Pectate lyase C from *Bacillus subtilis*: a novel endo-cleaving enzyme with activity on highly methylated pectin

Margarita Soriano, Pilar Diaz and Francisco I. Javier Pastor

Correspondence
Francisco I. Javier Pastor
fpastor@ub.edu

Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain

The gene *yvpA* from *Bacillus subtilis* was cloned and expressed in *Escherichia coli*. It encoded a pectate lyase of 221 amino acids that was denominated PelC. The heterologously expressed enzyme was purified by His-tag affinity chromatography and characterized. PelC depolymerized polygalacturonate and pectins of methyl esterification degree from 22 % to 89 %, exhibiting maximum activity on 22 % esterified citrus pectin. It showed an absolute Ca^{2+} requirement and the optimum temperature and pH were 65 °C and pH 10, respectively. The deduced amino acid sequence of PelC showed 53 % identity to pectate lyase PelA from *Paenibacillus barcinonensis*, which was also characterized. Similarly to PelC, purified PelA showed activity on polygalacturonate and pectins with a high degree of methyl esterification. The two enzymes cleaved pectic polymers to a mixture of oligogalacturonates, indicating an endo mode of action. Analysis of activity on trigalacturonate showed that PelC cleaved it to galacturonic acid and unsaturated digalacturonate, whereas PelA did not show activity on this substrate. PelC and PelA showed high homology to a few recently identified pectate lyases of family 3 and form with them a cluster of small-sized pectate lyases from non-pathogenic micro-organisms.

Received 4 October 2005
Revised 15 November 2005
Accepted 23 November 2005

INTRODUCTION

Pectin is a plant polysaccharide that contributes to the structure of plant tissues as a component of the middle lamella and primary cell wall. It consists of a backbone of D-galacturonic acid residues which are partially methyl esterified. The main chain of the polymer also contains rhamnose residues that can be highly substituted by arabinose and galactose side chains (Schols & Voragen, 2003). Pectin degradation requires the combined action of several enzymes, among which two main groups can be distinguished: pectinesterases, which remove methoxyl groups from pectin, and depolymerases (hydrolases and lyases) that degrade the backbone chain. Lyases cleave glycosidic bonds by β -elimination, giving rise to unsaturated products. Among these enzymes, pectate lyases are specific for unmethylated polygalacturonate (pectate), although they can be active on pectins with a low degree of methyl esterification (Benen & Visser, 2003; Tardy *et al.*, 1997). Pectate lyases are widely distributed among microbial plant pathogens, where they play an important role as virulence factors (Barras *et al.*, 1994; Herron *et al.*, 2000). They have also been found in saprophytic micro-organisms, including members of the genus *Bacillus* (Berensmeier *et al.*, 2004; Hatada *et al.*, 2000; Nasser *et al.*, 1993) and in some thermophilic bacteria (Kluskens *et al.*, 2003). Multiple sequence alignment of pectate lyases has been used to classify these enzymes into several subfamilies and to identify consensus sequences of

some of them (Coutinho & Henrissat, 1999; Shevchik *et al.*, 1997). The study of the three-dimensional folding of pectate lyase C from *Erwinia chrysanthemi* led to the finding of a novel protein structure, a parallel-right handed β -helix (Yoder *et al.*, 1993), that is found in pectate lyases of families 1, 3 and 9 (Jenkins *et al.*, 2004).

Pectin-degrading enzymes are widely used in the food industry for improving the yield and the clarification of fruit juices (Kashyap *et al.*, 2001). Alkaline pectinases are being introduced in the textile industry to release fibres from flax stems, as an alternative to conventional retting (Hoondal *et al.*, 2002). In these fields, finding new enzymes has special interest to improve the efficiency of the production systems.

In a previous study, pectate lyase PelA from *Paenibacillus barcinonensis* was identified and characterized (Soriano *et al.*, 2000). As a distinctive trait among known pectate lyases, the new enzyme showed high activity on both polygalacturonate and highly methylated pectins. Analysis of the PelA amino acid sequence showed that the enzyme did not display homology to pectate lyases from saprophytic micro-organisms, while high homology was found to the hypothetical protein deduced from the *yvpA* gene from *Bacillus subtilis* (Kunst *et al.*, 1997). In this article we describe the cloning of *yvpA*, and show that it encodes a pectin-degrading enzyme that has been denominated PelC. The new pectate lyase shares with PelA a high activity on highly

methyated pectins and defines, together with PelA, a cluster of family 3 pectate lyases from non-pathogenic micro-organisms.

METHODS

Bacterial strains and plasmids. *Bacillus subtilis* 168 was obtained from the Spanish Type Culture Collection (CECT 461) and cultivated at 30 °C in nutrient broth. *Escherichia coli* 5K (Soriano *et al.*, 2000) and *E. coli* BL21(DE3) (Studier & Moffatt, 1986) were cultivated at 37 °C in LB medium and used as recipient strains for recombinant plasmids. Plasmids pJF118HE (Fürste *et al.*, 1986) and pET28a (Studier & Moffatt, 1986) were used as cloning vectors. *E. coli*/pP22 has been previously described (Soriano *et al.*, 2000). It harbours recombinant plasmid pP22 containing the *pelA* gene from *Paenibacillus barcinonensis* (Sánchez *et al.*, 2005) cloned in pBR322.

Nucleic acid manipulation. Isolation of plasmids, restriction digestion of DNA, ligation of DNA and transformation of *E. coli* were carried out by standard methods. Chromosomal DNA from *B. subtilis* and *P. barcinonensis* was prepared as previously described (Soriano *et al.*, 2000). PCR amplification and sequencing of DNA were done according to standard methodology.

