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The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins *in situ*

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The nematophagous fungus *Verticillium chlamydosporium* secreted several proteases in submerged culture in which soya peptone was the sole carbon and nitrogen source. One protease, VCP1 (M_r 33000, pI 10·2), was purified 14-fold from culture filtrates to apparent homogeneity using preparative isoelectric focusing in free solution, and shown to rapidly hydrolyse the chymotrypsin substrate Suc-(Ala)$_2$-Pro-Phe-pNA and elastin. VCP1 had a K_m for Suc-(Ala)$_2$-Pro-Phe-pNA of $4{\cdot}3 \times 10^{-5}$ M and a k_{cat} of 5·8 s^{-1}. It was highly sensitive to PMSF and TPCK, but only moderately sensitive to chicken egg-white and soya bean trypsin inhibitors. VCP1 degraded a wide range of polymeric substrates, including Azocoll, hide protein, elastin, casein and albumin, and accounted for most of the non-specific protease activity detected in culture filtrates. The purified enzyme hydrolysed proteins *in situ* from the outer layer of the egg shell of the host nematode *Meloidogyne incognita* and exposed its chitin layer. VCP1 was secreted by several isolates of *V. chlamydosporium* and *V. lecanii*, pathogens of nematodes and insects respectively, but not plant-pathogenic species of *Verticillium*. These observations suggest that VCP1 or similar enzyme(s) may play a role in the infection of invertebrates.

Keywords: Chymotrypsin, chymoelastase, protease, nematophagous fungus, *Verticillium chlamydosporium*

INTRODUCTION

The hyphomycete fungus *Verticillium chlamydosporium* is a widespread pathogen of eggs and females of root-knot and cyst nematodes (Willcox & Tribe, 1974; Kerry & Crump, 1977; Morgan-Jones *et al.*, 1981). It plays an important role in the suppression of populations of *Heterodera avenae* in cereal monocultures (Kerry *et al.*, 1982). There is considerable interest in developing this fungus for control of nematodes in the field because it offers an environmentally benign alternative to chemical pesticides. Although some aspects of its ecology (De Leij, 1992) and taxonomy (Carder *et al.*, 1993) have been elucidated, its mode of action remains largely obscure (Morgan-Jones *et al.*, 1983). Successful exploitation of this fungus will, however, depend on a comprehensive understanding of its biology, in particular identification of those attributes which determine virulence.

Enzymes play a key role in fungal infection processes (Butt, 1990). Lipases (Paris & Ferron, 1979), chitinases (Jackson *et al.*, 1985) and proteases are considered to be virulence determinants of entomogenous fungi. The protease chymoelastase (Pr1), a major protein secreted by *Metarhizium anisopliae* and other hyphomycete fungi (St Leger *et al.*, 1987b), has been shown to be a key enzyme in the infection process (St Leger *et al.*, 1988). Proteases are also virulence determinants of the human-pathogenic fungi *Candida albicans* (Kwon-Chung *et al.*, 1985) and *Aspergillus fumigatus* (Frosco *et al.*, 1992). Little is known about the enzymes secreted by egg-parasitic nematophagous fungi but proteases may be important in host

Abbreviations: Bz, benzoyl; CBZ, carboxybenzoxy; CEW, chicken egg-white inhibitor, containing ovoinhibitor; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; MeOSuc, methoxysuccinyl; PMSF, phenylmethylsulphonyl fluoride; pNA, *p*-nitroanilide; STI, soybean trypsin inhibitor; Suc, *N*-succinyl; TEW, turkey egg-white inhibitor; TLCK, tosyl-Lys-chloromethyl ketone; TPCK, tosyl-Phe-chloromethyl ketone; VCP1, chymoelastase-like protease from *V. chlamydosporium*.

infection because a large part of the host egg shell and cuticle is composed of protein (Clarke *et al.*, 1967; Bird & McClure, 1976; Perry & Trett, 1986).

Proteolytic activity has been demonstrated for *V. chlamydosporium* (Kunert *et al.*, 1987; Carder *et al.*, 1993) and *V. suchlasporium* (Dackman *et al.*, 1989; Dackman, 1990), a closely related species with disputed taxonomic status (Carder *et al.*, 1993). Lopez-Llorca (1990) purified a 32 kDa serine protease from culture filtrates of *V. suchlasporium* and demonstrated that it was secreted during infection of cyst nematode eggs (Lopez-Llorca & Robertson, 1992). This paper describes the properties and possible role of a major protease secreted by an isolate of *V. chlamydosporium* which could be used in the selection of virulent strains of fungi for use in biocontrol programmes.

