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An Exo-Poly-α-D-Galacturonosidase Implicated in the Regulation of Extracellular Pectate Lyase Production in *Erwinia chrysanthemi*

ALAN COLLMER,†* CATHERINE H. WHALEN, STEVEN V. BEER, AND DURWARD F. BATEMAN‡

Department of Plant Pathology, Cornell University, Ithaca, New York 14853

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Pectic enzymes in the supernatants of *Erwinia chrysanthemi* cultures in late-logarithmic-phase growth on D-galacturonan were resolved into three components: two pectate lyase isozymes and an exo-poly-α-D-galacturonosidase previously unreported in this organism. The hydrolytic enzyme was purified to homogeneity by ammonium sulfate fractionation, preparative electrofocusing in Ultrodex gel, and gel filtration through Ultrogel AcA54. The enzyme had a specific activity of 591 μmol/min per mg of protein, a pI of 8.3, a molecular weight of 67,000, a pH optimum of 6.0, and a K_m of 0.05 mM for D-galacturonan. Analyses of reaction mixtures by paper chromatography revealed that the enzyme released only digalacturonic acid from D-galacturonan. The action of the hydrolytic enzyme on D-galacturonan labeled at the nonreducing end by partial digestion with pectate lyase revealed that it rapidly released 4,5-unsaturated digalacturonic acid from 4,5-unsaturated pectic polymers. The production of extracellular exo-poly-α-D-galacturonosidase was coordinately regulated with pectate lyase production. The action patterns of the two enzymes appeared complementary in the degradation of pectic polymers to disaccharides that stimulated pectic enzyme production and supported bacterial growth.

A study of extracellular pectate lyase (EC 4.2.2.2) regulation in the phytopathogen *Erwinia chrysanthemi* led to the discovery that the organism also secretes a hydrolytic pectic enzyme whose product effectively induces pectate lyase (A. Collmer and D. F. Bateman, Phytopathology **69**:1025, 1979). Production of hydrolytic pectic enzymes by *Erwinia* species in the carotovora group has been reported before. *E. carotovora* produces an extracellular polygalacturonase (EC 3.2.1.15) which randomly cleaves D-galacturonan (18); cell-free extracts of *E. aroideae* (= *E. carotovora*) contain an exo-poly-α-D-galacturonosidase (exoPG; EC 3.2.1.82) which releases digalacturonic acid from D-galacturonan (12). As will be shown below, the hydrolytic enzyme secreted by *E. chrysanthemi* strain 630 has the latter type of action pattern.

Extracellular degradation of D-galacturonan by these bacteria is unique in that enzymes possessing different reaction mechanisms may participate in the degradation of the same substrate. Research so far has dealt largely with the bacterial pectate lyase. This enzyme has a pH optimum of 8.5 and an absolute requirement for divalent cations, and it cleaves internal glycosidic bonds in D-galacturonan by β-elimination, generating a 4,5-unsaturated product (3). Because of the ability of highly purified pectate lyase to cause maceration and cell death in plant tissues (activities characteristic of endo-cleaving pectic enzymes of both reaction types), this enzyme has been implicated as a factor in the "soft rot" diseases caused by these bacteria (3). Pectate lyase in *Erwinia* species has been reported to be induced by D-galacturonan (23, 27) and subject to catabolite repression (6, 13, 17, 24). Induction on D-galacturonan is mediated by reaction products of extracellular pectic enzymes (6, 23).

Pectate hydrolases are distinguishable from lyases by their lower pH optima (typically less than 6.0), by their lack of a divalent metal requirement, and by their hydrolytic reaction mechanism (21). Their role (if any) in plant pathogenesis, bacterial nutrition, or pectic enzyme regulation remains to be elucidated.

The objective of this study was to determine in vitro the relationship between the activities of exoPG and pectate lyase in pectate degradation and pectic enzyme regulation in *E. chrysanthemi*. We report here (i) resolution of the extracellular pectic enzymes (and isozymes) produced by strain 630; (ii) characterization of the

† Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853.

‡ Present address: North Carolina Agricultural Research Service, North Carolina State University, Raleigh, NC 27650.

exoPG; (iii) a facile method of preparing digalacturonic acid; (iv) evidence of coordinate regulation of pectate lyase and exoPG; and (v) evidence that the activities of pectate lyase and exoPG are complementary in pectate lyase induction on substrates containing D-galacturonan, including isolated plant cell walls.

MATERIALS AND METHODS

Bacterium and Culture conditions. *E. chrysanthemi* CUCPB (Cornell University Collection of Phytopathogenic Bacteria; R. S. Dickey, Department of Plant Pathology, Cornell University, Ithaca, N.Y.) 630, originally isolated from carnation (9), was used in this study. Cultures were maintained on nutrient agar, grown on 0.5% (wt/vol) glycerol mineral salts (pH 7.0) medium (27) before all experiments, and incubated at 25°C with shaking (120 rpm) in mineral medium supplemented with specific carbon sources during the various experiments.

