Hydrolysis of Plant Cuticle by Plant Pathogens. Purification, Amino Acid Composition, and Molecular Weight of Two Isozymes of Cutinase and a Nonspecific Esterase from *Fusarium solani f. pisi*†

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ABSTRACT: The extracellular fluid of the plant pathogen, *Fusarium solani f. pisi,* grown on the plant cuticular polymer, cutin, was shown to contain cutinase and *p*-nitrophenyl palmitate hydrolase activities (R. E. Purdy and P. E. Kolattukudy (1973), *Arch. Biochem. Biophys. 159,* 61). From this extracellular fluid two isozymes of cutinase and a nonspecific esterase (*p*-nitrophenyl palmitate hydrolase) were isolated using Sephedex G-100 gel filtration, QAE-Sephadex chromatography, and SE-Sephedex chromatography. Phenolics contained in the extracellular fluid were found to be associated with the cutinase but not with the nonspecific esterase, and the phenolic materials were removed from cutinase at the QAE-Sephedex step. A 34-fold purification of the nonspecific esterase and a 6.5-fold purification of cutinase were achieved by the procedure described. The two isozymes of cutinase (I and II) and the nonspecific esterase were homogeneous as judged by polyacrylamide disc gel electrophoresis and sedimentation equilibrium centrifugation. Molecular weights of cutinase I, cutinase II, and the nonspecific esterase were determined by Sephedex G-100 gel filtration, sedimentation equilibrium centrifugation, amino acid composition, and sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. The values obtained with these techniques agreed with each other and were about 22,000 for both cutinases and 52,000 for the nonspecific esterase. The dodecyl sulfate gel electrophoresis indicated that a small portion of cutinase II contained proteolylic clips, near the middle of the polypeptide chain, and that the nonspecific esterase might also have undergone some proteolylic modification. The amino acid composition of cutinase I was similar to that of cutinase II except for the presence of a larger number of tryptophan residues in the latter, while the amino acid composition of the nonspecific esterase showed more differences from that of either cutinase.

In terrestrial organisms the cuticle consists of a polymeric structural component impregnated with waxy materials. Proteins or carbohydrates constitute the cuticular polymer on animals, while on plants, a hydroxy fatty acid polymer, cutin, serves this function. The fatty acids of cutin are usually n-C_{16} and n-C_{18} and contain one to three hydroxyl groups. Fatty acids containing other functional groups such as epoxides, aldehydes, and ketones are also found (Brieskorn and Boss, 1964; Crisp, 1965; Baker and Halloway, 1970; Kolattukudy and Walton, 1972; Hunneman and Eglinton, 1972; Walton and Kolattukudy, 1972; Croteau and Fagerson, 1972; Kolattukudy, 1972, 1973; Deas et al., 1974). Ester bonds predominate in the cutins thus far analyzed, and some indirect evidence for the possible presence of peroxide bridges and ether linkages has also been presented (Crisp, 1965). Cleavage of cutin with specific enzymes would be an ideal method for elucidation of the structure of the polymer. Such enzymes would also be useful tools in biosynthetic studies of this polymer. However, no such enzymes have been characterized.

Cutin is thought to play a key role in plant protection because it is a barrier against the entry of pathogens into plants. Therefore enzymatic degradation of the cutin has been suggested to be one of the first steps in the infection process. However, it is not known whether pathogens penetrate this barrier by physical force or by means of enzymatic degradation of cutin. Recent electron microscopic studies indicate that enzymatic degradation of cutin may be involved in fungal penetration into the plant (McKeen, 1974), although there is no conclusive evidence for such an enzymatic penetration. Some microorganisms, including plant pathogens, have been shown to live on cutin as their sole carbon source (Heinen and DeVries, 1966; Hankin and Kolattukudy, 1971, Purdy and Kolattukudy, 1973), and suggestive evidence for the production of cutinolytic enzymes by pathogens has been presented (Shishiyama et al., 1970; Heinen, 1960; Linskens and Haage, 1963).

The extracellular fluid of cutin-grown *F. solani pisi* was shown to catalyze depolymerization of cutin and hydrolysis of fatty acyl esters of *p*-nitrophenol (Purdy and Kolattukudy, 1973). However, little is known about cutin depolymerizing enzymes as no such enzyme has been purified.

In this paper we describe the purification, amino acid composition, and molecular weight of two cutinases and a *p*-nitrophenyl ester hydrolase (a nonspecific esterase)[1] isolated from the extracellular fluid of the pathogen *F. solani f. pisi* grown on cutin as the sole source of carbon.

Experimental Section

Materials. Golden Delicious apple cutin (0.5 g, 60 mesh) prepared as described previously (Walton and Kolattukudy,

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[1] The *p*-nitrophenyl palmitate hydrolase was shown to be a nonspecific esterase (Purdy and Kolattukudy, 1975). Therefore it is referred to as either PNP hydrolase or nonspecific esterase.

1972) was exposed to 5 Ci of tritium gas for 6 hr at room temperature and the labile ^3H was immediately removed by washing this material with water at New England Nuclear Corporation. This tritiated cutin was treated with cellulase and pectinase as described before (Walton and Kolattukudy, 1972), and the solids recovered from this treatment were thoroughly washed with water. This material released ^3H into several polar and nonpolar solvents. In order to remove the bulk of this soluble radioactive material, the cutin powder was subjected to Soxlet extraction with water, dioxane, and chloroform as solvents (1–3 days each). This cycle was repeated several times. The resulting cutin was diluted 60-fold by homogenizing it in water with unlabeled cutin in a Ten-Broeck homogenizer. The diluted cutin was extracted for 3 days with acetone in a Soxlet extractor, dried, and stored at −20° until used. Aquasol was obtained from New England Nuclear Corp. Bovine serum albumin, β-lactoglobulin, bovine pancreatic ribonuclease A (type III-A), myoglobin (type I), alcohol dehydrogenase, cytochrome *c*, and *p*-nitrophenyl palmitate were obtained from Sigma Chemical Company. Pepsin, chymotrypsin, and chymotrypsinogen A were obtained from Worthington Biochemical Corporation. All other chemicals were of reagent grade.