METHODS

Organisms and growth conditions. The fungi used in this study included five isolates of the nematophagous fungus *Verticillium chlamydosporium* (*Vc8, Vc10, Vc11, Vc26, Vc65*), three isolates of the entomophagous fungus *V. lecanii* (*Vl4, VlG, VlMy*) and one isolate each of the plant pathogens *V. albo-atrum* (*Va*) and *V. dahliae* (*Vd*). All the *V. chlamydosporium* isolates were originally collected from infected nematode eggs or from nematode-suppressive soils, and stored on silica gel at 4 °C in the Rothamsted culture collection. Isolate *VlG* (original name *Aln3*) was a gift of Dr G. Moritz (Martin-Luther University, Halle, Germany), *Vl4* and *VlMy* (Mycotal®) were obtained from Chr. Hansen's BioSystems (Denmark), and Koppert (the Netherlands) respectively. The plant-pathogenic isolates *V. albo-atrum* 1974 and *V. dahliae* 327, isolated from hop and strawberry, respectively, were gifts from Dr D. J. Barbara (East Malling, UK). All the isolates were maintained on potato dextrose agar at 23 °C in the dark.

Conidia and/or chlamydospores, harvested in sterile distilled water from 3-week-old cultures, were used to inoculate soya peptone medium (SPM: 10 g soya peptone, 0·3 g K_2HPO_4, 0·3 g $MgSO_4.7H_2O$, 0·15 g NaCl, 0·3 g $CaCl_2.6H_2O$, 0·8 mg $MnSO_4.6H_2O$, 0·2 mg $CuSO_4.5H_2O$, and 2 mg $FeSO_4.7H_2O$, made up to 1 l with distilled water). The final concentration of the inoculum was 10^5 conidia ml^{-1} and 3×10^3 chlamydospores ml^{-1}. Studies were done using 250 ml conical flasks containing 150 ml SPM. Isolate *Vc10* was also grown in four 2 l flasks each containing 1 l SPM. The flasks were incubated at 23 °C in a Gallenkamp orbital shaker (90 r.p.m.) in the dark for 7 d.

Comparative studies were done using the entomogenous fungus *Metarhizium anisopliae* (*V245*) originally isolated from Finnish soil and maintained on Sabouraud dextrose agar at 23 °C in darkness. Conidia from 2-week-old cultures were harvested in an aqueous solution of Tween 80 and 1 ml suspension containing 10^7 conidia ml^{-1} was added to a medium (100 ml) containing the same salts as SPM, but supplemented with beetle (*Phaedon cochleariae*) homogenate (10 mg ml^{-1}) (St Leger *et al.*, 1986). Incubation was as with *Vc10*.

The root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood was routinely maintained on *Solanum melongena* cv. Black Bell in greenhouse conditions (26 °C).

Chemicals. Most reagents were obtained from Sigma except for acrylamide (Protogel, National Diagnostics), neutralized soya peptone and Sabouraud dextrose agar (Oxoid), dialysis tubing (Medicell International), ammonium sulphate (Fisons), glycine (BDH), molecular mass markers and bovine serum albumin (Bio-Rad); ampholytes, GelBond and nitrocellulose were from Pharmacia.

Protein determination. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Preparation of crude enzyme concentrates. Cultures were harvested after 8 d by vacuum filtration through Whatman No. 1 filter paper on a Büchner funnel. Proteins were precipitated by adding solid ammonium sulphate to the culture filtrates (80% saturation) and collected by centrifugation (10000 ***g*** for 30 min). The pellet was resuspended in 0·05 M Tris, pH 7·9, and dialysed overnight at 4 °C against 300 volumes of buffer. This crude enzyme concentrate was used for further purification.

Preparative and analytical isoelectric focusing (IEF). Preparative IEF was done with a Bio-Rad Rotofor electrofocusing cell (Pichuantes *et al.*, 1989; Huang *et al.*, 1992). A mixture of 52 ml enzyme concentrate, 2 ml glycerol and 1·1 ml Pharmalyte 3-10 was injected in every other compartment of the focusing chamber. Ion exchange membranes were equilibrated and electrolyte solutions prepared according to the manufacturer's instructions. A ceramic cooling finger kept the sample at 4 °C during the whole run, which lasted 5 h, during which time the voltage gradually increased from 400 to 960 V. Twenty fractions were collected simultaneously under vacuum. The protein content, pI and proteolytic activity of each sample was determined. Maximum protease activity was recorded in the first five fractions (pI 8–10), which were pooled and re-fractionated using 2% (v/v) ampholytes, pH 8–10·5.

Analytical IEF was done using a Pharmacia Multiphor II apparatus at 5 °C for 30 min according to the manufacturer's instructions. Samples (20 µl) of culture filtrates and purified enzyme (i.e. fraction with highest activity) were applied alongside pI markers using paper strips to an ultrathin 5·4% (w/v) polyacrylamide gel containing 10% Pharmalyte 3-10.

Enzymoblotting. This was performed according to the method of Ohlsson *et al.* (1986) except that proteins from IEF gels were transferred to nitrocellulose by semi-dry electroblotting using the Novablot system (Pharmacia). The transfer buffer system used was as in Bjerrum & Schafer-Nielsen (1986).