Resolution of extracellular pectic enzymes. Three Fernbach flasks containing 1 liter each of 0.5% (wt/vol) D-galacturonan (product 3491 from Sunkist Growers Inc., Corona, Calif.) medium were inoculated with 20 ml of a stationary-phase, glycerol-grown culture. Pectate lyase in centrifuged culture supernatants was assayed during the ensuing growth period. When the sharp increase in pectate lyase activity which signals the end of the logarithmic phase was observed, the cultures were pooled and immediately centrifuged at 10,000 × *g* for 30 min. Centrifugation and all subsequent steps were performed at 0 to 8°C. Solid ammonium sulfate was slowly added to the supernatant to 80% saturation. After 1 h of continued slow stirring, the mixture was centrifuged at 10,000 × *g* for 30 min. The pellet was dispersed in 300 ml of water at 25°C, and then centrifuged as before to remove residual D-galacturonan that had been precipitated by the ammonium sulfate. The supernatant (172 ml) received 96 g of ammonium sulfate and, after 30 min of stirring, was centrifuged. This pellet was redissolved in 26 ml of water, desalted on a column of Sephadex G-50 fine (2.5 by 85 cm; Pharmacia Fine Chemicals AB, Uppsala, Sweden), and eluted with water at 25 ml/h (pumped). Fractions (4.5 ml) were collected and assayed for both pectate lyase and exoPG activities and for conductivity, using a YSI model 31 conductivity bridge (Yellow Springs Instruments, Yellow Springs, Ohio). Fractions 42 to 80, which contained most of the pectic enzyme activity, were pooled and concentrated to 88 ml on an Amicon ultrafiltration apparatus equipped with a UM-10 membrane (Amicon, Lexington, Mass.). The conductivity of this enzyme concentrate was 50 μmho.

The concentrated and desalted enzyme mixture was then electrofocused in an LKB flat-bed apparatus (LKB-Produkter AB, Bromma, Sweden), using Ultrodex (LKB) as a stabilizing medium. Thirteen milliliters of water was added to 82 ml of the enzyme preparation and mixed with LKB Ampholine carrier ampholytes of the following pH ranges: 3 ml of 3.5 to 10; 2 ml of 9 to 11; 0.2 ml of 4 to 6; and 0.2 ml of 6 to 8. A slurry prepared by adding 4 g of Ultrodex to the above mixture was then poured into the electrofocusing trough and partially dried under an air stream at 25°C until the slurry was stable when the trough was tilted at a 45° angle. The wicks were soaked in 10 ml of water containing Ampholines: 0.06 ml of pH 3.5 to 10 and 0.04 ml of pH 9 to 11. The proteins were electrofocused for 15 h, using an LKB 2103 power supply set at 8-W constant power with voltage and current limited to 1,500 V and 20 mA, respectively. The bed was partitioned with a metal grid, and individual fractions were packed into elution columns (LKB) and eluted twice with 1-ml portions of degassed, distilled water. The pH was immediately determined (meter previously standardized with cold buffers). The columns were then further eluted with 2 ml of 70 mM potassium phosphate (pH 7.0)–100 mM sodium chloride (PBS). The combined eluants from a given elution column were assayed for pectate lyase and exoPG activity.

The exoPG peak from the electrofocusing step was concentrated to 6 ml on an Amicon UM-2 membrane, applied to a column (2.5 by 83 cm) of LKB Ultrogel AcA54, and eluted in PBS at 25 ml/h (pumped). Fractions (2 ml) were collected and assayed for pectate lyase and exoPG. Pooled peak fractions were stored at −20°C. The pectate lyase peak was dialyzed against 50 volumes of 50 mM Tris-hydrochloride, pH 8.5, before storage because activity decreased if stored in phosphate buffer.

Gel electrophoresis. The homogeneity and molecular weight of the purified exoPG was determined by sodium dodecyl sulfate (SDS)-gel electrophoresis on a 12% acrylamide gel (16). The gel was run at 55 V until bromphenol blue reached the bottom. The gel was stained with Coomassie brilliant blue R. Molecular weight markers were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Analytical centrifugation. The molecular weight was also determined by sedimentation equilibrium in a Beckman model E/Scan ultracentrifuge. Four Yphantis cells were loaded with 0.085 ml of purified exoPG at a concentration of 0.13 mg/ml in 50 mM potassium phosphate, pH 6.5. After centrifugation at 28,000 rpm and 4°C for 24 h, photoelectric scans at 280 nm were taken and processed by an on-line computer system (7). A partial specific volume of 0.72 was used in the molecular weight calculations.

Purification and characterization of digalacturonic acid. Digalacturonic acid was enzymatically prepared from D-galacturonan, using purified exoPG. An aqueous suspension containing 8 g of D-galacturonan (molecular weight, 10,000 to 20,000) in 400 ml of water was titrated to pH 5.5 with sodium hydroxide and passed through a nitrocellulose filter (3-μm pore size), and sodium azide was added at a final concentration of 0.02% (wt/vol). Any 4,5-unsaturated residues in the native substrate were removed by partial digestion with 10 U of purified exoPG. When the reducing group concentration had increased by 3 mM, as determined with arsenomolybdate reagent (19), an equal volume of 95% redistilled ethanol was added, and the mixture was centrifuged 10,000 × *g* for 30 min. Unsaturated digalacturonic acid is soluble in 50% ethanol (15). The pellet containing the polymer was drained, and the ethanol was removed under reduced pressure at 50°C. This modified substrate was redissolved in the original volume of water, and then 0.02% sodium azide and 50 U of purified exoPG were added. After 20 h of incubation at 30°C, an equal volume of ethanol was added, and the mixture was centrifuged as before. The supernatant, which contained the digalacturonic acid,

was concentrated to about one-fourth of its original volume by evaporation at 50°C and reduced pressure and then distributed to centrifuge bottles. Four volumes of ethanol was then added to each bottle; the bottles were incubated at −20°C for several hours and then centrifuged at 10,000 × *g* for 30 min. After the ethanol had been removed by decantation and evaporation, the pellets were redissolved in a small volume of distilled water. When 0.06-mg samples of this material were analyzed on paper chromatograms stained with silver nitrate (1), faint spots representing larger oligomers were observed; the limit of detection for D-galacturonic acid is around 1 μg. Digalacturonic acid was further purified by gel filtration through a column (2.5 by 90 cm) of Bio-Gel P-2, minus 400 mesh (Bio-Rad Laboratories, Richmond, Calif.), eluted at 25 ml/h with 50 mM potassium phosphate, pH 7.0. The final yield of digalacturonic acid was 2.9 g.

