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Novel *Coprinopsis cinerea* Polyesterase That Hydrolyzes Cutin and Suberin▽†

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Three cutinase gene-like genes from the basidiomycete *Coprinopsis cinerea* (*Coprinus cinereus*) found with a similarity search were cloned and expressed in *Trichoderma reesei* under the control of an inducible *cbh1* promoter. The selected transformants of all three polyesterase constructs showed activity with *p*-nitrophenyl-butyrate, used as a model substrate. The most promising transformant of the cutinase CC1G_09668.1 gene construct was cultivated in a laboratory fermentor, with a production yield of 1.4 g liter^{-1} purified protein. The expressed cutinase (CcCUT1) was purified to homogeneity by immobilized metal affinity chromatography exploiting a C-terminal His tag. The N terminus of the enzyme was found to be blocked. The molecular mass of the purified enzyme was determined to be around 18.8 kDa by mass spectrometry. CcCUT1 had higher activity on shorter (C_2 to C_{10}) fatty acid esters of *p*-nitrophenol than on longer ones, and it also exhibited lipase activity. CcCUT1 had optimal activity between pH 7 and 8 but retained activity over a wide pH range. The enzyme retained 80% of its activity after 20 h of incubation at 50°C, but residual activity decreased sharply at 60°C. Microscopic analyses and determination of released hydrolysis products showed that the enzyme was able to depolymerize apple cutin and birch outer bark suberin.

Cutin and suberin are lipid-derived insoluble polyesters that serve as structural components of the outer barriers of plants. Significant amounts of cutin are present in different agricultural and food raw materials, such as cereals, berries, fruits, and vegetables, and their processing by-products, whereas the bark of cork oak and birch is rich in suberin. These polymers are estimated to be the third most abundant natural polymers after cellulose and lignin (22). The weight of an isolated cuticle ranges from 0.45 to 0.8 mg cm^{-2} (leaf cuticles) to 2 mg cm^{-2} (fruit cuticles), of which 40 to 80% is formed by cutin (23). Cutin is primarily composed of ester-linked fatty acids, of which mono-, di-, and trihydroxy and epoxy fatty acids of the C_{16} and C_{18} families are the most abundant (25). Suberin consists of both a polyaliphatic fatty acid domain similar to that of cutin, with epoxy, hydroxy, and carboxylic acid functionalities, and a polyphenolic domain (4). The main suberin monomers are aliphatic long-chain α,ω-diacids and ω-hydroxyacids, together with glycerol (20).

Cutin can be depolymerized by chemical cleavage of the ester bonds. However, epoxy, hydroxy, and carboxylic acid functionalities are not always retained when harsh chemical treatments are applied. Thus, enzymatic depolymerization of cutin is an attractive option and could result in formation of both monomers and oligomers with unmodified functionalities (26). Partial depolymerization of cork suberin by methanolysis has been found to form linear esters, glyceryl esters, and feruloyl esters, in which ferulic acid is linked to hydroxy acids (20).

Cutinases and suberinases are polyesterases which are able to degrade or partially depolymerize cutin and suberin. Cutinases (EC 3.1.1.74) are frequently produced by phytopathogenic fungi and are present in plant pollen. The fungal cutinases are involved in the disruption of the cuticular barrier of the host plant during the initial stage of fungal infection (25, 30). A cutinase (CUT1) from the plant pathogenic fungus *Fusarium solani* f. sp. *pisi* is the most studied cutinase hitherto (6), but cutinases have also been characterized in other fungi, such as *Magnaporthe grisea* (47), *Alternaria brassicicola* (52), *Botrytis cinerea* (18), *Monilinia fructicola* (53), *Venturia inaequalis* (27), and *Aspergillus oryzae* (32) and in bacteria such as *Thermomonospora fusca* (13) and certain *Pseudomonas* and *Streptomyces* species (11, 12).

Cutinases are the smallest members of the α/β-hydrolase fold family, which also contains other lipases and esterases (30). The active sites of all biochemically well-characterized cutinases are composed of serine, aspartate, and histidine residues, which form a catalytic triad similar in arrangement to those of serine proteinases and several lipases (5). A short consensus sequence around the active serine residue of cutinases is Gly-Tyr-Ser-Gln-Gly (5).

Suberinases are a poorly characterized group of enzymes, and hitherto only one suberin-degrading esterase from *Streptomyces scabiei* (SEST), a causal agent of the potato scab disease, has been characterized both at the DNA (44, 54) and protein levels (34). Suberin-degrading enzymes have also been shown to be produced by microorganisms belonging to the genera *Fusarium* and *Aspergillus* (10, 16), as well as by *Armillaria mellea* (58) and *Rosellinia desmazieresii* (37). An esterase

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TABLE 1. Primers used to construct overexpression strains of the three polyesterases from *C. cinerea*

Gene	Primer	
	Direction	Sequence
CC1G_09668.1 gene	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGTTCACCACTCTCGCC
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGTGGTGGTGGTGGTGGTGAACGCGAGCGGCGATCCATC
CC1G_07482.1 gene	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGTTTTCCGCCCTCGTC
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGTGGTGGTGGTGGTGGTGAACGCGGTCAGCGACCCAG
CC1G_05430.1 gene	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTCTCCAAATCACTCACCT
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGTGGTGGTGGTGGTGGTGACCACCAATCTTATCAAGAACAAA

of *Streptomyces diastatochromogenes* has been characterized both at the gene and protein levels, and its protein sequence was found to be similar to that of suberinase from *S. scabiei* (51). The *S. scabiei* esterase belongs to the SGNH hydrolases due to the presence of four strictly conserved residues, Ser-Gly-Asn-His, in four conserved blocks, and in addition it has a distinct Gly-Asp-Ser-(Leu) sequence edging the active serine residue, different from the GXSXG motif found in many lipases and cutinases (1, 54). The tertiary fold of these enzymes is also substantially different from that of the α/β-hydrolase family.

Despite the abundance of lipids and waxes in nature, only a limited set of lipid-modifying enzymes, other than conventional lipases, are commercially available. Moreover, classical lipases are generally not able to access the recalcitrant cutin and suberin polymers as they are typically highly active on water-insoluble lipids at the interface between the substrate and water. Cutinases could have potential in a wide variety of applications in, e.g., laundry and dishwashing applications to remove fats and cotton bioscouring and surface modification of synthetic polyester fibers (6, 9, 57). Additionally, cutinases could be used in plant by-product valorization processes and in depolymerization of plastics (24; K. E. Andersen, K. Borch, N. E. Krebs Lange, E. Steffen, S. Landvik, and K. M. Schnorr, patent application WO 2006/111163, 26 October 2006, World Intellectual Property Organization).

The aim of this work was to identify novel cutinolytic polyesterases that could be exploited in treating agricultural, food, and forest raw materials as well as their processing by-products. Genome analysis of the basidiomycete *Coprinopsis cinerea* was performed in order to identify genes encoding polyesterases. Three genes were cloned and expressed in *Trichoderma reesei*, and one of the esterases was produced in a laboratory fermentor and characterized.

