

**The nematophagous fungus
Verticillium chlamydosporium:
aspects of pathogenicity**

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Abstract

Verticillium chlamydosporium is a fungal pathogen of eggs and females of plant-parasitic nematodes. The fungus produced an alkaline serine protease in submerged culture. This enzyme, VCP1, was characterized as a class II subtilisin, based on amino acid sequence homology. Several of its characteristics, e.g. molecular mass (33 kDa), pI (ca 10) and broad substrate utilisation, are typical of fungal subtilisins. Although some immunological cross-reactivity existed with other enzymes of this class, an antigenic fingerprint was obtained that was distinct, even from the subtilisin that was its closest homologue based on amino acid sequence, Pr1 from the entomogenous fungus *Metarhizium anisopliae*.

There was circumstantial evidence, suggesting that this fungal protease was involved in the infection of nematode eggs, which have a largely proteinaceous eggshell. First of all, the enzyme was able to remove the outer protein layer from eggs of the susceptible root-knot nematode, *Meloidogyne incognita*, exposing the underlying chitin layer. Scanning electron microscopy revealed that fungal hyphae on the egg surface left an imprint, presumably through enzymatic action. There was also evidence of the protease weakening the eggshell, as enzyme-treated nematode eggs were more easily lysed and infected by the fungus than those not pre-incubated in the enzyme. A polyclonal antibody against VCP1 demonstrated protease production by the fungus, prior to, or concurrent with, penetration. The enzyme was associated with appressoria, i.e. fungal infection structures. In contrast to the susceptible root-knot nematode, VCP1 had little impact on the egg shell of the potato cyst nematode *Globodera rostochiensis*. It is suggested that the limited *in situ*

hydrolysis of *G. rostochiensis* egg shell proteins is a factor contributing to its relative resistance to the fungus.

Regulation studies in batch culture showed that production of the protease VCP1 was repressed by high carbon and nitrogen levels. Its basic regulatory mechanism was that of repression/derepression. However, the highest protease titre was obtained when *M. incognita* eggs were present in the medium, suggesting induction by the host. Collagen and chitin were possibly responsible for this inductive effect.

In conclusion, it is believed that VCP1 is a protease with a dual role for *V. chlamydosporium*. During saprotrophic growth, VCP1 would allow the fungus to scavenge nutrients from a wide range of protein sources. However, the enzyme also has a designated function in penetration of the host, which makes it a versatile tool for a fungus that can switch trophic modes during its life-cycle.

The achievements of this research include the first demonstration in a nematode-attacking fungus of:

- a well-characterized protease, including data on stability, kinetics and isoforms;
- a subtilisin-like protease in an egg-parasitic nematophagous fungus;
- a pathogenicity-related enzyme in *V. chlamydosporium*;
- a determinant of host specificity;
- enzyme regulation in general, and induction by the host, in particular.

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Abbreviations

A_{280} : absorbance at the wavelength (nm) indicated;
Ac: N-acetyl;
BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium;
Bz: benzoyl;
CBZ: carboxybenzoxy;
CEW: chicken egg-white inhibitor, containing ovoidinhibitor;
Da: dalton, unit of molecular mass;
DAB: 3,3'-diaminobenzidine tetrahydrochloride;
E-64: trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane;
EDTA: ethylenediamine tetraacetic acid;
 g : acceleration due to gravity;
IEF: isoelectric focusing;
IP₃: inositol 1,4,5-triphosphate;
IPTG: isopropyl-1-thio- β -D-galactopyranoside;
 k_{cat} : catalytic constant;
 k_{cat}/K_m : specificity constant;
 K_m : Michaelis-Menten constant;
 λ_{max} : wavelength of maximum absorbance;
LB: Luria-Bertoni medium;
MeOSuc: methoxysuccinyl;
MES: 4-morpholineethansulfonic acid;
MNA: 4-methoxy- β -naphthylamide;
 M_r : molecular weight or relative molecular mass
 β NA: β -naphthylamide;
ORF: open reading frame;
Orn: ornithine;
PAGE: polyacrylamide gel electrophoresis;

TLCK: tosyl-Lys-chloromethyl ketone;

TPCK: tosyl-Phe-chloromethyl ketone;

V_{\max} : maximum velocity of an enzymatic reaction;

VCP1: subtilisin-like, extracellular protease from *Verticillium chlamyosporium*;

v/v: volume/volume;

w/v: weight/volume;

WA: water agar;

X-gal: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

1.1 BIOLOGICAL CONTROL OF NEMATODE PESTS

Biological control is a broad subject and various workers, dealing with very diverse systems, hold different views on the terminology. A current definition of biological control of plant-parasitic nematodes is “a reduction of nematode populations which is accomplished through the action of living organisms other than nematode-resistant host plants, which occurs naturally or through the manipulation of the environment or the introduction of antagonists” (Stirling, 1991).

A considerable number of antagonists (natural enemies) of nematodes have been identified and new ones are continuously being found. They include viruses, rickettsia, bacteria, a range of invertebrate animals, mycorrhizae and other fungi. These organisms have been the subject of several recent reviews (Barron, 1977; Mankau, 1981; Jatala, 1986; Kerry, 1987; Gray, 1988; Morgan-Jones & Rodríguez-Kábana, 1988; Small, 1988; Nordbring-Hertz, 1988; Saxena & Mukerji, 1988; Saxena et al., 1991; Stirling, 1991; Dijksterhuis et al., 1994), and only fungi will be dealt with from here on.

Biological control is either natural or induced. In **natural control** the nematode population is kept below a certain threshold by an increase in density of indigenous antagonists. The establishment of an equilibrium between the populations of nematodes and antagonists may take place over several years in perennial crops or monocultures. Natural control below the economic threshold has been

demonstrated only for the cereal cyst nematode, *Heterodera avenae*, under continuous cereals in England. The hyphomycete *Verticillium chlamydosporium* was one of the responsible agents for this best documented case of natural biological control (Kerry *et al.*, 1982a,b). Data from Germany indicate increased abundance of *V. chlamydosporium*, parasitising the beet cyst nematode, *H. schachtii*, in fields where beet monoculture had been practiced (Tribe, 1977). However, in this case, the equilibrium population is above the economic threshold (Kerry, 1989). Other fungi that have been considered the causes of nematode suppressiveness include the egg-parasite *Dactylella oviparasitica* in a peach orchard in California where *Meloidogyne* infestation was unexpectedly low (Stirling & Mankau, 1978), and a sterile Ascomycete, Arkansas Fungus 18, that was isolated from eggs and second-stage juveniles in a continuously cropped soybean field where a natural decline in the *Heterodera glycines* population occurred (Kim & Riggs, 1992). In **induced biological control** the fungal parasites are introduced once or repeatedly. Natural biological control agents may be used in a scheme of induced biological control. *Verticillium chlamydosporium* has been found effective if introduced to soil, e.g. against *H. avenae* (Kerry *et al.*, 1984) and against *Meloidogyne* spp. (Godoy *et al.*, 1983; Rodríguez-Kábana *et al.*, 1984; de Leij & Kerry, 1991; de Leij *et al.*, 1993b). However, some effective natural control agents lack characteristics essential for a strategy of introduction to soil, e.g. *Nematophthora gynophila*, which cannot be grown *in vitro* (Kerry, 1987; Crump & Moore, 1990).

The main asset of *V. chlamydosporium* as a potential biological control agent, is probably the fact that it attacks nematode eggs and females (Morgan-Jones *et al.*,

1981b). This makes it potentially very suitable for the control of root-knot and cyst nematodes, as the females of these nematodes are sedentary, and their eggs remain aggregated.

1.2 ROOT KNOT (*MELOIDOGYNE* SPP.) AND CYST (*HETERODERA* AND *GLOBODERA* SPP.) NEMATODES

Nematodes are currently placed in a separate phylum, Nematoda (previously Aschelminthes) (Maggenti, 1976). They are vermiform, unsegmented, bilaterally symmetrical pseudocoelomates with a circumenteric nerve ring and no circulatory or respiratory organs. The plant-parasitic nematodes that are economically most important belong to the order of the *Tylenchidae*: apart from free-living species, they include root knot nematodes in the genus *Meloidogyne* and cyst nematodes in the genera *Heterodera* and *Globodera*.

Meloidogyne species have a worldwide distribution, extensive host ranges, and are involved with fungi and bacteria in disease complexes, which makes them of major economic importance in a wide range of crops. The most important species, largely because of their wide distribution and broad host range, are *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla* (Jepson, 1987). They get their popular name “root knot nematodes” from the galls they produce on the roots of infected plants, mainly by inducing hypertrophy of cortical cells. These galls may coalesce, and in the species named above, several females may be present in a single gall.

Infective 2nd stage juveniles hatch from eggs that are embedded in a gelatinous matrix that often still adheres to the root tissue of the previous host plant. After migration through the soil towards a suitable host plant, the root epidermis is

penetrated near the root tip. The juveniles migrate between cells, until they establish themselves with the anterior end in contact with the vascular cylinder, where giant cells are induced. These cells are multinucleate with dense cytoplasm and highly invaginated cell walls. The giant cell is induced and maintained by a continuous stimulus from the nematode (Bird, 1979). A gall develops, inside which the juveniles gradually assume a flask shape. Three moults occur, the last of which is a true metamorphosis for the occasionally occurring male, which appears as a long vermiform nematode folded inside the cuticle of the fourth juvenile stage. The adult female at first retains the same shape of the last larval stage, but, as it matures, it enlarges and becomes pyriform (De Guirian & Ritter, 1979). Reproduction is often by mitotic parthenogenesis. Insemination only rarely takes place as the females are virtually inaccessible to the males, and maturation of the oocytes of inseminated females has been found to be similar to that of non-inseminated females (Triantaphyllou, 1963). Female rectal gland cells produce the gelatinous matrix in which the eggs, usually 500-1000, are deposited. Cellulolytic activity from the gelatinous matrix allows the eggmass to protrude on the gall surface (Orion *et al.*, 1987). Depending on the environmental conditions, the nematode life-cycle can be completed in less than one month (De Guirian & Ritter, 1979).

The life-cycle of cyst nematodes (*Heterodera* spp. and *Globodera* spp.) is roughly similar to that of root knot nematodes, but no gall is produced. As the swelling female ruptures the root cortex, permanent exposure on the root surface allows fertilisation. The body of the female fills with eggs, although some are extruded from the female body in a gelatinous matrix. Females appear as white

nodules on the root surface, but as soon as egg production ceases, the cuticle sclerotizes, the female dies and its remains form the mature cyst. In cyst nematode species that depend on root diffusates for induction of hatching, such as the potato cyst nematodes, *G. pallida* and *G. rostochiensis*, eggs can remain viable for many years (Shepherd & Clarke, 1971). The cyst may protect the eggs against desiccation and parasites.

Because of their confinement inside host roots or the cyst, their large reproductive rates, and the wide array of host plants, these plant-parasitic nematodes are difficult to control. Nematicides have been generally used to reduce soil populations to below damage threshold levels during the early and most susceptible stage of plant growth. If they are to be used as sole control agents for nematodes that have several generations during a crop cycle, they will have to be applied repetitively because of the nematode's reproductive capacity. A major disadvantage is the cost involved (Rodríguez-Kábana, 1992), but the main reason why they have been largely abandoned is their inherent toxicity coupled with their potential to be environmentally hazardous. The persistence of the dichloropropane component of D-D, DBCP, EDB and the water solubility of others such as aldicarb are particularly troublesome (Thomason & Caswell, 1987).

There are many non-chemical methods which can contribute to nematode control. They include the use of resistant cultivars, hot water treatment of planting material, flooding, cultivating the soil during a dry period, solarization. Rotation involves growing a sequence of susceptible and resistant, non-host or poor host crops. Fallow can be very effective in rotation. While crop rotation programmes have

become less attractive to growers where economics demand specialisation and intensification, and virulent nematode pathotypes have developed to overcome resistant cultivars, interest has shifted towards biological control. In particular, research on the natural enemies of plant-parasitic nematodes has focused on fungi that attack nematodes (Kerry, 1987).

1.3 NEMATOPHAGOUS FUNGI

1.3.1 Different modes of action

Nematophagous fungi comprise those fungi that produce traps or adhesive spores to infect active vermiform nematodes, and those which colonize sedentary stages such as females and their eggs. Irrespective of the type of nematophagous fungus, the ultimate fate of the nematode will be the same, with fungal hyphae developing within the nematode and the body or egg contents used by the fungus. The biomass of soil nematodes is often considerable, and nematophagous fungi help to recycle carbon, nitrogen, and other important elements from this trophic stratum. For man however, the ability of these fungi to destroy nematodes has presented an interesting possibility of employing them as a biological control agents for these agricultural pests. Most workers have focused on plant-parasitic nematodes, but there are also good prospects for the development of biological control agents for animal parasitic nematodes (Waller, 1993).

Despite the diversity in the fungal adaptations involved, three main strategies are commonly recognized. These fungal adaptations are briefly reviewed here. The purpose is not to acquaint the reader with the subject as a whole, but rather to provide

the framework in which it will be possible to interpret the degree of specialization of the fungus of interest, *V. chlamydosporium*.

1.3.2 Nematode-trapping fungi

In general, trapping fungi produce extensive but rather sparse mycelia in the soil environment, with trapping devices at intervals along the hyphae. Nematodes are captured either mechanically or by adhesion, followed by rapid penetration by a narrow infection peg, formation of an infection bulb, from which mycelium develops to digest the prey (Shepherd, 1955). Nematodes are usually fairly quickly immobilized when captured, which suggests the production of a fungal nematotoxin by these fungi (Gray, 1988). Despite their ubiquity in nematode-infested soil, a correlation between the presence of nematode-trapping fungi and the suppression of nematode populations has not yet been clearly demonstrated (Nordbring-Hertz, 1988).

There is an amazing diversity in the trapping mechanisms, and these fungi can be found in quite diverse taxa. However, the taxonomy of some of these, e.g. *Arthrobotrys* Corda, *Dactylaria* Sacc., *Dactylella* Grove, *Monacrosporium* Oudem. is confused, and new characters are urgently required (Gray, 1988; Liou *et al.*, 1995).

Simple adhesive hyphae. This feature is largely confined to the Zygomycetes, such as *Cystopage* spp. and *Stylopage* spp., fungi which are unable to form intricate capturing structures as they are unseptate and do not anastomose (Barron, 1977).

Adhesive branches are produced by anastomosis of a limited number of cells and covered by a thin adhesive film. Apposition of several branches prevents the escape of the prey. An example of a fungus producing adhesive branches is *Monacrosporium cionopagum* (Drechsler) Subram. (Dowsett *et al.*, 1984).

Adhesive knobs are morphologically distinct cells, covered with a thin layer of adhesive. They are either sessile, as in *Monacrosporium phymatopagum* (Drechsler) Subram., or borne on a non-adhesive stalk, as in *M. ellipsosporum* (Grove) Cooke & Dick. Adhesive knobs can sometimes be formed directly from the conidium (Liou *et al.*, 1995), conferring ecological advantage on the fungus as it will not encounter fungistatic effects that might thwart vegetative growth. Moreover, if the knob is detached from the stalk or hyphae by the struggling nematode, it often breaks while remaining attached to the nematode and forming an infection hypha anyway (Gray, 1988).

Non-constricting rings. In this infrequently encountered trapping mechanism nematodes are passively captured as they wedge themselves in the 3 or 4-celled rings, borne on lateral hyphal branches. As the prey struggles, the ring often breaks off the hypha, but nevertheless remains wedged around the nematode body, resulting in subsequent infection. The most frequently isolated species with this form of trap is *Dactylaria candida*, which forms not only non-constricting rings, but also adhesive knobs (Barron, 1977).

Constricting rings develop similarly to non-constricting rings but are ultrastructurally much more complex. Nematodes that enter the ring stimulate the inner surface, causing the ring to close abruptly after 2 to 3 seconds. The best-studied

species forming these traps is *Dactylaria brochopaga* (Drechsler, 1937; Barron, 1977).

Adhesive trapping networks. *Arthrobotrys oligospora* Fres. is probably the best known trapping fungus. It is a ubiquitous species that forms a three-dimensional complex of anastomosed loops which are thinly coated with a fibrillar adhesive material. The host recognition process involves the binding of a lectin, present on the *A. oligospora* trap, to N-acetyl-galactosamine on the nematode cuticle (Nordbring-Hertz & Mattiasson, 1979; Borrebaeck *et al.*, 1984). Despite the design of the network, which ensures that the struggling nematode becomes entangled in other parts of the network, they are not very efficient at trapping nematodes, as the prey tends to escape (Gray, 1988).

Arthrobotrys oligospora conidia may germinate to produce directly adhesive traps when applied close to cow faeces on a water agar plate (Dackman & Nordbring-Hertz, 1992). These conidial traps are considered a survival structure: the fungus is enabled to overcome fungistasis by adhering to the passing nematode before penetration takes place. The conidial trap is interesting as it is functionally similar to the adhesive conidia of endoparasitic fungi, and thus constitutes an intermediate form between the endoparasitic and nematode-trapping fungi (Dackman & Nordbring-Hertz, 1992).

Commercial products, based on nematode-trapping fungi, have been developed (Cayrol *et al.*, 1978; Cayrol & Frankowski, 1979), but control with this type of fungus has been erratic (Morgan-Jones & Rodríguez-Kábana, 1987). Nematode-trappers, as a whole, are only capable of killing second-stage juveniles or

males that migrate through soil. Juveniles are short-lived and often abundant, and it is difficult to make the periods of nematode migration and fungal trap formation coincide (Kerry, 1980). In a critical review, Stirling (1991) points out that the complex three-dimensional networks, formed on agar, fill a volume much greater than the small and irregular pore spaces in most soils. There have been few studies on trap formation *in situ*, and results from trap formation *in vitro* cannot necessarily be extrapolated to soil.

1.3.3 Endoparasites with infective spores

Although all nematophagous fungi could be considered “endoparasitic”, this term traditionally refers to fungi whose infective unit is a spore. Most endoparasitic nematophagous fungi are obligate parasites. They exist mainly as conidia in the soil, and do not form extensive hyphal systems outside the nematode. Conidia are usually either i) flagellate zoospores, in lower fungi, that encyst on the cuticle; ii) adhesive and attach to the nematode surface, which they penetrate after germination; iii) ingested, after which they germinate in the digestive tract.

Encysting spores. Endoparasitic Chytridiomycetes or Oomycetes form flagellate zoospores that are attracted by nematode exudates. Upon reaching the target, encystment of *Catenaria anguillulae* spores is triggered by specific carbohydrate/lectin interactions (Jansson & Thiman, 1992) and infection occurs either through natural openings or by penetration through the cuticle.

Adhesive conidia are produced by a number of endoparasitic hyphomycetes. *Verticillium coronatum* forms conidia with a cluster of small, apical appendages, with

which they attach to the cuticle of the host nematode (Barron, 1989). A well studied species is the obligate parasite *Drechmeria coniospora* (Drechs.) Gams & Jansson, the conidia of which have an adhesive knob, which in turn forms an appressorium (Dijksterhuis et al., 1990). This attachment is required, but not sufficient for infection, as *Drechmeria* can attach to nematodes that are non-hosts (Jansson et al., 1987); a lectin-carbohydrate recognition event involving sialic acids has been demonstrated (Jansson & Nordbring-Hertz, 1983).

Ingested conidia. *Harposporium spp.* are remarkably adapted to parasitism of free-living nematodes. Their morphologically adapted conidia are eaten by the host and attach to an area of the digestive tract that is species dependent. Plant parasitic nematodes are stylet-bearing and cannot be infected by this type of fungus (Gray, 1988).

“Explosive” conidia. *Haptoglossa heterospora* has one of the most unusual and spectacular methods of infection of a nematode by spores. The shape of its spore is such that, on mechano-stimulation by a passing nematode, the spore's protoplasm is shot through the nematode's cuticle, anchoring itself between cuticle and epidermis. The whole process only takes a fraction of a second (Barron, 1977).

Nematoctonus leiosporus is a remarkable example of overlap between endoparasitic and trapping fungi. Its conidia are not adhesive as long as they remain attached to the parent hypha. When a spore is detached, a short extension grows from it, carrying a terminal adhesive bud. Barron (1977) believes that the endoparasitic species of *Nematoctonus* are basically trapping fungi that have developed adhesive knobs on conidia rather than on hyphae.

1.3.4 Parasites of eggs and females

Parasites of eggs and females have been the focus of work on nematophagous fungi during the last few years. By killing the female or reducing fecundity they are potentially more effective at controlling population growth than species that parasitise juvenile stages (Kerry, 1980).

Cysts and eggs of a wide range of plant-parasitic nematodes have been found to be infected by a number of fungal genera. They include obligate parasites such as *Nematophthora gynophila* Kerry & Crump, *Catenaria auxiliaris* (Kühn) Tribe and a range of facultative parasitic members of the genera *Cylindrocarpon*, *Exophiala*, *Fusarium*, *Gliocladium*, *Paecilomyces*, *Phoma* and *Verticillium* (which will be dealt with separately in this review).

The relatively consistent association between cysts and/or eggs and certain fungal genera does, however, not always imply a clear-cut parasite-host relationship. *Fusarium* species, for example, have been frequently found as colonizers of nematode cysts. There are conflicting results as to their nematophagous potential (Nigh *et al.*, 1980; Dackman *et al.*, 1989). Egg penetration by these “opportunistic fungi” may or may not require a predisposing factor, weakening the eggs. A possible ecological role for these fungi may be the long term degradation of cyst exocuticle in soil (Morgan-Jones *et al.*, 1981a; Gray, 1988; Carris *et al.*, 1989). Several authors erroneously consider the opportunistic/parasitic fungi found in cysts as “cyst parasites”. This term has to be rejected, as Jatala (1986) correctly points out, because it is a contradiction in terms: the cyst is not a living nematode stage. Morgan-Jones *et al.* (1981a) try to clarify the confusion by dividing the fungi found in cysts and

eggs in three categories. Firstly, fungi that are able to enter the cysts early, grow saprophytically on its contents, including the embedding mucilage. Secondly, *bona fide* pathogens that are able to penetrate the egg shell. Thirdly, a succession of fungi involved in long-term degradation of resistant structures.

Nematophthora gynophila Kerry & Crump is an obligate endoparasitic Oomycete that infects female *Heterodera* but not *Globodera* nematodes. It has also been isolated from root-knot nematodes that form small galls, when females are exposed on the root surface (Kerry, 1989). Motile zoospores are released from the infected female, the cuticle of which becomes soft and breaks down. The survival stage is represented by thick walled resting spores which are formed inside the female (Kerry & Crump, 1980). Attempts to culture *N. gynophila in vitro* have failed (Crump & Moore, 1990). *Dactylella oviparasitica* Stirling & Mankau has successfully decreased parasitism of *M. incognita* on peach, but the success was mainly dependent on the small egg numbers produced on this crop (Stirling & Mankau, 1978, 1979; Stirling *et al.*, 1979). The hyphomycete *Paecilomyces lilacinus* (Thom) Samson is considered to have strong potential as a biological control agent in subtropical and tropical regions, due to its temperature optimum (26-30°C), intrinsic virulence and antibiotic activity against bacteria and other fungi (Morgan-Jones *et al.*, 1984). Although a commercial product has been developed (Timm, 1987), a serious drawback is the potential of this species to cause mycoses in man (Gordon & Norton, 1985).

1.4 VERTICILLIUM SPP.

1.4.1 Taxonomic status

The separation of the genus *Verticillium* (subdivision Deuteromycotina, form-class hyphomycetes) was based on its characteristic conidiophore morphology (Isaac, 1967). The conidiophores are erect, septate, and branched, with the short branches forming typical whorls. The terminal branches of the conidiophores are usually flask-shaped and pointed at the tips. Conidia are borne terminally, either singly or in small clusters.

Recently, the genus was divided into four sections (Gams & van Zaayen, 1982). Section *Nigrescentia* contains *V. albo-atrum* Reinke & Berth. and *V. dahliae* Kleb., plant-parasites with a host range including herbaceous as well as woody plants, many of which are of economic importance (Melouk, 1992). Section *Albo-erecta* contains several parasites of mushrooms (Gams & van Zaayen, 1982). Section *Prostrata* is characterized by white or whitish cottony colonies and a strong tendency to form prostrate conidiophores, little differentiated from vegetative hyphae. The resting structures of some species are very characteristic hyaline, thick-walled, multicellular, stalked chlamydospores (Campbell & Griffiths, 1975). The most common species of section *Prostrata* is *V. lecanii* (Zimm.) Viégas, and nematophagous *Verticillium* species are also classified in this section (Gams & van Zaayen, 1982).

Verticillium chlamydosporium was originally isolated from garden soil in Michigan, U.S.A. (Goddard, 1913). Its teleomorph is *Cordyceps* sp., provisionally named as *Cordyceps ovoparasitica*, which occurs on egg masses of molluscs in

tropical forest soils of South America (H. Evans, Silwood Park, personal communication). Whereas Isaac (1967) conjectured that *V. chlamydosporium* may not be a true *Verticillium* because it only rarely forms verticillately branched conidiophores, its taxonomic position was consolidated by Gams (1988). Based on differences in morphology and physiology, *V. chlamydosporium* is regarded as a species complex by several authors (Bursnall & Tribe, 1974; Irving & Kerry, 1986). However, Gams (1988) distinguishes *V. suchlasporium* var. *suchlasporium*, which he believes often to have been confused with it. According to Gams (1988), *V. suchlasporium* is distinct from *V. chlamydosporium* because of its taller, mostly erect and more densely verticillate conidiophores and its chlamydo-spores mostly buried in the agar (the new name is an abbreviation of *V. subchlamydosporium*). *Verticillium suchlasporium* has a lower temperature optimum compared to *V. chlamydosporium* (Dackman & Baath, 1989). The newly erected species could, however, not be confirmed by enzymatic and genetic analyses (Carder *et al.*, 1993).

1.4.2 Biological control potential and ecological aspects of *V. chlamydosporium*

Verticillium chlamydosporium is a worldwide soil-inhabiting fungus, isolates of which have been found to be associated with various species of cyst and root knot nematodes (see Table 1.1). *Verticillium chlamydosporium* has been isolated, not only from nematodes, but has also been reported to parasitise the oospores of Oomycetes (Sneh *et al.*, 1977), to be a slow but effective mycoparasite of the plant-parasitic fungus *Rhizoctonia solani* Kühn (Turhan, 1990), and to destroy eggs of snails

(Barron & Onions, 1966). The significance of these observations is not always clear, e.g. Tribe (1977) considered the latter to be an exceptional record.

Table 1.1 Field isolations of *Verticillium* spp. from nematode eggs, females or cysts.

<i>Verticillium</i>	Nematode host	Reference
<i>V. chlamydosporium</i>	<i>Globodera</i> sp.	Crump (1989)
	<i>G. rostochiensis</i>	Roessner (1987); Morgan-Jones & Rodríguez-Kábana (1986)
	<i>H. avenae</i>	Graham & Stone (1975); Kerry (1975); Kerry & Crump (1977); Tribe (1979); Cayrol <i>et al.</i> (1982); Juhl (1982); Dackman & Nordbring-Hertz (1985); Lopez-Llorca & Duncan (1988, 1990)
	<i>H. carotae</i>	Kerry & Crump (1977)
	<i>H. cruciferae</i>	Kerry & Crump (1977)
	<i>H. glycines</i>	Gintis <i>et al.</i> (1983)
	<i>H. schachtii</i>	Burnsall & Tribe (1974); Willcox & Tribe (1974); Kerry & Crump (1977); Tribe (1979); Muller (1982); Crump & Kerry (1983); Crump (1987); Dackman <i>et al.</i> (1989); Saleh & Quadri (1989)
	<i>H. trifolii</i>	Kerry & Crump (1977)
	<i>M. arenaria</i>	Morgan-Jones <i>et al.</i> (1981b, 1983); Rodríguez-Kábana <i>et al.</i> (1984)
	<i>M. incognita</i>	Gaspard <i>et al.</i> (1990)
	<i>T. semipenetrans</i>	Walter & Kaplan (1990)
<i>V. lamellicola</i>	<i>H. glycines</i>	Morgan-Jones <i>et al.</i> (1981a); Godoy <i>et al.</i> (1982b)
	<i>M. arenaria</i>	Rodríguez-Kábana <i>et al.</i> (1984)
<i>V. lecanii</i>	<i>H. glycines</i>	Gintis <i>et al.</i> (1983)
<i>V. leptobactrum</i>	<i>H. glycines</i>	Godoy <i>et al.</i> (1982b)
<i>V. suchlasporium</i>	<i>G. rostochiensis</i>	Dackman (1990)
	<i>H. avenae</i>	Dackman & Baath (1989)

G.: *Globodera*; H.: *Heterodera*; T.: *Tylenchulus*; V.: *Verticillium*

Although some introductions of *V. chlamydosporium* isolates to soil have been relatively successful (Godoy *et al.*, 1983; Kerry *et al.*, 1984; Rodríguez-Kábana *et al.*, 1984; de Leij & Kerry, 1991; de Leij *et al.*, 1993b), significant failures (see Stirling, 1991) indicate that further development of the system is required. An

obvious question for the field worker is how to formulate the fungus. *Verticillium chlamydosporium* hyphae and conidia do not survive well when introduced to soil, unless a food base is included, which results in unwieldy inocula in the range of 2.5 tons ha⁻¹ (Kerry, 1988). However, when chlamydospores are introduced into soil without an added food base, the fungus survived and established (de Leij & Kerry, 1991). As yet, chlamydospores in large numbers can only be produced on solid media (Kerry *et al.*, 1993), but development of a submerged culture system might entice industrial participation in the development of *V. chlamydosporium* as a biological control agent.

Soil survival is an essential attribute of any nematophagous fungus, and it has been a limiting factor also for other systems. For example, *P. lilacinus* has been reported to have good potential against *Meloidogyne* species (Jatala, 1985), but later proved to be inadequate as it failed to survive after inoculation (Gomes Carneiro & Cayrol, 1991). The survival of *V. chlamydosporium* (de Leij & Kerry, 1991; de Leij *et al.*, 1993b) may be partly determined by its ability to form resting structures, chlamydospores (Campbell & Griffiths, 1975).

An important feature of successful isolates of *V. chlamydosporium* is their rhizosphere competence, i.e. the extensive colonization of the rhizosphere of the nematode's host plant. De Leij & Kerry (1991) found that the only isolate in their test that was effective against *M. arenaria* was the rhizosphere competent one. Rapid and abundant growth over the root surface may enhance the chances of nematode infection, and therefore provide a competitive advantage for the fungus. The fungus is more abundant on roots infected with root-knot nematodes, presumably because

of enhanced root exudation (de Leij *et al.*, 1992b). The isolates used by Clyde (1993), when given the choice, did not select for *H. schachtii* infected roots, but they were more prevalent near the root tip, which again may be exudation-related. Rhizosphere colonization never implies invasion of the root cortex and no detrimental effects on the plants have ever been observed (Kerry *et al.*, 1986, de Leij & Kerry, 1991). The plant species seems to have a significant effect on rhizosphere colonization, but better techniques to study colonization are called for (Bourne *et al.*, 1994). Many factors may contribute to rhizosphere competence (Ahmad & Baker, 1987; Weller, 1988; Weller & Tomashow, 1994), but the actual determinants for *V. chlamydosporium* are unknown.

There is little information on the competitiveness of *V. chlamydosporium*, apart from indirect observations, such as the fungus being much more abundant on the surface of sterile roots than on those grown in soil (Bourne *et al.*, 1994). Mycostasis by rhizosphere bacteria, a profound regulatory factor of fungal populations in soil (Garrett, 1956), has been observed in the case of the closely related species *V. suchlasporium* (Lopez-Llorca & Boag, 1990). This egg-parasitic fungus is, however, never completely inhibited by bacteria, which may be explained by the antibacterial activity of the fungus (Filipello-Marchisio, cited in Lopez-Llorca & Boag, 1990).

1.4.3 Ultrastructure of infection by *V. chlamydosporium*

Many pathogenic fungi form specialized infection structures, called appressoria, on germ tubes or hyphae, prior to penetration (Emmett & Parbery, 1975). Appressoria

are produced by fungi infecting widely different hosts, including other fungi (Jeffries & Young, 1994), plants (Nicholson & Epstein, 1991), arthropods (Boucias & Pendland, 1991), and nematodes (Barron, 1973; Stirling & Mankau, 1979; Nigh *et al.*, 1980; Dunn *et al.*, 1982; Lýsek & Krajčí, 1987; Lopez-Llorca & Claugher, 1990; Sjollemma *et al.*, 1993). Some model systems, e.g. the plant pathogens *Magnaporthe grisea* and *Uromyces appendiculatus*, and the insect pathogen *Metarhizium anisopliae*, are very well studied and much of the signalling and differentiation events leading to appressorium formation has been elucidated (Hoch & Staples, 1991; St. Leger *et al.*, 1991b; Read *et al.*, 1992; Talbot, 1995). The study of appressorium formation by egg-parasitic *Verticillium* species is comparatively poorly developed, with few observations (Lýsek & Krajčí, 1987; Lopez-Llorca & Claugher, 1990; Chapter 5).

It has been very difficult to obtain information about the early stages of infection, as the intact egg shell is extremely impermeable to embedding mixtures for transmission electron microscopy (Bird & McClure, 1976; Wharton, 1980; Morgan-Jones *et al.*, 1983). However, penetration pegs from appressoria breach the chitin and lipid layers of the egg shell, after which the mycelium branches. The juvenile cuticle is also disrupted, and after a proliferation stage, the fungus penetrates the egg inside-out, leaving the juvenile necrotic and disintegrated (Lýsek & Krajčí, 1987; Morgan-Jones *et al.*, 1983; Lopez-Llorca & Robertson, 1992b). Each author suggests the involvement of hydrolytic enzymes to explain areas of low-electron density around the penetration hyphae.

1.4.4 Factors influencing nematode susceptibility to fungal attack

Nematode genus. The vitelline and chitin layers of *M. incognita* eggs are relatively thin when compared to those of *G. pallida* and *Nacobbus aberrans*. This simplicity may account for the vulnerability of *M. incognita* eggs to fungal penetration, or to the action of their exopathic compounds (O'Hara & Jatala, 1985).

There is a correlation between relative dityrosine content of egg shells and cyst walls, and resistance to fungal attack, as *Heterodera avenae* and *H. schachtii* have lower dityrosine:tyrosine ratios compared to *Globodera rostochiensis* and *G. pallida* (Lopez-Llorca & Fry, 1989), and the former are more susceptible to fungal attack (Kerry, 1982).

Developmental stage of nematode. Cayrol *et al.* (1982) reported that *H. avenae* eggs were not susceptible to parasitism by *V. chlamydosporium* after they had developed beyond the three-cell stage. Although infection of mature eggs has been observed (Lopez-Llorca & Duncan, 1990; see Chapter 5), immature eggs are generally considered to be more susceptible to attack by *V. chlamydosporium* than are those containing second-stage juveniles (Morgan-Jones & Rodríguez-Kábana, 1985; Irving & Kerry, 1986; Roessner, 1987). The same held for *Dactylella oviparasitica* (Stirling & Mankau, 1979). The reason for this decreasing susceptibility with age may be the dityrosine polypeptide cross-linking which increases during maturation (Lopez-Llorca & Fry, 1989). On the other hand, no apparent differences in level of invasion among various developmental stages have been observed in the infection of *M. arenaria* eggs by *Paecilomyces lilacinus* (Morgan-Jones *et al.*, 1984).

Effect of aggregation of eggs in gelatinous matrix or cyst. More *M. incognita* eggs are infected by *D. oviparasitica* if the eggs are retained within the mucilage of the egg masses than if the eggs are dispersed on the fungal culture. It was concluded that, although this fungus derives little nutrition from the mucilage, it spreads more rapidly and killed more eggs when they remain aggregated (Stirling & Mankau, 1979). Whereas, similarly, *V. chlamydosporium* infected more *H. schachtii* eggs in females compared to dispersed eggs, the reverse was true with *H. avenae* eggs (Irving & Kerry, 1986). The authors were unable to explain the difference between the egg infection rates in females of *H. schachtii* and *H. avenae*, but maybe the background microflora in females of both species, that were obviously reared on different host-plants, presented different levels of competition.

1.5 ENZYMES AND OTHER METABOLITES PRODUCED BY NEMATOPHAGOUS FUNGI

1.5.1 Lipases

When comparing the eggshell ultrastructure of *H. schachtii* from the field (heavily contaminated with fungus) and from the greenhouse (virtually fungus-free), Perry & Trett (1986) were unable to detect an inner lipid layer in the former. This was attributed to fungal lipolytic activity, although no direct evidence was obtained. *Verticillium chlamydosporium* is claimed to produce lipases (Kunert & Lýsek, 1987), but these authors used Tween as substrate in their lipase assays, which are known to be also degraded by a range of proteolytic enzymes (Brokerhoff & Jensen, 1974). Apparently, there are no conclusive reports on lipolytic activity in *V. chlamydosporium*.

1.5.2 Chitinases

Chitinolytic activity appears to be widespread in nematophagous fungi (Table 1.2).

Table 1.2 Nematophagous fungi that are chitinolytic by virtue of their ability to clear colloidal chitin in agar plates.

Fungal species	Reference
<i>Dactylella oviparasitica</i>	Stirling & Mankau (1979)
<i>Paecilomyces lilacinus</i>	Gintis et al. (1983)
<i>V. chlamyosporium</i>	Gintis et al. (1983); Kunert et al. (1985); Dackman et al. (1989); Carder et al. (1993) ^a
<i>V. lamellicola</i>	Godoy et al. (1982a)
<i>V. leptobactrum</i>	Godoy et al. (1982a)
<i>V. suchlasporium</i>	Dackman et al. (1989); Dackman (1990); Carder et al. (1993) ^a

^a These authors established chitinase activity colorimetrically as N-acetyl- β -D-glucosaminidase

Many authors have observed the clearing of colloidal chitin on agar plates, and most of them immediately implicated chitinase activity in the infection process, since nematode egg shells contain chitin (Clarke *et al.*, 1967; Bird & McClure, 1976). However, no one has studied the enzymes involved in any great detail, and no chitinase from any nematophagous fungus has been purified and/or cloned.

1.5.3 Proteases

Proteases are probably the best studied enzymes from nematophagous fungi. A collagenase (M_r 28,000) has been isolated and purified from a culture filtrate of *Arthrobotrys amerospora*. The enzyme was more active against *Pratylenchus scribneri* than against bovine collagen (Thomas *et al.*, 1988), suggesting adaptation to the host. True collagenases are capable of hydrolyzing peptide bonds of the poly-proline type in the helical region of undenatured collagen. This restrictive definition

excludes a wide array of enzymes that readily cleave denatured collagen or slice it in its non-helical peripheral parts (Keil, 1979). Fungal collagenases usually have broad proteolytic activity, and the limited data provided by Thomas *et al.* (1988) provide insufficient evidence for a collagenase *sensu stricto*. More convincing data are provided on a collagenase from the same fungus by Schenk *et al.* (1980), but it is unclear whether both enzymes are the same.

Tunlid and co-workers recently purified and characterised an acidic subtilisin (M_r 35,000, pI 4.6) from *A. oligospora*, which they believed was involved in the infection process because it immobilized the free-living nematode *Panagrellus redivivus* (Tunlid *et al.*, 1994). Furthermore, trap-bearing mycelium that was incubated with a broad-spectrum serine protease inhibitor decreased the immobilisation of captured nematodes (Tunlid & Jansson, 1991).

An alkaline subtilisin (M_r 33,500, pI 10.2) from the egg-parasitic fungus *Paecilomyces lilacinus* was characterized recently (Bonants *et al.*, 1995). The molecular age is dawning on nematophagous fungi, as this is the first report of a cloned gene (see Appendix).

Elastin, an insoluble protein of unusual structure, was digested by the *V. chlamydosporium* isolates tested by Kunert *et al.* (1987). The same authors observed gelatinolytic and caseinolytic activity. A positive correlation between proteolytic activity and ovicidity against *Ascaris lumbricoides* L. has been demonstrated (Kunert *et al.*, 1987). Mutants of *V. chlamydosporium* in which proteolytic activity (measured on chocolate agar) was altered, were characterized by a similar correlation (Chapulová & Lenhart, 1984).

Verticillium suchlasporium is the source of a 32 kDa serine protease, purified by Lopez-Llorca (1990). The enzyme is thought to be involved in the infection process as it is able to degrade *G. pallida* cyst wall proteins *in vitro*, and also because it was detected immunologically during infection of *H. schachtii* eggs (Lopez-Llorca & Robertson, 1992a).

1.5.4 Other enzymes

There are a few studies on enzyme activities in the filtrates of submerged cultures, other than the ones already reported. Cellulase activity of *V. chlamydosporium* (Kunert *et al.*, 1982) may contribute to its rhizosphere competence (Weller, 1988). The quantitation of no less than 20 glycosidases, 10 esterases, one transferase and 56 different peptidases allowed Carder *et al.* (1993) to cast doubt on the newly erected species *V. suchlasporium*, as it could not be separated from *V. chlamydosporium*.

1.5.5 Inhibitory and stimulatory metabolites

There are reports suggesting toxic effects of nematophagous fungi, e.g. nematode immobilization and death by *Nematoctonus* sp. (Giurma & Cooke, 1971; Giurma *et al.*, 1973) and *Arthrobotrys oligospora* (Anke *et al.*, 1995). Potent nematotoxic activity has also been observed in the oyster mushroom, *Pleurotus ostreatus*, and other species in the genus (Thorn & Barron, 1987). However, reports are inconsistent, e.g. the results with *V. chlamydosporium* from Irving & Kerry (1986) and Morgan-Jones *et al.* (1984). Some of the inconsistencies may be caused by the apparent dependence of toxin production on the medium (Cayrol *et al.*, 1989). Unfortunately, little work

has been done on the characterisation of toxins from nematophagous fungi (Anke *et al.*, 1995). No biotechnologist has been enticed to take up their study.

1.6 TROPHIC MODES

There are three basic nutritional modes recognised amongst fungi, according to the way in which they exploit external resources (Thrower, 1966). These are: **saprotrophy**, in which non-living substrata, other than those that have been killed by the fungus itself, are utilised; **necrotrophy**, where host tissues are first killed then utilised saprotrophically; and **biotrophy**, where nutrients can be obtained from living cells only, death of the latter will terminate biotrophic activity.

Whereas the categories saprotrophy, necrotrophy and biotrophy refer to host-parasite physiology, the terminology parasite - saprophyte traditionally refers to the ability to grow in axenic culture. A **parasite** is an organism existing in intimate association with another living organism from which it derives an essential part of the material for its existence. Whereas a **facultative parasite** and a **saprophyte** can grow on artificial media in axenic culture, an **obligate parasite** cannot. However, as physiology progresses, more and more unculturable organisms are likely to be grown *in vitro*, and therefore culturability may have to be abandoned as a basis for classification (Lewis, 1973).

Trophic modes have often been used to classify fungi in ecological groups based on the fact whether they are obligately confined to a particular mode or whether they can facultatively acquire external resources via other modes. This type

of classification reveals enormous nutritional plasticity in fungi as nine main categories may be recognized (see Table 1.3).

Table 1.3 Econutritional groups of fungi according to trophic mode and ecological behaviour (after Cooke & Whipps, 1993).