The identification of digalacturonic acid was facilitated by reference to a homologous series of saturated oligogalacturonides that had been generated from D-galacturonan by the polygalacturonase complex of *Sclerotium rolfsii* (2). Lyophilized crude *S. rolfsii* enzyme was suspended at a concentration of 2 mg/ml in distilled water and dialyzed against water overnight in an animal membrane. Equal volumes of dialyzed enzyme, aqueous 2% (wt/vol) D-galacturonan (neutralized with sodium hydroxide), and 100 mM citrate-phosphate buffer, pH 4.5, were combined and incubated at 25°C for 5 h before assay of reaction products by paper chromatography.

The identification of digalacturonic acid was further aided by the use of "pectinase," a hydrolytic pectic enzyme preparation derived from *Aspergillus niger* (Sigma Chemical Co., St. Louis, Mo.), which converted the disaccharide to D-galacturonic acid. A preparation of pectinase (10 mg/ml in 100 mM citrate-phosphate, pH 5.5) was dialyzed against the same buffer overnight and then combined with an equal volume of 10 mM purified digalacturonic acid in 50 mM phosphate, pH 7.0, and 2 volumes of 50 mM sodium acetate, pH 4.5. The reducing group concentration was assayed at the beginning and after 2 h of incubation, and then 0.05 ml of the reaction mixture was immediately applied to chromatography paper for assay.

Paper chromatography. Reaction mixtures and purified pectic reaction products were analyzed by paper chromatography. Samples were spotted without prior treatment on Whatman no. 1 paper and developed with descending elution in butanol-acetic acid-water, 2:1:1 (vol/vol). Products were detected with silver nitrate reagent (1) or periodate-thiobarbituric acid (25).

Preparation of 4,5-unsaturated D-galacturonan. D-Galacturonan labeled at the nonreducing end with a 4,5-unsaturated uronic acid residue was prepared by incubating purified pectate lyase isozyme 2 (see Results) with 10 ml of 0.5% (wt/vol) D-galacturonan in 20 mM potassium phosphate, pH 7.0, and 0.5 mM calcium chloride. (Calcium chloride must be added before D-galacturonan.) After 30 min, the reducing group concentration in the reaction mixture had increased 1.89 mM from the initial concentration of 0.5 mM, as determined with arsenomolybdate reagent (19), and the reaction was terminated by adding 0.3 ml of 100 mM EDTA. Residual chains longer than five uronic acid units were found to be precipitated by addition of an equal volume of 95% ethanol; these were sedimented by centrifugation at 10,000 × *g* for 30 min, then redissolved in 100 ml of 20 mM potassium phosphate, pH 7.0, 2.0 mM EDTA, and precipitated three times again with ethanol. One-third of the D-galacturonan chains in the final washed preparation bore a 4,5-unsaturated nonreducing terminus, as indicated by the ratio of unsaturated residues to reducing groups which was determined by periodate-thiobarbituric acid (26) and arsenomolybdate reagent (19) assays, respectively.

ExoPG and pectate lyase assays. ExoPG activity was assayed by using the arsenomolybdate method (19) to determine the increase in reducing groups in reaction mixtures containing 0.5% (wt/vol) D-galacturonan, 50 mM potassium phosphate, pH 6.0, 2 mM EDTA (to inhibit pectate lyase), and a volume of enzyme constituting up to 25% of the volume of the reaction mixture. Samples (0.2 ml) were removed from reaction mixtures at intervals and immediately mixed with 0.3 ml of water and 0.5 ml of the first reagent in the arsenomolybdate assay (19). The progress of enzyme reactions in this assay was found to be linear between absorbancy at 500 nm values of 0.2 (substrate background) and at least 0.8; the amount of enzyme present in reaction mixtures was adjusted to stay within this range during an incubation period of 20 min or less. D-Galacturonic acid (Sigma Chemical Co.) was used as a standard in reducing sugar assays. D-Galacturonic acid gives standard values identical to those of oligogalacturonic acids in the arsenomolybdate reagent assay (20). Pectate lyase was routinely assayed by determining the increase in absorbance at 230 nm of reaction mixtures containing 0.07% (wt/vol) D-galacturonan, 30 mM Tris-hydrochloride, pH 8.5, 0.1 mM calcium chloride, and 6.7% (vol/vol) enzyme sample. Protein was determined by the method of Lowry et al. (14), with bovine serum albumin (Sigma Chemical Co.) as the standard.

RESULTS

Resolution of extracellular pectic enzymes. The purification scheme summarized in Table 1 was designed to resolve the components of the extracellular pectic enzyme complement of *E. chrysanthemi* and to contribute to the characterization of the exoPG. This enzyme was purified 45-fold and was homogeneous in SDS-gel electrophoresis (Fig. 1).

