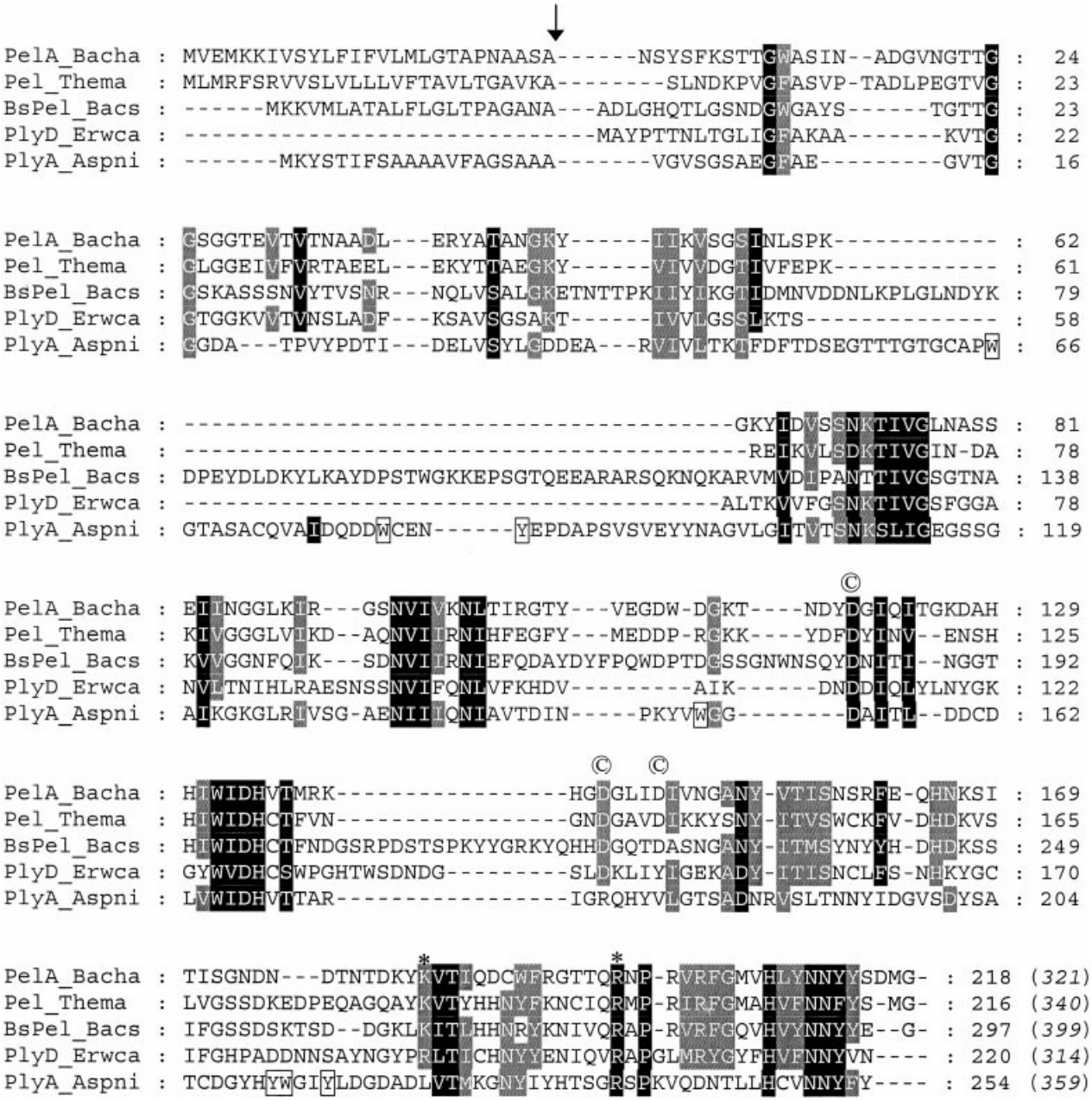

Figure 1 Multiple sequence alignment of pectate and pectin lyases belonging to family 1 of the polysaccharide lyases

