Cloning and structure determination of cDNA for cutinase, an enzyme involved in fungal penetration of plants

(cDNA cloning/nucleotide sequencing/amino acid sequencing/fungal cutinase)

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ABSTRACT The primary structure of cutinase, an extracellular fungal enzyme involved in the penetration of plants by pathogenic fungi, has been determined from the nucleotide sequence of cloned cDNA. Clones containing cDNA made from poly(A)⁺ RNA isolated from fungal cultures induced to synthesize cutinase were screened for their ability to hybridize with the [³²P]cDNA for mRNA unique to the induced culture. The 75 cDNA clones thus identified were screened for the cutinase genetic code by hybrid-selected translation and examination of products with anti-cutinase IgG. This method yielded 15 clones containing cDNA for cutinase, and Southern blots showed that the size of the cDNA inserts ranged from 279 to 950 nucleotides. Blot analysis showed that cutinase mRNA contained 1050 nucleotides, indicating that the clone containing 950 nucleotides represented nearly the entire mRNA. This near-full-length cDNA and the restriction fragments subcloned from it were sequenced by a combination of the Maxam-Gilbert and the phage M13–dideoxy techniques. cDNAs from two other clones, containing the bulk of the coding region for cutinase, were also completely sequenced, and the results confirmed the sequence obtained with the first clone. A peptide isolated from a trypsin digest of cutinase was sequenced and the amino acid sequence as well as the initiation and termination codons were used to identify the coding region of the cDNA. The primary structure of the enzyme so far determined by amino acid sequencing (≈40% of the total) agreed completely with the nucleotide sequencing results. Thus, the complete primary structure of the mature enzyme and that of the signal peptide region were ascertained.

Cutinase, a glycoprotein excreted by phytopathogenic fungi, catalyzes the hydrolysis of cutin, the structural polyester of the plant cuticle (1, 2). Cutinase was shown to be present at the site of fungal penetration of the host plant cuticle and specific inhibition of cutinase was shown to protect plants against fungal penetration and consequently infection (1, 2). The active site of cutinase is composed of a catalytic triad involving serine, histidine, and a carboxyl group (3). The enzyme contains one disulfide bridge, which is essential for the activity of the enzyme (2). Recently the amino acid sequence of the active-serine-containing tryptic peptide of cutinase was determined (4). An understanding of the structure and mechanism of action of cutinase could help in the development of effective inhibitors for use as antipenetrants to protect plants against fungal attack.

Recent evidence suggested that the infecting capacity of certain pathogenic fungi can be determined by the ability of the pathogen to produce cutinase (5, 6). Thus the regulation of expression of cutinase gene could be highly relevant to pathogenesis. To investigate these molecular aspects of host–pathogen interaction, labeled cDNA probes for cutinase would be highly beneficial. In this paper we report the

cloning of cutinase cDNA and the complete nucleotide sequence of the coding region. The amino acid sequence predicted from the nucleotide sequence was verified by amino acid sequence determination of a significant portion of the enzyme.

MATERIALS AND METHODS

Construction of a *Fusarium solani pisi* cDNA Library. Poly(A)⁺ RNA from *Fusarium solani* f. sp. *pisi*, T8 strain, was isolated as described (7). Single- (8) and double- (9) stranded cDNA was synthesized and the hairpin loops were cleaved with nuclease S1 and the termini were repaired to blunt ends with the large fragment of DNA polymerase I. A size fraction of duplex DNA >500 base pairs (bp) in length was obtained by preparative electrophoresis in a 1% agarose gel. Homopolymer tracts of dC were added to the 3' ends of this cDNA with calf-thymus terminal transferase (10). The oligo(dC)-tailed cDNA and oligo(dG)-tailed pBR322 plasmid were annealed (11), and this modified plasmid was used to transform *Escherichia coli* K-12 RR1. Colonies that contained pBR322 plasmid with cDNA inserts were selected by their tetracycline resistance and ampicillin sensitivity. The enzymes and plasmids used in this cloning procedure were purchased from New England Nuclear.

Isolation of Plasmid DNA. Plasmid DNA, extracted from selected clones by using either a Triton X-100 (12) or an alkaline lysis procedure (13), was purified by CsCl/ethidium bromide density gradient centrifugation.

