Hydrolysis of Plant Cuticle by Plant Pathogens. Properties of Cutinase I, Cutinase II, and a Nonspecific Esterase Isolated from *Fusarium solani pisi*†

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ABSTRACT: The properties of the homogeneous cutinase I, cutinase II, and the nonspecific esterase isolated from the extracellular fluid of cutin-grown *Fusarium solani f. pisi* (R. E. Purdy and P. E. Kolattukudy (1975), *Biochemistry*, preceding paper in this issue) were investigated. Using tritiated apple cutin as substrate, the two cutinases showed similar substrate concentration dependence, protein concentration dependence, time course profiles, and pH dependence profiles with optimum near 10.0. Using unlabeled cutin, the rate of dihydroxyhexadecanoic acid release from apple fruit cutin by cutinase I was determined to be 4.4 μmol per min per mg. The cutinases hydrolyzed methyl hexadecanoate, cyclohexyl hexadecanoate, and to a much lesser extent hexadecyl hexadecanoate but not 9-hexadecanoyloxyheptadecane, cholesteryl hexadecanoate, or hexadecyl cinnamate. The extent of hydrolysis of these model substrates by cutinase I was at least three times that by cutinase II. The nonspecific esterase hydrolyzed all of the above esters except hexadecyl cinnamate, and did so to a much greater extent than did the cutinases. None of the enzymes hydrolyzed α- or β-glucosides of *p*-nitrophenol. *p*-Nitrophenyl esters of fatty acids from C_2 through C_{18} were used as substrates and V's and K_m's were determined. The cutinases hydrolyzed, at significant rates, only the short-chained esters and the highest V was observed for *p*-nitrophenyl acetate, while the nonspecific esterase hydrolyzed all of the nitrophenyl esters, but showed the highest V for C_4 acyl moieties. The K_m's were in the range of 10^{-4} to 10^{-3} M. The cutinase isozymes showed no metal ion dependence, and they were not affected by thiol reagents such as *N*-ethylmaleimide, iodoacetamide, and *p*-chloromercuribenzoate. The classical serine hydrolase inhibitors, diisopropyl fluorophosphate (Dip-F) and paraoxon, severely inhibited all three enzymes. Treatment of the three enzymes with [^{3}H]Dip-F resulted in covalent attachment of the radioactive moiety to all of the three proteins. Cutinase I and II contained no more than one reactive serine per molecule and reaction of this serine residue with Dip-F completely inactivated these enzymes. Sodium dodecyl sulfate gel electrophoresis of the tritiated cutinase I, thus obtained, showed only one band of radioactivity which coincided with the single 21,800 protein band. A similar treatment of cutinase II showed one major band of radioactivity which coelectrophoresed with the 21,800 protein band, and a smaller amount of ^{3}H was found in the protein band at 10,600. When the nonspecific esterase was similarly treated, radioactivity was found in both the 54,000 and 50,000 bands, and a much smaller amount of ^{3}H was found in the low molecular weight region of the gel. Thus, it is probable that cutinase II and the nonspecific esterase contained small amounts of peptides modified by proteolytic clips, but these modified molecules appear to be enzymatically active. Using biosynthetically prepared ^{14}C-labeled apple cutin as the substrate, and Sephadex LH-20 column chromatography as the analytical technique, both cutinases were shown to hydrolyze cutin to oligomers and monomers. Time-course experiments, as well as direct tests, showed that the cutinases hydrolyzed the polymer as well as the oligomers generated from it, but the nonspecific esterase did not hydrolyze either cutin or the oligomers generated from cutin.

The cuticle of terrestrial plants consists of a structural biopolymer, cutin, impregnated with waxes (Mazliak, 1968; Martin and Juniper, 1970; Kolattukudy and Walton, 1972). Cutin is a naturally occurring polyester of *n*-C_{16} and *n*-C_{18} hydroxy fatty acids. The enzymatic depolymerization of cutin may be one of the first steps involved in the entry of pathogens into plants. Some microorganisms, including plant pathogens, can live on cutin as their sole carbon source (Heinen and DeVries, 1966; Hankin and Kolattukudy, 1971; Purdy and Kolattukudy, 1973). When the plant pathogen *Fusarium solani f. pisi* is grown on cutin, the extracellular fluid contains cutinolytic activity (Purdy and Kolattukudy, 1973). From the extracellular fluid two cutinase isozymes of about 22,000 daltons and an extracellular *p*-nitrophenyl palmitate (PNP)[1] hydrolase of about 52,000 daltons have been isolated and purified to homogeneity (Purdy and Kolattukudy, 1975). In this paper we describe some of the properties of these novel enzymes.

Experimental Section

Materials. [^{3}H]Diisopropyl fluorophosphate (10 Ci/mmol), [1-^{14}C]hexadecanoic acid (57.9 Ci/mol), and [1-^{14}C]acetate (62 Ci/mol) were purchased from Amersham/Searle. Aquasol and Omnifluor were obtained from New England Nuclear Corp. *p*-Nitrophenyl esters (C_4 through C_{18}), *p*-nitrophenyl α-D-glucoside, *p*-nitrophenyl β-D-glucoside, indoxyl acetate, *N,O*-bis(trimethylsilyl)acetamide, α,α′-dipyridyl, 8-hydroxyquinoline, 1,10-phenanthroline,

† From the Department of Agricultural Chemistry and the Program in Biochemistry and Biophysics, Scientific Paper No. 4377, College of Agriculture Research Center, Washington State University, Pullman, Washington 99163. *Received January 22, 1975.* This work was supported in part by Grants GB-23081 and GB-43076x from the National Science Foundation.

[1] Since the *p*-nitrophenyl esterase has been shown to be a nonspecific esterase it is referred to as either PNP hydrolase or nonspecific esterase.

N-ethylmaleimide, and diisopropyl fluorophosphate (Dip-F)[2] were obtained from Sigma Chemical Company. *p*-Nitrophenyl acetate was purchased from Aldrich Chemical Company. Cholesterol was obtained from the Hormel Institute. Hexadecanol and cyclohexanol were obtained from J. T. Baker Chemical Co. 9-Heptadecanone was from K and K Laboratories and ethylenediaminetetraacetate was from Fisher Scientific Corporation. Paraoxon and parathion were reference standards obtained from the Environmental Protection Agency. *p*-Chloromercuribenzoate was obtained from the National Biochemical Corp. Cinnamic acid was purchased from Matheson Co. Pharmacia was the source of Sephadex LH-20. Bio-Rad Laboratories was the source of Bio-Gel P-2. 9-Heptadecanol was prepared by $LiAlH_4$ reduction of 9-heptadecanone followed by thin-layer chromatography (TLC). [1-^{14}C]Hexadecanol was prepared by $LiAlH_4$ reduction of [1-^{14}C]hexadecanoic acid (57.9 Ci/mol) followed by thin-layer chromatography.

Thin-layer chromatography was performed with activated (110° for 12 hr) 0.5-mm thick silica gel G plates (20 × 20 cm). The chromatograms were visualized under uv light after spraying them with a 0.1% ethanolic solution of dichlorofluorescein.