Electrophoresis. SDS-PAGE was done according to the method of Laemmli (1970) using a 1 mm thick gel (12% resolving and 3·9% stacking gel). Following electrophoresis, proteins were incubated with Coomassie stain (0·2%, w/v, Coomassie Brilliant Blue R250 in destain) and destained using an aqueous mixture of 26% (v/v) ethanol and 10% (v/v) acetic acid.

Enzyme assays. Proteolytic activity of crude and purified enzymes was determined using a range of chromogenic substrates, including Azocoll, hide powder azure, elastin-Congo red, elastin-orcein, azocasein and azoalbumin. Each assay consisted of 500 µl substrate (10 mg ml^{-1}), 490 µl 0·1 M Tris pH 7·9, and 10 µl enzyme. Azocoll was prewashed according to the precautions outlined by Chavira *et al.* (1984). The mixture was incubated for 1 h on a rotary shaker at 37 °C, after which the protein was pelleted at 14000 r.p.m. in an Eppendorf microcentrifuge for 5 min. Undigested azocasein and azoalbumin were precipitated by adding 200 µl 20% (w/v) TCA, left to stand for 30 min then centrifuged in an Eppendorf microcentrifuge at 14000 r.p.m. for 5 min. The absorbance of the supernatants was read using a Hewlett Packard diode array spectrophotometer 8452A fitted with a temperature-controlled multicell unit at the following wavelengths: 520 nm (Azocoll), 595 nm (hide protein azure), 495 nm (elastin-Congo red), 592 nm (elastin-orcein), 336 nm (azocasein), and 326 nm (azoalbumin). Reference enzymes included final concentrations

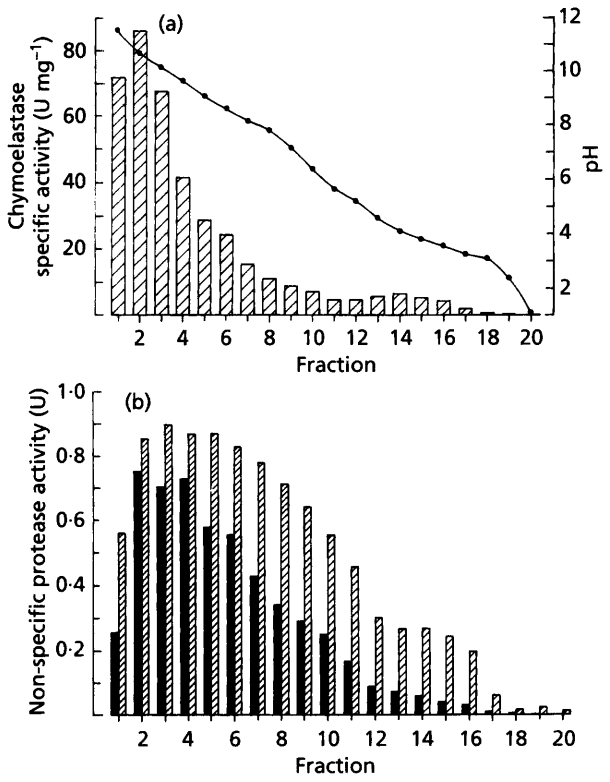

Fig. 1. (a) Chymoelastase activity measured using Suc-$(Ala)_2$-Pro-Phe-pNA. ●, pH of fractions. (b) Non-specific protease activity measured using Azocoll at pH 6 (■) and pH 9 (▨). Both activities were from fractions of crude enzyme concentrate separated by IEF using the Rotofor system (pH 3–10). One unit of activity for hydrolysis of Azocoll is defined as increase in A_{520} of 1 absorbance unit h^{-1}.

of 0·01 mg porcine pancreatic trypsin ml^{-1}, 0·01 mg bovine pancreatic chymotrypsin ml^{-1} and 0·05 or 0·2 mg porcine pancreatic elastase ml^{-1}. For elastolytic activity, standard curves were prepared measuring the absorbance of dilutions of elastin-Congo red that had been completely hydrolysed with porcine pancreatic elastase. One unit (U) of elastase activity is defined as the amount of enzyme that hydrolysed 1 mg elastin in 3 h at 37 °C.

Spectrophotometric assays for specific enzymes were done using *p*-nitroanilide oligopeptides as substrates. Crude and purified enzyme samples (10 µl) were added to 490 µl 0·1 M Tris/HCl buffer, pH 7·9, and the reaction, which was started by adding 500 µl substrate (2 mM in the same buffer), was followed by continuous recording of the increase in absorbance at 410 nm, at 37 °C. Chymotrypsin-like activity was assayed using Suc-$(Ala)_2$-Pro-Phe-pNA (Del Mar *et al.*, 1979), Suc-Phe-pNA (Nagel *et al.*, 1965) and Bz-Tyr-pNA (Bundy, 1962). Trypsin-like activity was assayed using Bz-Arg-pNA (Erlanger *et al.*, 1961) and Bz-Phe-Val-Arg-pNA (Svendsen *et al.*, 1972), while Suc-$(Ala)_3$-pNA (Bieth *et al.*, 1974) and MeOSuc-$(Ala)_2$-Pro-Val-pNA (Nakajima *et al.*, 1979) were used to detect elastase-like activity. Subtilisin-like activity was tested with CBZ-$(Ala)_2$-Leu-pNA (Stepanov *et al.*, 1977). One unit of activity is defined as the amount of enzyme releasing 1 µmol *p*-nitroaniline min^{-1}. The absorption coefficient was 10700 M^{-1} cm^{-1} at 410 nm.

