Purification and Characterization of Cutinase from *Venturia inaequalis*

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ABSTRACT

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Venturia inaequalis was grown in a culture medium containing purified apple cutin as the sole carbon source. After 8 wk of growth an esterase was isolated from the culture fluid and purified to apparent homogeneity. The enzyme hydrolyzed tritiated cutin and thus was identified as cutinase. The purified cutinase is a glycoprotein with a molecular mass of 21–23 kg/mol, as determined by various procedures. Remarkable structural features are a high content of glycine, a high content of nonpolar amino acids, two disulfide bridges, and a high degree of hydrophobicity. Cutin hydrolysis by cutinase from *V. inaequalis* is optimal at a pH of 6 and thus different from the alkaline pH-optimum reported for other purified cutinases. The hydrolysis of the model ester *p*-nitrophenyl butyrate was less affected by the pH. The esterase activity was strongly inhibited by diisopropyl fluorophosphate, and the phosphorylation of one serine was sufficient for complete inhibition. Thus, cutinase from *V. inaequalis* belongs to the class of serine hydrolases, a characterisitic shared with other fungal cutinases.

Plant-pathogenic fungi gain access into the aerial parts of plants by penetration of unwounded tissue (1). Some pathogens such as rust fungi invade the host via stomata (11), whereas others penetrate the intact leaf surface without regard to natural openings (1). The latter type encounters the plant cuticle as the first defensive barrier. The mechanism by which pathogens breach this barrier has been debated for almost a century. Penetration was long thought to be mechanical (43), but the involvement of cutinolytic enzymes secreted by the invading pathogens has often been postulated (39).

Venturia inaequalis (Cooke) Winter, the causal agent of apple scab, exemplifies this controversy. Infection structures originating from ascospores or conidia gain entrance through the intact cuticle (9,32), a step even accomplished in the interaction with apple cultivars resistant to scab (38). Whether the breaching of this first barrier is achieved by mechanical force or the enzymatic degradation of the cuticle has been debated for more than 70 years. Wiltshire (41) reported in 1915 that scab hyphae grew in the cuticle and that the cuticle above these hyphae was always thinner. This was taken as evidence for cuticle degradation; however, Nusbaum and Keitt could not confirm this observation (28). Furthermore, the fungitoxic action of fatty acids derived from hydrolyzed cutin was taken as evidence against an enzymatic penetration of the cuticle. It was speculated that these fatty acids, once liberated by the action of the enzyme, would suppress the growth of the invading scab fungus (25). However, transitory esterase activity detectable only during the early stage of infection (27) and the presence of cutin monomers in the culture media of *V. inaequalis* grown on cutin as the carbon source (35) provided the first circumstantial evidence for the involvement of cutinase in the penetration of apple leaves.

Conclusive proof for a decisive role of cutinase in plant infection would require the isolation and characterization of cutinase from a particular fungal pathogen, the demonstration that specific inhibition of cutinase activity (chemical inhibitors or monospecific antibodies) prevents penetration, and the isolation of cutinase-deficient strains that lack virulence on intact plants. This approach has been employed with two host-pathogen systems, namely *Fusarium solani* f. sp. *pisi* on pea epicotyls and *Colletotrichum gloeosporioides* on papaya fruits (12,14–17). Here, we report the isolation, purification, and characterization of cutinase from *V. inaequalis* grown on apple cutin as the sole carbon source. This report is the first step in elucidating the involvement of cutinase in the infection of apples by the scab fungus.

MATERIALS AND METHODS

Materials. Cutin from apple peels (cultivar mix) was isolated and purified according to Walton and Kolattukudy (40). [^{3}H]-Sodium borohydride (11.8 GBq mmol^{-1}) and [1,3-^{3}H]-diisopropylfluorophosphate (162.8 GBq mmol^{-1}) were obtained from NEN Research Products. QAE disks (Zetachrome 60) were purchased from Cuno, Inc. All other column materials and chemicals were from Sigma Chemical Company.

Culture conditions. *V. inaequalis*, strain Maine 8 (+), was grown and maintained on potato-dextrose agar at 20 C (36). Fungal material from six colonies (approximately 2 cm in diameter; mostly mycelium) was homogenized in a Potter-Elvehjem tissue grinder (10 ml of H_2O). The suspension was transferred to 50 roux bottles each containing 100 ml of Czapek's medium supplemented with 0.1% $CaCO_3$ and 1.5 g of powdered apple cutin as the sole carbon source (35). The cultures were incubated at 20 C for 8 wk.