Preparative electrofocusing in Ultrodex resolved pectate lyase into two isozymes: isozyme 1 had an apparent pI of 8.3 and cofocusd with exoPG; isozyme 2 had an apparent pI of 9.3. ExoPG and pectate lyase isozyme 1 were resolved by gel filtration through a column of LKB Ultrogel AcA54 (Fig. 2).

Characterization of exoPG. The enzyme had a pH optimum of 6.0 and exhibited no activity as the pH approached 3 and 9. ExoPG had no apparent divalent cation requirement since its activity was unaffected by 2 mM EDTA. The K_m of this enzyme on D-galacturonan was 0.05 mM, based on the reducing group assay (19) of substrate concentration. The molecular weight of

TABLE 1. Purification of exoPG from 3 liters of *E. chrysanthemi* culture supernatant

Purification step	Total protein (mg)	Total pectate lyase (U)[a]	Total exoPG (U)	ExoPG sp act (U/mg of protein)
Culture supernatant	120	21,672	1,618	13
Ammonium sulfate fractionation	34	4,799	1,036	30
Sephadex G-50	8.6	3,246	490	57
Electrofocusing	3.6	356	361	99
Ultrogel AcA54	0.28	0.0	166	591

[a] A unit of activity is defined as that amount of enzyme causing the formation of 1 μmol of product/min under optimal conditions as described in the text.

exoPG was determined to be 67,000 by SDS-gel electrophoresis, 43,000 by gel filtration, and between 58,000 and 65,000 by sedimentation equilibrium analytical centrifugation.

This enzyme possessed a principal characteristic of an exo-attacking enzyme: it released a single product (Fig. 3), rather than a series of oligomers, from its polymeric substrate. This was determined by paper chromatography of exoPG–D-galacturonan reaction mixtures sampled over the course of reactions. The failure of the product to give a positive reaction in the thiobarbituric acid assay or to have an absorbance maximum near 230 nm indicated that it lacked the unsaturation at carbons 4 and 5 characteristic of pectate lyase products. The identification of the product was aided by reference to a series of saturated oligogalacturonides that had been prepared by using the polygalacturonase complex of *S. rolfsii* (Fig. 3). As is indicative of a homologous series of oligomers, a linear relationship existed between the number of uronic acid residues in each product and the logarithm of its relative mobility (RgalUA). The exoPG product cochromatographed with the

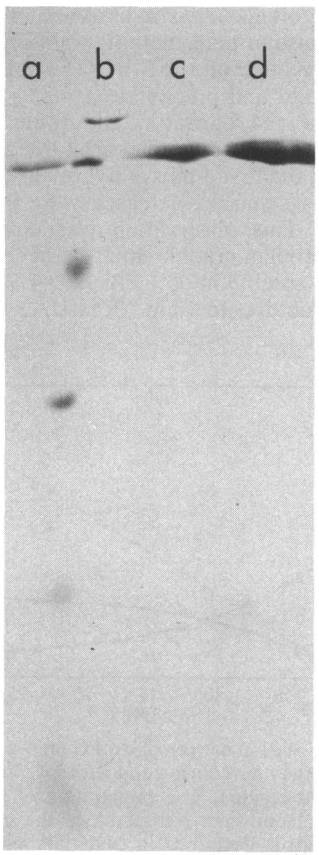

FIG. 1. SDS-gel electrophoresis of purified exoPG and molecular weight marker proteins. Electrophoresis on 12% acrylamide gels in the presence of SDS was performed according to Maizel (16). Lanes a, c, and d contained 2, 15, and 50 μg, respectively, of purified exoPG. Molecular weights of marker proteins in lane b are (from top to bottom): phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; and α-lactalbumin, 14,400.

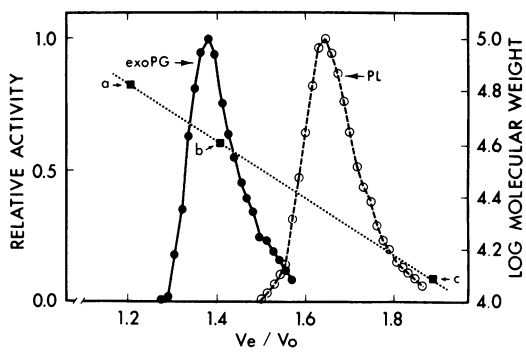

FIG. 2. Resolution of exoPG and pectate lyase (PL) isozyme 1 by gel filtration. The fractions from the electrofocusing step containing exoPG and pectate lyase isozyme 1 were pooled and concentrated by ultrafiltration, then applied directly to a column (2.5 by 83 cm) of Ultrogel AcA54, and eluted at 25 ml/h with 70 mM potassium phosphate, pH 7.0, and 100 mM sodium chloride. Ferritin marked the void volume. Molecular weights of other markers were: (a) bovine serum albumin, 67,000; (b) horseradish peroxidase, 40,000, and (c) cytochrome *c*, 12,300. The molecular weight of pectate lyase is between 30,000 and 32,400 (10). The relative activity of each enzyme is plotted independently.