Preparation of Derivatives. $LiAlH_4$ reduction was performed by refluxing the samples with powdered $LiAlH_4$ in tetrahydrofuran for 2–12 hr. The excess $LiAlH_4$ was carefully decomposed by the slow addition of the reaction mixture to water. After acidifying the reaction mixture with HCl, the products were recovered by extraction with chloroform. Methyl esters were prepared by refluxing the appropriate acid in 14% BF_3 in methanol for 2 hr. After the addition of water, the products were recovered by extraction with chloroform. Trimethylsilyl ethers and esters of hydroxy fatty acids were prepared by reaction with *N,O*-bis-(trimethylsilyl)acetamide (0.3 ml) at 90° for 20 min.

Protein Determination. Protein concentrations were routinely determined by the method of Lowry et al. (1951). Occasionally, the result obtained with this method was compared to that obtained from the amino acid analysis.

Enzyme Assays. Usually for cutinase assay, 0.8 μg of protein was incubated at 30° for 10 min with 4.2 mg of tritiated Golden Delicious apple cutin in 1 ml of 50 m*M* glycine–NaOH buffer (pH 10.0), and the radioactivity in the soluble products was measured as described in the preceding paper (Purdy and Kolattukudy, 1975). PNP hydrolase activity was measured spectrophotometrically as described before (Purdy and Kolattukudy, 1975).

Sodium Dodecyl Sulfate Electrophoresis. Dodecyl sulfate polyacrylamide disc gel electrophoresis was performed by the method of Weber and Osborn (1969), with the same conditions and standards as those used previously (Purdy and Kolattukudy, 1975).

Enzymatic Hydrolysis of Unlabeled Cutin. Cutinase I (12.8 μg) was incubated with 192 mg of unlabeled Golden Delicious apple cutin for 10 min at 30° in a gyrating water bath shaker at a rate sufficient to keep the cutin suspended in 16 ml of 50 m*M* glycine–NaOH buffer (pH 10.0). The reaction was stopped by the addition of about 20-fold excess of a 2:1 mixture of chloroform and methanol, and the products were extracted by the method of Folch et al. (1957). The products were silylated and analyzed by gas–liquid chromatography–mass spectrometry using a 0.32 × 152 cm stainless steel column packed with 5% SE-30 on 60–80 mesh Chromosorb W at 242° as described previously (Walton and Kolattukudy, 1972). The quantity of dihydroxyhexadecanoic acid was determined with an authentic standard.

Preparation of ^{14}C-Labeled Model Esters. 9-[1-^{14}C]Hexadecanoyloxyheptadecane, cholesteryl [1-^{14}C]hexadecanoate, hexadecyl [1-^{14}C]hexadecanoate, and cyclohexyl [1-^{14}C]hexadecanoate were prepared in the following manner. [1-^{14}C]Hexadecanoic acid (25 μCi, 0.43 μmol) was refluxed with 2 ml of trifluoroacetic anhydride for 30–60 min. After the removal of the trifluoroacetic anhydride with a stream of nitrogen, 1–2 mg of the appropriate alcohol in 1–4 ml of redistilled benzene was added and the reaction mixture was refluxed for 1 hr. The esters were purified by TLC using hexane–diethyl ether (19:1 v/v) as the developing solvent. Unlabeled pure standards prepared in a similar manner were identified by gas–liquid chromatography–mass spectrometry and were used for the identification of the labeled esters. [1-^{14}C]Hexadecyl cinnamate was prepared in the following manner. About 2 mg of cinnamic acid was refluxed with 1 ml of thionyl chloride for 1 hr. After removal of thionyl chloride with a rotary evaporator [1-^{14}C]hexadecanol (22 μCi, 0.38 μmol) was added in 1.5 ml of distilled benzene and the reaction mixture was refluxed for 1 hr. The products were recovered and purified as described above. Methyl hexadecanoate was prepared by heating [1-^{14}C]hexadecanoic acid (4 μCi, 73 nmol) with 3 ml of 14% BF_3 in methanol for 5 min on a steam bath. The ester was purified by TLC using hexane–diethyl ether–formic acid (40:10:1 v/v) as the developing solvent.

Determination of Radioactivity. A Berthold LB 2721 thin layer scanner was used to monitor thin-layer chromatograms for radioactivity. Radioactivity in liquid samples was measured with a Packard liquid scintillation spectrometer, Model 3003. [^{14}C]- and [^{3}H]toluene were used to determine counting efficiencies. The tritiated products from the cutinase assay were measured as described before (Purdy and Kolattukudy, 1975). Aliquots of soluble products and silica gel from thin-layer chromatograms were assayed for radioactivity in 15 ml of 30% ethanol in toluene containing 4 g of Omnifluor/l. With this fluor counting efficiencies were 12.5 and 76% for ^{3}H and ^{14}C, respectively. Aquasol (10 ml) was used to measure the radioactivity in dodecyl sulfate polyacrylamide gel slices which were dissolved in 0.5 ml of 30% hydrogen peroxide. The counting efficiency of this system was 11%. All radioactivity measurements were done with <3% standard deviation.

Inhibitors and Chelators. Cutinases I and II (7.8 μg/ml) were preincubated with each of the following compounds in 100 m*M* glycine–NaOH buffer (pH 10.0), for 30 min at room temperature prior to cutinase assay: 1 m*M* bipyridyl, 1 m*M* ethylenediaminetetraacetate, 1 m*M* 8-hydroxyquinoline, 1 m*M* phenanthroline, 1 m*M* *N*-ethylmaleimide, 1 m*M* iodoacetamide, 0.5 m*M* *p*-chloromercuribenzoate, 0.1 m*M* paraoxon, and 0.025 m*M* Dip-F. In the spectrophotometric assays, with *p*-nitrophenyl butyrate as the substrate, cutinase I (7.8 μg/ml), cutinase II (7.8 μg/ml), and the nonspecific esterase (1.7 μg/ml) were preincubated with Dip-F and paraoxon for 10 or 20 min prior to assay.

Enzymatic Hydrolysis of Model Esters. Substrate solutions were prepared by adding 14.8 mg of Triton X-100 in a few milliliters of diethyl ether to 6.8 nmol of the appropriate ester in a 15-ml graduated centrifuge tube. After removal of diethyl ether with a stream of N_2, 4 ml of water was added and the mixture was sonicated (needle probe of

[2] Abbreviation used is: Dip-F, diisopropyl fluorophosphate.

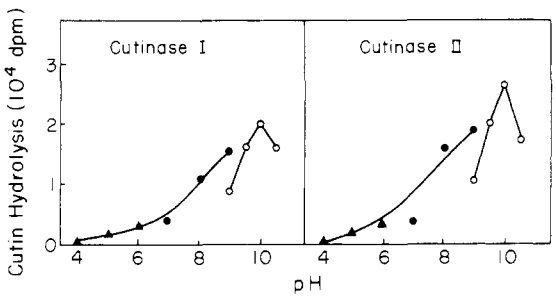

FIGURE 1: Effect of pH on the hydrolysis of tritiated cutin by cutinases I and II. The reaction mixtures contained 50 mM citrate-phosphate (△), Tris-HCl (●), or glycine-NaOH (○).

