

# Characterization and Cloning of Cutinase from *Ascochyta rabiei*

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*Ascochyta rabiei*, the causal agent of *Ascochyta* blight on chickpea plants, secretes a cutinase in the culture filtrate when it is induced by cutin or hydroxylated fatty acids. This cutinase is the main esterase in the culture fluids. The enzyme was purified to homogeneity by three successive chromatographic steps. It showed an apparent molecular weight of 22 kD in SDS-PAGE and cleaved ester bonds of <sup>3</sup>H-labelled cutin or *p*-nitrophenylbutyrate with maximal activities around pH 8. As a serine esterase, cutinase is strongly inhibited by organophosphorous compounds and the most effective inhibitor 2,3,5-trichloropyridine-6-(*O*-methyl-*O*-*n*-butyl)-phosphateester (MAT 9564) shows a *K<sub>i</sub>* value of 0.8 nM. The cutinase gene was cloned from a genomic cosmid library by screening with two oligonucleotides directed against cutinase consensus peptides. The gene was subcloned to a 1.7 Kb SalI/HindIII-insert and sequenced. The cutinase gene codes for a 223 amino acid protein with strong homology to other fungal cutinase sequences. The purified cutinase is encoded by a single copy gene.

## Introduction

*Ascochyta rabiei* is a severe pathogen of chickpea plants around the world causing blight disease on leaves and stems. Possible traits of virulence of *A. rabiei* previously determined are an exo-cleaving polygalacturonase, formation of the phytotoxic solanapyrones and fungal enzymes involved in phytoalexin detoxification under *in-planta* conditions (Tenhaken and Barz, 1991; Tenhaken *et al.*, 1991). Histological studies have provided good evidence for direct fungal penetration through the cuticle followed by subepidermal spread of the fungus between the cells (Pandey *et al.*, 1987; Höhl *et al.* 1990; Köhler *et al.*, 1995). The first barrier between the host plant and an attacking pathogen is the cuticle which is an important hydrophobic

defence layer. All aerial parts of plants are normally covered by cutin, a biopolyester of predominantly hydroxylated fatty acids impregnated with wax. Fungal penetration is often accompanied by the production of cutinase to overcome cutin layers. The cutinase weakens cutin polymers by cleaving ester bonds and this process may facilitate penetration of fungal hyphae. Fungal isolates of *Fusarium solani* and *Colletotrichum gloeosporioides* with a reduced cutinase activity appear to be also reduced in pathogenicity. Based on this observation many phytopathologists classified cutinase as an essential virulence factor for directly penetrating fungi (Köller *et al.*, 1982a; Dantzig *et al.*, 1986; Dickmann and Patil 1986; Dickmann *et al.*, 1989).

However, some successful penetrating leaf pathogenic fungi like the corn pathogen *Helminthosporium maydis* (*Cochliobolus heterostrophus*) are virulent on their host plants despite the low production of cutinase when compared to *Fusarium solani*. This low activity has also been found with *A. rabiei*, where the cutinase activity is two orders of magnitude lower than in *F. solani*. In addition, some cutinase producing fungi can successfully penetrate their host plants apparently without cutinase as essential virulence factor (Bonnen and Hammerschmidt, 1989). Most of this data showing a correlation between reduced cu-

**Abbreviations:** PNP, *p*-nitrophenol; OnI, oligonucleotide I; OnII, oligonucleotide II; bp, base pairs; Kb, kilo bases; LumigenPPD, 4-methoxy-4-(3-phosphatephenyl)-spiro(1,2-dioxetane-3,2'-adamantan); TLC, thin layer chromatography; SDS, sodium dodecylsulfate; IEP, isoelectric point; MAT 9564, 2,3,5-trichloropyridine-6-(*O*-methyl-*O*-*n*-butyl)-phosphateester; Tris, tris-(hydroxymethyl)-aminomethan.

The nucleotide and protein sequence data has been submitted to EMBL under accession number X65628.

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tinase activity and reduced pathogenicity were obtained with fungal mutants in which additional unknown genes may also be altered. This may lead to unexpected results when such mutants are used to evaluate the potential role of one factor, e.g. cutinase, for pathogenicity.

In view of these difficulties recent studies have used the techniques of specific transformation-mediated cutinase gene replacement (Stahl and Schäfer, 1992; Yao and Köller, 1995) or cutinase gene disruption (Sweigard *et al.*, 1992). However, the present results so far fail to unequivocally clarify the role of cutinase as a pathogenicity factor.

As part of our studies on *A. rabiei* – chickpea interaction we now report the characterization of the cutinase from *A. rabiei* and the cloning of the gene. Since *A. rabiei* is a strict leaf pathogen such investigations and subsequent gene replacement studies will contribute to molecular aspects of cutinase from this group of pathogenic fungi.

## Materials and Methods

### Fungal strains

*Ascochyta rabiei* isolates used for these studies have been described (Tenhaken *et al.*, 1991). The genomic DNA-library was prepared from isolate III, whereas the cutinase was purified from isolate X. Southern analyses were performed with DNA from isolates III, IV, VI, VII, X and XXI.

### Purification of cutinase

The fungus was grown without shaking in modified Richard's mineral salt medium (Tenhaken *et al.*, 1991), containing 0.05% sucrose and cutin hydrolysate (0.05%) or hydroxylated fatty acids (0.025%) as cutinase inducers. The culture filtrate was harvested after 3 to 4 weeks of growth at 22 °C. Cutinase in the medium was bound to phenyl-sepharose material by adding 0.5 M ammonium sulfate to the filtrate, gentle stirring the suspension for 30 min and then the adsorber was collected by filtration. The enzyme was eluted with 50% isopropanol in potassium phosphate buffer (50 mM, pH 7.2) and the solution concentrated 4 fold by evaporation. The cutinase preparation was chromatographed on a Superdex 75 gelfiltration column (1.6 x 60 cm) with sodium phosphate buffer (75 mM, pH 7.3, 25 mM NaCl) and a flow rate of

1 ml/min. Active fractions were pooled, adjusted to 1 M NaCl and loaded on a phenyl-superose HR 5/5 column (1 ml gel volume, FPLC-system). Unbound protein was removed with starting buffer (50 mM potassium phosphate buffer, pH 7.2) and cutinase was eluted as a single protein peak using an increasing gradient of isopropanol (0% to 35% isopropanol within 17 ml, initial flow rate 0.4 ml/min, within the gradient 0.25 ml/min) in the starting buffer.