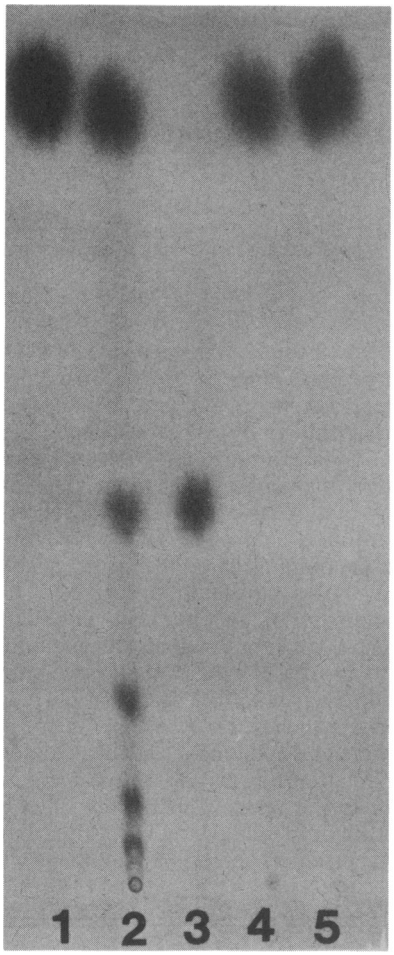

FIG. 3. Enzymatic and paper chromatographic evidence that the exoPG reaction product is digalacturonic acid. The homologous series of saturated oligogalacturonides in lane 2 was generated by digestion of D-galacturonan with the polygalacturonase complex of *S. rolfsii*. The exoPG product in lane 3 had been purified by gel filtration. After hydrolysis with Sigma pectinase, the exoPG product was converted to a new product (lane 4) which migrated with authentic D-galacturonic acid in lanes 1 and 5.

dimer in this series (Fig. 3). The exoPG product was enzymatically cleaved to D-galacturonic acid (Fig. 3) with a concomitant 1.7-fold increase in reducing groups.

Action patterns of exoPG and pectate lyase isozymes 1 and 2. To determine whether exoPG attacked the nonreducing end of D-galacturonan, and if so, whether it could release unsaturated digalacturonic acid from chains bearing a 4,5-unsaturated residue, the experiment depicted in Fig. 4 was performed. Several peculiar characteristics of pectic compounds facilitated this experiment: 4,5-unsaturation can be introduced by cleaving D-galacturonan with pectate lyase; 4,5-unsaturation constitutes a chemical label that can be selectively assayed with periodate-thiobarbituric acid; and the disaccharides released by exoPG are soluble in 50% ethanol, but residual polymers are not (15).

The washed 4,5-unsaturated D-galacturonan preparation (Materials and Methods) was redissolved in 50 mM potassium phosphate, pH 7.0–2 mM EDTA, and purified exoPG was added. At about 4-min intervals, 1-ml portions were removed to an ice bath, and 0.1 ml of 0.2 M glycine-sodium hydroxide, pH 10.5, was added to terminate the reaction. After removal of 0.1 ml for reducing group assays (19), 0.9 ml of 95% ethanol was added; the mixture was then stirred vigorously and centrifuged at 10,000 × *g* for 20 min. The supernatant (containing reaction products) was decanted, and the pellet (containing residual polymer) was redissolved in 1 ml of 50 mM potassium phosphate, 20 mM glycine-sodium hydroxide, and 2.0 mM EDTA, pH 9.7. Supernatant and precipitate samples were then assayed for 4,5-unsaturated uronic acid residues, using periodate-thiobarbituric acid (26).

During the first 4 min of hydrolysis, almost all of the disaccharides released were unsaturated (Fig. 4). This observation was confirmed by paper chromatographic analysis of the reaction products soluble in 50% ethanol; 4,5-unsaturated digalacturonic acid (RgalUA, 0.84) was

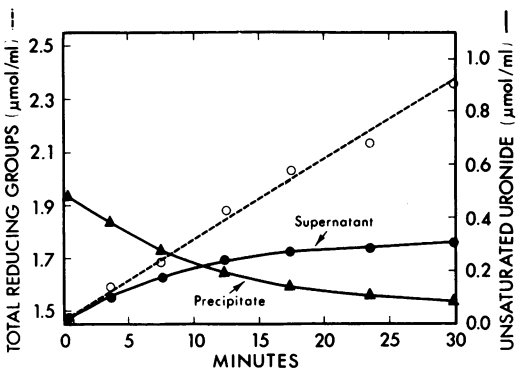

FIG. 4. Action pattern of exoPG on D-galacturonan labeled at the nonreducing end with a 4, 5-unsaturated uronic acid residue. The preparation of partially labeled D-galacturonan (one-third of the chains were unsaturated) is described in Materials and Methods. During incubation of this material with exoPG, equal portions of the reaction mixture were removed at intervals, precipitated with an equal volume of 95% ethanol, and then assayed with periodate-thiobarbituric acid (26) to determine if exoPG rapidly released the 4, 5-unsaturated residue (as ethanol-soluble 4, 5-unsaturated digalacturonic acid) by attack on the nonreducing end. The progress of the exoPG reaction was monitored with reducing group assays of subsamples removed before ethanol precipitation.

present in the sample taken at 4 min, but a similar level of digalacturonic acid (RgalUA, 0.48) did not appear before 8 to 12 min of incubation (based on intensity of spots detected with silver nitrate after development of 0.05-ml samples as described in Materials and Methods.) The immediate release of unsaturated digalacturonic acid from a population of pectic polymers in which only one-third of the chains bore an unsaturated residue at the nonreducing end suggests several features of the exoPG action pattern: (i) the enzyme attacks the nonreducing end of D-galacturonan, (ii) the enzyme has a preference for chains bearing an unsaturated residue, and (iii) the enzyme apparently attacks the nonreducing ends in a population of pectic polymers by a random multichain action pattern, rapidly releasing a substantial portion of the 4,5-unsaturated residues in the reaction mixture, rather than completely degrading individual polymers. This last conclusion is inferred from the observation that the unsaturated disaccharide rapidly accumulated to a detectable level even though there were several orders of magnitude more polymer molecules than enzyme molecules.