MATERIALS AND METHODS

Microbial strains. The fungal donor strain used in this study was *C. cinerea* VTT D-041011. The *Escherichia coli* strain used was DH5α (Gibco BRL, Gaithersburg, MD). The host strain used for cutinase production was a *cbh1* (encoding cellobiohydrolase 1) disruptant of *Trichoderma reesei* strain VTT D-00775, constructed by replacing the *cbh1* gene with an acetamidase marker gene (data not shown).

Construction of expression vectors. The genome of *C. cinerea* (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html) was analyzed by similarity searches based on known polyesterase (cutinase and suberinase) sequences. Three polyesterases (CC1G_09668.1, CC1G_07482.1, and CC1G_05430.1) were chosen to be cloned and expressed in *T. reesei*. These are referred to as 09668/CcCUT1, 07482, and 05430, respectively.

For isolation of chromosomal DNA, the *C. cinerea* strain VTT D-041011 was grown in liquid cultures on YP medium containing 20 g liter^{-1} of Bacto peptone and 10 g liter^{-1} of yeast extract. The spores were inoculated to 50 ml of YP medium and grown for 2 days at 24°C with shaking. The mycelia were harvested by filtration, and the genomic DNA was isolated by the method of Raeder and Broda (43). The genomic DNA was used as a template in PCR amplifications of the three polyesterase genes with primers which were designed to create a C-terminal His_6 tag and which carried phage lambda-based site-specific recombination sequences. The native signal sequences of the genes were used. Primers used in the PCR amplification are given in Table 1. The PCRs were performed with Phusion thermostable polymerase (Finnzymes, Finland) in a reaction mixture recommended by the manufacturer. The PCR program had an initial denaturation step of 30 s at 98°C, followed by 25 cycles of 10 s at 98°C, 30 s at 64°C, and 30 s at 72°C, after which the annealing temperature was decreased by 1°C per cycle until 50°C was reached. This was followed by a final elongation step of 10 min at 72°C. The amplified PCR products were recombined into the Gateway donor vector pDONR221 (Invitrogen) with the Gateway recombination kit (Invitrogen) and sequenced. The genes were transferred by LR recombination reactions from the pDONR221 vector to the *T. reesei* expression vector pMS186 with the kit, giving rise to the plasmids pAWP26 (09668), pAWP27 (07482), and pAWP28 (05430). The pMS186 vector contains the Gateway reading frame cassette C (RfC) inserted between the *cbh1* promoter and terminator and a hygromycin resistance cassette.

Expression of novel polyesterases in *Trichoderma reesei*. The polyesterase genes were expressed in *T. reesei* under the strongly inducible promoter of the major cellulase gene *cbh1*. Circular expression vectors (5 μg) were transformed into the *T. reesei* VTT D-00775 *cbh1*-negative strain by polyethylene glycol-mediated transformation, essentially as described by Penttilä et al. (41), and transformants were selected for hygromycin resistance on plates containing 125 μg ml^{-1} of hygromycin B (Calbiochem, EMB Biosciences Inc., La Jolla, CA). The transformants were streaked on the selective medium for two successive rounds and tested by PCR for integration of the expression constructs into the genome. Positive transformants were purified through single-spore cultures and were tested for cutinase activity in liquid cultures using *p*-nitrophenylbutyrate (*p*-NPB) as described below. Fifty milliliters of *Trichoderma* minimal medium (41) supplemented with 4% lactose, 2% spent grain, and 100 mM PIPPS [piperazine-*N*,*N*′-bis(3-propanesulfonic acid)] as the buffering agent (pH 5.5) was inoculated with 1×10^7 spores and grown for a maximum of 10 days at 28°C with shaking at 250 rpm.

Production of novel polyesterases in a laboratory fermentor. *T. reesei* was cultivated in a Biostat C fermentor (B. Braun Biotech, Germany) in 20 liters of a medium containing 60 g liter^{-1} lactose, 5 g liter^{-1} $(NH_4)_2SO_4$, and 5 g liter^{-1} KH_2PO_4 dissolved in an extract of distiller′s spent grain prepared by heating 60 g liter^{-1} spent grain at 115°C for 20 min in an autoclave, cooling, and centrifuging to remove the solid components. The cultivation temperature was 28°C, and pH was 5.0 to 5.5 (controlled by addition of ammonium hydroxide and phosphoric acid). Dissolved oxygen was maintained at >30% by agitation at 300 to 700 rpm, with a constant aeration of 8 liters min^{-1}. Foaming was controlled by automatic addition of Struktol J633 polyoleate antifoam agent (Schill & Seilacher, Germany). After the cultivation, cells were removed by centrifugation and the culture supernatant was concentrated 16-fold by ultrafiltration using Millipore (France) BioMax 10 membranes, with a nominal cutoff of 10 kDa.

Protein and enzyme activity determinations. The expression of cutinases in liquid cultures was followed by measuring carboxyl esterase (CAE) activity in the culture supernatant with a spectrophotometric assay (slightly modified from that of Davies et al. [8]) using 5 mM *p*-NPB (Sigma) as a model substrate. The

reaction was carried out for 10 min in 0.1 M sodium phosphate buffer (pH 7.0) using a final *p*-NPB concentration of 2.1 mM at 40°C, and the amount of released *p*-nitrophenol was measured at 340 nm, with commercial *p*-nitrophenol as the standard. This method enabled a convenient and rapid assay for nonspecific esterase activity.

Protein concentration was determined with the DC protein assay kit (Bio-Rad, Richmond, CA), with bovine serum albumin as a standard.

The expression of recombinant cutinases was also verified by Western blot analysis using a monoclonal antibody against the His tag (Trend Pharma & Tech Inc., Surrey, Canada). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ready Gel Cell; Bio-Rad, Hercules, CA) using 12% Tris-HCl Ready Gel (Bio-Rad), and proteins were electroblotted onto Protran BA83 nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany). The second antibody was an anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad). Protein bands were visualized by enzymatic color reaction and compared with a molecular weight marker (prestained SDS-PAGE standards; Broad Range; Bio-Rad).

Purification and molecular characterization of recombinant enzyme. The presence of a C-terminal His tag enabled a one-step purification of *C. cinerea* cutinase by immobilized metal affinity chromatography (IMAC). Concentrated culture supernatant (435 ml) was applied to a chelating Sepharose FF column (11.3 by 11 cm; Amersham Biosciences, Uppsala, Sweden) preloaded with Ni^{2+} and equilibrated with 50 mM sodium phosphate buffer containing 0.5 M NaCl and 5 mM imidazole, pH 7.2. The column was washed with equilibrating buffer supplemented with 50 mM imidazole at a flow rate of 200 ml min^{-1} in order to remove the unbound material. The bound protein was eluted with equilibrating buffer supplemented with first 200 mM and then 500 mM imidazole. Fractions were collected and screened for activity on *p*-NPB. The pooled cutinase fractions were finally concentrated, and buffer was exchanged for 50 mM sodium acetate buffer, pH 5, with a polyethersulfone desalting column (5.5 by 32.5 cm; Millipore) to remove the imidazole.