### Enzyme assays

Enzyme activity of cutinase was measured with the model substrate PNP-butyrate or with tritium labelled cutin. [<sup>3</sup>H]-cutin was prepared by Amersham Buchler (Braunschweig, Germany) from fine powdered apple cutin by the method of Köller *et al.*, (1982a). Labelled cutin was repeatedly washed with MeOH/chloroform and subsequently with test buffer to reduce background radioactivity. Enzyme assays (1 ml volume) were performed at 30 °C in Tris/Cl<sup>-</sup> buffer (0.1 M, pH 8, 0.1% Triton X 100, 0.05% sodium azide) containing 75 µl protein solution by occasionally agitating the cutin (2 mg/ml) suspension for 4 h. The assay was acidified with 5 N HCl and products were extracted with 5 volumes of diethylether. The ether solution was brought to dryness and analyzed in a scintillation counter with hydroluma (Baker, Groß Gerau, Germany) as scintillation cocktail. Alternatively, the hydrolysis products obtained with <sup>3</sup>H-cutin were separated by silica gel TLC (solvent = diethylether: acetone: acetic acid, 95:5:1, v/v) and detected by radio scanning.

As a control purified cutinase of *Fusarium solani* (donated by Dr. H. D. VanEtten, Tucson, Ar., USA) was used.

Enzyme assays (1 ml final volume) with PNP-butyrate (5 µl of a 50 mM stock solution in ethyleneglycol-monomethylether) were performed in sodium phosphate buffer (0.1 M, pH 7.5, 0.1% Triton X 100) in order to minimize non-enzymatic hydrolysis of the substrate. The absorption increase at 405 nm was continuously recorded with an Uvicon spectral photometer. The extinction coefficient for *p*-nitrophenolate at pH 7.5 was determined to be  $\epsilon = 15680 \times \text{mol}^{-1} \times \text{cm}^{-1}$ .

Cutinase activity with umbelliferyl butyrate as substrate was determined in a 1 ml assay in the

same buffer with 5  $\mu$ l of 20 mM substrate stock solution in ethyleneglycolmonoethylether. The excitation wavelength for liberated umbelliferone was 333 nm, while the fluorescence was recorded at 466 nm ( $\epsilon = 38200 \times \text{mol}^{-1} \times \text{cm}^{-1}$ ).

Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

#### *Inhibitor experiments*

Inhibitor experiments of the cutinase were performed with the PNP-butyrate enzyme assay. The organophosphorous inhibitor compounds were dissolved in isopropanol. All assays contained a final concentration of 5% organic solvent. The presence of 5% isopropanol in the assays had no inhibitory effect on the cutinase. The cutinase was preincubated with the inhibitor for 60 min at 20 °C and remaining enzyme activity was determined in a standard assay.

#### *Induction of cutinase*

Various commercial fatty acids were tested for the induction of cutinase in surface culture. 16-Hydroxy-hexadecanoic acid and 12-hydroxy-*cis*-9-octadecenoic acid (ricinoleic acid) well induced cutinase whereas no induction was found with 9,12-dihydroxy-octadecanoic acid, 10, 13-dihydroxy-octadecanoic acid, 10,12-dihydroxy-octadecanoic acid, *cis*-9, *cis*-12-octadecadienoic acid (linoleic acid) or hexadecanol. Routinely, cutinase was induced by a crude alkaline hydrolysate of tomato cutin, directly obtained with dried tomato skin without prior purification of the cutin.

#### *Isoelectric focussing and gel activity staining of esterases*

Isoelectric focussing was performed in rehydrated gels according to Westermeyer (1990). After polymerization of the polyacrylamidegel (0.5 mm, 7.5% PAA) on a solid surface the gel was washed twice in bidistilled water. After washing the gel with 2% (w/v) glycerol it was dried overnight at RT. Rehydration was carried out in 10% sorbitol containing 2.5% (w/v) Pharmalyte (Pharmacia, Freiburg, Germany) with a pH-range from 3–10. Gels were run at 14 mA and 2000 V for 30 min at 10 °C without samples and after sample

application for additional 1.5 h. The gel was stained for esterase activity in 25 mM indoxylacetate, dissolved in 0.1 M potassiumphosphate buffer, 0.1% Triton X-100, pH 7.5). Indoxylacetate was presolved in 1 ml methoxyethanol. Samples were prepared by the following procedure: 1 ml culture fluid from *A. rabiei*, grown on cutin hydrolysate as esterase inducer, was concentrated by evaporation to dryness. The evaporate was carefully resuspended in 100  $\mu$ l bidistilled water. 30  $\mu$ l of this concentrate was run on the gel.

#### *Southern blot with genomic DNA from A. rabiei*

Genomic DNA from *A. rabiei* was isolated according to Doyle and Doyle (1991). The DNA was restricted with the endonucleases Hind III, KpnI, and XhoI (Pharmacia, Freiburg, Germany) following the manufacturers instructions. Restriction fragments were separated for 18 h at 25 V in 0.7% agarose gels solved in 0.5\* TBE (0.04 M Tris, 0.04 M boric acid, 1 mM EDTA). Subsequent blotting was performed according to Chomczynski (1992) on nylon membrane (Quiagen, Düsseldorf, Germany).