The *yvpA* gene was PCR amplified with *Pfu* (Stratagene) with oligonucleotides 5'-ATCACCCGGAAGATCGATTTC-3' and 5'-CCGGAGCTCTGTCCATAAG-3'. These oligonucleotides are located 179 bp upstream and 141 bp downstream of the initiation and stop codons, respectively, of *yvpA* structural gene. The amplified fragment was ligated to plasmid pJF118HE previously digested with *Sma*I, and introduced by transformation into *E. coli* 5K. The recombinant strain containing the *yvpA* gene was denominated *E. coli*/pJYvpA.

Plasmids for overexpression of pectate lyases under the control of the T7 RNA polymerase system (Studier & Moffatt, 1986) were constructed as follows. *yvpA* was amplified with oligonucleotides 5'-TTGCTAGCGCTGACAAAGTGG-3' and 5'-CTAAGCTTGTTAA-AATTGAGTGTG-3'. The amplified fragment was digested with *Nhe*I and *Hind*III, inserted between the *Nhe*I and *Hind*III sites of plasmid pET28a, and transformed into *E. coli* BL21(DE3). The recombinant plasmid pET28a-YvpA obtained encoded PelC, devoid of its signal peptide, with an N-terminal His-tag. After removal of the His-tag, the amino acid sequence Gly-Ser-His-Met-Ala-Ser remained added to mature PelC. For expression of *pelA* from *P. barcinonensis*, a similar approach was used. The oligonucleotides used for *pelA* amplification were 5'-CCCATATGGCGCCAACCGTC-3' and 5'-GCAAGCTTTAATACTGTGTATTTCCG-3'. The amplified fragment was digested with *Nde*I and *Hind*III, and inserted between the corresponding sites of pET28a. The recombinant plasmid pET28a-PelA encoded a mature PelA with an N-terminal Gly-Ser-His-Met extension, after removal of the His-tag.

Purification of pectate lyases. Purification of PelC and PelA was performed by His-tag affinity chromatography using NTA-Ni agarose resin (Qiagen) essentially as described by the pET28a supplier (Novagen). Recombinant clones *E. coli*/pET28a-YvpA and *E. coli*/pET28a-PelA were cultivated at 37 °C in 1 litre of kanamycin-supplemented LB to OD₆₀₀ 0.5–0.6. Then 1 mM IPTG was added and the cultures were further incubated for 150 min. Cells were collected, resuspended in 20 ml buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed in a French press. Cell extracts were clarified by centrifugation and His-tagged proteins were purified from the extracts. The His-tag of purified proteins was removed using the Thrombin Cleavage Capture Kit from Novagen.

Enzyme assays. Pectate lyase activity was assayed spectrophotometrically by measuring the formation of unsaturated products from

pectic substrates at 232 nm (Collmer *et al.*, 1988). The standard assay mixture contained 0.2% (w/v) substrate (Sigma) in a final volume of 3 ml 50 mM glycine buffer pH 10.0, containing 0.5 mM CaCl₂ for PelA activity determination or 2 mM CaCl₂ for PelC activity determination. Assay mixtures were incubated for 2.5 min at 50 °C and the increase of A₂₃₂ was measured. The substrates used were polygalacturonic acid and pectins of various degrees of methylation (22, 64 and 89% esterified citrus pectin, Sigma). Apple pectin (68% esterification, Sigma) was also tested as a substrate. One unit of enzymic activity was defined as the amount of enzyme that produces 1 µmol unsaturated product per minute under the assay conditions described. To study the effect of pH on activity, assays were performed at 65 °C (PelC) or 55 °C (PelA), replacing 50 mM sodium glycine buffer pH 10.0 by the following buffers: 50 mM sodium citrate pH 3.0–4.0, 50 mM sodium acetate pH 4.0–6.0, 50 mM sodium phosphate pH 6.0–8.0, 50 mM Tris/HCl pH 8.0–9.0, and 50 mM sodium glycine pH 9.0–12.0. The effect of temperature was studied at pH 10 in the temperature range 30–85 °C. For inhibition studies, 1 mM different ions were added to the assay mixture and activity was determined as indicated.

For stability assays, enzymes were incubated at 50 °C in buffers at pH 7.0 or 10.0 and samples were taken at regular intervals. Residual activity after thermal treatment was determined by the standard assay.

Kinetic parameters V_{max} and K_m were determined by fitting hyperbolic Michaelis–Menten curves with the program SigmaPlot version 8.0 (SPSS). Kinetic parameters of PelC and PelA on 22% methyl esterified pectin and polygalacturonic acid were determined with concentrations of substrates from 0.25 to 6 mg ml⁻¹, at 65 °C and 55 °C, respectively, in buffers at pH 10.

Analytical procedures. SDS-PAGE was performed in 13% (w/v) gels, essentially as described by Laemmli (1970). Samples were heated for 10 min at 45 °C in sample buffer before being applied to gels. After electrophoresis, gels were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in 100 mM glycine buffer pH 10.0, 1.5 mM CaCl₂ for 30 min, and overlaid with a 1% (w/v) agarose gel containing 1% (w/v) pectin in the same buffer as above. After 30 min incubation at 45 °C, gels were stained with 0.05% (w/v) ruthenium red (Sigma) for 10 min and washed with water until pectate lyase bands became visible.

Analysis of PelC and PelA reaction products was performed by TLC on silica gel plates (Kieselgel 60 F₂₅₄, Merck). The solvent used was n-butanol/water/acetic acid (5:3:2, by vol.). Oligosaccharides were detected by spraying the dried plates with a solution of 3% (w/v) phosphomolybdic acid and 10% (v/v) sulphuric acid in ethanol, and heating at 125 °C for 5 min. Galacturonic, digalacturonic and trigalacturonic acids (Sigma) were used as size markers.

