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Affinity Purification and Characterization of a Cutinase from the Fungal Plant Pathogen *Monilinia fructicola* (Wint.) Honey

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Trifluoromethyl ketones (TFK) are potent inhibitors of a variety of serine hydrolases. The TFK inhibitor, 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone (MBTFP), was found to competitively inhibit cutinase activity ($I_{50} = 9.4 \times 10^{-3}$) from the fungal plant pathogen *Monilinia fructicola* and to serve as an effective affinity ligand for the purification of cutinases from culture filtrates. The TFK inhibitors, 3-*n*-octylthio-1,1,1-trifluoro-2-propanone (OTFP) and 3-*n*-pentylthio-1,1,1-trifluoro-2-propanone (PTFP), also inhibited cutinase activity with I_{50} values of 1.6×10^{-6} and 2.3×10^{-4} M, respectively. Buffer containing OTFP was the strongest eluant for cutinases of *M. fructicola* and provided the best purification factor and yield, although buffers containing OTFP, detergent, and salt were found to be effective for eluting cutinases bound to MBTFP-Sepharose. Buffer containing 0.5% Triton X-100 also selectively eluted cutinases from the affinity column. Two-dimensional electrophoretic analysis by SDS-PAGE and isoelectric focusing of the affinity-purified cutinase fraction indicated activity associated with proteins of p*I* 8.2 and molecular masses of approximately 18.6 and 20.8 kDa. These proteins hydrolyzed [^{3}H]cutin and artificial substrates such as *p*-nitrophenylbutyrate and related esters, typical of other cutinases, but differ from previously characterized cutinases in molecular mass. The two low-molecular-weight proteins resolved by 2-D gel electrophoresis were subjected to in-gel digestion with Lys-C and the resulting peptide fragments were separated by Microbore-HPLC. The amino acid sequences of several internal peptide fragments had high homology with cutinase sequences from other fungi, particularly the plant pathogen *Botrytis cinerea*. Our study illustrates the potential of TFK ligands for the affinity purification of cutinases and indicates that the cutinases from *M. fructicola* have novel features warranting further study.

Key Words: **affinity purification; cutinase; *Monilinia fructicola;* serine hydrolases; trifluoromethyl ketone.**

Cutinases are extracellular fungal enzymes that hydrolyze cutin, an insoluble lipid-polyester that forms a major component of the plant cuticle (1, 2). Although their function in plant pathogenesis is unresolved, cutinases have been shown to facilitate penetration of the cuticle by certain plant pathogenic fungi during the initial stage of infection (3), and some may have a role in saprophytic growth of fungi (4). Cutinases are serine esterases and contain the classical catalytic triad of Asp, Ser, and His, with the active serine in the consensus sequence Gly-His/Tyr-Ser-X-Gly, and an oxyanion hole formed in part by Gln and Ser residues (5). The sequence around the catalytic domain is highly conserved and provides a signature pattern for cutinases (1). Fungal cutinases are also glycoproteins that usually contain 3–6% carbohydrate (6) and have secretory signals (7).

The first cutinase to be purified and characterized was from the pea pathogen *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*) (8). Since then, cutinases from several fungal pathogens have been purified. The molecular masses of these previously characterized cutinases are typically between 21 and 60 kDa (4, 8, 9), except a cutinase from the plant pathogen *Botrytis cinerea* which has a molecular mass of approximately 18 kDa (10). In previous studies, classical purification techniques employing ion-exchange and size-exclusion chromatrography were used (4, 8–10). Although these methods are powerful and widely used for protein pu-

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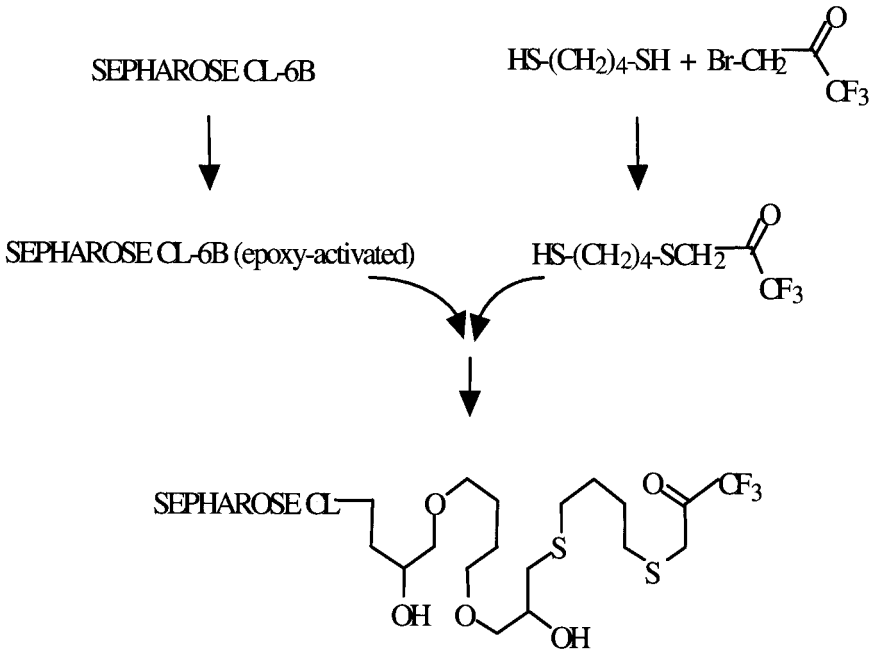