For preparation of the hybridization probe, the internal 0.27 kb PstI-fragment from pCutAr was eluted from agarose gels using the Jetsorb-Kit (Genomed, Bad Oenhausen, Germany) and approx. 100 ng were random primed with hexamer primers and labeled using the digoxigenin nonradioactive detection kit (Böhringer, Mannheim, Germany).

Prehybridization and hybridization were carried out at 37 °C with 50% formamide and subsequent 5\*, 3\* and 2\* SSC (20\*SSC: 3 M NaCl, 0.3 M Na<sub>3</sub> citrate) washing steps (remaining at each SSC concentration for 2\*15 min). Blocking of the membrane was prolonged to 1.5h.

LumigenPPD (Böhringer, Mannheim) was used to detect hybridizing fragments. The filter was exposed 1–3 days to X-ray film (Kodak XAR-100).

#### *DNA isolation for preparing the cosmid library*

DNA for preparing the cosmid library was isolated from 8 day old *A. rabiei* mycelium. The mycelium was homogenized in liquid nitrogen and the powder was added to prewarmed lysis buffer (15% saccharose, 50 mM EDTA, 50 mM Tris/Cl pH 8.0, 0.1 mg/ml proteinase K, 1% SDS and

0.05 mg/ml RNase) and incubated for 1 h at 60 °C. The preparation was centrifuged at 3000 x g (20 min) and 0.1 volume of potassium acetate (3 M, pH 4.2) was added to the supernatant and incubated on ice for 90 minutes. After centrifugation (30,000 x g, 30 min) DNA was precipitated with 0.1 volume sodium acetate (3 M, pH 4.8) and 2 volumes EtOH and carefully spooled on a pasteur pipette. DNA was washed with 70% EtOH, air-dried and dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Then, the dissolved DNA was again treated with proteinase K, SDS and RNase as described above and all subsequent steps were also performed as mentioned. Finally, the isolated high molecular weight DNA was checked with pulse-field agarose gel electrophoresis.

Plasmid DNA was isolated by using a Quiagen DNA purification kit according to the manufacturer's instructions (Diagen, Düsseldorf, Germany). Southern blots were incubated overnight with the respective oligonucleotide at 41 °C. Finally, the blots were washed three times in 5 x SSC at 41 °C.

#### *Cosmid library construction*

High molecular weight DNA was partially restricted with MboI and 35–45 kb DNA fragments were separated by ultracentrifugation (50000 x g, 15 h, vials with 3 M NaCl in TE buffer were three times frozen and thawed prior to use). These MboI fragments were ligated in the single Bam HI site of the cosmid pCosHyg 1 (Arnemann, 1991). The concatamer DNA was *in vitro* packaged with the Packagene-kit (Promega, Heidelberg, Germany) and transduced in *E.coli* DHI. About 150,000 recombinant clones were obtained and stored at -80 °C.

#### *Screening of the cosmid library*

The cosmid library was screened with a <sup>32</sup>P-labelled oligonucleotide (OnI) directed against the cutinase consensus sequence ANTKCP. The oligonucleotide (sequence: 3'GGI-TT(GA)-TGI-TT(TC)-AC(GA)-GG 5') contained either a mixture of the wobble bases or desoxyinosine in the indicated wobble codons. The oligonucleotide was labelled by T<sub>4</sub> kinase with γ-<sup>32</sup>P-ATP according to Arnemann (1991). Approximately 10,000 clones were screened by standard procedures (Sambrook

*et al.*, 1986). Hybridizations were performed in 6.6x SSC/0.1% SDS at 41 °C overnight and washed with 5 x SSC/0.1% SDS at 41 °C. Blots were autoradiographed at -70 °C for 1 to 7 days.

Southern blot analyses were carried out with the non-radioactive DIG-system (Boehringer, Mannheim, Germany). Restricted DNA was separated in agarose gels and transferred to nylon membrane (PAL Biodyne A) by alkali transfer and heat fixation. Cosmid clones derived from screening with OnI were rescreened with a second cutinase consensus oligonucleotide (OnII, 20 base pairs) directed against the amino acid sequence WI/VQGVGG. OnII (sequence: 3'ACC-T(C)AIGTT(C)-CCI-CAI-CCI-CC5') was designed with the same concept used for OnI. Only a single group of cosmid clones strongly hybridized to both OnI and OnII. The cutinase gene was subcloned on a 4.1 kb *Hind*III fragment out of the 40 kb cosmid clone cc6 into a pT<sub>7</sub>T<sub>3</sub> vector (Pharmacia, Freiburg, Germany) and finally deleted to a 1.7 kb *Sal*I/*Hind*III fragment. DNA sequences were determined at least twice from a set of subclones or with appropriate sequencing primers, using the dideoxy chain termination method (Sanger *et al.*, 1977) and a T<sub>7</sub>-sequencing kit (Pharmacia). Sequences were assembled and analysed using the HUSAR software package (DKFZ, Heidelberg, Germany).

For hybridization of restricted cosmid clones a PCR-fragment (pΔCut 22) from the *Fusarium solani* cutinase gene was also used (Stahl and Schäfer, 1992).

## **Results**

### *Purification and characterization of cutinase*

*Ascochyta rabiei* secretes pronounced esterase activity into the culture filtrate when the mineral salt medium is supplemented with cutinase inducers. Upon isoelectric focussing of this enzyme activity with subsequent activity staining one dominant and two minor bands were detected (Fig. 1). Cutin or an alkaline hydrolysate of cutin were the best esterase inducers. Weak enzyme activity was also obtained with 16-hydroxy-hexadecanoic acid but ricinoleic acid was only a poor inducer of the esterase. All other tested compounds (see Materials and Methods) failed to induce detectable esterase activities in *A. rabiei*. The enzyme production