RESULTS

Cloning of the pectate lyase

In a previous study a novel pectate lyase with high activity on both polygalacturonic acid and highly methyated pectin was cloned and characterized from *Paenibacillus barcinonensis* (Soriano *et al.*, 2000). The deduced amino acid sequence of the enzyme, PelA, did not show homology to pectate lyases from saprophytic micro-organisms, but it displayed high homology to the protein deduced from *yvpA* gene from *Bacillus subtilis*, of unknown function (Kunst *et al.*, 1997), indicating that it probably encoded a pectin-degrading enzyme. *yvpA* from *B. subtilis* was isolated by PCR

and cloned in *E. coli*. The recombinant clone obtained, *E. coli*/pJYvpA, produced haloes of substrate degradation when grown on polygalacturonic acid supplemented agar plates, showing that *yvpA* encoded a pectinase. Cell extracts from the recombinant clone showed lyase activity on polygalacturonic acid and pectin, while hydrolase activity on these substrates was undetectable. The results suggested that *yvpA* encoded a pectate lyase with similar substrate specificity to PelA.

Sequence analysis

The nucleotide sequence of the cloned gene was identical to that reported for *yvpA* (Kunst *et al.*, 1997). It encodes a protein of 221 amino acids with predicted molecular mass and pI of 24281.4 Da and 8.85, respectively. The deduced amino acid sequence shows an N-terminal region of 27 amino acids with the features of a typical *Bacillus* signal peptide (Nagarajan, 1993). The open reading frame starts with TTG as initiation codon. This start codon is frequently found in *Bacillus* genes and in some instances it has been correlated to translation efficiency (Vellanoweth, 1993). Seven nucleotides upstream of the start codon there is a GGG-GAGGA sequence, resembling that of a ribosome-binding (Shine-Dalgarno) site. A putative promoter sequence, similar to those recognized by the σ^A subunit of *Bacillus* RNA polymerase, showing a -35 (TTTACT) and -10 (TATGAT) region, was found upstream of the *yvpA* structural gene. An inverted repeat of 14 bp, that could act as a transcriptional terminator, was found in the 3' region of the structural gene.

The deduced amino acid sequence of the cloned enzyme was compared to protein sequences in the Swissprot and EMBL databases. Homology was found to pectate lyases belonging to family PL3, while no homology was found to pectate lyases of other families (Fig. 1). The highest homology was found to pectate lyase from *Bacillus* sp. P-2850 (accession no. Q8L0R5), the protein deduced from *yvpA* gene from *Bacillus licheniformis* (accession no. Q65EF5), pectate lyase from *Bacillus* sp. KSM-P15 (Hatada *et al.*, 2000) and PelA from *P. barcinonesis* (Soriano *et al.*, 2000), which showed 72, 72, 53 and 53 % identity, respectively, to the cloned enzyme. High homology was also found to open reading frames deduced from genes SCO1110 from *Streptomyces coelicolor* (Bentley *et al.*, 2002) and SAV6383 from *Streptomyces avermitilis* (Omura *et al.*, 2001), which showed 44 and 40 % identity to the cloned enzyme. Homology was also found to pectate lyases from phytopathogenic fungi and bacteria: Pl1 from *Fusarium oxysporum* (Huertas-González *et al.*, 1999), PelI from *Erwinia chrysanthemi* (Shevchik *et al.*, 1997), PelB from *Fusarium solani* (Guo *et al.*, 1995) and Pel3 from *Erwinia carotovora* (Liu *et al.*, 1994), which showed 43, 42, 37 and 35 % identity, respectively, to the cloned pectate lyase.

The cloned enzyme did not show homology to the two pectin-degrading enzymes, a pectate lyase (Nasser *et al.*, 1993) and a pectin lyase (Sakamoto *et al.*, 1996), characterized to date from *B. subtilis*. We named the cloned enzyme

PelC and propose the encoding gene to be designated *pelC* instead of *yvpA*. The amino acid sequence of PelC contains three out of the four blocks of conserved residues proposed as signatures of pectate lyases from family 3 (Shevchik *et al.*, 1997) (Fig. 1). Among the conserved amino acids, PelC shows an Arg residue (Arg-158) in an identical position to Arg-132 of the mature pectate lyase from *Bacillus* sp. KSM-P15, which is proposed to initiate proton abstraction during the β -elimination catalytic mechanism (Akita *et al.*, 2001). The three-dimensional structure of PelC was predicted by the Swiss pdb viewer program. The enzyme folds as an eight-turn right-handed parallel β -helix, similarly to family 3 pectate lyases.

Substrate specificity

The cloned pectate lyase was purified by His-tag affinity chromatography, the His-tag was removed and the properties of the enzyme were studied. Analysis by SDS-PAGE, IEF and zymograms showed that PelC has an apparent molecular mass of 23 kDa (Fig. 2) and a pI of 8.6 (data not shown), in good agreement with the predicted values of 24281.4 Da and 8.55, respectively. The influence of substrate methylation on enzymic activity was tested by evaluating the activity of the purified enzyme on polygalacturonic acid and on pectins of increasing degree of methyl esterification. Release of esterified methyl groups from pectin by saponification can take place after incubation at pH 10 for longer than 10 min (Brown *et al.*, 2001). To avoid this phenomenon, activity was determined in 2.5 min assays in which enzyme samples, substrates and buffer at pH 10 were mixed immediately before the assays. PelC exhibited activity on all substrates tested and showed maximum activity on pectin with a low degree of methylation (22 % esterified) (Fig. 3). On polygalacturonic acid, and pectins of 64 % and 89 % methyl esterification, the enzyme showed 29, 87 and 43 %, respectively, of maximum activity.