Where necessary, the alignment was adapted as described in [45]. PelA_Bacha (*Bacillus halodurans*, GenBank® accession no. AB041769), Pel_Thema (*Thermotoga maritima*, GenBank® accession no. AE001722) and BsPel_Bacs (*B. subtilis*, GenBank® accession no. D26349) are pectate lyases. PlyD_Erwca (*Erwinia carotovora*, GenBank® accession no. M65057) and PlyA_Aspni (*Aspergillus niger*, GenBank® accession no. X60724) are pectin lyases. An arrow indicates the predicted cleavage site between the signal peptide and the mature enzyme. ©, proposed Ca^{2+}-binding aspartates; *, catalytic residues. Overall homology is highlighted in black, partial homology is highlighted in grey. Aromatic residues expected to contribute to the substrate binding by PlyA_Aspni [32] are shown in boxes.

extracellular activity was detected in cultures grown on glucose (Figure 2, lane 3). It is therefore very likely that the *E. coli*-produced PelA and the secreted pectate lyase are identical. When analysed by gel filtration, PelA eluted similar to rabbit muscle aldolase at 158 kDa, indicating that PelA is probably a 151.2 kDa tetramer (the calculated molecular mass of the mature monomer is 37.8 kDa) (results not shown).

Initial activity of PelA was assayed by monitoring the increasing absorbance at 230 nm of Δ4,5-unsaturated reaction products. Following the standard assay by Collmer et al. [24] the activity of PelA was 422 units/mg. The K_m for PGA was found to be 0.06 mM. The effect of pH and temperature was examined. Optimal catalysis was observed over a broad temperature range, with an optimum at 90 °C. Activity increased moderately with an increasing pH until pH 9.0, after which it decreased rapidly (results not shown).

Since Ca^{2+} is known to be essential for the catalytic activity of pectate lyases, PelA activity was examined on PGA (0.25%,

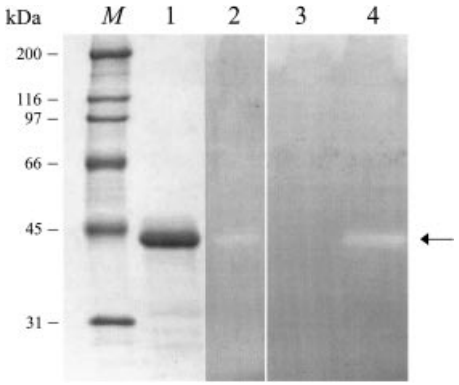

Figure 2 SDS-gel electrophoresis of recombinantly produced PelA juxtaposed to a PGA-zymogram analysis of recombinant PelA and the medium fraction of *T. maritima* grown on glucose and pectin

Samples were prepared as described in the Experimental section. The masses of the marker proteins (*M*) are shown on the left in kDa. The position of PelA is indicated by an arrow on the right. Lane 1, purified recombinant PelA, Coomassie-stained; lane 2, purified recombinant PelA, zymogramically stained; lane 3, medium fraction *T. maritima* culture, grown on glucose; lane 4, medium fraction *T. maritima* culture, grown on pectin.

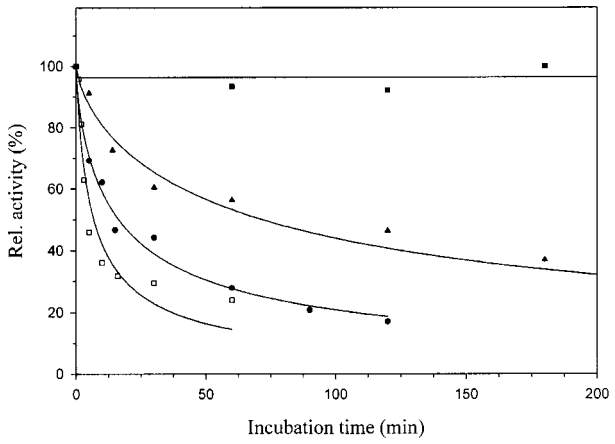

Figure 3 Thermostability of PelA examined by measuring the residual activity after incubation under standard conditions at 90 °C (■), 95 °C (▲), 100 °C (●) and at 90 °C in the absence of $CaCl_2$ (□)

The activity determined under standard conditions at 90 °C was defined as 100%. All data represent the means of duplicate assays.

w/v) in the presence of the chelator EGTA. Treatment with 1 mM EGTA completely eliminated PelA activity. The effect of Ca^{2+} was examined at different $CaCl_2$ concentrations. An increase in activity was detected until apparent saturation was reached at 0.6 mM $CaCl_2$. Further increase, up to 1 mM, had no significant effect on the activity of the enzyme (results not shown). The low-level activity detected in the absence of $CaCl_2$ might be attributed to residual trace amounts of Ca^{2+}, still present after purification.

