APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1985, p. 615–622
0099-2240/85/090615-08$02.00/0

Vol. 50, No. 3

Activity Stain for Rapid Characterization of Pectic Enzymes in Isoelectric Focusing and Sodium Dodecyl Sulfate-Polyacrylamide Gels†

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Received 27 February 1985/Accepted 28 June 1985

A system was developed for the rapid characterization of microbial pectic enzyme complexes and then tested on *Erwinia chrysanthemi* and *Sclerotium rolfsii*. Pectic enzymes in minute samples of crude culture filtrates were resolved by ultrathin-layer polyacrylamide gel isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then assayed with an ultrathin pectate-agarose overlay stained with ruthenium red. The simple procedure can be completed within 30 min after isoelectric focusing, can detect extremely low levels of pectate lyase (6.4×10^{-6} μmol of product per min), and is sufficiently sensitive to determine the pectate lyase isozyme profile of a single bacterial colony with a diameter of 4 mm. Pectate lyases and polygalacturonases can be distinguished by altering buffer conditions in the overlays. The assay system revealed additional isozymes not resolved by classical techniques and generally corroborated the previously published isoelectric points and molecular weights of the pectate lyase isozymes and exo-poly-α-D-galacturonosidase produced by *E. chrysanthemi* and the endopolygalacturonase and exopolygalacturonase produced by *S. rolfsii*.

Pectic enzymes play an important role in the interactions of microorganisms with higher plants. Pectic polymers (chains of 1,4-linked-α-D-galacturonic acid and methoxylated derivatives) are major structural constituents of plant middle lamellae and primary cell walls (27, 36). Purified pectic enzymes have multiple effects on plant tissues, including: (i) maceration and killing of cells (1, 3, 34), (ii) elicitation of disease defense reactions (12, 23), and (iii) rendering of cell walls more susceptible to attack by other microbial polysaccharidases (4, 14). Besides having an apparently important but still incompletely understood role in the development of plant diseases and the biodegradation of plant materials, pectic enzymes have several commercial uses (7).

Analysis of microbial pectic enzyme complexes and their interaction with plant tissue is limited by the techniques available for resolving and identifying the individual components. Various techniques have been reported for detecting pectic enzymes in electrophoretic gels by introducing pectic polymers into the gel before (11, 33) or after (24) electrophoresis or by activity staining a paper print of the resolved proteins (13), but none has met wide acceptance. Thus, in most studies of the pectate lyase isozymes produced by soft-rot erwinias, the time-consuming technique of isoelectric focusing in sucrose gradient columns has been used (for examples, see references 15, 16, 19, 26, 29, and 30).

Höfelmann et al. (18) have reported an alternative approach to the detection of depolymerases in isoelectric focusing gels through the use of an ultrathin-layer substrate-agarose overlay bonded to a plastic support sheet, and Bertheau et al. (5) have recently reported the use of a similar technique to detect pectic enzymes and other depolymerases in nondenaturing gels. We have developed the substrate-agarose overlay technique into a generalized system for rapidly analyzing microbial pectic enzymes in isoelectric focusing and sodium dodecyl sulfate (SDS)-polyacrylamide gels. In this report, we have used the previously characterized extracellular pectic enzyme complexes of *Erwinia chrysanthemi* CUCPB (Cornell University Collection of Phytopathogenic Bacteria) 1237 (10, 15) and *Sclerotium rolfsii* isolate 14 (2) to demonstrate the ability of the assay system to determine the isoelectric points and molecular weights of pectic enzymes in minute samples of crude protein mixtures.

We have used portions of this assay system to analyze the pectate lyase isozyme profiles of *Escherichia coli* clones containing *Erwinia chrysanthemi* genes (9).

(A preliminary report of this procedure has been published [J. L. Ried and A. Collmer, Phytopathology **74**:834, 1984].)

MATERIALS AND METHODS

Source, preparation, and assay of pectic enzyme samples. Concentrated, desalted proteins were prepared from culture supernatants of polygalacturonic acid-grown cultures of *Erwinia chrysanthemi* CUCPB 1237 as previously described (9). This strain is a spontaneous rifampin- and streptomycin-resistant derivative (8) of strain 307 characterized by Garibaldi and Bateman (16). Osmotic shock fluids (17) were also prepared for isoelectric focusing from two *Escherichia coli* clones harboring recombinant plasmids containing *Erwinia chrysanthemi* DNA (9). Alternatively, protein samples from recombinant bacteria were prepared for isoelectric focusing from single-colony supernatants. Colonies (diameter, 4 mm) of recombinant, pectolytic *Escherichia coli* clones, grown on LB (25), were suspended in 0.030 ml of Tris hydrochloride (pH 7.5) and centrifuged for 1 min at 12,000 × *g*. The supernatants were then applied to the surface of an isoelectric focusing gel. Extracellular proteins from *S. rolfsii* Sacc. isolate 14 grown on bean hypocotyls (2) were obtained from D. F. Bateman and C. H. Whalen. The lyophilized enzyme preparation was suspended in 50 mM Tris hydrochloride (pH 7.5) and diafiltered extensively against the same buffer with an immersible CX-10 membrane (Millipore Corp.).

Pectate lyase activity was determined by monitoring the

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† Scientific article no. A-4160, contribution no. 7145, of the Maryland Agricultural Experiment Station.

