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Purification and Characterization of an Extracellular Pectate Lyase from an *Amycolata* sp.

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The extracellular pectate lyase (EC 4.2.2.2) of a nonsporulating *Amycolata* sp. was purified to homogeneity by anion- and cation-exchange chromatographies followed by hydrophobic interaction chromatography. The enzyme cleaved polygalacturonate but not highly esterified pectin in a random endolytic transeliminative mechanism that led to the formation of a wide range of 4,5-unsaturated oligogalacturonates. As shown by high-performance anion-exchange chromatography and pulsed amperometric detection, these unsaturated oligogalacturonates were further depolymerized by the enzyme to the unsaturated dimer and trimer as final products. The pectate lyase had a molecular weight of 31,000 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a molecular mass of 30,000 Da determined by matrix-assisted laser desorption ionization mass spectrometry. The isoelectric point of the protein was 10. Maximum activity occurred at pH 10.25. Calcium was essential for activity, and EDTA inactivated the enzyme under standard assay conditions. Interestingly, EDTA did not inhibit the ability of the enzyme to cleave the native pectin (protopectin) of ramie (*Boehmeria nivea*) fibers. The K_m value with sodium polygalacturonate as the substrate was 0.019 g liter^{-1}. The purified enzyme lost its activity after a 1-h incubation at 50°C but was stabilized by calcium or polygalacturonate. The N-terminal sequence showed high similarity within a stretch of 13 amino acids to the N-terminal sequences of pectate lyases PLa and PLe from *Erwinia chrysanthemi*. The *Amycolata* sp. did not produce additional isozymes of pectate lyase but produced further activities of pectinesterase, xylanase, and carboxymethyl cellulase when grown in a medium with decorticated bast fibers from ramie as the sole carbon source.

Pectinolytic enzymes play an important role in the decomposition of plant residues and are widely distributed among soil and compost microorganisms such as actinomycetes (14). A nonsporulating, pectinolytic species of *Amycolata* was recently isolated from a soil sample from Thailand (3). To date, there are no reports available on the extracellular polysaccharide-degrading enzymes of this bacterium. However, screening of pectinolytic actinomycetes has revealed pectate lyases of the exo and endo types (3, 24, 29). Activities of pectinesterases were found only recently in these bacteria (3, 30). Some pectate lyases from *Streptomyces* spp. (21–23, 25, 26) and from *Thermomonospora fusca* (30) have been isolated and characterized, but no detailed studies of the products released by pectate lyases from actinomycetes have been carried out so far. Besides being produced by bacteria, pectate lyases are produced by fungi. A pollen protein exhibiting a pectate lyase activity was isolated only recently from Japanese cedars (32).

Pectate lyases cleave the α-1,4-glycosidic bonds in galacturonans by β elimination, thereby generating 4,5-unsaturated oligogalacturonates (1). The breakdown of pectin located in the middle lamella and primary cell wall leads to the maceration of plant tissues (4, 10). Endopectate lyases have much higher levels of maceration activity than exopectate lyases (9). Pectate lyases are known to be virulence factors in many plant pathogenic (soft-rotting) microorganisms (10). Because of their high pH optima (in general, pH 8 to 10.5), pectate lyases have not been used in the food industry, but they might be useful for retting and degumming processes in the textile industry (3, 19). Enzymatic retting of bast fibers from jute, flax, hemp, or ramie for the manufacturing of textiles may be faster and more reproducible than traditional methods which rely on spontaneous microbial processes. The availability of purified pectinolytic enzymes may help in the design of enzyme preparations highly efficient in the removal of noncellulose polymers from bast fibers.

This is the first report that describes the induction, purification, and characterization of an extracellular pectate lyase from an actinomycete of the genus *Amycolata.*

MATERIALS AND METHODS

Strain. The strain used in this work was isolated from a sand sample from Thailand as described previously (3). It was tentatively identified as a nonsporulating species of *Amycolata* by E. Wellington (University of Warwick, Coventry, United Kingdom). The strain was stored as suspension of spores and hyphae in a 25% (vol/vol) aqueous solution of glycerol at −70°C.

Growth of the organism. The organism was grown at 30°C in 300-ml Erlenmeyer flasks containing 100 ml of medium on a rotary shaker at 150 rpm. One liter of medium contained the following components: decorticated, cut ramie fibers, 15 g; yeast extract, 1 g; $(NH_4)_2SO_4$, 2.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; K_2HPO_4, 5.5 g; KH_2PO_4, 2.4 g; and trace element solution (3), 1 ml. Decorticated, dried fibers from ramie (*Boehmeria nivea*) were cut to 0.5 cm in length, sterilized separately in deionized water, and added to sterile double-strength medium after being cooled. The shake flasks were inoculated with 1 ml of a cell suspension harvested from a glucose-grown culture after 48 h.