To better understand the contribution of the exoPG to the total extracellular enzymatic digestion of pectic polymers, we investigated the action patterns of pectate lyase isozymes 1 and 2. These isozymes had previously been characterized with respect to their relative activities in several assays that differentiate endo- and exo-attacking pectic enzymes (10). Isozyme 2 was typical of an endo enzyme in causing rapid reduction in the viscosity of D-galacturonan and rapid maceration and killing of potato tuber disks with relatively little glycosidic bond cleavage. Isozyme 1 also caused these effects but at a slower rate relative to the number of bonds cleaved. To ascertain the patterns of product formation by these isozymes, a reaction mixture was prepared containing 0.5% (wt/vol) D-galacturonan, 50 mM Tris-hydrochloride, pH 8.5, 0.02% sodium azide, and sufficient isozyme 1 or isozyme 2 to cause formation of 0.116 μmol of 4, 5-unsaturated product/min per ml of reaction mixture. Portions of 0.1 ml were removed at intervals from the reaction mixture, boiled for 5 min, then spotted (0.02 ml) on chromatography paper, and developed. By 2 h, isozyme 1 had generated easily detectable (by silver nitrate) unsaturated dimer, trimer, tetramer, and pentamer. In contrast, isozyme 2 produced only faintly detectable unsaturated tetramer and pentamer by 2 h and only faintly detectable unsaturated dimer at 4 h. This observation confirms that isozyme 1 is indeed an endo-attacking enzyme and that it is relatively more efficient at generating lower oligomers than isozyme 2.

The efficiency with which exoPG and pectate lyase isozyme 1 generated disaccharide was similarly compared. Reaction mixtures containing 0.5% (wt/vol) D-galacturonan and 0.105 U of pectate lyase isozyme 1 per ml or 0.066 U of exoPG per ml (in their respective buffer systems) were sampled at intervals and analyzed by paper chromatography as described above. ExoPG and pectate lyase isozyme 1 generated equivalent levels of disaccharide after 30 and 120 min of incubation, respectively. ExoPG was thus approximately six times more efficient at generating disaccharide than was pectate lyase isozyme 1.

ExoPG and pectate lyase regulation. *E. chrysanthemi* could utilize 0.5% (wt/vol) D-galacturonan without the benefit of extracellular pectate lyase activity, as shown by the growth of a glycerol-grown culture that had been centrifuged and suspended in D-galacturonan minimal medium with 2 mM EDTA (Fig. 5). This ability appeared to result from exoPG activity. The sole oligomeric product found in supernatants of cultures in early logarithmic growth on D-galacturonan in the presence of EDTA was digalacturonic acid. These supernatants also contained exoPG activity. The stationary-phase optical density at 600 nm (OD_{600}) attained by the culture in Fig. 5 was only half that of a duplicate culture incubated on 0.5% (wt/vol) D-galacturonan without EDTA. This result is consistent with the

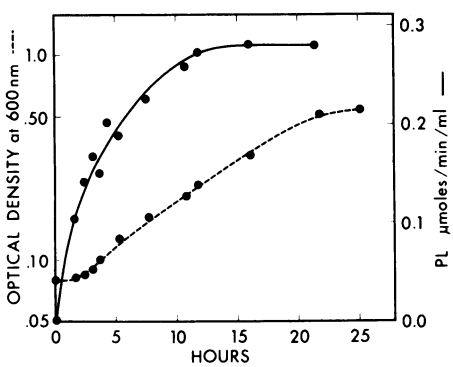

FIG. 5. Growth and pectate lyase (PL) production on D-galacturonan in the absence of pectate lyase activity. A glycerol-grown, early-stationary-phase culture was centrifuged, and the bacterial pellet was suspended in 0.5% (wt/vol) D-galacturonan mineral salts medium containing 2 mM EDTA, an inhibitor of extracellular pectate lyase activity. Equal portions of the culture were removed at intervals, assayed for bacterial growth (OD_{600}), and then centrifuged 10,000 × *g* for 15 min. Pectate lyase activity in culture supernatants was assayed in the presence of sufficient calcium chloride to completely reverse the inhibition by EDTA. Activity is expressed as micromoles of 4, 5-unsaturated product per minute per milliliter of culture supernatant.

observed failure of exo-cleaving pectic enzymes to completely degrade D-galacturonan (21). Although EDTA completely inhibits pectate lyase, substantial pectate lyase synthesis occurred during growth on D-galacturonan with EDTA (Fig. 5). Indeed, the differential rate of pectate lyase synthesis (increase in specific activity during an interval of logarithmic growth) was higher than in a duplicate culture without EDTA (data not shown).