FIG. 1. Outline of the synthesis of the MBTFP-Sepharose resin used for affinity chromatography.

rification, they can be tedious and time consuming, and may have poor yields of the protein of interest. In contrast, affinity chromatography provides one of the most rapid and efficient methods for protein purification. To our knowledge, this approach has not been used successfully for cutinase purification from any source.

Trifluoromethyl ketones (TFK)[2] are highly polarized inhibitors of serine esterases and proteases (11, 12). The inhibitory activity of these compounds appears to be due to their ability to mimic the transition state of ester hydrolysis by forming a hemiketal link with the serine at the enzyme's active site (12, 13). TFK have been shown to inhibit juvenile hormone esterases and some, such as 3-*n*-octylthio-1,1,1-trifluoro-2-propanone (OTFP), 8-mercapto-1,1,1-trifluoro-2-octanone (MTFO), and 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone (MBTFP), have been used as affinity ligands to purify juvenile hormone esterases from several insect species (14–16). The unusually high potency and selectivity of TFK to juvenile hormone esterase and other serine esterases (11, 12, 15) suggested to us that TFK could be used as affinity ligands for fungal cutinases. In this paper, we report the purification and partial characterization of cutinases from *Monilinia fructicola* cultures using affinity chromatography with MBTFP-Sepharose.

[2] Abbreviations used: TFK, trifluoromethyl ketone; OTFP, 3-*n*-octylthio-1,1,1-trifluoro-2-propanone; MTFO, 8-mercapto-1,1,1-trifluoro-2-octanone; MBTFP, 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone; PTFP, 3-*n*-pentythio-1,1,1-trifluoro-2-propanone; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; DTT, dithiothreitol; PNB, *p*-nitrophenyl butyrate.

METHODS AND MATERIALS

Fungal culture. A peach isolate of M. fructicola (AF32/81) was maintained at room temperature on V-8 juice agar medium. Conidia were obtained from 5- to 7-day-old cultures by adding 5 ml of sterile distilled water containing 0.001% Triton X-100 to the culture and then pipetting the conidial suspension through two layers of sterile cheesecloth. The inoculum concentration was adjusted to 10^6 spores/ml with a hemocytometer. One-half milliliter of spore suspension was transferred to 300-ml Erlenmeyer flasks containing 100 ml of modified Czapek-Dox broth (17). The liquid medium was composed of 1 g K_2HPO_4, 0.5 g KCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MgSO_4$, 1 g yeast extract, and 2 g apple cutin in 1 L deionized water. The pH of the medium was adjusted to 7.0 with NaOH or HCl prior to autoclaving for 20 min. Cultures were then incubated at room temperature for 2 weeks after inoculation. Cultures were harvested by filtration through Whatman No. 1 filter paper and then passage of the filtrate through a polycarbonate membrane (1 μm; Poretics Corporation, Livermore, CA). The resulting culture filtrate was flash-frozen and concentrated by lyophilization. The lyophilized culture filtrate was dissolved in 50 mM sodium phosphate buffer, pH 7.0, and the resulting solution was dialyzed against 50 mM sodium phosphate buffer, pH 7.0, and then stored at −80°C until use.

Synthesis of trifluoromethyl ketones and preparation of affinity matrix. 3-*n*-octylthio-1,1,1-trifluoro-2-propanone and 3-*n*-pentythio-1,1,1-trifluoro-2-propanone (PTFP) were synthesized according to procedures described by Shiotsuki *et al.* (16). The affinity matrix was prepared by reacting epoxy-activated Sepharose with 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone, as described elsewhere (18) (Fig. 1). MBTFP was synthesized from the reaction of 1,4-dimercaptobutane (Aldrich Chemical Co., Milwaukee, WI) with equimolar 3-bromo-1,1,1-trifluoroacetone (Aldrich), as described by Prestwich *et al.* (19, 20).

