Cutin Degradation by Plant Pathogenic Fungi

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ABSTRACT

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Sixteen plant pathogenic fungi were cultured on a V-8 juice broth medium for 3-5 days at 25 C. This medium then was replaced with either a minimum salts or a V-8 juice broth medium supplemented with 0.5% apple cutin. The cultures were allowed to incubate for an additional period of up to 14 days, during which time samples of the culture fluids were assayed periodically for cutinase. Cutinase activity was determined routinely by measuring the release of ether-soluble radioactivity from ^{3}H-cutin at pH 5.0 and 8.5. Cutinase activity was demonstrated in the culture fluids of the following fungi: *Botrytis cinerea*, *B. squamosa*, *Cladosporium cucumerinum*, *Colletorichum graminicola*, *Fusarium solani* f. sp. *phaseoli*, *F. solani* f. sp. *pisi*, *F. roseum*, *Gloeocercospora sorghi*, *Helminthosporium carbonum*, *H. maydis* (race T), *Pythium aphanidermatum*, *P. arrhenomanes*, *P. ultimum*, *Rhizoctonia solani*, *Stemphylium loti*, and *Sclerotium rolfsii*. The released of fatty acids from cutin by culture filtrates of 12 of the fungi studied was corroborated by gas-liquid chromatography of reaction products.

Additional key words: cutinase assay, plant cuticle, cutinase.

Many plant pathogenic fungi can penetrate plant cuticles directly (5, 39). The mechanism(s) involved in cuticular penetration has been a matter of controversy. The possible involvement of enzymes in digestion of plant cuticle during fungal penetration was raised by several investigators prior to 1900 (4, 21), but much of the work which followed focused on the possible involvement of mechanical pressure as the primary means of fungal ingress (1, 2, 8, 38). This body of information coupled with the lack of chemical evidence for production of cutinolytic enzymes prior to 1960 led to the generally accepted view that fungi breached plant surfaces by mechanical means (5, 39).

Since 1960 there have been several reports of fungal degradation of cutin (9, 25, 26, 31, 32), a polymeric layer of fatty acid derivatives within the plant cuticle (14). These studies plus recent histological studies of fungal penetration at the electron microscopic level (6, 19, 20, 22, 24, 27) indicate that enzymes may be involved in fungal ingress. Histochemical studies have demonstrated the presence of esterase activity as well as chemical modification of the cutin matrix at penetration sites during fungal ingress (16, 19, 23). Recently, the production of cutinase by *Fusarium solani* f. sp. *pisi* during penetration of host culticle has been confirmed by immunohistological procedures (29).

Chemical evidence supporting enzymatic degradation of cutin is quite limited. For the most part this evidence has been obtained by indirect methods for measuring enzyme activities on impure substrates (36). Recently, Purdy and Kolattukudy (26) have described an improved, sensitive method for measuring cutinolytic activity which involves measuring radioactivity released from ^{3}H-cutin. Since it is difficult to obtain pure cutin, all the released radioactivity measured by this assay may not be attributable to cutin degradation. In this study we improved Purdy and Kolattukudy's assay procedure and demonstrated the ability of several phytopathogenic fungi to produce cutinolytic enzymes.

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MATERIALS AND METHODS

Preparation of cutin substrates.—Apple cutin was prepared from 30 kg of fresh Golden Delicious apple peelings by a four-step procedure similar to that described by Walton and Kolattukudy (37). Apple peelings were immersed and constantly stirred in boiling aqueous 0.4% oxalic acid and 1.6% ammonium oxalate (10 liters/kg peelings). As soon as the cuticles could be separated from the underlying tissue (about 10-15 min), they were collected on a 2.5-mm (10-mesh) screen and rinsed thoroughly with large amounts of tap water (step 1). Then 50-g amounts of the 400 g of crude cuticles recovered in step 1 were treated for 18 hr at 30 C with 2 liters of 100 mM sodium acetate buffer (pH 4.5) containing 0.5% cellulase and 0.1% pectinase from *Aspergillus niger* (Lots 42C-1670 and 13C-2370, respectively, Sigma Chemical Company, St. Louis, MO 63178). The insoluble material that remained (cutin plus epicuticular waxes) was collected on a 2.5-mm screen and rinsed thoroughly with tap water (step 2). This material then was extracted with methanol (1 liter/50 g) followed by 2:1 chloroform-methanol (1 liter/50 g) at room temperature (stem 3). The remaining material (cutin) was subjected to Soxhlet extraction (three cycles/hr) with chloroform for 24 hr

(step 4). The cutin films obtained then were dried under a hood, and repurified twice (steps 2-4). About 250 g of purified cutin was obtained from the 30 kg of apple peelings. This purified cutin was ground in a Wiley mill equipped with a 0.64-mm (40-mesh) screen and used as a supplement in culture media, and for preparing substrates for enzyme assays.