To analyse the influence of saponification of pectins on activity evaluation, new assays were performed on substrates that had been kept with buffer at pH 10 for more than 1 h. Under these conditions PelC showed maximum activity on 64 % esterified pectin, while on polygalacturonic acid, 22 % and 89 % esterified pectins, 14, 42 and 97 %, respectively, of maximum activity was found. The results indicated that saponification can give rise to false high activity results on pectins, and for this reason standard activity assay conditions were established in which buffer at pH 10 and the substrates were mixed just before determination of activity.

Pectate and pectin lyases can be distinguished by the absolute Ca^{2+} requirement for catalysis of pectate lyases versus the lack of Ca^{2+} requirement by pectin lyases (Jurnak *et al.*, 1996). Analysis of the effect of Ca^{2+} on PelC activity showed that the enzyme exhibited maximum activity at 2 mM CaCl_2 , while without Ca^{2+} addition no activity on polygalacturonic acid and very little activity on pectins were found (Fig. 3). The activity found on pectin without

Fig. 1. Amino acid alignment, performed by CLUSTAL W, of several pectate lyases belonging to family 3. Numbering of the amino acids starts at the N-termini of the proteins. Gaps are indicated by dashes. Asterisks indicate identical amino acids; colons and dots represent conserved and semi-conserved residues, respectively. Blocks of conserved residues of family 3 pectate lyases are boxed. The sequences shown are: BliYvpA, protein deduced from *yvpA* gene from *Bacillus licheniformis*; BspP2850, pectate lyase from *Bacillus* sp. P-2850; BsuPelC, PelC from *Bacillus subtilis*; BspKSM15, pectate lyase from *Bacillus* sp. KSM-P15; PbaPelA, PelA from *Paenibacillus barcinonensis*; SavPel, protein deduced from gene SAV6383 from *Streptomyces avermitilis*; ScoPel, protein deduced from gene SCO1110 from *Streptomyces coelicolor*; FoxP11, P11 from *Fusarium oxysporum*; FsoPelB, PelB from *Fusarium solani*; EchPelI, Pell from *Erwinia chrysanthemi*; EcaPel3, Pel3 from *Erwinia carotovora*. Amino acids conserved in the pectate lyases of the subgroup, and not found in the other enzymes aligned, are shaded. The conserved arginine residue proposed to initiate proton abstraction is in bold.

exogenous Ca^{2+} could result from the presence of trace amounts of contaminant Ca^{2+} ions in this substrate. In fact, activity was not detected when the chelator EDTA was included in the assays. The absolute requirement of Ca^{2+} ions for activity indicates that PelC is a pectate lyase, although, unusually, it shows higher activity on pectins than on polygalacturonic acid and shows activity on highly methylated pectins.

Comparison to PelA from *P. barcinonensis*

The high activity of PelC on pectins resembled that of PelA from *P. barcinonensis*. This enzyme had been previously characterized from crude cell extracts from the recombinant clone *E. coli*/pP22 (Soriano *et al.*, 2000). To compare the two pectate lyases more accurately, PelA was purified by His-tag affinity chromatography, and its properties were analysed

Fig. 2. (a) Electrophoretic analysis of purified PelC and PelA. Lanes 1, Coomassie blue stained SDS-polyacrylamide gels; lanes 2, zymograms of SDS-polyacrylamide gels. The positions of molecular mass markers are indicated. (b) Effect of pH and temperature on enzyme activity. Assays of PelC activity were performed with 22% methylated citrus pectin as substrate, while PelA activity was determined on polygalacturonic acid. The results are means of triplicate experiments.

after removal of the His-tag (Fig. 2). Activity of purified PelA on substrates of different degree of methyl esterification was evaluated. PelA showed maximum activity on polygalacturonic acid, although very similar activity (99% of maximum) was found on 22% esterified pectin (Fig. 3). On highly methylated pectin (89% esterification) the enzyme showed 39% of maximum activity. Activity of the enzyme on all these substrates showed an absolute requirement of Ca^{2+} , as no activity was found in the presence of EDTA. Maximum activity was found when 0.5 mM CaCl_2 was added to the assays (Fig. 3).

The effect of metal ions others than Ca^{2+} was analysed by performing activity assays in the presence of metal ions at 1 mM concentration, without Ca^{2+} added. In the case of PelA, activity was undetectable or very low in all assays, indicating that none of the ions studied could replace Ca^{2+} . However, when PelC activity was assayed in the presence of 1 mM Hg^{2+} , similar activity to control assays was found, suggesting that Hg^{2+} could replace Ca^{2+} . When the assays were performed in the simultaneous presence of Ca^{2+} and Hg^{2+} , each at 1 mM, a stimulation of PelC activity (120% of control) was found.

The effect of pH and temperature on activities of PelC and PelA was determined. The two enzymes exhibited activity only in a narrow alkaline pH range, with maximum activity at pH 10 (Fig. 2). PelC showed maximum activity at 65°C, while the optimum temperature for PelA activity was 55°C (Fig. 2). Stability assays showed that PelC was stable for at least 24 h at 50°C in buffers at pH 7, whereas PelA showed only 40% of initial activity after incubation under these conditions. When stability assays were performed at 50°C in buffers at pH 10, PelC showed 58% of initial activity after

24 h incubation, while PelA was completely inactivated by this treatment.