Inhibition studies. Protease inhibitors (see Table 4) were used to characterize the purified enzyme and proteases in culture filtrates. Samples (10 µl) were preincubated with inhibitor at room temperature for 1 h before addition of 500 µl 2 mM Suc-$(Ala)_2$-Pro-Phe-pNA and buffer to make a final volume of 1 ml. Appropriate solvent controls were included.

Determination of kinetic constants. K_m and V_{max} values for Suc-$(Ala)_2$-Pro-Phe-pNA were determined using an iterative least-squares fit to the Michaelis–Menten equation (Leatherbarrow, 1990). Catalytic constant (k_{cat}) and specificity constant (k_{cat}/K_m) were calculated assuming that there was one active site per enzyme unit and that $V_{max} = k_{cat} \times [E]$, where [E] is the concentration of active sites. Duplicate measurements were made at eight substrate levels between 0·06 and 12 times K_m.

Proteolysis of *M. incognita* egg shells. Nematode eggs were collected by washing the roots of plants that had well-developed root-knots in 20% (v/v) domestic bleach (Teepol; available chlorine 4%, w/v), then immediately rinsed on a 30 µm mesh sieve. After separating eggs and debris by sugar centrifugation (47%, w/v, sucrose), eggs were sterilized in 0·1% $HgCl_2$ (Sijmons *et al.*, 1991). The effect of VCP1 on the egg shell was assessed by incubating 7500 eggs with 0·2 U VCP1 at 23 °C for 48 h; enzyme that had been denatured by boiling for 5 min

Table 1. Scheme for the purification of VCP1 from *V. chlamydosporium*

One unit (U) of chymoelastase activity is the amount of enzyme required to catalyse the production of 1 µmol *p*-nitroaniline from Suc-$(Ala)_2$-Pro-Phe-pNA min^{-1}.

Purification procedure	Total activity (U)	Yield (%)	Total protein (mg)	Specific activity (units mg^{-1})	Purification factor
Crude filtrate	4889·5	100	285·1	17·2	1·0
Ammonium sulphate precipitation	2053·6	42	70·3	29·2	1·7
Isoelectric focusing	286·7	5·9	2·2	130·3	7·6
Isoelectric focusing + refractionation	126·9	2·6	0·5	233·2	13·6

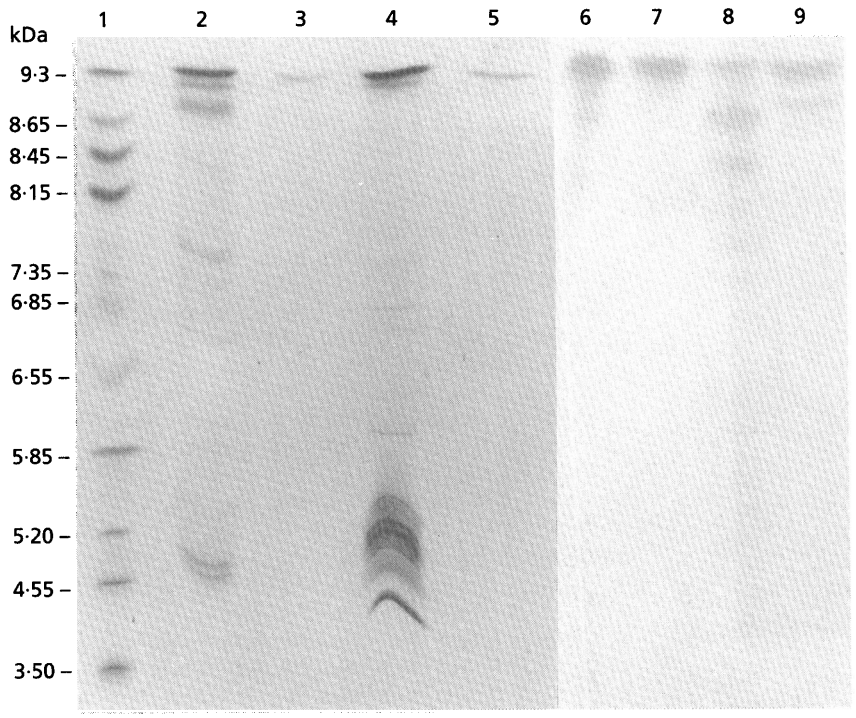

Fig. 2. Analytical IEF (pH 3–10) of purified chymoelastase of *V. chlamydosporium* (*Vc10*) and *Metarhizium anisopliae*. Lane 1, pI marker proteins; lane 2, *Vc10* culture filtrate; lane 3, purified VCP1; lane 4, *M. anisopliae* culture filtrate; lane 5, purified Pr1; lane 6, enzymoblot of *Vc10* culture filtrate; lane 7, purified VCP1; lane 8, *M. anisopliae* culture filtrate; lane 9, purified Pr1.