Biosonik III, 4 × 5 sec). The reaction mixtures for the enzyme assays containing 0.85 μM ester, 0.43 μg of nonspecific esterase, or 0.5 μg of either cutinase, and 3.7 mg of Triton X-100 in a total volume of 1.0 ml of 50 mM glycine-NaOH buffer (pH 10.0), or 50 mM phosphate buffer (pH 8.0), were incubated for 10 or 20 min at 30°. The reaction was terminated by the addition of a 20-fold excess of $CHCl_3$-MeOH (2:1). The lipid products were recovered in the usual manner and they were subjected to TLC with internal unlabeled standards of the expected products. Hexane-diethyl ether-formic acid (40:10:1 v/v) was used as the developing solvent. The radioactivity in the TLC fractions was determined as described above. Controls containing no enzyme were run, and the values (<1%) were subtracted from those obtained with the enzyme.

Treatment of the Enzymes with Radioactive Dip-F. Cutinase I or cutinase II (100 μg) was incubated with tritiated Dip-F (9.5 μM) in 0.3 ml of 10 mM sodium phosphate buffer (pH 7.0) for 15 min at 23°. The PNP hydrolase (50 μg) was similarly treated with Dip-F in a total volume of 0.15 ml. The reaction mixtures were dialyzed against 1 l. of the same buffer overnight at 0–4°. Cutinase I and II were divided into two portions which were incubated at 50 and 70°, respectively, for 12 hr with 1% dodecyl sulfate and 1% mercaptoethanol. These samples were subjected to dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The PNP hydrolase was similarly treated for 3 hr at 41°. The PNP hydrolase and cutinases were electrophoresed in 10 and 15% gels, respectively. The gels were stained with Coomassie Brilliant Blue. The gels were frozen and then sliced into 1–3-mm thick disks. These disks were placed in 0.5 ml of 30% hydrogen peroxide in glass scintillation vials and the caps were secured before incubation at 80° for 3–4 hr to dissolve the polyacrylamide. The radioactivity in these samples was determined as described above.

Cutinases I and II (60 μg) were incubated for 10 min at room temperature in 0.5 ml of 50 mM phosphate buffer (pH 7.0) containing 0, 2.9 μM, or 11.5 μM tritiated Dip-F. Cutinase II was also treated for 20 min with 11.5 μM Dip-F. The incubation mixtures were immediately chromatographed in Bio-Gel P-2 columns (1 × 35 cm) at 0–4° with 50 mM phosphate buffer (pH 7.0) and 1-ml fractions were collected. Radioactivity in each fraction was determined. The specific activities of the Dip-F treated and untreated enzyme obtained from these columns were determined with p-nitrophenyl butyrate as the substrate.

Release of Oligomers and Monomers from Cutin by the Cutinases. Radioactive cutin synthesized from [1-^{14}C]acetate by apple skin slices was isolated and purified as described before (Purdy and Kolattukudy, 1973). The specific activity was 2.7 × 10^5 cpm/mg as determined by reduction with $LiAlH_4$ followed by determination of the radioactivity in the chloroform soluble products. This labeled cutin was homogenized with a Ten-Broeck homogenizer in water and the solids were recovered by centrifugation. Water was added to give a final concentration of 2.3 mg of cutin/ml. This suspension (2.5 ml) was incubated with an equal volume of 100 mM glycine-NaOH buffer (pH 10.0) containing 3 μg of cutinase I at 30° in a gyratory water bath (260 rpm) for 10 or 120 min. The reaction was stopped by adding a 20-fold excess of a 2:1 mixture of chloroform and methanol. This mixture was acidified and the soluble lipids were recovered by the method of Folch et al. (1957). The chloroform soluble products were chromatographed on a Sephadex LH-20 column (1.8 × 57.5 cm) with chloroform containing 5% 2-propanol and 1% acetic acid as the eluent. Radioactivity in the eluent fractions (2.5 ml) was monitored and the appropriate fractions were pooled and evaporated to dryness under a stream of nitrogen. These radioactive fractions were subjected to TLC on silica gel G with ethyl ether-hexane-methanol-formic acid (40:10:2:1 v/v) as the developing solvent. The radioactive products were identified by comparison of their R_f values with those of authentic standards. Radio gas-liquid chromatography was also used to confirm the identity of some products (Purdy and Kolattukudy, 1973). In some cases $LiAlH_4$ reduction products were also identified by TLC with diethyl ether-hexane-methanol (8:2:1 v/v) as the developing solvent. The epoxy acid fraction from the LH-20 column was acetoxylated by refluxing it with glacial acetic acid overnight. The $CHCl_3$ soluble products recovered from this reaction mixture were reduced with $LiAlH_4$ and the products were identified by TLC with authentic standards.

Enzymatic Hydrolysis of Oligomers. All the post-void fractions which were eluted from the Sephadex LH-20 column before the monomers emerged were pooled and this material was designated as the oligomer fraction. A portion (3.5 × 10^5 dpm) of this material, dispersed in water (0.5 ml) by sonication, was incubated with 0.6 μg of cutinase I in a total volume of 1.0 ml of 50 mM glycine-NaOH buffer (pH 10.0) for 2 hr at 30°. An equal amount of the oligomer fraction, dispersed in water containing 3.7 mg of Triton X-100 by sonication, was incubated with 0.68 μg of PNP hydrolase in a total volume of 1 ml of 50 mM phosphate buffer (pH 8.0) at 30° for 2 hr. The reaction mixtures were mixed with a 20-fold excess of $CHCl_3$-MeOH (2:1) and the lipid products were recovered as indicated above. These products were subjected to column chromatography on Sephadex LH-20 as described above.

Results and Discussion

Effect of Time, Protein Concentration, and pH on the Rates of Hydrolysis by Cutinases and PNP Hydrolase. Two isozymes of cutinase and a PNP hydrolase isolated from the extracellular fluid of cutin-grown *F. solani pisi* have been purified to homogeneity (Purdy and Kolattukudy, 1975). The cutinases were characterized with the tritiated cutin described in the previous paper as the substrate. The rates of cutin hydrolysis by the two isozymes were similarly affected by changes in pH (Figure 1). Below pH 7.0 the rate of hydrolysis by either isozyme was very low but the rate increased as the pH was raised from 7.0 to 10.0, beyond which the hydrolysis rate decreased. At pH 9.0 both isozymes were more active in Tris-HCl buffer than in glycine-NaOH buffer. However, since the optimal pH was about 10.0 for both isozymes the enzymes were routinely