Enzyme assays. Pectate lyase was estimated spectrophotometrically by measuring the increase in A_{235} (1). The reaction mixtures contained 300 μl of 0.2 M glycine–NaOH buffer (pH 10.25) with 1 mM $CaCl_2$ (freshly prepared, free of carbonate), 300 μl of 0.5% sodium polygalacturonate (Sigma), and 25 μl of diluted enzyme solution. Measurements were carried out at room temperature with a UV-250 spectrophotometer (Shimadzu Scientific Instruments and Equipment, Kyoto, Japan). One unit of enzyme was defined as the amount of enzyme which forms 1 μmol of a product per min with a molar extinction coefficient (ε) of 4,600 liters mol^{-1} cm^{-1} (34).

Polygalacturonase, pectinesterase, xylanase, and carboxymethyl cellulase activities were measured as described previously (3).

Protein determination. Protein concentrations were determined by the

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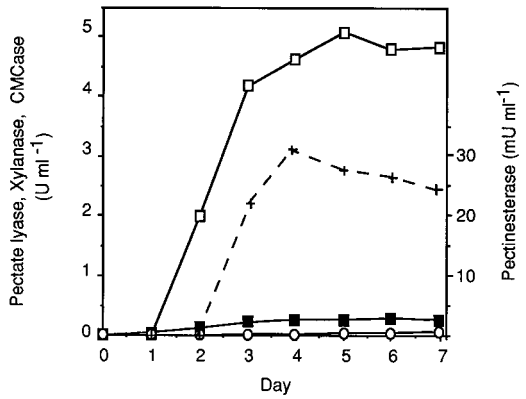

FIG. 1. Time courses of pectate lyase (□), xylanase (■), carboxymethyl cellulase (CMCase) (○), and pectinesterase (+) production by the *Amycolata* sp. grown in a medium with ramie fibers as the sole carbon source.

method of Bradford (2) with bovine serum albumin (Bio-Rad, Richmond, Calif.) as the standard.

Concentration of culture supernatant. The extracellular enzyme solution was recovered after centrifugation (30 min at 5,000 × *g*) by filtration through a glass fiber filter (Whatman GF/A). The crude enzyme solution was concentrated at 4°C by ultrafiltration in a stirred Amicon cell (Amicon, Danvers, Mass.) with an Amicon PM1 membrane (1-kDa cutoff). Approximately 30% of the total active pectate lyase passed through the membrane and was found in the ultrafiltrate when a PM10 membrane (10-kDa cutoff) was used. The concentrated enzyme solution was stored at −20°C.

Purification of pectate lyase. All steps of fast-protein liquid chromatography (FPLC) were carried out at room temperature.

(i) Anion-exchange chromatography. One milliliter of the concentrated crude enzyme was applied to a DEAE-Sepharose Fast Flow column (1 by 5 cm; Pharmacia, Uppsala, Sweden) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0). After the column was washed with the equilibration buffer (14 ml), the proteins were eluted with a linear NaCl gradient ranging from 0 to 1 M NaCl in 10 min. The flow rate was 1 ml min^{-1}. Fractions (1 ml) were collected, and active protein was pooled.

(ii) Cation-exchange chromatography. The pooled fractions from the DEAE-Sepharose chromatography were concentrated to a final volume of 1 ml with a microconcentrator unit (Skan, Basel, Switzerland; 1-kDa cutoff). The concentrated enzyme solution was applied to a Mono S HR 5/5 column (Pharmacia) that had previously been equilibrated with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 8.0). After the column was washed with 10 ml of equilibration buffer, the proteins were eluted with a linear NaCl gradient ranging from 0 to 1 M NaCl in 15 min. The flow rate was adjusted to 0.5 ml min^{-1}. Fractions (0.5 ml) were collected, and active protein was pooled.

(iii) Hydrophobic interaction chromatography. The active fractions from the Mono S column were concentrated in a microconcentrator (1-kDa cutoff), and the buffer was replaced by 50 mM phosphate buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$. The purification was carried out on a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$. The proteins were eluted with 15 ml of a decreasing salt gradient ranging from 1.7 to 0 M $(NH_4)_2SO_4$. The flow rate was adjusted to 0.5 ml min^{-1}. Fractions (0.5 ml) were collected, and active protein was pooled. The eluted protein could be stored at −20°C for several months without loss of activity.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with slab gels (11.5 by 14 by 0.1 cm) by the discontinuous buffer system of Laemmli as modified by Schägger and von Jagow (27). The separating gel (10%) was overlaid with a stacking gel (4%). The amount of proteins applied to the gel was between 0.5 and 5 μg. A standard protein mixture with six proteins ranging in size from 14,400 to 97,400 Da was purchased from Bio-Rad. The electrophoresis was performed at room temperature for 1 h at 30 V and then for 4 h at 100 V. Proteins in the SDS-polyacrylamide gels were silver stained by the method of Nielsen and Brown (12). SDS-PAGE for routine analytical testing was also performed on precast minigels (12.5% T, 8 to 25% C) with a Phast System from Pharmacia.