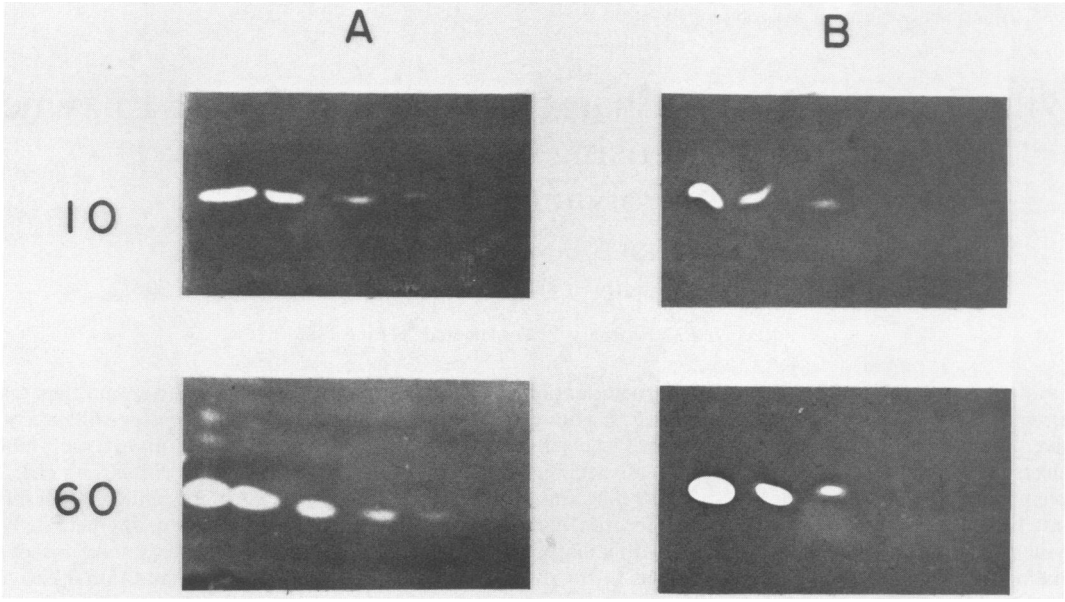

FIG. 1. The effect of overlay thickness on the sensitivity of the activity stain in detecting a pectate lyase isozyme in isoelectric focusing gels. Osmotic shock fluids from recombinant *Escherichia coli* clone CSR2 were resolved by ultrathin-layer isoelectric focusing and then incubated for either 10 or 60 min with 0.35-mm (A) or 0.8-mm (B) pectate-agarose overlays. Lanes in each panel received, from left to right, 4.0×10^{-3}, 8.0×10^{-4}, 1.6×10^{-4}, 3.2×10^{-5}, 6.4×10^{-6}, or 1.3×10^{-6} U of pectate lyase activity.

increase in A_{230} (32) of a reaction mixture containing 0.07% (wt/vol) polygalacturonic acid (product P21750 of Pfaltz and Bauer), 30 mM Tris hydrochloride (pH 8.5), 0.1 mM $CaCl_2$, and 1.7% (vol/vol) enzyme sample. Polygalacturonases were assayed by using the arsenomolybdate method of Nelson (28) to determine the increase in reducing groups in the reaction mixtures. For both types of enzymes, a unit of activity is defined as the amount of enzyme causing the formation of 1.0 μmol of product per min under optimal conditions at 25°C.

Preparation of ultrathin gels for isoelectric focusing and enzyme detection. Ultrathin (0.35-mm) polyacrylamide gels were cast for isoelectric focusing as described in the instructions of the manufacturer (FMC Corp.). Polyacrylamide solutions containing 5% acrylamide (Bio-Rad Laboratories), 0.17% *N*,*N*′-methylenebisacrylamide (Bethesda Research Laboratories), 20% (vol/vol) glycerol, 0.25 ml of Pharmalyte carrier ampholytes (pH 3 to 10; Pharmacia Fine Chemicals), 0.005 ml of *N*,*N*,*N*′,*N*′-tetramethylenediamine (Bethesda Research Laboratories), and 0.019 ml of 10% ammonium persulfate per 5.0 ml of total volume were cast by capillary action between two glass plates separated by spacers. On one of these glass plates, a gel support film (100 by 125 mm) for acrylamide (Bio-Rad Laboratories) was affixed by a thin film of water. Polymerization occurred within 15 min.

Ultrathin (0.35-mm) pectate-agarose overlay gels for enzyme detection were cast as described above except that gel support film for agarose (Bio-Rad Laboratories) was used, the agarose solution was heated to 95°C, and the gel mold was heated to 50°C before casting. The agarose solution contained 1% agarose (Bethesda Research Laboratories), 0.1% polygalacturonic acid, and the following buffers designed for differential detection of particular pectic enzymes: for *Erwinia chrysanthemi* pectate lyase, 50 mM Tris hydrochloride (pH 8.5)–1.5 mM $CaCl_2$; for *Erwinia chrysanthemi* exo-poly-α-D-galacturonosidase, 100 mM potassium phosphate (pH 6.5)–10 mM EDTA; and for *S. rolfsii* exo- and endo-polygalacturonases, 50 mM potassium acetate (pH 4.5)–10 mM EDTA. Although thicknesses of 0.35 mm for polyacrylamide and agarose gels were optimum for conservation of electrofocusing reagents and for assay sensitivity, gels 0.8 mm thick were easier to cast and provided greater reproducibility.