Enzyme assays. Esterase activity was determined as previously described (23) with *p*-nitrophenyl butyrate (PNB) as the model substrate. Enzyme activity was determined at 400 nm with a Uvikon 860 spectrophotometer. The molar extinction coefficient used for the calculation of enzyme activity (ϵ = 16,290 $M^{-1}cm^{-1}$) was determined with *p*-nitrophenol under assay conditions. For assays of esterase activities at various pH values, only the buffers were different: MES (2[N-morpholino] ethanesulfonic acid) for pH 5 and 6, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) for pH 7 and 8, and Tris (tris[hydroxymethyl] amino methane) for pH 9. Molar extinction coefficients were determined for each pH separately (ϵ = 170 $M^{-1}cm^{-1}$ for pH 5; 930 $M^{-1}cm^{-1}$ for pH 6; 6,830 $M^{-1}cm^{-1}$ for pH 7; 15,740 $M^{-1}cm^{-1}$ for pH 8; 19,280 $M^{-1}cm^{-1}$ for pH 9).

Cutinase activity was assayed with tritiated grapefruit cutin according to published procedures with slight modifications (19). In brief, cutin was isolated and purified from grapefruits as described elsewhere (7). Cutin powder (3 g) was suspended in 10 mM NaOH (50 ml) and treated with 925 MBq [^{3}H]-$NaBH_4$ at 20 C for 48 hr. The purification of the labeled cutin and the cutinase enzyme assay was done as described before (19), except that 6 mg of cutin was used per assay, and the assay buffer was 100 mM MES, pH 6.2. Buffers used at various pH values were 100 mM MES-NaOH (pH 5 and 6), 100 mM HEPES-NaOH (pH 7 and 8), and 100 mM glycine-NaOH (pH 9 and 10). Cutinase assays were

run in triplicates for 1 hr at 30 C with stirring. Cutinase activity is expressed as cutinase units (cu; kBq released from labeled cutin/min).

The hydrolysis products released by the action of cutinase (35 mg of labeled cutin; 6 ml of 100 mM MES pH 6.2; 3 μg of purified cutinase; cutin hydrolysis for 2 hr at 30 C) were analyzed by thin-layer chromatography (TLC) (Silica Gel 60; Merck 5610). The plates were developed with diethyl ether/acetone/formic acid (95:5:1, v/v/v) as the solvent (42). The radioactivity distribution was analyzed by proportional counting (31) with a Berthold 2842 TLC Linear Analyzer equipped with a Data Acquisition System.

Enzyme purification. Cultures of *V. inaequalis* grown on apple cutin were filtered twice through filter paper (Whatman Qualitative Filter Paper No. 4 and No. 1). The pH of the filtrate was adjusted to 8.5 with a concentrated solution of Tris. The crude solution was pumped through a QAE-disk (5 ml min^{-1} flow rate) equilibrated with 100 mM Tris-HCl, pH 8.5. The eluant was lyophilized; the residue was dissolved in 10 mM Tris-HCl, pH 8.5 containing 10 mM ascorbic acid and dialyzed overnight against the same buffer (3 L; one buffer change after 8 hr). Proteins in the dialyzed solution were precipitated with acetone (75% [v/v]). The precipitate was resuspended in 6 ml of citrate buffer (5 mM, pH 4.8), clarified by centrifugation, and dialyzed overnight against the same buffer.

The concentrated protein solution (15 ml) was applied to a S-Sepharose column (1.5 × 40 cm) equilibrated with 5 mM citrate buffer, pH 4.8. Cutinase was eluted with the same buffer (1 ml min^{-1} flow rate; 3-ml fractions). Additional proteins lacking esterase activity were eluted with a salt gradient (400 ml; 0–250 mM KCl). The fractions possessing esterase activity were pooled and applied to a Phenyl-Sepharose column (1 × 15 cm), which had been equilibrated with 10 mM sodium phosphate, pH 7.5. The proteins were eluted with two bed volumes of the same buffer, followed by an isopropanol gradient ranging from 0 to 55% (v/v) in 10 mM sodium phosphate, pH 7.5 (60 ml; 3 ml hr^{-1} flow rate; 1-ml fractions). The gradient was followed by an isopropanol solution representing the highest concentration of the gradient.