The homogeneity of the purified protein was determined by SDS-PAGE and reversed-phase chromatography. SDS-PAGE (12% Tris-HCl Ready Gel; Bio-Rad) was performed as described by Laemmli (29) using prestained SDS-PAGE standards (Broad Range catalog no. 161-0318 [Bio-Rad] or LMW catalog no. 17-0446-01 [GE Healthcare]) and Coomassie brilliant blue (R350; Pharmacia) for staining the proteins. Reversed-phase chromatography was performed on a 1- by 150-mm Jupiter C_4 column (5 μm, 300Å; Phenomenex Inc.) with a linear gradient of acetonitrile (0 to 100% in 60 min) in 0.1% trifluoroacetic acid. The flow rate was 50 μl/min, and detection was at 214 nm. The protein peak was collected and used for N-terminal sequence analysis on a Procise 494A sequencer (Perkin Elmer, Applied Biosystems Division, CA) and mass spectrometric analyses.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry was performed with an Ultraflex TOF/TOF instrument (Bruker Daltonik GmbH, Bremen, Germany), and electrospray ionization mass spectrometry was performed with a quadrupole TOF (Q-TOF) instrument (Micromass Ltd., Manchester, United Kingdom).

For alkylation, 2 nmol of protein was dissolved in 50 μl of 6 M guanidine-HCl, 0.5 M Tris-HCl, and 2 mM EDTA, pH 7.5, and 5 μl of 100 mM dithiothreitol was added. The mixture was kept for 30 min at room temperature, 1 μl of 4-vinylpyridine was added, and the mixture was again kept at room temperature for 30 min. Then, 5 μl of 100 mM dithiothreitol was added. The alkylated protein was desalted by reversed-phase chromatography as described above.

Cyanogen bromide cleavage of 1 nmol alkylated protein was performed in 50 μl 70% trifluoroacetic acid by adding BrCN (80 μg in 3 μl acetonitrile) and incubating at room temperature for 16 h. The digest was then diluted with four volumes of water and dried in a vacuum centrifuge. Dissolved fragments were separated by reversed-phase chromatography as described above and analyzed by electrospray ionization mass spectrometry.

Characterization of the novel polyesterase. The substrate specificity was determined with *p*-nitrophenols esterified with acetate (C_2), propionate (C_3), valerate (C_5), caproate (C_6), caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), and stearate (C_{18}), obtained from Sigma-Aldrich. The concentration of substrate dispersions was 5 mM. A lower concentration of *p*-nitrophenylstearate (2.5 mM) was used due to its lower solubility. Activity assays were performed as described for *p*-NPB (C_4) at pH 7 and 40°C.

Lipase activity was assayed with an olive oil emulsion as a substrate as described by Kontkanen et al. (28). The assay used for determination of cholesteryl esterase (CE) activity was based on the spectrophotometric determination of liberated cholesterol after hydrolysis of 4.3 mM cholesteryl oleate as described by Tenkanen et al. (50).

The temperature stability of cutinase was investigated by incubating the enzyme at 30 to 80°C for 1, 3, and 20 h at a protein concentration of 5 mg ml^{-1} and pH 5 (0.02 M sodium acetate buffer). After the incubations, the residual activity was measured, with *p*-NPB as a substrate (at pH 7 and 40°C).

The pH stability of cutinase was determined by incubating the purified enzyme at different pH values at room temperature and at 50°C for 20 h. The pH of the solution was adjusted with McIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid) at pH 2.2 to 8.0, 0.2 M Tris-HCl buffer at pH 7.2 to 9.1, or 0.2 M glycine-NaOH buffer at pH 8.6 to 10.6 at a protein concentration of 5 mg ml^{-1}. The residual activity was measured with *p*-NPB at pH 7 and 40°C.

The carboxyl esterase activity of purified cutinase preparation was measured at different pH values with McIlvaine buffer at pH 2.3 to 8.0, 0.2 M Tris-HCl buffer at pH 7.2 to 9.1, and 0.2 M glycine-NaOH buffer at pH 8.6 to 10.6, with *p*-NPB as a substrate. The reaction time was 10 min at 40°C.

Isolation and pretreatment of cutin and suberin. Apples (Royal Gala) were peeled by hand. The peelings rich in cutin were cooked for 3 h to remove remaining fruit flesh and treated as described by Pacchiano et al. (39) to remove connected carbohydrates and pectin as well as noncovalently attached lipophilic components. Briefly, the peelings were treated with Pectinex Ultra SP-L (50 nkat g^{-1}) (Novozymes A/S, Bagsvaerd, Denmark) and Econase CE (1.5 filter paper units [FPU] g^{-1}) (AB Enzymes, Rajamäki, Finland) for 48 h, washed with ethanol, and Soxhlet extracted successively with 70% methanol, ethanol (EtOH), and hexane-isopropanol (3:2). The dried and powdered sample was treated again with Pectinex Ultra SP-L (3,000 nkat g^{-1}) and Econase CE (150 FPU g^{-1}) for 48 h and extracted with EtOH and hexane-isopropanol (3:2). The sample was dried and stored in a desiccator at room temperature.

Birch outer bark (kindly provided by Christer Eckerman, Åbo Akademi University, Finland) was treated with high-pressure steam (17 bar, 206 to 208°C) for 5 min, after which the pressure was explosively discharged to atmospheric pressure. The steam-exploded bark was powdered, Soxhlet extracted with acetone, dried, and stored at room temperature.

Hydrolysis of polyester fractions and monitoring of polyester degradation. Cutin and steam-exploded suberin were suspended in 0.2 M sodium phosphate buffer, pH 8, at a concentration of 20 mg ml^{-1} and treated with cutinase for 20 h. The treatments were first performed at 45°C with and without addition of 0.1% Triton X-100 using enzyme dosages of 1,000 and 10,000 nkat g^{-1} substrate (CAE activity). Subsequently, enzyme treatments were performed at 50°C with higher enzyme dosages (10,000 and 100,000 nkat g^{-1} substrate) with and without addition of *Trichoderma reesei* hydrophobin II (HFBII) (36) at a final concentration of 0.1%.

Released hydrolysis products were primarily analyzed with an enzymatic colorimetric assay (free fatty acids; Roche Diagnostics Ltd.), both directly as free fatty acid monomers and after alkali hydrolysis to determine the amount of released oligomers. To test the applicability of the assay kit for this purpose, the main epoxy acid of birch outer bark suberin, 9,10-epoxy-18-hydroxy octadecanoic acid, was used as a standard sample. The hydrolysates were extracted three times with 2 volumes of methyl *tert*-butyl ether (MTBE) in order to recover released monomers and oligomers from the solid matrix. MTBE was evaporated, and free fatty acids were analyzed after dissolving them in EtOH and 6% Triton X-100 solution according to the kit instructions. The total amount of released fatty acids was analyzed after hydrolysis of released oligomers with 0.5 M KOH in EtOH-water (9:1, vol/vol) at 70°C for 1.5 h and neutralization of pH. The amount of released fatty acids (mmol $liter^{-1}$) was converted to weight units using the molecular mass of stearic acid (284.5 g mol^{-1}) in order to express the amount of released fatty acid as a proportion (%) of the amount of substrate.