Given that *T. maritima* is of marine origin, the effect of NaCl on the PelA activity was examined. When considering the activity of PelA without NaCl as 100%, PelA displayed its highest activity at 200 mM NaCl, exceeding the initial activity by more than five times (results not shown). Addition of more NaCl results in a reduction of the water activity, which in turn leads to gel formation between Ca^{2+} ions and PGA, causing the decrease in activity.

Thermostability of PelA

Purified PelA (0.05 mg/ml) was incubated in a 50 mM Tris buffer (pH 9.0), in the presence of 0.6 mM $CaCl_2$ at 90, 95 and 100 °C respectively, and without $CaCl_2$ at 90 °C. Samples taken at timed intervals were analysed at 80 °C for residual PelA activity. At 95 °C, the half-life of PelA was found to be 110 min, whereas at 100 °C PelA lost half of its activity after 15 min. The absence of Ca^{2+} caused a dramatic decrease in residual activity of PelA, losing half of its activity within 5 min of incubation at 90 °C (Figure 3). The presence of NaCl, up to a concentration of 1 M, appeared to have no effect on the thermostability (results not shown).

In addition, experiments were conducted to examine the melting temperature of PelA (0.3 mg/ml) by differential scanning calorimetry in the presence and absence of Ca^{2+}. They showed that after the addition of 0.6 mM $CaCl_2$, PelA thermally unfolded at a temperature of 102.5 °C. This melting temperature is 20 °C higher than that for incubation without Ca^{2+} (81.5 °C), thereby corroborating the importance of Ca^{2+} for the thermostability of PelA (results not shown).

Mode of action of PelA on substrate polymers

Degradation of PGA by PelA was monitored by HPSEC and HPAEC (Figure 4). HPSEC columns were calibrated with pectins and oligoGal*p*A, allowing the identification of the substrate polymer and produced oligogalacturonides. Activity on PGA resulted in the direct formation of an oligomer, which was identified as trigalacturonate when related to a standard set of oligoGal*p*A. Trigalacturonate in its turn is further processed into a dimer and monomer, both of which appear after 24 h (Figure 4A). The repositioning of the large polymer peak, in relation to the polymer in the blank reaction (dotted line), indicates that PelA displays exocleaving activity towards PGA.

The oligomers generated by PelA were examined in more detail on HPAEC using PAD [(un)saturated oligoGal*p*A] and UV detection (unsaturated oligoGal*p*A). The peak appearing in Figure 4(A) was clearly identified by UV detection as an unsaturated trigalacturonate (uG_3). Already, a slight formation of unsaturated digalacturonate (uG_2) can be identified (Figure 4B). Figure 4(C) shows the progression profiles of the amount of Δ4,5 unsaturated oligoGal*p*A (UV) generated as a function of time, and corrected for chemical β-elimination. uG_3 is immediately produced from the start of the reaction. These results illustrate that PelA attacks from the reducing end, since only then uG_3 can be generated. This mode of action is then followed by degradation of uG_3 into uG_2, as can be deduced from the increasing amount of released uG_2. The corresponding uG_1 (5-keto-4-deoxyuronate) cannot be detected by UV as a result of its open-ring structure, and is also relatively unstable [34]. Nevertheless, it has been shown in a previous study using HPSEC. From the progression profile, the specific activity for the generation of uG_3 from PGA over the first 4 h was calculated to be 9.9 units/mg.

When assayed on pectin with a different degree of methylesterification, the product formation indicates that a substantial amount of uG_3 is produced, despite the high DM (Figure 5). However, the specific activity of PelA decreased considerably, retaining only 41 and 2% of its activity on DM of 30 and 74%