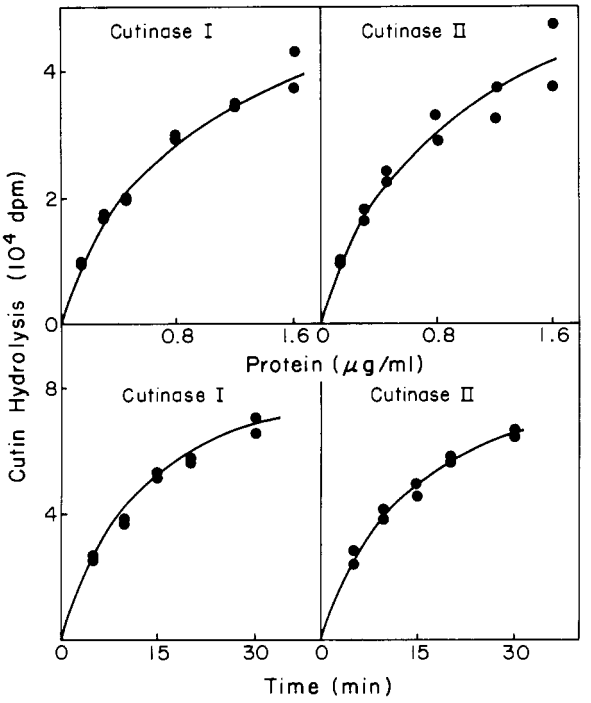

FIGURE 2: Effect of time and protein concentration on the hydrolysis of tritiated apple cutin by cutinases I and II.

assayed with glycine–NaOH buffer (pH 10.0). In contrast to the high pH optimum we observed for the isozymes of cutinase, it has been reported that a crude preparation from *Botrytis cinerea* released some acids from tomato cutin with a pH optimum of 5.0 (Shishiyama et al., 1970). The PNP hydrolase had a pH profile similar to that previously described for the crude preparation, and the pH optimum was at about 8.0 (Purdy and Kolattukudy, 1973). The optimal pH observed with the present fungal enzyme is within the range reported for mammalian carboxyl esterases (Krisch, 1971), but it is higher than that reported for intracellular esterase of *Bacillus stearothermophilus* (Matsunaga et al., 1974).

As the enzyme concentration increased the rate of cutin hydrolysis increased, but strictly linear increases were observed only up to 0.4 μg/ml of protein with either of the isozymes of cutinase (Figure 2). Similarly, linear rates of cutin hydrolysis were observed only up to 15 min of incubation with either of the isozymes of cutinase. Since the assay consists of only measurement of the release of radioactivity from the insoluble polymer into the soluble phase, and it cannot take into account the possibilities of release of a mixture of soluble oligomers and monomers, it is not surprising that linear rates are observed only within a short range of protein concentration and time. With PNP hydrolase, linear rates of hydrolysis were observed up to 3 min with 14 ng of protein/ml and only the initial rates were used for all calculations.

Even though the usual kinetic parameters such as K_m cannot be determined with an insoluble polymeric substrate such as cutin, the effects of the amount of cutin per reaction mixture on the rate of hydrolysis by the two isozymes were determined (Figure 3). The rate of hydrolysis increased linearly with increasing amounts of cutin in the reaction mixture up to about 8 mg/ml with either of the isozymes of cutinase. Further increases in the amount of cutin added resulted in no further increase in the rates of hydrolysis, resulting in identical substrate saturation patterns for both of

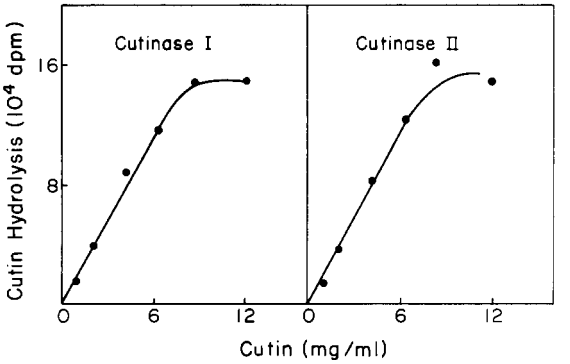

FIGURE 3: Effect of substrate concentration on the hydrolysis of tritiated apple cutin by cutinase I and cutinase II.

the isozymes of cutinase. As indicated earlier, these data provide only operational parameters.

Since the assay with the tritiated cutin reflects only the relative rates of hydrolysis, no conclusions regarding absolute rates can be drawn from such data. In order to determine, at least approximately, the absolute rate of hydrolysis, cutinase I was incubated with unlabeled cutin powder (12 mg/ml). The hydrolysis products recovered by solvent extraction were analyzed as their trimethylsilyl derivatives by combined gas–liquid chromatography–mass spectrometry. The quantity of dihydroxyhexadecanoic acid in the hydrolysate was determined with an authentic standard. Thus, it was found that the rate of release of this monomer by cutinase was 4.4 μmol per min per mg. Since this monomer represents about one-fourth of the total cutin (Walton and Kolattukudy, 1972), it appears that cutinase I released about 18 μmol of monomers per min per mg of protein. Since the two isozymes appear to be identical in their rates of hydrolysis, this value presumably holds good for cutinase II.

Substrate Specificity. In order to determine the specificities of the cutinases and PNP hydrolase, six labeled esters were prepared. These esters represent some of the possible types of linkages which might be present in cutin. Since both primary and secondary alcohols are found in cutin, ester linkages in cutin could contain either, or both, secondary and primary alcohols. Therefore, three esters of secondary alcohols and three esters of primary alcohols were used as model substrates. Cutinases I and II hydrolyzed methyl hexadecanoate, cyclohexyl hexadecanoate, and to a lesser extent hexadecyl hexadecanoate (Table I). Hexadecyl hexadecanoate contains a primary long chain alcohol and represents an ester linkage which should be similar to that present in cutin. The hydrolysis of the methyl and cyclohexyl esters of hexadecanoate was somewhat surprising, since they contain alcohols of shorter chains than those found in cutin; however, the rates of hydrolysis were low for all the model substrates except the cyclohexyl ester. The extent of ester hydrolysis by cutinase I was at least three times that of cutinase II. This was the first difference we observed in the catalytic properties of the two cutinases. The cutinases did not hydrolyze 9-hexadecanoyloxyheptadecane which resembles possible secondary alcohol esters which might be present in cutin. None of the three hydrolases catalyzed hydrolysis of either *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside under the same conditions used for *p*-nitrophenyl esters.

The PNP hydrolase catalyzed hydrolysis of 9-hexadecanoyloxyheptadecane, cholesteryl hexadecanoate, hexadecyl

Table I: Hydrolysis of Synthetic Esters by Cutinase I, Cutinase II, and PNP Hydrolase.[a]

Substrate	% Hydrolysis					
	Cutinase I		Cutinase II		PNP Hydrolase	
	pH 8.0	pH 10.0	pH 8.0	pH 10.0	pH 8.0	pH 10.0
9-Hexadecanoyloxyheptadecane (20 min)	0	0	0	0	88	8
Cholesteryl hexadecanoate (20 min)	0	0	0	0	95	23
Hexadecyl hexadecanoate (20 min)	6	4	1	1	66	25
Cyclohexyl hexadecanoate (10 min)	53	37	17	11	98	69
Methyl hexadecanoate (10 min)	12	18	4	6	97	50
Hexadecyl cinnamate (20 min)	0	0	0	0	0	0

[a] The esters (0.85 μM) along with 3.7 mg/ml of Triton X-100 were incubated with 0.43 μg of PNP hydrolase, or 0.5 μg of either cutinases in 1 ml of 50 mM phosphate buffer (pH 8.0) or 50 mM glycine–NaOH buffer (pH 10.0) at 30° for 10 or 20 min. The products were analyzed as described under the Experimental Section.