Culture Conditions. Fusarium solani f. pisi was maintained on potato dextrose agar at 23°. Suspensions of mycelia in sterile distilled water were used to innoculate cutin media which contained 0.5 g of 60 mesh Golden Delicious apple cutin per 100 ml of pH 7.2 mineral medium (Purdy and Kolattukudy, 1973). Cultures were grown on the cutin media for 12 days at room temperature, 23°.

Ammonium Sulfate Precipitation. The extracellular fluid from 60 to 70 cultures yielded after two vacuum filtrations 5–6 l. of fluid. The extracellular fluid (0–4°) was stirred as granular ammonium sulfate was slowly added until 50% saturation was achieved, and the suspension was stirred for 1.5 hr. The precipitate was recovered by centrifugation at 20,000*g* for 35 min and it was dissolved in 50 m*M* glycine–NaOH buffer (pH 10.0), before dialysis against 40 m*M* phosphate buffer (pH 8.0) at 0–4°. The remaining insoluble material was removed by centrifugation and the supernatant was concentrated with a UM10 Amicon ultrafiltration membrane in a Model 52 Amicon ultrafiltration cell.

Gel Filtration. For preparative purposes, the concentrated enzyme solution (6–7 ml) from the above step was applied to a Sephadex G-100 column (4.0 × 49 cm) which was previously equilibrated with 40 m*M* phosphate buffer (pH 8.0) and the proteins were eluted with the same buffer. The column effluent fractions were monitored for their o.d. at 280 nm with a Gilford spectrophotometer and they were assayed for cutinase and *p*-nitrophenyl palmitate (PNP) hydrolase activities. For molecular weight determination a similar gel filtration was carried out with a small column (2.2 × 85 cm) of Sephadex G-100. In this case bovine serum albumin (68,000), β-lactoglobulin (36,700), chymotrypsinogen (25,700), myoglobin (16,900), and ribonuclease A (13,700) were used as standards. In all cases the void volumes were determined with Dextran Blue 2000.

Ion Exchange Chromatography. The fractions containing both the PNP hydrolyzing activity and cutin hydrolyzing activity obtained from the G-100 column were pooled and concentrated with a UM10 Amicon membrane in an Amicon ultrafiltration cell, Model 52. The concentrate was dialyzed against 100 m*M* Tris-HCl buffer (pH 9.0) and it was applied to a QAE-Sephadex A-25 column (3 × 21.5 cm) which had been washed with 100 m*M* Tris-HCl buffer (pH 7.0) and then equilibrated with 10 m*M* Tris-HCl buffer (pH 9.0). After the application of the protein the column was washed with two bed volumes of the pH 9.0 buffer followed by two bed volumes each of pH 8.3 and 7.0 Tris-HCl buffers (100 m*M*). Cutinase and PNP hydrolase activities and the optical density at 280 nm of each fraction (10 ml) were measured. Fractions containing cutinase activity were pooled and concentrated by ultrafiltration. The concentrate was dialyzed against citrate–phosphate buffer (pH 5.0) (5 m*M* citrate–10 m*M* phosphate), and it was applied to an SE-Sephadex C-25 column (1.8 × 19.5 cm) which was equilibrated with the same buffer. The column was washed with at least two bed volumes of buffer before a linear gradient of 0–180 m*M* sodium chloride was applied in a total volume of 500 ml and 5-ml fractions were collected. Each fraction was assayed for cutinase activity and the optical density at 280 nm.

Electrophoresis. Cationic polyacrylamide disc gel electrophoresis was run as described by Gabriel (1971). The gels were prepared by filling 65 × 5 mm glass tubes to 45 mm with 7.5% separating gel containing acetate buffer (pH 3.8). After polymerization of the running gel, the stacking gel (acetic acid buffer, pH 5.0) was added to a 52-mm line. The running buffer was β-alanine (3.12 g/l.) and acetic acid (0.8 ml/l.) adjusted with NaOH to pH 4.5. Methylene Blue was used as the tracking dye. Up to 50 μl of sample in 10% sucrose solution was applied to the gels. Electrophoresis was run at 0–4° with 2 mA/tube until the tracking dye approached the end of the gel. The total length of the gel and the distance of migration of the tracking dye were recorded immediately after electrophoresis. Gels were stained with 1% Amido Black in 7% acetic acid for 2 hr and they were destained by soaking in 7% acetic acid. In order to determine the location of the enzymatic activity, the gels were cut into 2-mm slices. For the PNP hydrolase assay the gel slices were soaked in 50 m*M* phosphate buffer (pH 8.0) and for the cutinase assay the gel slices were soaked in 50 m*M* glycine–NaOH buffer (pH 10.0) at 0–4° overnight.

Enzyme Assays. In preparation for a cutinase assay the diluted labeled cutin described under the Experimental Section was homogenized in water and placed in a 15-ml graduated centrifuge tube. The solid material, recovered by centrifugation, was washed three times with water followed by three washes with acetone. Each time the solid material was suspended in the solvent and the solid was recovered by centrifugation. It was then soaked in acetone overnight, followed by a repetition of the above washing procedure with acetone followed by water. After the resulting residue was soaked for 2–3 hr in 50 m*M* glycine–NaOH buffer (pH 10.0), it was thoroughly washed with water. The specific activity of this cutin preparation was adjusted, usually by the addition of an equal weight of homogenized (Ten-Broeck) unlabeled cutin suspended in water. After such dilution steps, the solids were washed five times with water in order to remove extremely fine particles which could interfere with the measurement of soluble monomers released enzymatically.