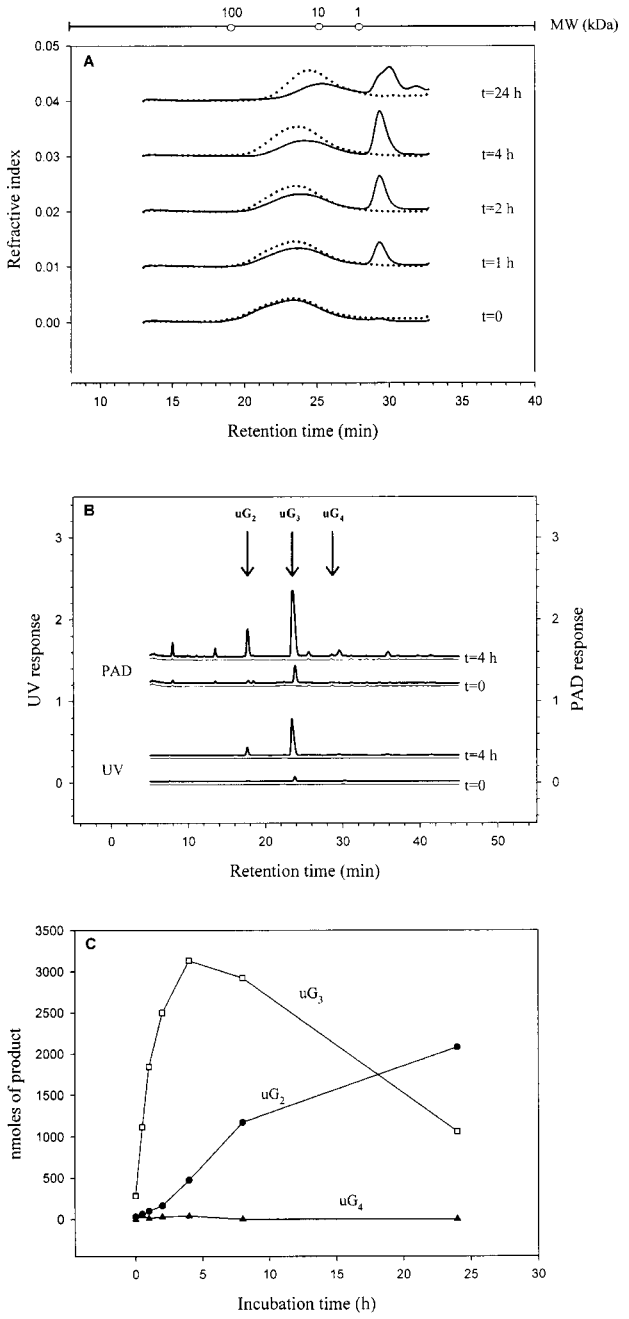

Figure 4 Degradation of PGA by PelA monitored over time by (A) HPSEC, (B) HPAEC (PAD and UV) and (C) progression profiles, derived from UV detection pattern

Broken lines indicate blank experiments (PGA incubation without PelA). The molecular-mass distribution (kDa) derived from a standard curve is given at the top. Unsaturated oligogalacturonides are indicated as follows: ●, uG_2; □, uG_3; ▲, uG_4.

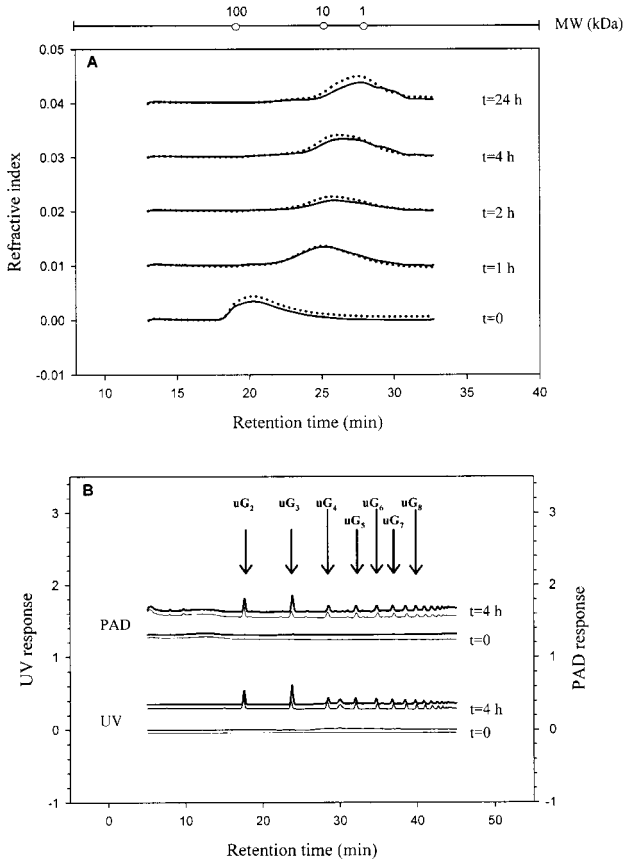

Figure 5 Degradation of pectin (DM = 74%) by PelA monitored over time by HPSEC (A) and at times $t = 0$ and $t = 4$ h by HPAEC (PAD and UV) (B)