served as a control. There were three replicates in both the control and enzyme treatments. Eggs were pelleted at 10000 r.p.m. in an Eppendorf microcentrifuge for 1 min, after which the protein content of the supernatant was determined (Bradford, 1976). Similarly treated eggs were stained for β-glucans with Calcofluor white M2R (Butt *et al.*, 1989) after 18 h incubation and examined with an Olympus BH-2 microscope fitted with epifluorescence attachments, including a 405 nm excitation filter and a 455 nm barrier filter. Photomicrographs of representative specimens were recorded on Ilford XP2 400 ASA film.

Unless stated otherwise, all experiments were at least duplicated. Purification steps were performed at least five times. Data are reported for a representative purification scheme.

RESULTS

Electrophoretic analyses

Protease activities present in enzyme concentrates prepared from culture filtrates of *V. chlamydosporium* were analysed using preparative isoelectric focusing in the Rotofor system. All the IEF fractions contained chymotrypsin-like and non-specific protease activity, against Suc-$(Ala)_2$-Pro-Phe-pNA and Azocoll, respectively, but these were greatest at pI 8–10 (Fig. 1). Chymotrypsin-like activity appeared to account for most of the non-specific protease activity in the alkaline fractions; however, the presence of non-chymotrypsin-like enzymes with intermediate pI values in the culture filtrate cannot be ruled out. Greater activity was measured at pH 9 in every fraction (Fig. 1b). The alkaline fractions were refocused using narrow-range ampholytes (pH 8–10·5), which resulted in a 14-fold enrichment of the chymotrypsin-like enzyme, VCP1 (Table 1). Both IEF and SDS-PAGE showed that VCP1 corresponded to a major protein in the culture filtrate (Figs 2 and 3). Analytical IEF and SDS-PAGE of the alkaline Rotofor

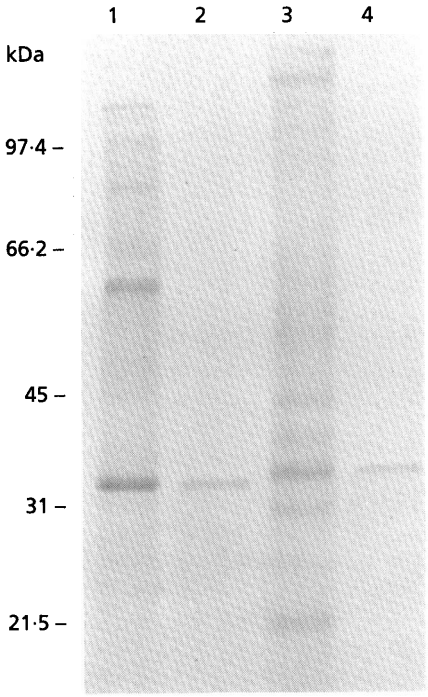

Fig. 3. SDS-PAGE of culture filtrates and purified chymoelastase of *Vc10* and *M. anisopliae*. Lane 1, *Vc10* culture filtrate; lane 2, VCP1; lane 3, *M. anisopliae* culture filtrate; lane 4, Pr1 from *M. anisopliae*.

Table 2. Substrate specificity of purified VCP1 and culture filtrates from *Vc10*, and reference proteases

All data are means of duplicate assays.

Substrate	Activity (% of max. specific activity) of:				
	Vc10 VCP1	*Vc10* filtrate	Trypsin	Chymo-trypsin	Elastase
Suc-$(Ala)_2$-Pro-Phe-pNA	100	100	15·8	100	100
Suc-Phe-pNA	0·1	0	0	0	0
Bz-Tyr-pNA	0	0	0	0	0
Bz-Arg-pNA	0	0·1	1·0	0	0
Bz-Phe-Val-Arg-pNA	8·3	5·5	100	0·2	0·5
Suc-$(Ala)_3$-pNA	0·2	0·6	0	0	21·3
MeOSuc-$(Ala)_2$-Pro-Val-pNA	0	0·1	0·1	0	8·5
CBZ-$(Ala)_2$-Leu-pNA	0·4	0·2	0	0	0
Max. specific activity (U mg^{-1})	19·2	13·2	7·1	53·6	0·3

Table 3. Substrate utilization of purified VCP1 and culture filtrates of *Vc10*

Activities are expressed as percentage of maximum specific activity per substrate. One unit of specific activity for hydrolysis of the following substrates is defined as follows. Azocoll hydrolysis, increase in A_{520} of 1 absorbance unit mg^{-1} min^{-1}; hide protein azure hydrolysis, increase in A_{595} of 1 absorbance unit mg^{-1} min^{-1}; elastin-Congo red hydrolysis, 1 unit of elastolytic activity solubilizes 1 mg elastin in 3 h at 37 °C; elastin-orcein hydrolysis: increase in A_{592} of 1 absorbance unit mg^{-1} h^{-1}; azocasein hydrolysis, increase in A_{336} of 1 absorbance unit mg^{-1} min^{-1}; azoalbumin hydrolysis, increase in A_{326} of 1 absorbance unit mg^{-1} ml^{-1}. All data are means of duplicate assays.