Ultrathin-layer isoelectric focusing of proteins. Isoelectric focusing was performed at 6°C on an LKB 2117 Multiphor apparatus modified by the use of adjustable electrofocusing electrodes (Bio-Rad Laboratories). The ultrathin polyacrylamide gel was trimmed to remove 2 mm of gel from the edges of the support film and affixed to the cooling plate by means of a thin film of water. Excess water was removed from underneath the support film by absorbent paper before preelectrofocusing to minimize distortions in the pH gradient resulting from gel contact with water. Electrode wicks (Bio-Rad Laboratories) for the anode and cathode were soaked for several minutes in 0.04 M aspartic acid and 0.5 M NaOH, respectively, and the excess electrolyte was removed by absorbent paper. Enhanced resolution in the alkaline region of the isoelectric focusing gel was obtained when the cathodic wick was then saturated with a 2% Pharmalyte carrier ampholyte solution (pH 8 to 10.5) before application to the gel. Electrofocusing was performed in a 100% N_2 atmosphere maintained in a plastic, rectangular food storage container modified to house the cooling plate and electrodes. This increased the uniformity of the pH gradient across the gel by eliminating condensation on the gel surface and also reduced cathodic drift resulting from absorption of CO_2. The gel was preelectrofocused for 30 min at a constant 2.0 W. Protein samples of up to 15 μl were applied directly onto the gel as a series of drops in each lane or indirectly via a small tab of electrode strip paper (Bio-Rad Laboratories). Subsequent electrofocusing was for 60 min at a constant 4.5 W with a maximum of 2,000 V; sample application tabs were removed 15 min after electrophoresis was begun.

SDS-polyacrylamide gel electrophoresis and enzyme renaturation. SDS-polyacrylamide gel electrophoresis was