Affinity chromatography. All procedures were carried out at 4°C in 50 mM sodium phosphate buffer, pH 7.0, containing 0.02% sodium azide unless otherwise noted. The TFK affinity gel (0.4 ml) was packed into a column attached with a fluoropolymer frit at the bottom (Econo-Column, Bio-Rad). The affinity matrix was then washed sequentially with EtOH, 50% EtOH, distilled water, and finally 50 mM sodium phosphate buffer, pH 7.0, twice, to remove unbound ligand and the storage solution (100% ethanol containing antioxidants such as butylated hydroxytoluene). The concentrated crude enzyme (4 ml) was mixed with the washed affinity matrix in a capped column at 4°C overnight by gently shaking on a shaker. After the gel settled, the supernatant solution was removed and enzymatic activity determined with PNB as a substrate. The affinity matrix was then washed with 50 column volumes (approximately 20 ml) of 50 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted from the affinity gel by gently shaking it with 8 ml of sodium phosphate buffer, pH 7, containing 0.5% Triton X-100, and then 8 ml of 0.1 M sodium acetate buffer, pH 5, containing 0.5 M NaCl for 4 h at 4°C. The eluates were concentrated by lyophilizing and dialyzed against 50 mM sodium phosphate buffer, pH 7, overnight at 4°C with two changes of buffer. The column was finally eluted by gentle shaking with 2.5 mM OTFP in 50 mM sodium phosphate buffer for 12 h. The OTFP eluate was concentrated by lyophilizing and dialyzed against 50 mM sodium phosphate buffer, pH 7.0, at 4°C for 5 days with 5 changes of dialysis buffer in SPECTRA/POR molecular porous membrane tubing (MW cutoff = 10,000, Spectrum Medical Industries, Inc., Houston, TX) to remove residual OTFP and reactivate enzymes.

Gel electrophoresis. SDS-PAGE was performed according to Laemmli (21) on 0.75 or 1 mm gels (12 or 14% acrylamide in running gel, 4.5% acrylamide in stacking gel). Proteins were visualized using a slight modification of the silver staining procedure described by Blum *et al.* (22). In our modified procedure to minimize gel shrinkage, methanol and ethanol concentrations were decreased from 50 to 35%, and wash times were reduced by half. Isoelectric focusing (IEF) was conducted for 3 h at 4°C on preparatory vertical native gels with a gradient from pH 3 to 10 using a Mini-Protein II system (Bio-Rad, Inc., Richmond, CA) as described by Robertson *et al.* (23). Electrophoresis was performed at 4°C for 1.5 h at 200 V constant voltage and then increased to 400 V constant voltage. The cathode solution was 25 mM NaOH and the anode solution 20 mM acetic acid. These solutions were cooled to 4°C prior to use. After electrofocusing, the gel was fixed overnight in a solution containing 30% methanol, 10% trichloroacetic acid, and 3.5% sulfosalicylic acid on a shaker, followed by at least 2 h in 30% methanol and 12% trichloroacetic acid, with three changes, and stained as above.

Two-dimensional gel electrophoresis was conducted by overlaying slices excised from the IEF gel on to the SDS gel, which is prepared using a 2-D preparative comb with one reference well. The IEF gel slice was incubated with two-dimensional gel buffer (24) before it was overlaid to the second dimensional gel. Electrophoresis was performed at room temperature at 120 V constant voltage until the dye front reached the edge of the gel. After electrophoresis, the gel was fixed and treated as above and visualized with Coomassie brilliant blue G-250 (25) or with silver stain.

Histochemical staining. Following electrophoresis in the presence of SDS, proteins were renatured by incubating the gel in sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 for 2 h at room temperature with shaking. The gel was then briefly rinsed with sodium phosphate buffer, pH 7, twice, prior to staining for esterase activity. The gels were stained in a solution containing 50 μg α-naphthyl acetate, 50 μg α-naphythyl butyrate and 10 mg Fast blue RR salt in 100 ml 0.1 M sodium phosphate buffer, pH 7, for 1 h at 30°C or until the desired color intensity (26). Native IEF gels were stained directly in the same solution at 30°C.