Enzyme	Activity (% of max. specific activity) using:					
	Azocoll	Hide protein azure	Elastin-congo red	Elastin-orcein	Azocasein	Azoalbumin
VCP1	100	100	36·3	80·2	88·8	53·3
Culture filtrate	29·3	18·4	27·2	24·3	100	100
Trypsin (0·01 mg ml^{-1})	30·9	14·8	2·6	3·8	55·9	26·6
Chymotrypsin (0·01 mg ml^{-1})	18·4	14·2	1·7	10·3	67·2	34·9
Elastase (0·05 mg ml^{-1})	16·4	11·3	5·3	12·9	27·5	13·1
Elastase (0·2 mg ml^{-1})	31·3	10·2	100	100	88·7	81·2
Max. specific activity (U mg^{-1})	5·0	16·6	6·2	5·6	4·2	5·3

fractions revealed a single band for VCP1, suggesting that they had been purified to homogenicity (Figs 2 and 3). The molecular mass of VCP1 was approximately 33 kDa, which corresponded with that of Pr1, purified from *Metarhizium anisopliae*.

Enzymoblotting of culture filtrates and purified VCP1 and Pr1, with Suc-$(Ala)_2$-Pro-Phe-pNA, showed that chymotrypsin-like activity was greatest at pI 10 but weaker activity was detected in culture filtrates at lower pI values (Fig. 2). *M. anisopliae* had at least three clearly distinct chymotrypsin-like enzymes, the most alkaline ones of which were difficult to separate.

Substrate specificity

Of the three chymotrypsin substrates assayed, VCP1 preferred Suc-$(Ala)_2$-Pro-Phe-pNA as opposed to Suc-Phe-pNA or Bz-Tyr-pNA (Table 2), suggesting that an aromatic residue in the P1 position (Schechter & Berger,

Table 4. **Effect of protease inhibitors on purified VCP1 and culture filtrates of *Vc10***

Residual activities were measured against Suc-$(Ala)_2$-Pro-Phe-pNA as the substrate. Ethanol-containing controls were used in the case of TPCK, pepstatin A and phenanthroline, and a 2-propanol-containing control for PMSF. Activity in the absence of inhibitor was 0·16 U (VCP1) and 0·12 U (culture filtrate). All values are means of duplicate assays and are expressed as percentage of the activities in the absence of inhibitor.

Inhibitor	Concn	Activity (%) of:	
		VCP1	Culture filtrate
Bowman-Birk	0·4 mg ml^{-1}	104·0	117·6
TEW	0·4 mg ml^{-1}	77·0	84·3
	1 mg ml^{-1}	80·5	77·7
CEW	0·4 mg ml^{-1}	56·2	73·2
	1 mg ml^{-1}	67·5	84·4
STI	0·4 mg ml^{-1}	68·3	86·0
	1 mg ml^{-1}	52·6	85·0
TPCK	10 μM	104·9	101·6
	100 μM	25·5	91·5
TLCK	10 μM	93·0	102·7
	100 μM	89·7	81·1
PMSF	1 mM	0·01	0
Elastatinal	250 μM	82·3	82·4
Leupeptin	0·1 mM	71·9	85·8
	0·5 mM	67·0	81·1
Pepstatin A	1 μM	85·8	80·6
E-64	10 μM	79·7	84·5
Phenanthroline	10 mM	114·2	99·7
EDTA	1 mM	68·7	76·2

Table 5. **Chymoelastase and non-specific protease activity of different *Verticillium* species**

Chymoelastase and non-specific protease activities measured using Suc-$(Ala)_2$-Pro-Phe-pNA, and Azocoll, respectively. Activities are expressed relative to those from *Vc10*. The maximum chymotrypsin-like and Azocoll degrading activities corresponding to 100 % were 0·40 U and 16·2 U, respectively. Units of activity for the nitroanalide are μmol min^{-1}, one unit of activity for hydrolysis of Azocoll is increase in A_{520} of 0·001 absorbance unit min^{-1}.

Species	Isolate	Chymotrypsin-like activity (% of *Vc10*)	Non-specific protease activity (% of *Vc10*)
V. chlamydosporium	*Vc8*	96·6	82·8
	Vc10	100	100
	Vc26	23·3	30·8
	Vc65	23·6	70·9
	Vc11	0·5	6·1
V. lecanii	*VlG*	18·0	33·3
	VlMy	32·7	86·1
	Vl4	23·2	38·9
V. albo-atrum		0·1	0
V. dahliae		0·3	1·5

1967) was insufficient for hydrolysis. VCP1 also exhibited weak trypsin, elastase and subtilisin-like activity since it was able to hydrolyse Bz-Phe-Val-Arg-pNA, Suc-$(Ala)_3$-pNA and CBZ-$(Ala)_2$-Leu-pNA, respectively (Table 2). The trypsin substrate Bz-Arg-pNA was not degraded. Substrate specificity of VCP1 and proteases in culture filtrate was similar, presumably because VCP1 accounted for most of the non-specific protease activity (Table 2).