Econutritional group	Characteristics
Obligate biotrophs	No capacity for saprotrophy or necrotrophy
Hemibiotrophs	Initially biotrophic but then becoming necrotrophic, saprotrophic potential as for obligate necrotrophs
Facultatively saprotrophic hemibiotrophs	Initially biotrophic but later becoming necrotrophic; a final saprotrophic phase then occurs
Obligate necrotrophs	Normally necrotrophic; any saprotrophic ability severely limited or restricted to survival in dead tissues
Facultatively saprotrophic necrotrophs	Normally necrotrophic but with some ability to become saprotrophic
Facultatively necrotrophic saprotrophs	Normally saprotrophic but with some ability to become necrotrophic
Obligate saprotrophs	No capacity for necrotrophy or biotrophy
Facultatively biotrophic saprotrophs	Normally saprotrophic but with some ability to become biotrophic
Facultatively saprotrophic biotrophs	Normally biotrophic but with some ability to become saprotrophic

It is tempting to evaluate the trophic modes saprotrophy, necrotrophy and biotrophy in an evolutionary context in terms of which is primitive and which is more evolved. Saprotrophy has often been considered as the most primitive state from which necrotrophy was derived, in turn giving rise to biotrophy. This complies with the long-standing view that parasites evolved from free-living ancestors, at the same time becoming increasingly specialized physiologically (Garrett, 1970; Lewis, 1973; Cooke & Whipps, 1993). Although there is consensus about this being the general rule, evolution has occasionally been found to be accompanied by the opposite trend, from biotrophy towards saprotrophy (Cooke & Whipps, 1980; Jeffries & Young, 1994). Based on the observed frequency of facultative behaviour, there is a far greater

extent of overlap between saprotrophy and necrotrophy than with biotrophy and any other mode (Cooke & Whipps, 1993). In general, the nutritional requirements of most necrotrophic and saprotrophic fungi are quite similar (Jeffries & Young, 1994). The strong connection between necrotrophy and saprotrophy is taken for granted, but it is the direction of evolutionary movement that is in doubt. Fungi are often not fixed within a single trophic mode but show flexibility during their life cycle. Cooke & Whipps (1993) conclude that the three trophic modes have arisen on many occasions throughout a range of systems as a response to changing environmental conditions, and therefore that the direction of nutritional evolution taking place at any time is determined by the nature of the environmentally imposed options presented to fungi, including, where appropriate, narrowing specialism within a single mode.

The ruling hypothesis as to “why” nematophagous fungi occupy their particular niche, is based on the ideas of Cooke (1962a,b). Although he worked only with nematode-trapping fungi, the concepts are equally valid for egg-parasites (see General Discussion). Trapping may be an adaptive mechanism for dealing with nutritional stress, imposed by competition with other microorganisms (Cooke, 1962a,b). Utilization of nematodes as an additional food source would give the fungus a competitive edge. Whereas the competition according to Cooke mainly revolved around carbon energy sources, Barron (1991) suggests nitrogen, and not carbon, as the limiting nutrient. In his view, the ability to trap nematodes might allow the fungus to utilize the carbon sources available more efficiently. There is experimental evidence that nematode-trapping species, grown with saprophytic

competitors in an artificial soil substrate, increase their trapping activity compared to control cultures, confirming Cooke's hypothesis (Quinn, 1987).

Building on the concept of trapping because of competition, Cooke (1964) went on to discuss specialization within nematophagous fungi. He found that net forming fungi are able to grow saprotrophically, even in conditions of competition, while constricting ring species and others, such as *Dactylaria candida* and *Monacrosporium cionopagum* (Tunlid *et al.*, 1992), adopt a nematode trapping habit especially when competition is severe. These stronger trappers only revert to a saprotrophic existence when ephemeral carbohydrates such as hexoses are available as energy sources (Satchuthananthavale & Cooke, 1967a,b). They are considered to be more advanced towards an obligately nematode-trapping mode than the net formers, particularly because constricting ring species have lost some requirements for successful saprophytism, such as a rapid growth rate and competitive saprophytic ability (Cooke, 1964). These findings were confirmed by Jansson & Nordbring-Hertz (1979) who concluded that the attraction of trapping fungi to their hosts increases with increasing parasitic behaviour and dependence on the nematode.

These principles have important consequences for biological control. For example, the facultative parasite *A. oligospora* was thought to be a most valuable regulator of nematode populations, as traps are induced by the presence of nematodes (Nordbring-Hertz, 1977). However, trapping activity is often more related to nutrient levels than to the presence of nematodes, as a low nutrient medium is essential (Cooke, 1962a; Nordbring-Hertz, 1973; see Chapter 6). An incomplete understanding

of the ecology involved may well in part explain why biological control attempts have often failed.

The previous scenarios have been contested very little, although Jaffee *et al.* (1992) recently contended that parasitism may be much more important in nematode-trapping fungi. On the other hand, Dijksterhuis (1994) studied the interaction between *A. oligospora* and the endoparasite *Drechmeria coniospora* and found that, when *A. oligospora* was added during later stages of infection with *D. coniospora*, it could still enter the host but its trophic hyphae collapsed upon contacting hyphae of *D. coniospora*. The latter may therefore have antagonistic capacities, suggesting that endoparasitic fungi may have a certain advantage over less specialized organisms with respect to their interaction with nematodes in the natural environment.

A final, remarkable, illustration of the nematophagous habit being a survival strategy, is *Macrobotrophthora vermicola* (McCulloch) Tucker. This fungus is a special case among the entomophthoralean fungi (Zygomycetes), being not an insect pathogen, but nematophagous, and also because the secondary spore, which is formed in unfavourable environmental conditions, is not forcibly discharged, but is passively detached and adhesive (Tucker, 1984). Primary spores are not adhesive, and they will germinate to form a saprotrophic mycelium in nutritive media, but in nutrient-poor media secondary spores are formed. The fungus switches to an endoparasitic mode, with these adhesive, secondary spores being the infective stage (Tucker, 1984).

1.7 PATHOGENICITY

Pathogenicity, or virulence, is a complex phenotype, in which a qualitative and a quantitative component can be distinguished (Caten *et al.*, 1984). The qualitative component is basic compatibility, or infectivity (the ability to grow in/on host tissue). The quantitative component, disease severity, has been called aggressiveness (Vanderplank, 1968).

What features make an organism pathogenic? It is generally assumed that virulence is based on the presence of certain genes, which have been called virulence-determining, pathogenicity-related, or simply pathogenicity genes. These genes are, by definition, not necessary for completion of the life cycle and are directly and intrinsically involved in pathogenicity under natural conditions (Schäfer, 1994). Putative pathogenicity-determinants have been identified in a number of host-pathogen interactions. Many infection processes require breaching of the host integument, e.g. the cuticle of plant and insect, by specialized structures. It is at these levels that several pathogenicity-determinants have been found, e.g. cutinase of *Fusarium solani* (Lin & Kolattukudy, 1978); subtilisin-like protease of *Metarhizium anisopliae* (St. Leger *et al.*, 1988a); hydrophobin in appressoria of *M. anisopliae* (St. Leger *et al.*, 1992b) and *Magnaporthe grisea* (Talbot *et al.*, 1993). Other pathogenicity-determinants come into play at later stages of infection, such as toxin production, as in *Fusarium sporotrichioides* (Desjardins *et al.*, 1989), or the inactivation of host responses, as in *Nectria haematococca*, the teleomorph of *Fusarium solani* f.sp. *pisi* (Van Etten *et al.*, 1989).

If pathogenicity genes are required for pathogenesis, disruption of these genes can be expected to knock out pathogenicity. It is an important test for their involvement. Positive results have been obtained for several of the pathogenicity-determinants mentioned (e.g. Desjardins *et al.*, 1992; Talbot *et al.*, 1993). However, in some cases, gene disruption of putative pathogenicity genes did not decrease pathogenicity to the extent expected, e.g. cutinase (Stahl & Schäfer, 1992; Sweigard *et al.*, 1992), and endopolygalacturonidase (Scott-Craig *et al.*, 1990), suggesting that pathogenicity, at least in these cases, is more complex than originally anticipated. Moreover, many workers have had great difficulty in correlating the virulence of fungal pathogens of plants, nematodes, insects or humans with any molecular marker (Kistler *et al.*, 1987; McDermott *et al.*, 1989; Nygaard *et al.*, 1989; Tedford *et al.*, 1994). A possible reason for this failure is that virulence may often be under polygenic control. This has been demonstrated in a number of cases (Emara & Sidhu, 1974; Caten *et al.*, 1984; Paris *et al.*, 1985; Calderone, 1994; Hawthorne *et al.*, 1994). Some genetic analyses even allowed an estimation of the number of pathogenicity-determining factors, e.g. between 3 and 15 in the pathogenicity of *Fusarium solani* on *Cucurbita* sp. (Hawthorne *et al.*, 1994).

1.8 AIMS OF THIS RESEARCH

Results of biological control trials with *V. chlamydosporium* have not been unequivocally positive. The basic lack of knowledge of the epidemiology, mode of action, and fungal gene products are some of the main problems (Kerry, 1990; Stirling, 1991). The molecular basis of pathogenicity in *V. chlamydosporium* has not

been investigated. Not a single compound has been purified from this fungus. Knowledge of pathogenicity-determinants may help in the selection of fungal isolates, and in the long term, may allow genetic manipulation of the respective traits, resulting in transformants with enhanced biological control potential.

The focus of this study is on extracellular proteases. These enzymes have been implicated in the pathogenicity of various other systems, e.g. *Metarhizium anisopliae* (St. Leger *et al.*, 1988a); *Candida albicans* (Ross *et al.*, 1990); *Pyrenopeziza brassicae* (Ball *et al.*, 1991) and *Arthrobotrys oligospora* (Tunlid *et al.*, 1994). Although proteolytic activity in *V. chlamydosporium* has been observed (Kunert *et al.*, 1987), no enzyme has been characterized. More specifically, this study aims to:

- purify and biochemically characterize the major protease produced by *V. chlamydosporium*;
- obtain its amino acid and nucleotide sequence;
- study the distribution of the enzyme among nematophagous, plant- and insect pathogenic, and saprotrophic fungi;
- examine the basic regulatory mechanism(s) of the protease;
- assess whether, and how, the enzyme is involved in the infection process.

This body of information will be interpreted in terms of the econutritional characteristics of the fungus: how pathogenic is *V. chlamydosporium*, and what, if any, is the contribution of proteases to its trophic modes.

Purification and biochemical characterization of the extracellular protease VCP1

2.1 INTRODUCTION

Despite some confusion in terminology, proteases or peptidases, i.e. the enzymes that hydrolyse peptide bonds, are commonly divided into two different groups, depending on whether they act on proteins (proteinases or endopeptidases), or on oligopeptide substrates (exopeptidases) (Barrett, 1986). In contrast to most other enzymes, their substrate specificities are invariably difficult to define, and do not provide an adequate basis for classification. For this reason, proteases are grouped according to their catalytic mechanism, into serine, cysteine, aspartic and metalloproteases (Barrett, 1986; 1994). Among these, serine proteases are the most numerous group; they are extremely widespread and diverse (Barrett, 1986). Over 20 families of serine proteases are currently recognized, but these can be classified in only a few structurally distinct clans (Rawlings & Barrett, 1994). The most important of these are the chymotrypsin- and subtilisin-like enzymes, named after the first well-characterized member of each group. These proteases are often extracellular (Rawlings & Barrett, 1994), and, in fungi, they typically serve nutritional purposes.

Fungi feed through extracellular digestion, and the production of extracellular hydrolytic enzymes is crucial to the resource capture for many of them (Cooke & Whipps, 1993). Fungal pathogens need to establish a nutritional relationship with their hosts, and hydrolases of various sorts have been identified as playing a role in fungal infection processes. Lipases (Paris & Ferron, 1979), chitinases (Jackson *et al.*,

1985) and proteases are considered to be virulence determinants of entomogenous fungi. The protease Pr1, a major protein secreted by *Metarhizium anisopliae* and other hyphomycete fungi (St. Leger *et al.*, 1987a,b), has been shown to be a key enzyme in the infection process (St. Leger *et al.*, 1988a). Proteases are also virulence determinants of the human pathogenic fungi *Candida albicans* (Ghannoum & Abu Elteen, 1986; Ross *et al.*, 1990; Calderone, 1994) and *Aspergillus fumigatus* (Markaryan *et al.*, 1994; Rhodes, 1995). The primary role of proteases in the infection process of some plant-pathogenic fungi has also been established (Ball *et al.*, 1991; Movahedi *et al.*, 1991; Rauscher *et al.*, 1995).

Little is known about the enzymes secreted by nematophagous fungi, in general, and the egg parasites in particular (see General Introduction, Section 1.5.3). Proteases are likely to be important in host 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; Lopez-Llorca, 1990), a closely related species with disputed taxonomic status (Carder *et al.*, 1993). This Chapter describes the properties of a major protease secreted by an isolate of *V. chlamydosporium* with promising biological control potential. It is a first step towards the biochemical and molecular dissection of pathogenicity in this fungus.

2.2 MATERIALS AND METHODS

2.2.1 Organisms and growth conditions

Isolate 10 of the nematophagous fungus *Verticillium chlamydosporium* Goddard was selected from the IACR-Rothamsted culture collection, based on its virulence against *Meloidogyne* spp. in assays done in pot and semi-field conditions, and its efficiency as a rhizosphere coloniser (de Leij & Kerry, 1991; de Leij *et al.*, 1992a,b, 1993a,b; Bourne *et al.*, 1994). Isolate 10 was stored on silica gel at 4°C, but for the purpose of experimentation, it was maintained on potato dextrose agar at 23°C in the dark. The fungus was never subcultured more than three times before returning to the original inoculum.

Conidia and chlamydo-spores, harvested in sterile distilled water from 3 week old cultures, were used to inoculate soya peptone medium (SPM), containing 10 g soya peptone, 0.3 g K₂HPO₄, 0.3 g MgSO₄·7H₂O, 0.15 g NaCl, 0.3 g CaCl₂·6H₂O, 0.8 mg MnSO₄·6H₂O, 0.2 mg CuSO₄·5H₂O, and 2 mg FeSO₄·7H₂O made up to 1 l. The final concentration of spores in the medium was 10⁵ conidia ml⁻¹ and 3x10³ chlamydo-spores ml⁻¹.

For purification purposes, isolate 10 was grown in four 2 l Erlenmeyer flasks each containing 1 l of SPM. The flasks were incubated at 23°C in a Gallenkamp Orbital Shaker (90 rpm), in the dark, for seven days. Large-scale cultivation was also done in a 10 l fermenter, containing 8 l of the same medium. Aeration was provided by bubbling air through the culture with an electric pump (air capacity, 900 in³ min⁻¹). There appeared to be no qualitative difference in the proteases obtained by both cultivation methods.

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

2.2.2 Preparation of crude enzyme concentrates

Cultures were harvested after 7 days 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 (10,000 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 the same buffer. This crude enzyme concentrate was used for further purification. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.2.3 Preparative and analytical isoelectric focusing (IEF)

Preparative IEF was done with a Bio-Rad Rotofor® electrofocusing cell (Egen *et al.*, 1984; Garfin, 1990). 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 this time the voltage gradually increased from 400 V 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 refractionated using 2% (v/v) ampholytes, pH 8-10.5.

Analytical IEF (Garfin, 1990) 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% polyacrylamide gel containing 10% (v/v) Pharmalyte 3-10.

2.2.4 Enzymoblotting

Enzymoblotting was done, essentially according to the method of Ohlsson *et al.* (1986). Briefly, proteins from IEF gels were transferred to nitrocellulose by semi-dry electroblotting using the Novablot system (Pharmacia). The transfer buffer system contained 48 mM Tris, 39 mM glycine, 1.3 mM SDS in 20% (v/v) methanol/water (Bjerrum & Schafer-Nielsen, 1986). After blotting, the nitrocellulose membrane was bathed in a solution of 1 mM of suc-(Ala)₂-Pro-Phe-pNA, prepared in 0.1 M Tris-HCl, pH 7.9. Immediately when the yellow colour of released *p*-nitroaniline became visible (*ca* 10-20 sec), the membrane was transferred to a solution of 0.1% (w/v) sodium nitrite in 1 M HCl for 5 min. After another 5 min in 0.5% (w/v) ammonium

sulfamate in 1 M HCl, the membrane was developed in a 0.05% (w/v) *N*-(1-naphthyl)ethylenediamine solution (Sigma) made up in 47.5% (v/v) ethanol (Ohlsson *et al.*, 1986). Pink-coloured bands became visible within 1 min, after which the membrane was washed in distilled water, and stored at -18°C.

2.2.5 SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel 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 visualized with Coomassie stain (0.2% [w/v] Coomassie Brilliant Blue R250 in 47.5% [v/v] ethanol and 10% [v/v] acetic acid) and gels were destained using an aqueous mixture of 26% (v/v) ethanol and 10% (v/v) acetic acid.

The glycoprotein staining method of Zacharius *et al.* (1969) was used to detect carbohydrate moieties in the *V. chlamydosporium* protease. Briefly, after running a near-pure enzyme preparation on an SDS-PAGE gel, the gel was immersed in 12.5% (w/v) TCA for 30 min, rinsed in distilled water, and immersed in 1% (v/v) periodic acid, in 3% (v/v) acetic acid. After washing thoroughly in water overnight, the gel was transferred to fuchsin-sulphite stain (Raymond A. Lamb, London) in the dark for 1 h, and washed three times in 0.5% (w/v) sodium metabisulphite. Overnight washing in water ensured removal of excess stain.

2.2.6 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 12,000 g in an Eppendorf micro-centrifuge for 5 min. Undigested azocasein and azoalbumin were precipitated by adding 200 μl 20% (w/v) trichloroacetic acid (TCA), left to stand for 30 min, then centrifuged in an Eppendorf microcentrifuge at 12,000 g for 5 min. The optical density of the supernatants was read using an Hewlett Packard Diode Array Spectrophotometer 8452A fitted with a temperature controlled multicell unit at the following wavelengths (λ_{max}): 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 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 by measuring the absorbance of dilutions of elastin-Congo red that had been completely hydrolysed with porcine pancreatic elastase. One unit of elastase activity is defined as the amount of enzyme that hydrolysed 1 mg of 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 ,

unless specified otherwise) were added to 0.1 M Tris-HCl buffer, pH 7.9, to make 500 μl , and the reaction, which was started by adding 500 μl substrate (stock solution of 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. Non-chymotrypsin-like subtilisin 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} ml^{-1} .

The dependence of proteolytic activity in the culture filtrate on pH was determined by an Azocoll assay as before, except that the Tris buffer was replaced with Britton-Robinson universal buffer (Dawson *et al.*, 1986), and assays were done between pH 3 and 10.

2.2.7 Determination of kinetic constants

The calculation of enzyme activity requires knowledge of ϵ , the absorption coefficient of the substrate. A review of the literature revealed that the value of ϵ for *p*-nitroaniline ranges between 8,800 (Erlanger *et al.*, 1961) and 10,800 $\text{M}^{-1} \text{cm}^{-1}$ (Tuppy *et al.*, 1962), in the wavelength range 400-410 nm. This variability necessitated the determination of ϵ in the local experimental conditions. A range of

concentrations of *p*-nitroaniline were established by assuring total hydrolysis of known concentrations of suc-(Ala)₂-Pro-Phe-pNA, bearing in mind a stoichiometry of 1:1 for the reagent and the hydrolysed product. The absorption coefficient is calculated as the slope of the change of absorbance with increasing *p*-nitroaniline concentrations.

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, as calculated by the software package Enzfitter (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 $V_{max} = k_{cat}[E]$, where $[E]$ is the concentration of active sites. Duplicate measurements were made at eight substrate levels between 0.06 and 12 times K_m .

2.2.8 Inhibition studies

Apart from substrates indicating particular specificities, inhibitors can be used to characterize the nature of a protease. Samples of the purified enzyme or the culture filtrate (10 μ l) were preincubated with inhibitor (see Table 2.4) at room temperature for 1 h before addition of 500 μ l 2 mM suc-(Ala)₂-Pro-Phe-pNA and buffer to make a final volume of 1 ml. Appropriate solvent controls were included for those inhibitors that were prepared in ethanol or isopropanol.

2.2.9 Enzyme stability

Sets of three replicates (10 μ l each) of the Rotofor fraction that contained pure enzyme were stored, unbuffered, at -20, 4, 23 or 37°C. The activity, remaining after different amounts of time, was measured with a standard suc-(Ala)₂-Pro-Phe-pNA assay (Section 2.2.6) and compared with the value obtained with the freshly harvested enzyme.

Attempts were made to find treatments that enhanced the stability of the enzyme. Aliquots of freshly harvested protease were incubated at 37°C with final concentrations of the following: CaCl₂, 20 mM; glycerol, 2 M; Tween 20, 15% (v/v); Triton X-100, 15% (v/v); and Tris pH 7.0, 0.1 M. After 4 h, the samples were placed on ice and immediately assayed, as above.

2.3 RESULTS

2.3.1 Electrophoretic analyses

Protease activities present in enzyme concentrates, prepared from batch cultures of *V. chlamydosporium*, isolate 10, 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 in the alkaline fractions with pH 9.5-10.3 (Figs. 2.1 and 2.2).

The substrate suc-(Ala)₂-Pro-Phe-pNA was designed as a substrate for chymotrypsin (Del Mar *et al.*, 1979). The parallel between the activity pattern with this substrate (Fig. 2.1) and that with Azocoll (Fig. 2.2), suggests that chymotrypsin-

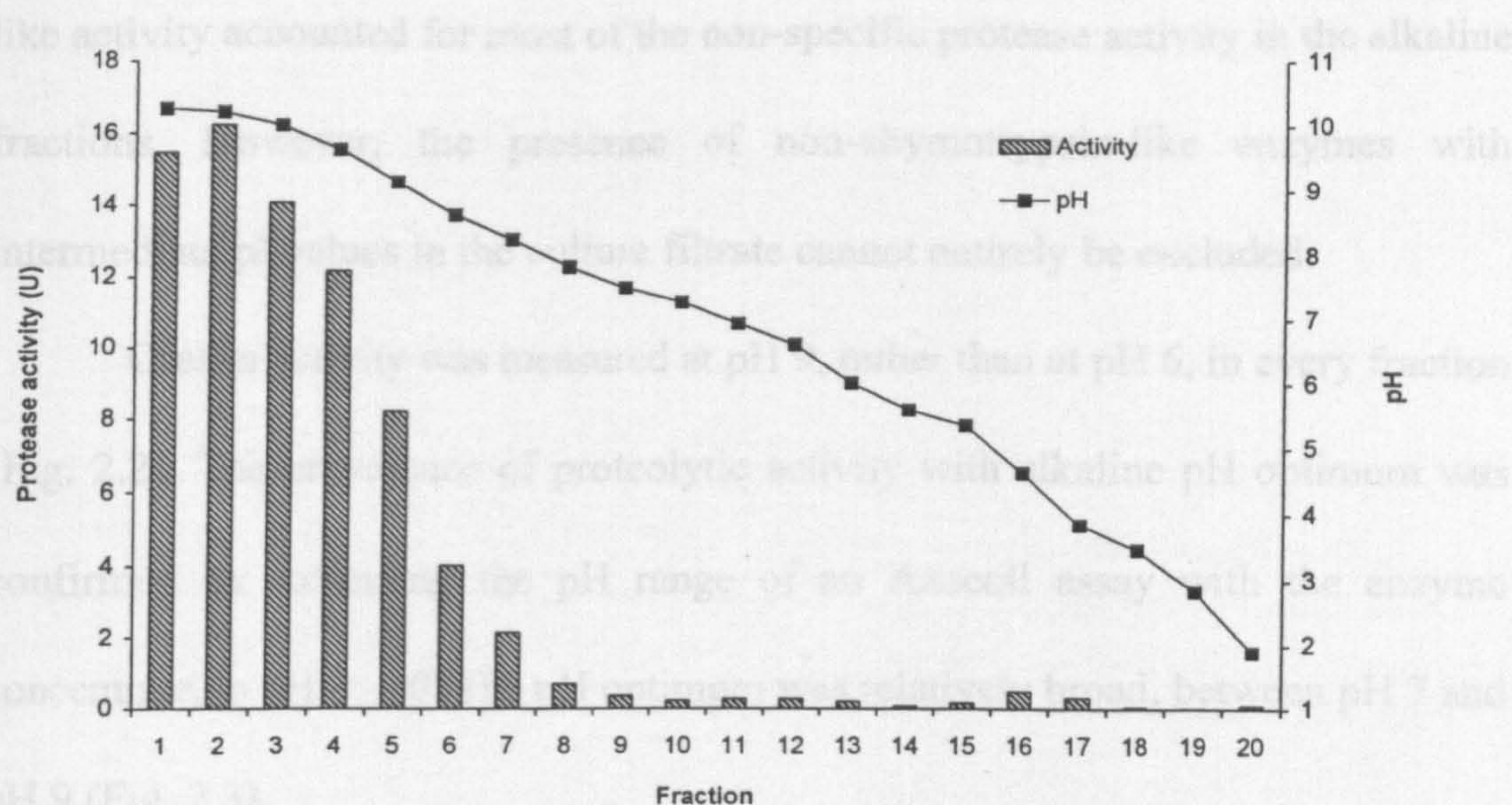


Fig. 2.1. Protease activity measured using suc-(Ala)₂-Pro-Phe-pNA, and pH of fractions of crude enzyme concentrate, separated by IEF using the Rotofor system (pH 3-10). One unit of activity is defined as the amount of enzyme releasing 1 μmol *p*-nitroaniline $\text{min}^{-1} \text{ml}^{-1}$.

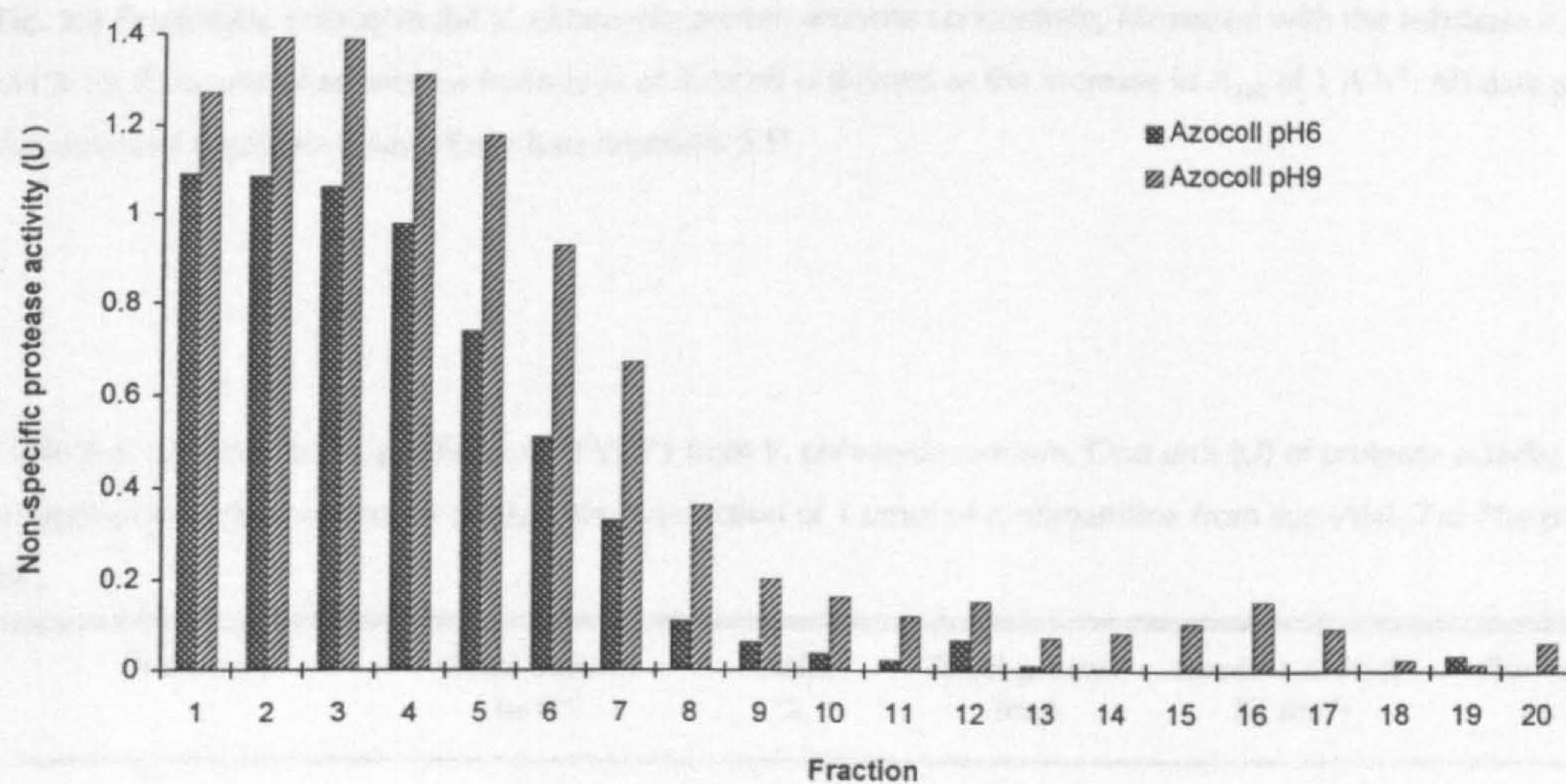


Fig. 2.2 Non-specific protease activity measured with Azocoll, at pH 6 and pH 9, of fractions of crude enzyme concentrate, separated by IEF using the Rotofor system (pH 3-10). One unit of activity for hydrolysis of Azocoll is defined as increase in A_{520} of 1 A h^{-1} .

like activity accounted 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 entirely be excluded.

Greater activity was measured at pH 9, rather than at pH 6, in every fraction (Fig. 2.2). The prevalence of proteolytic activity with alkaline pH optimum was confirmed by extending the pH range of an Azocoll assay with the enzyme concentrate, to pH 3 - 10. The pH optimum was relatively broad, between pH 7 and pH 9 (Fig. 2.3).

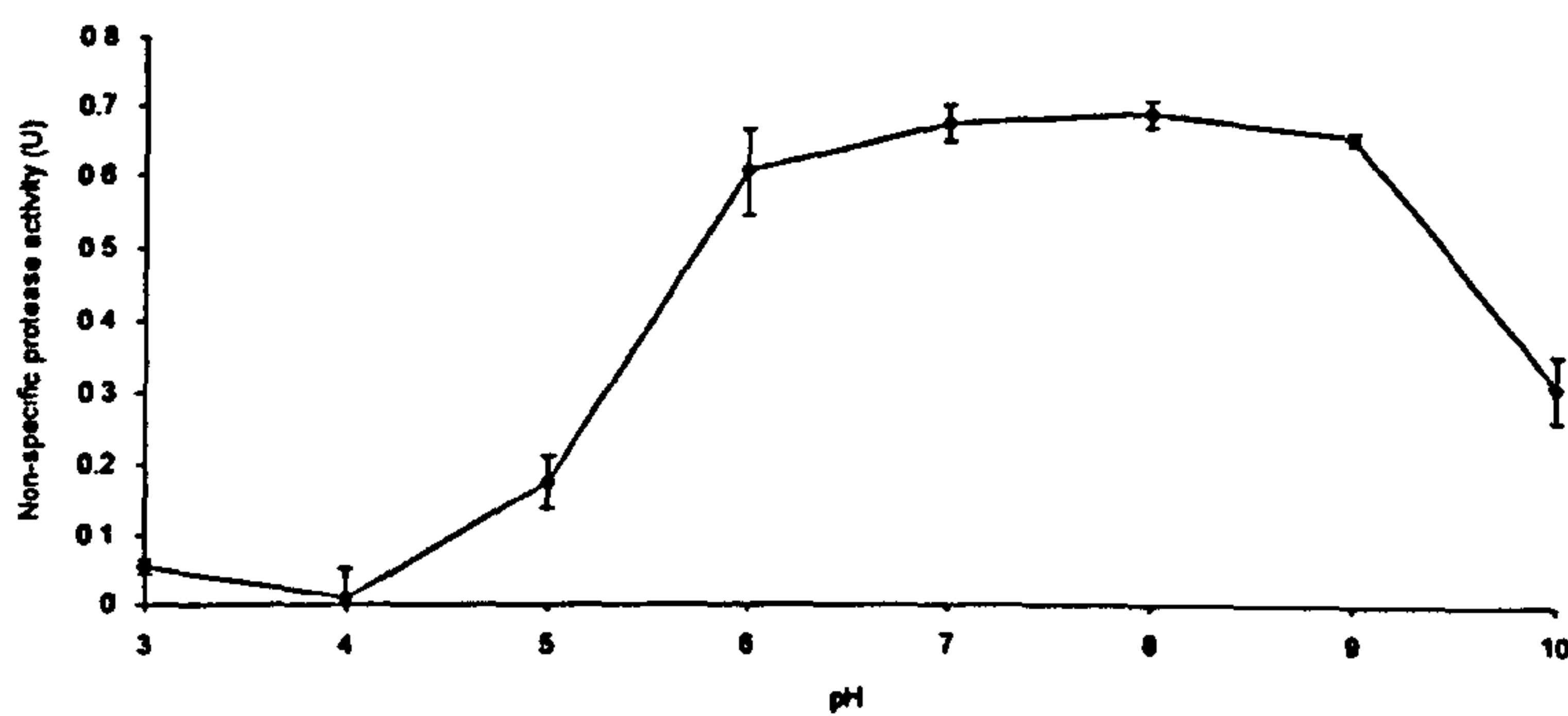


Fig. 2.3 Proteolytic activity in the *V. chlamydosporium* enzyme concentrate, measured with the substrate Azocoll at pH 3-10. One unit of activity for hydrolysis of Azocoll is defined as the increase in A_{520} of 1 A h^{-1} . All data points are the means of duplicate assays. Error bars represent S.D.

Table 2.1. Scheme for the purification of VCP1 from *V. chlamydosporium*. One unit (U) of protease activity is the amount of enzyme required to catalyse the production of $1 \mu\text{mol}$ of *p*-nitroaniline from $\text{succ-(Ala)}_2\text{-Pro-Phe-pNA}$ $\text{min}^{-1} \text{ ml}^{-1}$.

Procedure	Total activity (units)	Yield %	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor
Crude filtrate	4889.5	100.0	285.1	17.2	1.0
$(\text{NH}_4)_2\text{SO}_4$ precipitation	2053.6	42.0	70.3	29.2	1.7
IEF	286.7	5.9	2.2	130.3	7.6
IEF, refractionation	126.9	2.6	0.5	233.2	13.6

Fig. 2.4. Analytical IEF (pH 3-10) of purified chymotrypsin-like enzyme from *V. chlamyosporium* (isolate 10) and Pr1 from *Metarhizium anisopliae*. pI marker proteins (lane 1), *V. chlamyosporium* culture filtrate (lane 2), purified VCP1 (lane 3), *M. anisopliae* culture filtrate (lane 4) and purified Pr1 (lane 5). Enzymoblot of *V. chlamyosporium* culture filtrate (lane 6) and purified VCP1 (lane 7), *M. anisopliae* culture filtrate (lane 8) and purified Pr1 (lane 9).

Fig. 2.5. SDS-PAGE of culture filtrates and purified VCP1 from *V. chlamyosporium* (isolate 10) and Pr1 from *M. anisopliae*. Lane 1 shows *V. chlamyosporium* culture filtrate; lane 2, VCP1; lane 3, *M. anisopliae* culture filtrate; and lane 4, Pr1 from *M. anisopliae*.

45a

Fig. 2.4

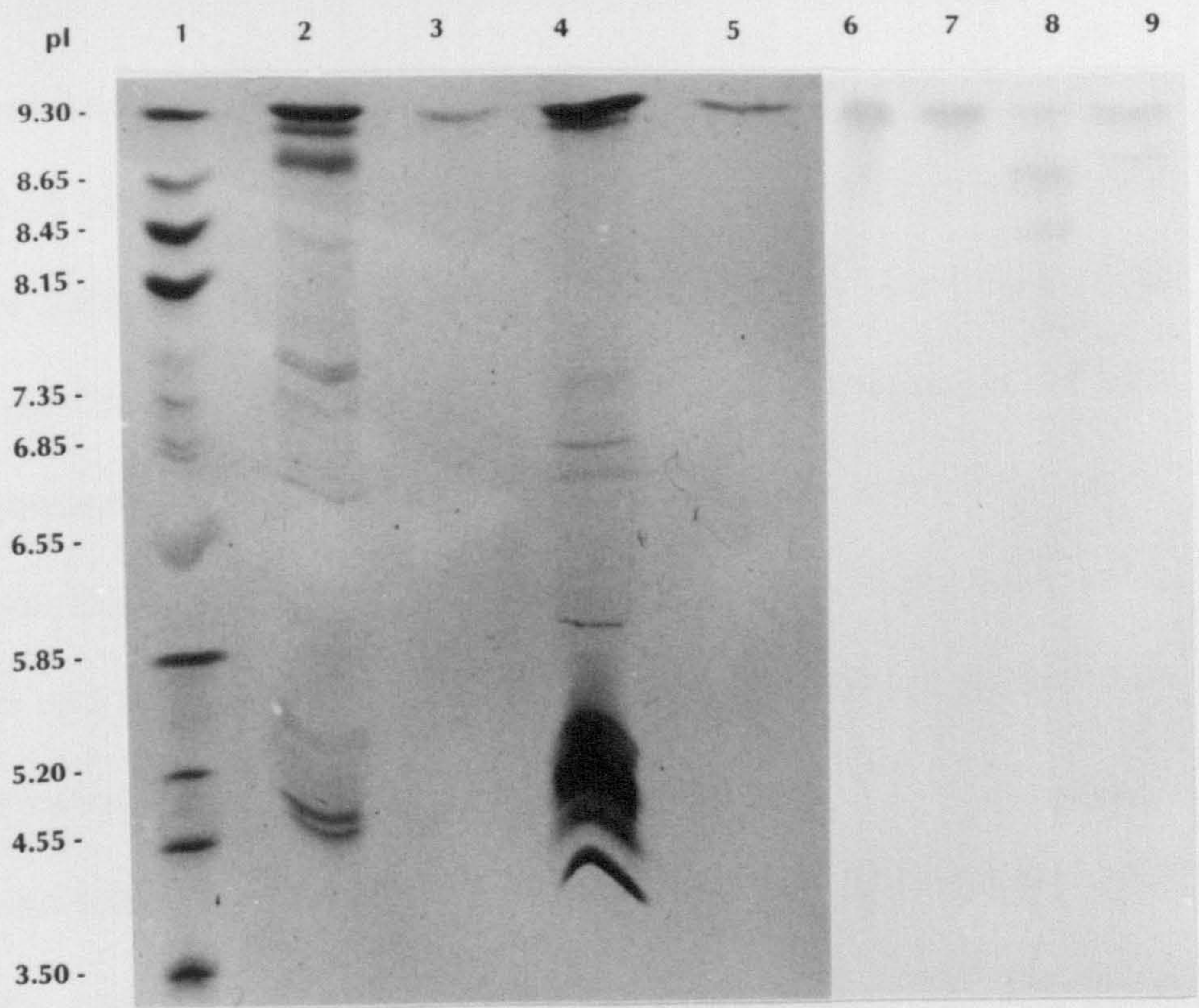
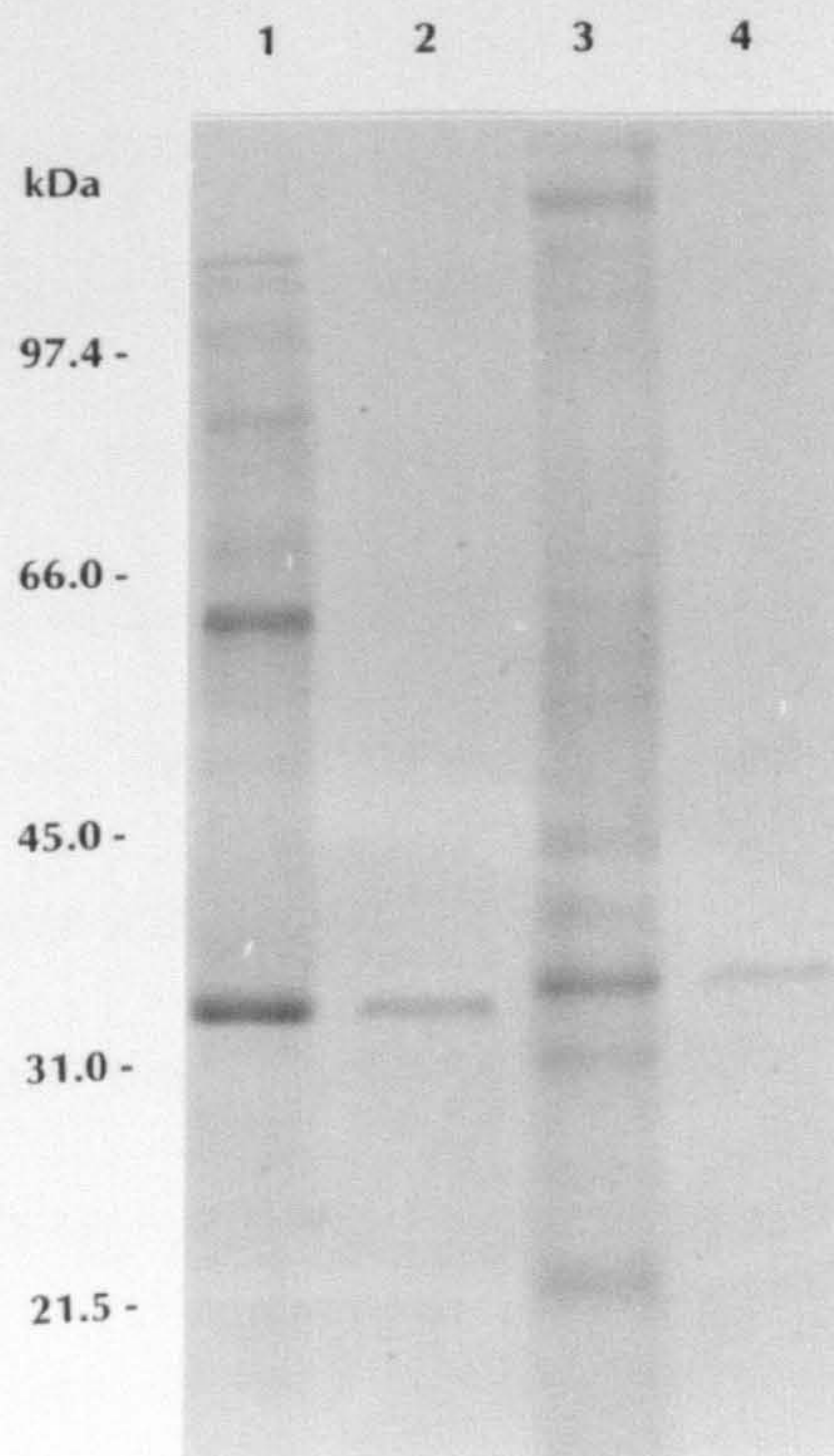


Fig. 2.5



The alkaline fractions were refocused using narrow-range ampholytes (pH 8-10.5) which resulted in a final 14-fold enrichment of the chymotrypsin-like enzyme (Table 2.1). Both IEF and SDS-PAGE showed that it corresponded to a major protein in the culture filtrate (Figs. 2.4 and 2.5). Analytical IEF and SDS-PAGE of the alkaline Rotofor fractions revealed a single band, suggesting the protein had been purified to homogeneity (Figs. 2.4 and 2.5). The enzyme was given the acronym, VCP1. The molecular mass of VCP1 was 33.3 ± 0.5 kDa (S.E., n=3), which corresponded with that of the extracellular protease Pr1, purified in parallel from *Metarhizium anisopliae*.

Enzymoblotting was the method of choice to localize proteolytic activity after electrophoretic separation. The method involves transfer to nitrocellulose, and incubation with a specific substrate. Enzymoblotting of culture filtrates and purified VCP1 and Pr1, with suc-(Ala)₂-Pro-Phe-pNA, showed that protease activity was greatest at pI 10, but weaker activity was detected in culture filtrates at lower pI values (Fig. 2.4). *Metarhizium anisopliae* had at least three clearly distinct enzymes in the enzyme concentrate, with affinity for the substrate. The most alkaline were difficult to separate, and were still present after purification (Fig. 2.4, lanes 8-9). Enzymoblotting confirmed that the single band, obtained in IEF, was indeed a protease (Fig. 2.4, compare lanes 3 and 7).