The kinetic parameters of purified pectate lyases on pectin and polygalacturonic acid were determined. PelC showed a $V_{\max}$ of 256.7 ± 23.9 units mg^{-1} and a K_m of 0.13 ± 0.03 mg ml^{-1} on 22% esterified pectin, while on polygalacturonic acid it showed a $V_{\max}$ of 59.6 ± 14.6 units mg^{-1} and a K_m of 0.15 ± 0.06 mg ml^{-1} (means $\pm$ SE, $n=3$). Kinetic parameters of PelA on 22% esterified pectin were $V_{\max}$ 102.4 ± 5.8 units mg^{-1} and K_m 0.14 ± 0.02 mg ml^{-1} , while on polygalacturonic acid they were $V_{\max}$ 95.4 ± 8.3 units mg^{-1} and K_m 0.16 ± 0.04 mg ml^{-1} (means $\pm$ SE, $n=3$).

Analysis of reaction products

To determine the mode of action of PelC and PelA, the reaction products obtained from various substrates were characterized by TLC. Both enzymes produced a mixture of oligomers from polygalacturonic acid (Fig. 4). The main product released from this substrate by PelC showed a mobility corresponding to unsaturated trigalacturonic acid. Unsaturated digalacturonic acid and oligomers of higher degree of polymerization were also released by the enzyme. PelA produced a mixture of reaction products from polygalacturonic acid similar to that produced by PelC, although the main products were oligomers longer than unsaturated trigalacturonate. Analysis of the reaction products from pectin showed that PelC and PelA released a mixture of oligomers with a similar pattern to that obtained from polygalacturonic acid. In contrast to the similar action of PelC and PelA on polygalacturonic acid and pectin, the enzymes behaved differently on trigalacturonate. PelC degraded trigalacturonate to galacturonic acid and unsaturated

Fig. 3. Enzymic properties of PelC and PelA. Left: influence of the degree of pectin methylation on activity. Activities of PelC and PelA on polygalacturonic acid (PGA), citrus pectins with degree of methyl esterification from 22% to 89%, and apple pectin (AP, 68% esterification) were determined by the standard assay. Activity of PelC on each substrate was expressed as a value relative to activity on 22% methylated pectin [358 units (mg protein)⁻¹], while activity of PelA on the different substrates was expressed as a value relative to activity on polygalacturonic acid [104 units (mg protein)⁻¹]. Right: influence of Ca²⁺ concentration on PelC and PelA activity. Activity on polygalacturonic acid and on 22% methylated citrus pectin was determined in the presence of different concentrations of CaCl₂. The results are means of triplicate experiments.

digalacturonate, whereas PelA was not active on this substrate.

DISCUSSION

We have identified and characterized a new pectin-depolymerizing enzyme from *B. subtilis* that cleaves bonds by β -elimination. The enzyme does not show homology to the two pectinases, a pectate lyase (Nasser *et al.*, 1993) and a pectin lyase (Sakamoto *et al.*, 1996), characterized up to now from this species. As no hydrolases acting on pectic polymers have been described in *B. subtilis*, the enzyme constitutes the third pectin depolymerase characterized in this species, and has been denominated PelC. The fact that until our work only two pectinases had been identified from *B. subtilis* contrasts with the pectin-depolymerizing activity of this species (Chesson & Codner, 1978) and the multiplicity of pectinases described in pectin-degrading microorganisms, which show complex sets of enzymes. In this way, at least eight pectate lyases have been cloned and

characterized in *Erwinia chrysanthemi* (Shevchik *et al.*, 1999b), while *Aspergillus niger* contains several pectin lyases, a pectate lyase and a family of polygalacturonases (Pařeniková *et al.*, 2000). We believe that *B. subtilis*, besides the three pectin-degrading enzymes characterized up to now, has several as yet unidentified pectinases and may exhibit a complex set of enzymes for pectin degradation.

One of the most notable features of PelC is its activity on both polygalacturonic acid and highly methylated pectin. Although many pectate lyases show maximum activity on polygalacturonic acid, some are most active on low methylated substrates (7–22% esterification) (Tardy *et al.*, 1997). The activity of these enzymes on pectin could indicate that they are pectin lyases rather than pectate lyases, although a clear difference between the two types of enzymes is the absolute Ca²⁺ requirement for catalysis of pectate lyases, while pectin lyases do not require Ca²⁺ (Jurnak *et al.*, 1996). Our results show that *B. subtilis* PelC requires added Ca²⁺ for activity on pectic substrates, indicating that it is a pectate lyase. The enzyme shows maximum activity on 22% methylated pectin, similar to what has been reported for *Er. chrysanthemi* PelB (Tardy *et al.*, 1997). However, in contrast to known pectate lyases, PelC shows high activity (43% of maximum activity) on 89% methylated pectins. This is a unique trait among pectate lyases characterized to date, which are not active or show very low activity on highly methylated pectins (Benen & Visser, 2003; Hugouvieux-Cotte-Pattat *et al.*, 1996), although some pectate lyases of family 3, such as PelI from *Er. chrysanthemi* and PelB from *Er. carotovora*, exhibit maximum activity on 45% and 68% esterified pectin, respectively (Shevchik *et al.*, 1998; Heikinheimo *et al.*, 1995). Pectate lyase PelA from *P. barcinonensis* (Soriano *et al.*, 2000), the enzyme that led to identification of PelC, resembles this enzyme in its high activity on highly esterified pectin (39% of maximum activity on 89% methylated pectin). This is a difference between these two enzymes (PelC and PelA) and the pectate lyases described up to now.