IEF. Analytical isoelectric focusing (IEF) was performed on 0.5% (wt/vol) agarose gels with a broad-range ampholyte (Pharmacia) ranging in pI from 3.5 to 10. Separations were carried out as recommended by Pharmacia (13). A protein calibration kit with pI values from 4.65 to 9.6 was purchased from Bio-Rad. IEF in the alkaline range with Servalyte 9-11 (Serva) was performed with a sealed flatbed apparatus flushed with nitrogen to minimize interference with atmospheric CO_2. All separations were done at 8°C. The pectate lyase was detected by the replica gel technique (17). Pectate-agarose overlays were placed in contact

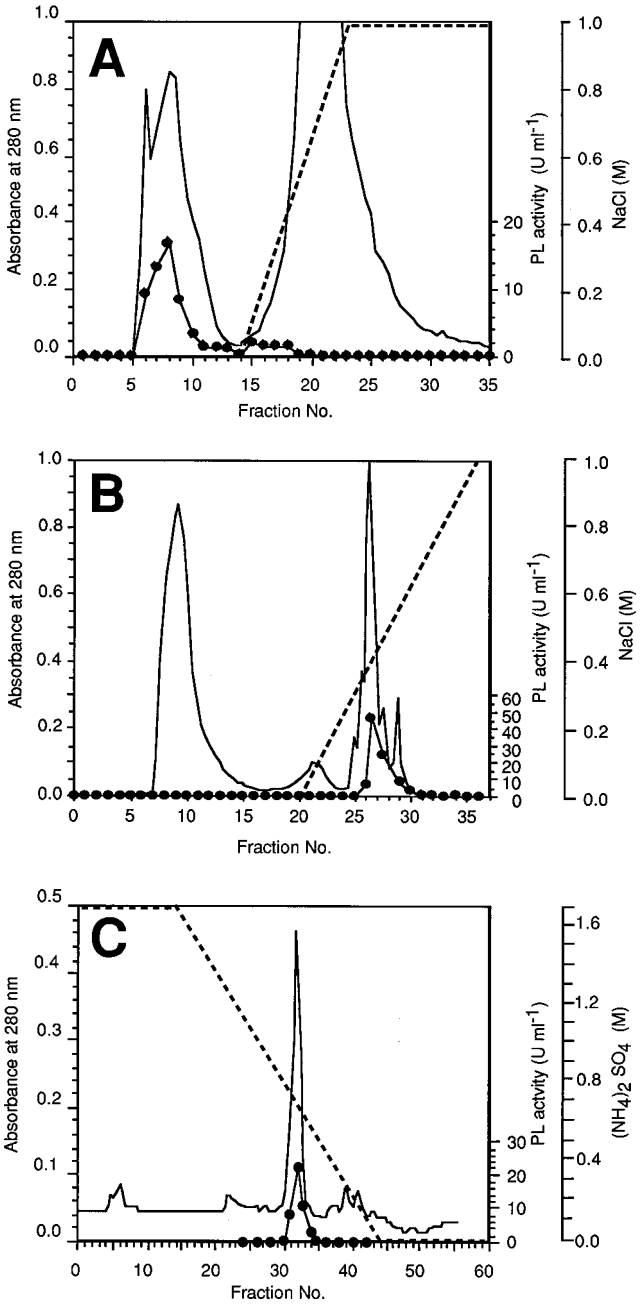

FIG. 2. FPLC of crude pectate lyase (PL) from the *Amycolata* sp. (A) Concentrated culture supernatant was applied to a DEAE-Sepharose Fast Flow column equilibrated with 10 mM Tris-HCl buffer (pH 8). Elution was carried out first with the equilibration buffer and then with a linear gradient of NaCl. (B) Active fractions from the DEAE-Sepharose column were adjusted to 10 mM HEPES (pH 8) and applied to a Mono S HR 5/5 column. Elution was carried out with an increasing NaCl gradient. (C) Active fractions from the Mono S column were adjusted to 1.7 M $(NH_4)_2SO_4$ and applied to a Phenyl-Superose HR 5/5 column. Elution was carried out with a decreasing linear gradient of $(NH_4)_2SO_4$. For each purification step, fractions were collected and assayed for pectate lyase activity (●), concentration of NaCl (A and B) or $(NH_4)_2SO_4$ (C) (– –), and A_{280} (——).

with the IEF gel for 12 h at room temperature before being stained with ruthenium red. Protein bands were also visualized by silver staining by the method of Vesterberg and Gramstrup-Christensen (33) or with Coomassie blue R-250 (13).