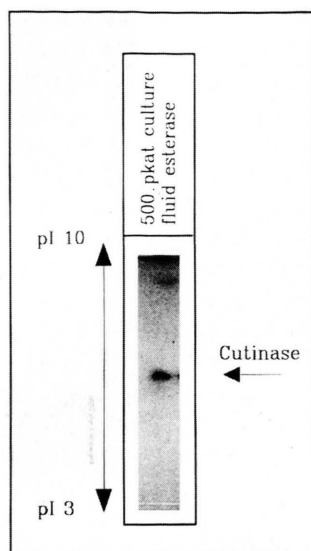


Fig. 1a. Isoelectric focussing and esterase activity staining of culture filtrates from *A. rabiei* grown for 3–4 weeks on cutin hydrolysate as esterase inducer. The filtrate was concentrated 10-fold by evaporation and 500 pkat total activity were run on the focussing gel. After isoelectric focussing, the gel was stained for esterase activity with indoxylacetate.

is repressed by glucose or sucrose (data not shown). As indicated by gelfiltration only one main esterase activity could be detected in *A. rabiei* culture filtrates (data not shown). This dominant esterase fraction measured with PNP-butyrate as substrate was assumed to be an inducible cutinase.

The esterase was purified to homogeneity by a three step procedure (Table I). The enzyme was concentrated out of the culture filtrate by batch absorption with phenyl sepharose material. The isopropanol eluate from the absorber was partially lyophilized and chromatographed on a Superdex 75 gelfiltration column. The enzyme eluted almost quantitatively in one peak when 40% isopropanol

was added to the buffer used for gelfiltration. Active esterase fractions were pooled, adjusted to 1 M NaCl and applied to a phenyl superose FPLC-column. In this step the esterase eluted as a single protein within the isopropanol gradient. The final enzyme preparation showed a single protein band at 22 kDa in a 15% SDS-Page after coomassie blue staining (Fig. 1b). In addition to PNP-substrates

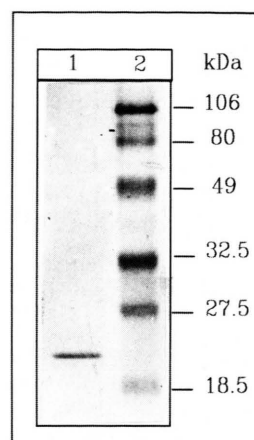


Fig. 1b. Sodiumdodecylsulfate-polyacrylamide gel electrophoresis of cutinase purified from culture filtrate of *Ascochyta rabiei*. Three microgramms were loaded on a gel containing 12.5% polyacrylamide. Gels were stained with coomassie. Lane 1: purified cutinase, lane 2: molecular weight marker.

the purified esterase as well as crude and partially purified protein preparations were also highly active when measured with [ $^3$ H]-cutin. The labelled reaction products were separated by TLC and shown to be identical with those liberated by purified cutinase from *Fusarium solani* (data not shown). Such data prove that the purified esterase of *A. rabiei* is a cutinase.

Attempts to obtain the N-terminal amino acid sequence failed because the cutinase was not accessible to automated Edman degradation using an Applied Biosystems A 470 machine.

Table I: Purification scheme of the cutinase from *A. rabiei*.

Chromatographic step	Volume [ml]	Protein [mg/ml]	Protein [mg]	Activity [nkat/mg protein]	Activity [nkat]	Yield %	Purification -fold
Culture filtrate	1000	0.025	25	124	3100	100	1
Phenyl-Sepharose (Batch)	15	0.025	4.1	454	1860	60	3.7
Superdex 75 (Gel filtration)	45	0.012	0.56	1821	1020	33	14.7
Phenyl-Superose (FPLC)	6	0.012	0.11	3364	370	12	27.1

Cutinase did not bind to concanavalin A sepharose (data not shown). We also failed to label cutinase on western blots with con A peroxidase and therefore assume that cutinase contains only a small sugar portion or it is not a glycoprotein. It is also remarkable that no ion-chromatography steps were possible with cutinase because no detectable enzyme activity could be retained. The reason for this phenomenon is not understood; all tested gel-materials (MonoS, MonoQ, MonoP, Pharmacia; DEAE EMD650, Merck) led to complete inactivation of the enzyme.

The  $K_m$ -value of the cutinase was 0.35 mM with PNP-butyrate and 0.9 mM with PNP-acetate. The hyperbolic activity curve was only weakly expressed with the latter substrate. The pH-optimum of *A. rabiei* cutinase was determined with [ $^3$ H]-cutin and two other model substrates (PNP-butyrate, umbelliferyl-butyrate) of cutinase. The curves were very similar with maximal activities around pH 8 (Fig. 2). The absorption of PNP at 405 nm, liberated from PNP-butyrate, is strongly pH dependent whereas the fluorescence (excitation 333 nm) of umbelliferone at 466 nm, liberated from the corresponding butyrate ester, is less variable with different pH values. All activities were pH-corrected with appropriate correction factors.

Cutinase belongs to the enzyme class of serine esterases and therefore it is strongly inhibited by organophosphorous compounds. We have tested three different cutinase inhibitors that differ in the side chain of the phosphorous esters (Fig. 3). Inhibition of cutinase was extremely pronounced with MAT 9564 (with a C4-side chain) where the molar ratio of inhibitor to cutinase was less than 5 fold. The same ratio in the inhibition pattern shown in Fig. 3, was found for the cutinase of *Fusarium solani* (D. Berg, Bayer AG, pers. communication) but the cutinase of *A. rabiei* is approximately 50 fold more sensitive to the inhibitors.