In-gel protein digestion and peptide recovery. In-gel protein digestion was carried out according to Hwang *et al.* (27) with modifications. After SDS-PAGE, the proteins were stained with Coomassie brilliant blue G-250 and destained with a solution containing 45% methanol and 10% acetic acid, and protein bands of interest were excised (those of approximately 2 μg protein or more estimated on the basis of color intensity). To remove acetic acid and SDS in the gel slice, the excised gel was washed in a 15 ml Corning tube with 10 ml of deionized water with gentle shaking for four 15-min intervals. After each interval, the wash solution was replaced with fresh water. The gel slice was then diced with a new razor blade to 1- to 2-mm squares and the resulting gel chips were transferred to a microtube (1.5 ml). The diced gel was dehydrated using a Speed-vac (Savant Instruments, Inc., Holbrook) for 1–2 h. The dried gel pieces were rehydrated with 50 μl rehydration buffer (0.1 M Tris-HCl, pH 9.0, with 0.05% SDS) containing 50 ng/μl Lys-C from *Achromobacter lyticus* (Wako Bioproducts, Richmond). Rehydration buffer was continually added until the gel was completely reswollen, providing a small volume above the gel pieces. The microtube containing the rehydrated gels was incubated at 30°C overnight and centrifuged at room temperature for 5 min. The supernatant was removed and transferred to a new microcentrifuge tube. The gel chips were covered with water and incubated for another 2 h, and the resulting supernatant was removed and saved. This extraction was repeated once and all the supernatants were combined in a microtube. To further extract peptides in the gel, 0.1% TFA in 80% acetonitrile was added just to the level where all gel chips were covered and the microtube was incubated at 30°C for 1 h. After centrifugation, the supernatant was transferred to a new microtube, and the extraction was again repeated and the resulting supernatant was combined with the first. The supernatant fraction was dried by Speed-vac, and the dried sample was stored at −20°C until further use.

The dried peptides in the microtube were dissolved in 25 μl 6 M guanidine-HCl, which is in 0.4 M Tris-HCl buffer, pH 8.2. One microliter of 450 mM DTT (in water) was added and the tube incubated for 45 min at 50°C. Two microliters of 500 mM iodoacetamide (in water and stored at 4–5°C) was added after warming up to room temperature and incubated for 15 min at room temperature. Seventy-two microliters of water was added to make a final concentration of 1.5 M guanidine-0.1 M Tris. The microtube was centrifuged for 5 min at maximum speed (14,000 rpm). The supernatant was removed to a new tube and then 25 μl of 0.1% TFA (in water) was added to the precipitate and vortexed. The microtube was centrifuged again and the supernatant was combined with the previous collection.

Peptide separation by reverse phase microbore-HPLC. Peptide fragment separation was performed by Microbore-HPLC on an Ultrafast Microprotein Analyzer (Michrom Bioresources, Inc., Pleasanton, CA) using a Model 760 solvent delivery system and a Model 112 oven/injector system and equipped with a Linear UV-VIS 200 detector (Linear Instruments Corporation, Reno, NV). Data were collected and analyzed using the Axxiom Chromatography 700 Series Data system (Axxiom Chromatography, Inc., Moorpark, CA). Peptide fragments were fractionated on a Rolliastil C18 reverse-phase column (1 mm × 15 cm, 5 μm, 300 A; Michrom Bioresources, Inc., Auburn, CA). The peptide fragments were fractionated using a linear gradient of 5% solvent A (1% TFA in 20% acetonitrile) to 70% solvent B (0.075% in 90% acetonitrile) in 90 min with a flow rate of 50 μl/min and UV detector range of 0–0.1 Å at 210 nm. The separated peptides were sequenced on a 470A ABI sequencer coupled to an ABI 120 A online HPLC. Data were collected and analyzed using ABI 610 software (ABI Applied Biosystems, Inc., Foster, CA).

Computer-assisted analysis of sequence and amino acid composition data. The amino acid sequence homology search was carried out using the BLAST program (National Center for Biotechnology Information) and further processed by the University of Wisconsin GCG program.

Enzyme assay and protein determination. Esterase assays were performed using *p*-nitrophenyl butyrate (PNB) in 96-well microtiter

plates (28). The reaction mixtures contained 167 μl 50 mM potassium phosphate buffer, pH 7, 13 μl 0.04% Triton-X 100, 10 μl enzyme solution, and 10 μl PNB stock solution (17.6 μl PNB in 1 ml acetonitrile). The reaction mixture was incubated at room temperature for 30 min and read at 405 nm on a Molecular Devices Vmax microtiter plate reader with Softmax data analysis software (Molecular Devices Corp., Sunnyvale, CA). Each experiment was carried out in triplicate. Cutinase activity was determined using ^{3}H-cucumber cutin, a gift of Dr. R. Hammerschmidt (4). The assay was done with triplicate samples at room temperature for 16 h. To analyze cutinase activity separated by isoelectric focusing, the gel was frozen briefly, and then a lane of interest was excised from the gel and diced into about 2-mm-wide pieces. The resulting gel pieces were assayed for cutinase activity as described above. The remainder of the gel was stained with silver for in-gel esterase activity as described above. To test for cutinase inhibitory activity of compounds, the purified enzyme was incubated with 50 mM sodium phosphate buffer, pH 7, containing a potential inhibitor for 30 min at room temperature prior to assay with PNB as substrate. The matrix binding experiments were performed by incubating the affinity matrix with the enzyme solution in a microtube at 4°C. The tube was centrifuged briefly and an aliquot of the supernatant was taken at different incubation periods to determine esterase activities and protein content. The binding capacity was evaluated by comparing esterase activities and protein in the supernatant with that of a control sample without inhibitor. The I_{50} values were calculated from the regression data on semilog plots for six or more different concentrations of inhibitors (16). Protein concentrations were determined by the method of Bradford using reagent supplied by Bio-Rad with bovine serum albumin as a standard (29).