Translation of Hybrid-Selected mRNA. From the total poly(A)⁺ RNA isolated from induced fungal cultures, specific mRNA was purified by hybridization to cloned DNA that had been denatured and immobilized on nitrocellulose filters (14). The translation products of the hybridized mRNA generated in a wheat germ translation system (15) with [³⁵S]methionine as the label were examined (7).

Colony Hybridizations. Colony hybridization was performed with [³²P]cDNA probes (16, 17). Two cDNA probes were generated from poly(A)⁺ RNA isolated from induced and noninduced fungal cultures with reverse transcriptase and [α -³²P]dATP (17). Hybridization probes were also made from cDNA inserts isolated from cutinase clones that responded positively to the hybrid-selected translation tests indicated above. Plasmid isolated from a cutinase cDNA clone was treated with *Pst* I restriction endonuclease and the digest was subjected to electrophoresis in 1% low-melting agarose. The agarose containing the DNA insert was cut from the gel and melted in 2 vol of 0.01 M Tris-HCl, pH 7.5, containing 0.001 M EDTA at 65°C, and the agarose was removed from the solution by phenol extraction. After the addition of NaCl to 0.2 M and 2 vol of ethanol, the DNA was precipitated by cooling the solution to –70°C for 15 min. A ³²P-labeled probe with a specific activity in excess of 10⁸

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Abbreviation: bp, base pair(s).

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dpm/ μ g was prepared by subjecting 1 μ g of the DNA insert to a nick-translation reaction with 60 μ Ci (1 Ci = 37 GBq) of [α - 32 P]dATP (800 Ci/mmol, New England Nuclear) as described (18).

Blot Hybridization Analysis of Inserted DNA and of Fungal RNA. Plasmid was isolated from cutinase cDNA clones by a rapid miniscreen procedure described for determination of the size of the inserted DNA (13). Restriction enzyme digests of the plasmid preparations were subjected to electrophoresis through 1% agarose gels, transferred to nitrocellulose filters by the method of Southern (19), and hybridized with a 32 P-labeled probe (17). The hybridized filter was subjected to autoradiography and the sizes of the labeled DNA inserts were determined by their mobilities as compared to those of DNA standards.

The size of cutinase mRNA was determined by subjecting 3.5 μ g of poly(A)⁺ RNA isolated from induced fungal cultures to electrophoresis through a 1.5% agarose gel containing 2.2 M formaldehyde (20). The formaldehyde-denatured RNA was transferred from the gel to nitrocellulose filters (21) and hybridized with a 32 P-labeled probe. The location of the hybridized mRNA was determined by autoradiography and the size of the cutinase mRNA was determined by comparison of its mobility to that of known DNA fragments generated by *Alu* I and *Hae* III digestion of pBR322.

Subcloning of DNA Fragments from Restriction Endonuclease Digests of Isolated Cloned Inserts. *Taq* I, *Hpa* II, and *Sau* 3A digests were selected for subcloning in cohesive *Acc* I, *Pst* I-*Acc* I, or *Bam*HI restriction sites of M13 mp10 and M13 mp11 bacteriophage. Conditions of ligation with T4 ligase, transformation of *E. coli* JM103 host, propagation of M13, isolation of both replicative form and single-stranded DNA, and subclone evaluation were as described by Messing and Vieira (22).

DNA Sequence Analysis. Isolated pUC9 plasmid containing the inserted DNA was digested with either *Bam*HI at the 3' end or *Hind*III at the 5' end and labeled with [α - 32 P]dGTP or [α - 32 P]dATP, respectively, by the action of the large fragment of DNA polymerase I (23). The end-labeled DNA insert was subjected to nucleotide sequence analysis by the method of Maxam and Gilbert (24). The single-stranded DNA prepared from the M13 subclones was used for sequence determinations by the dideoxy chain-termination methods of Messing *et al.* (25) and Sanger *et al.* (26), using an M13 pentadecamer primer (New England Biolabs) and reverse transcriptase (27).

Determination of Molecular Weight and Carbohydrate Content of the Enzyme. Molecular weights of the mature enzyme and primary translation product were determined as described (7). Carbohydrate content was measured as before (2).