Sequence comparison with proteins contained in databases revealed that PelC from *B. subtilis* and PelA from *P. barcinonensis* belong to family 3 pectate lyases. Sequence alignment of enzymes of this family has identified four blocks of conserved residues, proposed as signatures of pectate lyases of family 3 (Shevchik *et al.*, 1997). These blocks of residues are also found in pectate lyases from plant-parasitic nematodes (Huang *et al.*, 2005). However, the two pectate lyases characterized in this work, together with pectate lyase from *Bacillus* sp. P-2850 (accession no. Q8LOR5) and pectate lyase from *Bacillus* sp. KSM-P15 (Hatada *et al.*, 2000) do not show the fourth block of conserved residues. In the corresponding area of these four enzymes (residues 172–221 in the case of PelC) analysis with ProDom at NCBI (Servant *et al.*, 2002) has identified domain ID:PD346936, not found in other enzymes. The four pectate lyases show high homology (more than 50% identity in amino acid sequence), similar size (221–224 amino acids), and low content of cysteine

Fig. 4. Reaction products from polygalacturonic acid, pectin and trigalacturonate. Samples of 1.2 µg purified PelC or 2.6 µg purified PelA were incubated in 500 µl assay mixture containing 0.15% substrate at 55 °C (PelC) or 40 °C (PelA) and samples were taken for analysis of the reaction products at time 0 (lanes 0), 30 min (lanes 1), 3 h (lanes 2) and 24 h (lanes 3). PGA, polygalacturonic acid; pectin, 22 % methylated citrus pectin (PelC) or 89 % methylated citrus pectin (PelA); G₃, trigalacturonate. Lanes M contain size markers: galacturonic acid (G), digalacturonate (G₂) and trigalacturonate (G₃) (Sigma).

residues (1–3) compared to the rest of the enzymes of family 3, which show a higher cysteine content [12 residues in the case of PelB from *Fusarium solani* (Guo *et al.*, 1995)]. The results show that PelC from *B. subtilis* and PelA from *P. barcinonensis*, together with pectate lyase from *Bacillus* sp. P-2850 and pectate lyase from *Bacillus* KSM-P15, constitute a subgroup of homology among family 3 pectate lyases. A dendrogram of enzymes of family 3 generated with CLUSTAL W (Thompson *et al.*, 1994) showed that these four pectate lyases cluster separately from the rest of enzymes of family 3 (data not shown), and form a subgroup of pectate lyases from saprophytic bacteria in a family where most of the enzymes belong to pathogenic micro-organisms. Analysis of the hypothetical protein deduced from the *yvpA* gene from *B. licheniformis* (accession no. Q65EF5) reveals it shares all the common traits of the enzymes of the subgroup identified and clusters with them.

As mentioned above, PelC and PelA have as a distinctive character their high activity on pectin with a high degree of methyl esterification. This property is not shared by the other enzymes of the homology subgroup. While activity results of pectate lyase from *Bacillus* sp. P-2850 have not been reported, pectate lyase from *Bacillus* sp. KSM-P15 shows a clear preference for polygalacturonic acid and very low activity on methylated pectins (Kobayashi *et al.*, 1999). Despite their similarity, PelC and PelA show some differences regarding substrate specificity. PelC shows a clear preference for 22 % methylated pectin, whereas PelA shows similar activity on polygalacturonic acid and 22 % esterified pectin. Study of the mode of action on polymeric substrates showed that PelC and PelA have an endo mode of action, producing a mixture of degradation products. A similar mode of action has been reported for pectate lyases of family

1, such as pectate lyase A from *Clostridium cellulovorans* (Tamaru & Doi, 2001), family 3, such as PelI from *Er. chrysanthemi* (Shevchik *et al.*, 1997), and family 9, such as PelL from *Er. chrysanthemi* (Lojewska *et al.*, 1995). In contrast, PelX and PelW from *Er. chrysanthemi* (Shevchik *et al.*, 1999a, b), which belong to families 9 and 2, respectively, show an exo mode of action, releasing unsaturated digalacturonate as the only degradation product from polygalacturonic acid. PelA and PelC differed in their activity on trigalacturonate: PelA did not degrade this substrate, whereas PelC cleaved it to galacturonate and unsaturated digalacturonate. The lack of trigalacturonate degradation by PelA is similar to that reported for endopectate lyase A from *Clostridium cellulovorans* (Tamaru & Doi, 2001) and to the requirement for substrates longer than a trimer for effective catalysis of several endopectate lyases from *Er. chrysanthemi* (Roy *et al.*, 1999). However, degradation of trigalacturonate by PelC resembles the results described for exopectate lyases PelX and PelW of *Er. chrysanthemi* (Shevchik *et al.*, 1999a, b). The scarcity of reports about the use of trigalacturonate as a substrate by pectate lyases makes it difficult to understand the significance of the different behaviour of PelA and PelC on this oligomer.

In summary, we have purified and characterized two novel pectate lyases, PelC from *B. subtilis* and PelA from *P. barcinonensis*, which differ from known pectate lyases in their high activity on highly methylated pectin. The characterized enzymes, together with pectate lyase from *Bacillus* sp. P-2850 and pectate lyase from *Bacillus* sp. KSM-P15, constitute a subgroup of highly homologous enzymes in family 3. The biochemical properties of the four pectate lyases need to be studied in more detail to identify common features of this subgroup of enzymes, which to our knowledge are the

only pectate lyases from non-pathogenic micro-organisms classified in family PL3.