Gel filtration. The molecular weight of the purified protein was also estimated with an FPLC system by gel filtration with a Superose 12 HR 10/30 column

TABLE 1. Purification of pectate lyase from the *Amycolata* sp. used in this study

Procedure	Vol (ml)	Activity (U ml^{-1})	Protein (μg ml^{-1})	Sp act (U mg^{-1})	Yield (%)
Crude extract	1	86.4	1,808	47.8	100
DEAE-Sepharose	5	15.5	246	63.0	89.7
Mono S	1.5	40.2	303	132.7	69.8
Phenyl-Superose	1.5	21.1	121	174.4	36.6

(Pharmacia) previously equilibrated with a buffer containing 50 mM phosphate (pH 7) and 150 mM NaCl. The injected-sample volume was 0.2 ml, and the flow rate was adjusted to 0.5 ml min^{-1}. A low-molecular-weight calibration kit (Pharmacia) was used to calibrate the column.

Mass spectrometry. Matrix-assisted laser desorption ionization mass spectrometry of the purified pectate lyase was performed on an LDI-1700 mass spectrometer (Linear Scientific Inc., Reno, Nev.). Sinapinic acid (0.1 M) served as the matrix. The molecular masses of the unsaturated di- and trigalacturonates were determined by fast atom bombardment mass spectrometry on a VG ZAB-SEQ mass spectrometer (VG Instruments, Manchester, United Kingdom). Glycerol served as the solvent.

Effect of temperature. The temperature optimum of the purified pectate lyase was evaluated at temperatures ranging from 20 to 98°C under standard assay conditions at pH 10.25. The reaction was stopped by the addition of ice-chilled 1 M sodium acetate (pH 4) to the reaction mixture. Enzyme stability at different temperatures was measured by incubating the enzyme for up to 5 days in 10 mM phosphate buffer (pH 7.0) at temperatures ranging from 20 to 98°C.

Effect of pH. The effect of pH on the activity of the enzyme was determined under standard assay conditions. The pH of the reaction mixture was controlled with a pH meter. The stability of the enzyme in buffers with different pH ranges was also tested. The following buffers (each 50 mM) were used: citrate (pH 4 to 6), bis-Tris propane (pH 6 to 9), and glycine-NaOH (pH 9 to 11). Residual activity was measured after a 24-h incubation of the enzyme at room temperature.

Substrate specificity. The relative activity of the pectate lyase toward several pectins with different degrees of esterification (Sigma, St. Louis, Mo.) was tested under standard assay conditions. In addition to the determination of cellulase and xylanase activities, the following substrates were similarly tested: crystalline cellulose (Serva), gum arabic (Sigma), locust bean gum (Sigma), arabinogalactan (Jensen), arabinan (MegaZyme, North Rocks, Australia), and alginate (Fluka, Buchs, Switzerland). Crude pectate lyase and purified pectate lyase (0.33 U ml^{-1}) were incubated with each substrate [0.5% (wt/vol in 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer at pH 7.0] for 1 h at room temperature. Liberated reducing sugars were measured with copper-2,2′-bicinchoninate reagent (28).

Mode of action. The mode of action of the purified pectate lyase was determined by a viscosimetric assay described by Hasegawa and Nagel (6). The reaction mixture (20 ml) contained 1% (wt/vol) sodium polygalacturonate, 0.1 M glycine–NaOH buffer (pH 10.25), 0.5 mM $CaCl_2$, and 2.5 U of purified pectate lyase. The reaction was carried out at 30°C. Viscosity was measured with an Ubbelohde viscosimeter. The splitting of the glycosidic bonds was also monitored by determining the increase in A_{235} and was calculated on the basis of an ε of 4,600 liters mol^{-1} cm^{-1} (34).

Detection of carbohydrates. SDS-polyacrylamide gels were stained with Schiff's reagent as described by Zaccharius and Zell (36). In addition, a glycan differentiation kit (Boehringer, Mannheim, Germany) was used.

Determination of the N-terminal sequence. The N-terminal sequence of the purified pectate lyase, immobilized on a polyvinylidene difluoride transfer membrane (Millipore Corporation, Bedford, Mass.), was determined with an Applied Biosystems 475 protein sequencer.

Preparative isolation of unsaturated oligogalacturonates. Unsaturated oligogalacturonates in milligram quantities ranging in degree of polymerization (DP) from 2 to 5 were produced by a partial digestion of sodium polygalacturonate (Sigma) with the crude pectate lyase. The reaction mixture (200 ml) consisted of 0.5% sodium polygalacturonate in 0.1 M glycine–sodium hydroxide buffer at pH 10.25 containing 0.5 mM Ca and 4 U of the crude pectate lyase from the *Amycolata* sp. After 10, 20, 30, 60, and 120 min, 25-ml aliquots were taken from the reaction mixture and cooled to −25°C prior to lyophilization. The freeze-dried material was dissolved in 5 ml of deionized water. The enzyme was heat inactivated for 5 min at 98°C. A 1-ml aliquot was then applied to a pre-equilibrated column (2.5 by 135 cm) of 200/400 mesh packed with P2 gel (Bio-Rad). The reaction products were eluted with 0.1 M ammonium acetate (pH 4.5) at a flow rate of 0.23 ml min^{-1} at room temperature. The A_{235} was measured on line with a UV spectrophotometer (Shimadzu) equipped with a flowthrough cell. Fractions (4.6 ml) were collected. Fractions with a high A_{235} were selected for high-performance anion-exchange chromatography (HPAEC) analyses. Fractions containing individual unsaturated oligogalacturonic acids were pooled and lyophilized.