Broken lines indicate blank experiments (pectin DM = 74% incubated without PelA). The molecular-mass distribution (kDa) derived from a standard curve is given at the top.

respectively (4.1 and 0.21 units/mg). Assays for HPLC were performed at the more physiological pH of 7.0, to minimize non-enzymic demethylation and β-elimination when using pectin as a substrate [35]. Demethylation of pectin under growth conditions of *T. maritima* was not observed; however, non-enzymic formation of unsaturated oligomers evidently occurred under HPLC assay conditions (Figure 5). In course of time, oligomers with a DP up to 8 were generated as a result of temperature-induced β-elimination of pectin with a DM of 74%. Clear fragmentation of the substrate as a direct result of the assay conditions could be identified by a drastic repositioning of the polymer peak (Figure 5A). The generated oligogalacturonides were shown to be unsaturated as detected by UV (Figure 5B).

Mode of action on oligoGal*p*A

The mode of action of PelA on oligoGal*p*A (DP3–8) was examined by HPAEC, analogous to PGA as a substrate. Progression profiles were constructed and showed a linear formation of both the saturated and the corresponding unsaturated oligogalacturonides (hence avoiding secondary product formation). From these linear areas both the bond cleavage frequency and the activity rate were calculated (Table 1). Besides confirming its obvious preference for releasing Δ4,5 unsaturated trimers it shows that the specific activity rates calculated from the saturated and unsaturated product formation correspond well. Noteworthy is the leap in activity rate when the oligogalacturonide length is increased from $(Gal pA)_3$ to $(Gal pA)_4$, which is a 100-fold. Analysis of the intermediate products demonstrated that incubation of $(Gal pA)_n$ results in the parallel production of uG_3 and $G_{(n-3)}$. The fact that the saturated counterproduct $G_{(n-3)}$ is formed in equivalent amounts indicates that PelA follows a multiple-chain attack, as opposed to a single-chain–multiple attack mode that would result in the immediate degradation of

Table 1 Bond cleavage frequencies (BCFs) and rate of β-elimination of PelA on saturated oligogalacturonides

BCFs are calculated from the Δ4,5 unsaturated products as detected by UV method. Activity rates were determined from the produced Δ4,5 unsaturated oligogalacturonides (UV) and the corresponding saturated oligogalacturonides produced (PAD). The reducing end of the oligoGal*p*A is shown in **bold** type (**G**).

DP																		Activity (m-units/mg)	
																		UV	PAD
3													G	–	G	–	**G**	34.1	36.2
														100					
4										G	–	G	–	G	–	**G**	3326	3818	
											99		1						
5								G	–	G	–	G	–	G	–	**G**	8258	8492	
											99		1						
6						G	–	G	–	G	–	G	–	G	–	**G**	6905	6399	
									1		98		1						
7			G	–	G	–	G	–	G	–	G	–	G	–	G	–	**G**	5217	4478	
									2		97		1						
8	G	–	G	–	G	–	G	–	G	–	G	–	G	–	**G**	3584	3356		
								3		96		1							

the saturated intermediate. An increase in activity rates was found on increasing DP to 5, with a significant elevation from $(Gal pA)_3$ to $(Gal pA)_4$ of 100 times. Surprisingly, both rates decreased again when the DP is further augmented. From the bond cleavage frequencies and the activity rates on the subsequent oligogalacturonides, we deduced that PelA probably contains five subsites.

DISCUSSION

T. maritima has the ability to grow on a variety of polysaccharides, such as cellulose, starch and xylan, for which it possesses a huge amount of carbohydrate-active enzymes, mainly glycoside hydrolases [12–14]. In the present study, we have demonstrated that *T. maritima* is also able to grow on pectin, the methylated α-1,4-linked polymer of Gal*p*A. Activity of pectate lyase, one of the key enzymes in the degradation process, was detected in the medium fraction of a pectin-grown culture. Activity was displayed as one single band, which shows that the production of PelA is induced by growth on pectin. Also, it strongly implies that PelA is the only secreted pectinolytic enzyme.

A candidate gene (TM0433), designated PelA, for the pectinolytic degradation has been identified in the *T. maritima* genome [15], based on a high-sequence similarity with a family 1 pectate lyase from *B. halodurans* [P. M. Coutinho and B. Henrissat (1999) http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html]. PelA contains a signal sequence and lacks additional transmembrane helices, which coincides with its presence in the medium fraction.