The fatty acid compositions of hydrolysates were analyzed qualitatively (and semiquantitatively) by gas chromatography-mass spectrometry (GC-MS). Heptadecanoic acid (100 μg) was added to the hydrolysate samples as an internal standard prior to extraction with 1 ml of MTBE. The extracted samples were evaporated to dryness under nitrogen flow and trimethylsilylated with 120 μl of BSTFA [bis(trimethylsilyl)trifluoroacetamide] and 30 μl of trimethylchlorosilane (80°C, 2 h). The GC-MS instrument consisted of an Agilent 6890A gas chromatograph and a 5973N mass spectrometer. The column used was an Agilent HP-Ultra 2 with 5% phenylmethylpolysiloxane stationary phase. The temperature program was as follows: 70°C for 1 min, an increase to 100°C at 10°C/min, an increase to 280°C at 15°C/min, and then 9 min at 280°C. The data collected were in the mass range from *m/z* 40 to 800 amu. The results are semiquantitative only, because no calibration standards were analyzed.

Enzyme-treated cutin and suberin samples were also analyzed by light microscopy and compared with reference samples. The samples for analysis were prepared as previously described by Olkku et al. (38). Briefly, the samples were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7), dehydrated with EtOH, embedded in Historesin, and sectioned (2 μm) with a rotary microtome.

TABLE 2. Sequence identity between the *C. cinerea* cutinases based on conceptual translations of the gene models

Cutinase (no. of aa)[a]	% Identity to:					
	09668	03922	11503	07482	09365	05430
09668 (199)	100					
03922 (199)	88	100				
11503 (199)	75	76	100			
07482 (200)	60	59	57	100		
09365 (216)	53	53	49	53	100	
05430 (229)	29	30	25	25	24	100

[a] aa, amino acids.

For the microscopic examination, the sections were stained with 0.5% Oil Red O in 70% EtOH for 1 min.

Nucleotide sequence accession numbers. The nucleotide sequences of the *C. cinerea* polyesterase 09668/CcCUT1, 07482, and 05430 genes can be obtained from the EMBL nucleotide sequence database under accession numbers EU435153, EU435154, and EU435155, respectively.

RESULTS

Cloning and characterization of novel polyesterases from *C. cinerea*. *C. cinerea* was previously found to be a potential producer of suberin-degrading enzymes when cultivated in the presence of birch outer bark suberin (our unpublished results). The published genome of *C. cinerea* was subsequently subjected to homology searches with known cutinase and suberinase sequences, and as a result six different uncharacterized genes were found. All the genes were located on different contigs and are proposed to contain two short introns. The deduced protein sequences (199 to 229 amino acids long) contain the catalytic residues Ser, His, and Asp and the consensus sequence Gly-Tyr-Ser-Gln-Gly, including the active-site serine (see Fig. S1 in the supplemental material). The four conserved cysteines, forming the two disulfide bridges essential for the correct folding of the protein, are also present in all sequences (see Fig. S1 in the supplemental material). Analysis of the deduced protein sequences with the program SIGNALP (3) predicted that all the proteins have N-terminal signal sequences and hence should be secreted.

Homology analysis of the deduced protein sequences from the gene models predicted from the genome sequence revealed that five of the genes (CC1G_09668.1, CC1G_03922.1, CC1G_11503.1, CC1G_07482.1, and CC1G_09365.1 genes) showed highest sequence homology with cutinase genes and one (CC1G_05430.1 gene) shared higher homology with acetyl xylan esterase (AXE) genes, having, e.g., a sequence identity of 30% with the *Trichoderma reesei* AXEI gene. The sequence identities between the *C. cinerea* polyesterases analyzed with the CLUSTAL W multiple alignment program (7) are shown in Table 2. The cutinase-like proteins shared 49 to 88% mutual sequence identity, whereas the AXE-like protein shared only 24 to 30% sequence identity with the other proteins. The closest homologs to the *C. cinerea* cutinases were the CUT1 cutinase of the phytopathogenic fungus *Monilinia fructicola* (37 to 47% sequence identity) and the hypothetical cutinase of *Botryotinia fuckeliana* (35 to 48% sequence identity). Interestingly, the sequence identity to known cutinases from other basidiomycetes was lower than that to these cutinases from *Ascomycota*. The sequence identity of *C. cinerea* cutinases with the cutinase of *Fusarium solani* f. sp. *pisi* varied between 22% and 30%. The *C. cinereus* genome was also searched with the protein sequence of *Streptomyces scabiei* suberinase and with fungal sequences having similarity with *S. scabiei* suberinase (containing SEST-like domains), but no hits were found.

The two cutinase-like (09668 and 07482) polyesterases and the AXE-like (05430) polyesterase sharing the lowest mutual amino acid homology were selected for cloning and expression in *T. reesei*. The selected genes had the most favorable codon usage as well as suitable native signal sequences for the expression host. The genes were cloned by PCR and sequenced in order to verify the sequence given at the *C. cinerea* genome website. Most of the clones sequenced had differences at the third codon base compared to the genome. Only in two cases did this substitution lead to a change in the amino acid (indicated in boldface and underlined in Fig. S1 in the supplemental material) (05430, A→S; 09365, G→A). The published genome sequence is derived from a haploid strain, and therefore the sequences of the genes cloned from a dikaryotic strain may be allelic variants of the published genes of the genome sequence. A second possibility is that the substitutions are due to strain differences.

Heterologous expression in *T. reesei*. DNA fragments encoding the putative *C. cinerea* 09668, 07482, and 05430 polyesterases were cloned with primers designed to create a C-terminal His_6 tag and bearing phage lambda-based site-specific recombination sequences. Expression constructs were made by in vitro recombination of the cloned polyesterase genes into a *T. reesei* expression vector carrying the strong inducible *cbh1* promoter. The expression constructs were used to transform *T. reesei*, and the transformants were selected for hygromycin resistance. Two hundred transformants per construct were streaked on hygromycin plates, and about 25 transformants for each gene were selected and purified to uninuclear clones. The selected transformants were cultivated in shake flasks, and esterase activity in the culture supernatants was measured as a function of time, with *p*-NPB as a model substrate. Transformants of all three polyesterase constructs showed activity (results not shown), and subsequently the six transformants of each gene showing the highest activity were recultivated for more-thorough analysis. Transformants of the polyesterase 09668 were found to be the most promising, producing a maximum activity level of 23 nkat ml^{-1} by day 5 of the cultivation, whereas the activity levels produced by the transformants of 07482 and 05430 were considerably lower (0.7 nkat ml^{-1} and 1.1 nkat ml^{-1}, respectively, after 5 days of cultivation). The background activity produced by the parental *T. reesei* strain was 0.46 nkat ml^{-1} after 5 days of cultivation.

The transformant of 09668 yielding the highest level of esterase activity in shake flasks was cultivated in a laboratory fermentor. Esterase production was detectable after about 2 days of cultivation, and the cutinase production increased to a maximum of over 8,000 nkat ml^{-1} after 96 h and decreased to 7,400 nkat ml^{-1} by the end of the run at 120 h (Fig. 1).