Tritium labeling of the purified apple cutin (350 mg) was done by the Biochemical and Nuclear Corporation, Burbank CA 91503 by the method of Hembree et al (11). The labeled product was treated with cellulase and pectinase and washed with organic solvents as described by Purdy and Kollattukudy (26). Labeled cutin (200 mg, 4×10^6 dpm/mg) was diluted 50-fold with nonlabeled cutin in 200 ml of 200 mM tris (hydroxymethyl) aminomethane (Tris) buffer (pH 8.5) containing 0.02% Thimerosal and 0.025% Triton X-100 and stirred for 24 hr at 28 C. The cutin was collected on Whatman GF/A paper by vacuum filtration and washed sequentially with 1 liter of distilled water, 1 liter of acetone, and 500 ml of ethyl ether. The final labeled product (^{3}H-cutin) contained about 6×10^4 dpm/mg.

Nonlabeled cutin substrate for enzyme assays was prepared by grinding purified cutin (5 g) in a Wiley mill equipped with a 246-μm (60-mesh) screen. The resultant powder then was stirred with 250 ml of 200 mM Tris buffer (pH 8.5) and washed with water and solvents in the same way that we treated the diluted radiolabeled cutin.

Analysis of cutin substrates.—Samples of cutin films at steps 1, 2, and 4 of the first cycle of purification, and samples of purified cutin were examined by light and electron microscopy. Unless otherwise stated, all steps were carried out at 25 C. Segments, 1 cm^2, from several cutin films were fixed in 3% glutaraldehyde in 50 mM potassium phosphate buffer (pH 6.8) for 1 hr, postfixed in buffered 1% osmium tetroxide for 3 hr, and stained with aqueous 0.5% uranyl acetate for 3 hr. Dehydration was accomplished with an acetone series. The segments were stored overnight at 4 C at the 75% acetone step. After dehydration, the segments were placed in propylene oxide for 1 hr and then embedded in Spurr's low-viscosity mix E, and polymerized at 70 C for 2 days (33).

The embedded cutin segments were thin-sectioned (approximately 0.05 μm) transversely with a diamond knife on a Sorvall Porter-Blum Ultramicrotome (MT-2). The sections were placed on 74-μm (200-mesh) copper grids and stained for 10 min with 0.5% aqueous uranyl acetate followed by a 10-min staining with 0.25% lead citrate (K & K Labs, Plainview, NY 11803) in 0.1 N NaOH. Sections of cutin preparations at different stages of purification were examined with a Philips Model 200 electron microscope operated at 60 kV to determine the loss of cell wall constituents and other noncuticular materials. Transverse sections (10 μm thick) of plastic-embedded cutin segments also were examined with a Zeiss Photomicroscope II employing bright-field, interference contrast, and polarizing light optics.

The purified cutin was analyzed for C, H, O, S (Schwarzkopf Microanalytical Laboratory, Woodside, NY 11377) and N (R. Whipple, Department of Floriculture and Ornamental Horticulture, Cornell University, Ithaca, NY 14853). The cellulose content was estimated by the method of Updegraff (34). Noncellulosic cell wall carbohydrate was estimated by gas-liquid chromatography (GLC) of methanolysis products prepared by heating 5 mg of the cutin in methanolic 1 N HCl for 24 hr at 85 C as described by Chambers and Clamp (3). Cutin fatty acids were extracted by refluxing 100 mg of cutin powder in 100 ml of methanolic 3% KOH for 24 hr. The insoluble material (18 mg) which remained after refluxing was removed by vacuum filtration. The methanol was removed from the filtrate by vacuum distillation. The residue was dissolved in 20 ml of water and acidified by the dropwise addition of 12 N HCl. This aqueous solution then was extracted four times with 20 ml of ethyl ether. The pooled ether extracts were dried over anhydrous sodium sulfate. The constituents extracted by the ether were converted to trimethylsilyl ethers of methyl esters, as described below, which then were analyzed with a Finnigan Model 3300 gas-liquid chromatograph-mass spectrometer (Department of Chemistry, Cornell University, Ithaca, NY 14853).

Radiolabeled cutin (5 mg) was chemically hydrolyzed by refluxing in 50 ml of methanolic 3% KOH for 24 hr. The insoluble material remaining was removed by filtration through glass fiber paper (Whatman GF/A). The methanol was removed from the filtrate under partial vacuum and the residue was dissolved in 20 ml water. The resulting aqueous hydrolyzate was extracted four times with 20 ml of ethyl ether, acidified by the dropwise addition of 12 N HCl, and the acidified hydrolyzate was extracted four times with 20 ml of ether. The radioactivity of the four fractions obtained; ie, residue, alkaline ether, acidic ether, and aqueous fractions, was determined with a liquid scintillation counter as described below. The alkaline and acidified ether fractions were combined and dried over anhydrous sodium sulfate. The ether was removed with a nitrogen stream. The samples were dissolved in 1.0 ml of column solvent consisting of 5% 2-propanol and 1% acetic acid in chloroform, and applied to a 2.5×90-cm column of LH-20 Sephadex. The column was eluted in an ascending manner at a rate of 10 ml/hr at 4 C. Five-ml fractions were collected and the radioactivity was determined. The contents of each column fraction were examined by GLC.