To determine the ability of exoPG, acting in the absence of pectate lyase activity, to stimulate pectate lyase production in the presence of a complex, insoluble substrate containing D-galacturonan, we incubated *E. chrysanthemi* with isolated plant cell walls and EDTA. Cell walls were prepared from red kidney bean (*Phaseolus vulgaris*) tissue cultures by a procedure which yields an exhaustively washed, insoluble preparation (22). An early-stationary-phase glycerol-grown culture was centrifuged, and the bacterial pellet was resuspended in mineral medium at an OD_{600} of 1.0 and then distributed to equal volumes of mineral medium containing 2% (wt/vol) isolated plant cell walls and either 0.4 mM calcium chloride (to enhance pectate lyase activity) or 4.0 mM EDTA. One flask contained EDTA without cell walls. Equal portions were removed at intervals and centrifuged at 10,000 × *g* for 20 min. The supernatants were then passed through a nitrocellulose filter (0.22-μm pore size) and assayed for pectate lyase activity. Pectate lyase production in *E. chrysanthemi* was induced by isolated plant cell walls, but only slightly by cell walls in EDTA.

The relationship between exoPG and pectate lyase regulation was further examined by comparing the differential rates of synthesis of each enzyme during incubation with their respective products (Fig. 6). Bacteria in a glycerol-grown culture were resuspended in 0.5% (wt/vol) glycerol minimal medium supplemented with 0.5 mM digalacturonic acid or 0.5 mM 4, 5-unsaturated digalacturonic acid. Glycerol was used to support equivalent logarithmic growth in the cultures and thereby normalize the basal levels of both enzymes in each treatment. The results of the experiment indicated that exoPG and pectate lyase were regulated coordinately and that each enzyme was induced by its own product as well as by the product of the other (Fig. 6).

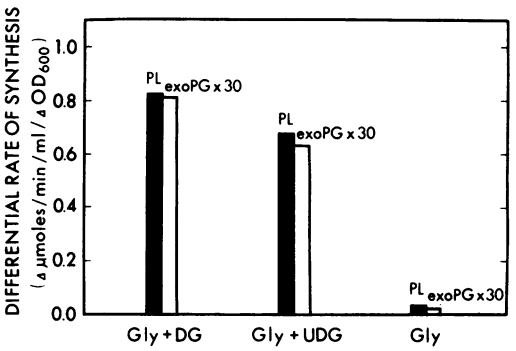

FIG. 6. Coordinate regulation of extracellular pectate lyase (PL) and exoPG in *E. chrysanthemi*. A glycerol-grown culture (0.87 OD_{600}) was centrifuged, and the bacterial pellet was suspended in a small volume of minimal medium and distributed (at 0.18 OD_{600}) to 0.5% (wt/vol) glycerol (Gly) medium supplemented as shown with 0.5 mM digalacturonic acid (DG) or 0.5 mM 4,5-unsaturated digalacturonic acid (UDG). Equal portions of the culture were removed at intervals, assayed for bacterial growth (OD_{600}), and centrifuged. The culture supernatants were then assayed for pectate lyase activity and for exoPG activity (in triplicate reactions). The differential rates of synthesis were determined during an interval of logarithmic growth (between 2.0 and 5.3 h of incubation), at the end of which the OD_{600} of each culture was ca. 0.5. The differential rate of synthesis (rate of increase of specific activity) is expressed as the increase in the rate of product formation (micromoles per minute per milliliter of culture supernatant) per increase in OD_{600}.

DISCUSSION

Two pectic enzymes, pectate lyase and exoPG, were resolved from the medium of *E. chrysanthemi* cultures in late-logarithmic-phase growth on D-galacturonan. ExoPG, which had not been reported before in this organism, was investigated with respect to its physical properties, action pattern, regulation, and biological role.

The purified enzyme was homogeneous on SDS-gels and comigrated with bovine serum albumin (molecular weight, 67,000) (Fig. 1). In gel filtration, the exoPG behaved as a protein with a molecular weight of 43,000 (Fig. 2). The accuracy of the calibration of the gel filtration column was confirmed by chromatographing purified exoPG and bovine serum albumin together through the column. Sedimentation equilibrium was used as a third method of determining the molecular weight of the exoPG. Although the values obtained of 58,000 to 65,000 are only an approximation because the partial specific volume is unknown, they clearly approach the molecular weight determined by SDS-gel electrophoresis. The anomalously slow elution in gel filtration may result from interaction of exoPG with polysaccharides in the Ultrogel matrix.

The *E. chrysanthemi* exoPG shares several features in its action pattern with exopolygalacturonase (EC 3.2.1.67), a hydrolytic enzyme which releases monomer galacturonic acid from D-galacturonan. Both enzymes attack the nonreducing end of the polymer, and both are unable

to completely degrade D-galacturonan (21). We have observed that approximately 50% of the D-galacturonan used in these studies remains precipitable by 50% ethanol after long-term digestion of the polymer with (i) purified exoPG, (ii) the pectic enzymes in concentrated culture supernatants (with EDTA added to inhibit pectate lyase), or (iii) bacterial cultures growing on D-galacturonan in the presence of EDTA (A. Collmer, C. H. Whalen, S. V. Beer, and D. F. Bateman, unpublished data). L-Rhamnose residues are known to occur in natural galacturonans (8), and the inability of exoPG to cleave the glycosidic bonds between galacturonosyl and occasional rhamnosyl residues may explain this observation.