Purification of cutinase. A His_6 tag was fused to the C terminus of *C. cinerea* cutinase in order to exploit a one-step purification protocol using IMAC. The 09668 cutinase produced by *T. reesei* was first studied by Western blotting with His tag-specific antibody in order to demonstrate the presence of intact cutinase in the culture supernatant and address the pos-

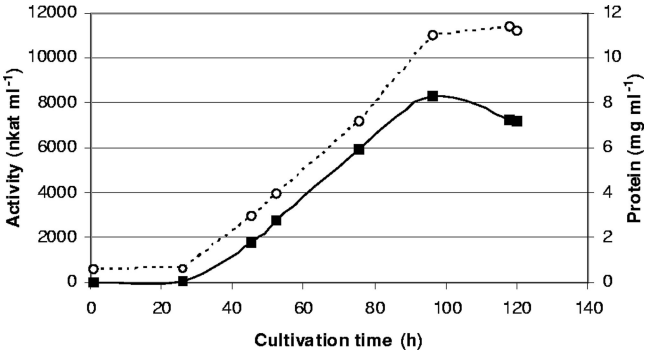

FIG. 1. Production of CcCUT1 in a 20-liter fermentor by cultivation on lactose-containing medium. Cutinase production was monitored by measuring carboxyl esterase activity (■). Production of soluble protein (○) is also shown.

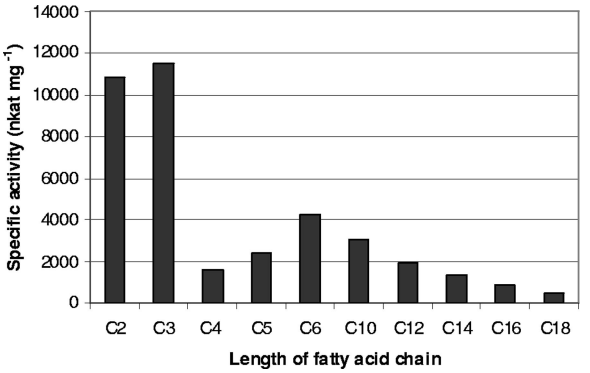

FIG. 3. Effect of fatty acid chain length on esterolytic activity of CcCUT1 measured with *p*-nitrophenyl esters at pH 7 and 40°C.

sibility of using affinity chromatography for purification. The antibody recognized one single protein band of about 20 kDa, whereas no band was detected in the *T. reesei* control samples (results not shown). Half of the total amount of culture filtrate concentrate was applied to a Sepharose FF column. The cutinase was effectively bound (82%, determined by monitoring enzyme activity), and it was eluted with the equilibrating buffer containing first 200 mM and then 500 mM imidazole, with yields of ca. 69% and 13%, respectively. The first peak showing homogeneity by SDS-PAGE with an apparent molecular mass of 20 kDa (Fig. 2) was pooled, concentrated, and buffer exchanged into sodium acetate buffer, pH 5, to remove imidazole. Approximately 10 g of purified cutinase was obtained, corresponding to a yield of ca.1.4 g liter^{-1} of culture. In reversed-phase chromatography the purified cutinase gave a single symmetric peak (data not shown). The purified 09668 cutinase was designated CcCUT1.

Characterization of the recombinant cutinase. The purified *C. cinerea* cutinase (CcCUT1) was biochemically characterized with respect to molecular size, substrate specificity, pH, and temperature characteristics. In MALDI-TOF mass spectrometry the purified CcCUT1 gave two masses, 18,721 Da and 18,859 Da (data not shown). N-terminal sequence analysis of the purified enzyme did not result in any signals, suggesting that its N terminus is blocked. In the more accurate electrospray ionization Q-TOF mass spectrometric analysis, the enzyme gave two masses, 18,726.0 Da and 18,863.0 Da, of which the higher mass corresponds to CcCUT1 with the six C-terminal histidines, two disulfide bridges, and an N terminus starting with Gln28 (see Fig. S1 in the supplemental material [indicated by a triangle]), which is cyclized to a pyroglutamic acid. The lower determined mass fits well with a similar molecule with only five histidines at the C terminus. To further confirm this structure, CcCUT1 was reduced and alkylated with 4-vinylpyridine (additional mass, 105.1 Da). The molecular masses of the alkylated enzyme, 19,149.0 Da and 19,286.0 Da, determined by electrospray ionization Q-TOF mass spectrometry, further suggested the presence of two disulfide bridges in the native enzyme. To confirm the blocked N terminus and the histidine heterogeneity in the C terminus, the alkylated CcCUT1 was cleaved with cyanogen bromide and the two fragments were separated by reversed-phase chromatography. Electrospray ionization Q-TOF mass spectrometric analysis gave the masses of the N-terminal fragment [6,982.0 Da; $Gln_{(28)}$ to homoserine $lactone_{(92)}$] and the C-terminal fragment {12,137.0 Da [$Ala_{(95)}$ to $His_{(204)}$] and 12,274.0 Da [$Ala_{(95)}$ to $His_{(205)}$]}. It can be deduced from the determined size that the introns of the CcCUT1 gene model seem to be correctly spliced in *T. reesei.* The determined CcCUT1 masses are close to those of most fungal cutinases, 22 to 25 kDa (5, 25). However, some cutinases are larger, such as *B. cinereus* cutinase, with a molecular mass of 40.8 kDa (18).

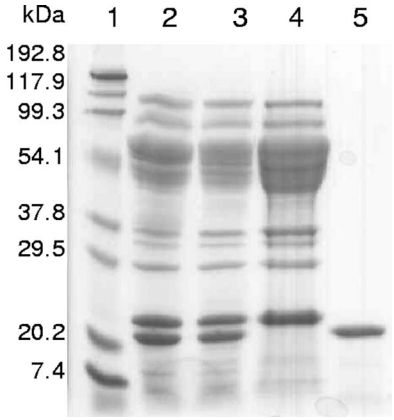

FIG. 2. SDS-PAGE analysis after fractionation of CcCUT1 with Sepharose FF-Ni^{2+}. Lane 1, molecular mass marker; lanes 2 and 3, culture supernatant; lane 4, flowthrough; lane 5, 7 μg of purified CcCUT1.

The activity profile of CcCUT1 was studied using *p*-nitrophenols esterified with fatty acids of different lengths (from C_2 to C_{18}). The specific activities on *p*-nitrophenyl esters are shown in Fig. 3. CcCUT1 had higher activity on shorter (C_2 to C_{10}) than on longer (C_{16} and C_{18}) fatty acids. Surprisingly, the activities on *p*-nitrophenylacetate (C_2) and propionate (C_3) were observed to be clearly higher than those on *p*-NPB (C_4). CcCUT1 also showed some lipase activity but no CE activity (Table 3).