Determination of radioactivity.—Radioactivity was determined (counting error $\leqslant$ 3%) with a Beckman Model LS-350 liquid scintillation counter. An external standard-channels ratio method was used to determine the counting efficiency (about 55%) of all samples except those which were particulate such as cutin powder. In the case of these latter samples, an internal standard-channels ratio method was used to determine counting efficiencies. Samples contained in organic solvents were placed under a hood until the solvent had evaporated prior to the addition of 10 ml of scintillation fluid [toluene containing 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazole) benzene]. Aqueous solutions and particulate samples were counted after addition of 10 ml of Aquasol (New England Nuclear, Boston, MA 02118).

Derivatization and gas-liquid chromatography (GLC).—Samples to be examined for cutin constituents by GLC were placed in 13×100-mm screw-cap tubes and the solvent was removed under reduced pressure and with a nitrogen stream. Ether extracts from aqueous solutions were first dried over anhydrous sodium sulfate prior to solvent removal. To prepare the methyl esters of fatty acids, 1.0 ml of benzene and 1.0 ml of 14% boron tri-

fluoride in methanol were added to each sample. Myristic acid was added to some samples to serve as an internal standard. A Teflon-lined cap was tightly affixed and the tube was heated to 85 C for 10 min in a heating block. After cooling, 10 ml of water was added. The benzene layer was removed and 0.5-ml amounts of benzene were layered onto the water and removed three times. The benzene washes were pooled in a 3-ml vial with a Teflon-lined cap and the solvent was removed partially under reduced pressure and completely by a stream of nitrogen. Overexposure (> 10 min) to drying in a nitrogen stream at any step significantly reduced the efficiency of recovery of the fatty acid methyl esters.

The trimethylsilyl (TMSi) ethers of the fatty acid methyl esters were prepared by adding 50 μliters of pyridine and 200 μliters of *N,O*-bis (trimethylsilyl) acetamide. In some samples, palmitic acid methyl ester was added with the pyridine to serve as an internal standard. After 20 min, 1 μliter of this solution was injected into a Perkin-Elmer Model 900 gas chromatograph equipped with dual flame ionization detectors and a linear temperature program (set at 4 C/min from 135 to 305 C). Glass columns (1.8 m long, 2 mm ID) packed with 3% SE-30 on 149-131 μm (100/120 mesh) Gas Chrom Q were used for gas chromatography of the TMSi ethers of fatty acid methyl esters. Column packing materials as well as standards and derivatization reagents were obtained from Supelco, Inc., Bellefonte, PA 16823.

With the GLC procedure described, derivatives of epoxy fatty acids and trihydroxy fatty acids could not be resolved. The quantities of these two classes of fatty acids are reported as a combined total.

Cultures of *Botrytis cinerea* Pers. ex. Fr., *B. squamosa* Walker, *Cladosporium cucumerinum* Ell. & Arth., *Colletotrichum graminicola* (Cesati) C. W. Wilson, *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyd. & Hans., *F. solani* (Mart.) Sacc. f. sp. *pisi* (F. R. Jones) Snyd. & Hans., *F. roseum* (Lk.) Snyd. & Hans., *Gloeocercospora sorghi* Bain & Edgerton, *Helminthosporium carbonum* Ullstrup, *H. maydis* Nisikado & Miyake (race T), *Pythium aphanidermatum* (Edson) Fitz., *P. arrhenomanes* Drechsler, *P. ultimum* Trow, *Rhizoctonia solani* Kühn, *Stemphylium loti* Graham, and *Sclerotium rolfsii* Sacc. were maintained on potato-dextrose agar (PDA) slants at 25 C. Three 1-cm-diameter disks from 3-day-old PDA plate cultures of these fungi were used to seed 50 ml of V-8 juice broth (in a 500-ml flask) prepared from 200 ml of V-8 juice filtrate, 36 ml 0.2 M sodium hydroxide, 200 ml 0.2 M glycine, and distilled water added to make one liter. Cultures were incubated at 25 C. When about two-thirds of the medium surface had been covered by a mycelial mat (about 3-6 days), the culture fluid was removed from beneath the fungal mats and replaced with 50 ml of V-8 juice broth supplemented with 0.5% apple cutin. At three different intervals during a 14-day incubation period, 4-ml samples of culture fluid were removed, centrifuged, and assayed for cutinolytic activity.

Cutinase assay.—Cutinolytic activity in fungal culture fluids was detected by measuring the radioactivity of ether-soluble materials released from ^{3}H-apple cutin. Reaction mixtures contained 5.0 mg ^{3}H-cutin wetted with 40 μliters of 1% aqueous Triton X-100, 0.5 ml 500 mM Tris (pH 8.5), or sodium acetate (pH 5.0) buffer, 1.0 ml culture fluid, and 75 μliters of 0.2% aqueous Thimerosal. Control mixtures contained culture fluid which was autoclaved for 1 hr. Reaction mixtures were incubated 6 to 24 hr at 28 C with stirring. Then the mixtures were acidified with two drops of 12 N HCl and extracted four times with 2 ml of ethyl ether. The ether extracts were filtered through Whatman GF/A paper and their radioactivity was determined.

