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

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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.

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* (Berensemeier et al., 2004; Hatada et al., 2000; Nasser et al., 1993) and in some thermophilic bacteria (Klusknens 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
methylated 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 pFJ118HE (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′-ATCACCCGGGAGATCGATT-3′ and 5′-CCGAGGTCTTGTCATAG-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 pFJ118HE previously digested with SmaI, and introduced by transformation into E. coli 5K. The recombinant strain containing the yvpA gene was denominated E. coli/pYPvA.

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′-TTGCTAGGCTGACAAAGTGG-3′ and 5′-CTAAGCTTGTATTTAATGTGTTT-3′. The amplified fragment was digested with Nhel and HindIII, inserted between the Nhel and HindIII sites of plasmid pET28a, and transformed into E. coli BL21(DE3). The recombinant plasmid pET28a-YvpA was obtained and 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-Val was added to mature PelC. For expression of pelA from P. barcinonensis, a similar approach was used. The oligonucleotides used for pelA amplification were 5′-CTCATATGGGGGCAAGGTC-3′ and 5′-GGAAGGTTATTACTTGTTATTCCG-3′. The amplified fragment was digested with Nhel and HindIII, 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, 50 mM sodium acetate pH 4–0–6, 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 Vmax and Km 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 25% (w/v) TCA 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/acidic 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 methylated 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 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 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 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 the ρ^b^ 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.

Homology was also found to open reading frames deduced from genes SCO1110 from *Streptomyces coelicolor* (Bentley *et al.*, 2002) and SA6383 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: P1 from *Fusarium oxysporum* (Huertas-González *et al.*, 1999), Pell 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 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

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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 analyzed.

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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*; FoxPel1, Pel1 from *Fusarium oxysporum*; FsoPelB, PelB from *Fusarium solani*; EchPel, Pel from *Erwinia chrysanthemi*; EcaPel3, Pel 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.

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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 Ca²⁺, as no activity was found in the presence of EDTA. Maximum activity was found when 0.5 mM CaCl₂ was added to the assays (Fig. 3).

The effect of metal ions others than Ca²⁺ was analysed by performing activity assays in the presence of metal ions at 1 mM concentration, without Ca²⁺ added. In the case of PelA, activity was undetectable or very low in all assays, indicating that none of the ions studied could replace Ca²⁺. However, when PelC activity was assayed in the presence of 1 mM Hg²⁺, similar activity to control assays was found, suggesting that Hg²⁺ could replace Ca²⁺. When the assays were performed in the simultaneous presence of Ca²⁺ and Hg²⁺, 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 Vₘₐₓ of 256.7 ± 23.9 units mg⁻¹ and a Kₘ of 0.13 ± 0.03 mg ml⁻¹ on 22% esterified pectin, while on polygalacturonic acid it showed a Vₘₐₓ of 59.6 ± 14.6 units mg⁻¹ and a Kₘ of 0.15 ± 0.06 mg ml⁻¹ (means ± SE, n = 3). Kinetic parameters of PelA on 22% esterified pectin were Vₘₐₓ 102.4 ± 5.8 units mg⁻¹ and Kₘ 0.14 ± 0.02 mg ml⁻¹, while on polygalacturonic acid they were Vₘₐₓ 95.4 ± 8.3 units mg⁻¹ and Kₘ 0.16 ± 0.04 mg ml⁻¹ (means ± 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 trigalacturonic acid. 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 trigalacturionate. PelC degraded trigalacturionate to galacturonic acid and unsaturated...
organisms, which show complex sets of enzymes. In this
text, the abundance of pectinases described in pectin-degrading micro-
organisms was noted, particularly for the B. subtilis species (Chesson & Codner, 1978) and the multi-
ple pectinases identified from this species. As no hydrolases acting on pectic
substrates were identified, the two pectinases, a pectate lyase (Nasser
et al., 1993) and a family of polygalacturonases (Parˇenikova´ et al., 2000), were determined. 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 methy-
lated 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 requirement for catalysis of pectate lyases, while pectin lyases do not require 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 Pel 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. barci-
nonensis (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. barci-
nonensis 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 nema-
todes (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 corres-
ponding 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

**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 micro-
organisms, which show complex sets of enzymes. In this way, at least eight pectate lyases have been cloned and
digalacturionate, whereas PelA was not active on this
substrate.

**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+ concentra-
tion 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.
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 \textit{Bacillus} sp. P-2850 have not been reported, pectate lyase from \textit{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 \textit{Clostridium cellulovorans} (Tamaru & Doi, 2001), family 3, such as Pell from \textit{Er. chrysanthemi} (Shevchik et al., 1997), and family 9, such as Pell from \textit{Er. chrysanthemi} (Lojkowska et al., 1995). In contrast, PelX and PelW from \textit{Er. chrysanthemi} (Shevchik et al., 1999a, b), which belong to families 9 and 2, respectively, show an exo mode of action, releasing unsaturated digalacturionate 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 galacturonic acid and unsaturated digalacturionate. The lack of trigalacturonate degradation by PelA is similar to that reported for endopectate lyase A from \textit{Clostridium cellulovorans} (Tamaru & Doi, 2001) and to the requirement for substrates longer than a trimer for effective catalysis of several endopectate lyases from \textit{Er. chrysanthemi} (Roy et al., 1999). However, degradation of trigalacturonate by PelC resembles the results described for exopectate lyases PelX and PelW of \textit{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 \textit{B. subtilis} and PelA from \textit{P. barcinonensis}, which differ from known pectate lyases in their high activity on highly methylated pectin. The characterized enzymes, together with pectate lyase from \textit{Bacillus} sp. P-2850 and pectate lyase from \textit{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.

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REFERENCES