The pH dependence and effects of temperature studied with *p*-NPB are shown in Table 3. CcCUT1 was shown to retain its activity over a wide range of pHs, including the acidic range. The residual activity of CcCUT1 was about 80% at a pH of 3

TABLE 3. Biochemical properties of CcCUT1

Property	Value
Molecular mass (kDa)	18.7/18.8[a]
No. of amino acids in mature protein	181
Thermostability[b] (pH 5) at:	
50°C	>20 h[c]
55°C	3 h
60°C	<1 h
pH stability (20 h) at:	
50°C	6–9
23°C	4–9
pH optimum (with *p*-NPB)	7–8
Activity (nkat mg^{-1})	
CAE[d]	1,570
Lipase	234
CE[e]	0

[a] Determined by MALDI-TOF mass spectrometry.
[b] Half-time of inactivation.
[c] The enzyme retained nearly 80% of its activity at 20 h.
[d] Carboxyl esterase activity measured with *p*-NPB.
[e] Cholesteryl esterase activity measured with cholesteryl oleate.

at room temperature, whereas the residual activity at 50°C was about 40% at pH 5 and about 100% at pH 6. The enzyme retained nearly 80% of its activity after 20 h at 50°C and over 90% after 1 h at 55°C but was no longer stable at 60°C. The pH optimum of CcCUT1 was around 7 to 8.

Hydrolysis of natural polyesters by cutinase. Isolated apple cutin and birch outer bark suberin were treated with CcCUT1 in order to demonstrate its capability to depolymerize these natural polyesters. The amounts of released fatty acids are shown in Table 4. CcCUT1 was able to liberate about 5.1 mmol liter^{-1} of monomeric fatty acids and 1.9 mmol liter^{-1} (7.07 to 5.13 mmol liter^{-1}) of oligomeric fatty acids from cutin with the highest enzyme dosage used. The total amount of released fatty acid monomers and oligomers corresponds to ca. 10.1% of the amount of substrate. Addition of Triton X-100 to the reaction mixture did not improve the hydrolysis efficiency of cutin. Suberin was clearly more resistant to CcCUT1, and with the highest enzyme dosage about 1.3 mmol liter^{-1} of fatty acids was released, corresponding to approximately 1.9% of substrate. In this case all the reaction products appeared to be in the monomeric form, as after alkaline hydrolysis no increase in the fatty acid content was observed. In contrast to what was found for cutin, the amount of hydrolysis products released from suberin was somewhat higher in the presence of Triton X-100.

TABLE 4. Effect of Triton X-100 on depolymerization of apple cutin and birch outer bark suberin with CcCUT1

Substrate	Dosage (nkat g^{-1})	Concn of Triton X-100 (%)[a]	Amt of:			
			Monomers		Monomers and oligomers	
			mmol liter^{-1}	%[b]	mmol liter^{-1}	%[b]
Cutin	0	—	0.17	0.2	0.36	0.5
	1,000	—	2.33	3.3	1.83	2.6
	10,000	—	5.13	7.3	7.07	10.1
	0	0.1	0.15	0.2	0.44	0.6
	1,000	0.1	1.07	1.5	1.18	1.7
	10,000	0.1	2.78	4.0	4.56	6.5
Suberin	0	—	0.03	0.0	0.05	0.1
	1,000	—	0.21	0.3	0.17	0.2
	10,000	—	1.34	1.9	1.23	1.8
	0	0.1	0.04	0.1	0.06	0.1
	1,000	0.1	0.36	0.5	0.32	0.5
	10,000	0.1	1.89	2.7	1.87	2.7

[a] —, no Triton X-100 added.
[b] Percentage of the amount of substrate.

TABLE 5. Effect of HFBII on depolymerization of apple cutin and birch outer bark suberin with CcCUT1

Substrate	Dosage (nkat g^{-1})	Concn of HFBII (%)[a]	Amt of:			
			Monomers		Monomers and oligomers	
			mmol liter^{-1}	%[b]	mmol liter^{-1}	%[b]
Cutin	0	—	0.03	0	0.42	0.6
	10,000	—	2.21	3.1	4.22	6.0
	100,000	—	5.13	7.3	8.61	12.2
	0	0.1	0.04	0.1	0.43	0.6
	10,000	0.1	3.96	5.6	5.43	7.7
	100,000	0.1	8.27	11.8	11.22	16.0
Suberin	0	—	0.00	0	0.00	0
	10,000	—	0.85	1.2	1.62	2.3
	100,000	—	1.57	2.2	2.63	3.7
	0	0.1	0.01	0	0.01	0
	10,000	0.1	1.06	1.5	2.24	3.2
	100,000	0.1	2.89	4.1	5.84	8.3

[a] —, no Triton X-100 added.
[b] Percentage of the amount of substrate.

In order to increase the degree of hydrolysis, polyesters were subsequently treated with CcCUT1 using only the higher enzyme dosages and additionally the effect of *T. reesei* hydrophobin (HFBII), an amphiphilic protein, on polyester degradation was studied. The use of hydrophobin as a hydrolysis enhancer has been previously studied by Takahashi et al. (48). The amount of enzyme had a significant effect on the degree of hydrolysis, even in the presence of hydrophobin. HFBII improved the hydrolytic effect of CcCUT1 on both substrates (Table 5), especially on suberin, although the degree of hydrolysis was still clearly lower with suberin than with cutin. The amounts of cutin-derived monomeric and oligomeric fatty acid products were maximally about 8.3 and 3.0 mmol liter^{-1}, respectively. The corresponding amounts released from suberin were 2.9 and 3.0 mmol liter^{-1}, respectively. Polyester degradation as a function of treatment time was also studied. However, increasing the incubation time had no significant effect on the degree of depolymerization (results not shown). Compared to the 24-h results, the total amounts of released cutin-based monomeric and oligomeric fatty acids were ca. 22 and 32% higher after 48- and 72-h incubations, respectively. The corresponding increases from suberin were ca. 31 and 41%, respectively.

GC-MS analysis of products from enzymatic and alkaline hydrolysis of cutin showed clear differences in the production pattern. 18-Hydroxy-16:0 acid and various dicarboxylic acids were the main products identified in the enzymatic hydrolysate. Surprisingly, 9,10-epoxy-18-hydroxy-18:0 acid was de-