HPAEC-pulsed amperometric detection (PAD) analyses were performed on a high-performance liquid chromatography system from Dionex (Sunnyvale, Calif.). The degradation products liberated by the enzymes were separated on a CarboPac PA1 column with a matching guard column and detected with a pulsed amperometric detector. Repeated sequences of potentials (P_1 = +0.05 V, P_2 = +0.75 V, and P_3 = −0.15 V) with times of 420 ms (t_1), 180 ms (t_2), and 360 ms (t_3) were applied to the gold electrode of a flowthrough electrochemical cell. If not otherwise stated, detector sensitivity was selected at 1 μA. Elution was done with a linear gradient of sodium acetate (200 to 900 mM) within 40 min. The flow rate was set at 1 ml min^{-1}. A 0.5 M NaOH "postcolumn" (Baker, Phillipsburg, N.J.) was added at 0.5 ml min^{-1}.

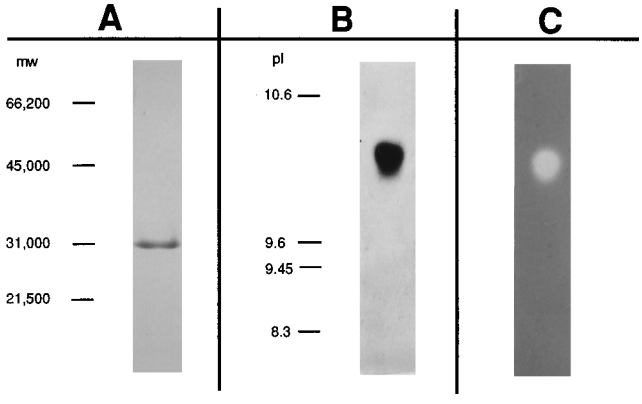

FIG. 3. Characterization of purified pectate lyase by SDS-PAGE and IEF. (A) SDS-PAGE of purified pectate lyase after silver staining. Molecular weight (mw) standards are indicated. (B) Analytical IEF of purified pectate lyase after silver staining. Isoelectric points of marker proteins are indicated. (C) Substrate-agarose overlay of the IEF gel after staining with ruthenium red.

RESULTS AND DISCUSSION

Enzyme production. The *Amycolata* sp. produced extracellular activities of pectate lyase, pectinesterase, xylanase, and carboxymethyl cellulase but not pectin lyase or polygalacturonase when grown on ramie fibers in shake flasks (Fig. 1). The different activities were present after the second day of cultivation and reached their maximum after the fourth day. Only cellulase activity, though induced at a very low level, still increased after the seventh day of cultivation. This result indicates that cellulose serves as a carbon source after degradation and utilization of the noncellulosic material. In addition to the polysaccharide-depolymerizing activities, a pectinesterase was produced. Pectate lyase was not detected in cultures when glucose was used as the sole carbon source. As shown recently, ramie leaves and sodium polygalacturonate induced high levels of pectate lyase activity, whereas citrus pectin was a poor inducer (3).

Enzyme purification. The cultures were harvested after 3.5 days. The starting material for the purification was 1 ml of the concentrated crude enzyme solution containing 1.8 mg of protein ml^{-1} with a specific pectate lyase activity of 86 U ml^{-1}. This crude enzyme solution was applied to a DEAE-Sepharose column (Fig. 2A). Most of the pectate lyase did not bind to the anion-exchange resin and eluted from the column with the starting buffer. A small fraction of pectate lyase bound weakly to the resin and was eluted at a very low NaCl concentration. IEF analysis showed that this pectate lyase had the same pI as the nonbound pectate lyase; therefore, it was discarded. Pigments bound to the resin were eluted at a higher NaCl concentration and thus could be easily separated from pectate lyase-containing fractions. Fractions 6 to 10, which had high levels of pectate lyase activity, were pooled, concentrated, and applied to a Mono S HR 5/5 column (Fig. 2B). The pectate

