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Purification and characterization of a 40.8-kDa cutinase in ungerminated conidia of *Botrytis cinerea* Pers.: Fr.

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Abstract

Cytoplasmic soluble proteins from ungerminated conidia of *Botrytis cinerea* exhibited cutinase activity. A 40.8-kDa cutinase was purified to homogeneity from this crude conidial protein extract. This cutinase does not correspond either to constitutive or to induced lytic cutin enzymes already described by other authors. The possible role of this constitutive cutinase in the induction of other cutinolytic proteins in the early stages of infection of plants by *B. cinerea* is discussed.

Keywords: *Botrytis cinerea*; Esterase; Cutinase; [^{3}H]Cutin

1. Introduction

Many plant pathogenic fungi penetrate the unwounded epidermal layer of their hosts by enzymatic hydrolysis of the cuticle [1]. Attachment of their spores to the plant surface is considered to be crucial for infection. Fungal esterases and particularly cutinases are involved in these phenomena [2]. A cutin-hydrolyzing enzyme was first described in *Fusarium-solani* f. sp. *pisi* [3]. Cutinases were characterized and purified from several fungal pathogens [4–7], bacteria [8], and pollen [9]. A culture filtrate of *Botrytis cinerea* Pers.: Fr. contained a cutinase induced by tomato cutin, which was the sole carbon source [10]. This extracellular cutinase was purified and the gene was cloned [11]. Recently it was demonstrated that ungerminated conidia of *B. cinerea* contained a constitutive cytoplasmic cutinase [12] which could initiate cutin degradation and release of cutin monomers. These fatty acids are known to induce cutinase gene transcription in germinated conidia of *F. solani* f. sp. *pisi* and enhance the pathogenicity of this fungus [13]. This paper describes the purification and characterization of a constitutive cutinase in ungerminated conidia of *B. cinerea*. The possible role of this enzyme in the early events of infection of grape berries is discussed.

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2. Materials and methods

2.1. Organism and growth conditions

B. cinerea Pers.: Fr., isolate P69, was cultivated in Petri dishes on oatmeal agar (OMA, Difco). Cultures were placed at 21°C under alternating light and dark (12 h each) for 1 week. Conidia were harvested according to the method of Pezet and Pont [14] and stored dry at −80°C until required.

2.2. ^3H-Labeled tomato cutin preparation

Pure tomato cutin was prepared according to Salinas et al. [10] and labeled with [^{3}H]$NaBH_4$ (3.7×10^9 Bq; 5.2×10^{11} Bq mmol^{-1}) according to Köller et al. [15]. Cutinase activity was determined in purified fractions using [^{3}H]tomato cutin according to Pascholati et al. [5].

2.3. Extraction and purification of a cytoplasmic constitutive cutinase

Cytoplasmic proteins were extracted from 15 g of ungerminated conidia with glass beads according to the method of Van Etten and Freer [16], slightly modified: buffer was replaced by distilled water. The crude protein extract, placed in dialysis tubing, was concentrated on polyethylene glycol (PEG 20 000) to 20 ml, then filtered (Akrodisk Nalgen, 0.2 μm) and stored at −20°C until use. Crude extract, dialyzed against piperazine-HCl buffer (PHB, 10 mM, pH 5.5), was loaded on a Superdex Prep Grade column (Pharmacia, 1.5×30 cm) and eluted with the same buffer. Non-specific esterase activity was measured in the eluted fractions using *para*-nitrophenylbutyrate (PNB) according to Pascholati et al. [6]. Active fractions were pooled and concentrated to 10 ml on PEG 20 000 as described before, then loaded on a DEAE Sepharose Cl-6B column (Pharmacia, 1.5×10 cm) suspended in PHB. A step gradient system of NaCl in the buffer (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 M) was used to elute eight fractions. The 0.2 M NaCl fraction contained non-specific esterase activity. This fraction was dialyzed against PHB, concentrated to 6 ml on PEG 20 000 and 30% (w/v) of $(NH_4)_2SO_4$ was added. It was loaded on a butyl Sepharose 4B column (Pharmacia, 1×15 cm) in PHB added with 30% (w/v) of $(NH_4)_2SO_4$. A decreasing step gradient system of $(NH_4)_2SO_4$ (30% to 0% in 5% steps) was used to elute seven fractions. These fractions were extensively dialyzed against distilled water and concentrated to 4 ml on PEG 20 000 before the PNB assay, where $(NH_4)_2SO_4$ interferes strongly. The active fractions (5% and 10% $(NH_4)_2SO_4$) were concentrated to 2 ml using microdialysis (Microsep, cut-off 10 000). Protein concentration was determined using the method of Bradford [18] (protein determination kit, Bio-Rad).