RESULTS

Purification and analysis of apple cutin.—Interference contrast microscopy showed that the cuticular material obtained after the oxalate treatment of apple peelings (step 1) was quite heterogeneous (Fig. 1-A). Epidermal tissue was attached to the underside of the cutin layer and remnants of cuticular waxes were present on the outer surface. Most of the surface wax present in the original sample probably was removed by the acetone used during the dehydration process.

Examination of cuticular material remaining after the enzyme and organic solvent treatments revealed a lack of epidermal tissue and cuticular waxes associated with the cutin layer. The purified cutin (Fig. 1-B) was more homogeneous. Also the outer surface of this layer was relatively smooth compared to the inner surface which was highly convoluted. The cutin layer ranged from 5 to 30 μm in thickness. After the oxalate treatment, the cuticular material exhibited a large amount of birefringence associated with the inner surface of the cutin layers which can be attributed to crystalline cellulose (Fig. 1-C). Birefringence also was observed at the outer surface of the cutin layer and may represent crystalline surface waxes or other materials at the junction of the wax and cutin layers. After subsequent steps in the purification process, the purified cutin segments did not contain birefringent materials (Fig. 1-D) except in areas where they were highly curled (Fig. 1-E).

Electron microscopy revealed that the noncurled areas of the purified cutin were remarkably free of contaminants (Fig. 2). The cutin matrix itself appeared to be a relatively homogeneous layer. Occasionally the highly convoluted undersurface of the cutin layer contained entrapped epidermal cell fragments (Fig. 2).

Cutin powder prepared from purified cutin films contained: 67.41% C, 9.61% H, 20.64% O, 0.74% N, and no detectable S. Analysis by the Updegraff procedure (34) showed that this cutin contained approximately 2.3% cellulose. Gas chromatography of methanolysis products from the cutin preparation revealed only trace amounts of neutral sugars (0.28% galactose, 0.19% glucose, and 0.31% xylose) and galacturonic acid (0.46%).

About 85% of the purified cutin preparation was solubilized by refluxing in 3% KOH in methanol. The ether extract of the acidified filtrate of this digest contained over 95% of the cutin constituents solubilized by the methanolic KOH treatment. The GLC-MS analysis of the silylated methyl esters of the acids in this extract revealed 16-hydroxyhexadecanoic acid, 18-hydroxyoctadec-9,12-dienoic acid, 18-hydroxyoctadec-9-enoic acid, 10,16-dihydroxyhexadecanoic acid, 9, 10, 18-trihydroxyoctadecanoic acid and 9,10,18-trihydroxy-octadec-12-enoic acid, 9,10-epoxy-18-hydroxyoctadec-