ACKNOWLEDGEMENTS

We thank Serveis Científic-Tècnics of the University of Barcelona for their support in nucleotide sequencing. This work was partially supported by the Spanish Ministry of Science and Technology, grant no. PPQ2000-0091-P4-04, the Generalitat de Catalunya (III Pla de Recerca, project ref. 2001SGR 00143), and Centre de Referència en Biotecnologia (CeRba). The experiments described in this article have been performed in compliance with current Spanish laws.

REFERENCES

- Akita, M., Suzuki, A., Kobayashi, T., Ito, S. & Yamane, T. (2001). The first structure of pectate lyase belonging to polysaccharide lyase family 3. *Acta Crystallograph D* **57**, 1786–1792.
- Barras, F., van Gijsegem, F. & Chatterjee, A. K. (1994). Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu Rev Phytopathol* **32**, 201–234.
- Benen, J. A. E. & Visser, J. (2003). Pectate and pectin lyases. In *Handbook of Food Enzymology*, pp. 1029–1041. Edited by J. R. Whitaker, A. Voragen & D. W. S. Wong. New York: Dekker.
- Bentley, S. D., Chater, K. F., Cerdano-Tarraga, A. M. & 40 other authors (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141–147.
- Berensmeier, S., Singh, S. A., Meens, J. & Buchholz, K. (2004). Cloning of the *pelA* gene from *Bacillus licheniformis* 14A and biochemical characterization of recombinant, thermostable, high-alkaline pectate lyase. *Appl Microbiol Biotechnol* **64**, 560–567.
- Brown, I. E., Mallen, M. H., Charnock, S. J., Davies, G. J. & Black, G. W. (2001). Pectate lyase 10A from *Pseudomonas cellulosa* is a modular enzyme containing a family 2a carbohydrate-binding module. *Biochem J* **355**, 155–165.
- Chesson, A. & Codner, R. C. (1978). The maceration of vegetable tissue by a strain of *Bacillus subtilis*. *J Appl Bacteriol* **44**, 347–364.
- Collmer, A., Ried, J. L. & Mount, M. S. (1988). Assay methods for pectic enzymes. *Methods Enzymol* **161**, 329–335.
- Coutinho, P. M. & Henrissat, B. (1999). Carbohydrate-active enzymes: an integrated database approach. In *Recent Advances in Carbohydrate Bioengineering*, pp. 3–12. Edited by H. J. Gilbert, G. J. Davies, B. Henrissat & B. Svensson. Cambridge: Royal Society of Chemistry.
- Fürste, J. P., Pansegrau, W., Frank, R., Bloecker, H., Scholz, P., Bagdasarian, M. & Lanka, E. (1986). Molecular cloning of the plasmid RP-4 primase region in a multi-host-range Tac-P expression vector. *Gene* **48**, 119–132.
- Guo, W., González-Candelas, L. & Kolattukudy, P. E. (1995). Cloning of a novel constitutively expressed pectate lyase gene *pelB* from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of the gene product expressed in *Pichia pastoris*. *J Bacteriol* **177**, 7070–7077.
- Hatada, Y., Saito, K., Koike, K., Yoshimatsu, T., Ozawa, T., Kobayashi, T. & Ito, S. (2000). Deduced amino-acid sequence and possible catalytic residues of a novel pectate lyase from an alkalophilic strain of *Bacillus*. *Eur J Biochem* **267**, 2268–2275.
- Heikinheimo, R., Flego, D., Pirhonen, M., Karlsson, M. B., Eriksson, A., Mäe, A., Kõiv, V. & Palva, E. T. (1995). Characterization of a novel pectate lyase from *Erwinia carotovora* subsp. *carotovora*. *Mol Plant Microbe Interact* **8**, 207–217.
- Herron, S. R., Benen, J. A. E., Scavetta, R. D., Visser, J. & Jurnak, F. (2000). Structure and function of pectic enzymes: virulence factors of plant pathogens. *Proc Natl Acad Sci U S A* **97**, 8762–8769.
- Hoondal, G. S., Tiwari, R. P., Tewari, R., Dahiya, N. & Beg, Q. K. (2002). Microbial alkaline pectinases and their industrial applications: a review. *Appl Microbiol Biotechnol* **59**, 409–418.
- Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J. & Hussey, R. S. (2005). Developmental expression and molecular analysis of two *Meloidogyne incognita* pectate lyase genes. *Int J Parasitol* **35**, 685–692.
- Huertas-González, M. D., Ruiz-Roldan, M. C., Garcia-Maceira, F. I., Roncero, M. I. & Di Pietro, A. (1999). Cloning and characterization of *plI* encoding an in planta-secreted pectate lyase of *Fusarium oxysporum*. *Curr Genet* **35**, 36–40.
- Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W. & Reverchon, S. (1996). Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu Rev Microbiol* **50**, 213–257.
- Jenkins, J., Shevchik, V. E., Hugouvieux-Cotte-Pattat, N. & Pickersgill, R. W. (2004). The crystal structure of pectate lyase Pel9A from *Erwinia chrysanthemi*. *J Biol Chem* **279**, 9139–9145.
- Jurnak, F., Kita, N., Garrett, M., Heffron, S. E., Scavetta, R., Boyd, C. & Keen, N. (1996). Functional implications of the three-dimensional structures of pectate lyases. In *Pectin and Pectinases*, vol. 14, pp. 295–308. Edited by J. Visser & A. G. J. Voragen. Amsterdam: Elsevier.
- Kashyap, D. R., Vohra, P. K., Chopra, S. & Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. *Bioresour Technol* **77**, 215–227.
- Klusens, L. D., Van Alebeek, G. J. W. M., Voragen, A. G. J., De Vos, W. M. & Van Der Oost, J. (2003). Molecular and biochemical characterization of the thermoactive family 1 pectate lyase from the hyperthermophilic bacterium *Thermotoga maritima*. *Biochem J* **370**, 651–659.
- Kobayashi, T., Koike, K., Yoshimatsu, T., Higaki, N., Suzumatsu, A., Ozawa, T., Hatada, Y. & Ito, S. (1999). Purification and properties of a low-molecular-weight, high-alkaline pectate lyase from an alkaliphilic strain of *Bacillus*. *Biosci Biotechnol Biochem* **63**, 65–72.
- Kunst, F., Ogasawara, N., Moszer, I. & 148 other authors (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Liu, Y., Chatterjee, A. & Chatterjee, A. K. (1994). Nucleotide sequence and expression of a novel pectate lyase gene (*pel-3*) and a closely linked endopolygalacturonase gene (*peh-1*) of *Erwinia carotovora* subsp. *carotovora* 71. *Appl Environ Microbiol* **60**, 2545–2552.
- Lojkowska, E., Masclaux, C., Boccara, M., Robert-Baudouy, J. & Hugouvieux-Cotte-Pattat, N. (1995). Characterization of the *pelI* gene encoding a novel pectate lyase of *Erwinia chrysanthemi* 3937. *Mol Microbiol* **16**, 1183–1195.
- Nagarajan, V. (1993). Protein secretion. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, pp. 713–726. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Nasser, W., Awadé, A. C., Reverchon, S. & Robert-Baudouy, J. (1993). Pectate lyase from *Bacillus subtilis*: molecular characterization of the gene, and properties of the cloned enzyme. *FEBS Lett* **335**, 319–326.
- Omura, S., Ikeda, H., Ishikawa, J. & 11 other authors (2001). Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci U S A* **98**, 12215–12220.