2.4. Gel electrophoresis analysis

SDS-PAGE (4%/12.5%) was performed according to Hames [19]. Protein bands were revealed by the silver staining method (Bio-Rad Silver Stain Kit) and esterase activity was revealed on renatured SDS-PAGE by incubation of the gel for 1 h at room temperature in Tris-HCl buffer (50 mM, pH 7.0+2% (w/v) Triton X-100) followed by incubation in the same buffer with α,β-naphthyl acetate according to Shaw and Prasad [17]. The molecular mass of cutinase was determined by SDS-PAGE using low-range molecular mass prestained standards (111–20.5 kDa, Bio-Rad). The isoelectric point of the purified protein was determined by IEF analysis on precoated gels (Serva, 3–6 pH gradient, 300 μm, 125×125 cm, p*I* marker protein test mixture 9) according to the manufacturer.

2.5. Immunoblotting

Proteins separated on SDS-PAGE were transferred to PVDF membranes (Immobilon-P, Millipore) for 1.5 h at 100 V in electrotransfer buffer (Tris 25 mM, glycine 192 mM, methanol 20% v/v, pH 8.3). The membranes were then soaked overnight at 4°C in blocking buffer (Tris saline blocking buffer (TSBB): Tris-HCl 10 mM pH 7.5; NaCl 100 mM; Tween-20 0.1% (v/v); BSA 5% (w/v)). After three 5-min washes (TSBB without BSA) they were exposed for 3 h at room temperature to primary antibody (10 μg ml^{-1} in TSBB containing 1% (w/v) BSA) raised against a constitutive esterase isolated from *B. cinerea* conidia [20] or an induced cutinase found in the culture filtrate [21] (mAb 14E5 and mAb 21C5 respectively, provided by Dr. A. Schots,

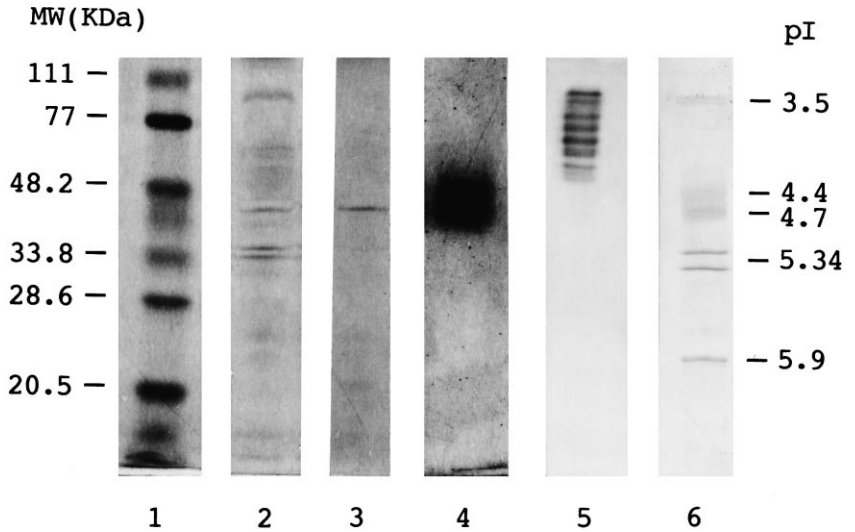

Fig. 1. Electrophoretic analysis of cutinase extracted from ungerminated conidia of *B. cinerea* Pers.: Fr.. Lane 1: Molecular mass standards (silver stain coloration). Lane 2: SDS-PAGE of the 10% $(NH_4)_2SO_4$ fraction. Lane 3: SDS-PAGE of the pure cutinase (5% butyl $(NH_4)_2SO_4$ Sepharose fraction, silver stain coloration). Lane 4: Renaturated pure cutinase on SDS-PAGE (5% butyl $(NH_4)_2SO_4$ Sepharose fraction, α,β-naphthyl acetate coloration). Lane 5: IEF of the pure cutinase (5% butyl $(NH_4)_2SO_4$ Sepharose fraction, α,β-naphthyl acetate coloration). Lane 6: p*I* standards (Coomassie coloration).