hexadecanoate, cyclohexyl hexadecanoate, and methyl hexadecanoate, and it hydrolyzed them to a much greater extent than did the cutinases. The PNP hydrolase showed little specificity, and it is, therefore, most probably a nonspecific esterase. It was previously shown that this nonspecific esterase was not a lipase (Purdy and Kolattukudy, 1973). The nonspecific esterase hydrolyzed these model ester substrates, yet it did not hydrolyze cutin. On the other hand, the cutinases hydrolyzed cutin, but they did not hydrolyze 9-hexadecanoyloxyheptadecane and they only slowly hydrolyzed hexadecyl hexadecanoate, although these esters should be similar to the type of ester linkages present in cutin. It should be pointed out that the assays with cutin were done without Triton X-100, while the model substrates were dispersed in the reaction mixture with Triton X-100. However, the same concentrations of the detergent did not affect the cutin hydrolyzing activity of the cutinases and therefore this difference in assay condition cannot explain the differences in the rates of hydrolysis observed. Therefore, it appears that the nonspecific esterase hydrolyzes only soluble substrates, while cutinases prefer insoluble substrates. In support of this conclusion it was found that cutinase catalyzed hydrolysis of [1-^{14}C]palmitoyl groups chemically attached to the cutin, while this acid was not released by the nonspecific esterase.

The hydrolysis of the model substrates was measured at pH 8.0, which is the optimum for the nonspecific esterase, and at pH 10.0, which is the optimum for the cutinases when cutin is used as the substrate. The cutinases showed higher hydrolysis rates of cyclohexyl hexadecanoate and hexadecyl hexadecanoate at pH 8.0 than at pH 10.0, but higher rates were observed at pH 10.0 for methyl hexadecanoate than at pH 8.0. The nonspecific esterase gave the highest percent hydrolysis at its pH optimum of 8.0 for the five esters it hydrolyzed.

Recently hydroxycinnamic acid was shown to be covalently attached to apple cutin (R. G. Riley and P. E. Kolattukudy, unpublished). It is possible that the nonspecific esterase, which is induced by growth on cutin, catalyzes hydrolysis of such naturally occurring esters of cinnamic acid derivatives. In order to test this possibility, [1-^{14}C]hexadecyl cinnamate was synthesized and this material was tested as a substrate for both cutinases and the nonspecific esterase. None of them catalyzed hydrolysis of this ester.

In an earlier study, p-nitrophenyl palmitate was used as the substrate for routine assays of the hydrolytic activity of the extracellular fluid of *F. solani pisi*. However, the cutinase activity was resolved from p-nitrophenyl palmitate hydrolyzing activity during the purification of these enzymes (Purdy and Kolattukudy, 1975). With the three purified enzymes, we investigated the chain length specificity for hydrolysis of p-nitrophenyl esters of fatty acids (Table II). For all of the esters (C_2–C_{18}) we examined with the three enzymes, linear Lineweaver–Burk plots were obtained and K_m and V for each ester were calculated by a least-squares computer program. Cutinase I and cutinase II showed a decrease in V as the chain length of the acyl moiety increased from C_2 to C_{12}. The hydrolysis rates for C_{14}, C_{16}, and C_{18} acyl esters of p-nitrophenol were too slow to calculate K_m and V for cutinases I and II. Even though V values for acetate and butyrate were similar for both cutinases, for longer esters, cutinase I showed significantly higher values than those obtained with cutinase II. It is noteworthy that cutinase I also hydrolyzed the labeled model substrates faster than did cutinase II. Thus the two cutinases do show discernible differences in their ability to catalyze hydrolysis of soluble substrates, while no differences could be detected in their ability to hydrolyze cutin. The hydrolysis of the nitrophenyl esters by the cutinases was measured at pH 8.0, while the pH optimum for these enzymes is 10.0 for hydrolysis of cutin. It is difficult to assess the effect of this difference in the pH on the comparison between the cutinases, because nonenzymatic hydrolysis of the nitrophenyl esters at pH 10.0 is too fast for an accurate measurement of the rate of enzymatic hydrolysis.

The nonspecific esterase showed higher V values than those obtained with the cutinases for all p-nitrophenyl esters. As was the case with the cutinases, V decreased as the chain length of the acyl moiety increased. However, the nonspecific esterase showed only less than fourfold decrease in V as the length of acyl moiety increased from C_4 to C_{12}, while cutinase I and cutinase II showed 125- and 600-fold decrease, respectively, for the same change in chain length. Thus, the cutinases are much more specific for the shorter chain esters than is the nonspecific esterase. In contrast to the specificities observed in the present paper, it was observed with an esterase isolated from *B. stearothermophilus* that V increased as the chain length of the acyl moiety increased from C_2 to C_6 (Matsunaga et al., 1974). The values of V reported for this enzyme were in the same range as those observed with the *Fusarium* enzyme, while the K_m values were about an order of magnitude lower for the *Bacillus* enzyme.

Active Site. Many esterases have been found to contain a serine residue at the active site. In order to determine whether the three enzymes from *F. solani pisi* are serine hydrolases, the effects of the classical serine hydrolase inhibitors on these enzymes were examined. Cutin hydrolysis by cutinase I and cutinase II was inhibited by Dip-F and paraoxon (Table III, experiment 1). Parathion was also shown to inhibit cutin hydrolysis by the isozymes. Parathion, generally, does not inhibit serine enzymes directly, but the samples used might have contained some paraoxon, or some paraoxon might have been generated under the prein-

Table II: K_m and V for the Hydrolysis of *p*-Nitrophenyl Esters by Cutinase I, Cutinase II, and PNP Hydrolase.[a]

Chain Length of Acyl Moiety	PNP Hydrolase		Cutinase I		Cutinase II	
	K_m ($M \times 10^4$)	V[a]	K_m ($M \times 10^4$)	V	K_m ($M \times 10^4$)	V
C_2			68.0	1.5	97	2.3
C_4	5.2	2.0	3.5	1.0	7.5	1.2
C_6	1.7	0.89	8.9	0.46	8.6	0.2
C_8	2.2	0.66	8.8	0.17	5.9	0.06
C_{10}	1.4	0.40	4.8	0.038	3.6	0.011
C_{12}	2.1	0.52	5.6	0.008	4.5	0.002
C_{14}	1.3	0.36				
C_{16}	1.0	0.31				
C_{18}	1.0	0.29				

[a] V is expressed as nmoles per min per mg of protein. Hydrolysis was measured spectrophotometrically (Purdy and Kolattukudy, 1973) in 105 m*M* phosphate buffer (pH 8.0).

Table III: Effects of Inhibitors and Chelators on the Activities of Cutinase I and Cutinase II.