Appropriate aliquots of the enzyme were incubated in a total volume of 1 ml of 50 m*M* glycine–NaOH buffer (pH 10.0) with 4.2 mg of tritiated cutin in a gyrating water bath shaker for 10 min at 30°. The reaction was terminated by force filtering the reaction mixture through a glass wool plug in a pasteur pipet. Aliquots (0.2 ml) of the filtrate were assayed for radioactivity in 5 ml of Aquasol with a

Packard 3003 liquid scintillation spectrometer. Controls for the enzyme assays were run with identical reaction mixtures containing no enzyme and the control values were subtracted from experimental values to give the rate of enzymatic hydrolysis. All measurements of radioactivity were done with an efficiency of 14% and a standard deviation of <3%. *p*-Nitrophenyl esterase activity was measured spectrophotometrically as described previously (Purdy and Kolattukudy, 1973), in 105 m*M* phosphate buffer (pH 8.0).

Thin-Layer Chromatography. Chromatography of reduced cutin monomers was carried out on 0.5-mm silica gel G thin layer plates using ethyl ether–hexane–methanol (8:2:1 v/v) as the developing solvent and the radioactivity in each thin-layer chromatographic fraction was determined as before (Purdy and Kolattukudy, 1973).

Amino Acid Analysis. The amino acids derived from the enzymes were analyzed with a Beckman automatic amino acid analyser, Model 121C, using the method of Spackman et al. (1958). Approximately 0.5-mg samples of the cutinases were hydrolyzed with 6 *M* HCl at 100° for 24 and 48 hr. Identical samples were used to measure the amounts of cysteine and methionine (Hirs, 1967) and tryptophan (Matsubara and Susaki, 1969). A sample containing 0.25 mg of the nonspecific esterase was hydrolyzed for 24 hr and the amino acids were determined on the expanded range of the analyser.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were performed on a Beckman Model E ultracentrifuge with the AnD rotor with a double sector cell at 20° using the Yphantis (1964) technique. All samples were run at a concentration of 0.5 mg/ml. The cutinases were run at 40,000 rpm. Cutinase I was run in 50 m*M* NaCl, and cutinase II was run in 60 m*M* NaCl. The nonspecific esterase was run at 28,000 rpm in 40 m*M* phosphate buffer (pH 8.0). The partial specific volumes were calculated from the amino acid composition of each enzyme.

Sodium Dodecyl Sulfate Electrophoresis. Dodecyl sulfate polyacrylamide disc gel electrophoresis was performed as described by Weber and Osborn (1969). Gels containing 10 and 15% polyacrylamide with a proportional increase in cross-linking were used. The standards were: γ-globulin (50,000 and 23,500), alcohol dehydrogenase (40,000), pepsin (35,000), α-chymotrypsinogen (25,700), ribonuclease (13,700), cytochrome *c* (11,700), and α-chymotrypsin (13,900 and 10,000). Cutinases I and II were treated with 1% dodecyl sulfate and 1% mercaptoethanol at 38, 50, or 70° for 12 hr before electrophoresis. The extracellular nonspecific esterase was maintained at 41° for 3 hr in 1% dodecyl sulfate and 1% mercaptoethanol or at 100° in 1% dodecyl sulfate and 50 m*M* dithioerythritol for 1 hr before electrophoresis.

Results and Discussion

Cutinase Assay. Previously, an ammonium sulfate precipitate obtained from the extracellular fluid from cutin-grown *F. solani f. pisi* was shown to release radioactive cutin monomers from ^{14}C-labeled cutin (Purdy and Kolattukudy, 1973). Such biosynthetically labeled cutin can be made only when rapidly growing apple fruits are available and the procedure is very expensive. Therefore, another suitable substrate was sought. Since apple cutin contains unsaturated monomers such as ω-hydroxyoleic acid and 9,10-epoxy-18-hydroxyoctadecenoic acid (Walton and Kolattukudy, 1972) the double bonds can be hydrogenated with tritium. Golden Delicious apple cutin was tritiated by exposing it to tritium gas. This material slowly released soluble radioactive materials and therefore required an extensive washing procedure to minimize the release of radioactive materials which would interfere with the enzyme assays. Thin-layer chromatography of the chloroform soluble products obtained by $LiAlH_4$ reduction of the tritiated cutin showed that it contained tritiated cutin monomers and a fairly large quantity of labeled unknown polar material that remained in the origin of the thin-layer chromatogram. A similar distribution pattern of ^3H was observed when the enzymatic hydrolysis products of cutin were examined by thin-layer chromatography after $LiAlH_4$ reduction. Even though unidentified polar compounds other than the well-characterized cutin monomers were contained in the tritiated cutin, the release of soluble tritiated products was a convenient assay for cutinase, and the results were comparable to those obtained when cutinase activity was measured by a tedious gas chromatographic method which consisted of measurement of monomers released from nonradioactive cutin.

Ammonium Sulfate Precipitation. Ammonium sulfate was used to salt out the proteins from the extracellular fluid of 12 day old *F. solani f. pisi* cultures grown on Golden Delicious apple cutin as the sole carbon source. The bulk of the protein precipitated between 40 and 50% ammonium sulfate saturation and this protein contained most of the PNP hydrolase and cutinase activities of the extracellular fluid. Therefore, 0–50% ammonium sulfate saturation was used as a concentrating device, since this technique did not result in resolution of the enzymes or significant purification. In contrast, a major part of the hydrolytic activities of the extracellular fluids from the same organism grown on cutin from Red Delicious apple was contained in the protein precipitated by 30% saturation with ammonium sulfate (Purdy and Kolattukudy, 1973). This difference appears to be due to the nature of the phenolics contained in the cutin preparation. It is probably for this reason that some variability in fractionation characteristics of the proteins are observed, even among enzyme preparations obtained from cultures grown on different batches of cutin from Golden Delicious apples. However, such variations are not found after removal of the phenolics from the enzyme preparations.