Laboratory of Monoclonal Antibody, Wageningen, The Netherlands). Positive reactions were revealed with a goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma, dilution 1/3000 in TSBB). Alkaline phosphatase activity was revealed with the fast red/naphthol method (Sigma Fast Red™/Naphthol AS-MX, N° F-4523) according to the manufacturer.

3. Results and discussion

Linskens and Haage [22] first provided evidence that penetration by *B. cinerea* in potato leaf cutin in vitro was linked to a cutinolytic activity. An 18-kDa cutinase was isolated by Salinas [21] in culture filtrates of *B. cinerea*. More recently, Comménil et al. [23] described a lipase excreted by *B. cinerea* which exhibited strong cutinolytic activity. Purified polyclonal antibody raised against this 60-kDa protein suppressed *B. cinerea* lesion formation on tomato leaves.

We have demonstrated previously that ungerminated conidia of *B. cinerea* contained a constitutive cytoplasmic cutinase [12], and we have now described its purification to homogeneity using gel filtration, ion exchange and hydrophobic interaction chromatography (Table 1). The 5% $(NH_4)_2SO_4$ fraction separated on butyl Sepharose contained a single

Table 1
Purification steps of the constitutive cutinase in ungerminated conidia of *B. cinerea* Pers.: Fr

Purification step	Volume (ml)	Total protein (μg μl^{-1})	Total enzyme[a] (μmol min^{-1})	Specific activity[a] (μmol min^{-1} μg^{-1})	Purification	Yield (%)
Crude extract	20	17.00	24.0	7.0	1.0	100
Superdex	10	0.40	7.9	98.7	14.1	32.5
DEAE (0.2 M NaCl)	6	0.04	3.6	446.0	63.7	14.9
Butyl Sepharose (5% $(NH_4)_2SO_4$)	2	0.01	1.2	640.0	91.4	5
Butyl Sepharose (10% $(NH_4)_2SO_4$)	2	0.03	0.7	466.0	66.5	2.9

[a]Determined with PNB as the substrate.

band with a molecular mass of 40.8 kDa revealed on SDS-PAGE by silver staining (Fig. 1) and with α,β-naphthyl acetate (Fig. 1). This fraction applied to ^{3}H-labeled cutin released ethyl acetate-soluble radioactive cutin monomers (Table 2). PNB hydrolytic activity (Table 1) and specific cutinase activity (Table 2) were also measured in the 10% $(NH_4)_2SO_4$ fraction (Table 2). Apart from the 40.8-kDa band, other unknown proteins were revealed in this latter fraction which will require further purification (Fig. 1). The constitutive 40.8-kDa cutinase does not correspond to other cutinases isolated from *B. cinerea*. Salinas [21] described a 110–111-kDa constitutive esterase isolated from *B. cinerea* conidia with an assumed cutinolytic activity and a 18-kDa cutinase purified from culture filtrates. mAb 14E5 raised against this constitutive esterase and mAb 21C5 raised against the excreted cutinase did not reveal the 40.8-kDa cutinase (Fig. 2). However, mAb 14E5 has revealed a 110-kDa protein present in a 2-week-old culture filtrate of *B. cinerea* (Fig. 2). Salinas [21] has described this 110-kDa constitutive conidial putative cutinase as a membrane-bound protein. The extraction protocols of conidial proteins used in this work did not allow the solubilization of such proteins. We can conclude that ungerminated conidia may contain two constitutive cutinolytic enzymes, one soluble (40.8 kDa) and another bound to membranes or cell walls (110 kDa), which are excreted at conidial germination and produced during mycelial growth. Further research will be necessary to elucidate this hypothesis.