Amino Acid Sequence Determination of Peptides. A tryptic digest of cutinase was subjected to gel filtration on Sephadex G-50 as previously described (4). The fraction with high absorbance at 280 nm was further purified by preparative HPLC on a 300-Å pore, C₁₈ reverse-phase column (250 × 10 mm, Synchropack RP-P; SynChrom, Linden, IN) with a 20–65% (vol/vol) linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid at a 1.0 ml/min flow. The fraction corresponding to the single peptide peak of high absorbance at 220 nm was collected, and aliquots were subjected to NH₂-terminal analysis (28, 29) and amino acid analysis. Sequence analysis of the peptide was performed by an automated Edman degradation sequencing system as described (4).

RESULTS

Screening of *F. solani pisi* cDNA Library for Possible Cutinase Sequences. Glucose-grown *F. solani pisi* can be induced

to produce cutinase by the addition of low quantities of chemically prepared cutin hydrolysate. Under such conditions cutinase is the major (>70%) extracellular protein (30). Therefore, the poly(A)⁺ RNA from such induced cultures, already enriched in cutinase-specific mRNA, could be readily used to generate cutinase cDNA by reverse transcribing the total poly(A)⁺ RNA. This inducibility was also useful in the initial selection of possible cutinase cDNA clones in the total cDNA library. Replica nitrocellulose filters containing lysed colonies of the fungal cDNA library were hybridized with labeled cDNA probes transcribed from poly(A)⁺ RNA isolated from either cultures induced to produce cutinase or control cultures. From 600 colonies selected as containing recombinant DNA, 75 colonies showed hybridization with the probe generated only from induced cultures. Screening for clones containing cutinase DNA sequences in these 75 colonies was done by hybrid-selected translation. The colonies were pooled into nine groups, and the plasmid DNA isolated from five of these groups hybridized with fungal mRNA that upon cell-free translation generated protein that cross-reacted with anti-cutinase IgG. Electrophoresis of the immunoprecipitated translation product in a NaDodSO₄/polyacrylamide gel revealed a single band with a molecular weight of 24,200 compared to the 21,600 molecular weight of the mature cutinase. Evaluation of the group of colonies that displayed the most efficient hybrid-selected translation showed two individual colonies that contained cutinase DNA sequences inserted in their plasmids. Agarose electrophoresis of the *Pst* I digest of the plasmid isolated from these two cDNA clones (C-16 and C-15) revealed inserts of 380 and 560 bp. The larger insert was isolated and labeled with [α - 32 P]dNTP by nick-translation for use as a probe to screen the other clones for cutinase sequences. DNA liberated from lysed colonies of the 75 clones was hybridized with the probe and 15 colonies appeared to contain cutinase recombinant cDNA (Fig. 1).

Selection of a Cutinase cDNA Clone for Sequencing. The lengths of the cloned inserts were determined by Southern blots of *Pst* I digests of isolated plasmid DNA from each clone (Fig. 2). The radioactive probe hybridized with cDNA inserts showing that the following clones had inserts of the sizes indicated by the number of base pairs in parentheses: C-4 (730), C-7 (815), C-8 (430), C-10 (795), C-15 (560), C-16 (380), C-30 (590), C-31 (950), C-32 (738), C-33 (738), C-34 (788), C-45 (279), C-49 (535), C-57 (770), and C-74 (700).

The cutinase cDNA probe was also used to locate cutinase

FIG. 1. Colony hybridization of clones containing cDNA unique to cultures induced to synthesize cutinase. The 75 such clones were numbered sequentially from left to right starting from the top, C-1 to C-75. The probe used was a 560-bp cloned cDNA for cutinase that was labeled with 32 P by nick-translation.

FIG. 2. Southern blot of *Pst* I restriction endonuclease digests of plasmids isolated from seven of the cDNA clones shown in Fig. 1. The clones used are from left to right, C-10, C-8, C-34, C-31, molecular weight marker, C-30, C-45, C-53, and C-49. Clone C-53, which did not hybridize with the probe in the colony hybridization, was included as a control. Hybridization was with a plasmid containing a cutinase cDNA insert 32 P-labeled by nick-translation. The center lane is an *Alu* I digest of the pBR322 plasmid, which generates fragments of the base pair sizes indicated at the right.

mRNA in a blot of electrophoretically separated poly(A)⁺ RNA isolated from induced fungal cultures. From a rectilinear semilogarithmic plot of the mobility of standards vs. their molecular size, the cutinase mRNA was determined to be 1050 nucleotides in length (Fig. 3). Thus, the largest cutinase cDNA clone (C-31), which contains 950 bp, represents nearly the full length of the mRNA, and thus it was selected for the first DNA sequence analysis. For confirmation of the cutinase cDNA sequence, clones C-4 and C-57, which contain 730 and 770 bp, respectively, were used.