Gel Filtration with Sephadex G-100. The protein obtained from the ammonium sulfate precipitation step was fractionated on a Sephadex G-100 column (Figure 1). The two large 280-nm peaks, at the void and near the end of the fractionation range, did not coincide with either of the enzymatic activities and apparently contained the bulk of the phenolics as indicated by the intense golden brown color. The optical density at 280 nm cannot be used as an indication of protein concentration in this fractionation because of the presence of the phenolics. Protein determination by the Lowry *et al.* (1951) method gave a similar profile. The PNP hydrolase activity was eluted as a fairly sharp peak soon after the void volume in the molecular weight range of 50,000–60,000. However, the cutinase activity eluted as a broad peak and this peak generally overlapped with the peak of PNP hydrolase activity. The breadth of the cutinase peak is probably due to binding of the enzyme with the phenolics. This purification step served to remove the bulk of the phenolics from the hydrolases. When this step was omitted the final cutinase preparation contained another protein of approximately the same size as cutinase. Thus during this gel filtration cutinase was resolved from this contaminating protein because cutinase was eluted earlier together

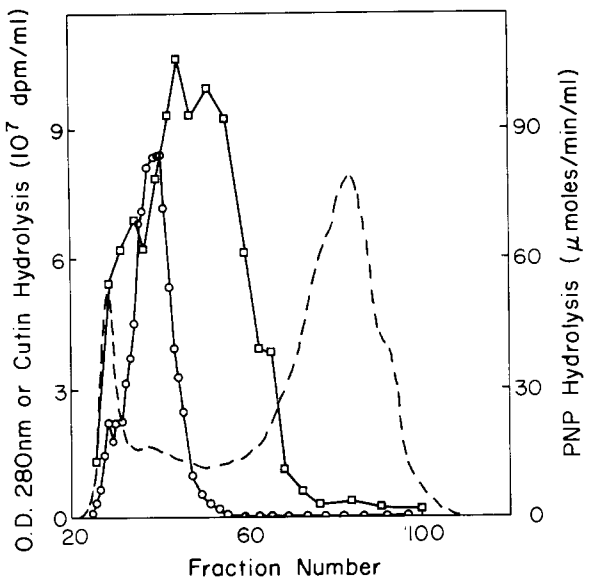

FIGURE 1: Sephadex G-100 gel filtration of the protein fraction precipitated at 0–50% saturation of the extracellular fluid of *F. solani pisi* with ammonium sulfate. A 4 × 49 cm column of Sephadex G-100 was equilibrated and eluted with 40 m*M* phosphate buffer (pH 8.0). Optical density at 280 nm (- - -); *p*-nitrophenyl palmitate (PNP) hydrolysis (○); and tritiated cutin hydrolysis (□) were measured as described in the Experimental Section.

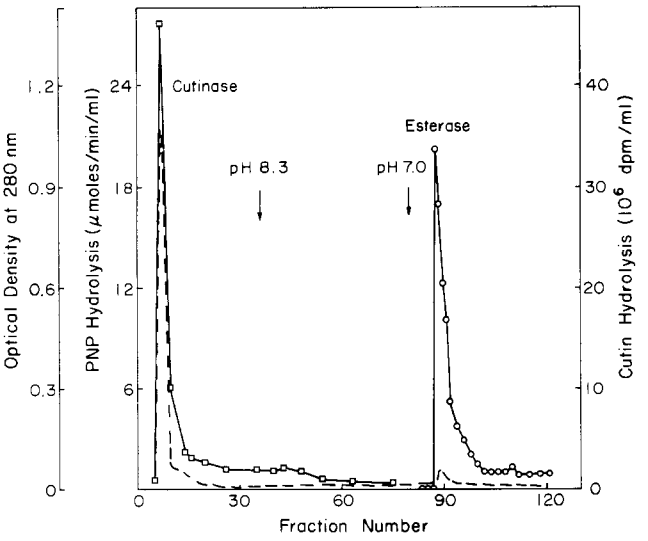

FIGURE 2: QAE-Sephadex chromatography of the protein fraction containing both cutinase and PNP hydrolase, obtained from the Sephadex G-100 step shown in Figure 1. The sample was applied in 100 m*M* Tris-HCl buffer (pH 9.0) and 100 m*M* Tris-HCl buffers of pH 8.3 and pH 7.0 were applied in batches. Optical density at 280 nm (- - -); *p*-nitrophenyl palmitate (PNP) hydrolysis (○), and tritiated cutin hydrolysis (□) were measured as described in the Experimental Section.

with the associated phenolics. Since the cutinase and PNP hydrolase could not be completely separated by this step, the fractions containing both of these activities were pooled and subjected to ion exchange chromatography.

QAE-Sephadex. The G-100 fractions containing both enzymatic activities were subjected to a QAE-Sephadex A-25 column chromatography (Figure 2). The cutinase did not bind to the column at pH 9.0 while the phenolics and PNP hydrolase were bound at this pH. Decreasing the pH of the eluting buffer to 8.3 resulted in the elution of only a small amount of protein which contained neither cutinase nor PNP hydrolase activity. The same buffer at pH 7.0 eluted a small amount of protein (280 nm) which contained PNP hydrolase activity. A significant amount of PNP hydrolase activity was spread out over many fractions which eluted after the peak of the activity. An attempt to avoid this trailing nature of the elution profile with the use of a KCl gradient (0–0.4 *M*) was unsuccessful. Since the phenolics were eliminated from the preparation at this step, absorbance at 280 nm became a reliable indicator of protein concentration as indicated by amino acid analysis, in further fractionation steps.