RESULTS

Binding capacity of the MBTFP affinity matrix. MBTFP is an optimized ligand for purification of juvenile hormone esterase from *Manduca sexta* (18), a serine hydrolase and a member of the α/β-hydrolase-fold family of proteins (30, 31). Because fungal cutinases are also members of this family of proteins, we hypothesized that MBTFP could also be used as a ligand for the purification of the cutinases from *M. fructicola*. Indeed, when an aliquot of a culture filtrate of *M. fructicola* was incubated with the affinity matrix, the specific esterase activity of the supernatant declined with time of incubation. After a 5-h incubation at 4°C, the matrix bound 16.1% of the total protein in the culture filtrate, which accounted for approximately 90.6% of the total esterase activity in the crude enzyme solution. When the incubation time extended beyond 5 h, there was no significant increase in the binding and removal of esterase activity from solution. When BSA was incubated with the affinity matrix, no significant reduction in the protein concentration was observed. Therefore, cutinases and/or other esterases in the fungal culture filtrate bound to the affinity ligand, indicating the potential for using the MBTFP ligand in the purification of cutinases from cultures of *M. fructicola*.

Cutinase inhibition by OTFP, PTFP, and the ligand. Since TFKs are slowly reversible covalent inhibitors of serine esterases (32), the proteins bound to the affinity gels usually can be eluted by competition with another soluble TFK inhibitor (13, 16, 32). Two TFK inhibitors, OTFP and PTFP, and the affinity ligand were tested for their ability to inhibit cutinase from the MBTFP affinity matrix. The I_{50}'s of OTFP, PTFP, and MBTFP for total PNB esterase activity in the crude culture filtrate are 1.6×10^{-6}, 2.3×10^{-4}, and 9.4×10^{-3} (M), respectively. The relative strengths of inhibition of OTFP, PTFP, and MBTFP on the *Monilinia* cutinase activity are similar to the respective effects of these ligands on juvenile hormone esterase activity (published I_{50}'s of 1.5×10^{-8}, 1.86×10^{-7}, and 1.5×10^{-6} (M), respectively; 16). However, all of the TFK ligands were stronger inhibitors of juvenile hormone esterase than of the fungal cutinase by 2–3 orders of magnitude (16, 18). Due to the small difference in the I_{50} of PTFP and MBTFP for *M. fructicola* cutinase, OTFP was chosen to elute the affinity column.

Elution of cutinase from MBTFP-Sepharose. The concentrated enzyme solution was incubated with the MBTFP affinity matrix overnight at 4°C in a capped column. The unbound proteins were drained from the column, and the column was then washed with 50 mM sodium phosphate buffer, pH 7. Several approaches, including elution with a low pH buffer containing NaCl and buffer containing detergent (Triton X-100), also were tried to elute bound proteins from the affinity gels. The affinity column was eluted sequentially with (1) 0.5% Triton X-100 in 50 mM sodium phosphate buffer, pH 7; (2) 50 mM sodium acetate buffer, pH 5, containing 0.5 M NaCl; and (3) 2.5 mM OTFP in 0.1 M phosphate buffer, pH 7. OTFP, which is the most potent inhibitor reported so far for juvenile hormone esterase, had been successfully used to elute other esterases from TFK columns (14, 15). However, elution of a significant fraction of juvenile hormone esterase from the affinity matrix with OTFP required a long time due to the slow but tight binding of this inhibitor (13, 14, 16). Therefore, the final elution step with OTFP was accomplished by shaking the column with OTFP in 50 mM sodium phosphate buffer for 12 h at 4°C. When the affinity gel was incubated with the elution buffer beyond 12 h, no significant measurable protein was detected in the eluate. The OTFP eluate was dialyzed for 5 days against several changes of 50 mM sodium phosphate buffer, pH 7. The other two fractions were concentrated by lyophilizing and dialyzed against 50 mM sodium phosphate buffer, pH 7, overnight with two changes of buffer. The purification results are shown in Table I. The specific activity of the OTFP eluate was the highest and 14 times that of the crude fraction, containing 65.6% of the total esterase activity recovered from the column. The specific activity of the phosphate buffer with Triton X-100 was over twice that of the crude enzyme fraction and contained 16.2% of total

TABLE I

Trifluoromethyl Ketone Affinity Purification of Cutinases from *M. fructicola*

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity	Total activity	Recovery (%)	Purification
Crude	4.0	0.93	38.45	35.68	—	—
Unbound fraction[a]	3.8	0.28	9.66	2.69	7.5	0.3
Phosphate	2.8	0.06	98.14	5.78	16.2	2.6
Acetate	10.4	0.02	117.23	2.31	6.5	3.0
Ligand	4.4	0.04	545.70	23.42	65.6	14.2