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#### Cloning of the cutinase gene

The cutinase gene was isolated from a genomic cosmid library using two different consensus oligonucleotides as probes (see Material and Methods). Upon screening with OnI approximately 20 clones were obtained out of 10,000 clones checked. Comparison of DNA restriction patterns and Southern blot analyses revealed four different groups of cosmid clones, all hybridizing to OnI (Fig. 4). This mini library with four representative cosmid

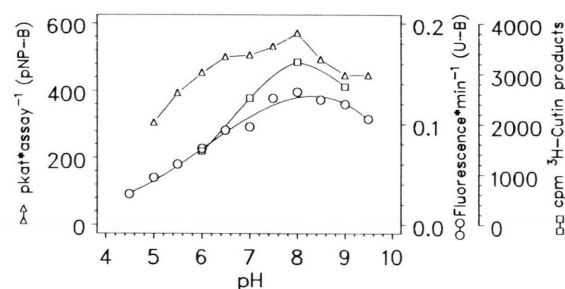


Fig. 2. pH-optimum of the *A. rabiei* cutinase determined with three different substrates, [ $^3$ H]-cutin ( $\square$ - $\square$ ), PNP-butyrate ( $\Delta$ - $\Delta$ ) or umbelliferyl-butyrate ( $\circ$ - $\circ$ ). The assays were performed in 0.1 M Tris-maleate or Tris-phosphate buffers. The absorption coefficients of the liberated *p*-nitrophenol and the fluorescence of the liberated umbelliferone were pH-corrected.

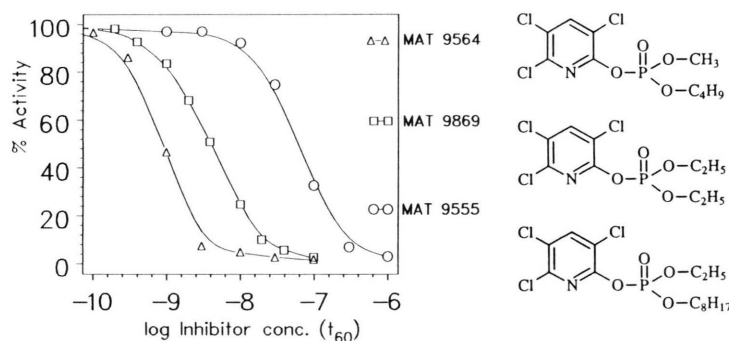


Fig. 3. Inhibition of the *A. rabiei* cutinase by three different organophosphorous compounds. (MAT 9564, ( $\Delta$ - $\Delta$ ), MAT 9869, ( $\square$ - $\square$ ) and MAT 9555, ( $\circ$ - $\circ$ )). The cutinase was preincubated with the appropriate amount of inhibitor for 60 min at room temperature and the remaining activity was measured in a standard enzyme assay with PNP-butyrate as substrate. All assays contained a final concentration of 5% isopropanol.

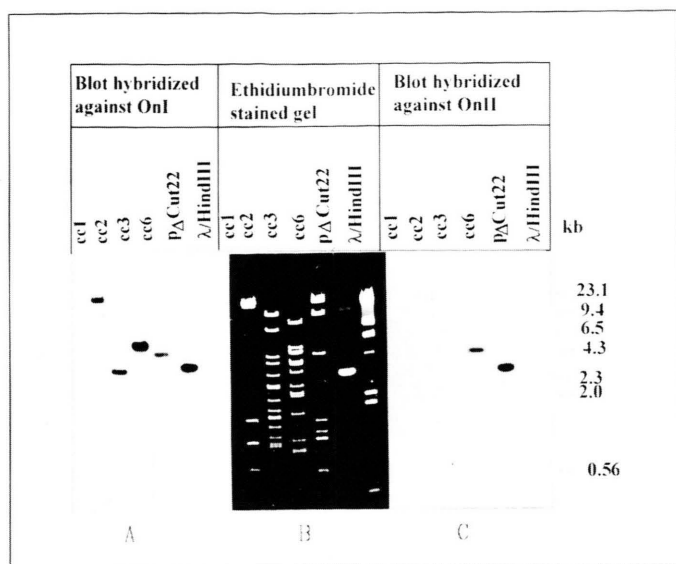


Fig. 4. Hybridization of the restricted cosmid clones cc1 (BamHI), cc2 (SalI), cc3(Hind III), cc6 (Hind III) and a PCR-fragment (pΔCut22) from the *Fusarium solani* cutinase gene (Stahl and Schäfer, 1992) with two different oligonucleotides (On I (A) and On II (C)). The oligonucleotides were labeled with [ $^{32}$ P]-ATP. All clones hybridized to On I, but only cc6 from *A. rabiei* and the PCR-fragment from *F. solani* hybridized to OnII.

clones was further screened with OnII and now only cc6 was labelled with both oligonucleotides (Fig. 4c). The cutinase gene was subcloned out of the 40 kb cosmid insert of cosmid clone cc6 on a 4.1 kb HindIII fragment into pT<sub>7</sub>T<sub>3</sub> standard vector. A detailed restriction map (Fig. 5) allowed to construct a set of different small subclones. The cutinase gene could be detected within the DNA fragments by hybridization with OnI or OnII. The 4.1 kb HindIII fragment was finally subcloned to a 1.7 kb SalI/HindIII construct and called pCutAr. The fragment was sequenced and found to contain the cutinase gene, 0.5 kb of the promotor and additional 0.4 kb of terminator sequences. DNA sequencing of pCutAr further showed one open reading frame for a 223 amino acid protein with a  $M_r$  of 23,423 kD (Fig. 6). The cutinase signal pep-

tide is cleaved between amino acid 15 and 16 as indicated by computer prediction (Von Heijne, 1986). This would result in a mature protein with a  $M_r$  of 21.8 kD and this value is in good agreement with the 22 kD found in SDS-PAGE.

The cutinase gene is interrupted by a 59 bp intron identified by homology comparison and consensus 5'-and 3'-sequences. The 5'-intron sequence is GTATGC (fungal consensus = GTANGT) and the intron is terminated by CAG (consensus = PyAG). The position of the intron is exactly conserved as in other fungal cutinase genes behind the amino acid sequence GNB (GNL in *F. solani*, Ettinger *et al.*, 1987) at position 64 of the *A. rabiei* amino acid sequence (Fig. 6). The translated amino acid sequence contained both consensus peptides which were used to design the oligonucleotides (underlined in Fig. 6). The PROSITE pattern for the active serine (GGYSQG) is also identical within the cutinase sequence from *A. rabiei*.