- Pařeniková, L., Benen, J. A. E., Kester, H. C. M. & Visser, J. (2000). *pgaA* and *pgbB* encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*. *Biochem J* **345**, 637–644.
- Roy, C., Kester, H., Visser, J., Shevchik, V., Hugouvieux-Cotte-Pattat, N., Robert-Baudouy, J. & Bennen, J. (1999). Modes of action of five different endopectate lyases from *Erwinia chrysanthemi* 3937. *J Bacteriol* **181**, 3705–3709.
- Sakamoto, T., Kawasaki, H. & Sakai, T. (1996). Molecular cloning and nucleotide sequence of the gene encoding phosphate-inducible pectin lyase of *Bacillus subtilis*. *FEBS Lett* **398**, 269–273.
- Sánchez, M. M., Fritze, D., Blanco, A., Spröer, C., Tindall, B. J., Schumann, P., Kroppenstedt, R. M., Diaz, P. & Pastor, F. I. J. (2005). *Paenibacillus barcinonensis* sp. nov., a xylanase-producing bacterium isolated from a rice field in the Ebro River delta. *Int J Syst Evol Microbiol* **55**, 935–939.
- Schols, H. A. & Voragen, A. (2003). Pectic polysaccharides. In *Handbook of Food Enzymology*, pp. 829–843. Edited by J. R. Whitaker, A. Voragen & D. W. S. Wong. New York: Dekker.
- Servant, F., Bru, C., Carrère, S., Courcelle, E., Gouzy, J., Peyruc, D. & Kahn, D. (2002). ProDom: automated clustering of homologous domains. *Brief Bioinform* **3**, 246–251.
- Shevchik, V. E., Robert-Baudouy, J. & Hugouvieux-Cotte-Pattat, N. (1997). Pectate lyase PelI of *Erwinia chrysanthemi* 3937 belongs to a new family. *J Bacteriol* **179**, 7321–7330.
- Shevchik, V. E., Boccara, M., Vedel, R. & Hugouvieux-Cotte-Pattat, N. (1998). Processing of the pectate lyase PelI by extracellular proteases of *Erwinia chrysanthemi* 3937. *Mol Microbiol* **29**, 1459–1469.
- Shevchik, V. E., Condemine, G., Robert-Baudouy, J. & Hugouvieux-Cotte-Pattat, N. (1999a). The exopolygalacturonate lyase PelW and the oligogalacturonate lyase Ogl, two cytoplasmic enzymes of pectin catabolism in *Erwinia chrysanthemi* 3937. *J Bacteriol* **181**, 3912–3919.
- Shevchik, V. E., Kester, H. C. M., Benen, J. A. E., Visser, J., Robert-Baudouy, J. & Hugouvieux-Cotte-Pattat, N. (1999b). Characterization of the exopolygalacturonate lyase PelX of *Erwinia chrysanthemi* 3937. *J Bacteriol* **181**, 1652–1663.
- Soriano, M., Blanco, A., Diaz, P. & Pastor, F. I. J. (2000). An unusual pectate lyase from a *Bacillus* sp. with high activity on pectin: cloning and characterization. *Microbiology* **146**, 89–95.
- Studier, F. W. & Moffatt, B. A. (1986). Use of bacteriophage T-7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Tamaru, Y. & Doi, R. H. (2001). Pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome. *Proc Natl Acad Sci U S A* **98**, 4125–4129.
- Tardy, F., Nasser, W., Robert-Baudouy, J. & Hugouvieux-Cotte-Pattat, N. (1997). Comparative analysis of the five major *Erwinia chrysanthemi* pectate lyases: enzyme characteristics and potential inhibitors. *J Bacteriol* **179**, 2503–2511.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Vellanoweth, R. L. (1993). Translation and its regulation. In *Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, pp. 699–711. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Yoder, M. D., Keen, N. T. & Jurnak, F. (1993). New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. *Science* **260**, 1503–1507.