No glycosylation was apparent in VCP1 when the protein was electrophoresed on an SDS-polyacrylamide gel, followed by PAS-staining (Fig. 2.6). The lack of glycosylation of VCP1 was confirmed (J.F. Peberdy, personal communication) with a DIG glycosylation kit and a lectin-glycan differentiation kit

Fig. 2.6 Glycoprotein staining of VCP1 after running ca 10 μ g of near-pure VCP1 on an SDS-PAGE gel, by the periodic acid - Schiff reagent (PAS) method (Zacharius *et al.*, 1969), with a) VCP1, Coomassie-stained; and b) VCP1, separated on the same gel, but PAS-stained.

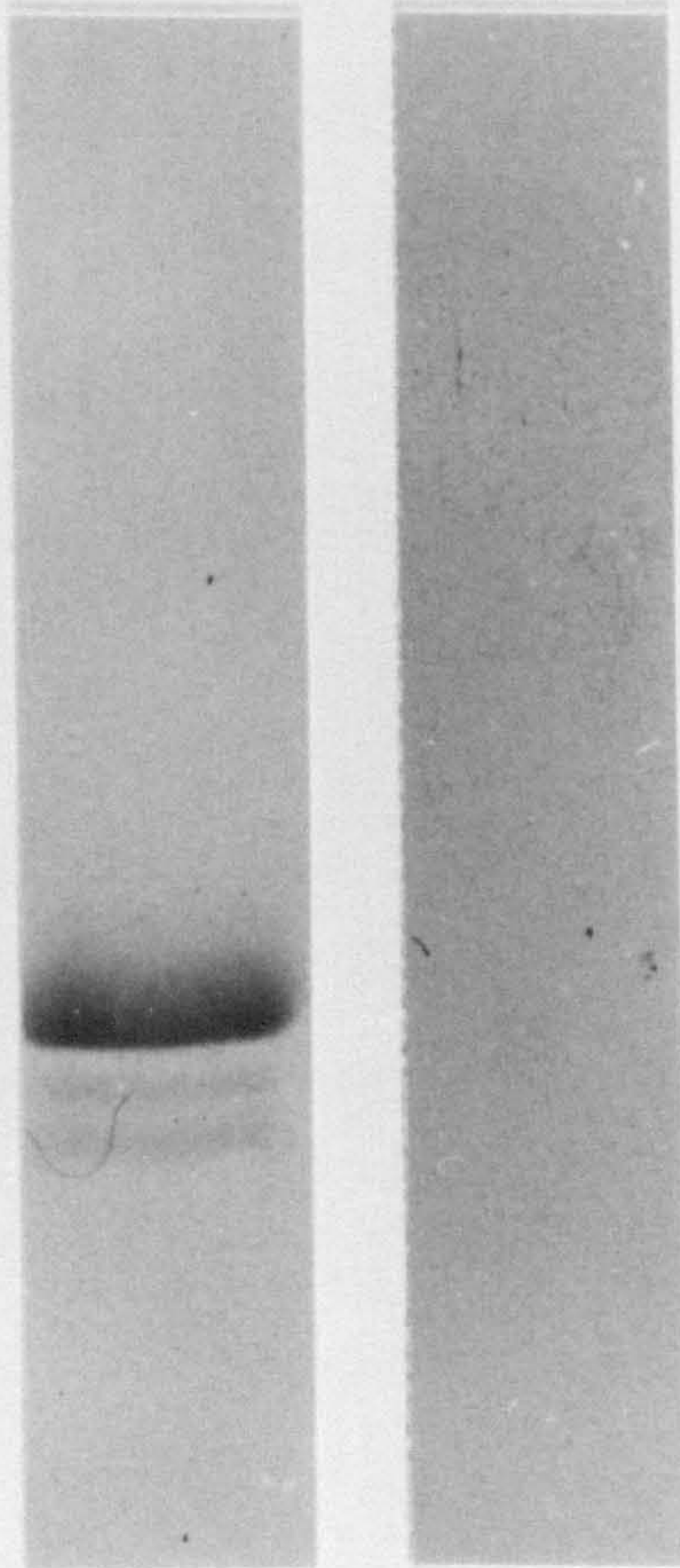
47a

1

2

kDa

33 -



(both from Boehringer-Mannheim). The lectin used in the latter assay was GNA, which binds to terminal mannose, and has proved effective with all other fungal glycans (J.F. Peberdy, personal communication).

2.3.2 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. Compared to the first substrate, suc-Phe-pNA resulted in a specific activity that was 1,000 times lower than the first substrate, while Bz-Tyr-pNA was not measurably hydrolysed (Table 2.2). This suggests that an aromatic residue in the P1 position (Schechter & Berger, 1967) was insufficient for hydrolysis by this enzyme, and that a longer peptide was required.

Table 2.2. Substrate specificity of purified VCP1, culture filtrate from *V. chlamydosporium*, and reference proteases. Activities are expressed as percentage of maximum specific activity for each enzyme preparation. All data are means of duplicate assays.

Substrate	Isolate 10 VCP1	Isolate 10 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
Maximum specific activity (100 %) expressed in U mg ⁻¹	(19.2)	(13.2)	(7.1)	(53.6)	(0.3)

VCP1 also exhibited weak trypsin-like activity, since it was able to hydrolyse Bz-Phe-Val-Arg-pNA. However, the specific activity with this substrate was only 8.3% of that with the nitroanalide hydrolysed with the greatest efficiency, and the trypsin

substrate Bz-Arg-pNA was not degraded (Table 2.2). No elastase activity of any significance was measured with the substrates suc-(Ala)₃-pNA and CBZ-(Ala)₂-Leu-pNA (Table 2.2). The substrate specificity of VCP1 and proteases in the culture filtrate was similar, confirming that VCP1 probably accounted for most of the non-specific protease activity (Table 2.2).

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 2.2). Porcine elastase hydrolysed both elastase (suc-(Ala)₃-pNA, MeOsuc-(Ala)₂-Pro-Val-pNA) and chymotrypsin (suc-(Ala)₂-Pro-Phe-pNA) substrates (Table 2.2). The specific activity of both VCP1 and porcine elastase for suc-(Ala)₃-pNA was low. VCP1, in contrast to the commercial proteases tested, was able to weakly hydrolyse the non-chymotrypsin-like subtilisin substrate CBZ-(Ala)₂-Leu-pNA (Table 2.2).

Table 2.3. Substrate utilisation of purified VCP1 and culture filtrates of *V. chlamydosporium*. Specific activities are expressed as percentage per substrate. One unit of specific activity for hydrolysis of the following substrates is defined as: Azocoll hydrolysis, increase in A_{520} of 1 A mg⁻¹ min⁻¹; hide protein azure hydrolysis, increase in A_{595} of 1 A mg⁻¹ min⁻¹; elastin-Congo red hydrolysis: one unit of elastolytic activity solubilizes 1 mg of elastin in 3 h at 37°C; elastin-orcein hydrolysis: increase in A_{592} of 1 A mg⁻¹ h⁻¹; azocasein hydrolysis, increase in A_{336} of 1 A mg⁻¹ min⁻¹; azoalbumin hydrolysis, increase in A_{326} of 1 A mg⁻¹ min⁻¹. All data are means of duplicate assays.

Enzyme tested	Azocoll	Hide Protein Azure	Elastin-Congo red	Elastin-orcein	Azo-casein	Azo-albumin
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 (100%) expressed in U mg ⁻¹	(5.0)	(16.6)	(6.2)	(5.6)	(4.2)	(5.3)

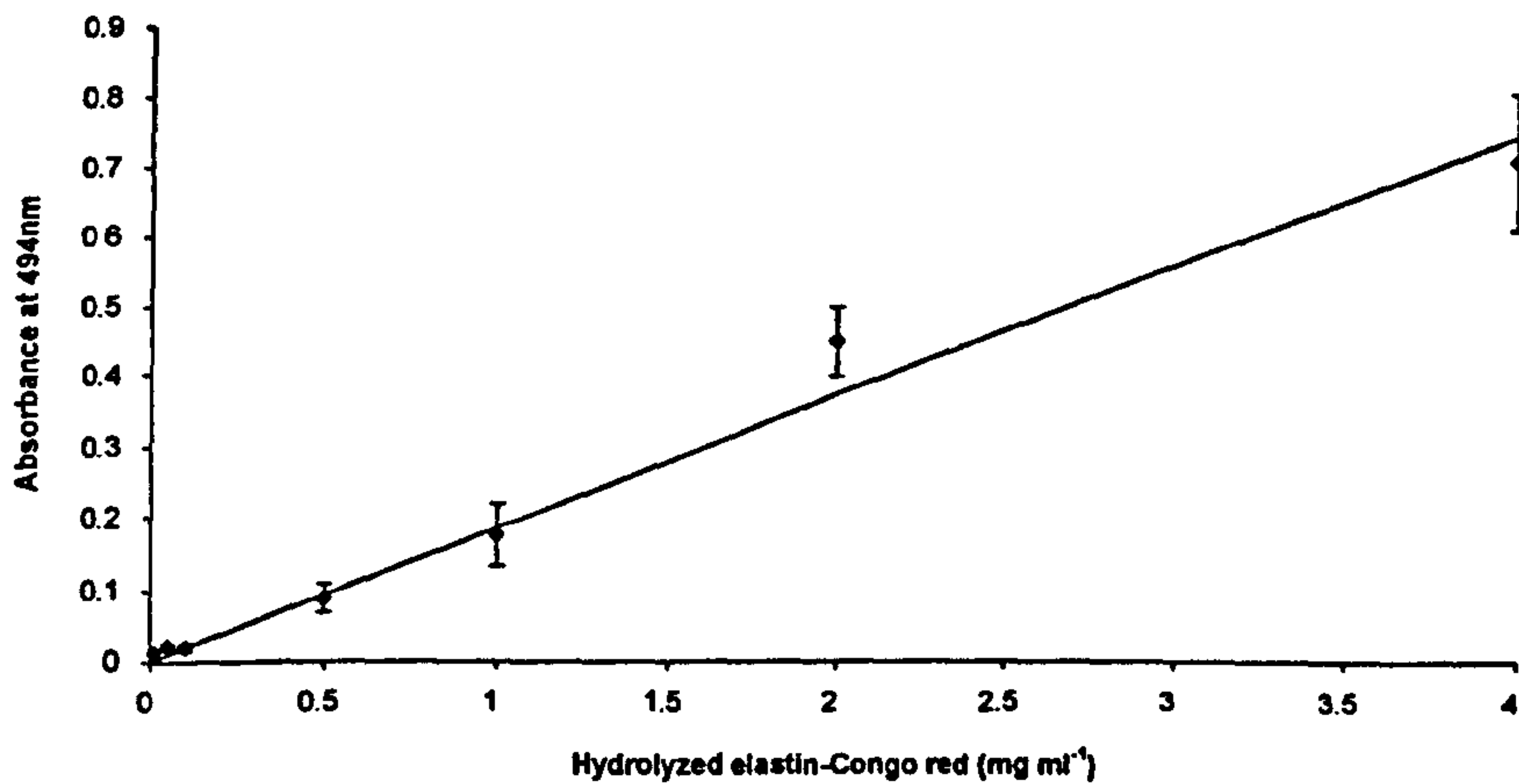


Fig. 2.7 Standard curve for the quantification of elastolytic activity against elastin-Congo red. The A_{494} values of a range of elastin-Congo red dilutions, completely solubilized with 20 mg porcine pancreatic elastase ml⁻¹, are indicated. Error bars represent S.D.

Both VCP1 and commercial-grade trypsin, chymotrypsin and elastase degraded a wide range of protein substrates, to varying degrees (Table 2.3). Whereas in Table 2.2 the hydrolysis of a range of nitroanilides could be compared per enzyme, the proteins listed in Table 2.3 are so diverse in their chromogenic leaving groups, that the unit of proteolytic activity depended on the substrate, and as a result, the effect of the different proteases was ranked per substrate. The unit of activity against elastin-Congo red was notably different from the others, as the elastase activity of VCP1 was expected to be an important feature in the comparison with data from other proteases in the literature. Rather than the fairly arbitrary change in absorbance over a given time, the hydrolysis of elastin-Congo red was quantified as the amount of elastin degraded, using a standard curve ($R^2 = 0.984$) that related concentration of elastin-Congo red to its absorbance at 494 nm (Fig. 2.7). Compared to trypsin, chymotrypsin and elastase, VCP1 was highly active against Azocoll and hide azure, and moderately active against casein, albumin and elastin. Activity of proteases from

the original culture filtrate on azocasein and azoalbumin was greater than that of the purified VCP1, suggesting that enzymes present in the composite culture filtrate, other than VCP1, were also acting on these substrates (Table 2.3).

2.3.3 Kinetic properties

Some kinetic aspects of the reaction of VCP1 with the substrate for which it had the highest activity, suc-(Ala)₂-Pro-Phe-pNA, were investigated. Firstly, quantification of enzyme activity in spectrophotometry can only be done if the machine-dependent absorbance units sec⁻¹ can be converted to actual concentration units. This conversion required the establishment under experimental conditions of ϵ , the absorption coefficient, of the chromogenic species, *p*-nitroaniline. The standard curve ($R^2 = 0.998$) that gives the relation between A_{410} and the concentration of *p*-nitroaniline had a slope of $\epsilon = 10,700 \text{ M}^{-1} \text{ cm}^{-1}$ (Fig. 2.8).

The reaction rate, v ($\mu\text{mol ml}^{-1} \text{ min}^{-1}$), of the hydrolysis of suc-(Ala)₂-Pro-Phe-pNA by VCP1, depended on the substrate concentration, $[S]$ (M), as described by the Michaelis-Menten equation, $v = V_{\text{max}}[S](K_m + [S])^{-1}$ (Fig. 2.9). In this equation V_{max} is the maximum velocity ($\mu\text{mol ml}^{-1} \text{ min}^{-1}$) at saturating substrate concentrations, and K_m is a reaction constant, known as the Michaelis-Menten constant, which is numerically equal to the substrate concentration (M) where the reaction velocity, v , is one-half of the maximum velocity V_{max} (Fersht, 1985). The non-linear Michaelis-Menten equation is often rearranged as a function of v^{-1} and $[S]^{-1}$, which is linear (Fig. 2.10). This is the Lineweaver-Burk plot, which is popular because of its linearity and because the intercepts indicate K_m and V_{max} directly. However, using the Lineweaver-

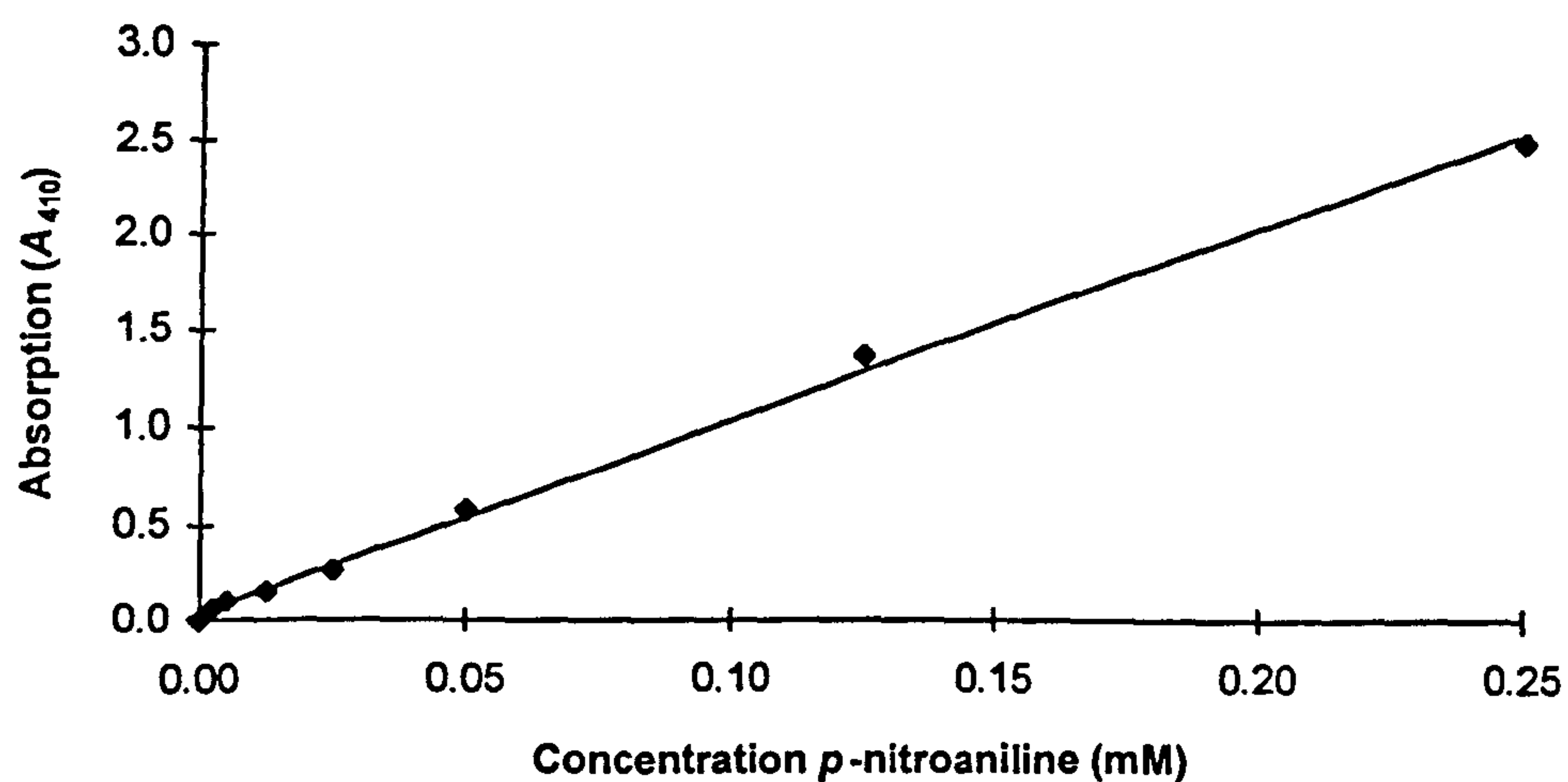


Fig. 2.8 Determination of the absorption coefficient, ϵ , of *p*-nitroaniline, which is the slope of the line between a range of concentrations of *p*-nitroaniline, obtained by complete hydrolysis of suc-(Ala)₂-Pro-Phe-pNA, and their A₄₁₀.

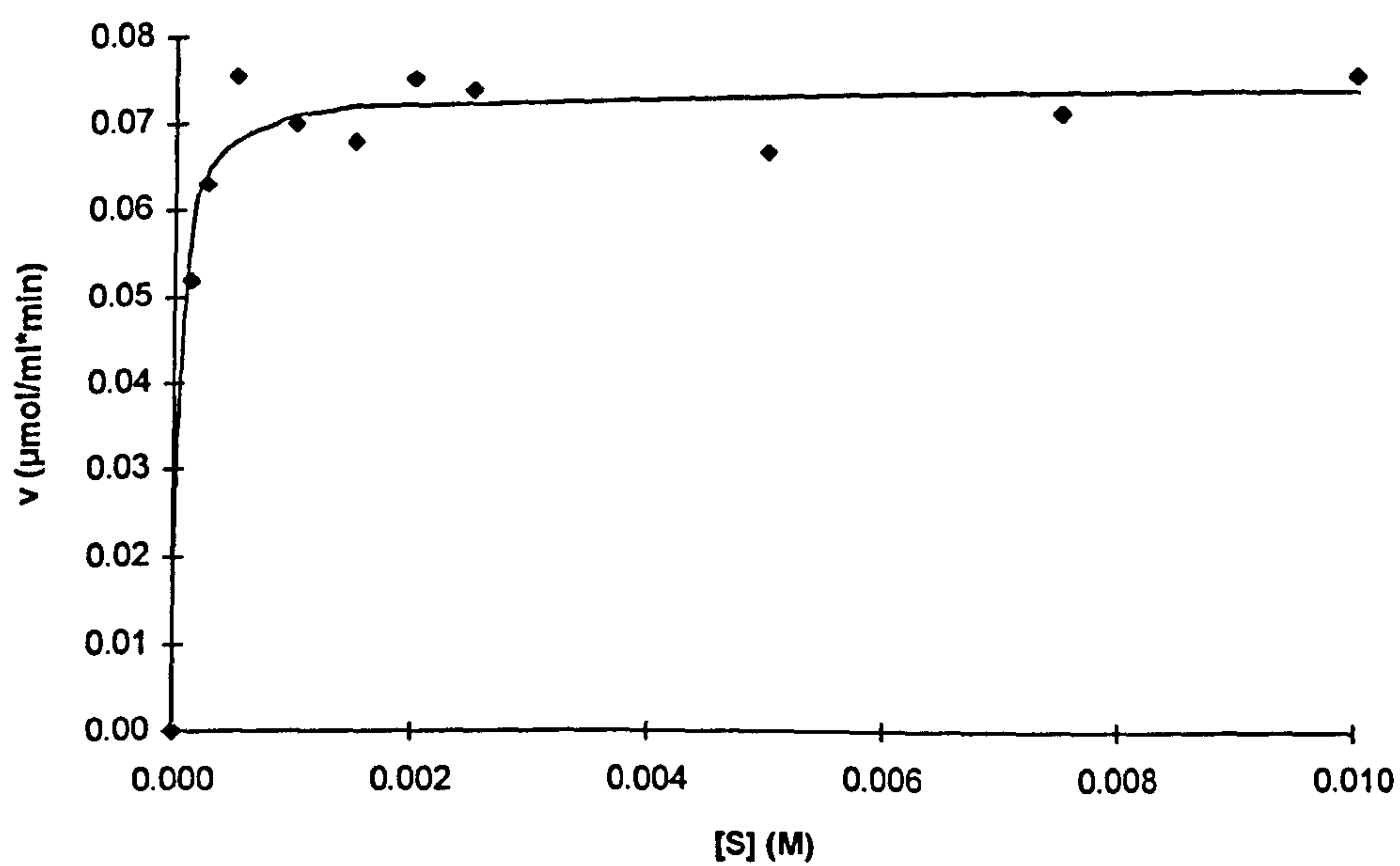


Fig. 2.9 Michaelis-Menten kinetics of the reaction between VCP1 and suc-(Ala)₂-Pro-Phe-pNA. The Michaelis-Menten constant K_m would be numerically equal to the substrate concentration [S] where the reaction rate is one-half of the maximum rate V_{max} .

Burk plot to determine these values is statistically unsound (Henderson, 1992), and it is only provided here as a graphical representation of the data. Rather, K_m and V_{max} were determined using non-linear regression (Leatherbarrow, 1990), which indicated that the reaction between VCP1 and suc-(Ala)₂-Pro-Phe-pNA had a Michaelis-Menten constant $K_m = 4.26 \times 10^{-5}$ M, which coincides exactly with the K_m value for bovine chymotrypsin and this substrate (Del Mar, 1979). The catalytic constant was determined as $k_{cat} = 5.76 \text{ s}^{-1}$ and the specificity constant was $k_{cat}/K_m = 1.35 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$.

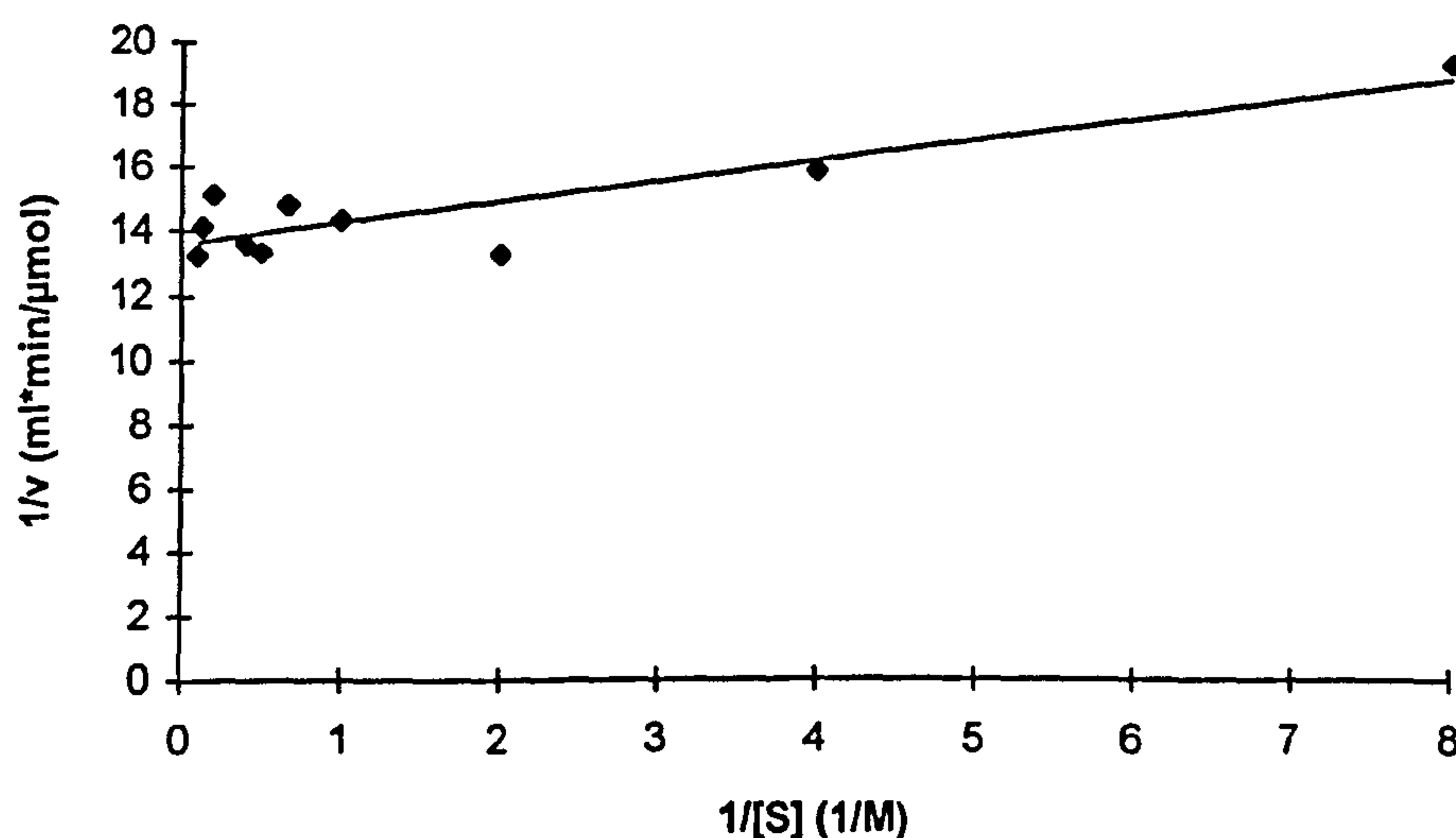


Fig. 2.10 Lineweaver-Burk plot of the reaction between VCP1 and suc-(Ala)₂-Pro-Phe-pNA. The intercepts with x- and y-axis would indicate reciprocal values of $-K_m$ and V_{max} , respectively.

2.3.4 Inhibitors

Thirteen different protease inhibitors were tested for their effect on the activity of VCP1 and the culture filtrate (Table 2.4).

Table 2.4. Effect of protease inhibitors on purified VCP1 and culture filtrates of *V. chlamydosporium*. Residual activities were measured against suc-(Ala)₂-Pro-Phe-pNA as the substrate and calculated using appropriate ethanol (†) or isopropanol (‡) containing controls, when required. 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	Concentration	VCP1	Culture Filtrate
Bowman-Birk ^a	0.4 mg ml ⁻¹	104.0	117.6
TEW ^a	0.4 mg ml ⁻¹	77.0	84.3
	1 mg ml ⁻¹	80.5	77.7
CEW ^a	0.4 mg ml ⁻¹	56.2	73.2
	1 mg ml ⁻¹	67.5	84.4
STI ^a	0.4 mg ml ⁻¹	68.3	86.0
	1 mg ml ⁻¹	52.6	85.0
TPCK † ^a	10 μM	104.9	101.6
	100 μM	25.5	91.5
TLCK ^a	10 μM	93.0	102.7
	100 μM	89.7	81.1
PMSF ‡ ^a	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 ^a	10 μM	79.7	84.5
Phenanthroline †	10 mM	114.2	99.7
EDTA ^a	1 mM	68.7	76.2

^a Bowman-Birk: trypsin/chymotrypsin inhibitor; CEW: chicken egg-white inhibitor, containing ovoinhibitor; E-64: trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDTA: ethylenediaminetetraacetic acid; PMSF: phenylmethylsulfonyl fluoride; STI: soybean trypsin inhibitor; TEW: turkey egg-white inhibitor; TLCK: tosyl-Lys-chloromethyl ketone; TPCK: tosyl-Phe-chloromethyl ketone.

The serine protease inhibitor PMSF completely inhibited VCP1. Tosyl-Phe-chloromethyl ketone (TPCK; chymotrypsin inhibitor), which has affinity for the histidyl group of the active site (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 ovoidinhibitor (CEW) and soybean trypsin inhibitor (STI) (Table 2.4).

2.3.5 Stability of VCP1

Freshly harvested IEF fractions, containing pure and unbuffered VCP1, were incubated at temperatures ranging from -20°C to 37°C , which had a marked effect on enzyme activity. Whereas VCP1, stored at -20°C for one month, retained 80% of the original activity, increasing the temperature significantly reduced its half-life. Approximately 80% of the activity was lost when the enzyme was stored at 23°C for 72 h, or at 37°C for only 1 h (Table 2.5).

Table 2.5 Stability of VCP1 as measured by residual protease activity after storage of pure enzyme at the temperatures, for the amounts of time indicated. Activity is expressed as units \pm S.E. ($n = 3$, independent samples). One unit (U) of activity is the amount of VCP1 that hydrolyses $1 \mu\text{mol}$ of $\text{succ-(Ala)}_2\text{-Pro-Phe-pNA}$ $\text{ml}^{-1} \text{min}^{-1}$.

Temp.	-	-20°C	4°C	23°C	37°C
Time	0	1 week	1 day	12 h	1 h
(U \pm S.E.)	20.3 ± 0.3	17.0 ± 0.6	13.0 ± 0.7	6.0 ± 0.1	4.4 ± 0.1
(%)	100.0	81.6	64.3	29.6	21.8
Time		1 month	3 days	24 h	4 h
(U \pm S.E.)		16.0 ± 0.4	11.9 ± 0.2	4.3 ± 0.1	2.8 ± 0.1
(%)		79.6	58.9	21.1	13.8
Time		7 days	72 h		8 h
(U \pm S.E.)		10.3 ± 0.1	3.5 ± 0.1		2.8 ± 0.03
(%)		51.0	17.3		13.7
Time				24 h	
(U \pm S.E.)				3.3 ± 0.1	
(%)				16.4	

The stability of the enzyme at 37°C could not be improved by storage in 20 mM Ca^{2+} ($P > 0.05$, t-test) and was reduced by storage in 15% (v/v) Triton X-100 (Table 2.6). On the other hand, 2 M glycerol ($P < 0.01$) and 15% (v/v) Tween 20

($P < 0.05$) resulted in slightly prolonged activity of VCP1. The most significant improvement in stability ($P < 0.001$) came from buffering the enzyme in 0.1 M Tris pH 7.0, which resulted in 68% of the pre-storage activity after 4 h at 37°C, while unbuffered VCP1 retained only 21% of the activity in these conditions (Table 2.6).

Table 2.6 Improvement of the stability of VCP1 by storing the enzyme in CaCl₂ (20mM), glycerol (2 M), Tween 20 (15% [v/v]), Triton X-100 (15% [v/v]), and 0.1 M Tris pH 7.0. Prior to storage at 37°C for 4 h, all samples (20 µl), including the controls, were diluted with 20 µl of one of the adjuvants or distilled water, respectively. All data are the means of three independent replicates.

	Activity	
	(U ± S.E.)	(% of control)
Time: 0 (control)	10.38 ± 0.17	100.0
Time: 4 h	2.18 ± 0.11	21.0
+ CaCl ₂	2.24 ± 0.17	21.6
+ Glycerol	2.98 ± 0.10	28.7
+ Tween 20	2.58 ± 0.07	24.9
+ Triton X-100	1.36 ± 0.09	13.1
+ Tris pH 7.0	7.11 ± 0.64	68.4

2.4 DISCUSSION

VCP1, a major alkaline protease secreted by *V. chlamydosporium* in soya peptone medium, was purified to homogeneity from culture filtrates, using preparative IEF. The enzyme was a serine protease, by virtue of its inhibition by PMSF. The data presented in this Chapter are insufficient to classify VCP1 in the chymotrypsin or subtilisin clan of serine proteases (Rawlings & Barrett, 1994). Inhibitor studies do not allow to make that distinction easily, and although suc-(Ala)₂-Pro-Phe-pNA was originally developed as a chymotrypsin substrate (Del Mar *et al.*, 1979), this nitroanalide is also degraded with great efficiency by numerous subtilisins (St. Leger *et al.*, 1987a; Burton *et al.*, 1993). The efficiency of the reaction between VCP1 and

suc-(Ala)₂-Pro-Phe-pNA was not due to its speed, as the catalytic constant k_{cat} was 5.76 s⁻¹, which is low (Cornish-Bowden & Wharton, 1988). However, the Michaelis-Menten constant K_m was only 0.043 mM, which suggests a low energy required for binding, or a low dissociation constant for the enzyme-substrate complex (Fersht, 1985). As a result, the reaction was efficient because the “slow” enzyme VCP1 had a high affinity for the substrate, suc-(Ala)₂-Pro-Phe-pNA.

VCP1 degraded elastin, but the two oligopeptide substrates for elastase that were tested, were not hydrolysed to any great extent. That is not exceptional. Suc-(Ala)₃-pNA is traditionally an elastase substrate, hydrolysed by porcine pancreatic elastase I (Bieth *et al.*, 1974; Kasafirek *et al.*, 1976), which is the enzyme that is used as the model for elastase type proteases. However, the elastolytic enzymes are very heterogeneous, and many of them have been reclassified in the chymotrypsin family (Rawlings & Barrett, 1994). Often, so-called elastases are functionally similar to chymotrypsin type enzymes, e.g. human pancreatic elastase II degrades suc-(Ala)₂-Pro-Phe-pNA (Del Mar *et al.*, 1980); porcine pancreatic elastase II is highly active on chymotrypsin substrates, but it does not degrade suc-(Ala)₃-pNA (Gertler *et al.*, 1977). The characteristics of the latter enzyme are similar to those of VCP1. Elastin-degrading enzymes with chymotrypsin-like activity have clearly been described before. They were invariably called elastase, presumably because of their important physiological implications in (mainly human) pathology. However, modern classification of proteases (Rawlings & Barrett, 1994) has downgraded the epithet elastase and, as a result, VCP1 was not given the trivial name elastase.

The enzyme, VCP1, 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 *ca* 10), molecular mass (*ca* 33 kDa), sensitivity to PMSF and the ability to hydrolyse chymotrypsin substrates and elastin. 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 similar but not identical to Pr1. By coining the trivial name, chymoelastase, for the *M. anisopliae* Pr1 enzyme, St. Leger *et al.* (1987a) made a compromise between chymotrypsin and elastase. However, Pr1 was later found to be a subtilisin-like enzyme (St. Leger *et al.*, 1992a), and the chymoelastase epithet is unlikely to be very persistent.

No glycosylation was detected in VCP1 by several biochemical methods. Potential glycosylation sites have often been detected in the genes encoding VCP1-like enzymes (see Chapter 3) (e.g. Moehle *et al.*, 1987; Jatou-Ogay *et al.*, 1992; St. Leger *et al.*, 1992a). Exceptionally, the related enzyme from *Beauveria bassiana* lacks these sites (Joshi *et al.*, 1995). There is however, little information on the actual glycosylation of most of these enzymes. Some of the related proteases that have potential glycosylation sites, e.g. those from *Fusarium* sp. (Morita *et al.*, 1994), and *Aspergillus oryzae* (Tatsumi *et al.*, 1989) are not glycosylated. In the latter example it was found that the potential N-linked glycosylation site is embedded in the molecule and can therefore not be glycosylated.

Unbuffered VCP1 was unstable at 37°C, as only *ca* 20% of the original activity could be recovered after 1 h. This lability could partly be attributed to the pH effect of the medium. Samples were stored unbuffered after harvesting from IEF, therefore the pH of the fraction containing VCP1 was *ca* 10 (Fig. 2.1). Lowering the pH during storage to pH 7 resulted in significant stabilisation (68% residual activity after 4 h at 37°C; $P < 0.001$). Under these conditions, VCP1 could be considered very stable. The protease is possibly autoproteolytic at pH 10, which is close to its pH optimum (Fig. 2.3), although conformational or chemical inactivation at this high pH is also possible (Abraham & Breuil, 1995). The physiological function of VCP1 is unlikely to be adversely affected by its thermolability, since 37°C is not a physiological temperature for *V. chlamydosporium* (Kerry *et al.*, 1986). However, thermostability is an important parameter for the industrial exploitation of proteases (Wells & Estell, 1988; Siezen *et al.*, 1991). Not all proteases that are related to VCP1 (see Chapter 3) have the same thermostability. Whereas proteinase K from *Tritirachium album* (Betzel *et al.*, 1990), and Pr1 from *M. anisopliae* (St. Leger *et al.*, 1987a) are heat-stable, even at pH 10, the related enzyme from *Ophiostoma piceae* (Abraham & Breuil, 1995) is very susceptible to autoproteolysis under conditions of elevated temperature or altered pH. Calcium and glycerol were also found to act as thermoprotectants in the latter case (Abraham & Breuil, 1995). Among the many possible measures that can be taken to protect enzyme activity (Price, 1992), buffering with Tris and/or the addition of glycerol or Ca^{2+} are relatively simple ways to retain the activity of VCP1.

3.1 INTRODUCTION

The major serine proteases of *Verticillium chlamydosporium* and *Metarhizium anisopliae* are VCP1 and Pr1, respectively. These enzymes share several attributes (e.g. similar molecular mass and charge) but differ slightly in their kinetics and sensitivity to some inhibitors (see Chapter 2).

Examination of the primary structure of Pr1 showed this enzyme to be related to the subtilisin proteinase K (St. Leger *et al.*, 1992a), but no structural data exist for VCP1. The aim of the work described in this Chapter is to determine the similarities between VCP1 and Pr1 based on surface characteristics determined by ion exchange and hydrophobic interaction chromatography, and serology. The partial amino acid sequence of VCP1 provides direct structural data, while more detailed substrate utilization data compare the functionality of both enzymes.

If VCP1 is a virulence-determinant, as is the case with Pr1, then detailed knowledge of the properties of the enzyme could help in the understanding of specificity and virulence in invertebrate mycopathogens.

3.2 MATERIALS AND METHODS

The proteases VCP1 and Pr1 were purified from the respective culture filtrates of *V. chlamydosporium*, isolate 10, and *M. anisopliae*, isolate V245, using preparative isoelectric focusing, as described previously (see Chapter 2).

3.2.1 Polyclonal antisera against VCP1 and Pr1

Rabbits were immunised either with VCP1 (150 µg) or Pr1 (200 µg) in Freund's complete adjuvant. Injections were repeated with antigen prepared in Freund's incomplete adjuvant after 4 and 15 weeks. Approximately 30 ml of blood was collected from a lateral ear vein and left to stand at room temperature for 1 hour to allow clotting. The serum was separated by spinning the blood at 3,600 rpm for 15 min and frozen until needed.

In order to estimate the antibody concentration required for a positive signal, the polyclonals were titrated. After coating round-bottomed ELISA plates (Sigma) with a dilution of VCP1, anti-VCP1 antibody dilutions ranging from 1/8 down to 1/256 were applied. The secondary antibody used was goat anti-rabbit, conjugated to alkaline phosphatase. The plates were developed with Tris-buffered *p*-nitrophenyl phosphate (Sigma), and the absorbance of the released chromogen recorded as A_{405} .

3.2.2 Western blotting

Western blots were done, not only with the antibodies against VCP1 and Pr1, but also with an antiserum to a 32 kDa protease from *V. suchlasporium*, obtained from Drs W. Robertson (SCRI, Invergowrie, Scotland) and L.V. Lopez-Llorca (Universidad

de Alicante, Spain), and an antiserum against an isoform of Pr1 specific to *M. anisopliae* isolate ME1, obtained from Dr. R. J. St. Leger (Boyce Thompson Institute, Ithaca, U.S.A.). The antibodies against VCP1 and Pr1 were also tested against commercial proteases (Sigma), including proteinase K, bovine elastase and chymotrypsin, and bacterial subtilisins from *Bacillus subtilis* KZ (Sigma, no. P8038) and *Bacillus sp.* (Sigma, no. P4789).

After SDS-PAGE (see Section 2.2.5), gels were soaked in transfer buffer (Bjerrum & Schafer-Nielsen, 1986) for 10 minutes and semi-dry electroblotted onto nitrocellulose using the NovaBlot system (Pharmacia), according to the manufacturer's instructions. Non-specific binding sites on the membrane were blocked with PBSTM (0.01 M phosphate buffer pH 7.4, containing 0.14 M NaCl, 0.1% [v/v] Tween 20 and 4% [w/v] skimmed milk powder). VCP1 pre-immune serum and VCP1-antiserum were diluted 1/20, Pr1 pre-immune serum, Pr1-antiserum, and *V. suchlasporium*-antiserum were diluted 1/200, while lyophilised ME1-antiserum was diluted 0.2% (w/v) in PBSTM and incubated with the antigens overnight at room temperature. The membranes were washed with PBSTM prior to incubation with goat anti-rabbit peroxidase or alkaline phosphatase conjugate (1/500 dilution) for two hours at room temperature. The excess secondary antibody was removed by washing the membranes with PBST before adding buffered 3,3'-diaminobenzidine tetrahydrochloride (DAB) or 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP/NBT, Sigma), substrates for peroxidase and alkaline phosphatase, respectively.

3.2.3 Elution patterns in HPLC

Surface properties of VCP1 and Pr1 were investigated by comparing their elution on a cation exchange and a hydrophobic interaction column in an HPLC-apparatus (Gilson). The equipment included a Rheodyne injection valve fitted with a 20 μ l injection loop, dual model 306 pumps, an 811B dynamic mixer, an 805 manometric module, and a model 759A dual beam detector (Applied BioSystems). The system was controlled, and detector output monitored, with version 712 software (Gilson). Columns were either a Hydropore™ strong cation exchange column (Rainin Instrument Co.; internal diameter, 4.6 mm; length 10 cm), with a mobile phase of 20 mM sodium acetate and the eluent 1 M NaCl (flow rate 1 ml min⁻¹), or a Hydropore™ hydrophobic interaction column (Rainin Instrument Co.; internal diameter, 4.6 mm; length 10 cm), in which case the mobile phase was 3 M (NH₄)₂SO₄ in 10 mM phosphate buffer, pH 7.0 (flow rate 0.5 ml min⁻¹).

The protease assay with suc-(Ala)₂-Pro-Phe-pNA was modified to accommodate large numbers of fractions. Ten μ l of each fraction was added to an ELISA plate well, containing 40 μ l of 0.1 M Tris pH 7.9, and the reaction started with 50 μ l of 2 mM suc-(Ala)₂-Pro-Phe-pNA, prepared in the same buffer. An ELISA plate reader recorded A_{405} after 1 min incubation at room temperature.

3.2.4 Amino acid sequencing

Proteins were separated by SDS-PAGE (15% acrylamide) and semi-dry electroblotted onto Fluorotrans membrane (Pall) in 10 mM CAPS pH 11.0, containing 10% (v/v) methanol. Following staining with 0.005% (w/v)

Sulphorhodamine B in 30% (v/v) aqueous methanol, containing 0.02% acetic acid, protein bands were excised, placed in the Blott™ reaction cartridge, and analysed on an Applied Biosystems 477A liquid-pulse sequencer, with an on line 120A PTH-analyzer and 610A data analysis system.

The following peptide sequence databases were searched for related sequences with BLASTP (Altschul *et al.*, 1990): the Brookhaven Protein Data Bank (Jan. 1994 release), Swiss-Prot (release 28.0), PIR (release 40.0), GenPept (release 82.0). The OWL database was searched using the SWEEP program (Akrigg *et al.*, 1988).