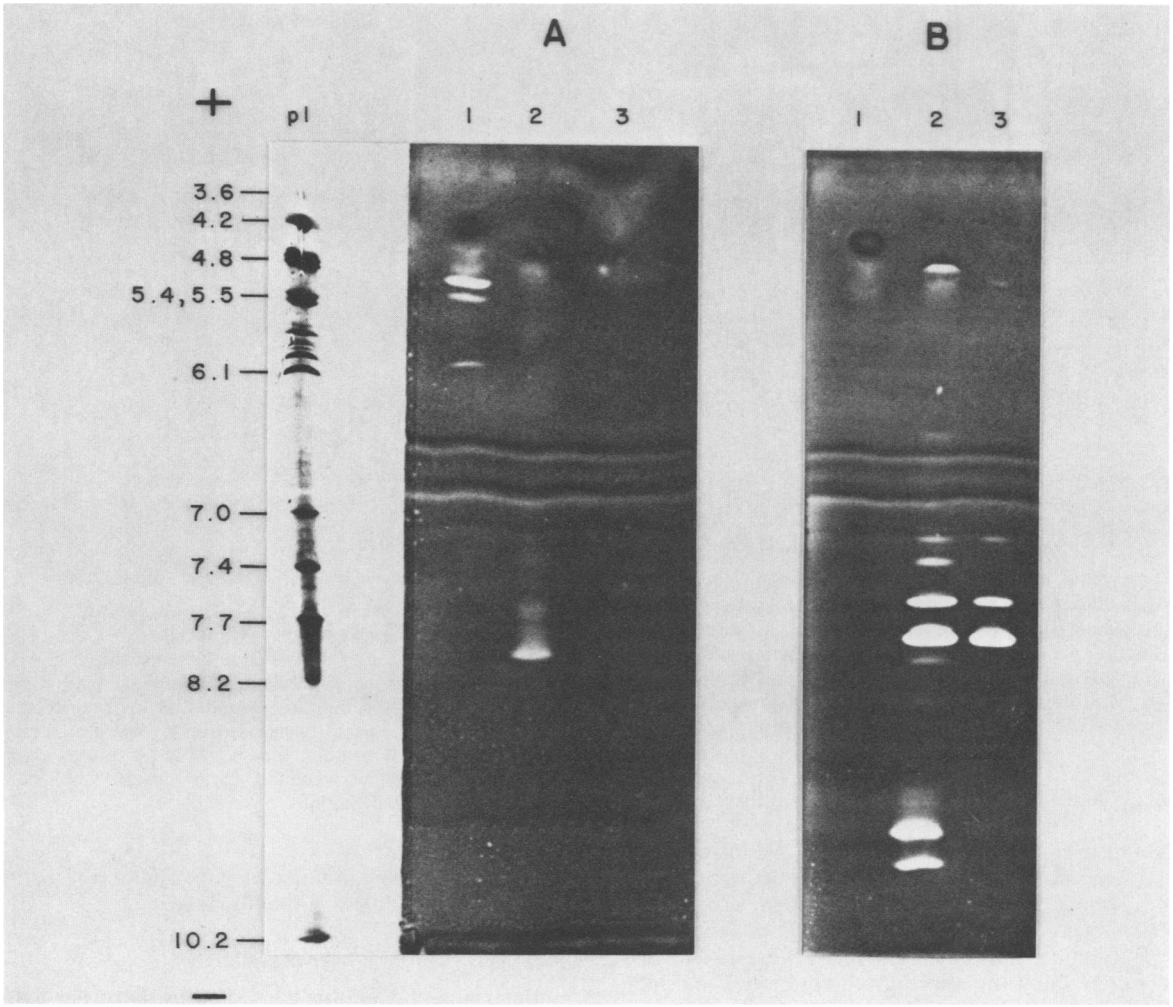

FIG. 2. Detection and differentiation of the pectic enzymes produced by *Erwinia chrysanthemi*, *S. rolfsii*, and recombinant *Escherichia coli* clone CSR50 after resolution by ultrathin-layer isoelectric focusing. Concentrated and desalted samples were electrophoresed on a single isoelectric focusing gel which was divided into three sections for processing. The lane containing pI markers (amyloglucosidase, glucose oxidase, ovalbumin, β-lactoglobulin, carbonic anhydrase, horse myoglobin minor and major bands, whale myoglobin minor and major bands, cytochrome *c*; FMC Corp.) was immediately stained for protein. The remaining lanes were incubated for 10 min with two diagnostically buffered pectate-agarose overlays. In panel A, overlays were buffered with 50 mM potassium acetate (pH 4.5) and 10 mM EDTA. In panel B, overlays were buffered with 50 mM Tris hydrochloride (pH 8.5) and 1.5 mM $CaCl_2$. Lane 1, *S. rolfsii*; lane 2, *Erwinia chrysanthemi*; lane 3, *Escherichia coli* clone CSR50.

performed by using the system of Laemmli (22) with a resolving gel containing 10% acrylamide, 0.33% bisacrylamide, and 20 μg of bovine serum albumin (Sigma) per ml to enhance renaturation of electrophoresed enzymes; the stacking gel contained 5% acrylamide–0.17% bisacrylamide. The SDS (Bio-Rad Laboratories) concentration was 0.1% in the gel and running buffer. Samples were held at 100°C for 2 min in sample buffer (2% SDS, 10% sucrose, 50 mM dithiothreitol [DTT], 125 mM Tris hydrochloride [pH 6.8], 0.003% bromphenol blue) before being cooled to 0°C. It was necessary to eliminate the DTT from the sample buffer for detection of depolymerizing activity from *S. rolfsii* protein samples. Samples were electrophoresed at 12.5 mA for 2.0 h through a 0.75-mm gel in a small, vertical slab unit (model SE 200; Hoefer Scientific Instruments). Gels that were activity stained for pectate lyase and exo-poly-α-D-galacturonosidase were incubated for 2 h with shaking in three 100-ml changes of 10 mM Tris hydrochloride (pH 7.5) (20). For *S. rolfsii* polygalacturonase detection, the gel was incubated with 10 mM potassium acetate (pH 4.5). Subsequently, these gels were protein stained by overnight immersion in a solution containing 0.1% (vol/vol) Coomassie brilliant blue R (Sigma), 10% (vol/vol) acetic acid, and 50% (vol/vol) methanol; the gels were destained in 10% acetic acid–50% methanol and then swelled to their original size in 10% acetic acid.

Activity stain overlay technique for detecting pectic enzymes after isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Pectate-agarose overlays constructed as described above were placed directly onto the gel to be assayed. Air bubbles trapped between the two gels were removed by gently rubbing the plastic backing of the overlay with the tip of a rounded glass stirring rod. The sandwiched gels were incubated for various times in a moist chamber at room temperature for isoelectric focusing or at 30°C for SDS-polyacrylamide gel electrophoresis (the temperature of incubation was not critical), and then the overlays were immersed in 0.05% (wt/vol) ruthenium red (Sigma) for 20 min, rinsed in distilled water and preserved by drying at 50°C for 45 min. The overlays were then placed between clear

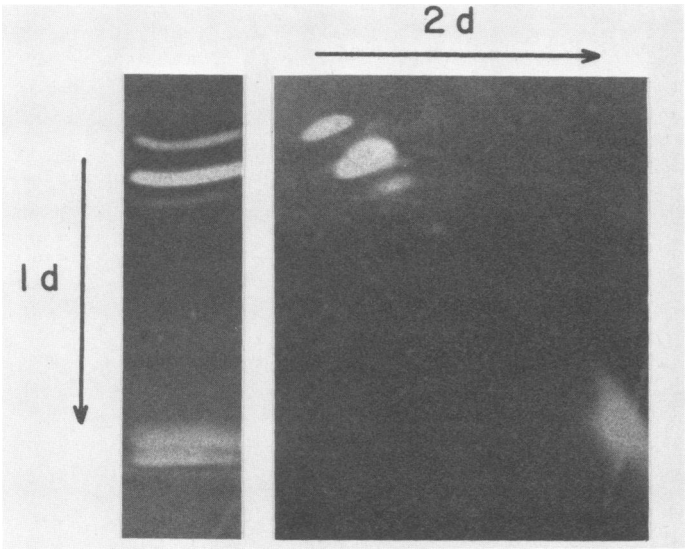

FIG. 3. Two-dimensional ultrathin-layer isoelectric focusing of the major *Erwinia chrysanthemi* pectate lyase isozymes. The first-dimension isoelectric focusing gel (ca. pH 7.0 to 10.0) was incubated for 10 min with a pectate-agarose overlay and then placed perpendicular to the pH gradient on a second ultrathin-layer isoelectric focusing gel. The electrofocused second dimension gel was then incubated with a pectate-agarose overlay for 15 min.