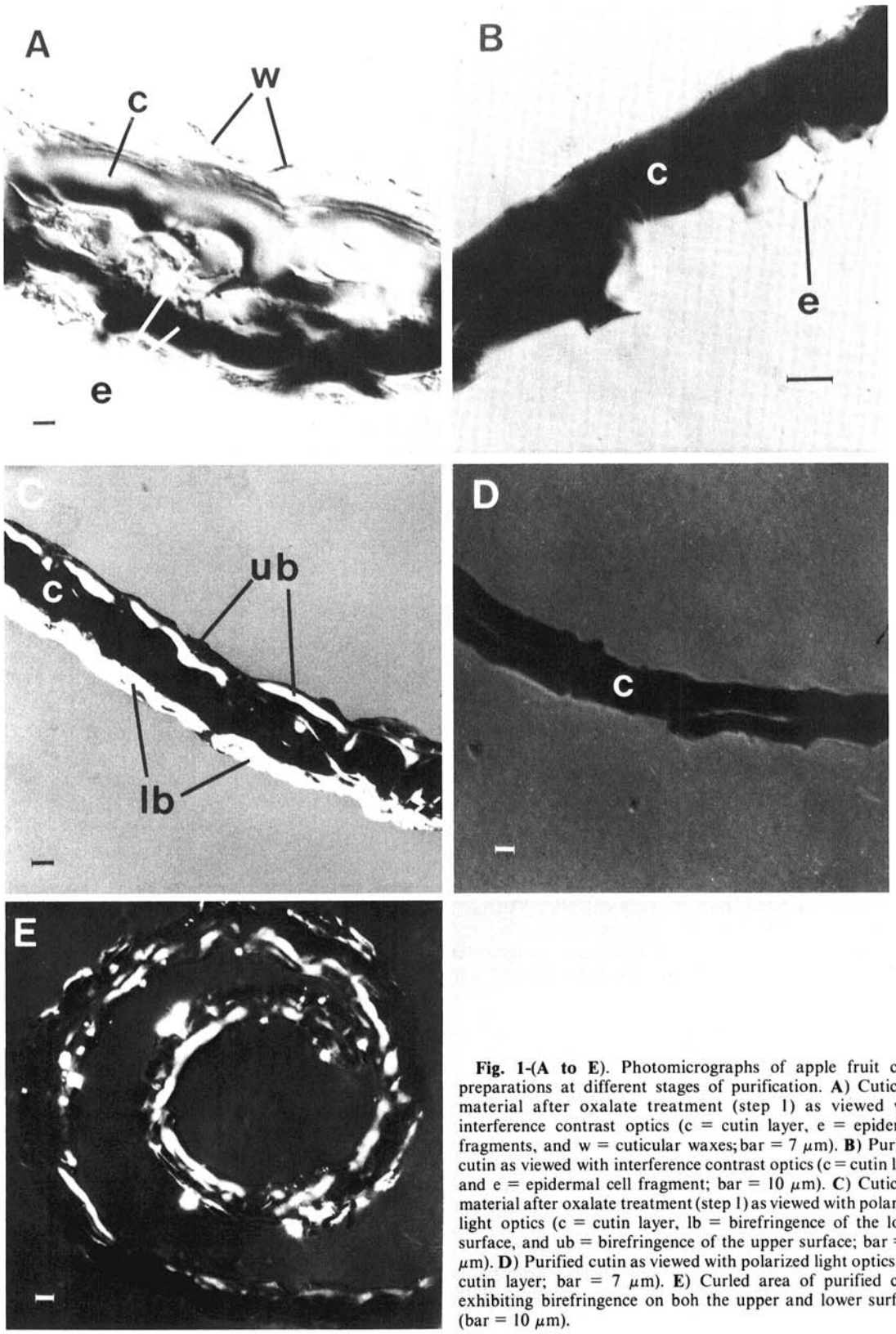

Fig. 1-(A to E). Photomicrographs of apple fruit cutin preparations at different stages of purification. **A)** Cuticular material after oxalate treatment (step 1) as viewed with interference contrast optics (c = cutin layer, e = epidermal fragments, and w = cuticular waxes; bar = 7 μm). **B)** Purified cutin as viewed with interference contrast optics (c = cutin layer and e = epidermal cell fragment; bar = 10 μm). **C)** Cuticular material after oxalate treatment (step 1) as viewed with polarized light optics (c = cutin layer, lb = birefringence of the lower surface, and ub = birefringence of the upper surface; bar = 10 μm). **D)** Purified cutin as viewed with polarized light optics (c = cutin layer; bar = 7 μm). **E)** Curled area of purified cutin exhibiting birefringence on boh the upper and lower surfaces (bar = 10 μm).

12-enoic acid, and 9,10-epoxy-18-hydroxyoctadecanoic acid. The latter two cutin acids were detected as their trihydroxy derivatives since the epoxide ring contained in each had been cleaved. The amounts of monohydroxy (19.5%), dihydroxy (25.5%), and trihydroxy (including epoxyhydroxy) fatty acids (55.0%) were similar to those previously reported for apple cutin (12).

The distribution of radiolabel among the four fractions obtained from ^{3}H-cutin depolymerized with methanolic 3% KOH (Table 1) showed that approximately 81% of the radiolabel remained in the aqueous fraction and that both ether fractions combined contained only 17% of the ^{3}H label. Since hydrolysis of nonlabeled apple cutin revealed that over 95% of the hydrolyzed cutin constituents were found in ether-soluble fractions, it appears that a great deal of ^{3}H was associated with water-soluble (but ether-insoluble) cutin contaminants. Fractionation of ether-soluble fractions from the hydrolyzed ^{3}H-cutin by column chromatography indicated a close correlation between peaks of radioactivity and cutin fatty acid constituents (Fig. 3).

Production of cutinolytic enzymes by phytopathogenic fungi.—The linearity of radioactivity released and an estimate of the maximum amount of radioactivity that can be released enzymatically in the cutinase assay were determined with culture fluids of *F. solani* f. sp. *pisi*. Two sets of reaction mixtures were stopped and extracted with ether after various periods of incubation up to 48 hr (Fig. 4). In one set of reaction mixtures the amount of radioactivity in the ether extracts was determined, while the other set of ether extracts was analyzed by GLC to determine the amount of cutin acids present. The release of radioactivity was relatively linear to about 125×10^3 dpm/5 mg ^{3}H-cutin, which corresponded to about 500 μg of cutin acids. After 48 hr, 160×10^3 dpm/5 mg ^{3}H-cutin was released; this amount appeared to be near the maximum that could be released enzymatically.

To determine whether the large amount of radioactivity in the water-soluble constituents of the ^{3}H-cutin (Table 1) would interfere with the cutinase assay, ^{3}H-cutin was incubated for 24 hr at 28 C with cell-wall-degrading enzymes from *S. rolfsii* (35) and the radioactivity in the ether- and water-soluble fractions was determined. The radioactivity in the ether and aqueous fractions was 29 and 123,480 dpm, respectively, after 24 hr. It was concluded that the release of water-soluble radioactivity by fungal cell-wall-degrading enzymes does not interfere with the cutinase assay used in this study.