3.2.5 Enzyme assays

Although some information on the substrate utilization of VCP1 was obtained in Chapter 2, the similarities between VCP1 and Pr1 warranted a more extensive comparison. Culture filtrates and pure proteases from *V. chlamydosporium* and *M. anisopliae* were tested using the API ZYM system (API BioMérieux UK, Hampshire). The kit consisted of assays for 59 arylamidases (series AP 1-6, β -naphthylamide derivatives of amino acids and peptides), 20 glycosidases (series OS, *p*-nitrophenyl derivatives of carbohydrates), 10 esterases (ES, β -naphthyl esters of fatty acids) and one transferase (β -naphthylamide). Culture filtrates and pure enzymes were diluted in 50 mM Tris-HCl, pH 7.0, and 60 μ l added to each well in the test galleries. The strips were incubated in humid chambers at 37°C, in darkness, for 4 hours. After incubation, one drop each of API ZYM reagent A (Tris, 25% [w/v]; HCl, 4% [v/v]; lauryl sulphate, 10% [w/v]), and reagent B (Fast Blue BB, 0.35%

[w/v] in 2-methoxy-ethanol) were added to each well in the AP and ES series. After 10 minutes, these strips were briefly placed under a bright light to bleach unbound Fast Blue dye, leaving the control colourless. In the OS series, one drop of 0.1 M NaOH was added to each well. Scoring was 1-5 according to the colour scale provided by the manufacturer.

3.2.6 Esterase activity in IEF gels

Isoelectric focusing (IEF) of VCP1 and Pr1 was as described previously (see Section 2.2.3). For visualisation of esterases, 4% (w/v) α - or β -naphthyl acetate, or α -naphthyl butyrate (Sigma) were dissolved in anhydrous acetone, and made to a final concentration of 0.04% (w/v) in 0.1 M Tris-HCl pH 7.9, in which 0.2 % (w/v) Fast Blue RR salt (Sigma) was dissolved. The gels were developed with these substrates at room temperature.

3.3 RESULTS

3.3.1 Western blotting

Titration of the anti-VCP1 polyclonal antibody, collected in the third bleed, revealed a relatively low titre, with *ca* 30 μg VCP1 ml^{-1} and a 1/64 dilution of the antibody required to result in an A_{405} equal to 1 (Fig. 3.1). Consequently, Western blots that were developed with a 1/20 dilution of the VCP1 antibody gave an adequate signal strength.

Fig. 3.2 Western blots of *V. chlamydosporium* culture filtrate and VCP1. After separation by SDS-PAGE (a), the *V. chlamydosporium* antigens were blotted onto nitrocellulose and probed with VCP1 antiserum (b), antiserum against *V. suchlasporium* protease (c), Pr1 antiserum (d), and *M. anisopliae* ME1 antiserum (e). Molecular weight markers were BSA (M, 66,200), ovalbumin (M, 45,000), carbonic anhydrase (M, 31,000) and soybean trypsin inhibitor (M, 21,500). Each panel has two lanes, *V. chlamydosporium* culture filtrate (lane 1) and purified VCP1 (lane 2).

Fig. 3.3 Western blots of *M. anisopliae* culture filtrate and Pr1. After separation by SDS-PAGE (a), the *M. anisopliae* antigens were blotted onto nitrocellulose and probed with Pr1 antiserum (b), antiserum against *V. suchlasporium* protease (c), VCP1 antiserum (d) and *M. anisopliae* ME1 antiserum (f). Molecular mass markers were as in Fig. 3.2. Each panel has two lanes, *M. anisopliae* culture filtrate (lane 1) and purified Pr1 (lane 2).

66a

Fig. 3.2

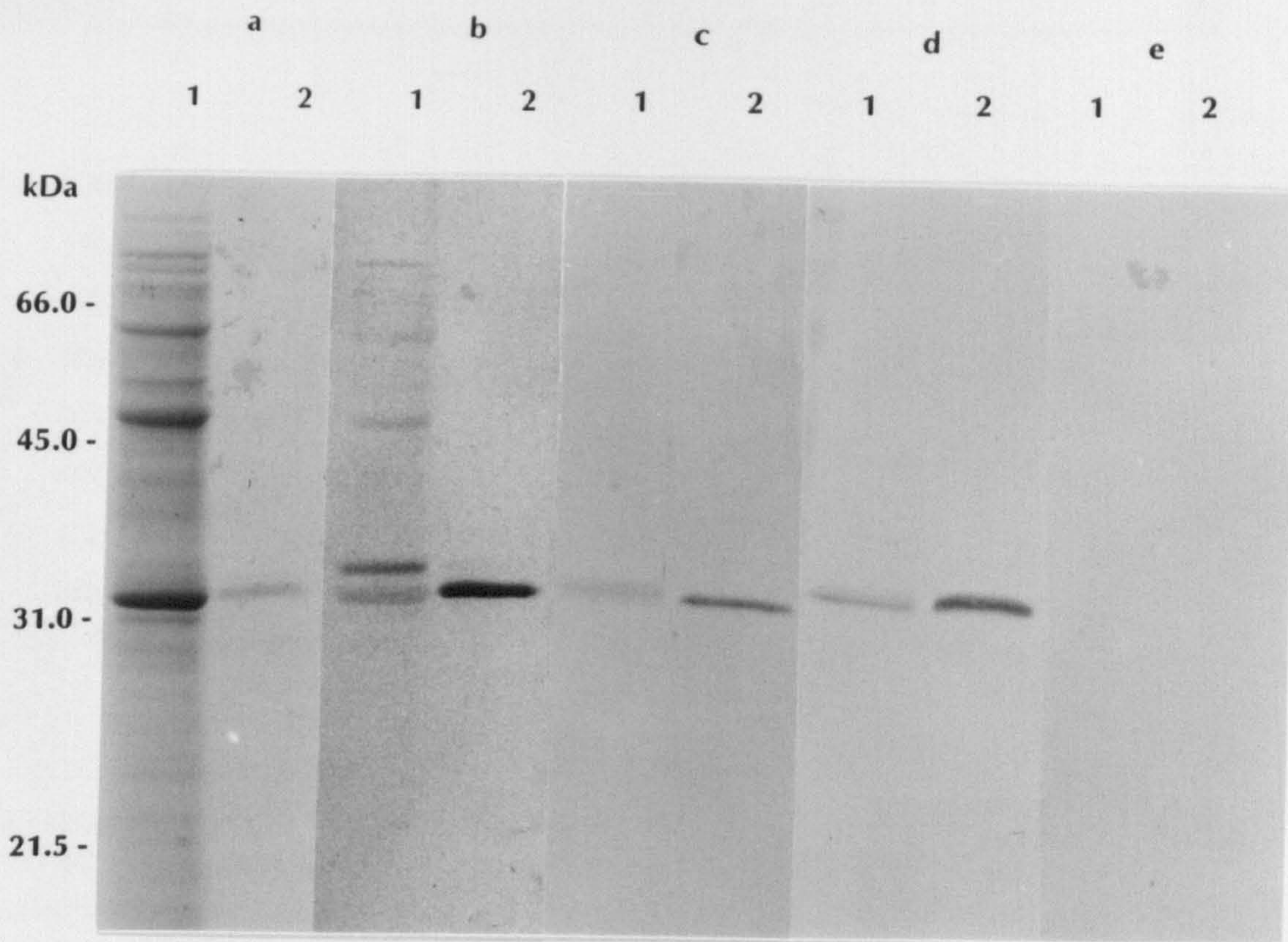
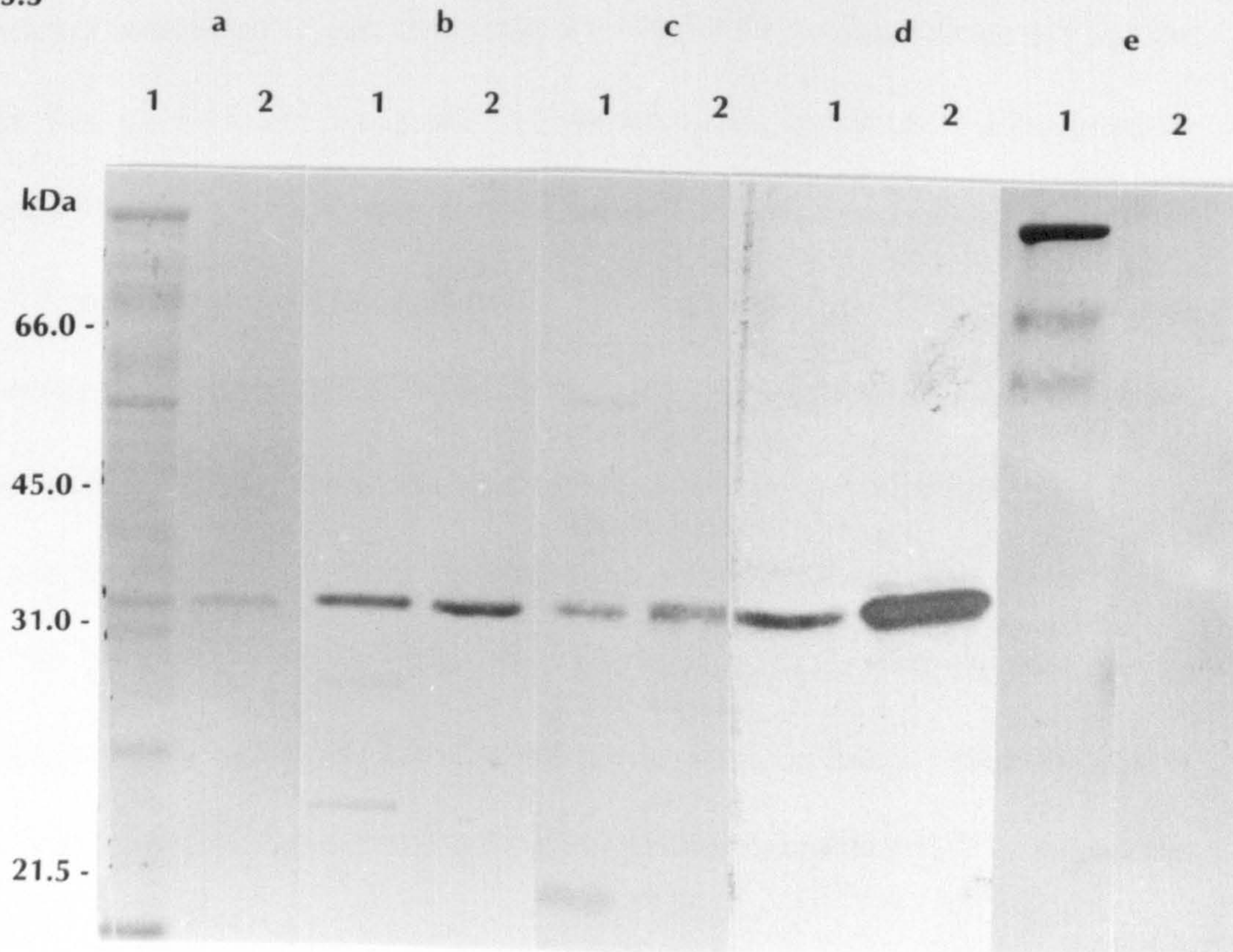


Fig. 3.3



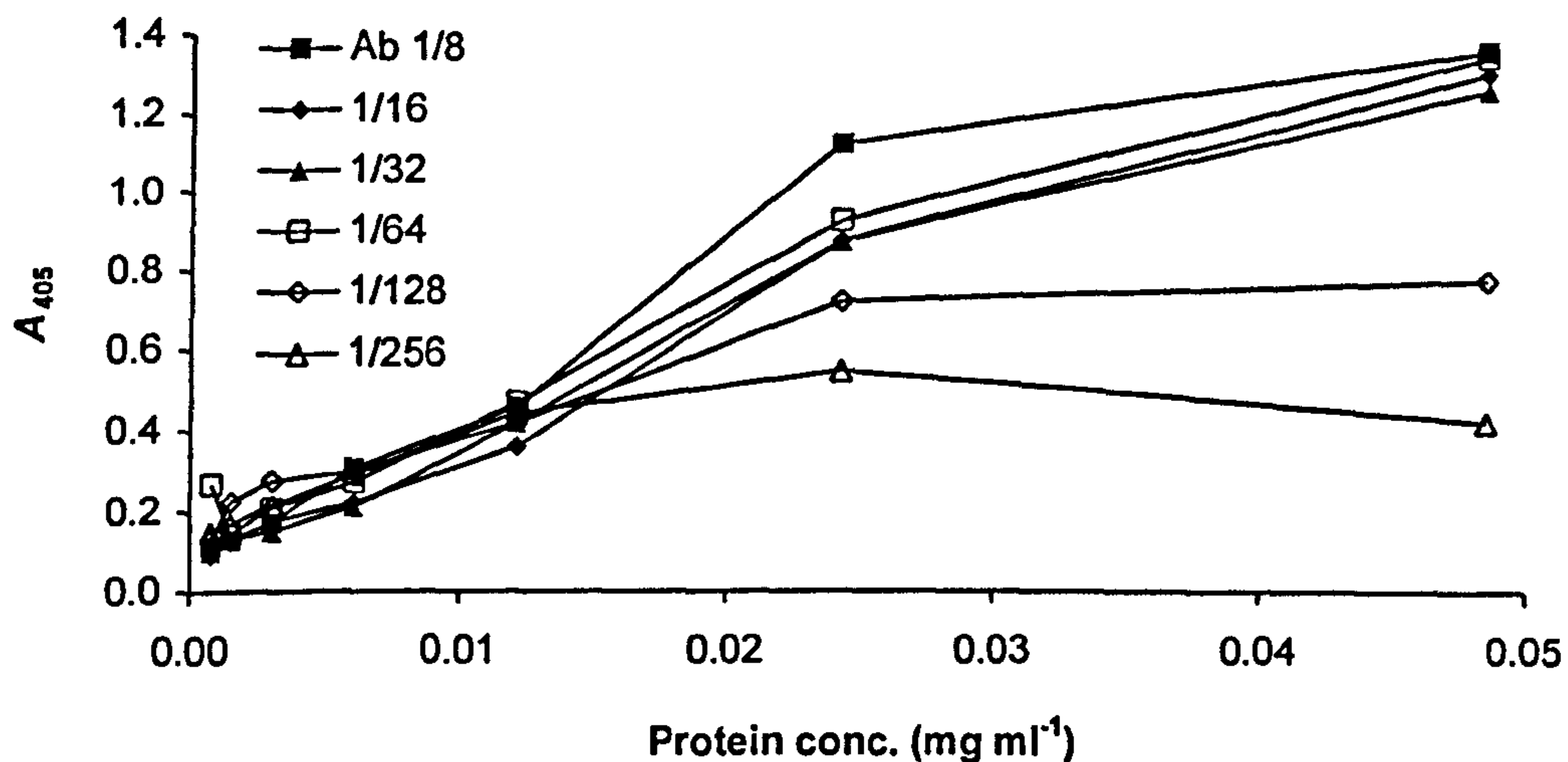
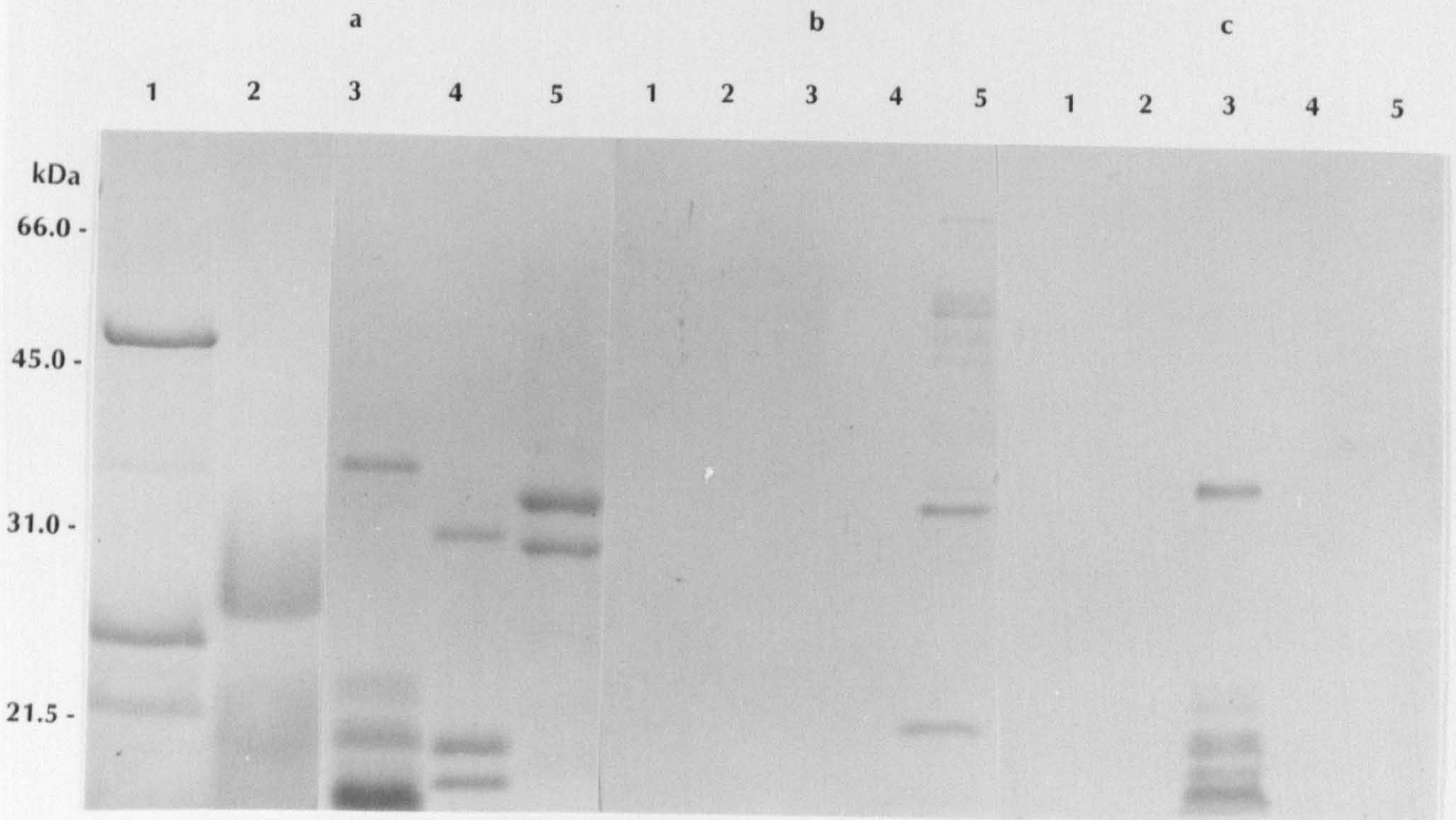


Fig. 3.1 Titration of anti-VCP1 antiserum by ELISA. A serial dilution of a polyclonal against VCP1 was allowed to bind to a dilution of antigen. A secondary anti-rabbit antibody, conjugated to alkaline phosphatase was applied, and the antibody was detected by reading the absorbance of released chromogen from *p*-nitrophenyl phosphate.

The antiserum to VCP1 bound to VCP1 (33 kDa), and cross-reacted with Pr1 (33 kDa) and elastase (31 kDa) (Figs. 3.2 and 3.4, panels b), whereas the Pr1-antiserum bound to Pr1, and cross-reacted with VCP1, and proteinase K (33 kDa) (Figs. 3.3, panel b; and 3.4, panel c). The polyclonal antiserum to a major serine protease from *V. suchlasporium* (Lopez-Llorca, 1990) also cross-reacted with VCP1 and Pr1, confirming the relatedness of all three enzymes (Figs. 3.2 and 3.3, panels c). However, the antiserum to the Pr1 isoform specific to isolate ME1 of *M. anisopliae*, failed to label VCP1, and also Pr1 from isolate V245 of *M. anisopliae* (Figs. 3.2 and 3.3, panels e). However, this polyclonal did bind to a protein in the culture filtrate from *M. anisopliae*, isolate V245 (Fig. 3.3, lane e). Apart from the fact that its molecular mass was just over 100 kDa, the nature of this protein is unknown. Neither of the antisera to VCP1 or Pr1 cross-reacted with bacterial subtilisins (Fig. 3.4, panels b-c).

Fig. 3.4 Western blots of commercial proteases with antibodies against VCP1 and Pr1. Bacterial subtilisins P4789 (lane 1) and P8038 (lane 2), proteinase K (lane 3), chymotrypsin (lane 4) and elastase (lane 5) were separated by SDS-PAGE (panel a), blotted onto nitrocellulose and probed with VCP1 antiserum (panel b), or with Pr1 antiserum (panel c). Molecular mass markers were as in Fig. 3.2.

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3.3.2 Elution from HPLC columns

VCP1 and Pr1 were separated from the culture filtrates of *V. chlamydosporium* and *M. anisopliae* using ion exchange, and hydrophobic interaction HPLC. Both enzymes had the same retention time in a strong cation exchange column, i.e. they eluted when the concentration of NaCl reached *ca* 0.35 M (Figs. 3.5a-b). In both cases the proteases eluted as a single peak, fairly well separated from other proteins. The separation of both enzymes from background protein on a hydrophobic interaction column was not as clear. The main protease activity in the *V. chlamydosporium* sample was associated with the first peak, which was eluted when the concentration of the mobile phase, $(\text{NH}_4)_2\text{SO}_4$, had reduced from 3 M to *ca* 0.45 M (Figs. 3.6a-b). The elution of protease activity in the *M. anisopliae* sample from this column was slightly different. Not only did the main activity elute earlier, when the concentration of $(\text{NH}_4)_2\text{SO}_4$ was still *ca* 0.66 M, but there was a much more pronounced tailing of the enzyme activity (Fig. 3.6a-b).

3.3.3 Amino acid sequence

N-terminal amino acid sequencing of the first 20 residues of VCP1 revealed considerable homology with regions in other fungal subtilisins (Table 3.1). The closest resemblance was with Pr1; there was 63% identity between VCP1 and Pr1. A highly conserved sequence G A P X G L in VCP1 (residues 7-12) corresponds to a helical structure in all known fungal subtilisins (Siezen *et al.*, 1991). The unknown residue X in VCP1 corresponds to tryptophan in all other subtilisins and the same is predicted for VCP1. Similarly, isoleucine in position 16 was a tentative assignment

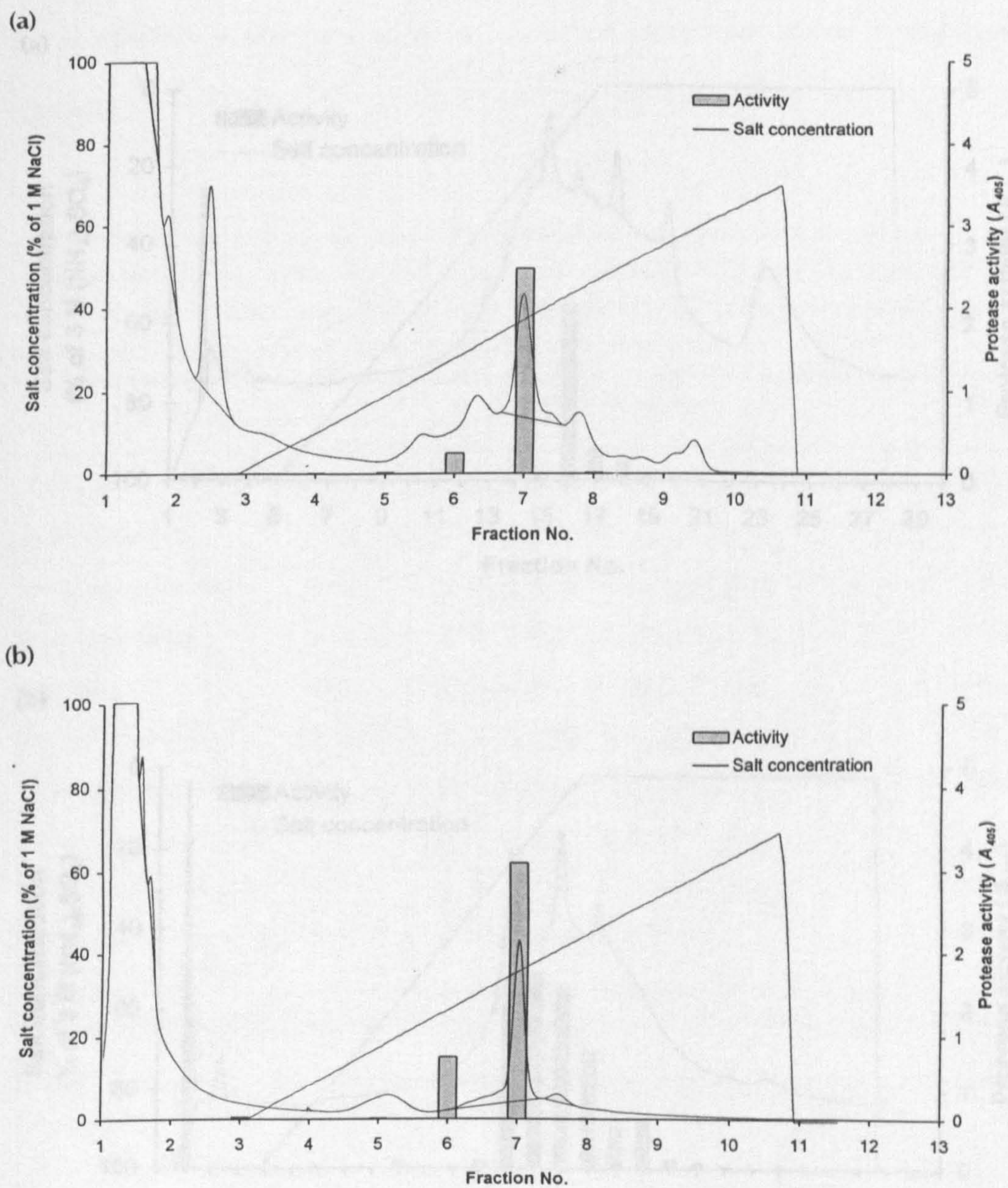


Fig. 3.5 Retention characteristics of a) VCP1 and b) Pr1 on a strong cation exchange HPLC column. Injection volume was 20 μ l of enzyme concentrate from *V. chlamydosporium* (1.0 mg ml⁻¹), and *M. anisopliae* (1.3 mg ml⁻¹), respectively. Elution was in a concentration gradient of NaCl. Bars represent protease activity against suc-(Ala)₂-Pro-Phe-pNA in collected fractions. Straight line indicates increasing concentration of NaCl (as % of 1 M NaCl). Trace is total protein, recorded by the detector as A_{280} , in arbitrary units.

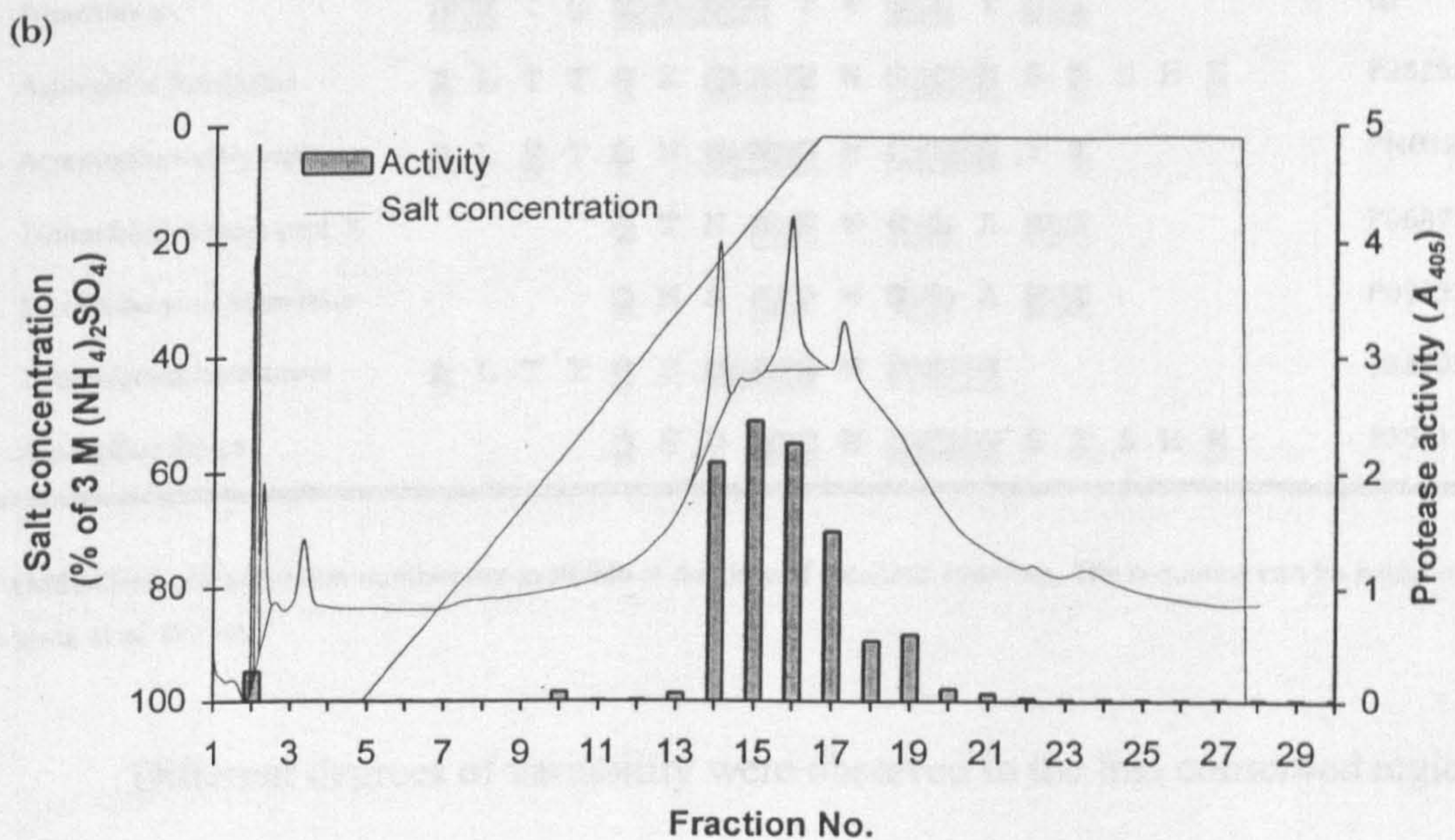
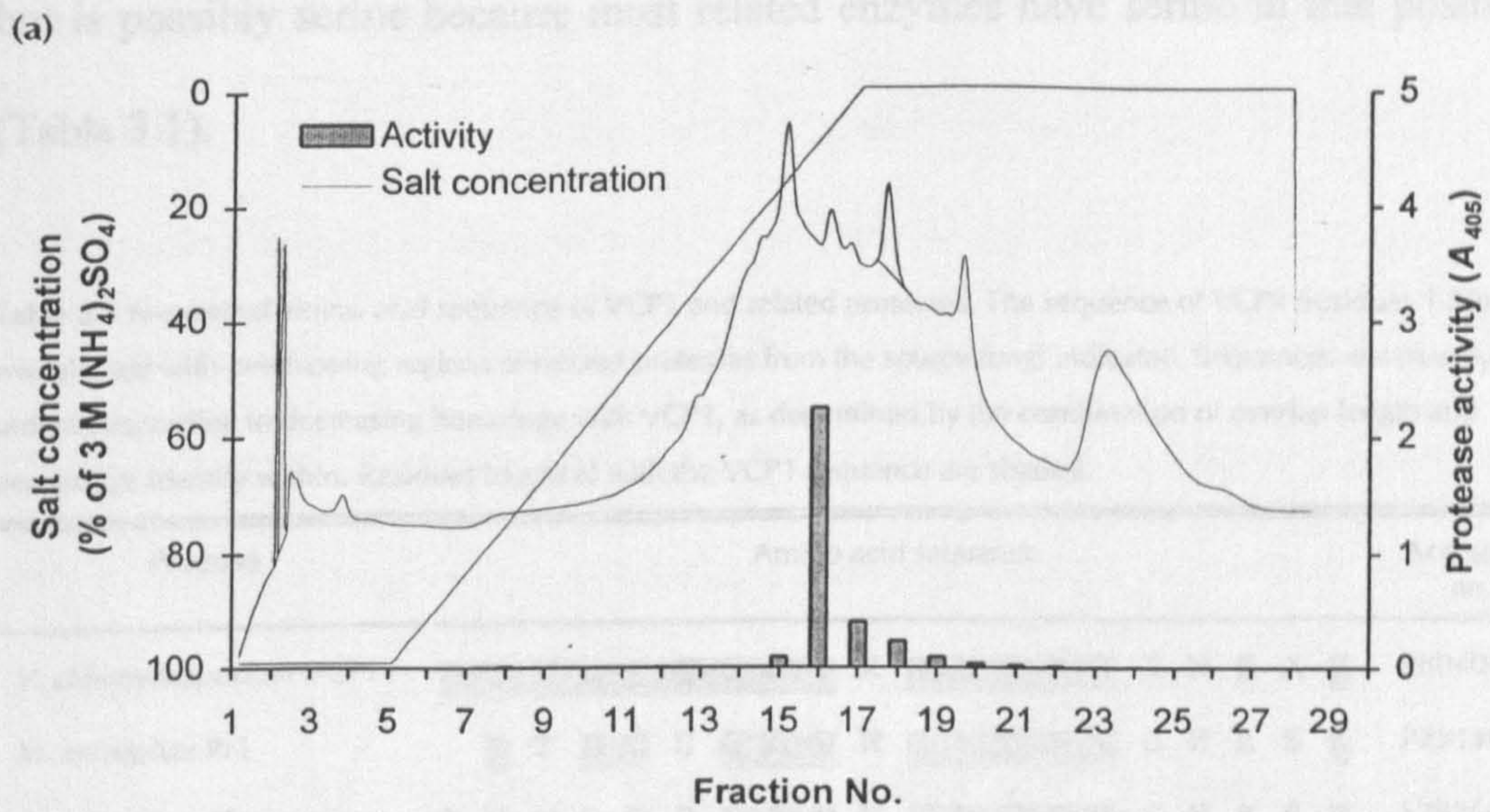


Fig. 3.6 Retention characteristics of a) VCP1 and b) Pr1 on a hydrophobic interaction HPLC column. Injection volume was 20 μ l of enzyme concentrate from *V. chlamydosporium* (1.0 mg ml⁻¹), and *M. anisopliae* (1.3 mg ml⁻¹), respectively. Elution was in a concentration gradient of (NH₄)₂SO₄. Bars represent protease activity against suc-(Ala)₂-Pro-Phe-pNA in collected fractions. Straight line indicates decreasing salt concentration (as % of 3 M (NH₄)₂SO₄, in 10 mM phosphate buffer, pH 7.0). Trace is total protein, recorded by the detector as A₂₈₀, in arbitrary units.

but is possibly serine because most related enzymes have serine in that position (Table 3.1).

Table 3.1 N-terminal amino acid sequence of VCP1 and related proteases. The sequence of VCP1 (residues 1-20) was aligned with overlapping regions of related proteases from the source fungi indicated. Sequences are crudely ordered according to decreasing homology with VCP1, as determined by the combination of overlap length and percentage identity within. Residues identical with the VCP1 sequence are shaded.

Protease	Amino acid sequence	Accession no.
<i>V. chlamyosporium</i> VCP1	A I V E Q Q G A P X G L G R I I N K X K	P80406
<i>M. anisopliae</i> Pr1	I T E Q S G A P W G L G R I S H R S K	P29138
<i>Paecilomyces lilacinus</i>	A Y T Q Q P G A P W G L G R I S H R S K	L29262
<i>Beauveria bassiana</i>	A V V R Q A G A P W G L G R I	U16305
<i>Fusarium</i> sp.	A I T Q Q Q G A T W G L T R I	(a)
<i>Aspergillus fumigatus</i>	A L T T Q K G A P W G L G S I S H K	P28296
<i>Acremonium chrysogenum</i>	A L V T Q N G A P W G L G T I	PN0129
<i>Tritirachium album</i> prot. K	Q T N A P W G L A R I	P06873
<i>Saccharomyces cerevisiae</i>	Q N S A P W G L A R I	P09232
<i>Trichoderma harzianum</i>	A L T T Q S G A P W G L G	S32905
<i>Aspergillus flavus</i>	Q S D A P W G L G S I S H K	P35211

^a EMBL/Genbank accession number not available at the time of databank searches. The sequence can be found in Morita et al. (1994).

Different degrees of variability were observed in the less conserved regions of the 20 amino acid sequence. The least variable was replacement of alanine for the closely related glycine (VCP1 residue 1), or valine for the polar threonine (VCP1 residue 3). More variable was asparagine (uncharged polar; VCP1 residue 17) which could be replaced in related sequences by either histidine (basic) or serine in the case of proteinase R and T (not shown). The most variable in this sequence was glutamine (VCP1 residue 6) which was replaced by various other amino acids (Table 3.1).

3.3.4 Substrate utilisation

Neither VCP1 nor Pr1 hydrolysed monoaminoacyl substrates. However, peptidases were present in culture filtrates which could utilize such substrates, e.g. Ala- β NA, β -Ala- β NA, Arg- β NA, Gln- β NA, Lys- β NA, Met- β NA, Phe- β NA, and Trp- β NA (Table 3.2). Certain aminopeptidases were produced in large amounts (score ≥ 2) only in culture filtrates of *V. chlamydosporium* (e.g. utilizing Orn- β NA, Ser- β NA) while others were highly active only in culture filtrates of *M. anisopliae* (e.g. utilizing Pro- β NA, hydroxy-Pro- β NA, His- β NA) (Table 3.2). N-terminally blocked monoaminoacyl β -naphthylamides (Bz-Leu- β NA, CBZ-Arg-MNA, Bz-Ala- β NA) were never degraded by either fungus indicating that, at least the corresponding non-blocked substrates, Arg- β NA and Ala- β NA were hydrolysed by true aminopeptidases, requiring free N-terminal residues.

Table 3.2 API ZYM scores for peptidase, esterase, glycosidase and transferase activity in *V. chlamydosporium* VCP1 and culture filtrate, and *M. anisopliae* Pr1 and culture filtrate. Scoring was 1 - 5 according to manufacturer's instructions. Means of two replicates.

		<i>V. chlamydosporium</i> isolate 10		<i>M. anisopliae</i> isolate 245	
		VCP1	Filtrate	Pr1	Filtrate
Peptidases (arylamidases)					
AP1.1	Tyr ^a - β NA ^b	0	1	0	1
AP1.2	pyroGlu- β NA	0	0	0	0
AP1.3	Phe- β NA	0	3.5	0	5
AP1.4	Lys- β NA	0	4.5	0	3
AP1.5	hydroxyPro- β NA	0	1	0	4
AP1.6	His- β NA	0	1	0	3
AP1.7	Gly- β NA	0	0	0	0
AP1.8	Asp- β NA	0	0	0	0
AP1.9	Arg- β NA	0	4.5	0	3
AP1.10	Ala- β NA	0	4.5	0	3
AP2.2	Bz ^c -Leu- β NA	0	0	0	0
AP2.3	S-Bz-Cys- β NA	0	1.5	0	1
AP2.4	DL-Met- β NA	0	2.5	0	2
AP2.5	Gly-Gly- β NA	0	1.5	0	0
AP2.6	Gly-Phe- β NA	0	5	0	5
AP2.7	Gly-Pro- β NA	1.5	2	0	5
AP2.8	Leu-Gly- β NA	0	1	0	0
AP2.9	Ser-Tyr- β NA	0	4.5	0	4
AP2.10	Control	0	0	0	0
AP3.1	CBZ ^d -Arg-MNA ^e	0	0	0	0
AP3.2	Gln- β NA	0	4.5	0	2

		<i>V. chlamydosporium</i> isolate 10		<i>M. anisopliae</i> isolate 245	
		VCP1	Filtrate	Pr1	Filtrate
AP3.3	α -Glu- β NA	0	0.5	0	0
AP3.4	Ile- β NA	0	1.5	0	0
AP3.5	Orn ^f - β NA	0	3	0	1
AP3.6	Pro- β NA	0	0	0	2
AP3.7	Ser- β NA	0	2	0	0
AP3.8	Thr- β NA	0	1.5	0	0
AP3.9	Trp- β NA	0	2	0	3
AP3.10	CBZ-Gly-Gly-Arg- β NA	0	0	4	3
AP4.1	β -Ala- β NA	0	3.5	0	3
AP4.2	Ala-Arg- β NA	0	0	0	1
AP4.3	Ala-Phe-Pro- β NA	0	2	0	3
AP4.4	Ala-Phe-Pro-Ala- β NA	4.5	4.5	5	3
AP4.5	Arg-Arg- β NA	0	1	0	1
AP4.6	α -Asp-Ala- β NA	0	3	0	1
AP4.7	α -Asp-Arg- β NA	0	0	0	0
AP4.8	α -Glu- α -Glu- β NA	0	0	0	0
AP4.9	α -Glu-His- β NA	0	0.5	0	1
AP4.10	Gly-Ala- β NA	0	5	0	2
AP5.1	Gly-Arg- β NA	0	0	0	0
AP5.2	Gly-Trp- β NA	0	0	0	0
AP5.3	His-Leu-His- β NA	1.5	1.5	4	1
AP5.4	His-Ser- β NA	0	1	0	0
AP5.5	Leu-Ala- β NA	0	4.5	3	2
AP5.6	Leu-Leu-Val-Tyr-Ser- β NA	0.5	3	1	1
AP5.7	Lys-Ala- β NA	0	4.5	0	3
AP5.8	Lys-Lys- β NA	0	2	0	0
AP5.9	Phe-Arg- β NA	0	2	0	1
AP5.10	Phe-Pro- β NA	1	2	1	5
AP6.1	Phe-Pro-Ala- β NA	0	1	1	0
AP6.2	Pro-Arg- β NA	0	0	0	0
AP6.3	Ser-Met- β NA	0	5	0	2
AP6.4	Val-Tyr-Ser- β NA	0	1	0	0
AP6.5	Bz-Ala-MNA	0	0	0	0
AP6.6	CBZ-Arg-MNA	0	0	0	0
AP6.7	CBZ-Gly-Gly-Arg- β NA	0	0	3	3
AP6.8	Ac ^e -Gly-Lys- β NA	0	0	0	0
AP6.9	His-Phe- β NA	0	2.5	0	1
AP6.10	Lys-Ser-MNA	0	1	0	0
Esterases					
ES1	2-naphthyl-butyrate (C4)	2.5	5	5	5
ES2	2-naphthyl-valerate (C5)	2	5	5	5
ES3	2-naphthyl-caproate (C6)	2	5	5	5
ES4	2-naphthyl-caprylate (C8)	2	5	1	5
ES5	2-naphthyl-nonanoate (C9)	2	5	1	5
ES6	2-naphthyl-caprate (C10)	2	5	1	5
ES7	2-naphthyl-laurate (C12)	0	1	0	0
ES8	2-naphthyl-myristate (C14)	0	1	0	0
ES9	2-naphthyl-palmitate (C16)	0	1	0	0
ES10	2-naphthyl-stearate (C18)	0	1	0	0
Transferases					
AP2.1	γ -glutamic acid- β NA	0	1	0	0

^a All amino acids and derivatives were L-configuration, unless indicated otherwise; ^b β -naphthylamide; ^c benzoyl; ^d N-carboxybenzoxy; ^e 4-methoxy- β -naphthylamide; ^f ornithine; ^g N-acetyl

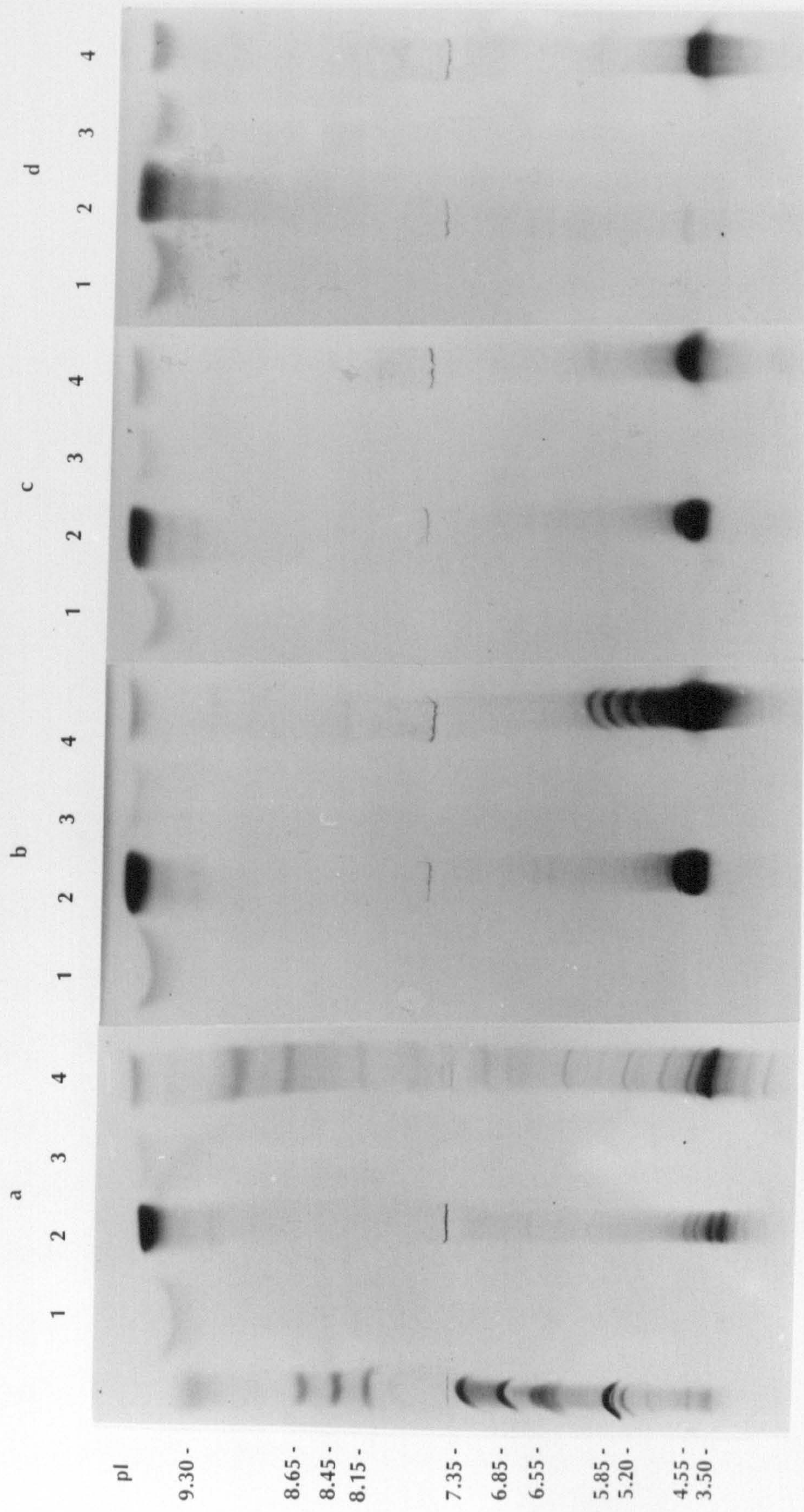
The pure enzymes exhibited restricted dipeptidyl peptidase activity with VCP1 and Pr1 being active against Gly-Pro- β NA and Leu-Ala- β NA, respectively (Table 3.2). Numerous dipeptidyl peptidases were, however, present in culture filtrates. These enzymes efficiently hydrolysed dipeptides with apolar penultimate groups, with the exception of Ser-Tyr- β NA, which has a polar penultimate residue, and Lys-Lys- β NA and Phe-Arg- β NA which have alkaline residues in the penultimate position. The dipeptidyl peptidases in the culture filtrates did not have a preference for the N-terminal residue (Table 3.2). Several dipeptidyl peptidases secreted in large amounts (score ≥ 2) by *V. chlamydosporium* were either absent or present in small amounts (score < 2) in *M. anisopliae* culture filtrates (Table 3.2).