The specific activity of VCP1 for 1 mM Suc-$(Ala)_2$-Pro-Phe-pNA (19·2 U mg^{-1}) was approximately a third of that of bovine chymotrypsin (53·6 U mg^{-1}), but more than 60 times greater than that of porcine elastase for this substrate (Table 2). Porcine pancreatic elastase hydrolysed both elastase [Suc-$(Ala)_3$-pNA, MeOSuc-$(Ala)_2$-Pro-Val-pNA] and chymotrypsin [Suc-$(Ala)_2$-Pro-Phe-pNA] substrates (Table 2). The specific activity of both VCP1 and porcine elastase for Suc-$(Ala)_3$-pNA was low (Table 2). VCP1, in contrast to the commercial proteases tested, was able to hydrolyse CBZ-$(Ala)_2$-Leu-pNA albeit weakly (Table 2), suggesting that it may belong to the subtilisin superfamily of serine proteases.

Both VCP1 and the commercial proteases degraded a wide range of protein substrates to varying degrees (Table 3). VCP1 was highly active against Azocoll and hide azure, and moderately active against casein, albumin and elastin. Activity of proteases from culture filtrates on azocasein and azoalbumin appeared to be greater than that of the purified VCP1, presumably because different enzymes were acting on these substrates (Table 3).

Kinetic properties

The reaction of VCP1 with Suc-$(Ala)_2$-Pro-Phe-pNA had a Michaelis–Menten constant, K_m, of $4{\cdot}26 \times 10^{-5}$ M, which coincides exactly with the K_m value for bovine chymotrypsin and this substrate (Del Mar, 1979). The catalytic constant, k_{cat}, was determined as 5·77 s^{-1} and the specificity constant, k_{cat}/K_m, was $1{\cdot}35 \times 10^5$ M^{-1} s^{-1}.

Inhibitors

Thirteen protease inhibitors were tested for their effect on the activity of VCP1 and the culture filtrate (Table 4). The serine protease inhibitor PMSF completely inhibited VCP1 and all proteolytic activity in culture filtrates. Tosyl-Phe-chloromethyl ketone (TPCK; chymotrypsin inhibitor), which has affinity for histidyl groups (Shaw *et al.*, 1965), was effective at 100 μM, causing 75 % inhibition of VCP1 and 8·5 % inhibition of culture filtrates. Similarly, the residual activity of VCP1 was less than that of the culture filtrates in the presence of leupeptin, chicken egg-white inhibitor containing ovoinhibitor (CEW) and STI (Table 4).

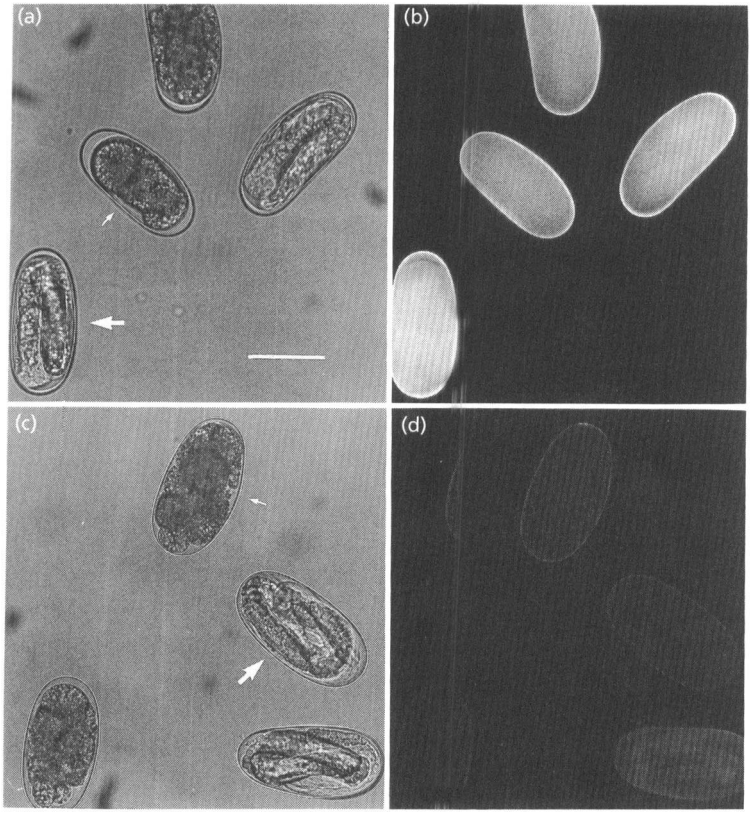

Fig. 4. Photomicrographs of *M. incognita* eggs stained with Calcofluor white M2R and treated with 0·2 U VCP1 for 18 h, recorded in bright field (a) and violet light (b), and eggs treated with the same amount of heat-denatured VCP1 in bright field (c) and violet light (d). Small arrows point at an immature egg; large arrows point at a mature egg. Bar, 20 μm.