A possible alignment of five cutinase amino acid sequences is shown in Fig. 7. Except for the precursor sequence the fungal cutinases show high overall homology within the amino acid sequence despite of the different enzyme properties of the various cutinases.

During chromatographic separations one dominant isoform of cutinase was detected. Identical results were obtained from Southern blot analyses

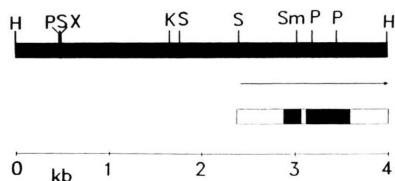


Fig. 5. Restriction map of a 4.1 kb HindIII fragment from the cosmid clone cc6, containing the cutinase gene from *A. rabiei*. A SalI/HindIII-fragment was further subcloned into a pT<sub>7</sub>T<sub>3</sub> vector leading to pCutAr (1.7 kb). The cutinase consists of two exons indicated by the two dark boxes in the second bar. (H: HindIII, K: KpnI, P: PstI, S: SalI, Sm: SmaI, X: XbaI).

1	TCGACGACAGCAAGTATTGTTGATCATGCAGTAAGAATAAGGTTTCGTCGTCTATCTTAG	60
61	CATAACGATGATCACAACCTTCGCATGAACGCACTCCAAACGATCATGCGCCGGTCCCTCC	120
121	CGTACCCTGATCCATGCACGGCATTGCACGGTACTTACCAGCTCCCAAGCGCTGAAACGC	180
181	GGCGCCTGTCCACTTCATCGTCATTCTGGCTCGAGGAGACGACGAACCGTAGAAGGGTA	240
241	TGCTTGTGGTCGATGACGACTTCGTCTAAGCTCTCTTGAATGGGGGATTGAGGCCTGAA	300
301	CCTTTGTTTTTCACTACCCTACCAGGGCGGGGCCAAGTGGGGCGGGATGATGGTGTCCG	360
361	TGATGTTGGGTCTGGAGGTTGTAGACATTGGATAAGTATAGCTTGAGGATTTTCCCCGTT	420
421	TACTCTATCATGGTCAAGAGTAACATCAAAGGGAAATTCAGCCCACACTCGCTCTCTTCT	480
481	TACCTTGTTTTGTCCATATCATCGCCTGCATCATGAAGTTTTTTCGCTTTCTCCATGCTTA	540
1	M K F F A F S M L I	10
541	TTGGCGAGGCCCTCGCCAATCGTACTGGCCCTGCGGCGGACAACGCTGGAAGTCCGCCAGC	600
11	G E A S P I V L A L R R T T L E V R Q L	30
601	TCGACCCGATCATCAGGTGAGABTCGAGCAGGGCAGCAGCTCGAGCTGTCCAAAGGCCA	660
31	D P I I R S E L E Q G S S S S C P K A I	50
661	TCTTAATTTTTCGCCGGGGCAGCACCAGATCGGCAATATGGTATGCCCTACTCACCTG	720
51	L I F A R G S T E I G N M	63
721	CACCCACACACATGCACGTCTTCTAACACTTACACCAGGGGTCTCCGCCGGGCCGGC	780
64	G V S A G P A	70
781	CGTGGCCAGCGCCCTCGAGGCCTATGGCGCCGACCAATCTGGGTCCAAGGCGTCGGCGG	840
71	V A S A L E A Y G A D Q I W V O G V G G	90
841	CCCCTACACTGCAGATCTCCCTCCAACTTCTGCCCAGGCGGCACCTCGCAATCCGCCAT	900
91	P Y T A D L P S N F L P G G T S Q S A I	110
901	CAACGAAGCGGTGCGCCTGTCAACGAAGCCAAACCAATGCCCTCGACCCCATCGT	960
111	N E A V R L F N E A N T K C P S T P I V	130
61	GGCCGGCGGATACAGCCAGGGAACGGCCGTCATGGCCGGCGCAATCCCCAACTCGACGC	1020
131	A G G Y S Q G T A V M A G A I P K L D A	150
1021	CGTGCAGCGCCAGGTCGTGGGCACGGTGTCTGTTGGGTACACGCAGAACCAGCAGAATAA	1080
151	V R A R V V G T V L F G Y T Q N Q Q N N	170
1081	CAAGGGGATCAAAGATTACCCCCAGGAGGATCTGCAGGTGTATTGTGAAGTGGGCGATCT	1140
171	K G I K D Y P Q E D L Q V Y C E V G D L	190
1141	GGTTGTGACGGTACGCTGATCATCACTGTGTGCACTTTTGTACTTGGAGGAGGCTGC	1200
191	V C D G T L I I T V S H F L Y L E E A A	210
1201	GGGACCGGCGCGGAGTTTCTGAAGAGCAAGATTGGGGCTTGAGCTTGAGCTTGAGCTTG	1260
211	G P A P E F L K S K I G A	223
1261	AGCTTGAGCTTGAGCTTGAGCTTGGGGTTAGGGTTGGGGTTGTCTGAACTCAAGGGCTAG	1320
1321	GAGATGACGGTGGGAGGCAGGAGATTCCGATAGGGGCATGACGGGGTTGGATGGGGGCT	1380
1381	TTCTCTTCTCCTCTTTAGCGTCTTCATTTCTATAATATTTACTGACTCATGACATGTTGA	1440
1441	CGTTGGCTGGCTAGCCTCAAACGCCACTTTCTACCTGCTCCGTGTACTGAGTTGATGTTT	1500
1501	TGAAAGAATGGTAAAGTGAGAGGTTGAGAACGATTCTACACAGACCCCTAAGACTCATGTG	1560
1561	TTTTGATGAATGGAATTTGACTGTCAATCTTTGTAGTCTACGATCGAGATTGCAAGCTAC	1620
1621	TAGCTTATCGTAGTGAGTCGCACTCAAGGACCATGTTTTGTA	1662

Fig. 6. Sequence of pCutAr containing the cutinase gene from *A. rabiei*. The gene consists of two exons (513 to 701= exon I and 761–1240=exonII) that are interrupted by a 59 bp intron (702–760). The consensus sequences used to design the oligonucleotides OnI and OnII are underlined as well as possible promotor elements.