Identification of an active serine. Purified cutinase (128 μg) in 0.1 ml of phosphate buffer (10 mM, pH 7.5) containing 60% (v/v) isopropanol was diluted with 0.1 ml of phosphate buffer (0.1 M, pH 8; 30% [v/v] isopropanol) and incubated with 102 nmol of diisopropyl fluorophosphate (DFP). The radioactive DFP was 51-fold diluted with nonlabeled inhibitor before incubation with cutinase. After 2 hr of treatment at 23 C the enzyme solution was applied to a column of Sephadex G-10 (0.5 × 8 cm) equilibrated with 50 mM phosphate buffer (pH 7.5) containing 20% (v/v) isopropanol, and labeled cutinase was separated from free DFP by elution with the same buffer (8 ml hr^{-1} flow rate; 0.15-ml fractions).

Other methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was employed to determine the homogeneity of the enzyme preparation and the molecular mass of cutinase. Electrophoresis (Bio-Rad Protean II slab electrophoresis cell) was done with 14% polyacrylamide gels according to Köller et al (22). Protein standards were bovine serum albumin (66 kg mol^{-1}), ovalbumin (45 kg mol^{-1}), glyceraldehyde-3-phosphate dehydrogenase (36 kg mol^{-1}), carbonic anhydrase (31 kg mol^{-1}), trypsinogen (24 kg mol^{-1}), trypsin inhibitor (20.1 kg mol^{-1}) and α-lactalbumin (14.2 kg mol^{-1}). Proteins were stained with Coomasie Blue R-250.

The molecular mass of native cutinase was determined by gel permeation chromatography (2). Cutinase (30 μg) and protein standards (1 mg each) were dissolved in 0.5 ml of 50 mM phosphate, pH 7.5, containing 10% (v/v) isopropanol and 5% (v/v) glycerol. The protein mixture was applied to a column of Sephadex G-100-50 (1.5 × 40 cm; 2 ml hr^{-1} flow rate; 0.5-ml fractions) equilibrated with the same buffer. Standards were blue dextran (V_0), bovine serum albumin (66 kg mol^{-1}), carbonic anhydrase (31 kg mol^{-1}), cytochrome c (12.4 kg mol^{-1}), and aprotinin (6.5 kg mol^{-1}).

Amino acid analysis was performed by reversed-phase high-performance liquid chromatography with phenylisocyanate precolumn derivatization (10) after hydrolysis with 6N HCl. The recovery of amino acid residues was determined by comparison with the number of residues present in ribonuclease A after hydrolysis and amino acid analysis under identical conditions.

Protein content was determined by using bicinchoninic acid (33) with bovine serum albumin as the protein standard. The carbohydrate content was estimated by the sulfuric-acid method (6) with glucose as the standard. Radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 4640). Absolute radioactivity was calculated by a calibration program based on the channels ratio method.

RESULTS

Enzyme purification. The Maine 8 isolate of *V. inaequalis* (36) was identified as the most efficient esterase producer among 17 different isolates grown on apple cutin. After 8 wk of growth, the esterase activity in the culture media from different isolates ranged from 80 to 0.8 nkat ml^{-1} when assayed with PNB. These large differences in esterase activity were not necessarily related to the amount of fungal mycelium. The lower enzyme levels observed in various cultures are most likely still sufficient to fully support the slow fungal growth from fatty acids derived from hydrolyzed cutin. Inhibition of fungal growth by cutin monomers, as suggested by Martin (25), was not observed.

The culture filtrate obtained from the Maine 8 isolate after 8 wk of growth on purified cutin was filtered through a solid anion exchange support. Brown phenolic material produced by all fungal cultures grown on apple cutin (18) was efficiently removed by this procedure. Material with a slight yellowish color, however, was not retained on the column and thus remained present in the enzyme preparation. The material greatly interfered with protein assays subsequent to the concentration step. It caused positive readings in the protein assay, was not precipitated with trichloroacetic acid (TCA), and prevented the TCA precipitation of proteins present in the concentrated solution. Thus, the protein content given in Table 1 for this purification step reflects both protein and accompanying contaminant. The esterase and the contaminant were not bound to S-Sepharose and eluted at the void volume. Two additional proteins lacking esterase activity were eluted with a linear salt gradient. A reliable determination of the protein content in the pooled fractions containing the esterase activity was again hampered by the accompanying material. Therefore, this step was omitted from Table 1.

The final purification of the esterase was accomplished by hydrophobic chromatography on Phenyl-Sepharose. The colored contaminating material with a strong absorbance at 280 nm was not retained and eluted at the void volume of the column. The esterase was eluted at the end of an isopropanol gradient (Fig. 1). The enzyme was stable under these conditions. A decrease of the isopropanol concentration to values lower than 40% led to a reversible precipitation of the enzyme at higher protein concentrations, once the accompanying material was removed. This observation together with high concentration of detergents (0.5% Triton X-100 or 5 mM octyl glucoside) required to elute the enzyme from Phenyl-Sepharose indicates a high degree of hydrophobicity.