Nucleotide Sequence of cDNA and Amino Acid Sequence of Cutinase. The cutinase recombinant cDNA was removed from pBR322 plasmid by *Pst* I restriction endonuclease and subcloned in the *Pst* I site of the more convenient pUC9 vector. This vector provided a *Bam*HI site just ahead of the 5' end of the insert and an *Hind*III site just behind the 3' end,

FIG. 3. Molecular size of cutinase mRNA as determined from a blot hybridization. The electrophoretic mobilities of DNA fragments of *Alu* I and *Hinc*II digests of pBR322 plasmid were plotted with their known molecular sizes on a logarithmic scale. The broken line represents the extrapolated size of cutinase mRNA (1050 nucleotides) from its measured electrophoretic mobility.

and these sites could be individually end-labeled for sequence analysis by the method of Maxam and Gilbert (24). The sequences of the first 250 nucleotides at the 5' end and about 200 nucleotides at the 3' end were determined.

A strategy for sequencing the remainder of the cutinase cDNA insert was devised after detailed restriction endonuclease mapping of the insert with a battery of enzymes (Fig. 4). *Alu* I, *Ava* I, *Hae* III, *Hpa* II, *Hinf* I, *Sau*3A, and *Taq* I cleaved the isolated insert, whereas *Bam*HI, *Bgl* II, *Eco*RI, *Hinc*II, *Hind*III, *Kpn* I, *Sal* I, and *Xba* I did not. Treatment of the insert with *Taq* I and *Hpa* II produced three fragments each, which could be completely sequenced, and *Sau*3A provided fragments for the necessary confirmatory overlapping sequences. *Taq* I, *Hpa* II, and *Sau*3A cutinase cDNA fragments were subcloned in M13 mp10 or M13 mp11 bacteriophage-producing vectors, and the extruded single-stranded DNAs of the selected subclones were sequenced by the method of Sanger *et al.* (26). By overlapping sequences from the subclones and from the end-labeling sequence analysis, the complete nucleotide sequence of the cutinase cDNA insert was constructed (Fig. 5).

An initiation codon and a termination codon were found 702 nucleotides apart in the sequence. This reading frame translated into a protein with a molecular weight of 23,951 and with the amino acid sequence shown in Fig. 5. To verify this sequence the amino acid sequence of a portion of the protein was determined directly. Since the protein contains only one tryptophan, we could identify the tryptophan-containing peptide by its absorbance at 280 nm. An HPLC peptide map of trypsin-digested cutinase showed a highly UV-absorbing peptide and Sephadex G-50 gel filtration gave a fraction enriched in this peptide. Preparative HPLC employing a *C*₁₈ reverse-phase column was used to isolate the UV-absorbing peptide (Fig. 6). Analytical HPLC of the purified peptide showed a single peak and NH₂-terminal analysis showed glycine as the only NH₂-terminal amino acid. The complete amino acid sequence of 37 residues contained in this peptide was determined by automated Edman degrada-

FIG. 4. Restriction map and strategy for sequence determination of the cutinase cDNA insert isolated from the selected clone. (A) cDNA from clone C-31, beginning at the *Pst* I site of the 5' untranslated region (5'-UT). The cDNA was originally inserted into the *Pst* I site of a pBR322 vector and later transferred into the *Pst* I site of a pUC9 vector, which contains the indicated *Hind*III and *Bam*HI restriction sites. (B) Restriction enzyme map of the C-31 cDNA insert. (C) Strategy for sequencing the entire cDNA insert. Arrows indicate direction and extent of sequence determination.