Ar	MKFFAF-SMLIGE--ASPI-VLALRRTT---LEVRQLDPIIRSELEQGSS	43
Fs	MKFFALTTLAATASALPTSNPAQE-----LEARQLGRTRRDDLINGNS	44
Cc	MKFLSI-ISLAVSLVAAA-PVEVLDTGVANLEARQ--SSTRNELESGSS	46
Cg	MKFLSV-LSLAITL-AAAAPVEVE--TGVA-LETRQ--SSTRNELETGSS	53
Mg	MQFITVALTLIALALASPIATNVEKPSE---LEARQL-NSVRSDLISGNA	46
Ar	SSCPKAILIFARGSTEIGNMGVSAGPAVASALE-AYGADQIWVQGVGGPY	92
Fs	ASCADVIFIYARGSTETGNLG-TLGPSIASNLESAGKDGWVIQGVGGAY	93
Cc	SNCPKVIYIFARASTEPGNMGISAGPIVADALESRYGASQVWVQGVGGPY	96
Cg	SACPKVIYIFARASTEPGNMGISAGPIVADALERIYGANNVWVQGVGGPY	93
Mg	AACPSVILIFARASGEVGNMGLSAGTNVASRLEREF-RNDIWVQGVGDPY	95
Ar	TADLPSNFL-PGGTSQSAINAEAVRLFNEANTKCPSTPIVAGGYSQGTAVM	141
Fs	AATLGDNAL-PRGTSSAIREMLGLFQQANTKCPDATLIAGGYSQGAALA	142
Cc	SADLASNFIIEGTSRVAINAEAKRLFTLANTKCPNSAVVAGGYSQGTAVM	146
Cg	LADLASNFL-PDGTSSAINEARRLFTLANTKCPNAAIVSGGYSQGTAVM	142
Mg	DAALSPNFL-PAGTTQGAIDEAKRMFTLANTKCPNAAVVAGGYSQGTAVM	144
Ar	AGAIPKLD-AVRARVVGTVLFGYTQNNQNNKGIKDYPOEDLQVYCEVGBL	190
Fs	AASIEDLDSAIRDKIAGTVLFGYTKNLQNRGRIPNYPADRTKVFCNTGDL	192
Cc	ASSISELSSTIQNQIKGVVLSAITKNLQNLGRIPNFSTSKTEVYCALADA	196
Cg	AGSISGLSTTIKNQIKGVVLFYTKNLQNLGRIPNFETSKTEVYCDIADA	192
Mg	FNAVSEMPAAVQDQIKGVVLFYTKNLQNRGRIPDFPTEKTEVYCNASDA	194
Ar	VCDGTLIITVSHFLYLEEAAGPAPEFLKSKIGA	223
Fs	VCTGSLIVAAPHLAYGPDARGPAPEFLIEKVRVARGSA	230
Cc	VCYGTFLFILPAHFLYQADAATSAPRFLAARIG	228
Cg	VCYGTFLFILPAHFLYQTDAAVAAPRFLQARIG	224
Mg	VCFGTLFLLPAHFLYTTTESSIAAPNWLIRQIRAA	228

Fig. 7. Alignment of five cutinase amino acids sequences from phytopathogenic fungi. The precursors of these exo-proteins are variable. In contrast, the mature proteins show high homology (shaded sequences) in all parts of the enzyme. (Ar, *Ascochyta rabiei*; Fs, *Fusarium solani*; Cc, *Colletotrichum capsici*; Cg, *Colletotrichum gloeosporioides*; Mg, *Magnaporthe grisea*).

of genomic DNA, restricted with HindIII, KpnI and XbaI. In all cases only a single DNA fragment was labelled upon hybridization with the internal Pst I fragment of the cutinase gene (Fig. 8). This indicates a single copy gene of cutinase in *A. rabiei*. These results were confirmed with five additional fungal isolates (IV, VI, VII, X, XXI), all showing the same hybridization pattern as *A. rabiei* isolate III (data not shown).

## Discussion

The phytopathogenic fungus *A. rabiei* secretes a cutinase into the culture filtrate, when grown with cutin or hydroxylated fatty acids, the major monomeric structures of cutin. Although the composition of cutin is species- and development - dependent (Riederer and Schönherr, 1988)  $\omega$ -hydroxylated fatty acids have been identified as the major compounds of cutin. The 16-hydroxy-

hexadecanoic acid was shown to be a better inducer of cutinase than the  $C_{18}$ -ricinoleic acid, whereas structurally related compounds (see Material and Methods) failed to induce any cutinase activity. The induction pattern of the *Fusarium solani* cutinase (Podila *et al.*, 1988) was similar for the fatty acids that were tested in both fungal systems. Podila *et al.*, (1988) have identified trans-acting protein factors in nuclear run off experiments that bind to DNA and specifically activate cutinase transcripts in the presence of hydroxylated fatty acids. A 135 bp fragment of the cutinase promotor was shown to bind the transcription factors (Bajar *et al.*, 1991).

We were able to purify the cutinase from *A. rabiei* in a three step procedure yielding an active enzyme with a  $M_r$  of 22 kD. This size is in good agreement with the cutinases of *F. solani* (Purdy and Kolattukudy, 1975), and *Colletotrichum gloeosporioides* (Dickmann *et al.*, 1982).