Purified VCP1 and Pr1 readily hydrolysed the tetrapeptide Ala-Phe-Pro-Ala- β NA but not the tripeptide Ala-Phe-Pro- β NA. However, enzymes present in culture filtrates were able to hydrolyse the latter substrate as well (Table 3.2). The N-terminally blocked CBZ-Gly-Gly-Arg- β NA was degraded by Pr1 but not by VCP1, nor was activity detected in the culture filtrate of *V. chlamydosporium* (Table 3.2). The tripeptide His-Leu-His- β NA was hydrolysed by both pure proteases. The pentapeptide, (Leu)₂-Val-Tyr-Ser- β NA, was hydrolysed by enzymes in culture filtrates of *V. chlamydosporium* but not those of *M. anisopliae* (Table 3.2).

VCP1 and Pr1 hydrolysed short (C4-C6) and medium (C7-C10) chain esters to a different extent. VCP1 was highly active on all these substrates while Pr1 was highly active against the short chain esters only (Table 3.2). Esterase activity was high in culture filtrates of both *V. chlamydosporium* and *M. anisopliae* (Table 3.2).

Fig. 3.7 Esterase electrophoresis. Analytical IEF-gel, stained for general protein using Coomassie blue (panel a), and esterase activity against α -acetate (panel b), β -acetate (panel c) and α -butyrate (panel d). Each gel was loaded with *M. anisopliae* Pr1 (lane 1) or culture filtrate (lane 2), *V. chlamydosporium* VCP1 (lane 3) or culture filtrate (lane 4). Marker proteins with corresponding pI values are indicated on panel a.

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Esterase activity was also visualised after electrophoretic separation of culture filtrates, providing more information on the enzymes involved. Activity was detected in IEF gels at pI 10, corresponding with Pr1 and VCP1, while several bands were also detected within the pI range of 4.5 - 6 (Fig. 3.7). The alkaline (including VCP1 and Pr1) and acidic esterases of *V. chlamydosporium* hydrolysed all three substrates tested. However, the acidic esterases of *M. anisopliae* hydrolysed α - and β -acetate but not α -butyrate (Fig. 3.7). *Verticillium chlamydosporium* had a prominent acidic enzyme (pI ca 4.5) that degraded all three substrates but also several other enzymes of higher pI, which were particularly active against α -acetate (Fig. 3.7). In culture filtrates of *M. anisopliae*, one minor band was discerned just above the major esterase (pI 4.5).

3.4 DISCUSSION

The proteases VCP1 and Pr1 are structurally and functionally related, as revealed by N-terminal amino acid sequencing, Western blotting, HPLC analysis and substrate specificity. These data corroborated the results from Chapter 2, that VCP1 and Pr1 are related but not identical enzymes. The amino acid sequence allowed VCP1 to be classified unambiguously as a subtilisin-like serine protease. Similar to all subtilisins isolated from fungi to date, VCP1 was a class II subtilisin, which are enzymes with a high degree of sequence homology, even in most of the variable regions, and a low incidence of insertions/deletions relative to the benchmark enzyme of this class, proteinase K (Siezen *et al.*, 1991). Although model structures can be derived directly from proteinase K, deviant structural features, e.g. disulphide bridging and

glycosylation patterns have been described (Joshi *et al.*, 1995). Recently, St. Leger *et al.* (1992a) showed that Pr1 was closely related to proteinase K, a fact confirmed in this study by Western blotting. In contrast, VCP1-antisera labelled Pr1 but not proteinase K, suggesting greater structural differences between VCP1 and proteinase K, than between Pr1 and proteinase K.

The limited data provided by Lopez-Llorca (1990) on the major protease secreted by *V. suchlasporium*, did not allow a comparison with VCP1 on the basis of the biochemical properties discussed in Chapter 2, but the serological results in this Section suggest that the two enzymes were immunologically related. Moreover, the *V. suchlasporium*-antiserum also reacted with Pr1, confirming the relatedness of all three enzymes. The antibody to an isoform of Pr1 specific to isolate ME1 of *M. anisopliae* (St. Leger *et al.*, 1994), failed to label Pr1 from isolate 245 of the same species, which suggests intraspecific variation in *M. anisopliae*. This could be due to the absence of epitopes, or the absence altogether of this particular isoform in isolate V245. Although no detailed comparison of host specificities in the case of these two isolates exists, differences in the proteolytic complex, as observed here, could theoretically contribute to variation in host specificity between isolates of *M. anisopliae*.

Due to their very alkaline pI, both VCP1 and Pr1 could be separated from contaminating proteins in the culture filtrate by cation exchange at pH 7.0. The elution patterns in these conditions were identical, suggesting similar electrostatic interactions between the bonded sulfonyl groups of the column, and positively charged areas of the protein surface. The excellent separation on this column

paralleled the ease with which these enzymes could be purified by preparative IEF. The success of both methods relied on the presence in the culture filtrates of apparently few protein species with a similar highly charged nature. The hydrophobic interaction column separates proteins according to their degree of hydrophobic interaction with the polyethylene glycol matrix. The fact that VCP1 eluted later than Pr1 suggests a higher degree of hydrophobicity of the surface of the *V. chlamydosporium* enzyme. In the culture filtrates of both *V. chlamydosporium* and *M. anisopliae*, minor enzyme activity eluted after the main peak, but this tailing off of activity was much more pronounced in the case of *M. anisopliae*. The tailing phenomenon might indicate the presence of more than one active enzyme species, possibly isoforms, with the minor activities due to more hydrophobic forms. The fact that the tailing in *M. anisopliae* was more extended than in *V. chlamydosporium* suggests that in the former case, the putative isoforms were either more concentrated, or they were more efficient at hydrolysing the substrate suc-(Ala)₂-Pro-Phe-pNA than those in *V. chlamydosporium*.

Subtilisins are generally regarded as being relatively non-specific, but individual enzymes can differ in their activity on selected substrates (Wells & Estell, 1988). This was also true for VCP1 and Pr1, which showed both qualitative and quantitative differences in the substrates they could hydrolyse in the API ZYM kit. This may be partially explained by point mutations, which can have a profound effect on substrate specificity and kinetic parameters (Wells & Estell, 1988). Such mutations have probably occurred, as witnessed by the amino acid sequence reported here, and they may explain some of the differences between Pr1 and VCP1.

A noted difference between VCP1 and Pr1 was that the former exhibited dipeptidyl peptidase activity against Gly-Pro- β NA, whereas the latter did not. However, *M. anisopliae* secretes dipeptidyl peptidases, one of which has been partially purified and found to have activity against this substrate (St. Leger *et al.*, 1993).

Staining IEF gels for esterase activity confirmed the esterolytic nature of VCP1 and Pr1 that was also observed with the API ZYM system. The esterase systems in both *V. chlamydosporium* and *M. anisopliae* consisted of enzymes of extreme charges, both alkaline and acidic ones. The esterases produced by *V. chlamydosporium*, in particular, appeared to be a rather complex group, as seen after their electrophoretic separation (Fig. 3.7). One or more of these enzymes must have been responsible for the activity against the esters of long-chain fatty acids (C12-C18), as that was not due to VCP1 (Table 3.2). The esterases that are produced by *M. anisopliae* (isolate ME1) appressoria during infection of *Manduca sexta* cuticle are all β -esterases (St. Leger *et al.*, 1991a). The current study, using the filtrate of batch cultures, indicated that *M. anisopliae*, isolate 245, had esterases that could also degrade the α isomer of acetate. This leaves the question whether α and β -esterases would have different physiological roles.

Speculating on the exact physiological role of the enzyme complexity seen here, would be premature. However, the diversity of enzyme activities seen in the API ZYM kit and the esterase electrophoresis presumably reflected the nutritional versatility of these fungi. It is tempting to speculate that mycopathogens secrete other enzymes which are able to degrade substrates that the major endoproteases, like

VCP1 and Pr1, cannot. This would enable the pathogens to exploit diverse nutrient sources and may also influence virulence and host range. Alternatively, the proteolytic complex may consist of a number of enzymes that act in concert to break substrates down in a progressive manner, as has been suggested for *M. anisopliae* (St. Leger, 1995). The further hydrolysis by exopeptidases, of peptides that are generated by endopeptidases, and their subsequent consumption, would also prevent the build-up of catabolic products that might otherwise repress the production of VCP1 (see Chapter 6) and Pr1 (St. Leger *et al.*, 1988b).

The aminopeptidase activity recorded in a range of isolates from the *V. chlamydosporium/V. suchlasporium* complex (Carder *et al.*, 1993) was similar to the one reported here for isolate 10. Exceptions were the high activities against Pro- β NA and hydroxy-Pro- β NA, which were absent in the current isolate, and the low activities against Gln- β NA and Trp- β NA, which were high this time.

Verticillium chlamydosporium, isolate 10, can now be fitted in the set of *Verticillium* species and varieties tested by Carder *et al.* (1993). When the five-level scoring system, used in the current experiments, was converted to three-level scoring (0 or 1 becomes 0; 2 or 3 becomes 1; and 4 or 5 becomes 2), and the number of matches between the transformed scores of isolate 10, and those of Carder *et al.* (1993) was enumerated, then the closest relative of isolate 10 was *V. chlamydosporium* var. *chlamydosporium*, isolate CBS 103.65 (27 matches). It remains to be seen whether Dr W. Gams, who proposed some of the species and varieties concerned here (Gams, 1988), would agree with this diagnosis.

4.1 INTRODUCTION

Subtilisin-like serine proteases, once thought to be confined to prokaryotes, are increasingly being isolated from a much wider range of sources, including archae, bacteria, yeasts, fungi, and higher eukaryotes (Markland & Smith, 1971; Siezen *et al.*, 1991). In a comprehensive review, Siezen *et al.* (1991) divided these enzymes in classes I and II, based on characteristic amino acid sequence patterns. All fungal subtilisins characterized to date, are homologous with proteinase K, from *Tritirachium album* Limber, and are grouped in class II. Various physiological roles have been suggested, or demonstrated, for subtilisin-like proteases. Class I subtilisins are extracellular in many bacteria (Markland & Smith, 1971), while eukaryotic proteases of this class typically carry out compartmentalized proteolysis within cells, processing prohormones and/or other protein precursors (Tanguy-Rougeau *et al.*, 1988; Roebroek *et al.*, 1991; Smeekens *et al.*, 1991). Typically, class II subtilisins are extracellular and have a nutritional role (Gunkel & Gassen, 1989; Monod *et al.*, 1991; Burton *et al.*, 1993), although exceptionally, a vacuolar class II subtilisin is present in baker's yeast (Moehle *et al.*, 1987).

Subtilisins are major proteins secreted by some invertebrate mycopathogens, fungi that breach the proteinaceous integument of hosts, such as nematodes and insects. In some of these fungi there is evidence of a significant contribution of these enzymes to penetration of the host (St. Leger *et al.*, 1988a; Tunlid *et al.*, 1994).

The subtilisin-ancestral gene must be old in evolutionary terms, as it probably appeared before prokaryotes and eukaryotes diverged. However, the extent of variation in subtilisin quantity and quality in current mycopathogens of invertebrates is unclear. The subtilisins secreted by the nematode and insect-pathogens, *Verticillium chlamyosporium* and *Metarhizium anisopliae*, are VCP1 and Pr1, respectively. As established in this study, these were immunologically related proteins with a similar charge (pI *ca* 10) and molecular mass (*ca* 33 kDa). However, they differed in their sensitivity to certain inhibitors, substrate utilization and N-terminal amino-acid sequence (see Chapters 2 and 3). Variability of this kind has not been established between isolates of *V. chlamyosporium*, but may hold a clue to differences in virulence, and also has the potential to affect host specificity. Furthermore, subtilisins from species that are taxonomically closely related, but occupy different niches, have not been compared. *Verticillium* is a particularly interesting genus in this respect, as it contains nematophagous, entomogenous, phytopathogenic and saprotrophic species.

4.2 MATERIALS AND METHODS

4.2.1 Organisms and growth conditions

Five isolates of the nematophagous fungus *Verticillium chlamyosporium* were selected from the Rothamsted culture collection, based on their rhizosphere colonization of barley, virulence against three test nematodes in an *in vitro* bioassay

(Irving & Kerry, 1986), and chlamydospore formation. All isolates differed in their combination of these properties (Table 4.1).

A range of additional nematophagous, entomogenous, phytopathogenic and saprotrophic isolates, of several species, were also studied, and their origins are summarized in Table 4.2.

Table 4.1 Origin, virulence, root colonization and chlamydospore formation of selected *V. chlamydosporium* isolates. Virulence is expressed as percentage of eggs infected in an *in vitro* bioassay according to Irving & Kerry (1986), using eggs of the following test nematodes: *Globodera rostochiensis*; *Heterodera avenae*, and *Meloidogyne incognita*. Rhizosphere colonization was recorded on barley roots (% of root length colonized), and chlamydospore formation on corn meal agar (after Kerry, unpublished).

Isolate	Original host	Virulence against			Rhizosphere colonization	Chlamydo-spores
		<i>H. avenae</i>	<i>G. rostochiensis</i>	<i>M. incognita</i>		
8	<i>H. avenae</i> eggs	36	30	53	0	unclear
10	<i>M. incognita</i> eggs	23	29	41	83	good
11	<i>H. schachtii</i> eggs	24	15	25	0	no
26	<i>H. cruciferae</i> soil	21	27	63	100	no
65	<i>H. avenae</i> eggs	31	19	14	100	no

4.2.2 Protein electrophoresis and blotting

All isolates were grown in liquid medium, containing soya peptone and beetle (*Phaedon cochleariae*) homogenate (4 mg ml⁻¹), as described in Chapter 2. Culture filtrates were assayed for VCP1-like activity using suc-(Ala)₂-Pro-Phe-pNA as the substrate, and the enzymes involved characterized by isoelectric focusing (IEF), SDS-PAGE, enzymoblotting and Western blotting by the methods described in Chapters 2 and 3. The antibody unit at IACR-Rothamsted generated polyclonal antibodies against the subtilisin-like proteases from *V. chlamydosporium*, isolate 10

Table 4.2 Fungal isolates used in this study, their host and origin.

Species	Isolate	Number	Host	Origin
<i>Verticillium chlamydosporium</i>	Vc8	1	<i>Heterodera avenae</i> eggs	UK
	Vc10	2	<i>Meloidogyne incognita</i> eggs	UK
	Vc11	3	<i>H. schachtii</i> eggs	UK
	Vc26	4	<i>H. cruciferae</i> infested soil	UK
	Vc65	5	<i>H. avenae</i> eggs	UK
<i>Verticillium lecanii</i>	Vl4	6	Unidentified aphid (Homoptera: Aphididae)	Denmark ^a
	VlG	7	<i>Macrosiphoniella sanborni</i> (Homoptera: Aphididae)	UK ^b
	VlMY	8	<i>Trialeurodes vaporariorum</i> (Homoptera: Aleyrodidae)	UK ^c
<i>Verticillium nigrescens</i>	1880	9	<i>Solanum tuberosum</i>	UK ^d
<i>Verticillium nubilum</i>	162	10	<i>Lycopersicon esculentum</i>	UK ^d
<i>Verticillium tricorpus</i>	44	11	<i>Lycopersicon esculentum</i>	UK ^d
<i>Verticillium albo-atrum</i>	Va luc SW	12	<i>Medicago sativa</i>	UK ^d
	Va1974 (ATCC 96521)	13	<i>Humulus lupulus</i>	UK ^d
<i>Verticillium dahliae</i>	Vd327	14	<i>Fragaria x ananassa</i>	UK ^d
	Vd1764 (ATCC 96522)	15	<i>H. lupulus</i>	UK ^d
<i>Paecilomyces lilacinus</i>		16	<i>M. incognita</i> females	Malaysia ^e
<i>Paecilomyces fumoso-roseus</i>	V80	17	<i>Cydia nigricana</i> (Lepidoptera: Tortricidae)	IACR-Rothamsted
<i>Acremonium</i> sp.		18	<i>Globodera rostochiensis</i> females	UK ^e
<i>Beauveria bassiana</i>	V77	19	Unidentified ladybird (Coleoptera: Coccinellidae)	UK ^f

<i>Metarhizium anisopliae</i>	V86 (ARSEF 252)	20	<i>Leptinotarsa decemlineata</i> (Coleoptera: Chrysomelidae)	USA
	V208	21	Orthoptera: Tettigoniidae	Brazil
	V245	22	Soil	Finland
	ME1	23	pecan weevil	USA ^a
<i>M. anisopliae</i> var. <i>majus</i>	V319	24	<i>Oryctes rhinoceros</i> (Coleoptera)	New Zealand ^b
<i>Metarhizium flavoviride</i>	V260	25	<i>Oedaleus nigeriensis</i> (Orthoptera: Acrididae)	Benin ^c

^a Obtained from Chr. Hansen's BioSystems (currently Koppert), Denmark.

^b Obtained from Dr G. Moritz (Martin-Luther University, Halle, Germany). The original isolate name was Aln3.

^c Obtained from Koppert (the Netherlands) as Mycotal[®].

^d Obtained from Drs J.H. Carder and D.J. Barbara (Horticulture Research International, Wellesbourne).

^e Obtained from Dr D.H. Crump (IACR-Rothamsted, Harpenden).

^f Obtained from Dr J.A. Whiteway (MAFF, Cambridge).

^g Obtained from Dr R.J. St. Leger (Boyce Thompson Institute, Ithaca, USA).

^h Collected by Dr R. Hall (CCRI, Littlehampton).

ⁱ Obtained from IIBC (Silwood Park, Ascot).

(VCP1); *M. anisopliae*, isolate 245 (Pr1); *Paecilomyces fumoso-roseus*, isolate 80; and *Beauveria bassiana*, isolate 77. Prior to injection in rabbits, all antigens were purified to apparent homogeneity by preparative IEF, as described in Section 2.2.3.

4.2.3 Amino acid sequencing of *V. chlamydosporium subtilisins*

Proteins were cut out from IEF gels and electro-eluted in a Biotrap BT1000 elution unit (Schleicher & Schuell) in a buffer containing 0.05 M Tris and 0.025% (w/v) SDS at pH 8.0. Their N-terminal sequence was determined with an Applied Biosystems 477A liquid-pulse sequencer, by Dr J.N. Keen in the Department of Biochemistry and Molecular Biology at the University of Leeds, using the procedure described in Section 3.2.4.

4.2.4 RFLP studies

Mycelium for DNA extraction was grown in liquid shake cultures using a modified Czapek-Dox basal medium, consisting of 0.01 g l⁻¹ FeSO₄·7H₂O, 0.5 g l⁻¹ MgSO₄·7H₂O, 0.05 g l⁻¹ KCl, 2 g l⁻¹ NaNO₃, 3 g l⁻¹ KH₂PO₄, 40 µg l⁻¹ ZnSO₄·7H₂O, 8 µg l⁻¹ CuSO₄·5H₂O, 4 µg l⁻¹ MnSO₄·4H₂O, 4 µg l⁻¹ NaB₄O₇·10H₂O, 2 µg l⁻¹ (NH₄)₆Mo₇O₂₄·4H₂O, 375 µg l⁻¹ vitamin B1, with 15 g l⁻¹ sucrose as the carbon source, and adjusted to pH 5.9 with NaOH (Carder *et al.*, 1987). Mycelium was harvested after 7 days growth at 22°C on an orbital shaker, washed with distilled water, blotted gently to remove excess liquid, lyophilised and stored at -20°C until required. DNA was extracted from lyophilised mycelium, ground in a mortar and pestle together with a small amount of acid-washed sand, using procedures described

by Murray & Thompson (1980), as modified by Manicom *et al.* (1987). Yields of DNA varied between 30 and 100 $\mu\text{g g}^{-1}$ mycelium, as estimated by visual comparison between the ethidium bromide fluorescence of fungal samples and DNA standards on agarose gels.

Fungal DNAs (*ca* 2 μg each) were digested for 16 h at 37°C with 10 units of *EcoRI* following the supplier's (BRL Ltd.) protocol. Digested samples were separated by electrophoresis in agarose (0.7% [w/v]) gels and transferred overnight by alkaline capillary blotting onto nylon membranes (Hybond-N, Amersham) (Sambrook *et al.*, 1989). Membranes were rinsed briefly in 2 x SSC (SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) and baked at 80°C in a vacuum oven for two hours to fix DNA onto the membrane. Membranes were pre-hybridized in pre-hybridization buffer (6 x SSC, 5 mM EDTA and 0.25% skimmed milk powder) at 65°C in an hybridisation oven for two hours.

Two probes were used on Southern blots: (i) pVARNA12, which contains a *ca* 8 kb complete ribosomal RNA gene repeat from *V. albo-atrum* isolate 1974 (Morton *et al.*, 1995); and (ii) 1.2 kb PCR products produced by amplifying genomic DNA from *Metarhizium anisopliae*, isolates 245 and 47, using PCR with primers based on the published sequence of the *Pr1* gene from *M. anisopliae*, isolate ME1 (St. Leger *et al.*, 1992a). Primers were as follows: METPR2, 5' AGG TAG GCA GCC AGA CCG GC3' and METPR5, 5' TGC CAC TAT TGG CCG GCG CG3'. These PCR products, kindly provided by Miss S.C.M. Leal (IACR-Rothamsted), were purified using the Prep-A-Gene DNA purification system (Bio-Rad) and verified on a 0.7% (w/v) agarose gel. Approximately 70 ng of purified PCR product

was labelled by nick-translation (Gibco-BRL) with α - ^{32}P dCTP (3000 Ci mmol⁻¹, Amersham). The labelled DNA was separated from unincorporated nucleotides with a spun-column procedure, on 2 ml Sephadex G50 gel permeation columns (Carder & Barbara, 1991). Labelled, denatured probes were added to fresh pre-hybridization solutions to give at least 10⁶ cpm ml⁻¹. Following hybridization at 65°C (at least overnight), membranes were washed in 2 x SSC and 0.1% (w/v) SDS at the same temperature, gently blotted dry, wrapped in Saran[®] wrap (Dow Chemical Co.), and exposed to Kodak X-OMAT XAR-5 film, between intensifying screens, at -80°C for an appropriate amount of time, depending on the intensity of labelling.

Occasionally, radiolabelled probe was removed from the nylon membranes to allow rehybridization with a different probe. To this end, blots were incubated in 0.4 M NaOH at 45°C for 30 min, drained, and transferred to 0.1 x SSC, 0.1 % (w/v) SDS in 0.2 M Tris-HCl, pH 7.5 at the same temperature for 30 min. Stripped blots were dried under an infrared lamp and stored at 4°C, until hybridized with a new probe.

4.3 RESULTS

4.3.1 Gene expression: quantitative enzyme assay

Significant inter- and intraspecific variation was observed in the protease activity, against suc-(Ala)₂-Pro-Phe-pNA, of a range of nematophagous, entomogenous and phytopathogenic fungi (Table 4.3). Within *V. chlamydosporium*, isolate 10 produced the most (147.2 units), and isolate 11 the least (0.2 units). Isolate 10 produced a larger

biomass than 11 (5.1 g *versus* 1.3 g oven dry weight), but this difference was not commensurate with the respective proteolytic activities. There was no apparent correlation between VCP1-like activity of the five *V. chlamydosporium* isolates (Table 4.3) and their virulence against three test nematodes, or their rhizosphere colonization of barley in an *in vitro* assay (Table 4.1; Kerry, unpublished).

Whereas the three entomogenous *V. lecanii* isolates produced relatively small amounts of VCP1-like activity (3.7 - 9.8 units), all isolates of the plant pathogens *V. albo-atrum* and *V. dahliae* produced no, or virtually no, protease with VCP1-like specificity in this soya peptone / beetle homogenate medium (Table 4.3). *Verticillium nigrescens* and *V. tricorpus* were as *V. albo-atrum* and *V. dahliae*, but a large amount of protease (98.3 units) was present in the culture filtrate of *V. nubilum*.

An undescribed *Acremonium*, possibly a new species, isolated from females of the potato cyst nematode, *Globodera rostochiensis*, by Dr D.H. Crump (IACR-Rothamsted), produced no detectable VCP1-like activity. An isolate of *Paecilomyces lilacinus*, recovered in Malaysia from a female of the root-knot nematode, *Meloidogyne incognita* (Dr. D.H. Crump, IACR-Rothamsted), produced an amount of protease that was twice as large as that of the most productive *V. chlamydosporium* isolate (i.e. 287.3 units). In comparison, the amount of enzyme detected in the culture filtrate of the entomogenous hyphomycete *P. fumoso-roseus*, was moderate (12.6 units). While the VCP1-like activity in *Beauveria bassiana* isolates varied between 7.1 and 40.4 units, some isolates of *Metarhizium* were exceptionally productive, e.g. *M. anisopliae*, isolates ME1 and 245, produced 591.0 and 256.7 units, respectively (Table 4.3).

Table 4.3 Protease activity in culture filtrates, assayed with suc-(Ala)₂-Pro-Phe-pNA at pH 7.9. All values are means of two replicates. One unit (U) of activity is defined as the amount of enzyme releasing 1 μ mol *p*-nitroaniline min⁻¹. Summary of Western blot analysis of culture filtrates, using polyclonal antibodies against purified subtilisins from *V. chlamydosporium*, isolate 10 (VCP1); *M. anisopliae*, isolate 245 (Pr1); *Paecilomyces fumoso-roseus*; and *Beauveria bassiana*, isolate 77. Hybridisation with a protein of ca 32 kDa is indicated as "+", weak hybridisation as "(+)", and lack of hybridisation as "-".

Fungal species and isolate	Biomass (g)	Protease activity (U \pm S.D.)	Antigenicity with antibody against			
			Vc10 ^a (VCP1)	Ma245 ^b (Pr1)	<i>P. fumoso-roseus</i>	<i>B. bassiana</i> 77
<i>V. chlamydosporium</i> 8	1.9	86.2 \pm 8.2	+	+	-	-
<i>V. chlamydosporium</i> 10	5.1	147.2 \pm 0.1	+	+	-	-
<i>V. chlamydosporium</i> 11	1.3	0.2 \pm 0.2	+	-	-	-
<i>V. chlamydosporium</i> 26	1.9	12.5 \pm 1.5	+	-	-	(+)
<i>V. chlamydosporium</i> 65	2.0	7.6 \pm 1.5	(+)	-	-	(+)
<i>V. lecanii</i> 4	2.3	9.8 \pm 0.1	-	-	-	+
<i>V. lecanii</i> G	1.5	6.0 \pm 0.1	(+)	-	-	-
<i>V. lecanii</i> My	1.4	3.7 \pm 0.5	-	-	-	-
<i>V. albo-atrum</i> luc	2.0	0.1 \pm 0.1	-	-	-	(+)
<i>V. albo-atrum</i> 1974	1.8	0	-	-	-	(+)
<i>V. dahliae</i> 327	1.8	1.2 \pm 0.1	-	-	-	(+)
<i>V. dahliae</i> 1764	1.7	0	-	-	-	-
<i>V. nigrescens</i>	0.6	1.3 \pm 0.3	(+)	(+)	-	-
<i>V. nubilum</i>	1.7	98.3 \pm 4.9	-	-	-	-
<i>V. tricorpus</i>	1.9	0.3 \pm 0.1	-	-	-	-
<i>Acremonium</i> sp.	1.0	0	-	-	-	-
<i>Paecilomyces lilacinus</i>	1.7	287.3 \pm 5.2	-	-	(+)	+
<i>P. fumoso-roseus</i>	2.7	12.6 \pm 0.2	-	(+)	+	+
<i>Beauveria bassiana</i> 77	1.7	40.4 \pm 3.9	-	-	+	+
<i>B. bassiana</i> 86	1.4	7.1 \pm 0.6	-	-	(+)	+
<i>M. anisopliae</i> ME1	1.7	591.0 \pm 8.3	+	+	-	+
<i>M. anisopliae</i> 208	2.1	67.5 \pm 6.4	+	+	+	+
<i>M. anisopliae</i> 245	2.0	256.7 \pm 23.2	-	+	(+)	+
<i>M. anisopliae majus</i> 319	2.3	161.8 \pm 10.5	-	+	-	-
<i>M. flavoviride</i> 260	1.9	219.9 \pm 41.4	-	+	-	-
Proteinase K	N.A. ^c	N.D. ^d	-	-	-	-
Porcine elastase	N.A.	N.D.	(+)	-	-	+

^a Vc10: *V. chlamydosporium*, isolate 10

^b Ma245: *M. anisopliae*, isolate 245

^c N.A.: not applicable

^d N.D.: not determined.

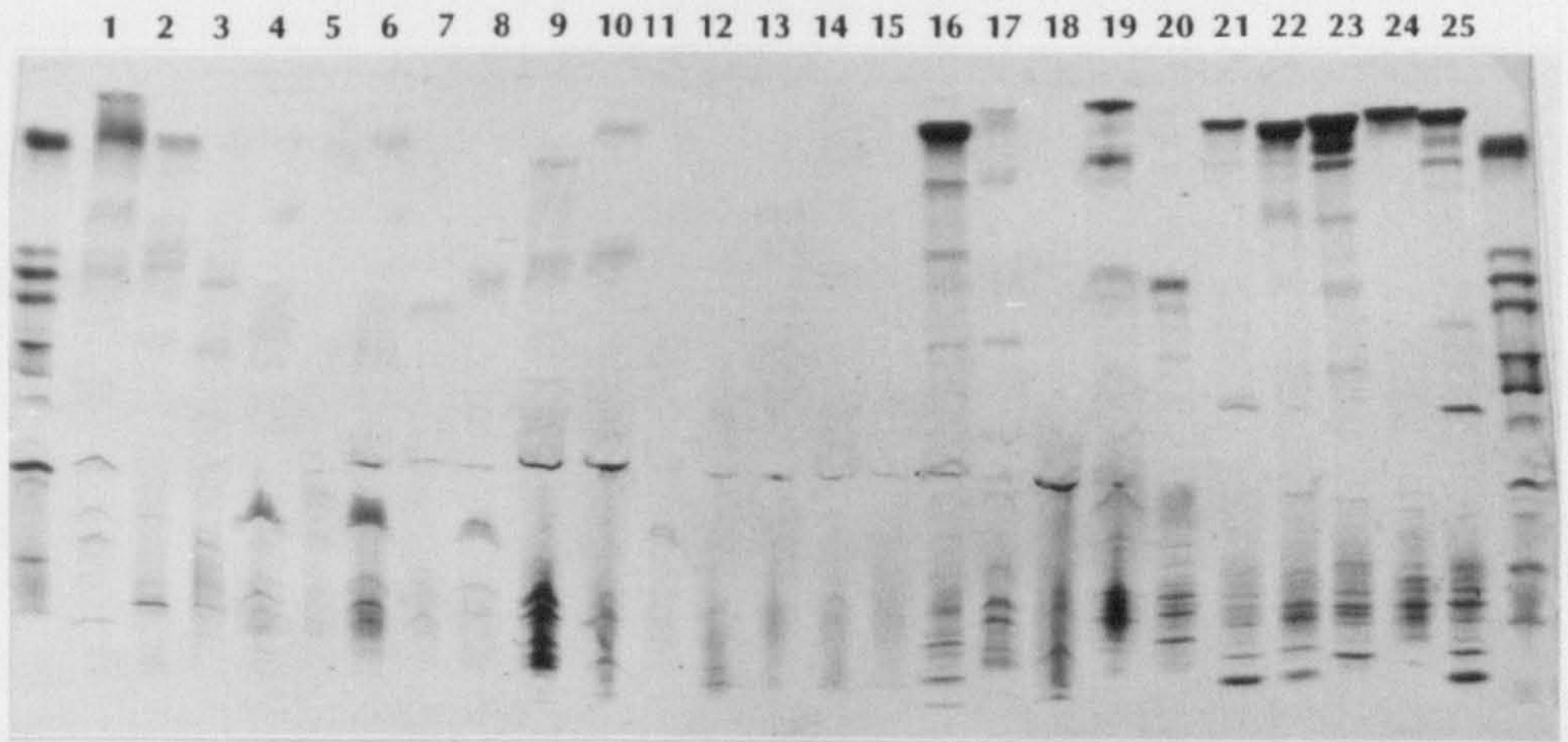
4.3.2 Gene expression: qualitative assessment with protein blotting techniques

The qualitative differences between the VCP1-like proteases of the same series of fungi was assessed by electrophoretic separation of the culture filtrates, using IEF,

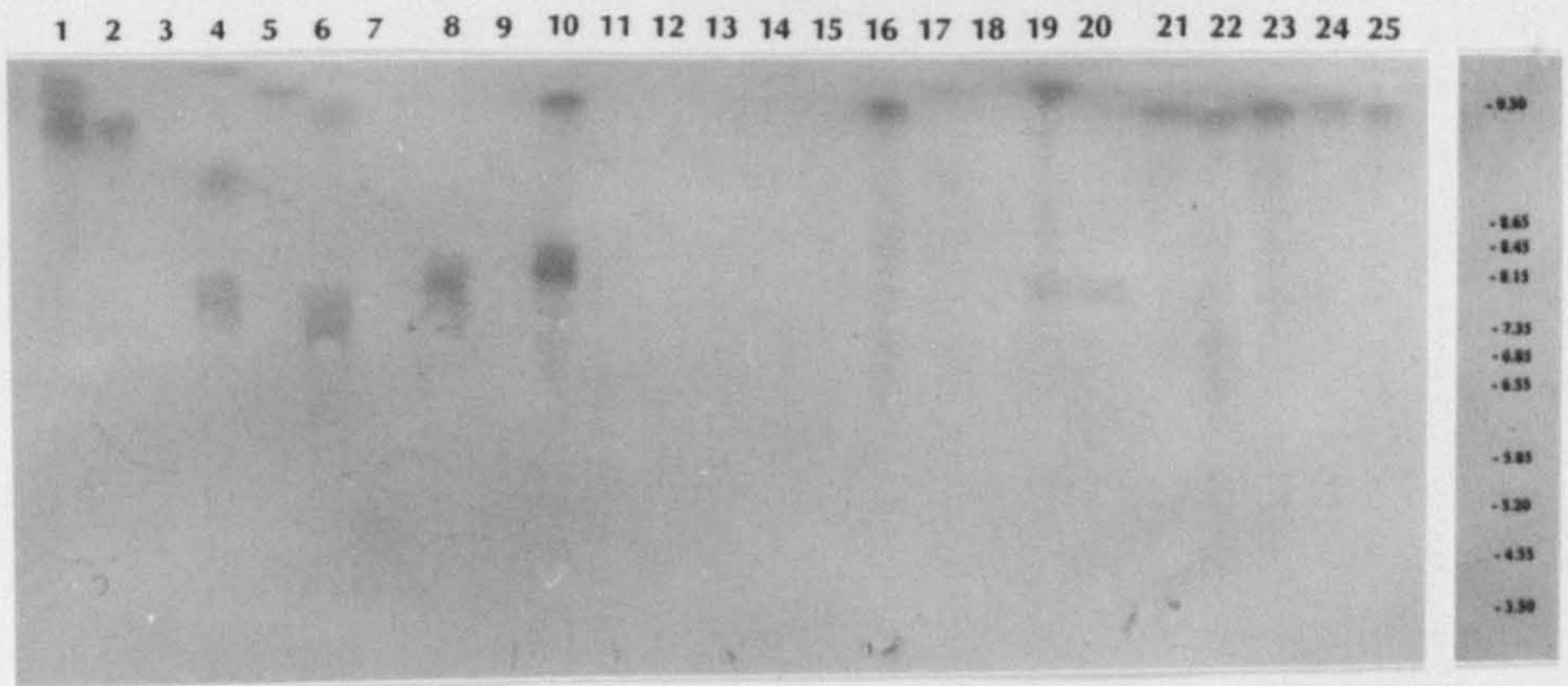
Fig. 4.1 IEF gel (pH 3-10) of the culture filtrates of 25 fungi, (a) stained with Coomassie blue; and (b) enzymoblotted onto nitrocellulose and developed with the substrate suc-(Ala)₇-Pro-Phe-pNA, using the procedure of Ohlsson (1986). (c) Similar enzymoblot, with *V. chlamydosporium* isolates only. Both outer lanes in (a) were marker proteins; corresponding pI values are indicated alongside (b) and (c). Lane numbers in each panel refer to fungal species and isolates, listed in Table 4.2. These were: *V. chlamydosporium*, isolate 8 (lane 1); isolate 10 (lane 2); isolate 11 (lane 3); isolate 26 (lane 4); isolate 65 (lane 5); *V. lecanii*, isolate 4 (lane 6); isolate G (lane 7); isolate My (lane 8); *V. nigrescens* (lane 9); *V. nubilum* (lane 10); *V. tricorpus* (lane 11); *V. albo-atrum*, isolate luc. SW (lane 12); isolate 1974 (lane 13); *V. dahliae*, isolate 327 (lane 14); isolate 1764 (lane 15); *P. lilacinus* (lane 16); *P. fumoso-roseus* (lane 17); *Acremonium* sp. (lane 18); *B. bassiana*, isolate 77 (lane 19); isolate 86 (lane 20); *M. anisopliae*, isolate 208 (lane 21); isolate 245 (lane 22); isolate ME1 (lane 23); *M. anisopliae* var *majus* (lane 24); *M. flavoviride* (lane 25).

92a

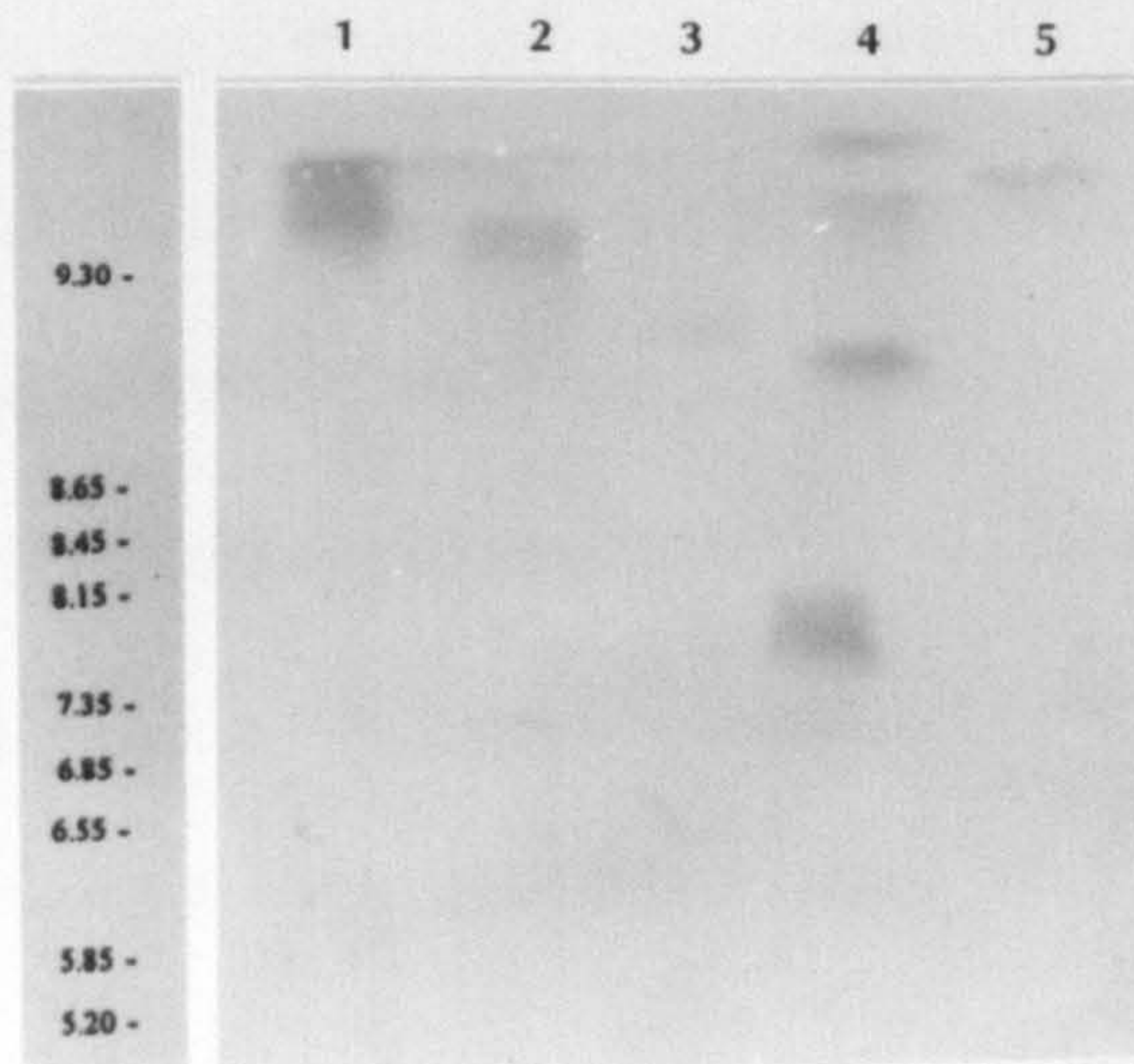
(a)



(b)



(c)



followed by enzymoblotting with suc-(Ala)₂-Pro-Phe-pNA as the substrate (Fig. 4.1). Despite concentrating (Amicon Centriprep) the more dilute samples in order to balance protein loadings on the gel, large differences in the intensity of the alkaline proteins were obtained after staining for total protein with Coomassie blue (Fig. 4.1a). This reflected the quantitative differences in protease production revealed by the enzyme assay in the previous Section.