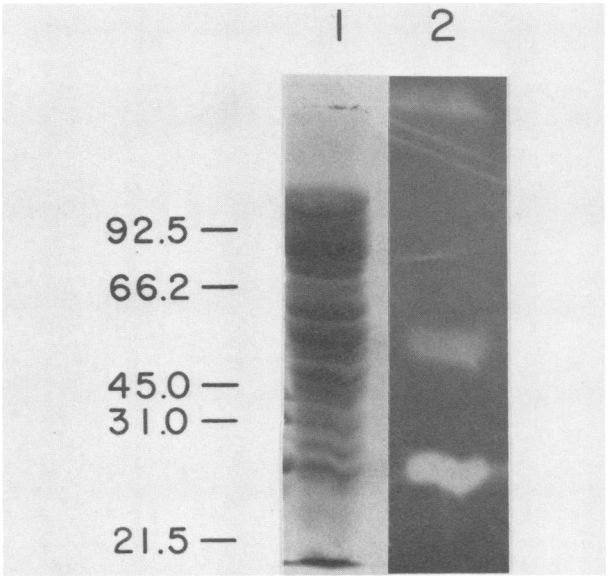

FIG. 5. Detection in SDS-polyacrylamide gels of the polygalacturonase and exopolygalacturonase produced by *S. rolfsii*. Gels were developed as described in the legend to Fig. 4 except that a 50 mM potassium acetate (pH 4.5)–10 mM EDTA buffer was used in the overlay. The sample contained 10.0 U of hydrolase, and the incubation with the pectate-agarose overlay was for 60 min. Lane 1, Coomassie-stained protein; lane 2, activity-stained overlay. The positions of molecular weight markers (described in the legend to Fig. 4) are shown at the left.

plastic sheets and photographed with a green filter (Wratten no. 58).

Detection of pectolytic bacterial colonies. We have applied the overlay technique to the detection of pectolytic *Escherichia coli* colonies which have been transformed by recombinant plasmids containing *Erwinia chrysanthemi* DNA (9). Putative pectolytic *Escherichia coli* cells were plated onto agar plates containing the appropriate antibiotics and incubated until colonies were about 2 mm in diameter. Whatman no. 1 filter paper was briefly laid onto each plate, removed (taking most of each colony), and then placed onto the agarose overlay. The resulting sandwich was covered with plastic wrap and incubated at 30°C for 30 to 60 min. The filter paper was then removed, and the overlay was vigorously washed in running water to remove adhering bacteria and then stained in 0.05% ruthenium red.

RESULTS

Determination of the limit of sensitivity for detection of pectate lyase in ultrathin-layer isoelectric focusing gels. The sensitivity of the pectate-agarose overlay technique was determined with osmotic shock fluids from recombinant *Escherichia coli* clone CSR2 because this strain produces a single major pectate lyase isozyme. The gel was incubated with overlays of different thicknesses for different periods of

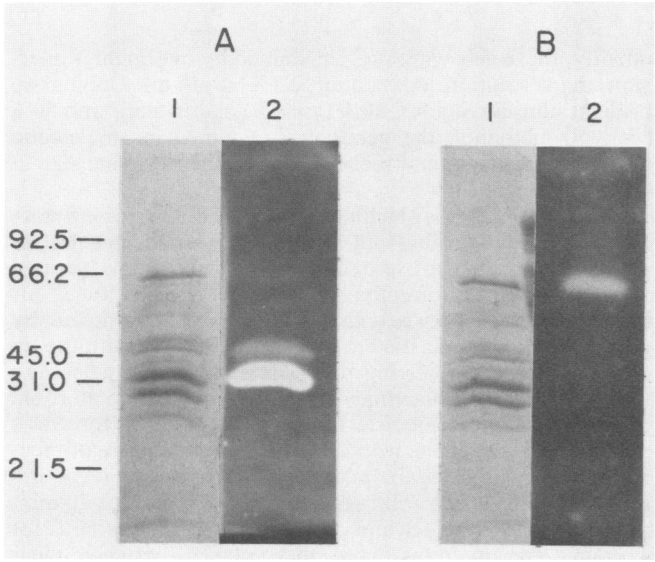

FIG. 4. Detection in SDS-polyacrylamide gels of the pectate lyase and exo-poly-α-D-galacturonosidase produced by *Erwinia chrysanthemi*. Both protein samples contained 1.6 U of pectate lyase and 0.19 U of exo-poly-α-D-galacturonosidase. Extracellular proteins of *Erwinia chrysanthemi* were resolved by SDS-polyacrylamide gel electrophoresis. The SDS was removed, and the gel was incubated at 30°C with a pectate-agarose overlay before protein staining. (A) Pectate lyase detection after 60 min of incubation with an overlay containing 50 mM Tris hydrochloride (pH 8.5) and 1.5 mM $CaCl_2$. Lane 1, Coomassie-stained protein; lane 2, activity-stained overlay. (B) Exo-poly-α-D-galacturonosidase detection after 120 min of incubation with a pectate-agarose overlay containing 100 mM potassium phosphate (pH 6.5) and 10 mM EDTA. Lane 1, Coomassie-stained protein; lane 2, activity-stained overlay. The positions and sizes (in thousands) of molecular weight markers (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor; Bio-Rad Laboratories) are shown at the left. In our system, carbonic anhydrase (M_r, 31,000) migrated anomalously relative to the remaining standards. For this reason, this protein was not used in calculating the molecular weights of the unknown proteins.