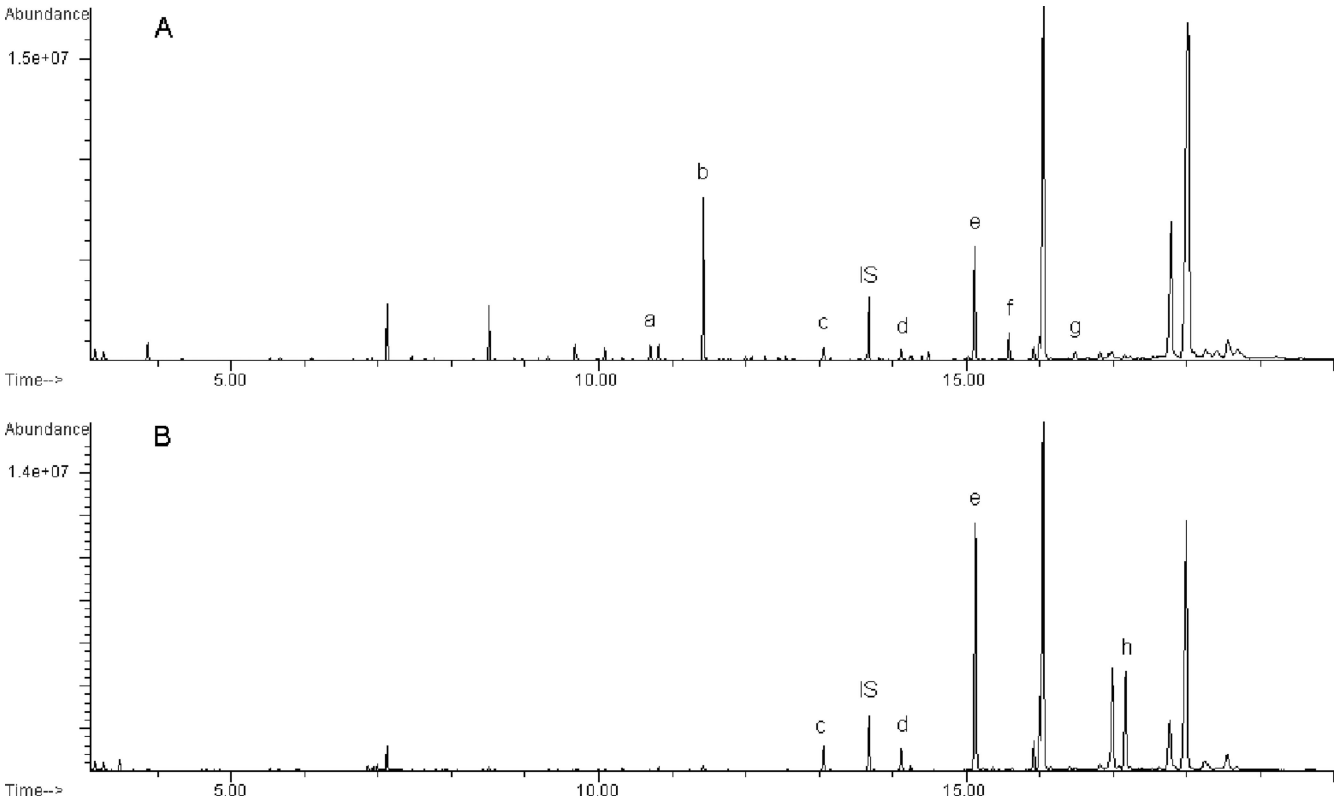

FIG. 4. Total ion chromatogram of the derivatized cutin hydrolysis products. Shown is enzymatic hydrolysis with CcCUT1 (100,000 nkat g^{-1}) (A) and total hydrolysis in 0.5 M KOH (B). a, octanedioic acid (suberic acid); b, nonanedioic acid (azelaic acid); c, hexadecanoic acid; d, octadecanoic acid; e, 18-hydroxy-16:0 acid, f, 1,16-dioic-16:0 acid; g, 1,18-dioic-18:1 acid; h, 9,10-epoxy-18-hydroxy-18:0 acid; IS, heptadecanoic acid.

tected only in the alkaline hydrolysate. The dioic acids were absent in the alkaline hydrolysate (Fig. 4).

Enzymatic modifications of cutin and suberin were also analyzed by light microscopy. The sections were stained with a lipid-soluble dye, Oil Red O, that is typically used for the staining of all lipidic compounds. The applicability of Oil Red O for cutinaceous substances in barley has previously been studied (38). Cutin and suberin were clearly visualized in the reference samples. After treatment with CcCUT1 the cutin structure was destroyed and only small fragments were observed. In the case of suberin, depolymerization could be visualized, albeit to a lesser extent than for cutin (Fig. 5). The microscopy analysis supports the conclusion that an increase in amounts of released fatty acid products is indicative of degradation of polyesters.

DISCUSSION

A selection of fungi were previously screened in our laboratory for their ability to produce enzymes modifying cutin and suberin, and *C. cinerea* demonstrated the most potential for polyesterase production in the conditions tested. To identify the genes encoding polyesterases, the sequenced and publicly available genome of *C. cinerea* was analyzed by similarity searches based on known cutinases and suberinases. Six different polyesterase genes were found, five of which showed high sequence homology with cutinase genes, and one shared higher homology with AXE genes. All six *C. cinerea* polyesterases showed the characteristic features of the primary protein sequences of cutinases, having well-conserved consensus sequences (GYSQG), catalytic triad residues, and the four cysteine residues required for the disulfide bridges. All sequences were predicted to encode a secretion signal (3).

Three polyesterase genes sharing the lowest mutual homology were cloned and expressed in *T. reesei.* The recombinant strain producing the highest activity level in shake flask cultivations was also cultivated in a laboratory fermentor. The *C. cinerea* cutinase 09668 (CcCUT1) was successfully produced, and an over 300-fold-higher production level was achieved in a fermentor than in shake flasks, corresponding to 1.4 g $liter^{-1}$ purified CcCUT1. This level of production in a simple batch process on only 60 g $liter^{-1}$ carbon source indicates a potential for very high productivity in commercial fed-batch processes with modified production strains and high medium concentration.

The CcCUT1 expressed in *T. reesei* is posttranslationally modified. The N-terminal signal sequence is cleaved off after Arg27; consequently the mature protein starts with Gln28. Many secreted endogenous *T. reesei* proteins, including the major cellulases, have an N-terminal Gln that is blocked (see, e.g., references 40 and 49). The endoprotease Kex2 has been reported to cleave off a propeptide from a number of secreted fungal enzymes on the carboxylic side of dibasic sequences

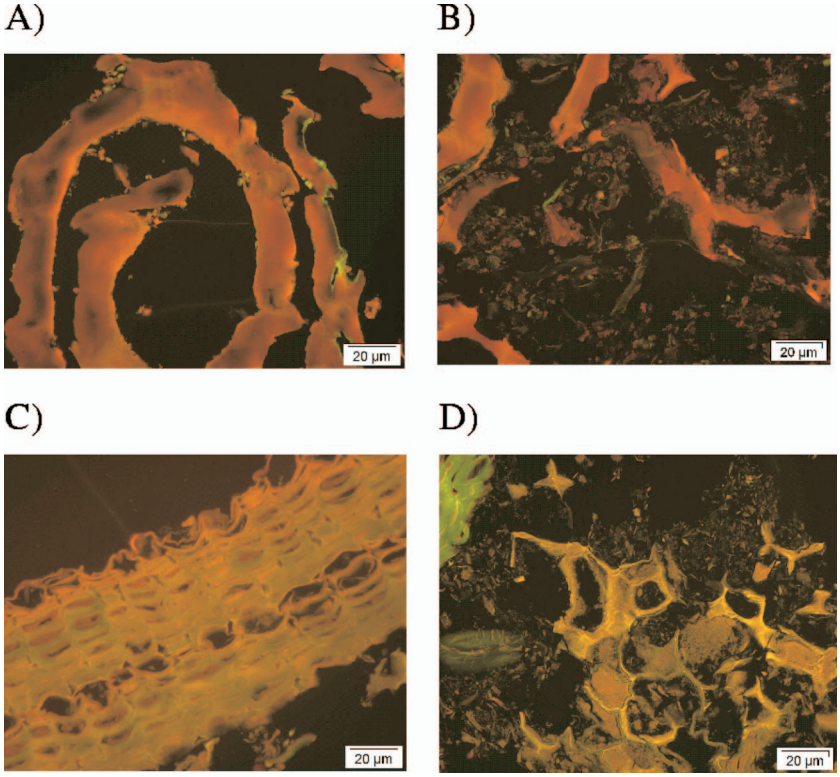

FIG. 5. Analysis of cutin and suberin after CcCUT1 treatment (100,000 nkat g^{-1}, 50°C, pH 8, 24 h, 0.1% HFBII) by fluorescence microscopy. Shown is the cutin reference (A), cutin treated with CcCUT1 (B), the suberin reference (C), and suberin treated with CcCUT1 (D).