Corresponding enzymoblots indicated differences in charge between the proteases of the different species, but also between isolates of the same species (Fig. 4.1b-c). A pattern, distinct for each isolate, was seen in *V. chlamydosporium*. Whereas VCP1 from *V. chlamydosporium*, isolate 10, was present as a single band with pI 10.0, the enzyme(s) of isolate 8 appeared as a diffuse zone with pI 10.0-10.4, possibly as a result of multiple bands with similar pI values. Whereas the pI of the enzyme in isolate 65 was in the same range (pI 10.2), that of isolate 11 was lower (pI 9.24). At least four enzyme forms were seen in isolate 26, ranging from very alkaline (pI 10.50, 10.15 and 9.15) to almost neutral (diffuse band with pI 7.80-7.35) (Fig. 4.1b-c). Near-neutral subtilisin isoforms were also seen in isolates of *V. lecanii* and *V. nubilum* (Fig. 4.1b). The plant pathogens *V. albo-atrum* and *V. dahliae* apparently secreted very little protein in the medium, as few bands could be stained with Coomassie (Fig. 4.1a), and no protease was visualized on the enzymoblot (Fig. 4.1b). All *Metarhizium* isolates had dominant alkaline proteases with small, but detectable, differences in charge (Fig. 4.1b), while *B. bassiana*, isolate 77, had the most alkaline subtilisin-like protease of all entomogenous isolates tested (pI ca 10.5).

Additionally, polyclonal antisera were generated against purified subtilisins from four fungal species, viz VCP1 from *V. chlamydosporium*, isolate 10; Pr1 from *M. anisopliae*, isolate 245; and the alkaline subtilisins from *Paecilomyces fumosoroseus*, and *Beauveria bassiana*, isolate 77 (Table 4.3). The hybridization pattern on Western blots of SDS-PAGE gels with culture filtrates of these fungi, indicated that seemingly similar enzymes did not necessarily share the same epitopes. Whereas the VCP1-antiserum hybridized to all *V. chlamydosporium* isolates, the Pr1-antiserum from *M. anisopliae* bound only to isolates 8 and 10 of *V. chlamydosporium* (Table 4.3). The response of the latter antibody could, theoretically, be related to the concentration of antigen, as isolates 8 and 10 produced the largest amounts of protease in *V. chlamydosporium*. However, the antiserum to the protease from *B. bassiana* recognized only *V. chlamydosporium* isolates 26 and 65, which produced approximately one tenth of the amount of isolates 8 and 10 (Table 4.3). As reported in Section 3.3.1, there was reciprocal cross-reactivity between antisera and proteases from *V. chlamydosporium* and *M. anisopliae*. However, extending the range of isolates indicated the isolate-dependence of this similarity, i.e. the VCP1-antiserum did not recognize the subtilisins of all *Metarhizium* isolates, and *vice versa*. It is noteworthy that the antisera to VCP1 and Pr1 were not the same ones used in the previous Chapter, and the two sets reacted slightly differently. According to the data presented in the previous Chapter, the VCP1- and Pr1-antisera hybridized only to elastase and proteinase K, respectively, but the antisera generated for the purpose of the current experiments never bound to either antigen (Table 4.3).

4.3.3 Gene expression: N-terminal amino acid sequencing

Proteins from culture filtrates of five *V. chlamydosporium* isolates were separated on an analytical IEF gel. The bands corresponding to proteins with $pI \geq 9$ were excised, electro-eluted and sequenced. Whereas limited amounts of protein, and machine-related problems, precluded sequencing of proteins from isolate 11, between 9 and 20 N-terminal residues were determined for all proteins of the other isolates (Table 4.4). Surprisingly, in the case of isolates 8 and 10, two distinct sequences could be extracted from what appeared as a single band in IEF, suggesting that different proteins had co-migrated. Every protein, with marginal variation in pI and/or molecular mass (32 - 34 kDa), had a unique sequence (Table 4.4). Proteins were given code names, based on the isolate number, followed by a second digit reflecting the relative pI (protein with highest pI in each isolate was given "1").

Table 4.4 Aligned N-terminal amino acid sequence of alkaline proteins ($pI > 9$) from *V. chlamydosporium* isolates. Within each isolate the proteins are listed according to decreasing pI . The letter "x" indicates an unidentified residue, while lowercase letters signify tentative assignments.

Isolate no.	Protein Code	Sequence	pI	Mol. wt. (kDa)	Presumed function
8	8_1	ALTTQTPSTGGLAVs	9.6	34	unknown
	8_2	AYVEQPGAP	9.4	34	subtilisin
	8_3	AYVEQPGAPWGLARVSA	9.3	33	subtilisin
	8_4	AIVEQPGAPWGLARI	9.3	32	subtilisin
10	10_1	AYVEQPGAPWGLARV	9.35	33	subtilisin
	10_2	AIVEQQGAPxGLgRIiNkxk	9.35	32	subtilisin
	10_3	AIVEQQGAPWGLARISNRQK	9.3	32	subtilisin
	10_4	ALTTQTPsTWGLARV	9.3	32	unknown
26	-	sYTTQQNAVwGLArIs	9.0	32	subtilisin
65	-	GIVEQSGAPWGLgRII	9.45	33	subtilisin

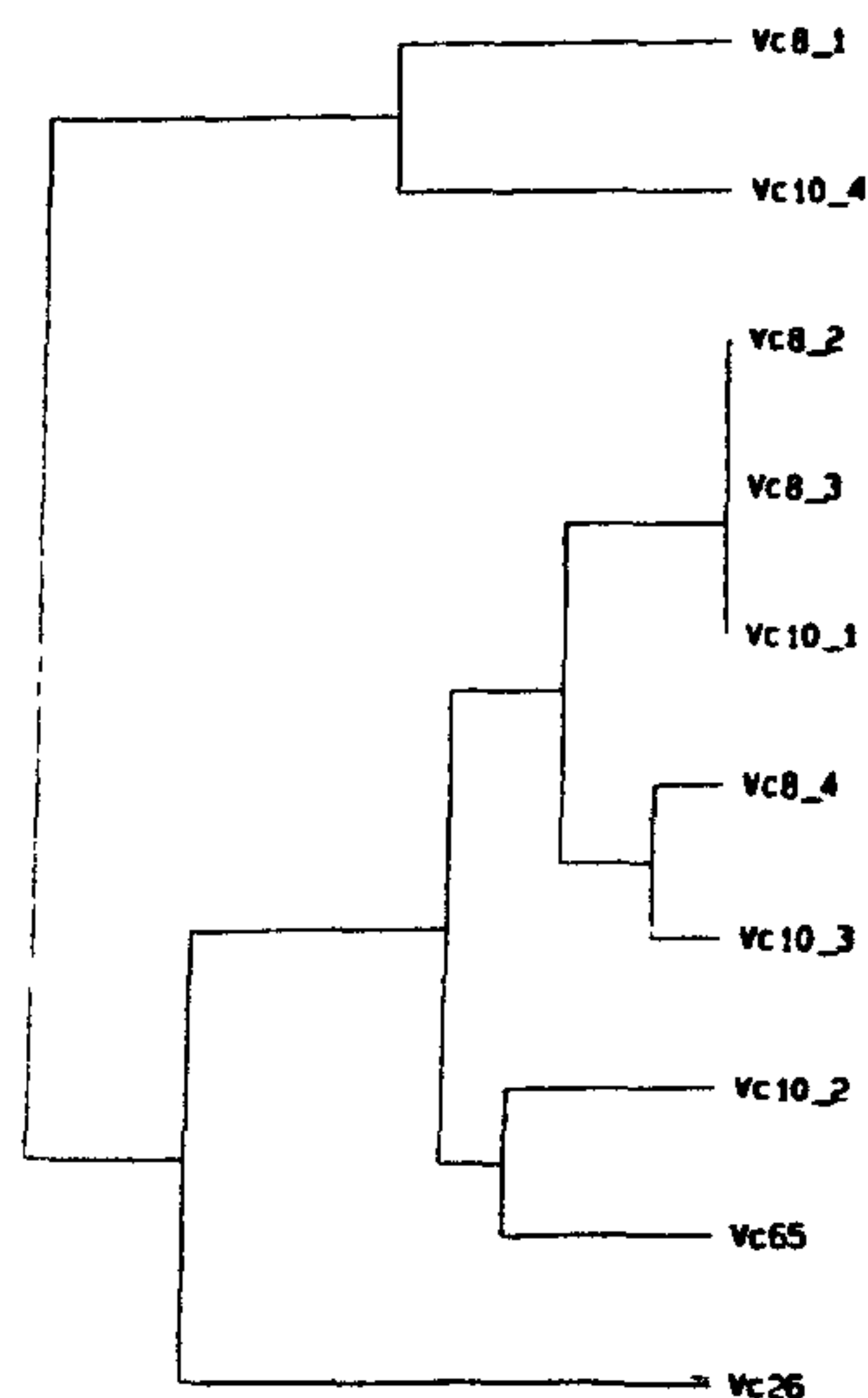


Fig. 4.2 Sequence similarity dendrogram of putative subtilisin-isoforms in isolates of *V. chlamydosporium*, according to the GCG program PILEUP.

The sequences obtained from these isolates appeared to be variants of two basic motifs (Fig. 4.2). Eight out of ten had Q . G / N . A P / V W G L A / G R V / I, in which “.” represents a variable residue, and “/” one of two residues (Table 4.4). This particular motif is typical of fungal subtilisins (Siezen *et al.*, 1991), and databank searches with any of these N-terminal sequences resulted in more or less the same list of homologous enzymes. The other sequence motif was ALTTQTPSTG / WGLA, which occurred in proteins 8_1 and 10_4 (Table 4.4). In this case, the homology was less obvious. The pattern ALTTQ does occur in some subtilisins (e.g. *Trichoderma harzianum*, EMBL/Genbank accession no. Q03420; and *Aspergillus fumigatus*, P28296), but the BLASTP program (Altschul *et al.*, 1990), from the GCG software package, did not consider this homology as significant. Irrespective of the true nature of proteins 8_1 and 10_4, the presence of multiple subtilisin-isoforms in at least two isolates of *V. chlamydosporium*, and sequence variability between all isolates, was established by this sequencing experiment.

4.3.4 Detection of subtilisin-like genes: hybridisation studies

The quality of the DNA isolated from a range of nematophagous, entomogenous and phytopathogenic fungi (Table 4.1), and its digestion by *EcoRI*, appeared to be adequate as seen after agarose electrophoresis and staining with ethidium bromide. (Fig. 4.3). These digests were blotted, and hybridized to a ribosomal RNA gene repeat unit, pVARNA12, that was cloned from *V. albo-atrum*, isolate 1974 (Morton *et al.*, 1995). This isolate was included in the study, and the restriction fragment length polymorphisms (RFLPs) obtained, not only provided comparative information on the fungi in question, but also served as an additional quality control of the blot. Apart from *B. bassiana*, all species had a DNA fragment of *ca* 3.2 kb that hybridized to the ribosomal probe from *V. albo-atrum* (Fig. 4.4). This 3.2 kb band has been seen before in *Verticillium* (Carder & Barbara, 1991; Typas *et al.*, 1992), and probably relates to fairly conserved *EcoRI* sites in the 5.8S and 25S ribosomal subunits (Garber *et al.*, 1988). The pattern seen here in *B. bassiana* could be due to incomplete digestion, but it seems more likely that these isolates did not have an *EcoRI* site in their ribosomal repeat unit. Among the isolates of *V. chlamydosporium*, isolate 26 differed in that it missed the second band of *ca* 4.7 kb. The third band seen in isolate 8 may be due to incomplete digestion of its DNA, the largest band being a fragment equal in size to the two smaller ones combined. The DNA from *V. lecanii*, isolate My, was possibly also not digested to completion, for the same reason (Fig. 4.4). The plant pathogens *V. albo-atrum* and *V. dahliae* had a distinct pattern in common, and shared only one (*V. nigrescens* and *V. nubilum*), or two bands (*V. tricorpus*) with the other plant pathogenic species of the genus. The double banding pattern of *V.*

Fig. 4.3 Genomic DNA, restricted with *Eco*RI and stained with ethidium bromide following separation on a 0.7% agarose gel, from the following fungi: *V. chlamydosporium*, isolate 8 (lane 1); isolate 10 (lane 2); isolate 11 (lane 3); isolate 26 (lane 4); isolate 65 (lane 5); *V. lecanii*, isolate 4 (lane 6); isolate G (lane 7); isolate My (lane 8); *V. albo-atrum*, isolate 1974 (lane 9); *V. dahliae*, isolate 1764 (lane 10); *V. nigrescens* (lane 11); *V. nubilum* (lane 12); *V. tricorpus* (lane 13); *P. lilacinus* (lane 14); *P. fumoso-roseus* (lane 15); *Acremonium* sp. (lane 16); *B. bassiana*, isolate 77 (lane 17); isolate 86 (lane 18); *M. anisopliae*, isolate 208 (lane 19); isolate 245 (lane 20); isolate ME1 (lane 21); *M. anisopliae* var *majus* (lane 22); *M. flavoviride* (lane 23).

Fig. 4.4 Southern blot of the gel seen in Fig. 4.3, hybridized with ³²P-labelled pVARNA12, a full length ribosomal repeat unit from *V. albo-atrum* (Morton et al., 1995). Numbering of lanes as in Fig. 4.3.

98a

Fig. 4.3

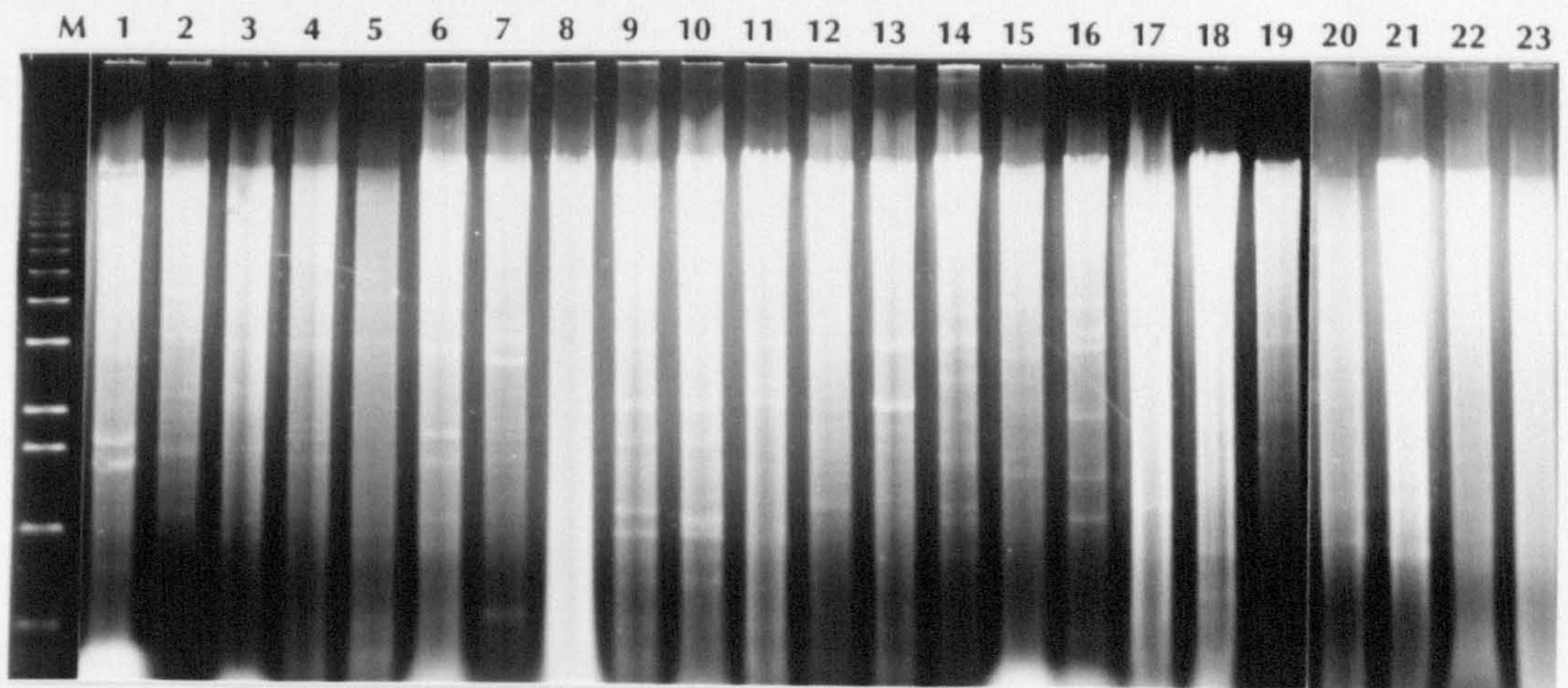


Fig. 4.4



chlamydosporium reoccured in both species of *Paecilomyces* and in most isolates of *Metarhizium* (Fig. 4.4).

Using as a probe a fragment of the *Pr1* gene from *M. anisopliae*, isolates 245 or 47, RFLPs were observed that suggested qualitative differences in the subtilisin genes within and between species. After moderately stringent washes (2 x SSC and 0.1% SDS, at 65°C) following hybridisation with the *Pr1* probe from *M. anisopliae*, isolate 245, a large number of bands appeared in the *EcoRI*-restricted DNA of *V. chlamydosporium* and *V. lecanii* (Fig. 4.5). Apparently, most of these were weak homologies with the probe, as few bands remained after more stringent washing (0.2 x SSC) of this blot (Fig. 4.6). Under these conditions, *V. chlamydosporium* isolates 11 and 65 had the same pattern, with hybridizing fragments of *ca* 6.0 and 1.4 kb. While in isolate 26 a single band was still clearly visible under the stringent washing conditions, very weak hybridisation of DNA from isolates 8 and 10 suggested weaker homology with the *Pr1*-probe; isolate 10 had bands of *ca* 8 and 1.2 kb (Fig. 4.6). Interestingly, none of the phytopathogenic *Verticillium* species had DNA fragments with sufficient homology to the subtilisin, *Pr1*, to withstand higher stringency washing (Fig. 4.6). Positives were obtained for *P. lilacinus* (fragment larger than 12 kb), *B. bassiana*, and all *Metarhizium* isolates. Considerable variation was seen in the banding patterns of *Metarhizium* spp., with the homologous isolate 245 resulting in a single hybridizing fragment of *ca* 3 kb (Fig. 4.6).

When a PCR-generated fragment of *Pr1* from *M. anisopliae*, isolate 47 was radiolabelled and used as a probe, banding patterns were obtained, essentially similar

Fig. 4.5 Southern blot of the gel seen in Fig. 4.3, hybridized with a PCR product generated from genomic DNA from *M. anisopliae*, isolate 245, using primers based on the published sequence (St. Leger et al., 1992a) of Pr1 from *M. anisopliae*, isolate ME1. Lanes as in Figs. 4.3-4.4: *V. chlamydosporium*, isolate 8 (lane 1); isolate 10 (lane 2); isolate 11 (lane 3); isolate 26 (lane 4); isolate 65 (lane 5); *V. lecanii*, isolate 4 (lane 6); isolate G (lane 7); isolate My (lane 8); *V. albo-atrum*, isolate 1974 (lane 9); *V. dahliae*, isolate 1764 (lane 10); *V. nigrescens* (lane 11); *V. nubilum* (lane 12); *V. tricorpus* (lane 13); *P. lilacinus* (lane 14); *P. fumoso-roseus* (lane 15); *Acremonium* sp. (lane 16); *B. bassiana*, isolate 77 (lane 17); isolate 86 (lane 18); *M. anisopliae*, isolate 208 (lane 19); isolate 245 (lane 20); isolate ME1 (lane 21); *M. anisopliae* var *majus* (lane 22); *M. flavoviride* (lane 23).

Fig. 4.6 Southern blot as in Fig. 4.5, but washed at higher stringency (0.2 x SSC).

Fig. 4.7 Southern blot of *EcoRI*-restricted DNA, hybridized with a PCR product generated from genomic DNA of *M. anisopliae*, isolate 47, using primers based on the published sequence (St. Leger et al., 1992a) of Pr1 from *M. anisopliae*, isolate ME1. Lanes as in Fig. 4.5.

100a

Fig. 4.5

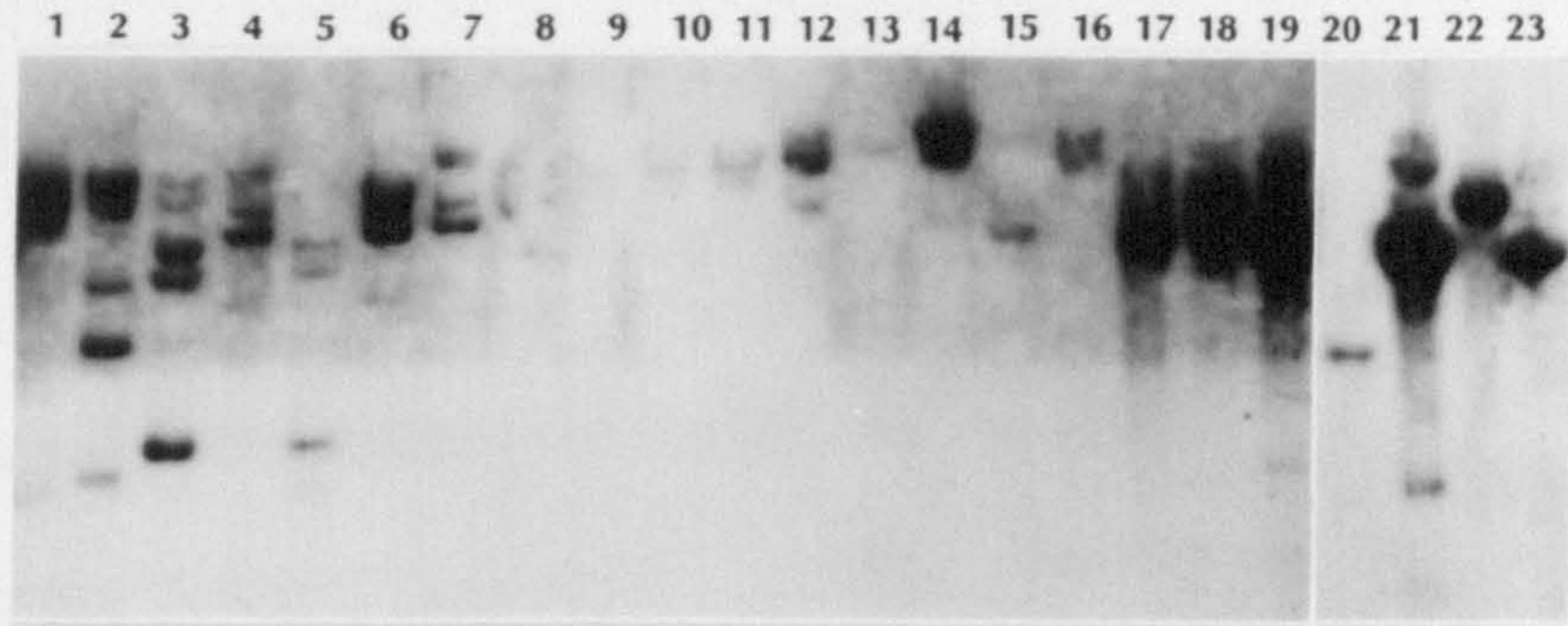


Fig. 4.6

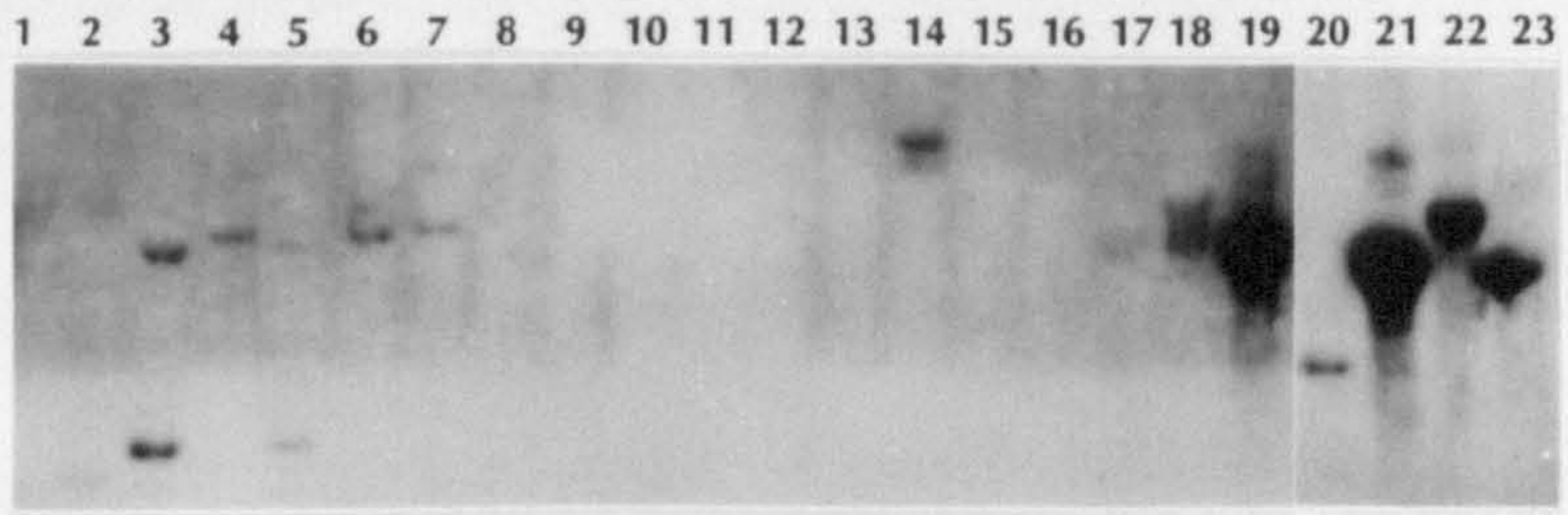


Fig. 4.7



to those with *Pr1* from isolate 245 (compare Figs. 4.7 and 4.5). The similarity in banding complexities with two related probes increased the reliability of the results.

4.4 DISCUSSION

The choice of *V. chlamydosporium*, isolate 10, as the source of the extracellular subtilisin-like protease VCP1, characterized in the previous Chapters, was based on the biological control potential of this particular isolate, and a growing body of available ecological data. In terms of total activity, isolate 10 had the highest yield of VCP1-like activity, in a soya peptone based medium, among the five *V. chlamydosporium* isolates tested. On the other hand, isolates 11 and 65 of *V. chlamydosporium* produced very little VCP1. However, genes, or gene fragments, with homology to the subtilisin *Pr1* from *M. anisopliae* were detected by Southern hybridisation, indicating that a similar gene, or genes, must be present in these isolates. These data can only be reconciled by assuming that (a) a poorly functioning gene product was secreted in isolates 11 and 65, (b) the enzyme had a different substrate specificity, that was not detected with the substrate suc-(Ala)₂-Pro-Phe-pNA, or that (c) regulatory controls limited its secretion in the medium in which these fungi were grown. Four polyclonal antibodies against fungal subtilisins also reacted differently within the set of *V. chlamydosporium* isolates, indicating that epitopes were not as conserved among the enzymes of different isolates as might have been expected.

The immunological data suggested intraspecific structural differences between proteases, which are likely to be determined at the level of their primary structure. In this respect, results from Southern analysis provided corroborative data. Using two different subtilisin-derived DNA probes, three different groups of *V. chlamydosporium* isolates could be discerned. Firstly, isolates 8 and 10 had a similar pattern that, above all, was characterized by the faintness of the signal, suggesting weak homology with the subtilisin gene from the two *M. anisopliae* isolates that had provided the probe. Secondly, a two-band pattern identical in isolates 11 and 65, and thirdly, a single band in isolate 26, distinct from all the others. A certain correlation existed between this grouping, based on *EcoRI*-generated DNA fragments, and the protease activity of these fungi, isolates 8 and 10 producing the most, or having the most active enzyme forms, isolates 11 and 65 the least, and 26 being intermediate. Most subtilisins share conserved regions, e.g. around the catalytic triad, but the connections between these segments can differ considerably, both in length and in sequence. These variable regions almost always are located on the external surface of the protein (Siezen *et al.*, 1991) and can confer different properties, such as hydrophilicity (Coleman & Whitby, 1993) and ionic interactions (Betzl *et al.*, 1992). There are reports of intraspecific structural differences between proteases, e.g. isolate-specific proteolytic activities have been described in *Candida albicans* (Rüchel *et al.*, 1982) and *Tritirachium album* (Samal *et al.*, 1990). There is also apparent variability between the *Pr1*-like genes from various *M. anisopliae* isolates. Digestion of genomic DNA with appropriate restriction enzymes and hybridisation to *Pr1*-cDNA revealed a single band, suggesting the presence of only one form of the

gene. However, European and Australian isolates had different sized fragments to those of American isolates (including ME1) (St. Leger *et al.*, 1992a). Further kinetic and sequence data on the subtilisins of the different isolates clearly are required to elaborate on the structure-function relationship in the VCP1-like enzymes. Such studies can lead to a profound understanding of enzyme properties, and are achievable, as illustrated by the comparative study of the alkaline proteases from *Aspergillus fumigatus* and *A. oryzae*. Their proteases are homologous (17% amino acid substitutions) (Jaton-Ogay *et al.*, 1992), and differences in pI, optimum pH for collagen and elastin digestion, and specific activity for both substrates have been mapped to these substitutions (Monod *et al.*, 1991). Another example is *Dichelobacter nodosus*, the causative bacterium of ovine footrot, virulent and benign isolates of which are immunologically distinguishable by their proteases, which apparently only differ in a single amino-acid (Riffkin *et al.*, 1995).

It has to be borne in mind that a variable number of bands on Southern blots from different isolates need not necessarily indicate the presence or absence of restriction sites in a single gene. The presence of more than one gene, homologous with the probe, could equally result in multiple bands. Moreover, the comparison of hybridisation data, published for *M. anisopliae* (St. Leger *et al.*, 1992a) and *B. bassiana* (Joshi *et al.*, 1995) and Southern blots from the current study provides additional evidence for the presence of multiple forms of subtilisin-like genes. In the first report (St. Leger *et al.*, 1992a), a *Metarhizium* Pr1 cDNA plasmid insert hybridized to a single *EcoRI* fragment of ca 6 kb in *M. anisopliae*, isolate ME1, and to three fragments smaller than 2 kb in *V. lecanii*, isolate 313. The *Beauveria* cDNA

probe, derived from isolate ARSEF252, on the other hand, hybridized to a single *EcoRI* fragment of 3.5 kb in ME1 and a single fragment of *ca* 1.4 kb in *V. lecanii*, isolate 313 (Joshi *et al.*, 1995). The amino acid sequences of the two cDNA translation products are 54% identical, and the nucleotide sequences 58%. It is impossible to predict what sequence similarity is needed to permit hybridisation at the relatively high stringencies used in the two experiments, but most of the base similarity is in short stretches of less than 10 bp, which will reduce rather than increase the likelihood of hybridisation. Joshi *et al.* (1995) did not compare the two sets of data, but these reports seem to suggest that the two cDNA probes are each hybridising to different fragments in both *M. anisopliae*, isolate ME1, and *V. lecanii*, isolate 313. In the current study, it was found that *EcoRI*-cut DNA from *B. bassiana*, isolate 86 (which is identical to ARSEF252, used by Joshi *et al.*, 1995), fragment size 6-9 kb hybridized to *Pr1* probes, derived from *M. anisopliae*. This fragment size was clearly different from the reported single band of 4.5 kb with the *B. bassiana* *Pr1* cDNA (Joshi *et al.*, 1995). In conclusion, the combination of data from two literature reports and Southern blots from this Chapter, suggest the presence of at least two forms of subtilisin-like genes in both *M. anisopliae*, *V. lecanii* and *B. bassiana*. Whereas the presence of multiple protease genes in the genome of *V. lecanii* has been acknowledged, based on Southern analysis (St. Leger *et al.*, 1992a), it is the recent discovery of isoforms that has suggested the presence of at least two distinct genes in *M. anisopliae* (St. Leger *et al.*, 1994). A second subtilisin-like gene has recently been cloned from *M. anisopliae* (R.J. St. Leger, Ithaca, personal communication), confirming this analysis.

When analytical IEF gels were blotted onto nitrocellulose, following the electrophoretic separation of the extracellular proteins of five *V. chlamydosporium* isolates, and incubated with the substrate suc-(Ala)₂-Pro-Phe-pNA, between one and four enzymes with different pI values became visible. To find out which enzymes were responsible for these activities, alkaline proteins were rescued from analytical IEF gels and the N-terminal regions were sequenced. Surprisingly, a range of sequences with subtilisin homology were found, each isolate being characterized by a unique set. One protein, with little homology to subtilisins, had a variant in isolates 8 and 10. Based on the current data, it cannot be concluded that these were subtilisin-isoforms. However, four distinct, but related, isoforms were seen in isolate 10, suggesting four related genes. Isolate 8 was characterized by two different subtilisin-like sequences, a third protein having an identical N-terminal sequence, but a slightly greater pI and molecular weight. At least two forms of the gene may be present in this isolate. St. Leger *et al.* (1994) only studied one isolate of *M. anisopliae*, and found three alkaline isoforms of Pr1, with an identical N-terminal sequence GITEQSGAPW, and one less alkaline isoform, with the sequence DLTTQESAPWGLAAI. Therefore, the Pr1-like enzymes in *M. anisopliae*, isolate ME1, are probably coded for by two distinct genes (St. Leger *et al.*, 1994). The recent isolation of a Pr1-like gene, encoding a protein different from the reported isoforms (R.J. St. Leger, Ithaca, personal communication), brings this number to three.

The subtilisins, of at least isolate 10, of *V. chlamydosporium* were remarkably polymorphic. The potential physiological implications of this enzyme complexity are considerable. Facultative pathogens and saprotrophs, exposed to diverse substrates

and environments, usually express much greater genetic variation than obligate parasites and highly specialized pathogens, which have uniform substrate and environment (adaptive polymorphism; Micales *et al.*, 1992; Wildman, 1995). The apparent plethora of subtilisins in *V. chlamydosporium* could enhance the saprotrophic abilities of the fungus, but also virulence and/or host specificity. Although additional characteristics of the isoforms await further investigation, there could be differences in substrate utilisation and regulation of enzymes from different isolates, while the presence of multiple isoforms in a single isolate potentially provides the fungus with the tools to hydrolyse additional substrates.

Although the main focus of this study was on *V. chlamydosporium*, other species were included for comparison, and to study the distribution of VCP1-like activity. St. Leger *et al.* (1987b) studied the alkaline proteases from *V. lecanii*, *B. bassiana* and *M. anisopliae*, and found their inhibitor sensitivity and substrate specificity very similar, apart from the secondary subsite specificities. The same authors showed that a polyclonal antiserum against Pr1 from *M. anisopliae* cross-reacted only with enzymes from two *M. anisopliae* isolates; the proteases from two other isolates, and those from *V. lecanii* and *B. bassiana* did not cross-react in Ouchterlony diffusion tests. However, more sensitive ELISA assays do suggest the presence of common antigens among the basic proteases from *B. bassiana*, *P. fumoso-roseus*, and *M. anisopliae* (Shimizu *et al.*, 1993), a conclusion which was confirmed by the Western blot analysis of the current study.

There is little information on protease production by *Verticillium* species other than *V. chlamydosporium* and *V. lecanii*. *Verticillium suchlasporium*, closely

related to *V. chlamydosporium* (Gams, 1988; Carder *et al.*, 1993), secretes a 32 kDa serine protease in semi-liquid gelatin medium (Lopez-Llorca, 1990). Although no sequence data exist for this enzyme, considerable similarity with VCP1 is anticipated based on the Western blot analysis in Section 3.3.1 of this study. *Verticillium fungicola*, not included in the current study, is a pathogen of the cultivated mushroom *Agaricus bisporus*, and also secretes an alkaline serine protease, of unidentified nature (Kalberer, 1984).

The genus *Verticillium* contains a number of plant pathogens, including *V. albo-atrum*, *V. dahliae*, *V. nigrescens*, *V. nubilum* and *V. tricorpus* (Domsch *et al.*, 1980). This is an interesting series of fungi, because it represents a gradient in pathogenicity and saprotrophic ability (Table 4.5).

Table 4.5 Pathogenicity and ecological characteristics of five phytopathogenic *Verticillium* species used in this study. Data were compiled from Isaac (1953, 1967), Garrett (1956) and Domsch *et al.* (1980).

Species	Virulence	Ecological category
<i>V. albo-atrum</i>	High	Root inhabitator ^a
<i>V. dahliae</i>	High	Root inhabitator
<i>V. tricorpus</i>	Between <i>V.dahliae</i> and <i>V.nubilum</i>	Intermediate
<i>V. nigrescens</i>	Between <i>V.dahliae</i> and <i>V.nubilum</i>	Soil inhabitator ^b
<i>V. nubilum</i>	Weak	Soil inhabitator

(^a) Root inhabitators have an "expanding parasitic phase on the living host tissue and a declining saprophytic phase after its death", while (^b) Soil inhabitators can "survive indefinitely as soil saprophytes and parasitism is incidental to their saprophytic existence in soil" (Garrett, 1956).

While virtually no VCP1-like activity was recorded in *V. albo-atrum*, *V. dahliae*, *V. nigrescens* and *V. tricorpus*, a relatively large amount was produced by *V. nubilum*. There was no immunological cross-reactivity of the *V. nubilum* protease with antisera

against subtilisins from four source fungi, nor did its *EcoRI*-restricted DNA hybridize with probes derived from *Pr1* of two *M. anisopliae* isolates, suggesting limited homology with the *V. nubilum* enzyme, and its coding gene, respectively. Interestingly, *V. nubilum* is probably the species with the highest degree of saprotrophy, being a limited pathogen and surviving well in soil. These data suggest that VCP1 is redundant in the phytopathogenic habit, but contributes to the saprotrophic mode of *Verticillium* species.

Globally, proteases of plant pathogenic fungi have received much less attention than other hydrolases, e.g. cutinases and endopolygalacturonidases. Although proteases have been detected during infection of plant tissue by several types of fungi (Wijesundera *et al.*, 1989; Rauscher *et al.*, 1995), in few cases is there significant evidence for a primary role in infection (Ball *et al.*, 1991; Movahedi *et al.*, 1991). In a taxonomic study of the genus *Verticillium*, plant pathogenic isolates (*V. albo-atrum* only) could be separated from seventy other *Verticillium* isolates, partly because of their lack of protease activity (Jun *et al.*, 1991). However, there are anecdotal records of proteolytic activity by a serine-type enzyme in *V. albo-atrum* and *V. dahliae* (Mussell & Strouse, 1971; Lambert & Pujarnisclé, 1984). An isolate of *V. dahliae*, grown on skimmed milk medium, produces a trypsin-like protease, molecular mass *ca* 30 kDa (K. Dobinson, London, Ontario, Canada, personal communication).

An unidentified *Acremonium* sp., used in this study, did not produce VCP1-like activity, which is remarkable for several reasons. Firstly, one of the enzymes most homologous with VCP1 is from a species of the same family, *A. chrysogenum*

(see Section 3.3.3). Secondly, the taxonomically revised genus *Acremonium* Link ex Fr. currently includes most former *Cephalosporium* Corda species. Remaining species from *Cephalosporium* were moved to *Verticillium* Nees ex Link section *Prostrata*. Furthermore, *Paecilomyces* Bain. “is very similar to *Acremonium*” (Domsch *et al.*, 1980), suggesting close relationships with some positive protease-producers included in this study. Thirdly, the current *Acremonium* sp. was isolated from *G. rostochiensis*, and appears to have some biological control potential for this nematode (D.H. Crump, IACR-Rothamsted, personal communication). Similarly, *A. strictum* (Nigh *et al.*, 1980) and *A. sordidulum* (Schuster & Sikora, 1992) infect cyst nematode eggs. Unfortunately, the mode of action of the *Acremonium* spp. that are pathogens of cyst nematodes has not been established, nor have their proteolytic activities, if any, been studied. In view of the proposed role of VCP1 produced by *V. chlamydosporium* in the infection process of nematode eggs (see Chapter 5), such data are eagerly awaited.

5.1 INTRODUCTION

The nematophagous fungus *Verticillium chlamydosporium* is one of the main causes of the natural decline of the cereal cyst nematode, *Heterodera avenae* Wollenw., in monocultures of susceptible crops (Kerry *et al.*, 1982). One particular isolate of *V. chlamydosporium*, number 10, is considered a potential agent for the biological control of root-knot nematodes (de Leij & Kerry, 1991; de Leij *et al.*, 1993b). The infection process of nematode eggs and females by this fungus has been outlined in Section 1.4.3.

Verticillium chlamydosporium, isolate 10, however, is a poor parasite of *Globodera* species (Kerry & Crump, unpublished), and there are few reports of isolates infecting the economically important potato cyst nematodes *Globodera pallida* (Stone) Behr. and *G. rostochiensis* (Wollenw.) Behr. (Roessner, 1987; Hiemer & Sikora, 1988; Crump, 1989; Crump & Irving, 1992). Furthermore, few other fungi show promise as biological control agents against eggs and/or females of these particular nematodes (Willcox & Tribe, 1974; Clovis & Nolan, 1983; Morgan-Jones & Rodríguez-Kábana, 1986; Roessner, 1987). The underlying mechanisms for this apparent resistance of potato cyst nematode eggs to fungal infection are not understood. It has been proposed that the size and structure of the vulval aperture in the cyst wall in *Globodera* spp. would make them less susceptible, as many fungi probably use the natural orifices for penetration (Tribe, 1980). It is also possible that no compatible fungal biotypes are yet present in areas where *Globodera* is not native

(Morgan-Jones & Rodríguez-Kábana, 1988). Still, the question remains as to why *V. chlamydosporium*, isolate 10, is inefficient at infecting *Globodera* spp.