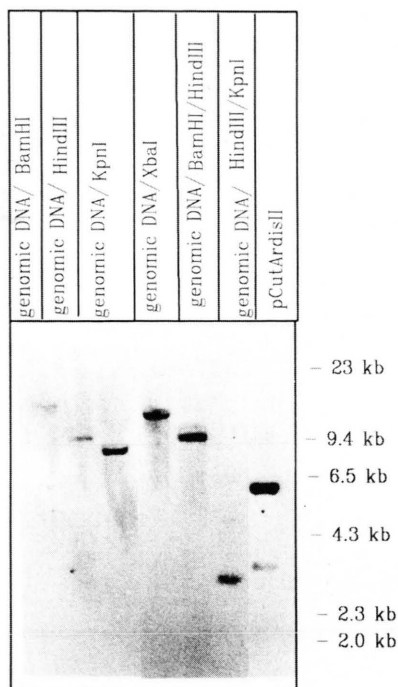


Fig. 8. Southern hybridization with genomic DNA from *A. rabiei* XXI. The DNA was totally restricted with the enzymes BamHI, HindIII, KpnI, or XbaI (lanes 1–4). Lanes 5 and 6 present a double restriction with the enzymes BamHI/HindIII and HindIII/KpnI. The blott was probed against the internal PstI-fragment (0.27 kb, cf. Fig. 5, restriction map of pCutAr) of the cutinase gene. In all single and double restrictions, only one band hybridized to the gene probe, indicating a single copy gene.

The purified cutinase represents the predominant esterase activity in the culture fluid of *A. rabiei*. The two minor bands detected by indoxyl staining on IEF gels (Fig. 1) occurred in only very low amounts in relation to the cutinase. Therefore, their enzyme specificity and their functional role cannot be evaluated.

Many different agrochemicals have been used to study cutinase because this serine esterase may be easily inhibited by organophosphorous compounds (Fig. 3). Köller *et al.*, (1982b) and Dickmann *et al.*, (1983), using commercially available phosphorous insecticides, reported on the essential role of the sidechain ester groups at the phosphorous atom. Substitution of the methyl groups in Fospirate (trade name) by ethyl groups led to a 50 fold lower  $K_i$ -value for cutinase inhibition of *F. solani*. An analog with butyl ester groups was, how-

ever, not tested in these studies. Therefore, it remains unclear whether inhibition of cutinase would possibly have been achieved by even lower concentrations as was found for the *A. rabiei* cutinase with the MAT 9564 inhibitor with butyl sidechains versus MAT 9869 with ethyl groups (Fig. 3). In general cutinase prefers molecules with a C4-chain and this preference is corroborated by the inhibition caused by MAT 9564 with a  $K_i$ -value of 0.8 nM. This inhibition is over 200 times more sensitive than the inhibition by nonspecific serine esterase inhibitors such as diisopropylfluorophosphate.

The pH optimum of cutinase as a possible determinant of plant tissue specificity of phytopathogenic fungi is a matter of debate, since this hypothesis was published by Köller and Parker (1989) and Trail and Köller (1990). Leaf pathogenic fungi were supposed to have a cutinase with a slightly acidic pH optimum around pH 6 and root and stem pathogens a cutinase showing optimal activity in the alkaline range between pH 9–10. The cutinase of *A. rabiei* is a clear example against this hypothesis due to its optimum around pH 8. This exception agrees with the cutinase from *Alternaria alternata* which shows optimal activity at pH 9 produced from a fungus attacking the leaves of Japanese pear (Tanabe *et al.*, 1988). Recently, a cutinase with a pH optimum of 9.5 was found in the leaf localized bean rust fungus *Uromyces appendiculatus* (Deising *et al.*, 1992). In summary we assume that there is no direct correlation between the pH optimum of a fungal cutinase and the tissue-specificity of the producing microorganism.

As a prerequisite for further studies on the functional role of *A. rabiei* cutinase we have cloned the cutinase gene employing a simple strategy. Although cutinase genes are highly homologous to each other, heterologous hybridization often did not give clear results (Ettinger *et al.*, 1987). Furthermore an antibody against *F. solani* cutinase did not recognize cutinase from species other than *Fusarium* (Lin and Kolattukudy, 1980). Therefore we have used two oligonucleotides directed against cutinase consensus sequences found in three published cutinase sequences of *F. solani*, *Colletotrichum capsici* and *C. gloeosporioides* (Ettinger *et al.*, 1987) for screening an *A. rabiei* genomic cosmid library. As a result of this screening procedure a single group of cosmid clones was obtained (Fig. 4) which hybridized to both oligonu-

cleotides. The amino acid sequence of the subcloned gene perfectly matched the peptides, used to design the oligonucleotides (Fig. 6). The *A. rabiei* cutinase sequence is homologous to the aforementioned published cutinase amino acid sequences (Fig. 7). The position of the 59 bp intron in the *A. rabiei* cutinase gene is also exactly conserved as in all other cutinases (Fig. 6) indicating a fungal ancestor cutinase gene. Only the *M. grisea* gene contains an additional intron.

The typical transcription initiation sites of fungal genes, TATAAA and CAAT, are not found in an identical sequence in the cutinase gene. But a TATA sequence at the position – 116 to – 113 from the translation start may fulfill this function in the *A. rabiei* gene. In addition, a CAAG sequence is in close proximity at position – 178 to – 175. Interestingly the stop codon of the cutinase gene is followed by a TTGAGC motif of unknown function, which is repeated six times at positions 1240 to 1275. Another repeating motif (AGAGG) subsequent to the stop codon was found in *Cladosporium fulvum* by RNA sequencing and this motif is not the polyadenylation site (van Kan *et al.*, 1991). In general the strategy used in this study to clone the cutinase gene from *A. rabiei* may be helpful for cloning cutinases from other fungi; this will fur-

ther contribute to our understanding of the biological role of these enzymes.

In order to better prove the role of cutinase more plant-fungus systems, especially with leaf pathogens, should be investigated. In case of *Ascochyta* blight this question is presently under investigation in our laboratory using *A. rabiei* transformants with a deleted cutinase gene.

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