TABLE 1. Purification of cutinase from the culture fluid of *Venturia inaequalis*

			Total activity		Specific activity		
						PNBase	
Step	Volume (ml)	Protein (mg)	Cutinase (cu)[a]	PNBase (μkat)	Cutinase (cu/mg)	(μkat/mg)	Ratio[b]
Culture fluid	3,360	47.0[c]	408.2	273.0	8.69	5.81	1.50
QAE-step[d]	15	77.4	248.6	239.8	3.21	3.10	1.04
Phenyl-Sepharose	9.6	5.87	119.6	133.5	20.41	21.74	0.96

[a] Definition of cutinase unit (cu) is given in Materials and Methods.
[b] Cutinase/PNBase.
[c] After precipitation with trichloroacetic acid.
[d] After QAE-filtration, freeze drying, acetone precipitation, and dialysis.

The purity of the enzyme was examined by SDS-PAGE. Only one protein band was visible when a high amount of protein (0.1 mg) was applied to the gel, indicating apparent homogeneity of the enzyme (Fig. 2).

The increase of the specific PNBase activity showed a 2.3-fold purification of the enzyme. Thus, almost half of the protein excreted by *V. inaequalis* when grown on cutin could be attributed to this enzyme. This high proportion was in good agreement with the peptide pattern obtained after SDS-PAGE.

Cutin hydrolysis. The radioactive cutin used to assay cutinase activity was prepared by chemical reduction of grapefruit cutin (19). This cutin is unique with respect to the presence of midchain oxo groups (7,8), which are selectively reduced by $NaBH_4$ (8,19). The chromatographic analysis of the products enzymatically released from reductively labeled cutin is shown in Figure 3. The bulk of the radioactivity (60%) migrated with an R_f corresponding to trihydroxyfatty acids (42). This major labeled component was expected and derived from 9,16-dihydroxy-10-oxo-hexadecanoic acid, a novel cutin monomer (8). The minor cutin component is particularly prone to reduction with $NaBH_4$ due to the presence of an activated oxo group (8,19). Less radioactivity (35%) migrated to the position of dihydroxy acids. This component originated from the reduction of 16-hydroxy-10-oxo-hexadecanoic acid and positional isomers, the major component present in grapefruit cutin (7,19). The result clearly indicates that the radioactivity enzymatically released from tritiated cutin corresponds to the labeled cutin monomers present in grapefruit cutin. This cutin hydrolyzing activity of the purified esterase from *V. inaequalis* provides clear evidence for the cutinase nature of the enzyme.

pH Dependency of hydrolase activities. The pH dependency of the hydrolytic activity of *V. inaequalis* cutinase was evaluated with [^{3}H]-cutin as the natural substrate, and PNB as a model substrate. The cutinolytic activity was highest at slightly acidic pH, with an optimum close to pH 6 (Fig. 4). The rate of cutin hydrolysis was

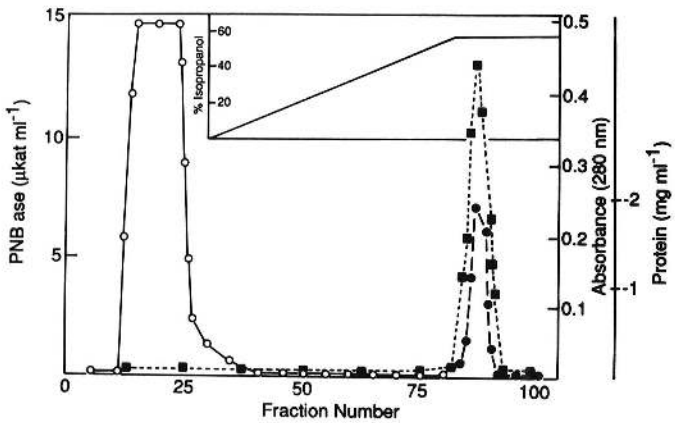

Fig. 1. Phenyl-Sepharose hydrophobic chromatography of the pooled esterase fractions obtained after the S-Sepharose step. The absorbance at 280 nm (o—o) or the protein concentration (•—•), and the PNBase activity (■----■) were monitored.