Verticillium suchlasporium Gams & Dackman, a member of the *V. chlamydosporium/V. suchlasporium* species complex (Carder *et al.*, 1993), produces appressoria and a VCP1-like protease (see Chapter 3) when infecting eggs of the beet cyst nematode *Heterodera schachtii* Schmidt (Lopez-Llorca & Claugher, 1990). Appressoria are also produced by other fungi parasitic on eggs of *Meloidogyne* spp. (Stirling & Mankau, 1979; Dunn *et al.*, 1982). Little, however, is known about the growth of *V. chlamydosporium*, isolate 10, on the surfaces of eggs of *Meloidogyne incognita* (Kof. & White) Chitw. and *G. rostochiensis*. The mycelium/egg interface is relevant, as *V. chlamydosporium* infects nematode eggs by hyphal penetration (Morgan-Jones *et al.*, 1983; Lýsek & Krajčí, 1987; Lopez-Llorca & Duncan, 1991; Lopez-Llorca & Robertson, 1992b). In saprotrophic mode, *V. chlamydosporium*, isolate 10, produced a subtilisin, VCP1. Its relative abundance and broad substrate utilization (see Chapter 2) makes this protease a candidate-enzyme assisting fungal penetration by hydrolysing the proteinaceous nematode egg shell, i.e. during pathogenic mode.

Elucidation of the underlying mechanisms for specificity could help identify the attributes of successful pathogens and determine the barriers to infection. Ultimately this knowledge could help in the selection of compatible, virulent strains for use in biological control programmes. The aim of the experiments described in this Chapter was to determine, using microscopy techniques and bioassays, whether the subtilisin, VCP1, could be involved in the infection of *M. incognita* and *G.*

rostochiensis eggs by *V. chlamydosporium*, isolate 10, and to investigate whether VCP1 was implicated in the relative host specificity of the fungus.

5.2 MATERIALS & METHODS

5.2.1 Nematodes and growth conditions

The potato cyst nematode, *Globodera rostochiensis*, pathotype Ro1, was cultured on *Solanum tuberosum* L. cv. Désirée, while the root-knot nematode, *Meloidogyne incognita*, was grown on *Solanum melongena* L. cv. Black Bell, both in the glasshouse. Mature *G. rostochiensis* cysts were extracted from the soil after the plants had died, using a fluidising column (Trudgill *et al.*, 1972). Subsequently, the cysts were crushed with forceps to release their egg contents. All *G. rostochiensis* eggs contained dormant, fully developed second stage juveniles. Eggs were surface-sterilized in 0.1% (w/v) HgCl₂ (Sijmons *et al.*, 1991). Eggs of *M. incognita*, which contained embryos and juveniles in various stages of development, were collected by brief but vigorous washing of infected roots with 20% (v/v) domestic bleach (4% [w/v] available chlorine) to release eggs from the egg masses on the root surface. They were immediately rinsed on a 30 µm aperture sieve, separated from plant and soil debris by sugar centrifugation (47% [w/v] sucrose, 600 g for 30 sec), rinsed, and sterilized as above.

5.2.2 Proteolysis of egg shell proteins *in situ*

Surface-sterilised eggs of *M. incognita* and *G. rostochiensis* (ca 1,200) were incubated with 20 $\mu\text{g ml}^{-1}$ of the following proteases: VCP1, purified from *V. chlamydosporium*, isolate 10; Pr1 from *M. anisopliae*, isolate 245; or proteinase K (Sigma). Each enzyme, denatured by boiling for 10 min, served as a control, and all eggs were incubated at 23°C for 48 h. Eggs were pelleted in an Eppendorf micro-centrifuge at 12,000 g for 1 min and solubilised protein was determined, using bovine serum albumin as a standard (Bradford, 1976). There were four replicates per assay.

In order to visualize proteolysis, *M. incognita* eggs, similarly treated with VCP1, were stained for β -glucans with Calcofluor white M2R (Butt *et al.*, 1989) after 18 hours 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.

5.2.3 Bioassays

Water agar (WA, 1% [w/v], Fisons, UK) plates were inoculated with *V. chlamydosporium* using a suspension of 9×10^5 conidia and 4×10^4 chlamydospores in 0.2 ml sterile distilled water per plate. In all experiments, the fungus was allowed to grow for 6 days before nematode eggs were added; all assays were undertaken at 20°C. In preliminary experiments, fluorescence microscopy verified that exposing eggs of *G. rostochiensis* and *M. incognita* to bleach did not remove their vitelline layer, or increase the susceptibility of this layer to proteolysis by VCP1 (data not

shown). *Meloidogyne incognita* or *G. rostochiensis* eggs (ca 1,000) were added to the fungal cultures or to WA without the fungus (control).

To investigate the role of VCP1 in fungal pathogenesis, similar numbers of eggs were pretreated with $20 \mu\text{g ml}^{-1}$ purified enzyme at 23°C for 24 h. To remove the enzyme, the eggs were washed three times with intervening centrifugation using an Eppendorf microcentrifuge at 2,000 g for 2 min. In order to denature proteins and/or make the egg shell more permeable, eggs were also boiled for 3 min, before adding them to the fungus. A final treatment consisted of eggs, killed using 0.1% (w/v) sodium azide. This treatment arrests metabolic processes without gross destruction of surface proteins. The azide was removed by washing as before. All treatments consisted of two replicate plates. The washing and pelleting procedures did not influence host susceptibility, as demonstrated by preliminary bioassays.

Infection and fungal growth on the egg surface were recorded after 6 and 14 days. A 1 cm^2 agar block of each treatment was cut out aseptically, stained with Calcofluor white M2R (Sigma) and examined with an Olympus BH-2 microscope as described in the previous Section. Fifty eggs per replicate, i.e. 100 per treatment, were examined at x500 and x1250 magnification, using a combination of bright field illumination and fluorescence. An egg was considered to be infected when either (a) penetrating hyphae were visible, (b) when there was abundant hyphal growth inside, or (c) when hyphae emerged from the egg. Fungal growth on the egg surface was quantified, and the number and types of appressoria recorded. Infection frequencies, were analysed using Fisher's exact tests, while mean surface growth per egg was

compared between treated and untreated eggs using Wilcoxon rank sum tests (SAS Institute, 1985).

5.2.4 Indirect immunofluorescence

Protease production by the fungus during the early stages of infection was demonstrated by indirect immunofluorescence. *Verticillium chlamydosporium*, isolate 10, was grown on WA and surface-sterilized *M. incognita* eggs were added as described for the bioassays. Six days after adding eggs to the fungus, eggs were collected from these plates and fixed in 2% (v/v) formaldehyde for 1 h at 23°C. After washing with PBS, eggs were incubated with 2% (v/v) anti-VCP1 serum (see Chapter 3) for 2 h. After removing excess primary antibody, a goat anti-rabbit antibody, conjugated with tetramethyl rhodamine isothiocyanate (TRITC, Sigma), was applied at 2.5% (v/v). Samples were examined with a fluorescence microscope as described before, using a filter set appropriate for TRITC.

5.2.5 Low Temperature Scanning Electron Microscopy (LTSEM)

Samples from each treatment were examined by LTSEM to visualise the fine structure of the pathogen, eggs and host-pathogen interactions. Specimens were mounted on copper stubs, plunged in liquid nitrogen, and sputter-coated with gold in an EMscope SP2000 Sputter-Cryo Cryogenic-Preparation System using the procedures described by Beckett & Read (1986) and examined in the partially freeze-dried state, with a Philips 501B scanning electron microscope.

5.3 RESULTS

5.3.1 Proteolysis of egg shell proteins *in situ*

Intact eggs of the root-knot nematode, *M. incognita*, and the potato cyst nematode, *G. rostochiensis*, were incubated with homogeneous VCP1. Protein assays indicated that VCP1 released significantly more peptides from eggs of *M. incognita* ($38.8 \mu\text{g ml}^{-1}$) than from those of *G. rostochiensis* ($13.7 \mu\text{g ml}^{-1}$, S.E.M. = $6.5 \mu\text{g ml}^{-1}$) (Fig. 5.1). However, the homologous proteases Pr1 and proteinase K were significantly less effective on *M. incognita* eggs ($26.6 \mu\text{g ml}^{-1}$ and $21.1 \mu\text{g ml}^{-1}$, respectively), but slightly more effective on those of *G. rostochiensis* ($20.0 \mu\text{g ml}^{-1}$ and $20.3 \mu\text{g ml}^{-1}$, respectively), compared with VCP1.

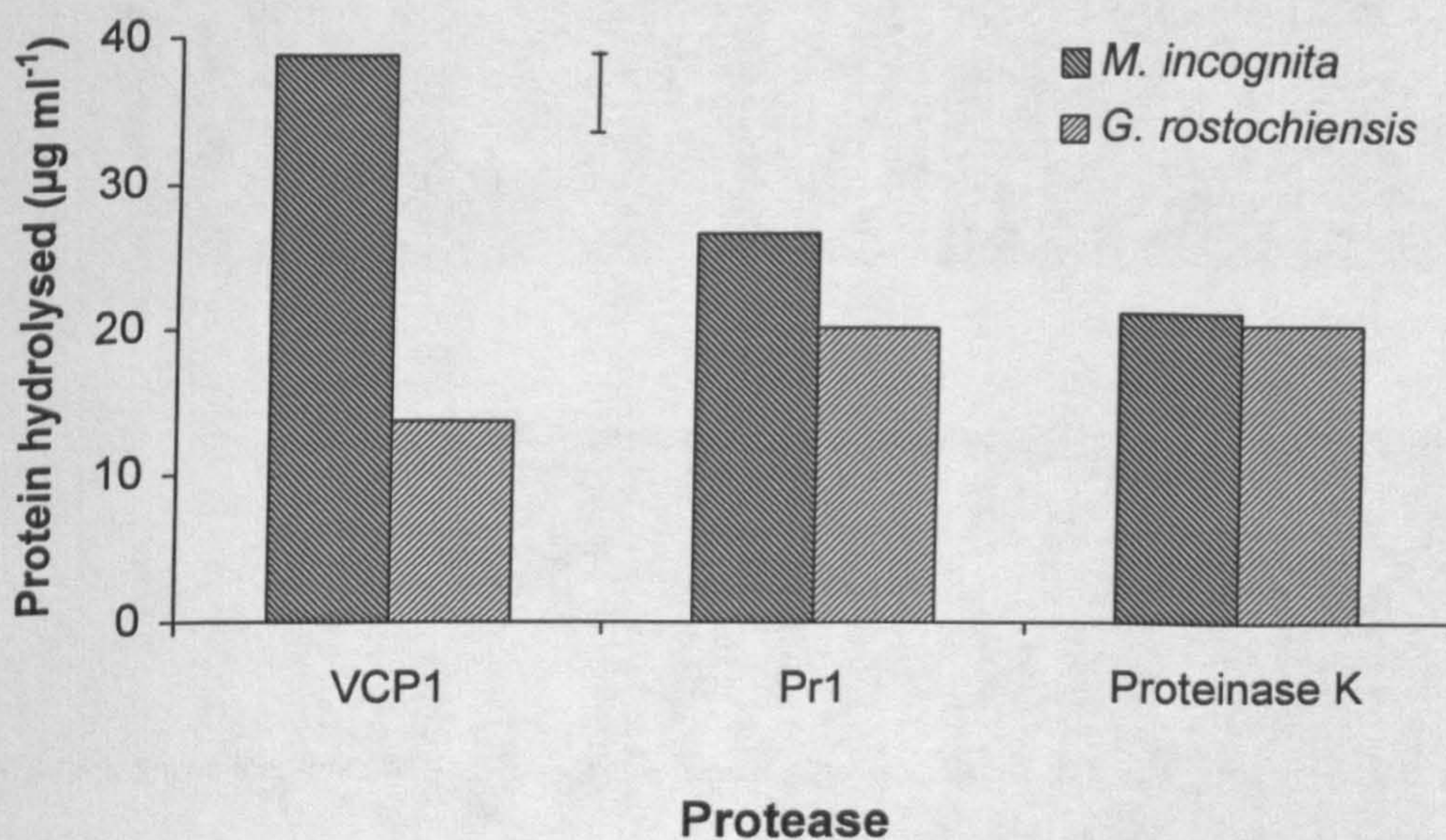


Fig. 5.1 Amount of protein hydrolysed from intact *M. incognita* and *G. rostochiensis* eggs by $20 \mu\text{g ml}^{-1}$ of homogeneous VCP1 from *V. chlamydosporium*, isolate 10; Pr1 from *M. anisopliae*; or proteinase K. Hydrolysis products were quantified using a protein assay according to Bradford (1976), using BSA as a standard. Means of three replicates, corrected for background protein. Error bar represents S.E.D. = $6.5 \mu\text{g ml}^{-1}$.

Fig. 5.2 Photomicrographs of *M. incognita* eggs, stained with Calcofluor white M2R, following treatment with 0.2 units of VCP1 for 18 hours, 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). Bar, 20 μm . Small arrow points at immature egg, large arrow at mature egg.

Fig. 5.3 Photomicrograph of *G. rostochiensis* eggs, stained with Calcofluor white M2R, following similar treatment with VCP1, recorded in violet light. Bar, 20 μm .

117a

Fig. 5.2

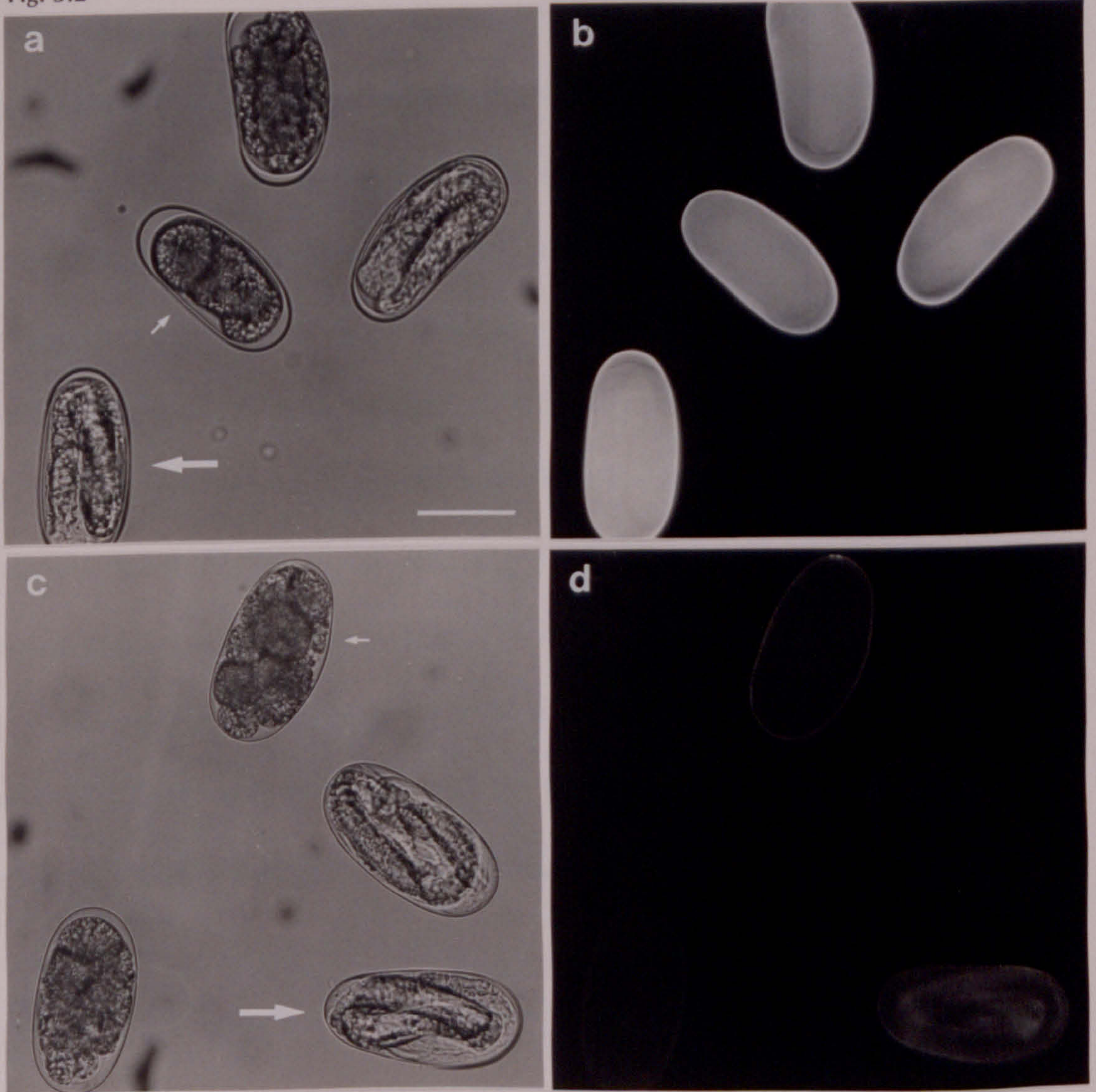
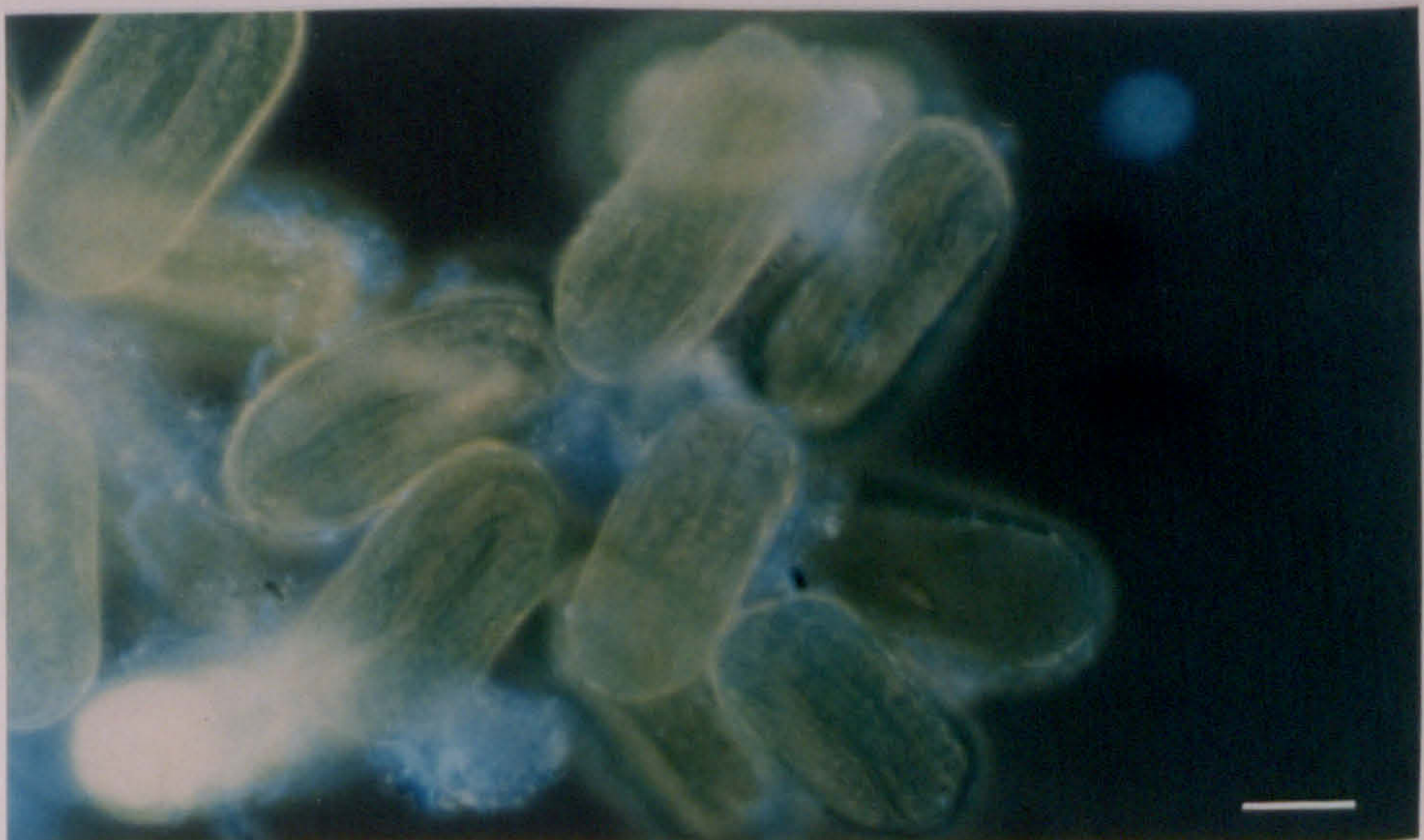


Fig. 5.3



The removal of the outer protein layer from the *M. incognita* egg-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. 5.2d), but bright blue fluorescence in VCP1-treated eggs (Fig. 5.2b). Since protein and chitin are main structural components of nematode egg-shells, chitin being covered by protein (Clarke *et al.*, 1967, Bird & McClure, 1976), this result suggests that the underlying chitin fibrils had been exposed. Eggs contained embryos in various developmental stages (Fig 5.2a,c), but the fluorescence intensity of the egg shells was similar for all eggs (Fig. 5.2b,d). However, enzyme-treated *G. rostochiensis* eggs did not stain. Although all *G. rostochiensis* eggs had some degree of yellow background fluorescence with the filter set required for Calcofluor, the fluorescent dye did not stain the eggs of this species (Fig. 5.3)

5.3.2 Infection rates

Verticillium chlamydosporium, isolate 10, was highly pathogenic for eggs of *M. incognita* but weakly pathogenic for those of *G. rostochiensis*, irrespective of whether observations were made 6 or 14 days post-inoculation. After 14 days, 79.7% of *M. incognita* eggs were infected (Table 5.1). Infection of *G. rostochiensis* eggs by this fungus (6.7 and 9.0% after 6 and 14 days, respectively) was not significantly greater than in the treatment with no fungus added reflecting background infection in *G. rostochiensis* eggs (2.8 and 5.6% after 6 and 14 days, respectively). When *M. incognita* eggs were pretreated with VCP1, sodium azide or boiled, infection was accelerated, reaching 100% 14 days post-inoculation and with most eggs colonised

after 6 days (Table 5.1). Although more eggs of *G. rostochiensis* also became infected following these treatments, the increases were comparatively small, with only VCP1 significantly enhancing infection (from 9.0 - 29.2%, $p < 0.01$).

Table 5.1 Infection frequencies (% eggs infected) of *M. incognita* and *G. rostochiensis*, 6 and 14 days after adding eggs to *V. chlamyosporium*, isolate 10. Eggs were either untreated (control) or were pretreated by suspending in protease (VCP1), by heat-denaturing, or by suspending in sodium azide. Infection of uninoculated eggs never exceeded 5% (not shown). Treatments were compared to untreated eggs (control) using 2-tailed Fisher's exact tests.

Time (day)	<i>M. incognita</i>				<i>G. rostochiensis</i>			
	Control	Protease	Heat	Azide	Control	Protease	Heat	Azide
6	23.7	92.9 ***†	94.6 ***	77.8 ***	6.7	11.0 NS	10.2 NS	16.4 NS
14	79.7	100.0 ***	100.0 ***	100.0 ***	9.0	29.2 **	20.3 NS	7.5 NS

† NS, *, ** or *** : $p > 0.05$, $p < 0.05$; $p < 0.01$, or $p < 0.001$, respectively. These significance levels relate to comparison between treated and untreated (control) eggs within each species and time.

Table 5.2 Surface growth (mean length of hypha [in μm], visible on egg surface) on *M. incognita* and *G. rostochiensis* eggs inoculated with *V. chlamyosporium*, isolate 10. Eggs were either untreated (control) or were pretreated by suspending in protease (VCP1), by heat-denaturing, or by suspending in sodium azide. Eggs with surface growth exceeding 400 μm were excluded since those measurements were increasingly inaccurate.

Time (day)	<i>M. incognita</i>				<i>G. rostochiensis</i>			
	Control	Protease	Heat	Azide	Control	Protease	Heat	Azide
6	44.4	172.6 ***†	141.4 ***	52.6 NS	25.8	39.0 NS	34.6 NS	30.2 NS
14	110.0	112.8 NS	105.8 NS	150.0 NS	18.8	153.6 ***	71.4 ***	48.6 *

† Significance levels indicated (see Table 5.1) relate to comparison between treated and untreated eggs within each nematode species and number of days post-inoculation.

5.3.3 Morphological aspects of the infection process

Healthy eggs of *M. incognita* and *G. rostochiensis* were similar in size but the former had thinner and more translucent egg shells. The shell of the *M. incognita* eggs often collapsed over the juveniles inside, while eggs of *G. rostochiensis* at a similar stage of development remained rigid.

Infection rates appeared to be correlated with the amount of surface growth (see Tables 5.1 and 5.2). In all treatments, *V. chlamydosporium* colonized the egg surface, to different degrees, but the external colonisation of *M. incognita* eggs was faster and more extensive than that of *G. rostochiensis* (compare growth at 6 and 14 days post-inoculation). Hyphal growth over egg surfaces 6 days post-inoculation was particularly extensive in heat- or VCP1-treated *M. incognita* eggs. In contrast, sodium azide, a metabolic poison, had no apparent effect (Table 5.2). After 14 days, there was no significant difference between surface growth on untreated and treated eggs of *M. incognita*. While the fungal growth on the surface of *G. rostochiensis* control eggs did not increase between 6 and 14 days post-inoculation, eggs killed by heat, or pretreated with protease, had significantly more external growth after 14 days ($p < 0.001$; Table 5.2).

Appressoria of different shapes and sizes were produced by *V. chlamydosporium* either at the ends of long hyphae, or from lateral branches. These consisted of prominently swollen or little differentiated structures which developed on eggs of both *M. incognita* and *G. rostochiensis* (Figs. 5.4-5.7). In general, few appressoria were formed (Table 5.3). When examining infected eggs, the penetration site could often not be identified, although the presence of the fungus in the egg was clear (Figs. 5.8 and 5.9). This suggests that appressoria may not always be produced, or overlooked because they were inconspicuous or present against a highly fluorescent background. Although strong fluorescence of heavily infected eggs prevented quantification of at least some appressoria, there was a difference in the number and type produced on the two species. More swollen appressoria developed

Fig. 5.4 Fluorescence micrograph of a terminal appressorium (arrow) of *V. chlamyosporium*, isolate 10, attached to the surface of an egg of *Meloidogyne incognita*. Note the colonization hyphae (small arrow). Stained with Calcofluor White M2R. Bar = 10 μm .

Fig. 5.5 Scanning electron micrograph of lateral appressorium of *V. chlamyosporium*, isolate 10, formed on the surface of the egg of *M. incognita*. Bar = 2 μm .

Figs. 5.6a-c Fluorescence micrographs of three optical sections of a single hypha which has produced two appressoria on the surface of an egg of *M. incognita*. A penetration hypha has been produced by each appressorium. A circular bore hole denotes the site of penetration (arrows).

Fig. 5.7 Fluorescence micrograph of the relatively little developed appressoria on the surface of a *G. rostochiensis* egg. At this stage, there were no signs of actual infection of the egg. Bar = 10 μm .

121a

Fig. 5.4



Fig. 5.6

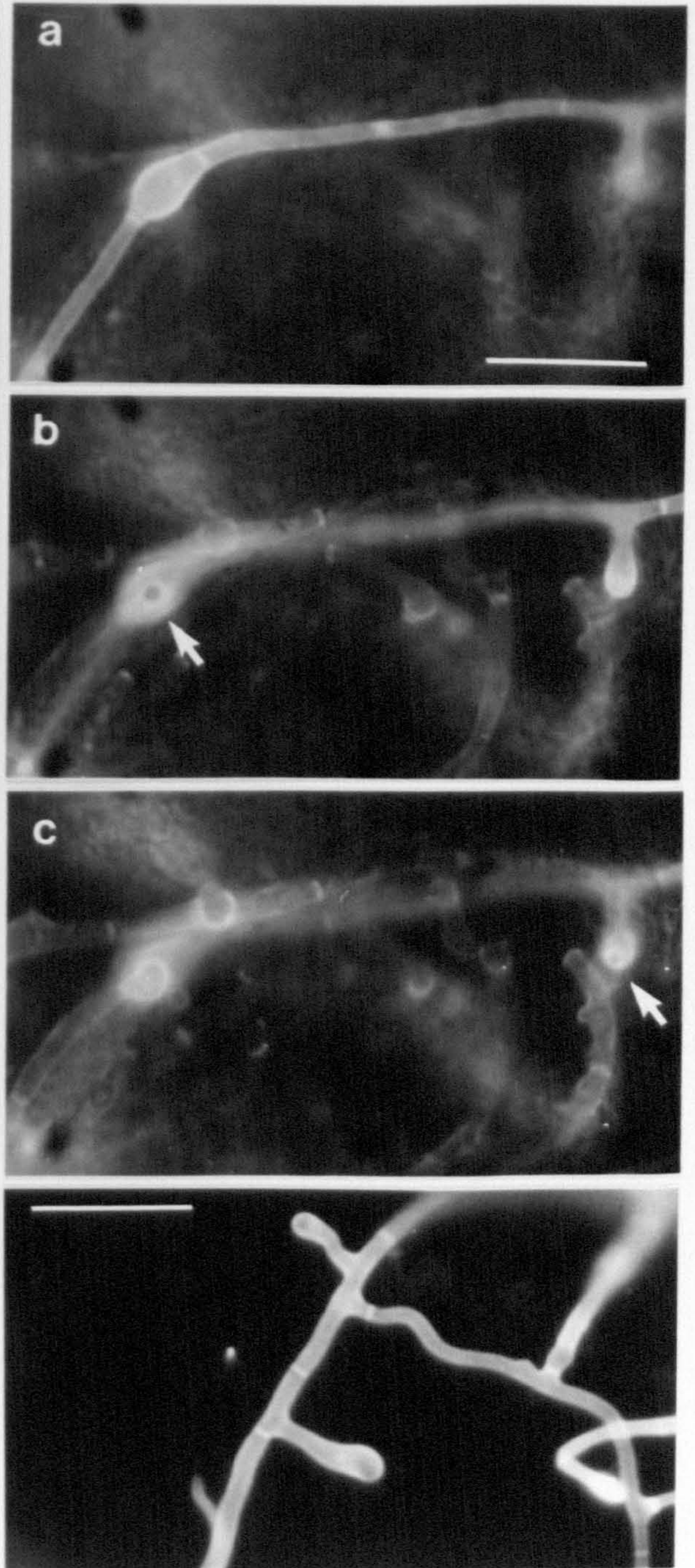


Fig. 5.5

Fig. 5.7

on eggs of *M. incognita* than on eggs of *G. rostochiensis*, e.g. 6 days post-inoculation, there were on average 1.53 lateral, swollen appressoria on each VCP1-pretreated *M. incognita* egg, but none on *G. rostochiensis* eggs ($p < 0.01$) (Table 5.3). These differences corresponded to the relative amounts of fungal growth on the egg surfaces of both nematode species (Table 5.2). More than one appressorium could be produced by the parent hypha on either nematode species (Figs. 5.5-5.8). Appressoria were produced on any part of the egg.

Table 5.3 Appressoria (mean number per egg) on *M. incognita* and *G. rostochiensis* eggs. Eggs were either untreated (control) or were pretreated by suspending in protease (VCP1), by heat-denaturing, or by suspending in sodium azide. Appressoria (App.) were either prominent, swollen, occurring terminally (T) or laterally (L) on hyphae, or inconspicuous, non-differentiated terminal (NDT) or lateral (NDL) structures. Excluded were eggs with surface growth exceeding 400 μm .

Time (day)	App. type	<i>M. incognita</i>				<i>G. rostochiensis</i>			
		Cont.	Protease	Heat	Azide	Cont.	Protease	Heat	Azide
6	T	0.04	0 NS [†]	0.14 NS	0.10 NS	0.01	0 NS	0 NS	0 NS
	L	0.31	1.53 ***	0.95 **	0.68 NS	0.02	0 NS	0 NS	0.02 NS
	NDT	0.01	0 NS	0 NS	0 NS	0	0.02 NS	0	0
	NDL	0.10	0.47 **	0.14 NS	0.15 *	0.01	0.22 *	0.02 NS	0.20 *
14	T	0	0	0	0	0	0	0	0
	L	0.56	0.78 NS	1.56 *	1.00 NS	0.03	0.13 *	0.01 NS	0.02 NS
	NDT	0.12	0 NS	0 NS	0 NS	0	0.05 NS	0	0
	NDL	0.08	0 NS	0 NS	0 NS	0.06	1.59 ***	0.37 ***	0.14 NS

[†] Significance levels indicated (see Table 5.1) relate to comparison between treated and untreated eggs within each nematode species and number of days post-inoculation.

Eggs were penetrated by short, narrow penetration pegs which became swollen immediately after breaching the egg shell. The first indication of peg formation was the appearance of a bright, circular ring beneath the appressorium. These circles were seen on both *M. incognita* and *G. rostochiensis* eggs suggesting

Fig. 5.8 Internal colonization of a *M. incognita* egg by *V. chlamydosporium*. Note the appearance of the fungus as hyphae with short, bulbous fragments. Bar, 10 μm .

Fig. 5.9 Internal colonization of a *M. incognita* egg by *V. chlamydosporium*. The fungus, colonizing the pseudocoel, follows the outline of the juvenile. Bar, 10 μm .

Fig. 5.10 Late stage of infection of *M. incognita* eggs by *V. chlamydosporium*; "break-out" hyphae are visible. Bar, 40 μm .

Fig. 5.11 Late stage of infection of *M. incognita* eggs by *V. chlamydosporium*; morphological transition from bulbous, internal mycelium to slender, post-emergence hyphae with larger intersegmental distance. Bar, 5 μm .

Fig. 5.8

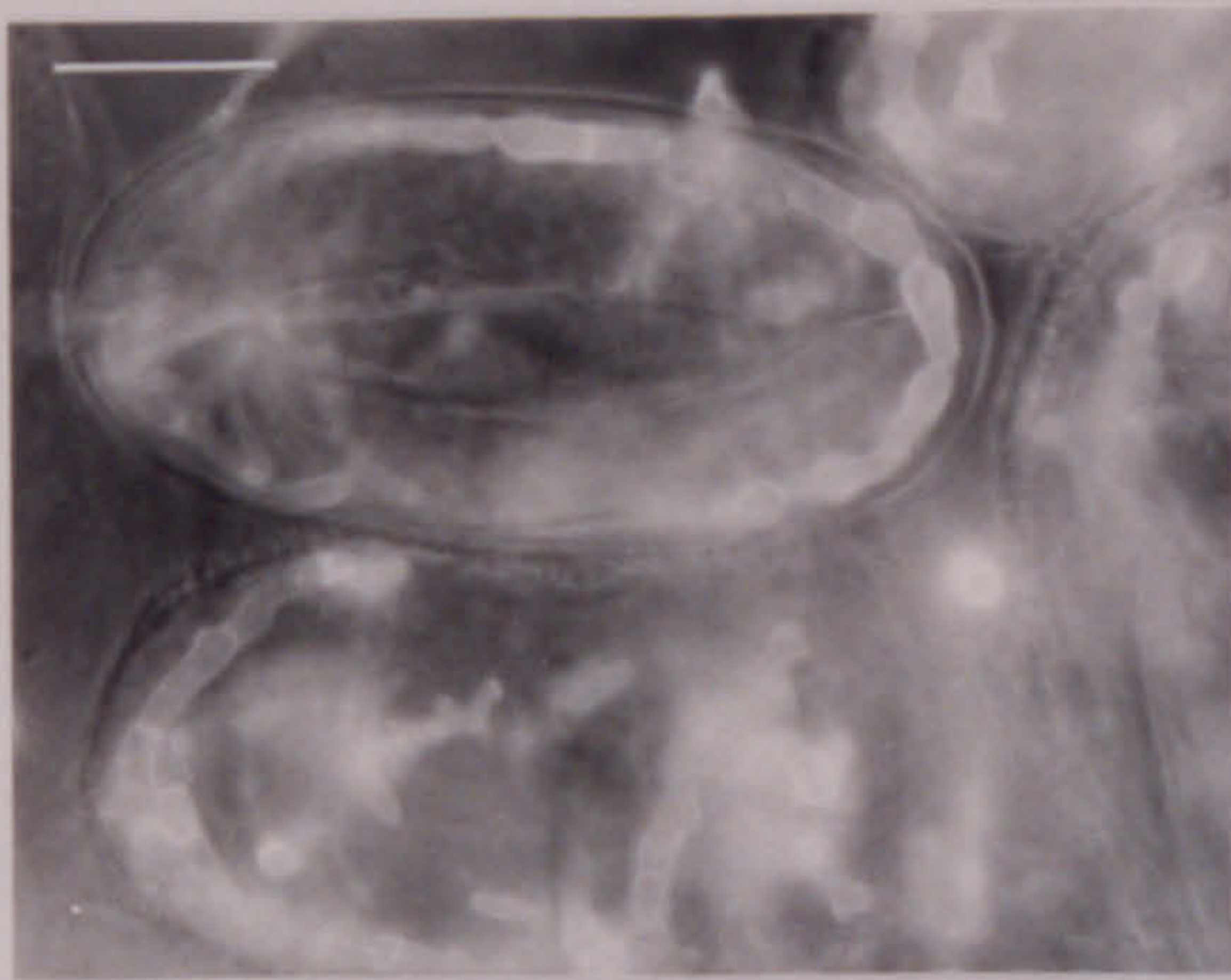


Fig. 5.9

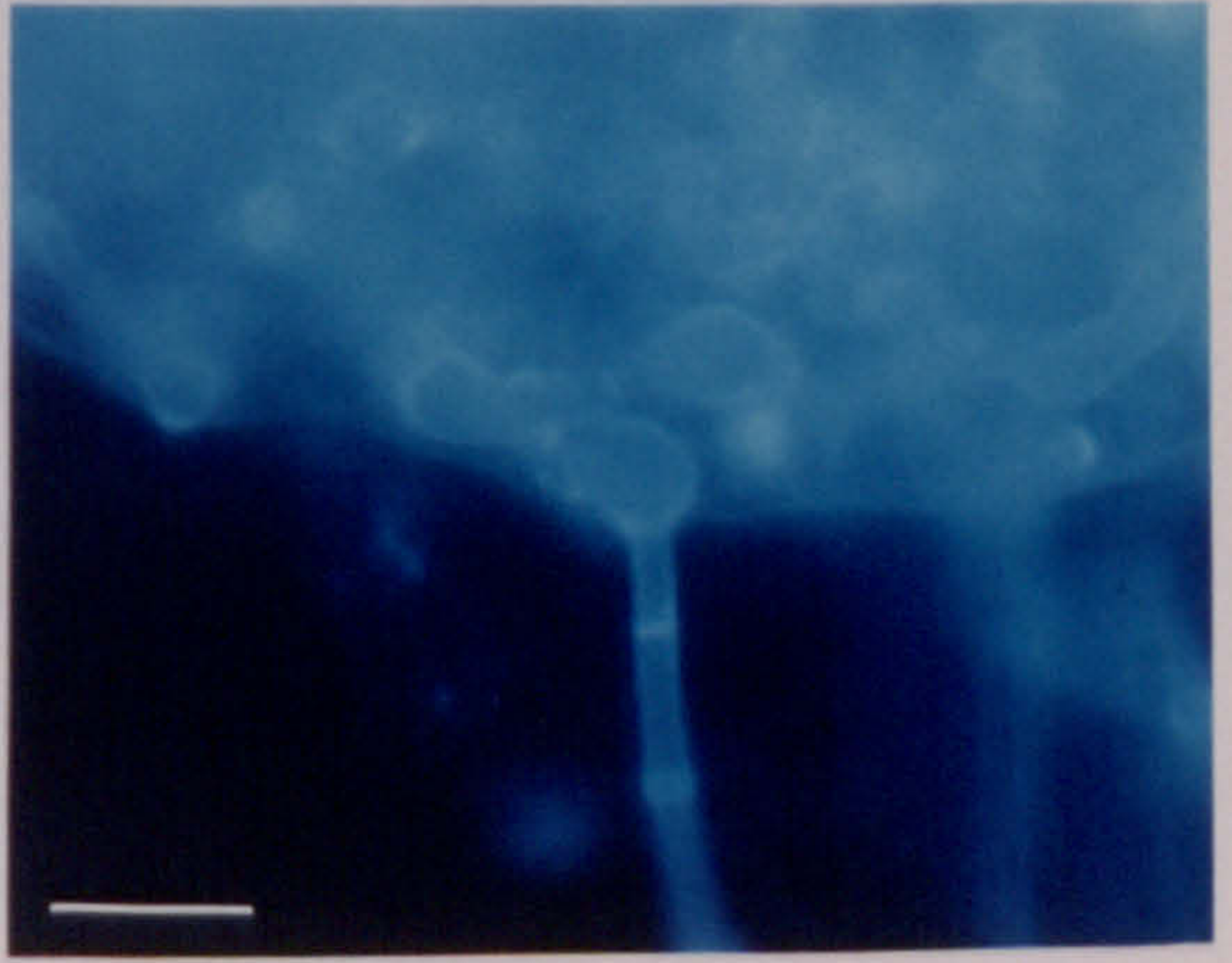
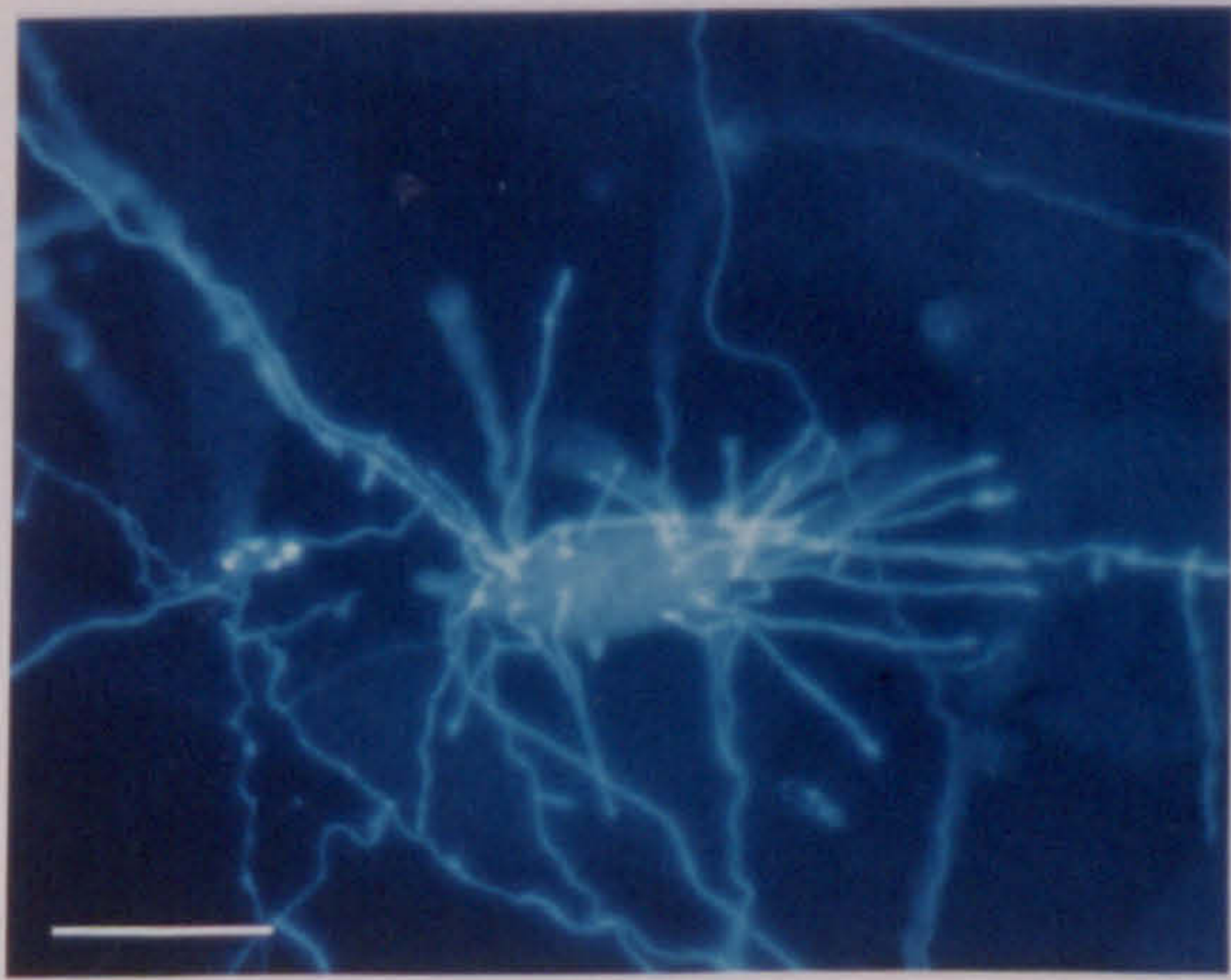
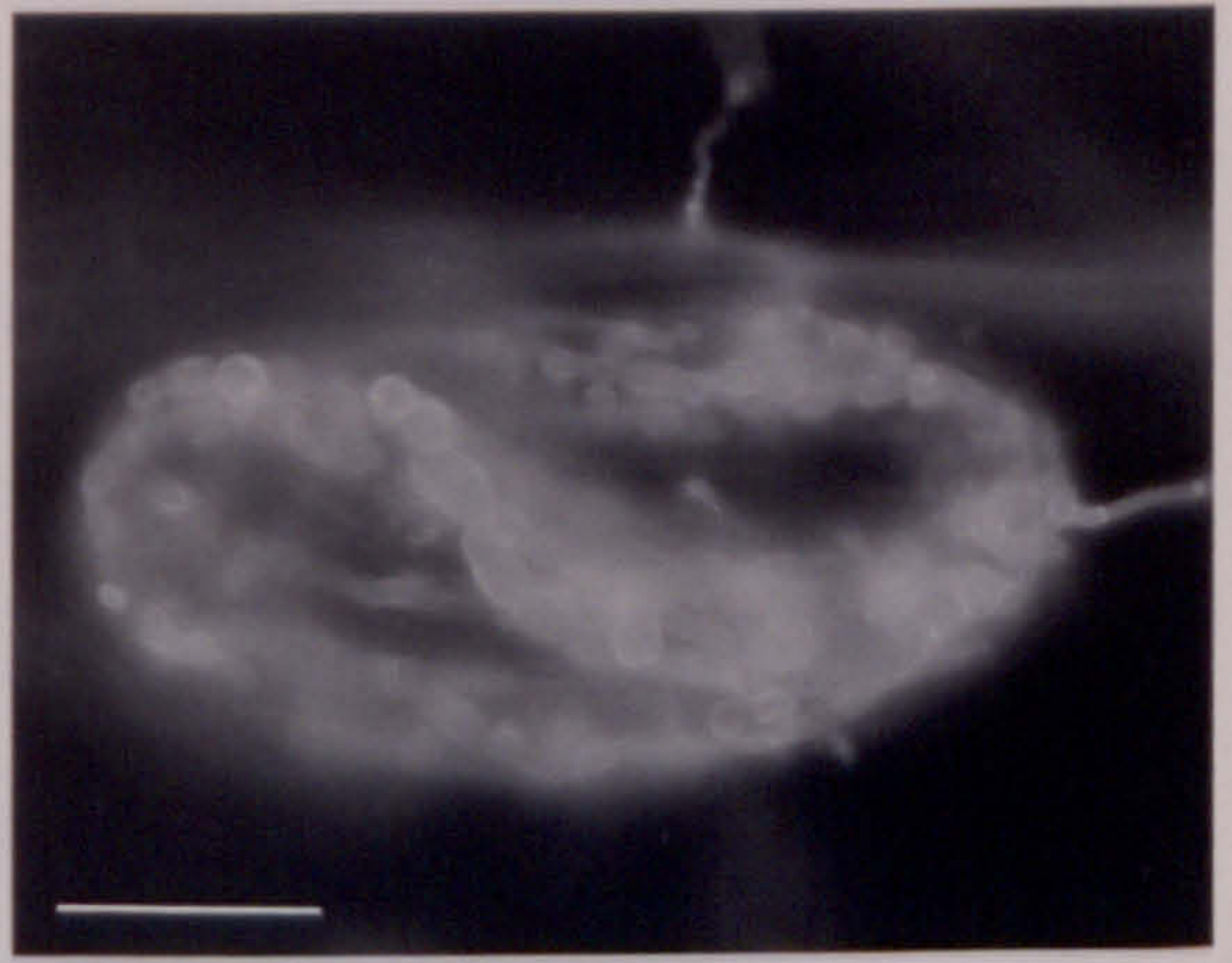