[a] The unbound fraction was combined with flowthrough and 20 ml of wash buffer. The fractions indicated above are in order of the sequence used for column elution (see Materials and Methods).

esterase activity. The specific activity of the acetate buffer fraction was three times that of the crude enzyme and higher than that of the phosphate buffer/ Triton X-100 fraction; however, it only contained 6.5% of the total esterase activity. It should be noted that the specific activity of the OTFP eluate could be an underestimate because of the inhibited catalytic activity of the purified enzyme due to the lipophilicity of the OTFP and its high affinity for serine esterases (14, 33). Full recovery of OTFP-eluted juvenile hormone esterase was reported to take about 2 weeks of extensive dialysis (34).

Electrophoretic analysis. The eluates from the affinity column were resolved in the SDS-PAGE gel. The fraction eluted with OTFP yielded five bands corresponding to molecular weights 18.6, 20.8, 25.0, 26.7, and 28.3 kDa (Fig. 2). To evaluate purity of the proteins, the OTFP fraction was further analyzed with two-dimensional SDS-PAGE/IEF and native isoeletric focusing gels. IEF resolved two bands, one band corresponding to p*I* 8.2 and the other to p*I* 6.1 (Fig. 3A). When the native gel was stained in a solution containing Fast blue RR salt, α-naphthyl acetate, and α-naphthyl butyrate, the proteins with p*I* 8.2 showed much stronger esterase activity than other proteins in the gel (Fig. 3B). Gel slices excised from the native IEF gel were subjected to cutinase activity assay using [^{3}H]cutin (4). Gel slices corresponding to p*I* 8.2 and 6.1 had cutinolytic activity and hydrolyzed [^{3}H]cutin (Fig. 3C). The two-dimensional gel resolved five bands. Two of these had a p*I* of 8.2 with molecular weights of approximately 18.6 and 20.8 kDa. Three of the bands had a p*I* of 6.1 and molecular weights of 25.0, 26.7, and 28.3

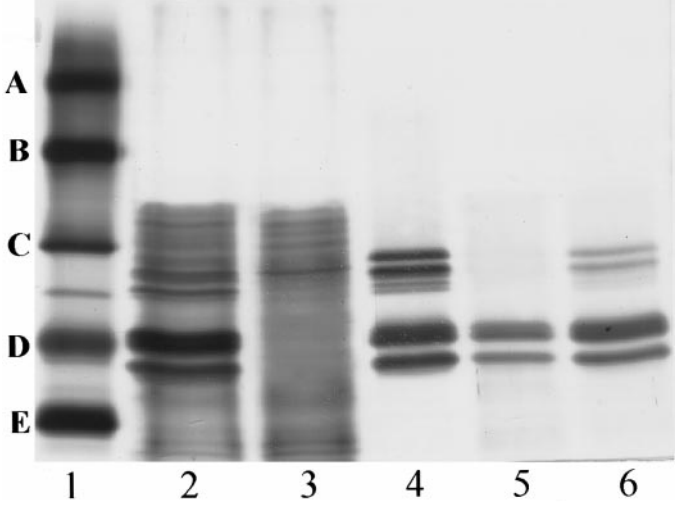

FIG. 2. SDS-PAGE gel stained with $AgNO_3$ of MBTFP affinity-purified cutinases from a cutin-amended culture of *Monilinia fructicola*. Lane 1, protein markers (A, bovine serum albumin, 66 kDa; B, ovalbumin, 46 kDa; C, carbonic anhydrase, 30 kDa; D, trypsin inhibitor, 21.5 kDa; E, lysozyme, 14.3 kDa); Lane 2, culture filtrate; Lane 3, unbound fractions; Lane 4, OTFP ligand; Lane 5, phosphate buffer, pH 7, with 0.5% Triton X-100; Lane 6, acetate buffer, pH 5, with 0.1 M NaCI.