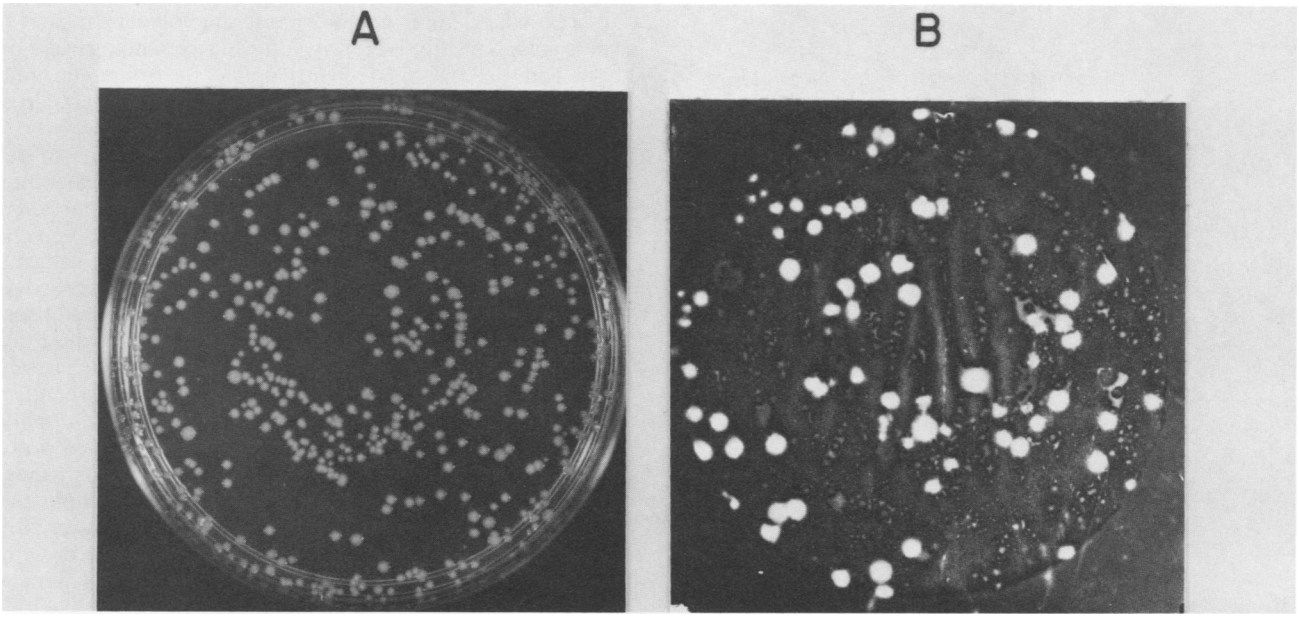

FIG. 6. Detection of pectolytic recombinant *Escherichia coli* clones in a background of 90% nonpectolytic *Escherichia coli*. (A) Colonies before assay; (B) agarose overlay of A.

time after isoelectric focusing of a dilution series of the osmotic shock fluid preparation (Fig. 1). The 0.35-mm agarose overlay was most sensitive, detecting 6.4×10^{-6} U of pectate lyase after 60 min of incubation and 3.2×10^{-5} U after 10 min (Fig. 1A). The 0.8-mm overlay detected only 1.5×10^{-4} U after both 10- and 60-min incubations (Fig. 1B). Overlays substantially less than 0.35 mm thick did not stain well. Resolution of pectate lyase isozymes diminished with increased sample activity and overlay incubation time (Fig. 1).

The sensitivity of the agarose overlay technique with SDS-polyacrylamide gels is about 0.10 U of pectate lyase after 60 min with 0.35-mm agarose gels (data not shown). This reduction in sensitivity compared with isoelectric focusing necessitates the use of a proportionately larger sample volume or an increased concentration of the enzyme.

Differential detection of polygalacturonic acid depolymerizing enzymes after isoelectric focusing. The composition of the agarose overlay can be varied to differentially detect pectate lyases (β-eliminative enzymes which have high pH optima and require a divalent cation) or polygalacturonases (hydrolytic enzymes which have acidic pH optima and are not inactivated by EDTA) (31). Figure 2 shows areas of pectolytic activity in identical sets of samples subjected to isoelectric focusing. In panel A, the overlay was buffered at pH 4.5 and contained 10.0 mM EDTA to optimize detection of *S. rolfsii* polygalacturonases and inhibit *Erwinia chrysanthemi* pectate lyases. Lane 1 shows three major bands of hydrolytic activity for *S. rolfsii* in the acidic area of the gel corresponding to pH's 5.1, 5.5, and 6.0. Lane 2 shows a single band of hydrolytic activity for *Erwinia chrysanthemi* at pH 8.0. In lane 3, a pectolytic *Escherichia coli* transformant previously testing negative for hydrolytic activity shows no depolymerase activity. In panel B, the same set and concentration of samples was focused, but the overlay had a pH of 8.5 and contained calcium. Under these conditions, the *S. rolfsii* polygalacturonases were completely inactive (lane 1), whereas the pectate lyases of both *Erwinia chrysanthemi* (lane 2) and the *Escherichia coli* transformant (lane 3) were active. The *Escherichia coli* transformant did not produce as many bands as *Erwinia chrysanthemi* apparently because not all of the pectolytic genes of *Erwinia chrysanthemi* were cloned. The faint band at pH 8.0 in lane 2, panel B, is probably the exo-poly-α-D-galacturonosidase of *Erwinia chrysanthemi* since it has the appropriate pI value and is not produced by the *Escherichia coli* transformant (Fig. 2A, lane 3).