VCP1 and non-specific protease activity of other *Verticillium* spp.

The proteolytic activity in culture filtrates of five *V. chlamydosporium* isolates (*Vc8, Vc10, Vc26, Vc65, Vc11*) differed significantly, with *Vc10* and *Vc11* exhibiting maximum and minimum activity, respectively (Table 5). In *Vc10*, VCP1 activity accounted for the bulk of the non-specific protease activity but this was not the case for all isolates (e.g. *Vc65, VlMy*). Both non-specific protease and chymotrypsin-like activities of *V. lecanii* (18–33%) were consistently lower than *Vc10* and were almost absent in *V. albo-atrum* and *V. dahliae* (Table 5).

Effect of VCP1 on host nematode egg shells

Protein and chitin are main structural components of nematode egg shells, chitin being covered by protein (Clarke *et al.*, 1967; Bird & McClure, 1976). The soluble protein content of the supernatant of *M. incognita* eggs treated with purified VCP1 was 60 μg ml^{-1}, whereas eggs treated with the same amount of denatured protease gave an average reading of 31 μg ml^{-1} (one-way ANOVA, $F = 9{\cdot}51$, $P = 0{\cdot}037$), indicating that the protease hydrolysed proteins from the nematode eggs. The removal of the protein cover from the eggs shells was further evidenced by staining similarly treated eggs for chitin with Calcofluor, resulting in very low background fluorescence in the eggs incubated in denatured protease (Fig. 4d), but bright blue fluorescence in VCP1 treated eggs (Fig. 4b). This suggested that the underlying chitin fibrils had been exposed. Eggs contained embryos in various developmental stages (Fig. 4a, c), but the fluorescence intensity of the egg shells was indiscriminately uniform (Fig. 4b, d).

DISCUSSION

VCP1, a major alkaline protease secreted by *V. chlamydosporium* (*Vc10*) in soya peptone medium, was purified from culture filtrates to homogeneity using preparative IEF. The enzyme shared several characteristics with Pr1, a major protease secreted by the insect pathogen *Metarhizium anisopliae* (St Leger *et al.*, 1987a). These included similar charge (pI approx. 10), size (approx. 33 kDa), sensitivity to PMSF and the ability to hydrolyse chymotrypsin and elastase substrates. However, VCP1 was less sensitive to CEW and TEW inhibitors (St Leger *et al.*, 1987a) but more sensitive to TPCK. Furthermore, VCP1 had a greater affinity for Suc-$(Ala)_2$-Pro-Phe-pNA than Pr1, but a smaller catalytic constant, although the specificity constant for this substrate was similar. These observations suggest that VCP1 is a chymoelastase similar but not identical to Pr1. An extracellular serine protease of *V. suchlasporium* has a size of 32 kDa, which is similar to VCP1, but its preliminary characterization (Lopez-Llorca, 1990) does not allow a fair comparison.

This paper provides the first clear evidence of a chymoelastase-like enzyme in a nematophagous fungus. Other hyphomycete fungi also produce alkaline chymoelastase-like enzymes, including *Beauveria bassiana*, *V. lecanii*, *Nomuraea rileyi*, *Aschersonia aleyrodis* (St Leger *et*

al., 1987b) and *Paecilomyces farinosus* (Samuels *et al.*, 1990). Antibodies against the chymoelastase Pr1 from *M. anisopliae* cross-react only with enzymes from some isolates of the same species (St Leger *et al.*, 1987b) but more sensitive assays have demonstrated that the basic proteases of *B. bassiana*, *P. fumosoroseus* and *M. anisopliae* share common epitopes (Shimizu *et al.*, 1993).

It is clear from this study that different isolates of *V. chlamydosporium* produce different amounts of chymoelastase, which corroborates the findings of St Leger *et al.* (1987b) for *V. lecanii*. The absence of such activity in plant-pathogenic members of the genus *Verticillium* suggests that this particular enzyme is restricted to invertebrate pathogens. These observations support the proposition of St Leger *et al.* (1987b) that these enzymes are required by invertebrate pathogens for nutrition, and as this paper shows, this would include the nematophagous fungus *V. chlamydosporium*. However, the overall success of fungal pathogens is dependent upon a complex interaction of several factors (Charnley & St Leger, 1991), including host recognition and the production of the right set and quantity of enzymes (Butt, 1990). In this respect VCP1 can be seen as a potent but broad-spectrum protease that not only has the potential to provide the facultative parasite *V. chlamydosporium* with nutrients from a wide range of protein sources, but may also have a role in pathogenesis, as it dissolves the outer protein barrier from *M. incognita* eggs.

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