Inhibitor (m*M*)	% Inhibition	
	Cutinase I	Cutinase II
Experiment 1[a]		
Dip-F (0.025)	100	100
Paraoxon (0.1)	100	100
N-Ethylmaleimide or iodoacetamide (1.0)	0	0
p-Chloromercuribenzoate (0.5)	0	0
Chelators (1.0)	0	0
Experiment 2[b]		
Dip-F (0.002)	68	43
Paraoxon (0.004)	59 (43)	16 (5)

[a] Cutinase I and II (7.8 μg/ml) were preincubated with an inhibitor or chelator for 30 min at room temperature in 100 m*M* glycine–NaOH buffer (pH 10.0) prior to the assay with the tritiated cutin as described under the Experimental Section. The chelators used were bipyridyl, ethylenediaminetetraacetate, 8-hydroxyquinoline, and *o*-phenanthroline. [b] Cutinase I and II (7.8 μg/ml) were preincubated with Dip-F for 10 min and with paraoxon for 20 min at room temperature in 50 m*M* glycine–NaOH (pH 10.0) prior to enzyme assays. The inhibition obtained with *p*-nitrophenyl butyrate (assay at pH 8.0) is shown in the table and the inhibition of tritiated cutin hydrolysis (assay at pH 10.0) is shown in parentheses.

cubation conditions. *N*-Ethylmaleimide, iodoacetamide, and *p*-chloromercuribenzoate did not inhibit either cutinase, indicating that the cutinases are not sulfhydryl hydrolases. The relative rates of inactivation of the two cutinases by Dip-F and paraoxon were compared using *p*-nitrophenyl butyrate hydrolysis as an assay for the two isozymes (Table III, experiment 2). Cutinase I was inhibited 68% after preincubation with 2×10^{-6} *M* Dip-F for 10 min, while under the same conditions cutinase II was inhibited only 43%. Cutinase I was inhibited 59% with 4×10^{-6} *M* paraoxon, while cutinase II was inhibited only 16% under the same conditions. Thus, cutinase I was found to be more sensitive to the two serine hydrolase inhibitors, and this difference is consistent with the previous observation that cutinase I hydrolyzed the model esters more rapidly than did cutinase II. The reason for these differences is unknown. The proteolytic clips contained in cutinase II might, in part, be responsible for these differences.

In order to determine whether the hydrolysis of *p*-nitrophenyl butyrate by the cutinases reflected accurately their ability to hydrolyze cutin, the effects of inhibitors on the two activities of the cutinases were compared. The values in

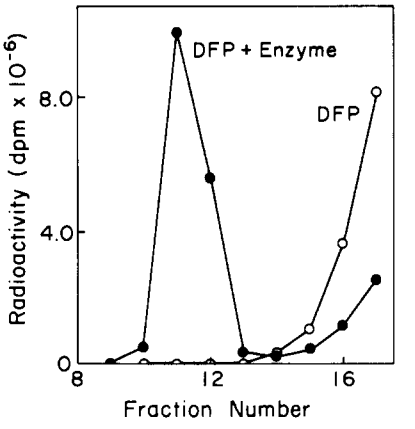

FIGURE 4: Bio-Gel P-2 chromatography of cutinase II after treatment with [^{3}H]Dip-F. Cutinase II was treated with 2.9 m*M* [^{3}H]Dip-F as described in text (●).

parentheses (Table III) show the effect of paraoxon treatment on the cutin hydrolyzing activity of cutinase I and cutinase II. These results show that the effect of paraoxon treatment on the cutin hydrolyzing activity of the isozymes is quite similar to that on the *p*-nitrophenyl butyrate hydrolyzing activity. The difference in paraoxon sensitivity of the two isozymes was obvious with either assay, and these results quite clearly show that the spectrophotometric assay with *p*-nitrophenyl butyrate is justifiable for both isozymes of cutinase. Preincubation of the nonspecific esterase with 1×10^{-5} *M* Dip-F or paraoxon for 10 min, under the same conditions as those used with the cutinases, resulted in 14 and 73% inhibition, respectively, when assayed with *p*-nitrophenyl butyrate. The nonspecific esterase appears to be less sensitive to Dip-F than to paraoxon unlike the cutinases which are more sensitive to Dip-F than to paraoxon. Thus, the cutinase isozymes and the nonspecific esterase are inhibited by Dip-F and paraoxon, as are several other serine hydrolases (Krish, 1971) and, therefore, the *Fusarium* enzymes are probably serine hydrolases. Recently, other ester hydrolases have been shown to be inhibited by Dip-F or paraoxon (Matsunaga et al., 1974; Haugen and Suttie, 1974; Hipps and Nelson, 1974).

If the three enzymes from *Fusarium* are indeed serine hydrolases, Dip-F treatment should result in the covalent attachment of the diisopropyl phosphate moiety to the serine at the active site (Cohen et al., 1959). In order to examine this possibility, the enzymes were treated with tritiated Dip-F and the reaction mixtures were subjected to gel filtration with Bio-Gel P-2 (Figure 4). In all cases, the protein

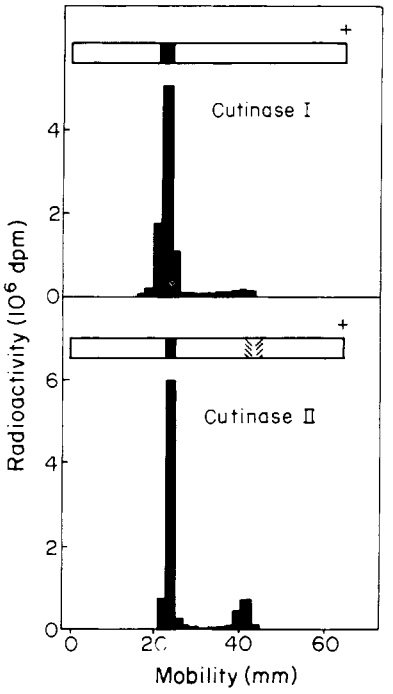

FIGURE 5: Sodium dodecyl sulfate polyacrylamide disc gel electrophoresis of [^{3}H]Dip-F-treated cutinases I and II. The 15% polyacrylamide gels were cut into 2-mm slices and the radioactivity was determined as described in text.

fraction contained radioactivity showing that the diisopropyl moiety was attached to the protein. In order to determine whether the diisopropyl moiety was covalently attached, the radioactive proteins were treated with dodecyl sulfate and mercaptoethanol, and they were subjected to dodecyl sulfate polyacrylamide disc gel electrophoresis. In the case of cutinases I and II, the major portion of radioactivity was found in the band corresponding to 21,400 (Figure 5). Therefore, it is clear that the diisopropyl moiety was attached very tightly, most probably covalently, to these enzymes. In a previous report (Purdy and Kolattukudy, 1975), we had shown that dodecyl sulfate polyacrylamide electrophoresis of cutinase II separated the major protein band (mol wt 21,800) from two minor bands corresponding to 10,600 and 9400. The two minor bands presumably originated from a proteolytic clip in the cutinase. Tritiated Dip-F labeled the 10,600 fragment but not the other fragment (Figure 5). Thus, the active site serine is retained in the larger fragment, and, presumably, the proteolytic clip did not destroy the activity of the enzyme. Furthermore, the observation that the diisopropyl moiety was covalently attached to this fragment strongly supports the hypothesis that the two smaller peptides originated by a proteolytic clip of the enzyme.