Demonstration that the multiple isozymes of pectate lyase are not artifacts of isoelectric focusing. The basic portion of an isoelectric focusing gel of *Erwinia chrysanthemi* culture supernatants was subjected to isoelectric focusing in a second dimension (Fig. 3). Only a single band of activity was generated in the second dimension for each band resolved in the first dimension, indicating that the multiplicity of isozymes is not an artifact generated by isoelectric focusing. They arise from different structural genes or posttranslational modification or both.

Differential detection of pectate lyase and polygalacturonase after SDS-polyacrylamide gel electrophoresis. The pectate-agarose overlay technique detected renatured pectate lyase and polygalacturonase after SDS-polyacrylamide gel electrophoresis (Fig. 4 and 5). Pectate-agarose overlays revealed two major bands of activity corresponding to molecular weights of 36,000 and 44,000 for *Erwinia chrysanthemi* (Fig. 4A) and a single band of exo-poly-α-D-galacturonosidase activity (Fig. 4B). Not all of these enzymes renatured equally. The 44,000-molecular-weight band of activity required a threefold longer buffer wash after electrophoresis for enzyme detection than did the 36,000-molecular-weight band. For *S. rolfsii*, the overlay detected two bands of polygalacturonase activity corresponding to molecular weights of 52,000 and 29,000 (Fig. 5). The presence of DTT in the sample buffer prevented the detection of polygalacturonase, indicating the presence of essential disulfide linkages.

The use of isopropanol for renaturing enzymes after SDS-polyacrylamide gel electrophoresis has been reported to increase activity for certain enzymes (6). We found no increase in the sensitivity of the assay when isopropanol was used. In agreement with other workers (21), we found that

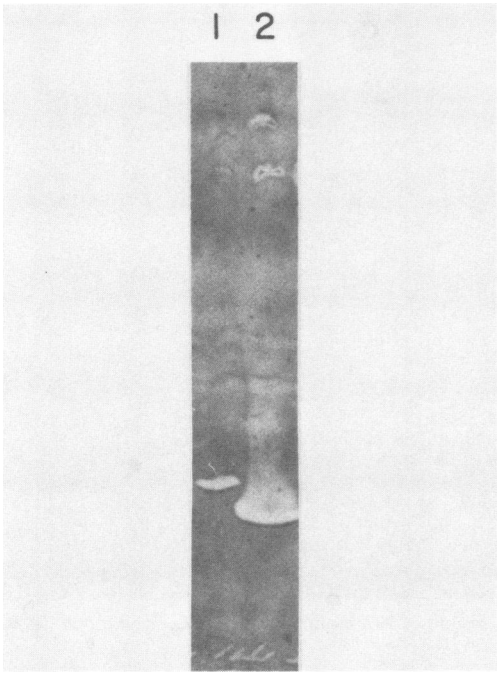

FIG. 7. Resolution of pectate lyase isozymes from single-colony supernatants. Samples (up to ,15 μl) of single-colony supernatants (see the text for details) were electrophoresed on ultrathin isoelectric focusing gels, then assayed with a pectate-agarose overlay buffered with 50 mM Tris hydrochloride (pH 8.5)–1.5 mM $CaCl_2$. Lanes 1 and 2, pectolytic *Escherichia coli* clones producing two different isozymes of pectate lyase. The anode is at the top of the figure, and the cathode is at the bottom.

the source of SDS was critical for detecting enzymes; in this case, SDS from Sigma completely inhibited enzyme activity with or without DTT.

Detection of pectolytic bacterial colonies and resolution of pectic enzymes from single colony supernatants. The pectate-agarose overlay technique has been adapted for detecting pectolytic *Escherichia coli* colonies grown on standard microbiological media. After the filter paper was removed from the pectate-agarose overlay, vigorous rinsing of the overlay was required to remove bacteria that adhered to the surface; otherwise, these bacteria would prevent staining in that area. Pectolytic colonies produced a clear area (Fig. 6). Growth of the assayed colonies in the original petri plate, followed by proper alignment of the overlay with the petri plate, allowed pectolytic colonies to be identified and isolated from a background of nonpectolytic colonies.

A profile of the pectate lyase isozymes produced by *Escherichia coli* colonies could be obtained by suspending colonies grown on petri plates in buffer and subjecting the supernatant to isoelectric focusing (Fig. 7).

DISCUSSION

The combination of electrofocusing in ultrathin-layer polyacrylamide gels and SDS-polyacrylamide gel electrophoresis in small slab gels provides a rapid, high resolution method for analyzing a complex mixture of proteins. We have developed an activity-staining system for use in conjunction with these electrophoretic techniques which permits facile detection and preliminary characterization of pectic enzymes in crude protein mixtures. The detection technique is based on the ability of pectic enzymes diffusing from bands in a polyacrylamide gel to degrade the polygalacturonic acid in a diagnostically buffered, ultrathin pectate-agarose overlay and on the subsequent ability of ruthenium red to precipitate and stain (35) the surrounding, undegraded substrate.

This detection system provides a rapid means to determine pI profiles and molecular weights of polygalacturonic acid-degrading enzymes. Our results are summarized and compared with previously obtained values for *Erwinia chrysanthemi* and *S. rolfsii* in Tables 1 and 2. The pI values that we report corroborate and extend the enzyme complexity for each organism (Table 1). Significant differences are observed with SDS-polyacrylamide gel electrophoresis of the *Erwinia chrysanthemi* pectate lyase isozymes (Table 2). However, our values are in close agreement with those recently obtained for a different strain of *Erwinia chrysanthemi* by SDS-polyacrylamide gel electrophoresis of purified proteins (19). The polygalacturonase values from *S. rolfsii* that we report were obtained without DTT in the sample buffer because it prevented detection of enzyme activity. Nevertheless, the protein patterns appeared similar with or without DTT (data not shown), and our values are consistent with those previously reported (Table 2).