Second Sephadex G-100 Gel Filtration. Since the QAE step appeared to have removed the phenolics from the enzyme preparation, this step should have changed the filtration properties of the enzymes if the phenolics affected such properties. In order to test this possibility, the PNP hydrolase and cutinase obtained from the QAE fractionation were subjected to gel filtration on a Sephadex G-100 column (2.2 × 85 cm). The gel filtration of the cutinase obtained from QAE showed only one major protein peak and it coincided with the peak of cutinase activity (Figure 3). The elution profile indicated that the cutinase had decreased in size from the 40,000 to 60,000 range indicated by the first G-100 gel filtration to about 22,000. Furthermore, in contrast to the broad peak observed in the first G-100 gel filtration, the gel filtration after the QAE step gave a sharp

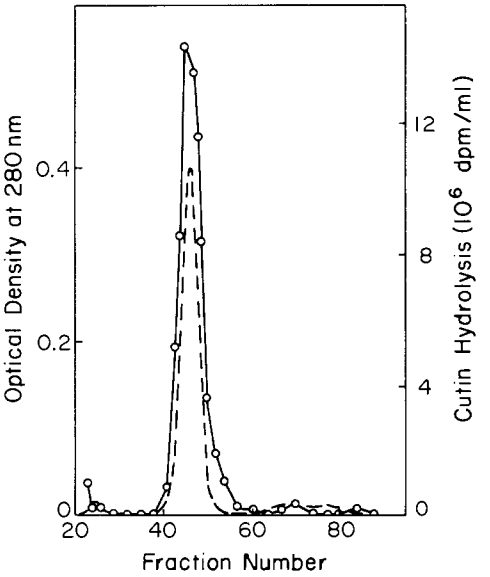

FIGURE 3: Sephadex G-100 gel filtration of cutinase from the QAE-Sephadex step shown in Figure 2. A 2.2 × 85 cm column was used and the effluent was monitored for tritiated cutin hydrolysis (○) and optical density at 280 nm (- - -) as described under the Experimental Section.

symmetrical peak of protein and enzymatic activity (Figure 3). The most probable reason for this change is the separation of the enzyme from the complex mixture of phenolics which were probably associated with the enzyme in the crude preparation. The elution profile of PNP hydrolase showed one major protein peak and two minor peaks. The major peak coincided with the peak of PNP hydrolase activity. This elution profile strongly suggested that the molecular size of this protein was the same as that indicated by the first G-100 gel filtration. Thus, it appears that the gel filtration characteristics of this enzyme were not affected by the phenolics present in the crude preparation. Therefore, this enzyme was probably not associated with the phenolics.

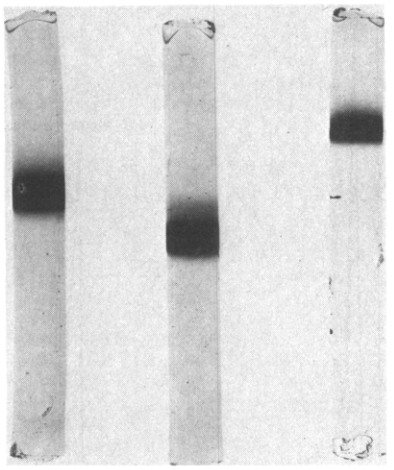

FIGURE 4: Polyacrylamide disc gel electrophoretograms of cutinase I, cutinase II, and PNP hydrolase. 30 μg of cutinase I (left) and 30 μg of cutinase II (middle) and 34 μg of PNP hydrolase (right). The gels were stained with Amido Black.

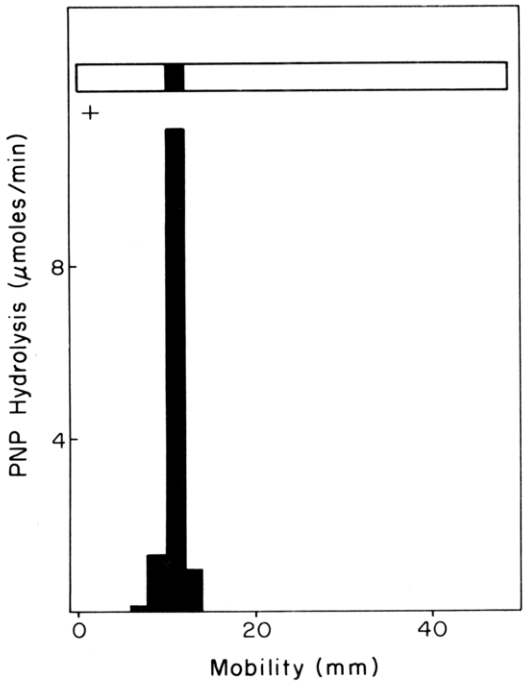

FIGURE 5: Polyacrylamide disc gel electrophoresis of PNP hydrolase obtained from the Sephedex G-100 step shown in Figure 3. PNP hydrolase (34 μg) was electrophoresed on two gels. One gel was stained (top) and the other was sliced and assayed for activity as described under the Experimental Section.

Electrophoresis. The purity of the PNP hydrolase was examined using polyacrylamide disc gel electrophoresis. Only one protein band was visible (Figure 4) and all of the PNP hydrolase activity coelectrophoresed with this only protein band (Figure 5). Electrophoresis of cutinase from the second G-100 gel filtration showed that this preparation contained only two closely migrating, but separable, proteins. The two proteins could be soaked out of gel slices and both of them catalyzed hydrolysis of cutin. Furthermore all of the cutinase activity of the preparation was contained in these two bands which represent isozymes of cutinase.