When fungal cultures were grown first on V-8 juice broth and then transferred to a minimum salts medium supplemented with 0.5% cutin, cutinase activity was found in culture fluids of most of the fungi tested (Table 2). *Fusarium solani* f. sp. *pisi* produced the greatest activity (27,515 dpm/6 hr). *Botrytis cinerea*, *F. solani* f. sp. *phaseoli*, *F. roseum*, *G. sorghi*, and *R. solani* produced significant levels of activity (>4,000 dpm/6 hr). *Helminthosporium carbonum*, *H. maydis*, *P. aphanidermatum*, and *P. arrhenomanes* produced low levels of activity (<2,000 dpm/6 hr), and little or no activity (<100 dpm/6 hr) could be detected in culture fluids of *P. ultimum* and *S. rolfsii*.

Some fungi, for example *H. maydis*, *P. ultimum*, and *S. rolfsii*, did not grow after they were transferred to the cutin-basal salts medium. In addition, little or no cutinase activity could be detected in cultures of these fungi. Additional experiments were carried out in which test fungi were transferred to V-8 juice broth containing 0.5% cutin after the initial growth period (Table 3). With this system, *H. maydis*, *P. ultimum*, and *S. rolfsii* continued to

TABLE 1. Distribution of radioactivity among the various fractions of depolymerized purified ^{3}H-cutin from Golden Delicious apple fruit

Extraction fraction[a]	Radioactivity	
	dpm/mg	Distribution (%)
Alkaline ether	1,968	1.0
Acidic ether	30,336	16.0
Aqueous	154,737	81.5
Insoluble residue	2,759	1.5

[a]The ^{3}H-cutin sample was depolymerized by refluxing in 3% methanolic KOH for 24 hr. The insoluble fraction was removed by filtration onto Whatman GF/A paper. Methanol was removed from the filtrate under partial vacuum and the residue was dissolved in water. The resulting aqueous solution was extracted four times with ethyl ether (alkaline ether fraction). The remaining solution was acidified with 12 N HCl and extracted four times with ether (acidic ether fraction). The radioactivity in each of the four fractions was determined with a liquid scintillation counter.

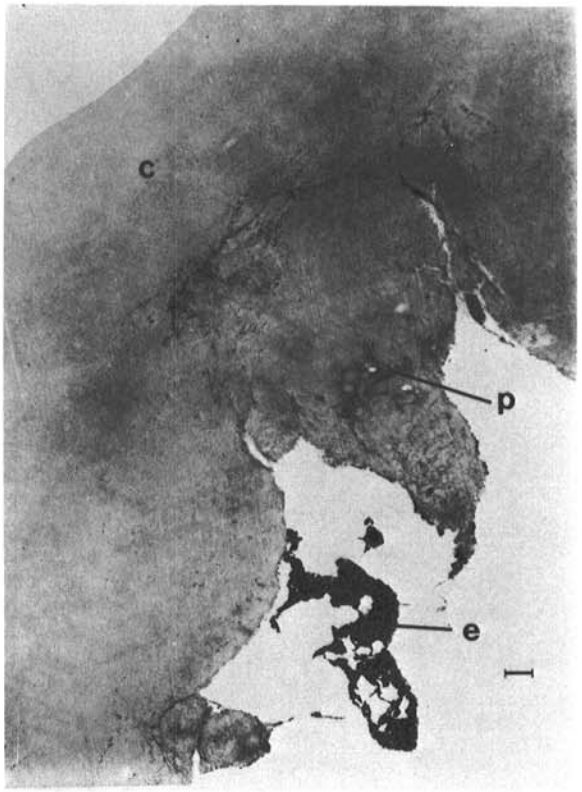

Fig. 2. Electron micrograph of a section of purified cutin in a noncurled region. Legend: c = cutin matrix, e = epidermal cell fragment, and p = electron-opaque material. Bar = 1 μm.

grow in the presence of cutin and produced cutinolytic activity. Three additional fungi that were tested (*B. squamosa*, *C. cucumerinum*, and *C. graminicola*) produced cutinase.

Dialyzed culture fluids from cultures grown under the latter culture regime as well as from cultures of *F. roseum* and *F. solani* f. sp. *phaseoli* grown under the first culture regime were incubated with nonlabeled cutin at pH 5 and 8.5, and the reaction mixtures were assayed by GLC. The major cutin constituents were released by culture fluids of all the fungi except for *H. carbonum*.

It was apparent from this study (Tables 2 and 3) that some pathogens, such as *B. cinerea* and *B. squamosa*, produce cutinases that are more active under acidic conditions, but the cutinases produced by others such as *F. solani* f. sp. *pisi* and *G. sorghi* exhibited greatest activity in an alkaline environment.