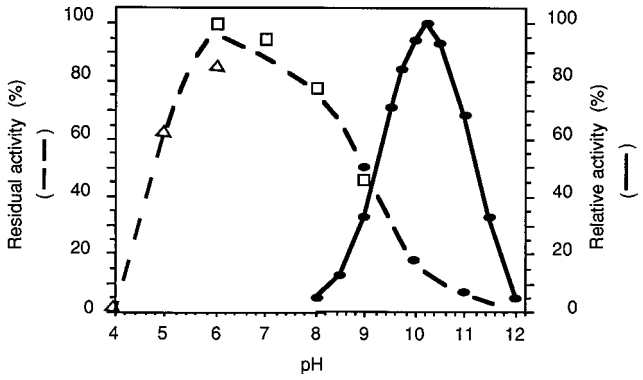

FIG. 4. Effect of pH on the activity and stability of purified *Amycolata* pectate lyase. ——, activity at different pHs under standard assay conditions; – – –, activity remaining after incubation of the purified enzyme in different buffers for 24 h at room temperature. The buffers used were glycine-NaOH (●), bis-Tris propane (□), and citrate (△).

lyase eluted in a single peak approximately at 0.4 M NaCl. Active fractions were pooled and concentrated, and the elution buffer was replaced by 50 mM phosphate (pH 7.0) with 1.7 M $(NH_4)_2SO_4$. The last purification step was performed by hydrophobic interaction chromatography on a phenyl-Superose column (Fig. 2C). The pectate lyase was eluted in a single, symmetrical peak at approximately 0.6 M $(NH_4)_2SO_4$. As summarized in Table 1, purification of the pectate lyase resulted in a fourfold increase in specific activity with a good recovery of about 37%. All chromatographic steps were carried out at room temperature. Shifting the temperature from room temperature to 4°C resulted in reduced resolution and loss of eluted activity during hydrophobic interaction chromatography.

SDS-PAGE and IEF of the purified pectate lyase showed a single band at a molecular weight of 31,000 (Fig. 3A) and a single band at approximately pH 10 (Fig. 3B), respectively. Pectate lyase activity was confirmed to be attributable to the purified protein by the zymogram technique (Fig. 3C). Matrix-assisted laser desorption ionization mass spectrometry revealed a molecular mass of 30,000 ± 30 kDa. Surprisingly, size exclusion chromatography on a Superose 12 column indicated a molecular weight of only 19,000. Since no carbohydrates were found to be associated with the purified enzyme, the low molecular weight measured by size exclusion chromatography might be due to an affinity of the enzyme for the matrix of the Superose 12 column used. High affinities of the enzyme for the gel of a PD 10 gel filtration column (Bio-Rad) and for membranes of microconcentrators designed for the desalting of protein samples were also observed. Therefore, desalting of the purified protein by these commonly used techniques led to a striking loss of activity. Affinities of pectate lyases for synthetic polymers have been reported by other investigators (18, 20) and were found to be related to the chemical nature and charge of membrane polymers (5).

Enzyme characterization. The purified protein exhibited a higher level of catalytic activity on sodium polygalacturonate than on highly esterified pectin. Viscosimetric measurements showed that the relative viscosity was reduced to 50% by cleavage of fewer than 1% of the glycosidic bonds of the substrate. Therefore, the enzyme was classified as an endopectate lyase (EC 4.2.2.2). No activity towards cellulose, xylan, galactomannan, arabinan, arabinogalactan, or alginate was seen. Calcium was essential for activity of the pectate lyase. A minimal concentration of 0.2 mM Ca^{2+} was required for maximum activity of the enzyme under the standard assay conditions. Salts of mercury or cadmium at 0.5 mM did not inhibit the enzyme in the presence of 0.5 mM calcium. Some divalent cations tested were able to replace calcium but did not lead to a fully active pectate lyase. Among the tested cations, magnesium and strontium showed activation levels of 41 and 21%, respectively, of the level obtained with calcium. The effect of pH on the activity and stability of the enzyme was investigated (Fig. 4). The pH optimum was found at 10.25, whereas stability was best in the range of pH 6 to 8. Only a pectate lyase from *T. fusca* has been reported to have a higher activity optimum (pH 10.45 [30]). In general, pH optima of these enzymes are in the range of pH 8 to 10, which is much higher than the pH optima of other pectinolytic enzymes (34). However, the discrepancy between the physiological pH of growth for the organism (about pH 7) and the pH optimum for maximum pectate lyase activity (pH 10.25) awaits clarification. Maximum activity was measured at 70°C, though the stability of the purified enzyme was very poor at this temperature. At 50°C, the crude pectate lyase was inactivated after 8 h, whereas the purified enzyme lost its activity after 1 h. Calcium and sodium polygalacturonate improved thermal stability significantly. For sodium polygalacturonate, a K_m value of 0.019 g liter^{-1} was determined with a Lineweaver-Burk plot. Thus, the affinity of the enzyme for polygalacturonate seems to be similar to that reported for a pectate lyase from *T. fusca* (30). The corresponding V_{max} was found at 158 μmol min^{-1} mg^{-1}. The N-terminal sequence of the pectate lyase from the *Amycolata* sp., comprising 18 amino acids, was obtained by gas phase sequencing. Comparison with protein sequences of the SwissProt data bank (115,821 sequences searched) showed high similarity within 13 amino acids to the N-terminal sequences of the pectate lyases PLa and PLe from