Resolution of Cutinase Isozymes. In order to resolve the two isozymes of cutinase, the enzyme preparation from the second G-100 gel filtration was subjected to ion exchange

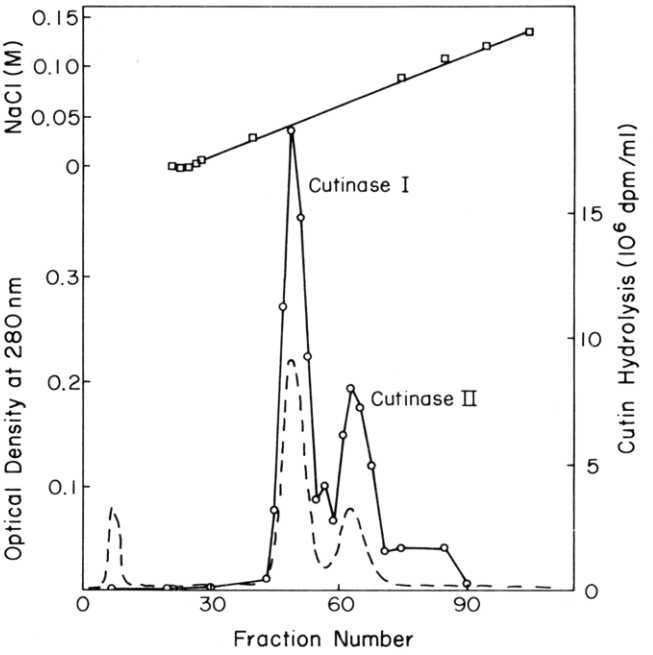

FIGURE 6: SE-Sephadex chromatography of cutinase obtained from the Sephadex G-100 gel filtration step shown in Figure 3. Conditions of chromatography and measurement of optical density at 280 nm (– – –) and cutin hydrolysis (O) are described under the Experimental Section.

chromatography on SE-Sephadex (Figure 6). A small amount of 280-nm absorbing material was not retained on the SE-Sephadex, but it did not have any cutinase activity. A linear gradient of NaCl eluted only two protein peaks which coincided with the only two peaks of cutinase activity eluted from the column. The first peak is referred to as cutinase I and the second as cutinase II. These enzymes are probably the two isozymes indicated by the electrophoresis referred to in the previous section. In order to confirm this conclusion, cutinase I and cutinase II were subjected to polyacrylamide disc gel electrophoresis and each showed only one protein band (Figure 4). To further show their purity, gels were sliced and soaked in buffer followed by measurement of cutinase activity in the extract of each gel slice. The protein band in each case matched precisely with the location of the only cutinase activity present in the gel (Figure 7). Cutinase II migrated slightly farther toward the cathode and bound tighter to SE-Sephadex than cutinase I, therefore cutinase II is more cationic than cutinase I.

Extent of Purification. The purification of PNP hydrolase from the extracellular fluid to apparent homogeneity involved a 34-fold purification (Table I). The purification procedures used resulted in substantial loss of total activity of this enzyme mainly at the QAE step. Only a 6.5-fold purification from the extracellular fluid resulted in apparently homogeneous cutinases. Since these cutinase isozymes are produced by the fungus in response to the presence of cutin as the sole carbon source, it is not surprising that the cutinases constitute a substantial portion of the proteins in the extracellular fluid. From 66 cultures containing a total of 33 g of cutin, 48 mg of cutinase I and 20 mg of cutinase II can be obtained using the procedures described. No attempt has been made to maximize the production of the cutinases or the PNP hydrolase.

Amino Acid Analysis. The pure cutinase isozymes and PNP hydrolase were hydrolyzed and their amino acid compositions were determined (Table II). There appears to be

Table I: Purification of Cutinase and PNP Hydrolase.

	% Total Activity		Specific Activity	
	Cutinase	PNP Hydrolase	Cutinase[a] (10^4 dpm/μg	PNP Hydrolase[b] (μmol/mg)
Extracellular fluid	100	100	1.2	11
0–50% $(NH_4)_2SO_4$ ppt	67	40	2.0	12
1st Sephadex G-100	61	26	4.1	17
QAE-Sephadex	36	5	7.6	123
2nd Sephadex G-100		5		340
SE-Sephadex	30(I) 13(II)		7.8	

[a] Assayed as described in text using tritiated cutin as substrate. [b] Assayed as described in text using *p*-nitrophenyl palmitate as substrate.

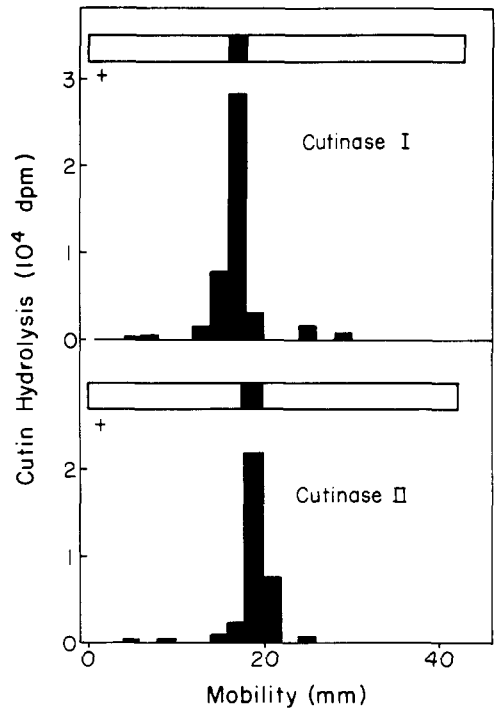

FIGURE 7: Disc gel electrophoresis of cutinase I and cutinase II obtained from SE-Sephadex step shown in Figure 6; 30 μg each of cutinase I and cutinase II were electrophoresed. The gels were stained or assayed for tritiated cutin hydrolysis as described under the Experimental Section.

little difference between the amino acid compositions of the two cutinases. The slightly higher cationic character of cutinase II with respect to cutinase I is not apparent in comparing the amino acid composition as both cutinases contain the same number of the basic amino acids. Even though both cutinases contain a similar number of acidic amino acids there might be significant difference between the two isozymes in the number of amide groups present in them. The number of amide groups has not been determined, but cutinase II routinely gave a greater amount of ammonia than cutinase I on acid hydrolysis. The significant difference between the two cutinases is in the higher number of tryptophan residues present in cutinase II. The amino acid composition of PNP hydrolase is significantly different from that of the cutinase isozymes. The main differences are in the content of alanine, valine, and phenylalanine. The amino acid composition of no other extracellular esterase appears to have been examined. However, the amino acid compositions of two extracellular lipases from the fungus *Mucor lipolyticus* and two from *Rhizopus arrhizor* have been reported (Nagoaka and Yamada, 1973; Benzonana, 1974), and they differ significantly from the amino acid compositions of cutinases and the PNP hydrolase from *F. solani pisi*. The lipases contain significantly fewer aliphatic amino acids than either the cutinases or the PNP hydrolase. The *Mucor* lipases had 34–35% aliphatic amino acids (Ala, Gly, Leu, Ile, and Val) and the *Rhizopus* lipases had 35–36% aliphatic amino acids, while the cutinase isozymes had 45–46%, and the PNP hydrolase had 41%.