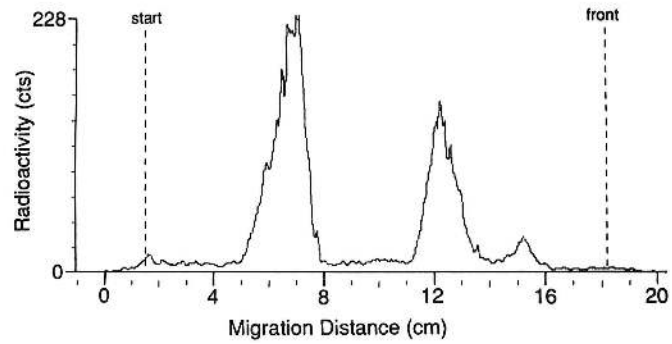

Fig. 3. Distribution of radioactivity after chromatography of fatty acids derived from radiolabeled grapefruit cutin after hydrolysis with cutinase from *Venturia inaequalis*. Radioactivity is expressed as accumulated counts (cts).

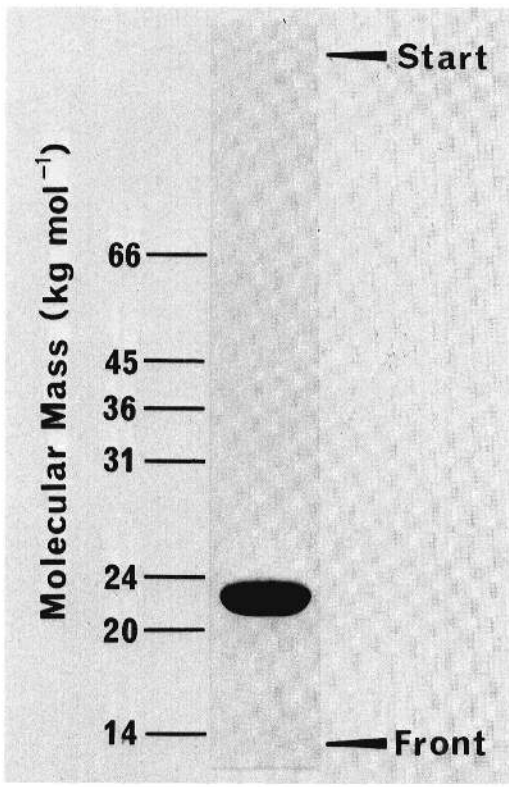

Fig. 2. Sodium dodecyl sulfate polyacrylamide (14%) gel electrophoresis of cutinase from *Venturia inaequalis* obtained after hydrophobic chromatography on Phenyl-Sepharose. 0.1 mg of protein was applied.

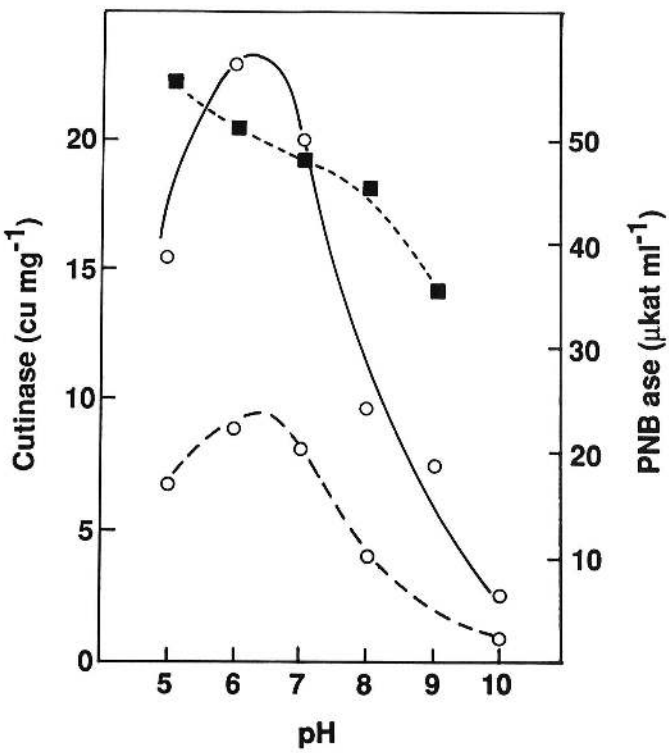

Fig. 4. Effect of pH on the hydrolytic activities of *Venturia inaequalis* cutinase. Cutin hydrolysis by the purified enzyme (o—o) or the culture fluid of *V. inaequalis* after growth on cutin (o----o), and the hydrolysis of the model substrate PNB (■—■) was determined.

low at pH 8 or above. Both homogeneous cutinase and the crude enzyme preparation showed similar pH dependency. The PNBase activity was only slightly influenced by the pH (Fig. 4). It was highest at pH 5, almost constant from pH 6 to 8, and lowest at pH 9. Assays with PNB as the substrate at pH-values lower than 5 (the molar extinction coefficient is too low) and higher than 9 (nonenzymatic hydrolysis is too high) are not feasible.