Isoelectric focusing analysis of the purified 40.8-kDa cutinase has revealed seven isoforms with acidic properties (p*I* 3.5–4.2) (Fig. 1). This constitutive cutinase might be excreted during the early events of conidial germination. It could release, through cutin degradation, sufficient amounts of cutin monomers, such as 16-hydroxyhexadecanoic acid, to activate the *cutA* gene expression, described by Van der Vlugt-Bergmans [11], which is responsible for the synthesis of the 18-kDa induced cutinase. Both 40.8-kDa con-

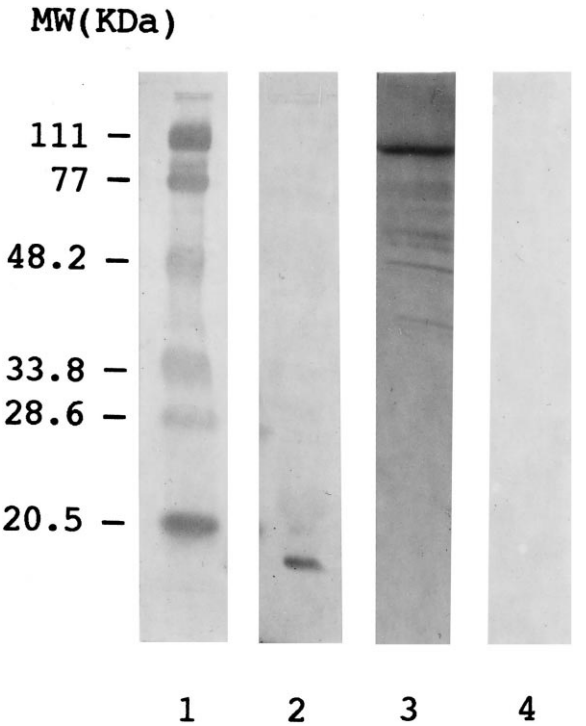

Fig. 2. Demonstration by immunoblotting that the 40.8-kDa cutinase does not react with mAb 14E5 and mAb 21C5 raised against a constitutive esterase in ungerminated conidia of *B. cinerea* and an induced 18-kDa cutinase purified from culture filtrates of *B. cinerea*. Lane 1: Prestained molecular mass standards. Lane 2: Detection of an 18-kDa cutinase with mAb 21C5 in 2-week-old cultures of *B. cinerea*. Lane 3: Detection of a 110-kDa constitutive conidial esterase with mAb 14E5 in 2-week-old cultures of *B. cinerea*. Lane 4: Pure 40.8-kDa cutinase (5% butyl $(NH_4)_2SO_4$ Sepharose fraction) is not detected by either mAb 21C5 or mAb 14E5.

Table 2
Radioactivity released from ^{3}H-labeled cutin by the pure 40.8-kDa constitutive cutinase contained in the butyl Sepharose (5% $(NH_4)_2SO_4$) fraction and in the partially purified 40.8-kDa cutinase (10% $(NH_4)_2SO_4$), compared to radioactivity released by cutinolytic activity in the crude extract

Extract	CPM ($\times 10^5$)	Specific activity (CPM $\times 10^4$ μg^{-1} protein)
Control	12.0 ± 0.4	0.00 ± 0.00
Crude extract	38.0 ± 0.7	0.21 ± 0.02
Butyl Sepharose (5% $(NH_4)_2SO_4$)	52.0 ± 0.6	436 ± 2.09
Butyl Sepharose (10% $(NH_4)_2SO_4$)	41.0 ± 0.4	273 ± 1.21

stitutive and 18-kDa induced cutinase could be strongly implicated in early infection processes by *B. cinerea* preceding latent stages and final plant colonization. The role of the 110-kDa cutinase is still unknown. The raising of polyclonal antibodies against the 40.8-kDa cutinase is in progress and such antibodies should be useful to study its role during conidial germination, its development during mycelial growth, and to explain some aspects of the complex enzymatic events of the infection process of *B. cinerea*.

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