(19). It is possible that this endoprotease cleaves CcCUT1 in *T. reesei* although there is only one basic residue on the N-terminal side of the cleavage site. Such a Kex2 cleavage site with only one basic amino acid from the beta-mannanase of *T. reesei* has been reported (46). Furthermore two different masses of CcCUT1 were obtained by MALDI-TOF mass spectrometry, corresponding to CcCUT1 with either five or six C-terminal histidines. Despite a shorter His tag in part of the CcCUT1, the enzyme was effectively bound and purified by IMAC, in agreement with His_5 tags being used. The His tag was placed at the C terminus of CcCUT1 as it can be presumed from the solved three-dimensional structure of *Fusarium solani* cutinase (33) that the His tag cannot interfere with the active site of CcCUT1, because the C terminus is on the opposite side of the enzyme from the active site. His tags are commonly left on the expressed and purified proteins, presuming that the small size and charge of the tag will not alter the properties of the protein. The His tag has been shown to have no effect on the activity of some proteins (31, 55), whereas the addition of a His tag to other proteins has been reported to alter enzyme activity (2, 15, 21) or dimerization state (56). The His tag is, however, a very useful tool for the detection and purification of hard-to-express novel proteins.

Cutinases commonly display hydrolytic activity on a broad variety of esters such as insoluble long-chain triglycerides and soluble synthetic *p*-nitrophenyl esters (30). The purified *C. cinerea* cutinase (CcCUT1) also showed lipolytic activity on olive oil but displayed no activity on cholesteryl oleate (steryl ester), which is more hydrophobic than triglycerides and probably therefore a preferable substrate for classical lipases and specific steryl esterases. Substrate specificities of CcCUT1 on *p*-nitrophenyl esters with various fatty acid chain lengths were also compared. CcCUT1 catalyzed the hydrolysis of *p*-nitrophenyl esters of acetate and propionate most effectively. Surprisingly, activity on *p*-NPB was clearly lower than that on the shorter (C_2 and C_3) and longer (C_5 to C_{12}) fatty acids. However, activity on long-chain *p*-nitrophenylpalmitate and *p*-nitrophenylstearate was the lowest. Typically, cutinases have high activity on C_2 to C_8 fatty acids and extremely low activity on C_{16} and C_{18} fatty acids (25, 52). The activity profile obtained for CcCUT1 supports this prevailing knowledge, with the exception of low activity on C_4 fatty acid. Low activity of cutinases on substrates with long-chain fatty acids has been explained by the length of the fatty acid binding site, which is significantly shorter in *Fusarium solani* cutinase (7.8 Å) than in lipases from *Candida rugosa* and *Rhizomucor miehei* (22 Å) (42). Furthermore, cutinases do not have a lid covering the active-site serine, and therefore they do not display the interfacial activation mechanism typical for classical lipases (33).

The pH optimum of CcCUT1 was around 7 to 8 when measured on *p*-NPB. Most fungal cutinases have an alkaline pH optimum of 9 to 10 when tested, e.g., with *p*-NPB, apple cutin, or polycaprolactone (6, 25, 35), although a slightly acidic pH (pH 6) has previously been reported to be optimal for *Venturia inaequalis* cutinase on grapefruit cutin (27). The pH optimum for bacterial cutinases has been reported to vary between 8.5 and 11 (13, 14). The purified suberinase from *Streptomyces scabiei* also showed maximum esterase activity at

pH 8.6 (34). However, it is noteworthy that optimum pH depends on the substrate, since pH may affect ionization of amino acids both inside and outside of the active site, subsequently affecting the stability of the active conformation of the enzyme and also the interaction between the enzyme and different substrates.

CcCUT1 retained its activity over a pH range of 4 to 10 for 20 h at room temperature. The cutinase of *Aspergillus oryzae* is reported to be stable over the pH range 6 to 11 for 30 min at 37°C (32), but pH stabilities for other cutinases have generally not been reported. CcCUT1 retained nearly 80% of its activity after 20 h at 50°C. *A. oryzae* cutinase was reported to be stable up to 40°C for 30 min (32), but cutinases produced by thermophilic bacteria that are stable at up to 70°C have also been found (13, 14).

Degradation of cutin has typically been studied with radioactively (tritium) labeled cutin, and the amount of released label has been expressed as disintegrations per minute (8, 45). However, this value does not describe the degree of hydrolysis, and therefore results cannot be compared unambiguously. In some cases, the degree of hydrolysis has been expressed as the ratio between released cutin monomers quantified by high-performance liquid chromatography and the amount of substrate. The culture supernatants of *Thermomonospora fusca* degraded 7 to 16% of substrate, depending on the origin of cutin (13), whereas *Fusarium* cutinase and certain unpurified *Streptomyces* cutinases hydrolyzed ca. 15% (17) and 3.8% (12) of apple cutin, respectively. The degree of suberin degradation has been clearly lower and degradation has been slower, e.g., only up to 1% of raspberry suberin was degraded when treated for 16 h by esterase from *Armillaria mellea* (58) and up to 9.8% of potato suberin when incubated for 2 months with *Rosellinia desmazieri* cells (37).

In this work hydrolysis products were analyzed as free fatty acids and after alkali hydrolysis of released oligomers using an enzymatic colorimetric method. The degree of hydrolysis by CcCUT1 was found to be strongly dependent on enzyme dosage and could also be improved by addition of *T. reesei* hydrophobin II (HFBII). In the presence of HFBII, CcCUT1 could maximally hydrolyze 16.0 and 8.3% of cutin and suberin, respectively. Improved enzymatic action in the presence of HFBII can be explained by the reduced surface and interfacial tensions between substrate and enzyme as well as accelerated diffusion of released hydrolysis products away from the hydrophobic interface. The effect of hydrophobin (*Aspergillus oryzae* RolA) on degradation of biodegradable polyester polybutylene succinate-coadipate (PBSA) has previously been studied by Takahashi et al. (48). Preincubation of PBSA with RolA was shown to stimulate PBSA degradation by *A. oryzae* CutL1, whereas simultaneous addition of CutL1 and RolA had no effect on PBSA degradation.

The results obtained in this paper show that CcCUT1 can act on plant cutin and suberin. Further studies concerning the characteristics and action of CcCUT1 on different natural polyesters and materials will provide information on its usefulness in food, textile, or forest raw material processing.

ACKNOWLEDGMENTS

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