The nonspecific esterase was also labeled by tritiated Dip-F. In this case dodecyl sulfate polyacrylamide gel electrophoresis showed that the major band at 54,000 was labeled (Figure 6). In addition, a less intense band was found at 50,000 and this protein was also labeled by treatment with tritiated Dip-F. Therefore, it is concluded that both of these proteins are serine hydrolases and that the 50,000 protein originated from the 54,000 enzyme by proteolysis. A significant amount of ^{3}H was found in the region of the gel where proteins of much lower molecular weight would be present. Presumably, proteolysis is responsible for this finding. A proteolytic clip in another extracellular fungal

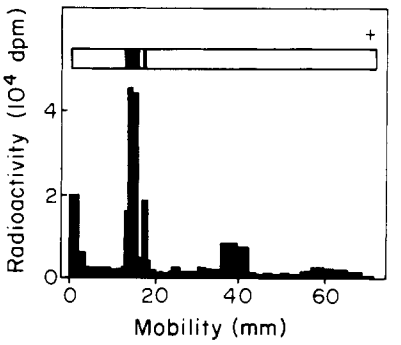

FIGURE 6: Dodecyl sulfate polyacrylamide disc gel electrophoresis of [^{3}H]Dip-F-treated extracellular nonspecific esterase. The 10% polyacrylamide gel was cut into 1-3-mm slices and the radioactivity was determined as described in text.

enzyme was recently reported (Benzonana, 1974). It is probably a coincidence that a bacterial extracellular glycerol ester hydrolase which was also labeled with tritiated Dip-F appeared at the 54,000 region when subjected to dodecyl sulfate polyacrylamide gel electrophoresis (Fulton et al., 1974).

In order to determine the number of reactive serines present in the cutinases, both enzymes were treated with [^{3}H]Dip-F at concentrations which would partially or completely inhibit the enzymes. The preparations were immediately chromatographed on Bio-Gel P-2 as indicated in Figure 4. All these operations were carried out at pH 7.0 to minimize the possibility of an elimination reaction which might generate anhydrocutinase in a manner similar to that observed with chymotrypsin (Feinstein and Feeney, 1966; Ako et al., 1974). Fractions (at the void) containing the enzyme which was free of noncovalently bound [^{3}H]Dip-F were located by monitoring the radioactivity of the effluent. The enzymatic activity contained in the labeled protein fraction was determined with *p*-nitrophenyl butyrate and the protein concentration was determined by the Lowry method while the amount of covalently attached Dip-F was determined from the amount of radioactivity. In the case of cutinase I, incorporation of 0.6 mol of Dip-F/mol of enzyme resulted in 87% inhibition of the enzyme, while with cutinase II, under conditions which resulted in incorporation of 0.6 mol of Dip-F/mol of enzyme, 80% inhibition of the hydrolase activity was observed (for this calculation molecular weight of 22,000 was used for both enzymes). Making corrections for the enzymatic activity remaining after the Dip-F treatment, it is calculated that the number of moles of Dip-F attached per molecule of inactivated enzyme is 0.7 and 0.75 for cutinase I and cutinase II, respectively. Neither higher concentrations of Dip-F nor longer periods of incubation gave substantially higher incorporation of Dip-F. Therefore, it is concluded that, in both cutinases, there is probably one reactive serine per molecule. There are at least two possible explanations for the fact that values obtained for the number of moles of Dip-F attached per mole of the enzyme are lower than 1.0. Label in the isopropyl moiety could have been partially lost by hydrolysis or the Dip-F derivative might have undergone elimination reaction during the procedures. In fact, values much lower than 0.7 were obtained when Dip-F treatment was conducted at pH 8.0 or higher. In any case, it appears that one reactive serine/molecule is present in both cutinases.

The cutinase isozymes apparently have no metal ion requirement since preincubation of the enzymes with chela-

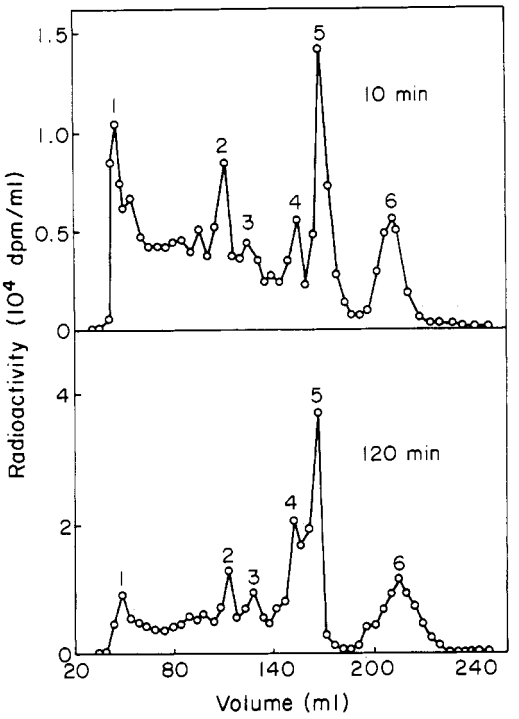

FIGURE 7: Sephadex LH-20 radiochromatograms of soluble products released by cutinase I from biosynthetically labeled apple cutin. Enzymatic hydrolysis was performed for 10 min, or 120 min, and the chromatography and identification of products were done as described in the text. (1) Void; (2) ω-hydroxy C_{18} acid, and probably 18-oxo-9,10-epoxyoctadecanoic acid; (3) ω-hydroxy C_{16} acid; (4) 9,10-epoxy-18-hydroxy C_{18} acid; (5) dihydroxy C_{16} acid; (6) trihydroxy C_{18} acid.

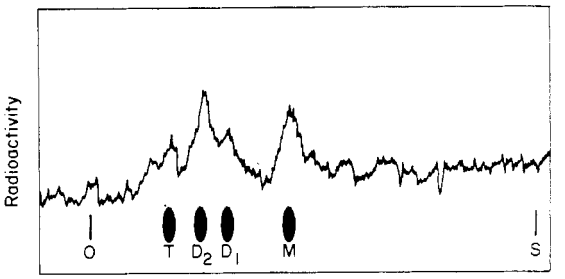

FIGURE 8: Radio thin-layer chromatogram of the reduction products of the oligomer fraction obtained from ^{14}C-labeled cutin by cutinase I. The oligomers were obtained by pooling the 41–96-ml fractions of the 10-min incubation shown in Figure 7. Chromatographic conditions are described under the Experimental Section. O, origin; T, 1,9,10,18-tetrahydroxyoctadecane; D_2, 1,7,16-trihydroxyhexadecane; D_1, 1,9,18-trihydroxyoctadecane; M, alkane-α,ω-diol; S, solvent front.

tors did not inhibit these enzymes (Table III). Similarly no metal ion requirement could be detected for a soil malathion hydrolase (Satyanarayana and Getzin, 1973) and a rat liver microsomal esterase (Haugen and Suttie, 1974). On the other hand, a liver lysosomal esterase (Mahadevan and Tappel, 1968) and an extracellular tannin acyl hydrolase of large molecular weight (Iibuchi et al., 1968) were inhibited by chelators.