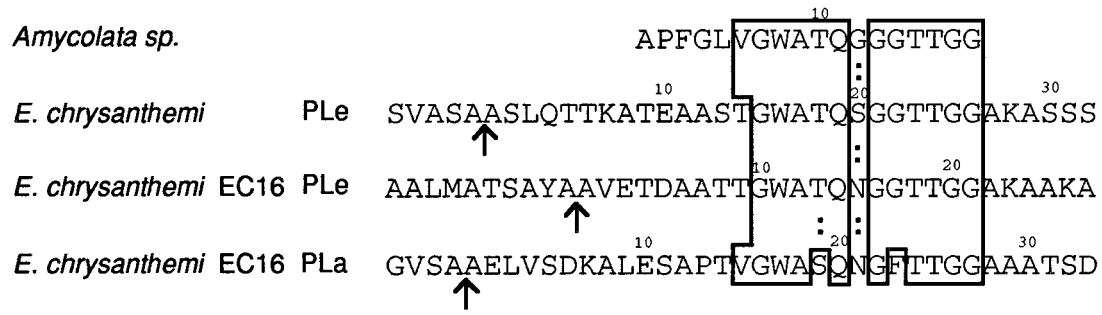

FIG. 5. Comparison between N-terminal sequences of pectate lyases from the *Amycolata* sp. and *E. chrysanthemi*. Boxed sequences indicate 100% homology. Colons connect functionally homologous amino acids. Arrows show the putative cleavage sites of the leader peptidase. Numbers refer to amino acid positions in the mature proteins. The protein sequences of the pectate lyases from *E. chrysanthemi* are derived from the respective nucleic acid sequences (8, 16, 31).

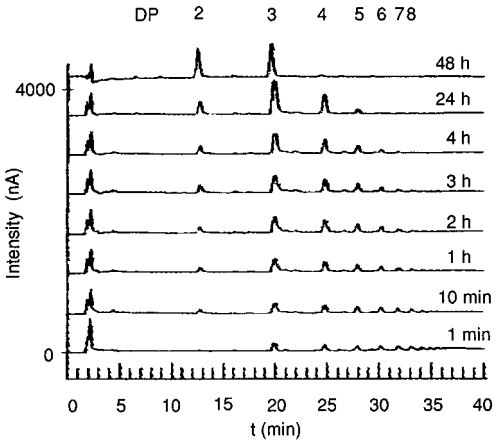

FIG. 6. Degradation of polygalacturonate by purified pectate lyase under standard assay conditions, as analyzed by HPAEC-PAD. After 24 h, new pectate lyase was added to the reaction mixture, which was then incubated for another 24 h. The signal at about 2 min is due to injection. Peaks are labeled according to the DPs of the unsaturated oligogalacturonates. No transelimination was observed in the absence of the enzyme (data not shown). Detector sensitivity was selected at 10 μA.

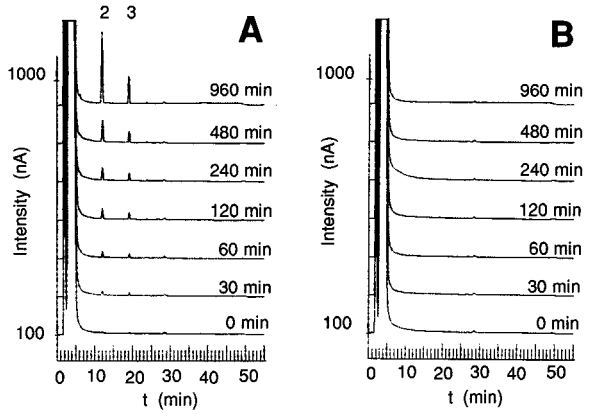

FIG. 7. Degradation of a highly esterified pectin (degree of esterification, 93%) by crude (A) and purified (B) pectate lyases in 50 mM TES buffer at pH 7.0, as analyzed by HPAEC-PAD. Peaks are labeled according to the DPs of the unsaturated oligogalacturonates. No degradation products were released by the purified pectate lyase.

Erwinia chrysanthemi (Fig. 5). The signal peptide sequences of PLa and PLe from *E. chrysanthemi* are cleaved by a signal peptidase between two A residues, leading to a mature protein with an alanine at the first position. One alanine could be part of the cleaving site for the leader peptidase in the *Amycolata* sp. as well.