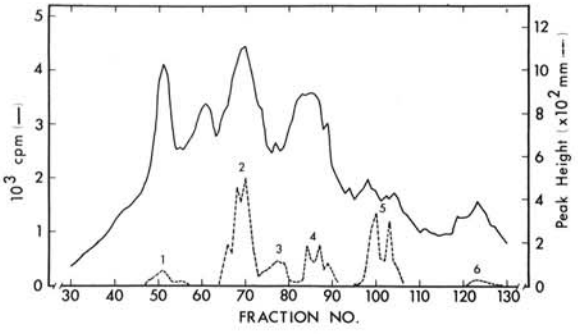

Fig. 3. Elution profile from a LH-20 Sephadex column of cutin acids obtained from depolymerized purified ^{3}H-cutin. The ether-soluble cutin constituents from depolymerized ^{3}H-cutin were dissolved in column solvent (5% 2-propanol and 1% acetic acid in chloroform) and applied to a 2.5 × 90-cm column of LH-20 Sephadex. The column was eluted in an ascending manner at a flow rate of 10 ml/hr at 4 C. Five-ml fractions were collected and the radioactivity of each fraction was determined with a liquid scintillation counter. In a separate column run the contents of each column fraction was derivatized and assayed by gas-liquid chromatography (GLC). The relative amount of cutin constituents in each fraction is represented by the combined GLC peak heights for the cutin constituents observed in each fraction. The cutin constituents in the various fractions were: (i) minor unidentified constituents, (ii) 18-hydroxyoctadec-9, 12-dienoic acid and 18-hydroxyoctadec-9-enoic acid, (iii) 16-hydroxyhexadecanoic acid, (iv) 9, 10-epoxy-18-hydroxyoctadecanoic acid and 9, 10-epoxy-18-hydroxyoctadec-12-enoic acid, (v) 10, 16-dihydroxyhexadecanoic acid, and (vi) 9, 10, 18-trihydroxyoctadecanoic acid and 9, 10, 18-trihydroxyoctadec-12-enoic acid.

DISCUSSION

This investigation has demonstrated that the ability of plant pathogenic fungi to produce cutinolytic enzymes is widespread. Pathogens capable of penetrating root, leaf, and stem tissue were shown to produce cutinases when grown under appropriate conditions.

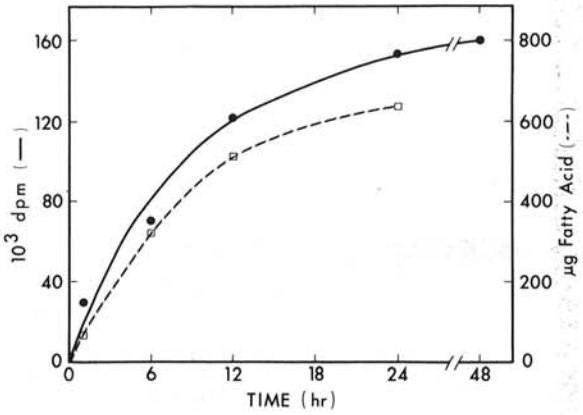

Fig. 4. Release of radioactivity and cutin constituents by culture fluids of *F. solani* f. sp. *pisi* in reaction mixtures containing ^{3}H-cutin. Reaction mixtures were acidified and extracted with ether after the indicated periods of incubation up to 48 hr. The radioactivity in the ether extracts (●——●) was determined with a liquid scintillation counter. The amount of cutin acids present in the extracts (□- - -□) was determined by GLC and expressed as µg of fatty acid.

TABLE 2. Cutinase activity in culture fluids of plant pathogenic fungi grown 3-5 days on V-8 juice broth and then transferred to a basal salts medium supplemented with 0.5% purified apple cutin

Culture[a] age (days)	pH	Radioactivity[b] released from ^{3}H-cutin (dpm/ml of culture fluid/6 hr) by[c]: Bc	Fr	Fsph	Fspi	Gs	Hc	Hm	Pap	Par	Pu	Rs	Sl	Sr
0[d]	5.0	4,135	155	395	1,100	225	0	315	0	185	0	1,255	505	0
	8.5	0	805	1,650	3,685	410	0	0	0	1,140	0	2,155	200	0
2	5.0	...	1,035	925	2,275	15	0	...	...	235	0	1,170	...	...
	8.5	...	1,055	2,610	6,960	360	0	...	...	630	0	2,370	...	...
7	5.0	4,467	5,125	1,895	8,240	0	0	...	420	130	0	4,300	21	...
	8.5	1,280	6,765	6,075	27,515	1,720	0	...	118	131	0	8,649	264	...
14	5.0	...	5,911	6,160	10,156	2,922	572	88	...	60	0	1,146	...	0
	8.5	...	10,866	6,227	...	4,996	191	0	...	72	85	4,527	...	0

[a]Designates age of cultures after transfer to medium containing cutin.
[b]Determined using the standard cutinase assay.
[c]Abbreviations for fungus names: *Botrytis cinerea* (Bc), *Fusarium solani* f. sp. *phaseoli* (Fsph), *F. solani* f. sp. *pisi* (Fspi), *F. roseum* (Fr), *Gloeocercospora sorghi* (Gs), *Helminthosproium carbonum* (Hc), *H. maydis* (Hm), *Pythium aphanidermatum* (Pap), *P. arrhenomanes* (Par), *P. ultimum* (Pu), *Rhizoctonia solani* (Rs), *Stemphylium loti* (Sl), and *Sclerotium rolfsii* (Sr).
[d]Culture fluid from the initial growth period on V-8 juice broth.