Mechanism of Enzymatic Hydrolysis of Cutin. Cutin was shown to be depolymerized to its monomers after a fairly long incubation with a concentrate of the extracellular fluid from *F. solani f. pisi* (Purdy and Kolattukudy, 1973). The cutinase isozymes and the nonspecific esterase might function as endo and exo hydrolases during the cutin depolymerization process. They might free dimers, trimers, or oligomers which might be useful in determining the structure of cutin as well as in understanding the mechanism of action of these enzymes. In order to investigate these possibilities, gel filtration with Sephadex LH-20 was employed to fractionate the soluble lipid products released by the enzymatic hydrolysis of cutin, which was labeled biosynthetically with [1-^{14}C]acetate. It is known that all classes of monomers of this cutin contain label (Kolattukudy et al., 1973). Elution profiles of products from 10- and 120-min incubations of cutinase I with the biosynthetically labeled cutin is shown in Figure 7. The major products were identified by a combination of techniques. Peak 6 was identified as 9,10,18-trihydroxy C_{18} acid by TLC with authentic standards. Peak 5 was identified as dihydroxy C_{16} acid by direct TLC, and TLC after $LiAlH_4$ reduction, with authentic standards. Peak 4 was identified as 18-hydroxy-9,10-epoxy C_{18} acid by TLC of its $LiAlH_4$ reduction product, as well as TLC of the product obtained by $LiAlH_4$ reduction of the acetoxylated product. Peak 3 was identified as 16-hydroxy C_{16} acid by TLC, as well as radio gas–liquid chromatography of the acetylated methyl ester. Peak 2 showed two components (TLC) of which one was identified as 18-hydroxy C_{18} acid by TLC and by radio gas–liquid chromatography of the acetylated methyl ester. The other component contained in peak 2 was tentatively identified as 18-oxo-9,10-epoxy C_{18} acid by its R_f value. The structure and composition of these enzymatically released monomers are the same as those found in the chemical hydrolysate of the polymer (Walton and Kolattukudy, 1972). Therefore, it is clear that cutinase I catalyzed hydrolysis of the polymer to the monomers. Similar experiments with cutinase II gave results identical with those shown in Figure 7. Therefore, it appears that there was no difference in specificity or mode of action between the two cutinases.

Peak 1 (Figure 7) appeared at the void and therefore this fraction probably consists of oligomers. The fractions between peak 1 and peak 2 also contained substantial amounts of label, presumably because these fractions also contained oligomeric materials of varying sizes. These fractions, as well as peak 1, upon $LiAlH_4$ reduction, gave rise to all of the known monomers of apple cutin (Figure 8). These results show that peak 1, and the fraction between peaks 1 and 2, contained monomers linked together with $LiAlH_4$-susceptible bonds, most probably ester bonds. Therefore, the sum of these fractions is referred to as the oligomer fraction. The soluble material released by short periods of treatment of cutin with the cutinases contained a much larger proportion of oligomers than that observed after longer periods of incubation (Figure 7). Therefore, it appears that the cutinases hydrolyze both cutin and oligomers to monomers. On the other hand, the nonspecific esterase did not release any radioactivity from the labeled cutin, either in the presence or absence of Triton X-100 which had been shown to stimulate this enzyme (Purdy and Kolattukudy, 1973). It is possible that the role of the nonspecific esterase is to hydrolyze the oligomers released by the cutinases. In order to investigate this possibility, the oligomer fraction released by a 10-min treatment of labeled cutin with cutinase I was treated with either the nonspecific esterase or cutinase I. Fractionation of the products by LH-20 chromatography showed that cutinase I almost completely hydrolyzed the oligomers to the monomers while the nonspecific esterase released very little monomers from the oligomers (Figure 9). These results clearly show that cutinase itself hydrolyzes the oligomers, and that the role of the non-

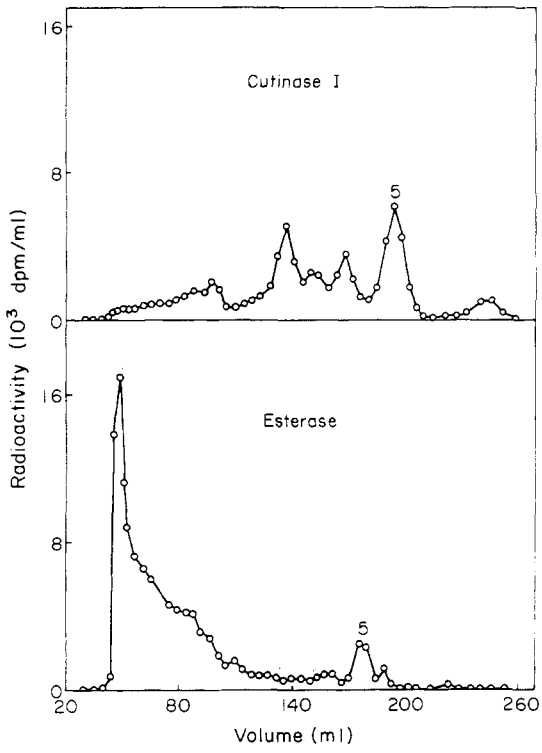

FIGURE 9: Sephadex LH-20 radiochromatograms of products obtained from the treatment of labeled oligomers with cutinase I and the nonspecific esterase. The oligomers were obtained from the 41–96-ml fractions of the 10-min incubation shown in Figure 7. The incubation conditions and chromatography are described under the Experimental Section. 5, dihydroxy C_{16} acid.

specific esterase is not hydrolysis of oligomers. At the pres- matter of controversy for many years. Recent electron-microscopic investigation into the infection of *Vicia faba* leaves by the facultative parasitic fungus *Botrytis cinerea* indicated that the mode of penetration of the fungus into the plant was enzymatic (McKeen, 1974). In an attempt to detect the postulated cutinase, indoxyl acetate was used as a cytochemical means of detecting an esterase type activity, and such an activity could be located at the penetration area. Both cutinases and the nonspecific esterase hydrolyze indoxyl acetate (data not shown). With the first isolation, purification, and characterization of cutinase reported here, ent time the role of the nonspecific esterase, which is also induced by growth of the fungus on cutin (Purdy and Kolattukudy, 1973), is unknown. It is possible that the nonspecific esterase is involved in the hydrolysis of the cuticular wax esters, and that this esterase is coinduced with the cutinase.

The mode of entry of pathogens into plants has been a matter of controversy for many years. Recent electron-microscopic investigation into the infection of *Vicia faba* leaves by the facultative parasitic fungus *Botrytis cinerea* indicated that the mode of penetration of the fungus into the plant was enzymatic (McKeen, 1974). In an attempt to detect the postulated cutinase, indoxyl acetate was used as a cytochemical means of detecting an esterase type activity, and such an activity could be located at the penetration area. Both cutinases and the nonspecific esterase hydrolyze indoxyl acetate (data not shown). With the first isolation, purification, and characterization of cutinase reported here, it is now possible to determine whether such enzymes are, in fact, involved in fungal penetration into plants.

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