Table II: Amino Acid Composition of Cutinase I, Cutinase II, and Nonspecific Esterase.[a]

	No. of Residues per Molecule[b]			Mole %		
Amino Acid	Cutinase I	Cutinase II	Nonspecific Esterase	Cutinase I	Cutinase II	Nonspecific Esterase
Asx	22 (20)	23 (18)	60	10.9 (11.1)	10.4 (10.2)	12.8
Thr	13 (11)	17 (11)	24	6.6 (6.3)	7.7 (6.5)	5.0
Ser	12 (9)	14 (10)	37	6.0 (5.3)	6.7 (5.5)	7.9
Glx	12 (11)	13 (10)	31	6.1 (6.3)	6.1 (5.9)	6.6
Pro	9 (10)	10 (9)	31	4.5 (5.7)	4.7 (5.1)	6.7
Gly	25 (22)	29 (23)	53	12.4 (12.4)	13.5 (13.0)	11.4
Ala	28 (23)	28 (22)	29	13.8 (13.3)	13.0 (12.3)	6.2
Cys	3 (3)	3 (3)		1.5 (1.7)	1.4 (1.7)	
Val	8 (8)	7 (7)	33	3.8 (4.3)	3.4 (3.7)	7.0
Met	1 (1)	1 (1)		0.5 (0.5)	0.4 (0.5)	
Ile	14 (14)	13 (13)	24	7.0 (7.7)	6.2 (7.2)	5.2
Leu	19 (16)	20 (20)	52	9.2 (9.2)	9.2 (11.4)	11.1
Tyr	7 (4)	5 (4)	16	3.4 (2.5)	2.4 (2.0)	3.5
Phe	7 (5)	7 (6)	32	3.4 (2.9)	3.4 (3.3)	6.8
Lys	6 (6)	6 (5)	23	3.0 (3.3)	3.0 (2.9)	4.9
His	2 (1)	2 (1)	4	0.8 (0.5)	0.8 (0.8)	0.8
Arg	13 (11)	13 (11)	19	6.4 (6.3)	6.0 (6.0)	4.0
Try	1 (1)	4 (4)		0.6 (0.6)	1.7 (2.1)	

[a] The values reported were determined after 24-hr treatment with 6 *M* HCl at 100°, except for values in parentheses which were determined after 48 hr. [b] The values were calculated assuming that there were three cysteine residues per molecule of cutinase I and II while PNP hydrolase was assumed to have four histidine residues per molecule.

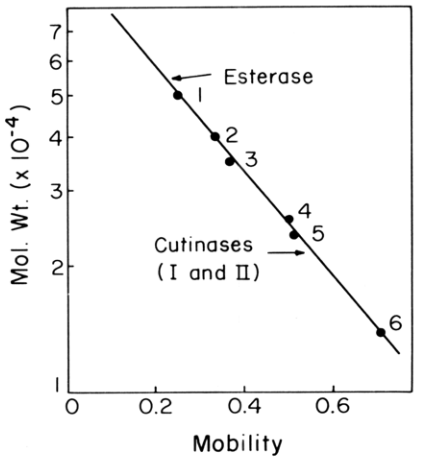

FIGURE 8: Molecular weight determination of cutinases I and II and PNP hydrolase with dodecyl sulfate gel electrophoresis in 10% gels. (1) Heavy chain of γ-globulin; (2) alcohol dehydrogenase; (3) pepsin; (4) chymotrypsinogen; (5) light chain of γ-globulin; (6) ribonuclease.

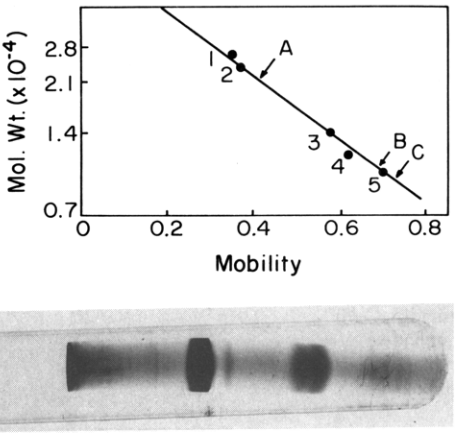

FIGURE 9: Molecular weight determination of cutinase I and cutinase II with dodecyl sulfate gel electrophoresis on 15% gels. (A) 21,800 band of cutinase I and cutinase II; (B) 10,600 band of cutinase II; (C) 9400 band of cutinase II; (1) chymotrypsinogen; (2) light chain of γ-globulin; (3) heavy chain of chymotrypsin; (4) cytochrome *c;* (5) light chain of chymotrypsin.