The purification factor of cutinase as judged from the specific enzyme activity with cutin as the natural substrate under optimal conditions is 3.7 and thus higher than indicated by the PNBase activity (Table 1). Comparison of both activities (Table 1) shows that an apparent decrease of specific activity occurred subsequent to the removal of the brown phenolic material and the dialysis of the concentrated enzyme preparation. Thus, the culture fluid appears to contain a compound with slight activating properties, which is removed during the purification of the enzyme.

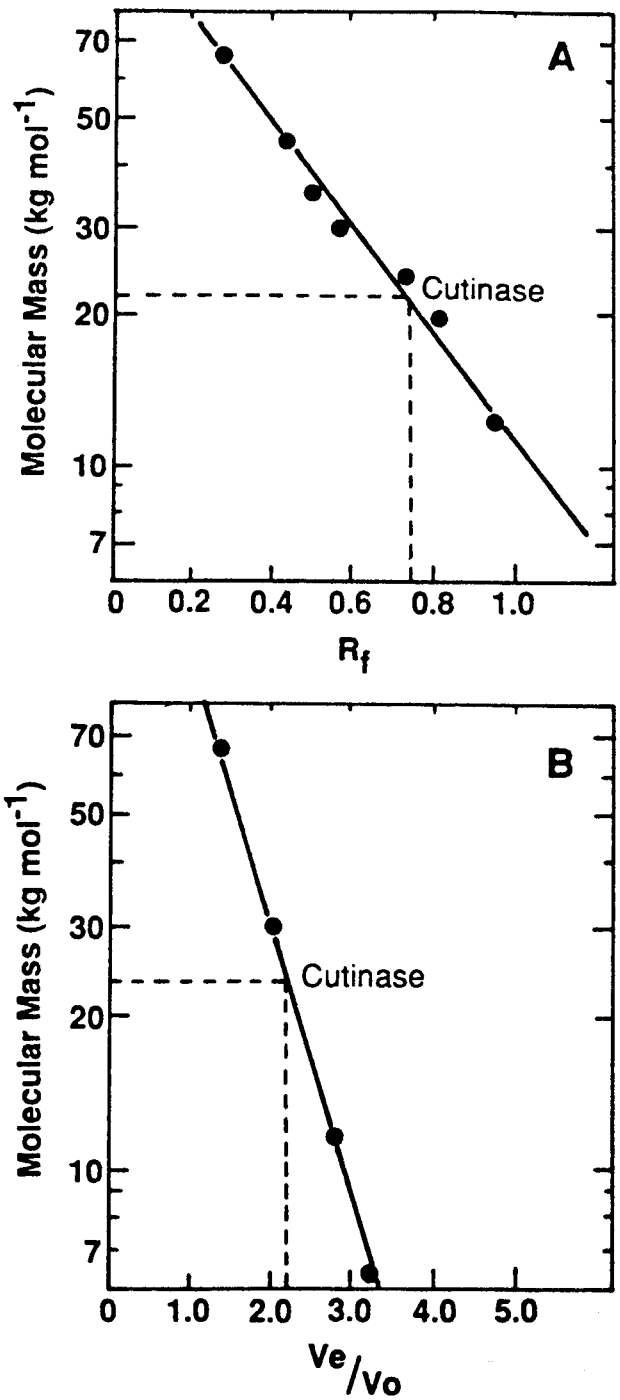

Fig. 5. Molecular mass of cutinase from *Venturia inaequalis* as determined by, **A**, SDS polyacrylamide gel electrophoresis and, **B**, gel permeation chromatography.

Structural properties of cutinase. The molecular mass of cutinase, as determined by SDS-PAGE and gel permeation chromatography, was 21,700 and 23,000 g/mol, respectively (Fig. 5). The pure enzyme contained 5.4% carbohydrate. The molecular mass calculated from the amino acid composition was 20,900 g/mol, including the carbohydrate portion. Isoenzymes reported for cutinases derived from *Fusarium* spp. (24) were not detected. Only one peak was eluted from a SP-Sephadex column loaded with pure cutinase when a salt gradient was applied (data not shown).

The high hydrophobicity of cutinase from *V. inaequalis* described above was not reflected by an increase of hydrophobic amino acids (e.g., valine, leucine, isoleucine, or phenylalanine) as compared to other cutinases (Table 2). However, the number of polar amino acids (arginine, proline, and tyrosine) is proportionally decreased, and the glycine content is considerably higher than the values previously reported (Table 2).