The activity staining system demonstrated with *E. chrysanthemi* and *S. rolfsii* pectic enzymes has at least four advantages over previously described methods. (i) Speed. Assay results are obtained in 30 to 80 min (depending on the desired sensitivity) after the completion of isoelectric focusing (Fig. 1) or 3 to 4 h after SDS-polyacrylamide gel electrophoresis, and each of the electrophoretic techniques is complete within 2 h. The activity-stained overlay can be immediately photographed or oven-dried for a permanent record. In the similar procedure of Bertheau et al. (5), a longer incubation of the substrate-agarose overlay with the isoelectrically focused gel (1 to 4 h versus 10 to 60 min) is recommended, as is a longer incubation of the overlay with the staining reagent (overnight in 1% cetyltrimethylammomium bromide versus 20 min in 0.05% ruthenium red).

(ii) Sensitivity. Figures 1 and 2 demonstrate the extreme sensitivity of the activity stain by showing its ability to detect an isoelectrically focused band containing only 6.4×10^{-6} U of pectate lyase activity (Fig. 1). Based on a specific

TABLE 1. Isoelectric points of major isozymes as determined by present and previous techniques

Enzyme	Isoelectric point of enzyme from:			
	Erwinia chrysanthemi		*S. rolfsii*	
	Present[a]	Previous	Present[a]	Previous
Pectate lyase	9.4 9.2 7.8 7.5	9.1,[b] 9.3[c] 7.9,[b] 8.3[c]	—[d]	—
Exo-poly-α-D-galacturonosidase	8.0	8.3[c]	—	—
Polygalacturonases	—	—	5.1 5.5 6.0	5.2[e]

[a] Determined by activity-stained ultrathin-layer isoelectric focusing.
[b] Determined by sucrose gradient isoelectric focusing (15).
[c] Determined by granulated gel bed isoelectric focusing (10).
[d] —, Not detected in this organism.
[e] Determined by sucrose gradient isoelectric focusing (2).

TABLE 2. Molecular weights of enzymes as determined by present and previous techniques

Enzyme	Molecular weight of enzyme from:			
	Erwinia chrysanthemi		*S. rolfsii*	
	Present[a]	Previous	Present[a]	Previous
Pectate lyase	36,000 44,000	30,000–32,400[b]	—[c]	—
Exo-poly-α-D-galacturonosidase	68,000	67,000[d]	—	—
Polygalacturonases	—	—	29,000 52,000	28,000–31,000[e] 46,000–48,000[e]

[a] Determined by activity-stained SDS-polyacrylamide gel electrophoresis.
[b] Determined by gel filtration chromatography and sucrose density gradient centrifugation (15).
[c] —, Not detected in this organism.
[d] Determined by SDS-polyacrylamide gel electrophoresis of purified protein (10).
[e] Determined by gel filtration chromatography and sucrose density gradient centrifugation (2).

activity for pectate lyase of 300 U/mg of protein (1; Collmer and Whalen, unpublished results), this is equivalent to 20 pg of pectate lyase protein. This technique is sufficiently sensitive to determine the isozyme profile of single-colony supernatants (Fig. 7). This high sensitivity can be attributed to the low concentration (0.1%) of polygalacturonic acid, the intense differential staining by ruthenium red (35), and the thinness of the overlay (Fig. 1).

(iii) Resolution. The superior resolution achieved by activity-stained ultrathin-layer isoelectric focusing gels is shown by the clean separation of several isozymes of pectate lyase present in *Erwinia chrysanthemi* culture supernatants. These results, which corroborate those of Bertheau et al. (5), are in sharp contrast to earlier work with sucrose gradient isoelectric focusing, in which only two peaks of activity were reported (15, 16). The remarkable ability of pectate-agarose overlays to resolve closely spaced pectic enzymes, even when one band contains far more activity than another, can be attributed to the assay mechanism of detecting zones of enzymatic degradation in a background of relatively slowly diffusing substrate rather than detecting a diffusible product. High resolution is also favored by the rapidity of the assay (Fig. 1).

(iv) Adaptability. Because the substrate-containing overlay is separate from the polyacrylamide gel or other enzyme source to be assayed, the activity-staining system is adaptable to a wide variety of applications. We have demonstrated its usefulness for detecting pectolytic *Escherichia coli* colonies on petri plates (Fig. 6). In addition, alternate lanes of a single polyacrylamide gel can be assayed with overlay strips containing different buffers for the detection of different enzymes (Fig. 2, 4, and 5). Proteins remaining in the polyacrylamide gel can then be visualized with standard protein stains (Fig. 4 and 5) or electrophoresed in a second dimension (Fig. 3). We have used second-dimension isoelectric focusing of pectic enzymes after SDS-polyacrylamide gel electrophoresis to determine pI values for specific, pectolytic bands (9). These manipulations allow the rapid assignment of an isoelectric point and a molecular weight to each component of a pectic enzyme complex.

ACKNOWLEDGMENT

The work was supported by grant 84-CRCR-1-1366 from the Competitive Research Grants Office of the U. S. Department of Agriculture.

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