AACACACATACCTTCACTTCATCAACATTCACTTCACTTCTTCGCTCTTCCTTTCACTCTTTATCATCCTCACC

MET LYS PHE PHE ALA LEU THR THR LEU LEU ALA ALA THR ALA SER ALA LEU PRO THR SER
 ATG AAA TTC TTC GCT CTC ACC ACA CTT CTC GCC GCC ACG GCT TCG GCT CTG CCT ACT TCT

ASN PRO ALA GLN GLU LEU GLU ALA ARG GLN LEU GLY ARG THR THR ARG ASP ASP LEU ILE
 AAC CCT GCC CAG GAG CTT GAG GCG CGC CAG CTT GGT AGA ACA ACT CGC GAC GAT CTG ATC

ASN GLY ASN SER ALA SER CYS ARG ASP VAL ILE PHE ILE TYR ALA ARG GLY SER THR GLU
 AAC GGC AAT AGC GCT TCC TGC CGC GAT GTC ATC TTC ATT TAT GCC CGA GGT TCA ACA GAG

THR GLY ASN LEU GLY THR LEU GLY PRO SER ILE ALA SER ASN LEU GLU SER ALA PHE GLY
 ACG GGC AAC TTG GGA ACT CTC GGT CCT AGC ATT GCC TCC AAC CTT GAG TCC GCC TTC GGC

LYS ASP GLY VAL TRP ILE GLN GLY VAL GLY GLY ALA TYR ARG ALA THR LEU GLY ASP ASN
 AAG GAC GGT GTC TGG ATT CAG GGC GTT GGC GGT GCC TAC CGA GCC ACT CTT GGA GAC AAT

ALA LEU PRO ARG GLY THR SER SER ALA ALA ILE ARG GLU MET LEU GLY LEU PHE GLN GLN
 GCT CTC CCT CGC GGA ACC TCT AGC GCC GCA ATC AGG GAG ATG CTC GGT CTC TTC CAG CAG

ALA ASN THR LYS CYS PRO ASP ALA THR LEU ILE ALA GLY GLY TYR SER GLN GLY ALA ALA
 GCC AAC ACC AAG TGC CCT GAC GCG ACT TTG ATC GCC GGT GGC TAC AGC CAG GGT GCT GCA

LEU ALA ALA ALA SER ILE GLU ASP LEU ASP SER ALA ILE ARG ASP LYS ILE ALA GLY THR
 CTT GCA GCC GCC TCC ATC GAG GAC CTC GAC TCG GCC ATT CGT GAC AAG ATC GCC GGA ACT

VAL LEU PHE GLY TYR THR LYS ASN LEU GLN ASN ARG GLY ARG ILE PRO ASN TYR PRO ALA
 GTT CTG TTC GGC TAC ACC AAG AAC CTA CAG AAC CGT GGC CGA ATC CCC AAC TAC CCT GCC

ASP ARG THR LYS VAL PHE CYS ASN THR GLY ASP LEU VAL CYS THR GLY SER LEU ILE VAL
 GAC AGG ACC AAG GTC TTC TGC AAT ACA GGG GAT CTC GTT TGT ACT GGT AGC TTG ATC GTT

ALA ALA PRO HIS LEU ALA TYR GLY PRO ASP ALA ARG GLY PRO ALA PRO GLU PHE LEU ILE
 GCT GCA CCT CAC TTG GCT TAT GGT CCT GAT GCT CGT GGC CCT GCC CCT GAG TTC CTC ATC

GLU LYS VAL ARG ALA VAL ARG GLY SER ALA @@@ GGAGGATGAGAATTTAGCAGGCGGGCCTGTTAAT
 GAG AAG GTT CCG GCT GTC CGT GGT TCT GCT TGA

TATTGCGAGGTTTCAAGTTTTCTTTTGGTGAATAGCCATGATAGATTGGTTCAACACTCAATGTACTACAATGCCTCCC

CCCCCCCCCCCC

FIG. 5. Nucleotide sequence of the cutinase cDNA clone C-31 and its translation into amino acid sequence from an ATG start codon to a termination codon. The portions of the sequence confirmed by amino acid sequencing of the peptides are indicated by the bold lines. The active serine, two cysteines labeled by iodoacetamide, and the single tryptophan residue are underlined. Residue 32, glycine, is thought to be the NH₂ terminus of the mature protein. C-57 and C-4 indicate the beginning of the cDNA sequences of two additional clones which were fully sequenced.

tion. This amino acid sequence matched precisely with a segment of the amino acid sequence predicted from the cDNA nucleotide sequence.

To confirm the results obtained with clone C-31 and to test whether the mRNA population is homogeneous, inserts from two other clones, C-4 and C-57, were sequenced. In this case

the clones were chosen in such a way that the 5' end was within the coding region of the presumed signal sequence. The restriction enzymes used for subcloning these two inserts were the same as those used for C-31. The entire nucleotide sequence of clones C-4 and C-57 was in complete agreement with that obtained with clone C-31. Although the relationships among the different forms of the enzyme isolated from the same species of fungus remain obscure, the cloning and sequencing described here firmly establish the primary structure of one major isozyme. No microheterogeneity could be detected.