Identification of an active serine. Treatment of 128 μg of purified cutinase with 102 nmol diisopropyl fluorophosphate resulted in almost complete inhibition (90%) of the PNBase activity. The amount of inhibitor used represents a 17-fold molar excess over cutinase based on a molecular mass of 21,500 g mol^{-1}. The number of residues phosphorylated by DFP, a chemical probe for active serines present in serine hydrolases, was calculated after the removal of free DFP from the inhibitor-treated cutinase (Fig. 6). The amount of radioactivity bound to the enzyme and recovered in the peak fraction (3.29 kBq) indicated that 1.02 nmol of inhibitor was bound to 1 nmol (21.5 μg) of cutinase. Thus, the phosphorylation of one active serine residue per cutinase molecule led to full inhibition of the enzyme activity.

DISCUSSION

The results presented in this paper demonstrate that cutinase can be readily isolated and purified from cultures of *V. inaequalis* grown on purified apple cutin. The enzyme shows both similarities and dissimilarities to cutinases purified from other fungal sources. Cutinase from *V. inaequalis* is, like all other cutinases, composed of one polypeptide with a molecular mass of approximately 22,000 g/mol. The values previously reported range from 22,000 to 26,000 g/mol (14–17). The enzyme from *V. inaequalis* was found to be a glycoprotein with a carbohydrate content of 5.4% that compares well with other cutinases (14–17). Only for cutinase purified from *C. gloeosporioides* has a higher carbohydrate content been

TABLE 2. Amino acid composition of fungal cutinases

Residue	*Venturia inaequalis*	*Colletotrichum gloeosporioides*[a]	*Helminthosporum sativum*[a]	*Fusarium solani (B)*[a]	*Fusarium culmorum*[a]
Aspartic acid	18	18	26	24	21
Glutamic acid	12	17	14	15	16
Serine	16	17	17	20	21
Glycine	42	23	24	27	26
Histidine	2	1	2	1	2
Arginine	6	10	8	14	11
Threonine	12	15	20	10	13
Alanine	20	31	27	23	34
Proline	8	13	12	11	10
Tyrosine	4	11	7	6	9
Valine	14	11	17	12	13
Methionine	2	2	1	1	1
Cysteine[b]	4	2	2	2	4
Isoleucine	10	14	14	9	17
Leucine	10	23	20	18	23
Phenylalanine	8	11	13	6	6
Lysine	8	9	9	5	6
Tryptophan	ND[c]	1	ND	1	1

[a] Data taken from Kolattukudy (14).
[b] One or two disulfide bridges.
[c] Not determined.

determined (5). The amino acid composition shows an increased number of glycine residues and a higher percentage of nonpolar amino acids than previously reported. This difference in amino acid composition explains the relatively high hydrophobicity of the purified enzyme. However, common structural elements such as 2 histidine, 2 methionine, or 4 cysteine residues in disulfide linkage (14–17) are well preserved in the enzyme (Table 2).

The pH dependency of both hydrolytic activities, the hydrolysis of cutin and PNB, is different from cutinases investigated so far. Cutin hydrolysis by cutinases isolated from *F. solani* f. sp. *pisi* (29) and *F. roseum culmorum* (34) was optimal at an alkaline pH 10, with very little activity at pH 6. The cutinolytic activity of a crude preparation derived from *Rhizoctonia solani* Kühn was also highest at alkaline pH (3). A slightly acidic pH was determined to be optimal for the *V. inaequalis* cutinase described in this report. This pH optimum is very similar to the value reported for a crude cutinase preparation isolated from *Botrytis cinerea* (30). Cutinases from *F. roseum sambucinum*, *Helminthosporium sativum*, *Ulocladium consortiale* (24), and *C. gloeosporioides* (5) were purified previously and assayed for cutinase activity with radioactive cutin as the substrate. Unfortunately, the enzyme assays were only done at alkaline pH in analogy to the enzyme from *F. solani*, and the pH optima remain unknown. The optimal hydrolyzing activity at a slightly acidic pH observed for cutinase from *V. inaequalis* closely corresponds to the environmental conditions encountered by a penetrating pathogen on a moist leaf surface, and both pathogens with cutinases most active at acidic pH are leaf pathogens. A pH optimum around 10, as demonstrated with cutinases from *Fusarium* spp., might require an additional factor that adjusts for environmental conditions more suitable for cutinase action. Interestingly, the cutinases with alkaline pH optima were all derived from pathogens (*Fusarium* spp. and *R. solani*) infecting roots and stem bases. Comparative studies of other pathogens are required to substantiate this potential difference between pathogens that infect either leaves or roots.