TABLE 3. Cutinase activity in culture fluids of plant pathogenic fungi grown 3-5 days on V-8 juice broth and then transferred to V-8 juice broth supplemented with 0.5% purified apple cutin

Culture[a] age (days)	pH	Radioactivity[b] released from ^{3}H-cutin (dpm/ml of culture fluid/24 hr) by[c]:										
		Bc	Bs	Cc	Cg	Fspi	Gs	Hc	Hm	Pu	Rs	Sr
1	5.0	...	...	...	...	...	...	...	...	1,940	...	...
	8.5	...	...	...	...	...	...	...	...	4,515	...	...
3-4	5.0	2,200[d]	4,685[e]	6,070[e]	...	11,800[d]	270[d]	75[e]	400[e]	0[e]	135[d]	175[e]
	8.5	250	1,245	8,610	...	41,690	2,460	1,330	1,240	1,000	1,155	1,075
7	5.0	4,700	1,425	3,950	...	9,970	1,725	0	0	0	...	0
	8.5	433	990	3,155	5,300	45,020	6,455	625	620	450	8,735	290
10	5.0	6,050	865	2,695	...	10,910	2,680	355	70	...	0	0
	8.5	622	735	2,980	...	53,960	3,430	596	515	...	1,005	270

[a]Designates age of cultures after transfer to medium containing cutin.
[b]Determined using the standard cutinase assay.
[c]The abbreviations for the fungi tested are *Botrytis squamosa* (Bs), *Colletotrichum graminicola* (Cg), *Cladosporium cucumerinum* (Cc); the others are given in Table 2.
[d]Culture fluids were assayed after 3 days of growth on the cutin-supplemented medium.
[e]Culture fluids were assayed after 4 days of growth on the cutin-supplemented medium.

The purity of cutin substrates, culture procedures, and the cutinase assay employed, all are of critical importance in studies of the cutinolytic ability of microorganisms. Cutin from apple fruits was chosen as a substrate for fungal cultures and enzyme assays because it contains representative amounts of all known major cutin constituents (7, 12) and can be prepared easily in large quantities. The purified apple cutin was relatively free of cellular debris and carbohydrates. Others have reported the occurrence of small quantities of carbohydrates in purified apple and tomato cutin preparations (13, 30). The elemental analysis of our purified cutin was very similar to that reported by Schönherr and Bukovac (28); it contained 21% oxygen which is characteristic of a material composed of cutin acids. The nitrogen content of the purified apple cutin indicates that as much as 4.6% protein may have been present. It has been suggested that the nitrogen in cutin may be due to enzymes that have become occluded within the cutin matrix (28). Another source of protein in cutin preparations might be fragments of epidermal cells that occasionally adhere to the highly convoluted undersurface of the cutin layer (Fig. 2).

The presence of noncutin constituents in apple cutin preparations was taken into consideration in modifying the cutinase assay described by Purdy and Kolattukudy (26). Only ether-soluble radioactive material was measured in order to avoid interference by the radioactivity released from carbohydrate and protein contaminants by enzymes present in the fungal culture fluids.

Although several fungi have been reported to grow on media containing cutin as a sole carbon source (9, 26, 31), in the present study attempts to culture several plant pathogenic fungi on a salts-cutin medium failed. An inhibitory effect of cutin on fungal growth has been reported by Martin (18) who found that cutin acids obtained by the chemical hydrolysis of citrus lime leaf cutin were toxic to *Gloeosporium limetticola*. This inhibition may be in part due to the presence of epoxy acids (12), which are toxic to microorganisms (15). The best culture regime tested for production of cutinase by fungi was to culture them first on a V-8 juice medium for 3-5 days, and then replace the medium with V-8 juice medium containing 0.5% apple cutin.

The pH optima for different cutinases differ depending upon their source. The cutinases of *F. solani* f. sp. *pisi* are most active in an alkaline environment (26) whereas those from *B. cinerea* (31) and *Penicillium spinulosum* (9, 10) are most active under acid conditions. In this study, the cutinases produced by *B. cinerea* and *B. squamosa* were most active in acidic conditions and those produced by the other fungi tested exhibited greatest activity in an alkaline environment.

The possibility that cutinolytic enzymes are involved in the penetration of plant cuticles by plant pathogens has not gained much support due to the lack of sufficient evidence to demonstrate the presence of these enzymes. This study has provided direct evidence of cutin degradation by a wide range of plant pathogenic fungi. The results of this and previous studies of cutinases (9, 17, 25, 26, 31, 32) in conjunction with recent histological (6, 19, 20, 22, 24, 27) and histochemical studies (16, 19, 23, 29) strongly suggests a possible role for these enzymes in fungal ingress. We suggest that the respective roles of enzymes and mechanical force in the penetration of plant tissues by fungi need to be reexamined.

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