The exoPG partially purified from acetone-treated cells of *E. aroideae* also attacks the nonreducing end of the polymer, as shown by paper chromatographic analyses of digestions of unsaturated and oxidized pectic substrates (11). It differs from the *E. chrysanthemi* exoPG in having a higher pH optimum (7.2) and in degrading unsaturated pectic oligomers more slowly than saturated oligomers (11).

The exoPG and pectate lyase secreted by *E. chrysanthemi* possess action patterns which are complementary in the degradation of D-galacturonan. Pectate lyase cleaves internal bonds in D-galacturonan by β-elimination. Low-molecular-weight oligomers, which can be assimilated by the bacteria, are generated only after extensive degradation by pectate lyase. ExoPG hydrolytically releases dimers from the new nonreducing ends that pectate lyase generates and, by its apparent preference for polymers bearing a 4, 5-unsaturated residue, contributes to the formation of 4,5-unsaturated digalacturonic acid (Fig. 4). In the absence of pectate lyase activity, exoPG activity is limited by the number of nonreducing ends in the starting substrate. Digalacturonic acid becomes the sole exoPG product under this condition. Every cleavage by exoPG yields a product that can be assimilated by *E. chrysanthemi*.

ExoPG appears to be regulated coordinately with pectate lyase, being produced in the same proportion (by activity) to pectate lyase (ca. 1:30) during bacterial growth on different substrates (Fig. 6). The possible role of this "minor" enzyme in the biology of *E. chrysanthemi* is considered in the context of three functions: plant pathogenesis, utilization of pectic substrates, and regulation of pectate lyase.

A priori, exoPG would not likely have a significant direct role in plant pathogenesis. Pectic enzymes that attack their substrates in an exo manner are poor agents of plant tissue maceration and cell death compared with endo-attacking enzymes (3). Furthermore, Chatterjee and Starr (5) reported that a mutant of *E. chrysanthemi* EC16 that lacked pectate lyase activity, though still possessing the activity of an (uncharacterized) hydrolytic pectic enzyme, was unable to macerate plant tissues.

The activity of exoPG may contribute significantly to the ability of *E. chrysanthemi* to utilize pectic polymers. Indeed, the bacteria can grow on D-galacturonan when pectate lyase is inhibited by EDTA (Fig. 5). The low growth rate under this condition apparently reflects the low level of exoPG in the medium, because EDTA had little effect on the rate of growth of strain 630 on 0.5% (wt/vol) glycerol, and the specific growth rate on digalacturonic acid was close to that observed with 4,5-unsaturated digalacturonic acid (6). However, during growth on D-galacturonan without EDTA, exoPG could complement pectate lyase activity by more efficiently releasing disaccharides for utilization by the bacteria.

This last consideration suggests the exoPG may be particularly important in the process of pectate lyase induction. There is some evidence that induction depends on conversion of part of the polymer to lower oligomers, particularly dimers (6). Pectate lyase activity does not immediately yield these products, but exoPG activity does. Furthermore, exoPG activity permits induction of pectate lyase under environmental conditions where pectate lyase activity is, itself, too low to generate reaction products to serve as inducers. Evidence for this is found in the induction of pectate lyase during incubation of *E. chrysanthemi* with 0.5% (wt/vol) D-galacturonan and 2 mM EDTA (Fig. 5). However, only slight induction of pectate lyase was observed during incubation of the bacteria with 1% (wt/vol) isolated plant cell walls and 2 mM EDTA. We attribute this difference to differences in availability of substrate to exoPG. D-Galacturonan is a covalently bound structural constituent of the plant cell wall (22). The lengths of the pectic polymers in the primary cell wall are still unknown, although some apparently contain at least 900 residues (8). A high degree of polymerization would diminish the relative number of nonreducing ends in cell wall galacturonans. Furthermore, the mesh of polymers in the cell wall is able to exclude macromolecules with a radius larger than that of a 17,000-molecular-weight protein (4). ExoPG may be excluded from cell wall substrates by its large size. These limitations do not occcur during incubation with purified D-galacturonan because the substrate is soluble and present at a molar concentration 10-fold higher than the K_m of exoPG.

An intriguing but less certain regulatory function of exoPG might be to serve as a monitor of extracellular pectate lyase activity. Since extracellular enzymes function in environments dif-

fering widely in their effects on enzyme activity and longevity, efficient regulation demands that the cell monitor the activity of the enzyme in the environment. One mechanism is self-catabolite repression by high concentrations of products (24), and it appears that this affects pectate lyase production in *E. chrysanthemi*: 4,5-unsaturated digalacturonic acid exerts cyclic AMP-reversible repression of pectate lyase (6), and pectate lyase production on D-galacturonan is stimulated by exogenous 5 mM cyclic AMP (Collmer et al., unpublished data). The activity of exoPG may increase the sensitivity of self-catabolite repression toward changes in pectate lyase activity by continuously "sampling" the nonreducing ends in the pectic polymer population, releasing predominantly 4,5-unsaturated digalacturonic acid if pectate lyase is active or digalacturonic acid if pectate lyase has become less active (Fig. 4). At concentrations above 0.05 mM, digalacturonic acid stimulates higher levels of pectate lyase synthesis than does the unsaturated disaccharide, apparently because it exerts less catabolite repression (6). The action pattern of exoPG and the differing effects of the two disaccharides on pectate lyase regulation tempt speculation that the ratio of the disaccharides informs the bacterium of changes in the activity of pectate lyase in the environment. These postulated functions of exoPG await testing by a mutant deficient in exoPG but not pectate lyase.

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