DISCUSSION

It is generally agreed that extracellular proteins require an NH₂-terminal sequence that functions as a signal for recognition by the appropriate components involved in secretion of the protein and is cleaved off during protein transport. The NH₂-terminal residue of extracellular cutinase was determined to be glycine with its amino group linked to glucuronic acid by an amide bond (31). From the amino acid sequence deduced from the nucleotide sequence, the first glycine in the primary translation product would be at position 32; this indicates that the signal sequence is 31 residues long, with a molecular weight of 3317. This value is in agreement with that calculated from the molecular weights of the primary translation product (24,200) and mature extracellular enzyme (21,600), taking into account its 4% carbohydrate content. In most other signal sequences there is an NH₂-terminal hydrophilic segment that varies from 1 to 7 residues in

FIG. 6. (Left) Preparative HPLC of the highly UV-absorbing fraction obtained from Sephadex G-50 gel filtration of a trypsin digest of cutinase (7). HPLC conditions were described in the text. (Right) Analytical HPLC of the purified peptide.

length and usually contains a basic amino acid that precedes the central hydrophobic core of at least 12 residues (32, 33). Cutinase signal sequence contains lysine as the second amino acid followed by 15 hydrophobic residues that comprise the hydrophobic core. The remaining 14-residue segment of the postcore sequence is much longer than the normally observed 4–8 residues (33) and the model signal-sequence cleavage site (34) is not evident. Perhaps the unique processing of the NH₂-terminus of cutinase that might involve introduction of a glucuronamide, possibly by a transpeptidation reaction (2), requires a slightly unusual postcore segment. However, until these post-translational events are elucidated, the functional significance, if any, of this region of the signal peptide cannot be determined.

The primary structure of cutinase deduced from the nucleotide sequence was verified by direct amino acid sequencing of a considerable portion of the protein. The sequence of 37 amino acid residues around the only tryptophan (residue 85) contained in this enzyme was matched exactly with the amino acid sequence predicted from the nucleotide sequence of the cDNA, and this region was near the NH₂-terminus of the enzyme. The amino acid sequence around the active serine (residue 136) involved in catalysis had previously been determined (4). This sequence of 30 amino acid residues matched exactly with another region of the amino acid sequence predicted from nucleotide sequencing, and this region was located toward the middle of the polypeptides. A cysteine-containing peptide was recently isolated and the sequence of 10 residues around this cysteine (residue 187) was determined (unpublished results). This sequence matched with the predicted amino acid sequence of a region near the COOH terminus. These three segments are indicated in Fig. 5. Excluding the NH₂-terminal 31 residues that presumably form the "signal" region, which is cleaved off during post-translational processing, the enzyme should contain 199 amino acid residues. Within this region almost 40% of the amino acid sequence predicted from the nucleotide sequence was already verified by direct determination of the amino acid sequence of the protein. Thus, there is little doubt that the cloned cDNA represents the authentic cutinase gene. These cDNA clones for cutinase should be very useful as probes to study the regulation of cutinase synthesis.

Comparison of the primary structure of cutinase with that of other hydrolytic enzymes containing the classical active serine catalytic triad revealed no significant homology. As observed for most active serine enzymes, the serine residue in cutinase is also a considerable distance away from the histidine (residue 204) of the triad (35). The disulfide bridges play an important role in maintaining the catalytically active structure of other active serine enzymes and in the present enzyme the only disulfide bridge contained in it is also essential for catalytic activity.

One discrepancy between the nucleotide sequence of the cutinase cDNA and the chemical information concerning the mature enzyme involves the number of potential SH groups. The native mature enzyme contains no free SH groups and upon reduction with dithioerythritol two SH groups are found (2). Nucleotide sequence shows two additional cysteine codons (residues 47 and 194). The occurrence of these codons was confirmed in three clones; the presence of a single disulfide bridge and absence of any SH groups in the mature enzyme were confirmed by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) and by labeling with radioactive iodoacetamide. Therefore, it appears possible that two cysteine residues were post-translationally modified. However, further work is required to clarify this aspect of the structure of cutinase.

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