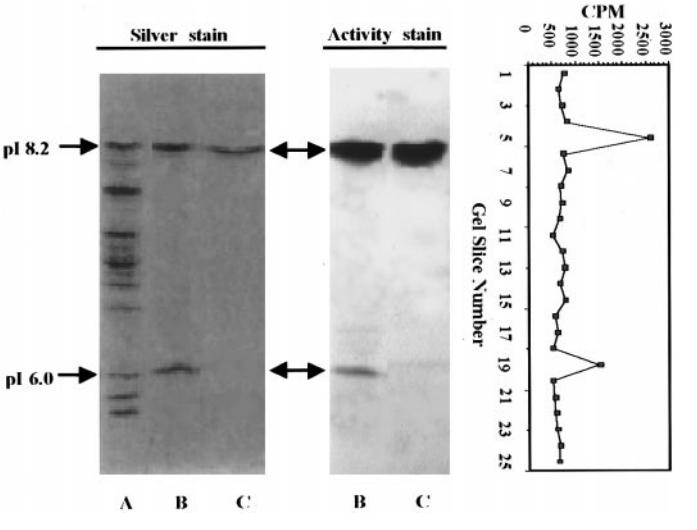

FIG. 3. Native IEF gel of MBTFP affinity-purified cutinases from cutin-amended culture of *M. fructicola* and in-gel cutinolytic activity of the OTFP-eluted fraction. Lane A, IEF standard; lane B, OTFP eluate; and lane C, phosphate buffer/ 0.5% Triton X-100 eluate. Left panel, silver-stained gel; middle panel, activity-stained gel; and right panel, cutinase activity determined with [^{3}H]cutin of different gel slices.

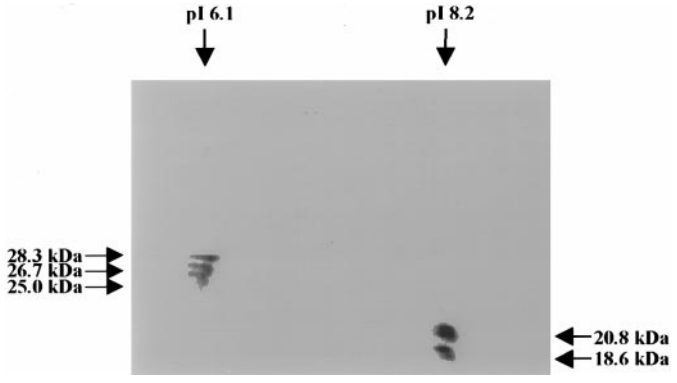

FIG. 4. Two-dimensional gel electrophoresis of MBTFP affinity-purified cutinases from *M. fructicola* culture filtrate. p*I* and molecular weight are indicated.

kDa, the same as in the SDS PAGE gel (Fig. 4). Since the proteins of p*I* 8.2 accounted for most of the esterase activity, and had higher cutinase activity by the [^{3}H]cutin assay, further characterization focused on these.

The acetate buffer fraction yielded similar bands as the OTFP fraction in the SDS gel, except that the 25.0-kDa protein band was absent. This fraction contained only a small portion of total esterase activity, so further analyses were not carried out. The phosphate buffer-detergent eluate yielded two bands with p*I* 8.2 and showed strong esterase activity (Fig. 3B). A native IEF gel slice containing these proteins also showed *bona fide* cutinolytic activity with [^{3}H]cutin.

Peptide map and amino acid sequences. The OTFP fraction was resolved by SDS-PAGE and stained with Coomasie blue R-250. Two proteins of MW 18.6 and 20.8 kDa were excised from the gel and subjected to in-gel digestion with the protease Lys-C, and the resulting peptides were separated by HPLC. The peptide

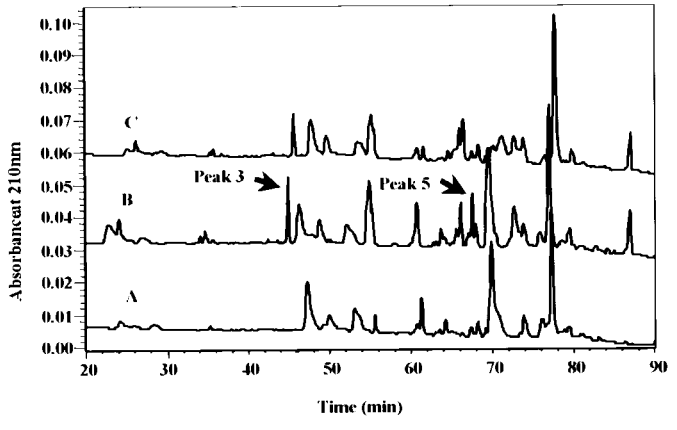

FIG. 5. Peptide maps of two cutinases digested with Lys-C. A, Lys-C; B, 20.8-kDa cutinase + Lys-C; C, 18.6-kDa cutinase + Lys-C. Peaks 3 and 5 of the 20.5-kDa cutinase were sequenced.

TABLE II

Amino Acid Sequences of Cutinase Peptides Derived from *in Situ* Protease Digestion

Samples	Amino acid sequence
Peptide A	LVISGYSQGGQLVHNAAK
Peptide B	TMNGVPYAADVPGFLKGGD

maps of both proteins are similar, and the fingerprint peaks are identical (Fig. 5). These two proteins could represent differences in posttranslational processing of a common precursor, or represent distinct isozymes. The peptide fragments of the 20.8-kDa protein corresponding to peaks 3 and 5 were sequenced, and 18 and 19 amino acid residues of peptide fragments A and B, respectively, were defined (Table II). Data base search results indicated that these two sequences share a high percentage identity with cutinases from *Botrytis cinera* (35) (Fig. 6).