Subsequent characterization of the purified, *E. coli* produced PelA validated its classification as a pectate lyase, displaying the highest activity on non-methylated PGA with an absolute requirement for Ca^{2+}. PelA cleaves the substrate by means of β-elimination optimally under alkaline conditions (pH 9.0). Detailed examination of the reaction products demonstrated that PelA is an exo-cleaving pectate lyase (EC 4.2.2.9) after multiple-chain attack, making it one of the few exopectate lyases from family 1; others are classified in families 2 and 9. Based on the activity rates, the substrate-binding cleft most probably consists of five subsites, three of which interact with the reducing end of the substrate. A large increase in activity rate was observed with an increase in DP from 3 to 4. This emphasizes the high affinity that subsite +3 has for the Gal*p*A monomer and relates well to the predominantly formed product uG_3. PelA generates uG_3 as the first product, thereby showing that the enzyme attacks the polymer from the reducing end. To the best of our knowledge this is the only exopectate lyase able to cleave off trimers (trigalacturonate) as opposed to digalacturonate which is usually observed in bacteria and fungi [36,37]. Unquestionably, the recent crystallization of *T. maritima* PelA [38] will give more insight into the exact mode of action of the enzyme.

When PGA is replaced by pectin, the activity of PelA strongly decreases as a direct result of a higher DM. Nevertheless, the organism is able to use highly methylated pectin (up to 89 %) as a carbon source. No pectin methylesterases could be identified in the genome sequence from *T. maritima* and also no esterase activity was detected in the medium fraction of pectin-grown cells. Unsaturated oligogalacturonides, generated by chemical β-elimination of highly methylated pectin, might serve as a substrate that is more ideal for PelA under growth situations. It is obvious that, to a certain extent, elevated temperatures play a role in enabling growth of *T. maritima* on pectin. It is therefore tempting to speculate on the absolute necessity of PelA under the extreme *in vivo* conditions, since the oligogalacturonides liberated by PelA could be generated equally well via chemical β-elimination. To do so, we need to know whether these oligogalacturonides still contain methyl groups or not and to what extent PelA could accept them. So far, the rate of tolerance for methylesters in the substrate-binding cleft of PelA is not known.

T. maritima PelA is the first heterologously expressed pectate lyase originating from a hyperthermophilic organism. With its temperature optimum at 90 °C, it is the most thermoactive pectate lyase known to date. Compared with other extracellular polymer-degrading enzymes from *T. maritima* like xylanases, mannanases and cellulases, PelA shows comparable properties with respect to thermoactivity and thermostability [39]. Based on gel filtration results, we found PelA to be a tetramer. With the exception of pectate lyase b from the thermophilic bacterium *T. italicus* (dimer) [10], all pectate lyases characterized to date are monomeric. A higher degree of oligomerization is observed more frequently in thermophilic enzymes than in their mesophilic counterparts, and is regarded as a potential stabilization factor [40]. The large effect that Ca^{2+} ions have on the thermostability

of pectate lyases has been described frequently for pectate lyases of both hyperthermophilic and mesophilic origin.

Enhancement of pectate lyase activity by NaCl has been stated above. Previous studies [41,42] have described the total dependence of the activity on NaCl; other pectate lyases are only stimulated [43] or not influenced at all. Benen et al. [44] have proposed that the positive effect of NaCl on the activity is caused by a discharge of loosely bound Ca^{2+} from pectate chains, leading to an increase in availability of substrate and free Ca^{2+}. Since $CaCl_2$ concentrations higher than 0.6 mM did not significantly stimulate PelA activity, the NaCl-induced enhancement of the activity is probably due to an increase in substrate accessibility. The decrease in activity when the NaCl concentration exceeds 250 mM can be ascribed to gel formation, caused by a reduction of the water activity. The NaCl concentration at which PelA is most active is much lower than the salt concentration of seawater, the natural environment of *T. maritima*, which contains approx. 500 mM NaCl. However, pectate in nature is generally methylated, which reduces its capacity as a Ca^{2+}-pectate gel.

Growth on pectin of the hyperthermophilic bacterium *T. maritima* appears to be initiated by the secretion of PelA, an extracellular exopectate lyase. Together with a putative exopolygalacturonosidase [15], the organism holds at least two pectinases, both active in the initial process of pectin catabolism. With the genome sequence available, the road towards the elucidation of additional genes related to pectin degradation appears to be wide open. For this reason, DNA microarray analyses on the *T. maritima* genome are currently being carried out, with the intention to unravel pectin catabolism.

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