A clear pH optimum for the hydrolysis of PNB at pH 10 or 8.5 was reported for cutinase from *C. gloeosporioides* (5) and *B. cinerea* (30), respectively. No typical pH optimum could be observed for the enzyme from *V. inaequalis*. The activity was relatively uniform over a wide range. A similar pattern has been reported for cutinase from *F. solani* when assayed in the absence of detergents (13). However, it should be pointed out that the extinction coefficient of *p*-nitrophenol is strongly dependent on the pH, and calculations of enzyme activities have to be based on the molar extinction coefficient at the particular pH employed. Apparently, these restrictions were not considered for the cutinases reported to exhibit a pH optimum in the alkaline range (5, 30). Corresponding corrections of these data indicate that the relatively small effect of the pH on PNB hydrolysis might well be a common but unique feature of cutinases. This phenomenon could be related to a conformational change of the active site observed after binding of SDS to the enzyme (13). A similar conformational change might also occur after binding of cutinase to the insoluble polymer cutin, and differences between the monomeric model substrate PNB and the polymeric substrate cutin would not be surprising.

The catalytic properties of cutinase purified from *F. s. pisi* have been investigated in great detail. The enzyme mechanism of ester bond cleavage was shown to depend on a catalytic triad consisting of one active serine, one histidine, and one carboxylic acid residue, a characteristic feature found in the class of serine hydrolases (23). The presence of the active serine residue, as indicated by the active serine probe DFP, was confirmed for all fungal cutinases investigated so far (12,14–17). This common feature is preserved in the cutinase isolated from *V. inaequalis*. The esterase activity of the pure cutinase was inhibited by the binding of 1 mol of DFP to 1 mol of the enzyme.

The availability of purified cutinase from *V. inaequalis* and the identification of an active serine residue involved in ester bond cleavage is a necessary step toward future goals, namely to clarify the role of cutin hydrolysis in infection of apples by scab, and to evaluate and refine the design of cutinase inhibitors with activity as a specific protectant compound useful in the management of apple scab. The idea of assigning cutinases considerable potential as a target for antipenetrants in plant protection has been successfully evaluated in the past (4,19,20). One of the organophosphorus esterase inhibitors used in these initial studies, diethyl trichloropyridyl phosphate, was shown to act both as a highly efficient inhibitor of cutinases derived from *F. solani* and *C. gloeosporioides* and as an antipenetrant protecting plants from disease (4,21). The compound was also reported to show activity as a protectant of apples from scab infection (12). More recently, the potency of this inhibitor has been successfully optimized by a rational inhibitor design (26). A modern and specific scab fungicide with a protective action would be highly desirable, since many of the sterol biosynthesis inhibitors currently in use for scab control lack sufficient protective activity (37).

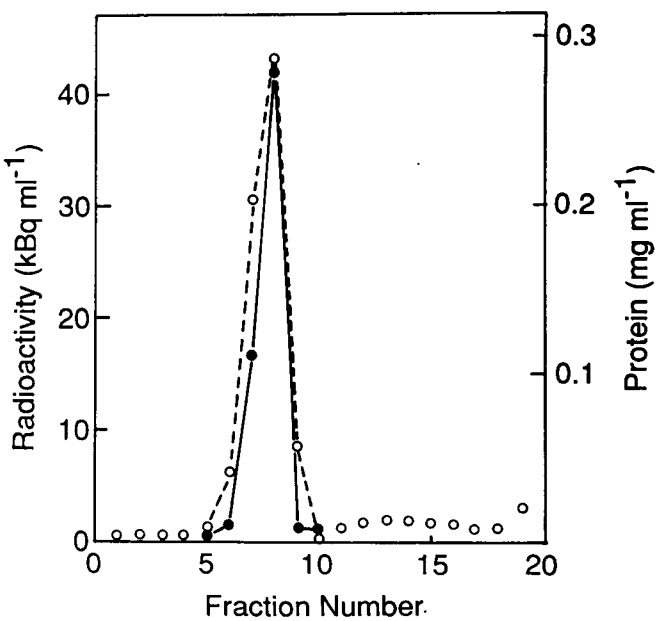

Fig. 6. Gel permeation chromatography of *Venturia inaequalis* cutinase after inhibition with [^{3}H]-diisopropyl fluorophosphate. Radioactivity (•—•) and protein content (o----o) were monitored.

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