DISCUSSION

This study demonstrated the feasibility of using TFK affinity chromatography in combination with preparative SDS-PAGE for the purification of fungal cutinases, and also provides a foundation for more comprehensive studies on the structure and regulation of cutinases from *M. fructicola*. Although affinity chromatography with TFKs did not yield pure homogeneous cutinase preparations from culture filtrates, this method in combination with preparative gel electrophoresis provides a rapid method for purification of these cutinases.

Trifluoromethyl ketones have been shown to be excellent ligands for purification of a number of esterases, providing high recoveries and comparable purification factors (13, 15, 16, 32, 33, 36). Juvenile hormone esterases and cutinases are serine hydrolases belonging to the α/β-fold superfamily of proteins (5, 30, 31). Some cutinases catalyze the hydrolysis of ester bonds of triglycerides (37) and emulsified triacylglycerol as efficiently as lipases (5). However, unlike the true lipases, cutinases show no enhancement of activity in the presence of a lipid-water interface (interfacial activation) (38). Therefore, cutinases are thought to bridge a link between acyl esterases and true lipases

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MFCUT1   3 VNGVPYAADVPGFLKGGD 20     1 LVISGYSQGGQLVHNAAK 18
            +NGV Y ADVPGFL+GGD          LVISGYSQGGQLVHNAAK
BOCI    71 MNGVDYPADVPGFLQGGD 88   111 LVISGYSQGGQLVHNAAK 128
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FIG. 6. Comparison of cutinase internal fragment sequences of MFCUT1 from *M. fructicola* and BOCI from *B. cinerea* (10). Numbers for BOCI indicate the relative position within the protein. The deduced amino acid sequences of MFCUTI and BOCI share 67% identity and 72% similarity.

(39). Similar to cutinases, juvenile hormone esterases are not predicted to display interfacial activation, due to the absence of a hydrophobic "lid," which is a hydrophobic sheet covering the catalytic residues (5, 31, 40).

Differences in the affinity of TFKs for different serine esterases arise from differences in substrate docking with, and the architecture of, the active site (5, 31). Hence, it is possible that other TFKs not tested in our study may provide better affinity ligands for cutinase purification than MBTFP. Also, in light of the reported variation in substrate acyl chain length preferences among fungal cutinases (8, 41, 42), different cutinases may display different affinities for a given TFK ligand. Although not examined in our study, ligand density increases both the capacity and the apparent affinity of the matrix. In addition to ligand selection and density, elution conditions can strongly influence the efficiency of TFKs as affinity ligands, and include detergent concentration, ionic strength, pH, and inhibitor concentration. Acidic conditions greatly accelerate the formation and decomposition of hemiketals, which can accelerate dissociation of an enzyme from its TFK inhibitor, a feature that was exploited to restore the catalytic activity of an OTFP-eluted juvenile hormone esterase (36). Indeed, low pH buffer in the present study increased the elution of cutinase from the affinity gel, but the recovery of enzymatically active protein was low (Table I). In our study, phosphate buffer containing 0.5% Triton X-100 selectively eluted two cutinases from the affinity column and provides an alternative eluant to one containing a TFK.

The cutinases from *M. fructicola* were found to be quite stable, retaining considerable (about 40%) esterase activity even after boiling for 15 min. OTFP was a strong competitive ligand that eluted both cutinases from the MBTFP affinity column. Although the OTFP fraction was dialyzed for 5 days with multiple changes of the phosphate buffer, dialysis apparently did not completely restore catalytic activity. This was expected since for other enzymes OTFP was shown to be a slow but tight-binding inhibitor, and its lipophilic nature would tend to retard its passage through dialysis membranes. Other TFK ligands (16) and extended dialysis times at low pH are parameters that should be investigated for optimizing affinity purification of cutinases.

Cutinases have been purified from a number of plant pathogenic fungi, including *Alternaria brassicicola* (43), *Botrytis cinerea* (10, 44), *Colletotrichum gloeosporioides* (41), *Fusarium solani* f.. sp. *pisi* (8), and *Venturia inaequalis* (4). The *M. fructicola* cutinases reported in this study have lower molecular weights (18.6 and 20.8 kDa) relative to most other fungal cutinases heretofore described and a high p*I* of 8.2. The other proteins eluted from the affinity ligand were not further characterized in this study, although some of them are cutinase isozymes similar to those induced by cutin in other plant pathogens (8, 42). The presence of multiple isoforms of cutinases in plant pathogenic fungi has complicated attempts to ascribe functional roles of these enzymes in pathogenesis. It is likely that the functional roles of the cutinases present in *M. fructicola* will also be complex.

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