Table III: Molecular Weight of Cutinase I, Cutinase II, and PNP Hydrolase from *F. solani f. pisi.*

Methods	Molecular Weight		
	Cutinase I	Cutinase II	Esterase
Gel filtration	21,800	21,800	52,500
Sedimentation equilibrium	23,400	20,400	52,000
Dodecyl sulfate /electrophoresis	21,400	21,400	54,000
Amino acid analysis	21,200	22,400	50,300

Molecular Weight Determination. The molecular weight of each enzyme was determined on a calibrated Sephadex G-100 column. From the linear plots of $(V_e - V_0)/(V_t - V_0)$ vs. log molecular weight the apparent molecular weight for a mixture of cutinase I and cutinase II from the QAE-Sephadex was found to be 21,800. In comparison to these results, the sedimentation equilibrium method of Yphantis gave a value of 23,400 for cutinase I, and 20,400 for cutinase II (Table III). The partial specific volumes used in the computation of these results were obtained from the amino acid composition and they were 0.730 for cutinase I, and 0.727 for cutinase II. Molecular weight of each enzyme was also calculated from the amino acid composition. For this calculation it was assumed that there are three cysteine residues per molecule for cutinase I and cutinase II. Molecular weights of 21,200 for cutinase I and 22,400 for cutinase II were obtained. These values should be taken as approximate values since the calculations were based on amino acids of low concentrations. However, these values agree very closely with those obtained from the Sephadex G-100 gel filtration and sedimentation equilibrium techniques (Table III). Also in agreement are the molecular weight values obtained by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. This method gave molecular weights of 21,400 for both cutinase I and cutinase II (Figure 8). Cutinase II also showed other minor bands when subjected to dodecyl sulfate gel electrophoresis (Figure 9). Cutinases I and II were treated with 1% dodecyl sulfate and 1% mercaptoethanol at 50 and 70° for 12 hr in order to be sure that the proteins dissociated into single polypeptide chains. Cutinase I showed only one polypeptide with a molecular weight of 21,400. Cutinase II differs from cutinase I in that it gave, in addition to a major band at 21,400, two minor bands corresponding to smaller molecular weight values (Figure 9). To determine the molecular weights of the small peptides, 15% gels were used and molecular weights of 10,600 and 9400 (Figure 9, B and C) were obtained. Since the sum of these values is quite close to the molecular weight of the major band, the two small bands probably represent fragments produced by a proteolytic clip in the major peptide. A similar, apparent proleolytic clip has been reported for an extracellular lipase (Benzonana, 1974). The observation that all of the fractionation techniques used in the purification of cutinase II failed to remove these fragments from the major peptide strongly suggests that the two fragments were held together. The two fragments are presumably held together by a disulfide bridge (s) similar to that found in chymotrypsin. Since treatment of cutinase II with dodecyl sulfate and mercaptoethanol or dithioerythritol (0.1 *M*) under harsher conditions (100°, 1 hr) did not show any decrease in the major band, or any increase in the minor bands, it appears that only a small fraction of the enzyme contains the proteolytic clip. The clipped molecules appear to be enzymatically active as the 10,600 fragment reacted with radioactive diisopropyl fluorophosphate (Purdy and Kolattukudy, 1975).

The molecular weight of PNP hydrolase was found to be 52,500 according the Sephadex G-100 gel filtration technique. The sedimentation equilibrium method gave a value of 52,500 using the partial specific volume of 0.738 which was calculated from the results of the amino acid analysis. Assuming that there are four residues of histidine per molecule of PNP hydrolase, a molecular weight of 50,300 was calculated from the amino acid composition. This value is only an approximate one, as values for the sulfur containing amino acids and tryptophan were not obtained because sufficient quantities of this protein were not available. However, the molecular weight calculated is in fairly good agreement with those obtained by the other techniques (Table III). Treatment of the PNP hydrolase with mercaptoethanol and dodecyl sulfate followed by dodecyl sulfate polyacrylamide gel electrophoresis showed a major protein band corresponding to a molecular weight of 54,000. A

minor band was observed at 50,000. Treatment of PNP hydrolase with 1% dodecyl sulfate and 50 m*M* dithioerythritol at 100° for 1 hr also gave an identical pattern. Since disc gel electrophoresis gave only one band, the results of the dodecyl sulfate gel electrophoresis indicate that the enzyme is a mixture of one major peptide (54,000) and a minor peptide (50,000). The smaller peptide might have been derived from a small amount of the enzyme which might have contained a proteolytic clip, such as that suggested to be present in cutinase II. If such was the case, the small amount of the smaller fragment might have gone undetected. It is also possible that the 50,000 moiety was present with the 54,000 moiety and the other fractionation techniques failed to separate them. Since these two moieties would be very similar in their properties they would have been copurified. In any case, both the 54,000 moiety and the 50,000 moiety were apparently enzymatically active as radioactive diisopropyl fluorophosphate labeled both of them (Purdy and Kolattukudy, 1975).

The results discussed above show that the molecular weight of the cutinases is about 22,000 and that of PNP hydrolase is about 52,000. The molecular weight of PNP hydrolase is within the size range of the mammalian liver microsomal esterases, which vary from 45,000 to 70,000 depending upon the conditions of isolation (Haugen and Suttie, 1974; Ljungquist and Augustinson, 1971; Krisch, 1971), although the mammalian enzyme tends to aggregate (Aune, 1973). The size of PNP hydrolase from *F. solani pisi* is fairly close to that of an intracellular esterase isolated from *Bacillus stearothermophilus* (Matsunaga et al. 1974), an esterase isolated from the midgut of cockroach (Hipps and Nelson, 1974), and a glycerol ester hydrolase isolated from *Corynebacteruim acnes* (Fulton et al., 1974). The molecular weights of the extracellular lipases thus far isolated from fungi (Nagaoka and Yamada, 1973; Benzonana, 1974) are not similar to those obtained for the cutinases or the PNP hydrolase of *F. solani pisi*. The amino acid composition of the enzymes from *Fusarium* are quite different from those of the fungal extracellular lipases. Furthermore, no extracellular esterase which could be similar to the cutinases appears to have been reported. Thus, it is unlikely that some esterase which has been previously isolated might have been a true cutinase. However, it should be pointed out that the possibility of proteolytic modification of extracellular enzymes should be carefully considered in comparing the molecular size of such enzymes. In any case, the first isolation and purification of the cutinases reported in this paper provide an opportunity to investigate the role of such enzymes in pathogenesis.

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