The degradation of polygalacturonic acid by the pectate lyase from the *Amycolata* sp. was studied by HPAEC-PAD. The limit products formed by the pectate lyase were preparatively isolated by size exclusion chromatography and identified by fast atom bombardment mass spectrometry. As shown in Fig. 6, the pectate lyase released a wide spectrum of unsaturated oligogalacturonates at the beginning of the incubation with polygalacturonate, thus confirming a random endolytic attack by the enzyme. Smaller unsaturated oligogalacturonates accumulated over time, whereas larger oligomers finally disappeared. The unsaturated digalacturonate and trigalacturonate accumulated as limit products in approximately equal amounts. The pectate lyase from the *Amycolata* sp. cleaved unsaturated tetragalacturonate into unsaturated digalacturonates, whereas the unsaturated pentamer was cleaved into the unsaturated dimer and the unsaturated trimer. Transglycosylation was not observed under the assay conditions. A random endolytic depolymerization of polygalacturonate, generating mainly unsaturated di- and trigalacturonate, was also reported for the acidic pectate lyase PLa from *E. chrysanthemi*, though depolymerization was less complete (15). A concomitant generation of unsaturated and saturated oligogalacturonates by pectate lyase PLc from *E. chrysanthemi* was reported recently (7). However, degradation of sodium polygalacturonate by the pectate lyase from the *Amycolata* sp. at pH 5 to 10 did not show the formation of saturated oligogalacturonates.

Unlike the crude pectate lyase from the *Amycolata* sp., the purified pectate lyase was unable to degrade a highly esterified pectin with a degree of esterification of 93%, as shown in Fig. 7. The crude enzyme contained a weak pectinesterase activity which rendered the pectin susceptible to the pectate lyase. In contrast to the nonesterified substrate, no unsaturated oligogalacturonates with a DP of >3 were generated by the crude pectate lyase, indicating that there were only short blocks of nonesterified carboxyl groups present in the galacturonan backbone. The pectinesterase left in the crude pectate lyase seems to limit the action of the pectate lyase on highly esterified pectins.

The effect of EDTA on the purified and crude pectate lyases from the *Amycolata* sp. was tested when a deesterified pectin (sodium polygalacturonate) and a native pectin (bast fibers from ramie) were used as the substrates. As expected, the enzyme was not active in the presence of EDTA when sodium polygalacturonate was used as the substrate (data not shown). HPAEC-PAD analysis of the degradation products released after treatment of ramie bast fibers with the crude and purified pectate lyases revealed the formation of a wide range of unsaturated oligogalacturonates. Surprisingly, addition of 10 mM EDTA, a concentration which was expected to inhibit the pectate lyase, abolished the formation of the unsaturated digalacturonate but did not reduce the overall activity of the pectate lyase on native ramie pectin (Fig. 8). Although some calcium might still be bound by the pectin of the ramie fibers, thus supplying the reaction mixture with calcium, the lack of formation of unsaturated digalacturonate is unexpected. Addition of EDTA shifted the product formation towards higher oligogalacturonates. Most of the biochemical data on pectate

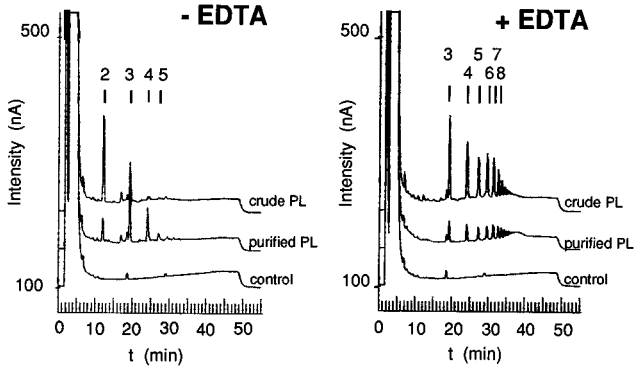

FIG. 8. Liberated unsaturated oligogalacturonates analyzed by HPAEC-PAD after incubation of ramie fibers with crude or purified pectate lyase (PL) in the absence (−) and in the presence (+) of 10 mM EDTA. Twenty-five milligrams of ramie fibers was treated with crude or purified pectate lyase (6 U ml^{-1}) in 50 mM TES buffer (pH 7.0) for 3 h. Fibers treated with the buffer alone served as the negative control. Peaks are labeled according to the DPs of the oligomers.

lyases have been obtained with water-soluble polygalacturonates. However, polygalacturonate does not occur in plants and fruits as a separate entity but is a constituent of pectins (smooth region). Therefore, pectate lyases may have biochemical effects on native substrates that are different from their effects on water-soluble, modified substrates such as sodium polygalacturonate. A calcium-binding site in PLc and PLe from *E. chrysanthemi* has been postulated (11, 35). The results with the *Amycolata* pectate lyase indicate that the effect of calcium is substrate dependent.

The pectin-depolymerizing activity of a species of *Amycolata* was due to the presence of a single pectate lyase. The endopectate lyase had a high pI and a high pH optimum. The high pI of the pectate lyase is considered beneficial for an efficient breakdown of polygalacturonate because of the reduced electrostatic repulsion between the enzyme and the negatively charged substrate. The results of this work indicate that pectate lyases might have different biochemical properties on soluble and native pectins. Therefore, increased attention should be given to the degradation of native pectins.

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