Fig. 5.10

Fig. 5.11

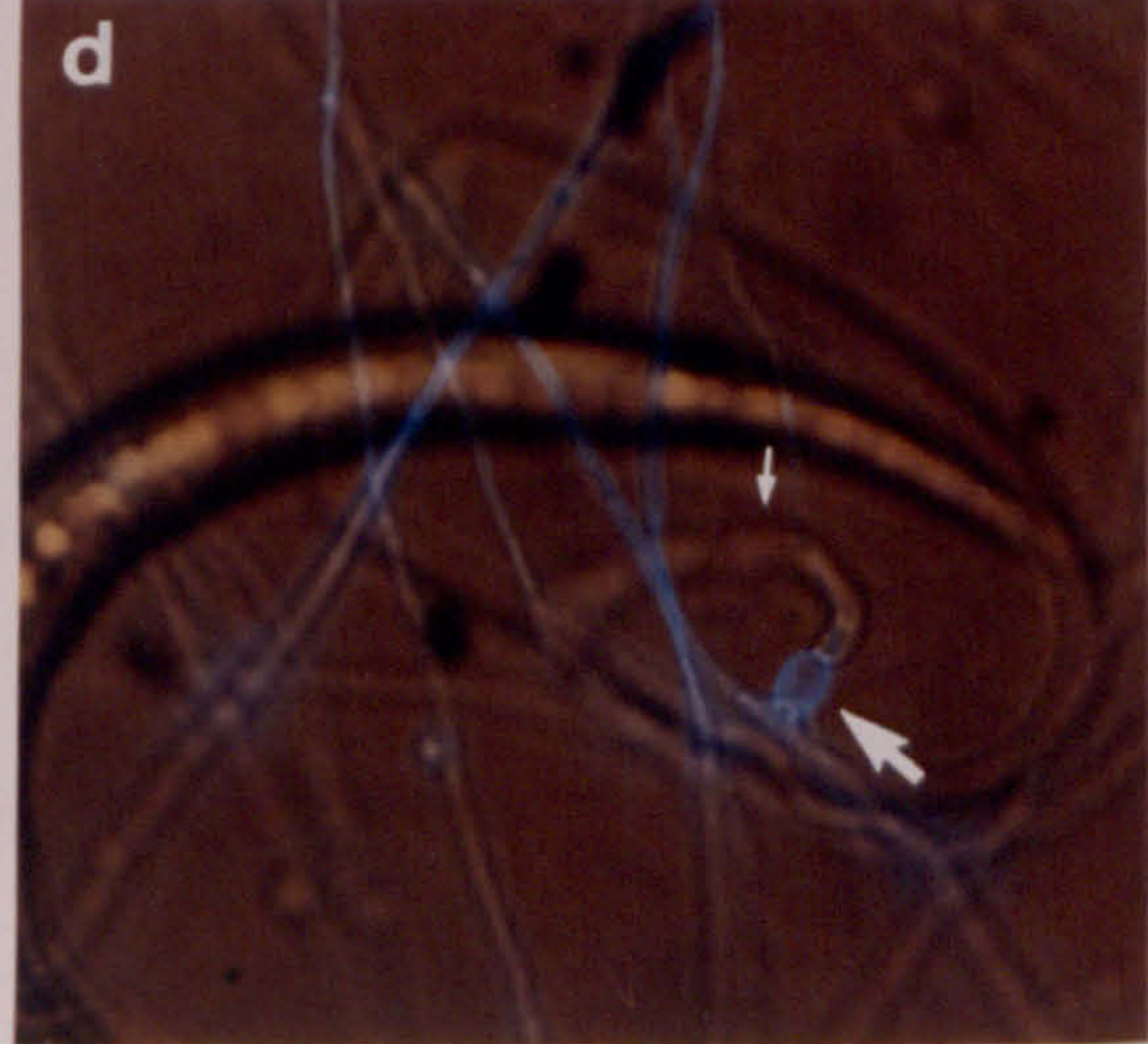
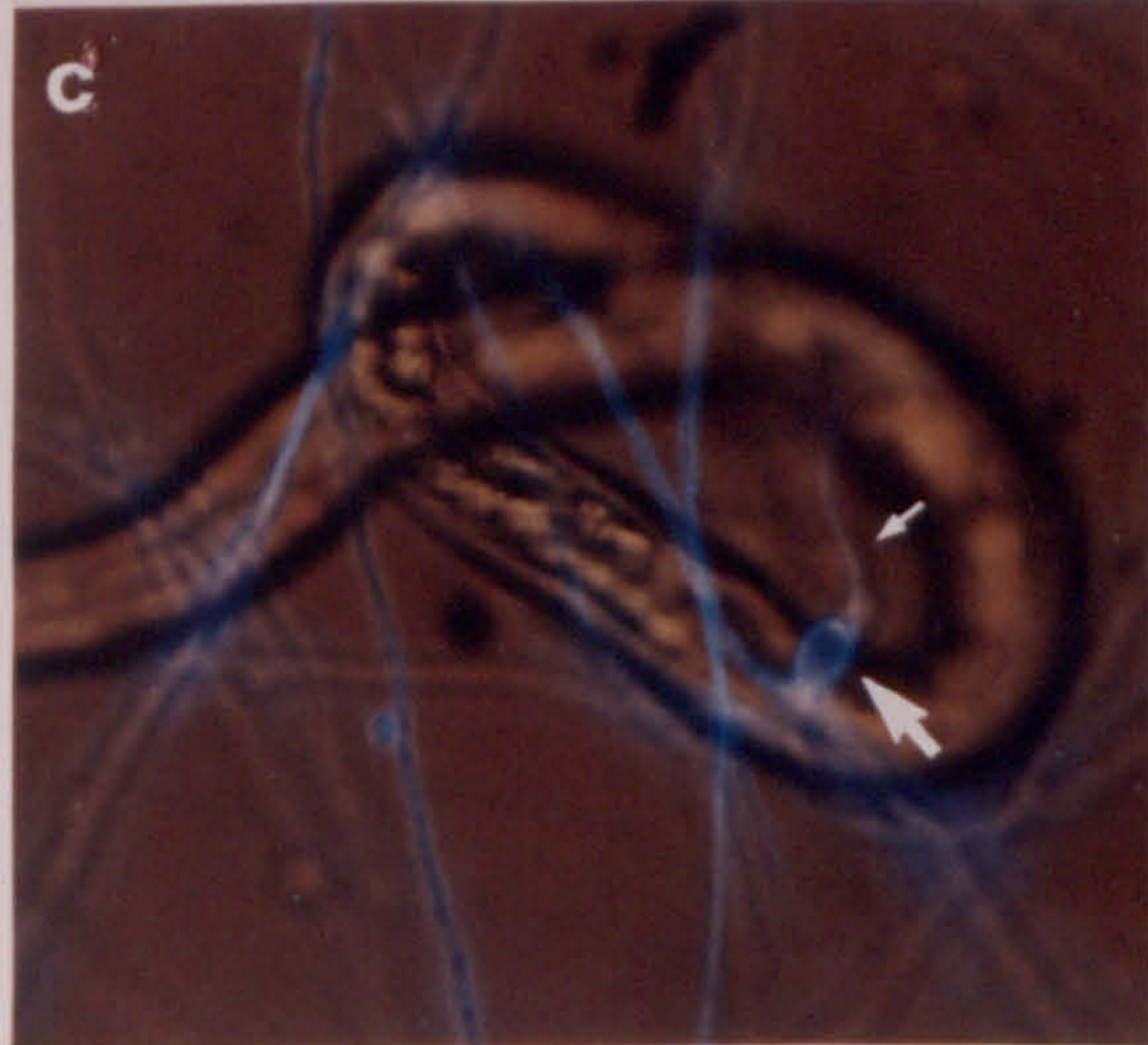
similar mechanisms of entry. Occasionally, the penetration pegs failed to enter the egg of either species, in which case they either grew on the surface of the egg (Fig. 5.6) or aborted after limited growth.

Verticillium chlamyosporium not only penetrated the egg shell, but the juvenile cuticle as well, as it often developed in the nematode's pseudocoel (Fig. 5.9). At this stage, the hyphal morphology was typically altered, from narrow pre-penetration hyphae with large inter-septal distances, to a post-penetration mycelial system with short, bulbous segments. There was no evidence of fragmentation of the mycelium into the yeast-like hyphal bodies (blastospores) that are formed by some entomopathogenic fungi to disperse through the insect haemocoel (Hall & Papierok, 1982; Charnley, 1984). In a rare event, a juvenile was seen escaping a penetrated egg shell before becoming infected (Figs. 5.12). The pressure of the coverslip may have caused it to "hatch". These observations indicate that *V. chlamyosporium* was able to initiate the infection process of nematode eggs that were still vital, confirming its pathogenic nature. The attempted infection of this fully-fledged second-stage juvenile also suggests that *V. chlamyosporium* was not confined to eggs containing embryos in the early developmental stages, which was confirmed by numerous other instances where the fungus had clearly colonised juveniles in eggs.

While a single hypha could infect an egg, numerous "break-out" hyphae usually emerged in the late stages of infection (Fig. 5.10). A typical "break-out" event is shown in Fig. 5.11. The sudden morphological transition from bulbous, internal mycelium to post-emergence hyphae resembled that at the beginning of the infection process. The bulbous segment that often developed on the inside of the egg

Fig. 5.12 (a) Second-stage juvenile of *M. incognita*, moving inside its egg and ready to hatch. (b), (c) Juvenile has broken through the egg shell. (d) Juvenile migrating away, leaving empty shell with appressorium on the outside (large arrow), and penetration hypha on the inside (small arrow). (a), (b) Bright field, and (c), (d) combined bright field and fluorescence (Calcofluor staining). Bar, 20 μm .

125a.



shell was somewhat homologous with an appressorium. However, no infection peg was observed during the outwards penetration of the egg shell, which may indicate that the egg shell had lost some integrity at that stage (Fig. 5.11). In the conditions of the assay, there was very limited sporulation post-emergence, and the fungus continued to grow as mycelium.

5.3.4 Enzymes in the early stages of infection

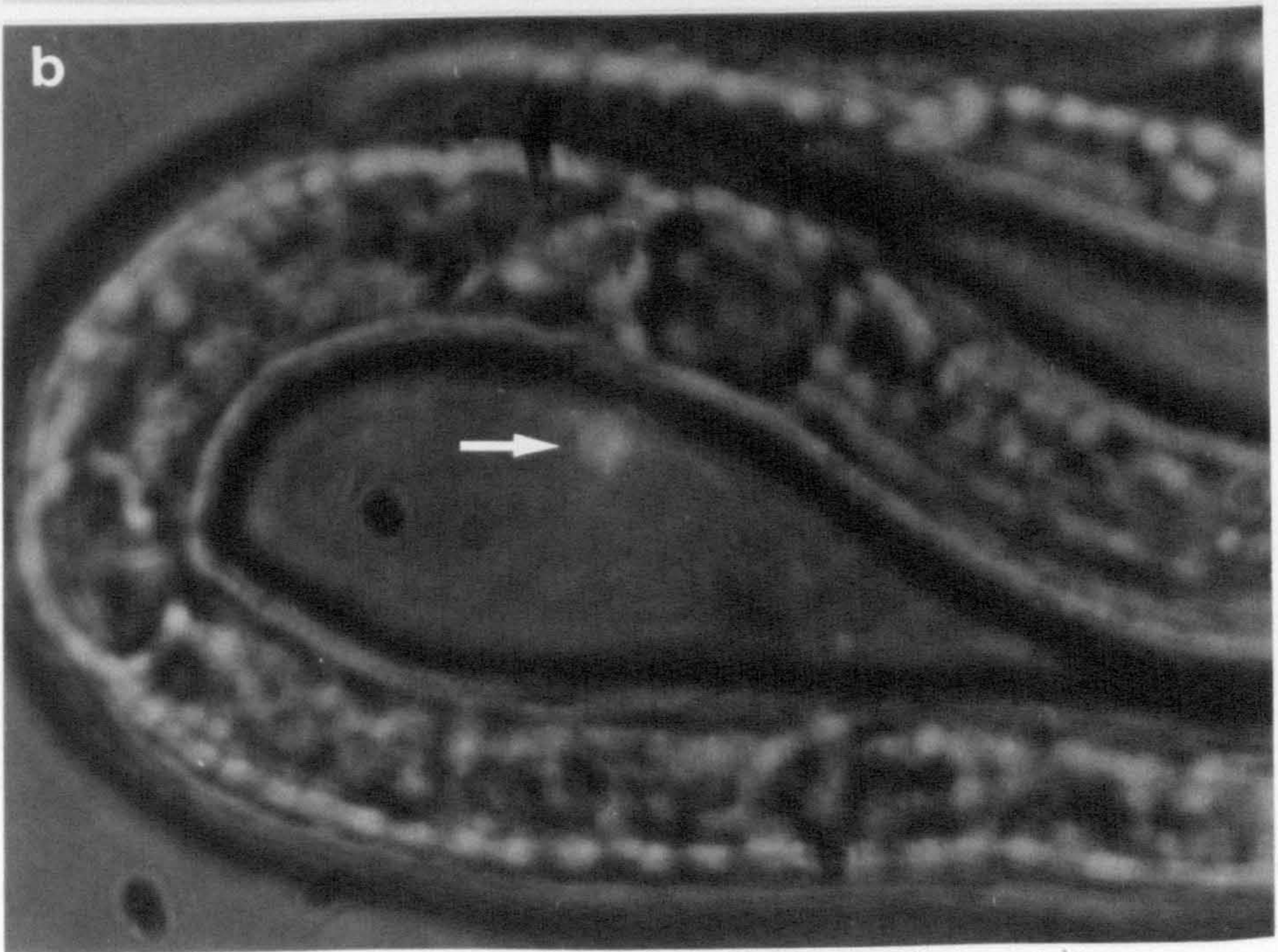
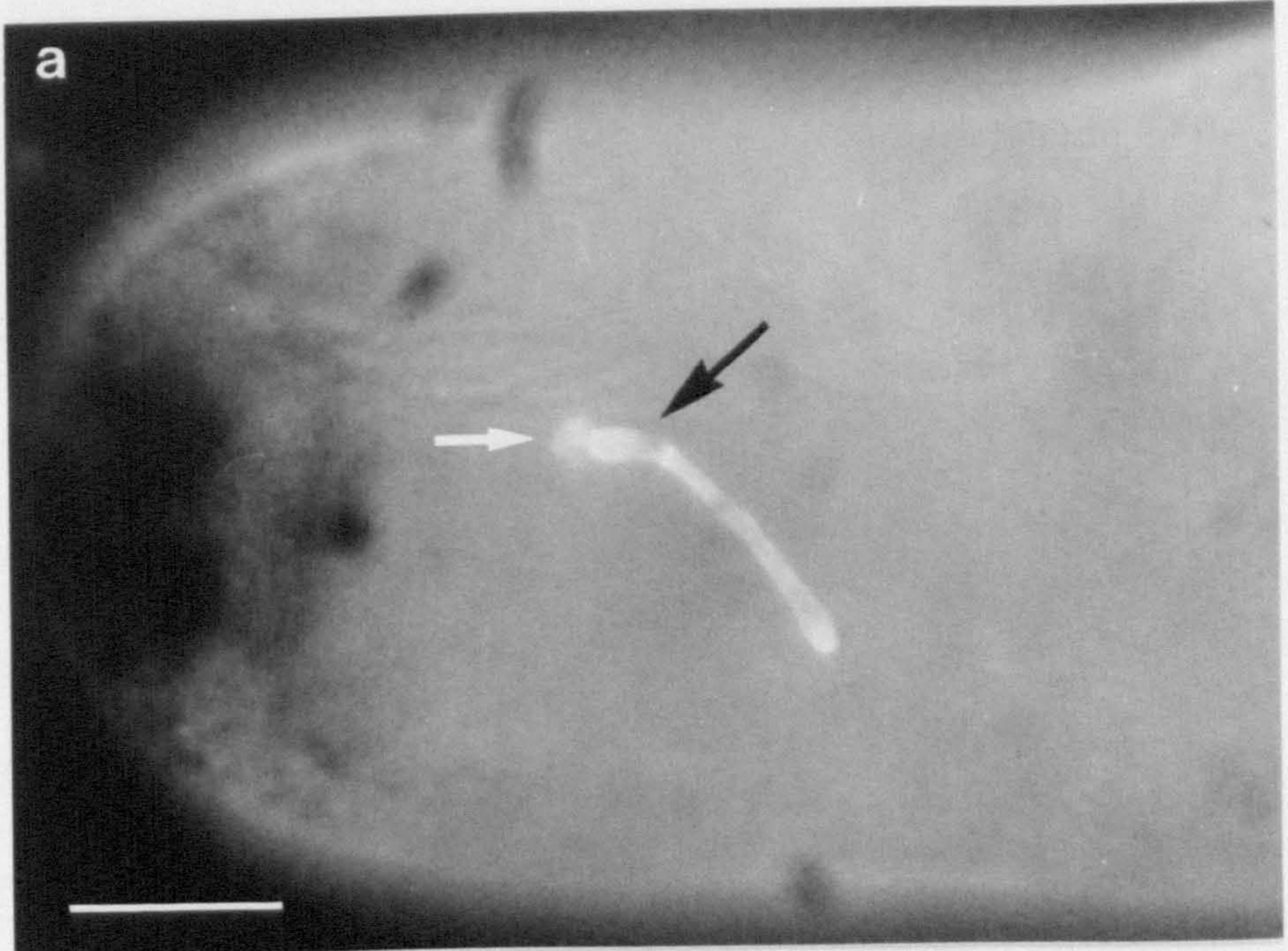
Extracellular fungal enzymes appeared to play a role in the infection processes of *V. chlamydosporium*, isolate 10, as indicated by imprints of hyphae on the *M. incognita* egg surface (Fig. 5.14). The localization of VCP1, by immunofluorescence, around appressoria suggested that the protease was produced by infection structures prior to, or concurrent with, penetration (Fig. 5.13a,b). Pitting and/or lesions in eggs treated with VCP1 were more extensive in the presence of the fungus than in its absence, suggesting that the protease treatment had weakened the egg shell of *M. incognita* (Fig. 5.15). In contrast, no lesions were detected on *G. rostochiensis* eggs treated with the enzyme, with or without the pathogen (Fig. 5.16).

5.4 DISCUSSION

The experiments in this Chapter confirmed that *V. chlamydosporium*, isolate 10, was highly pathogenic for the root-knot nematode, *M. incognita*, as reflected by rapid egg penetration and colonization of a large proportion of eggs exposed to the fungus in an *in vitro* bioassay. Not only was the purified fungal protease, VCP1, able to

Figs. 5.13 Fluorescence (a) and combined bright field and fluorescence (b) images of a single egg of *M. incognita*, not yet penetrated, containing a second-stage juvenile of *M. incognita*. (a) Calcofluor staining of a fragment of a hypha with terminal appressorium (black arrow) and fluorescent lesion (white arrow). (b) Immunolocalization of VCP1 on the same egg using anti-VCP1 antibody and rhodamine-conjugated secondary antibody. The fluorescence corresponds with the site of the lesion (white arrow). Bar = 10 μm .

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hydrolyse proteins from the outer layer of *M. incognita* eggs, but eggs that had been exposed to the protease were also quickly lysed and degraded by the fungus, suggesting that removing, or damaging, the vitelline layer physically weakened the egg shell. A bioassay confirmed the higher susceptibility of these protease-treated eggs. Immunofluorescence microscopy detected the protease on the surface of egg shells, where it was produced by appressoria. Together, these data suggest a role for VCP1 in pathogenesis. The protease may also work in concert with other secreted enzymes, as lesions in VCP1-treated eggs were far more extensive in the presence of the fungus than in its absence. Some of these enzymes have already been reported (Carder *et al.*, 1993), and they may facilitate infection and utilization of the egg contents as a source of nutrients for growth.

On the other hand, *V. chlamydosporium*, isolate 10, was much less pathogenic for the potato cyst nematode, *G. rostochiensis*. The prime difference in fungal growth on *M. incognita* and *G. rostochiensis* eggshells was the limited surface growth on the latter, combined with a higher proportion of little-differentiated appressoria. The factors governing these growth and differentiation patterns must be determined by the respective eggshells.

Clearly, the egg shell of *M. incognita* is thinner than that of *G. rostochiensis* (O'Hara & Jatala, 1985; Jatala, 1986), which was also evident in this study. Although egg shell thickness may be a resistance factor, it does not explain the differences in fungal growth and differentiation seen on eggs of the two species. Rather, these differences could be based on nutritional availability on the egg surface, intrinsic recognition patterns, or fungistatic compounds. The nutritional status on the egg shell

Fig. 5.14 Scanning electron micrograph of hypha of *V. chlamydosporium*, isolate 10, growing on the surface of an egg of *M. incognita*. Part of the hypha was broken off (arrow) during preparation exposing the underlying imprint on the egg surface presumably due to enzyme action. Bar = 2 μm .

Fig. 5.15 Scanning electron micrograph of an egg of *M. incognita* pretreated with VCP1 and colonized by *V. chlamydosporium*, isolate 10. Note the numerous large holes and extensive colonization of the egg surface by the pathogen. Bar = 20 μm .

Fig. 5.16 A similarly treated *C. rostochiensis* egg is intact and there is little growth on the egg surface. Bar = 20 μm .

Fig. 5.14

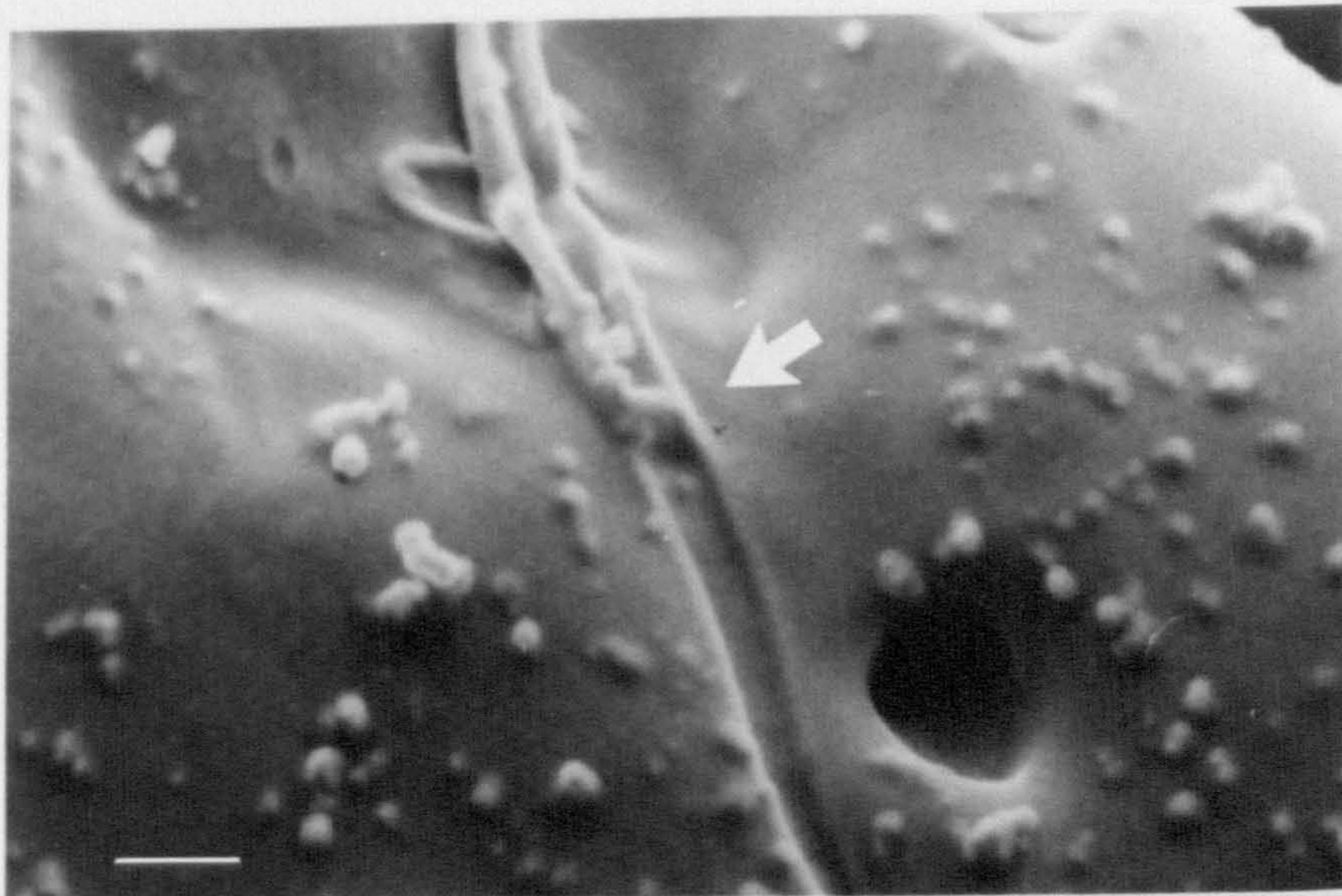


Fig. 5.15

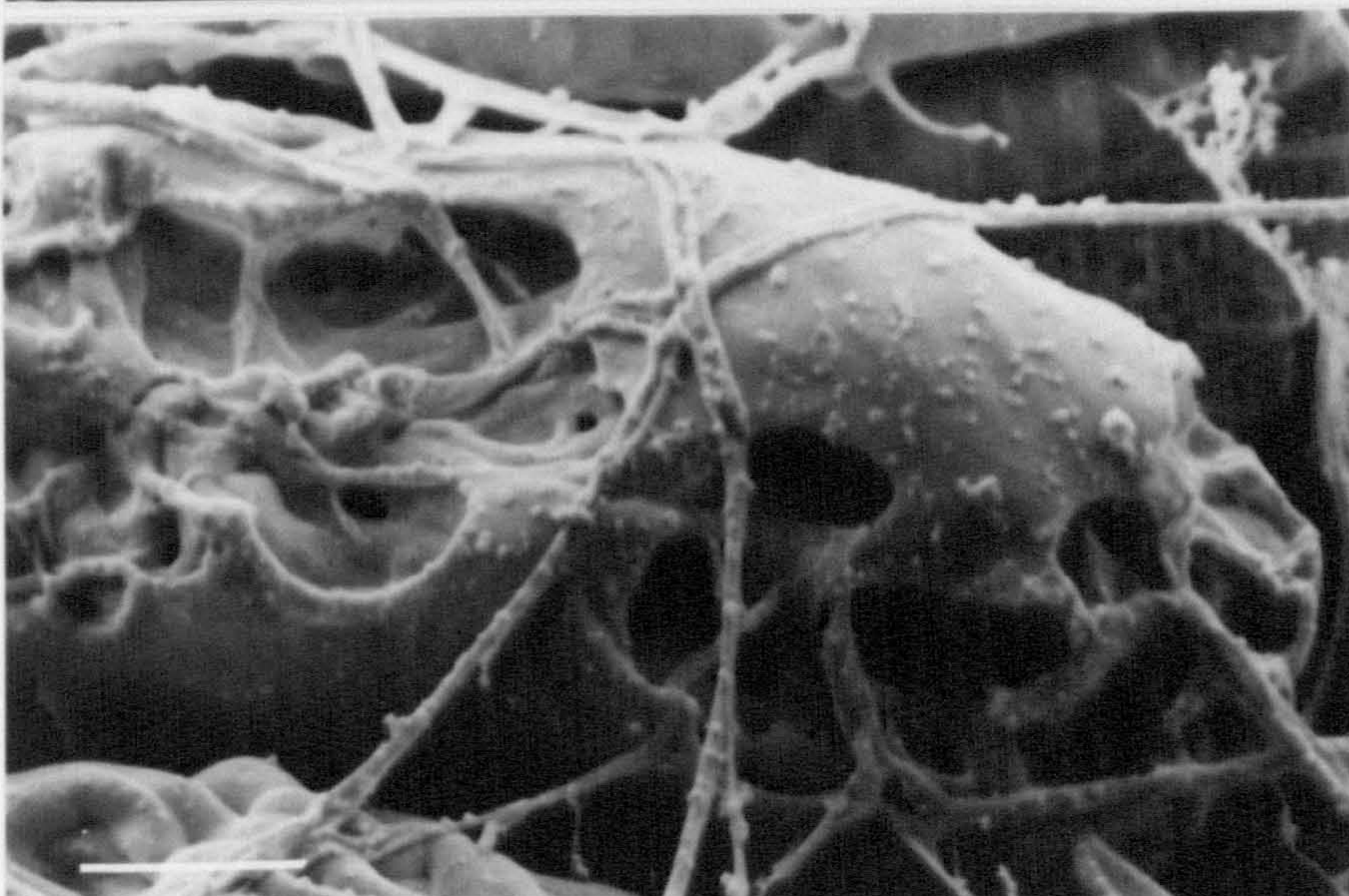
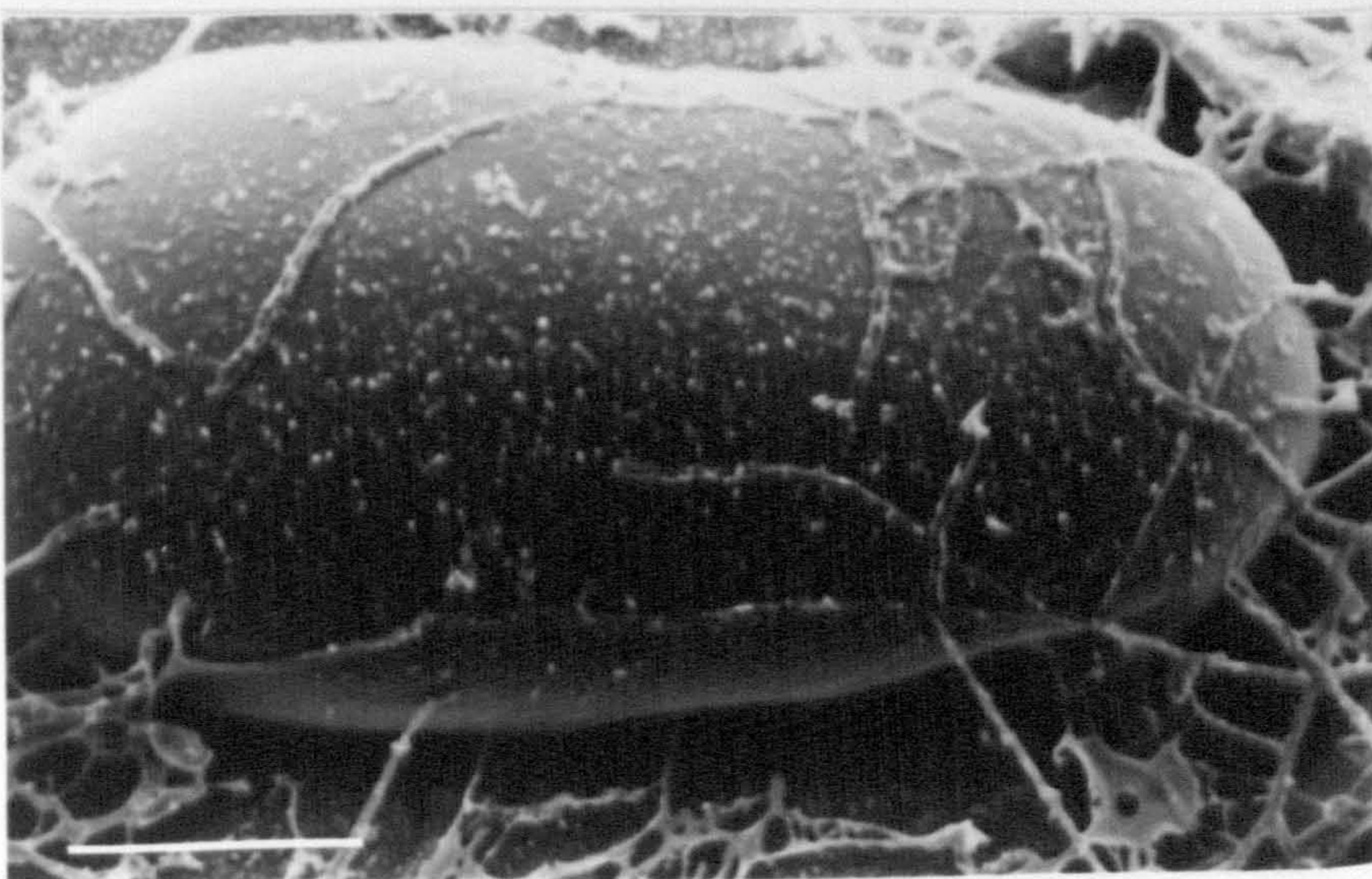


Fig. 5.16



of *M. incognita* may be determined by the apparent ability of the fungal protease to inflict damage on the outer layer(s), making hydrolysis products available for growth. Eggs of both *M. incognita* and *G. rostochiensis* have an outer vitelline layer (Bird & McClure, 1976; Perry *et al.*, 1982) but the lack of hydrolysis of the latter suggests a different composition. A potential resistance mechanism, proposed by Lopez-Llorca & Fry (1989) is tyrosine cross-linking of proteins, which occurs in *G. rostochiensis* egg shells. However, it is unclear whether this protein tanning occurs in *M. incognita*. Specific recognition phenomena, as yet undocumented, may account for the advanced differentiation of many appressoria on eggs of *M. incognita*, while, equally hypothetical, fungistasis by components of the *G. rostochiensis* eggshell may be responsible for the limited surface growth on these eggs.

It is conceivable that the heat treatment of eggs resulted in modified host surface properties by denaturation of surface proteins, but increased infection rates did not suggest that impeded host recognition was the main effect of heating. It is plausible that the overriding effect was increased permeability of the eggshell, as observed with *M. javanica* after exposure to 60°C (Bird & McClure, 1976). Heating may cause more nutrients to leak from the egg, explaining the enhanced surface growth of the fungus in this treatment. The rather limited effect of the heat treatment on the susceptibility of *G. rostochiensis* suggests a very rigid eggshell in this nematode.

VCP1 hydrolysed only a small amount of protein from *G. rostochiensis* eggs. Enzyme-treated *G. rostochiensis* supported a larger fungal biomass on the egg surface, and more ensuing infections, compared to control eggs, suggesting that the

pretreatment with the protease must have inflicted some damage on the egg shell surface. Although less active than VCP1 on *M. incognita* eggs, the fungal subtilisins, Pr1 and proteinase K, released more protein from *G. rostochiensis* eggs than VCP1. Both Pr1 and proteinase K are related to VCP1 and, if *G. rostochiensis* eggs could be demonstrated to be more susceptible to infection after treatment with these enzymes, it may be a worthwhile exercise to transform *V. chlamydosporium*, isolate 10, with the gene for either Pr1 or proteinase K; this could increase the pathogenicity towards *G. rostochiensis*.

Verticillium chlamydosporium infects nematode eggs by hyphal penetration. A feature of this mode of infection was that several appressoria could be produced by the same hypha, theoretically increasing the chances of infection. This appears not to have been reported for any other nematophagous fungus. It is unclear whether *V. chlamydosporium* isolates differ in their ability to form multiple appressoria per hypha, what influences this branching pattern and how it could be manipulated. Appressoria differentiated on dead as well as live eggs, suggesting that their formation was not limited to the parasitic state of the fungus. Dead root-knot nematode eggs were more susceptible to infection than eggs that were added to the fungus when they were still alive, suggesting that the saprotrophic mode of *V. chlamydosporium* was at least as successful as the necrotrophic mode at colonizing eggs. However, the infection rate of dead potato cyst nematode eggs was not significantly greater than that of vital eggs for the duration of the bioassay, corroborating the resilience of the egg shell structure in this nematode.

In conclusion, the subtilisin-like protease VCP1 was produced by appressoria of *V. chlamydosporium*, isolate 10, during infection of *M. incognita* eggs, which are susceptible to the fungus. The protease removed part or the whole of the vitelline layer of the *M. incognita* eggs, which physically weakened the egg shell. It is suggested that the fungus derived nutrients from the surface of these eggs, allowing it to build up biomass, and to differentiate well-developed appressoria. On the other hand, little protein was removed from the egg shells of *G. rostochiensis*, which is a poor host to the fungus. These eggs have a thicker egg shell, but limited growth and differentiation of the fungus on these surfaces may be linked to the inability of the fungal protease to hydrolyse its proteins *in situ*. As such, VCP1 may have contributed to the relative host specificity of *V. chlamydosporium*, isolate 10.

6.1 INTRODUCTION

Enzymes that are produced at the same rate, irrespective of the presence of substrate in the environment, are described as constitutive. Some industrially important bacterial amylases and proteases are produced constitutively, but it is more common that hydrolytic enzymes are regulated. Inducible enzymes are synthesized at a low, basal rate in the absence of substrate. When the substrate, or a derivative thereof, is present in the medium, there is a dramatic increase in the rate of synthesis of the corresponding catabolic enzyme(s). Synthesis continues at this amplified rate until the inducer is removed whereupon it returns to the basal rate (Priest, 1984). The product of an enzyme's activity may repress its synthesis. It is energetically favourable for the fungus to repress the utilisation of complex carbon or nitrogen sources when more convenient sources are available, such as glucose and ammonium, respectively. Catabolic genes of a certain pathway are often coordinately repressed, although, at the molecular level, mechanisms involved in carbon and nitrogen catabolite repression by various components can be radically different (Marzluf, 1981; Hueck & Hillen, 1995; Scazzocchio *et al.*, 1995). The effects of excess glucose or other simple carbon sources can reach much further than the repression of proteases only; profound physiological changes are brought about, by modifying the fluxes in many pathways that generate energy and metabolic intermediates. In fungi, sporulation can also be repressed (Skromne *et al.*, 1995) and hyphal formation disrupted (Paigen & Williams, 1970).

In the regulation of proteases in filamentous fungi, two “classical” type cases are commonly discerned. The first one is represented by *Aspergillus nidulans* and *A. niger*, in which starvation for any nutrient derived from protein (C, N or S) results in biosynthesis and secretion of extracellular protease. No protein inducer is required, and thus “relaxation of repression”, or derepression, is involved (Cohen, 1981; Jarai & Buxton, 1994). The second type case is represented by *Neurospora crassa*, which unlike these *Aspergillus* spp., requires a protein inducer to effect synthesis of extracellular proteases under conditions where cells are starved for either C, N or S. In this case, induction (a positive form of control) is balanced by repression (a negative form of control) (Cohen & Drucker, 1977). Considerable progress has been made in understanding catabolite repression in *A. nidulans* and *N. crassa* at the level of its transcriptional control, i.e. several activator and repressor proteins, together with their DNA target sites, have been identified (MacKenzie *et al.*, 1993). However, the way in which a signal is generated from external nutrient concentrations and transduced, is largely unknown (Ronne, 1995; Scazzocchio *et al.*, 1995).

The study of the regulation of extracellular enzymes is relevant for understanding *in vitro* production systems as it is an economic imperative to have an enzyme system inducible at low cost and resistant to high levels of catabolic end-products (Priest, 1984; Rao *et al.*, 1988). The sensitivity of hydrolytic enzymes to catabolite repression can be important in the infection process. Catabolite repression is responsible for disease resistance in some plant-pathogen interactions (Holz & Knox-Davies, 1986a,b), and treatments that result in increased soluble carbohydrates in the plant, decrease enzyme production and alleviate disease symptoms (Horton &

Keen, 1966; Patil & Dimond, 1968). Appressoria of the entomogenous fungus *M. anisopliae* synthesize the cuticle-degrading protease Pr1 during infection, unless exogenous nutrients repress its production (St. Leger *et al.*, 1989a). As yet, there are no studies of enzyme regulation in any nematophagous fungus. It is an intriguing question how an extracellular protease, likely to be involved in the infection process (see Chapter 5), would be regulated in a fungus that is a rhizosphere colonizer.

6.2 MATERIALS AND METHODS

6.2.1 Relation between fungal growth and protease production

The time-course of protease production of *V. chlamydosporium* isolate 10 in soya peptone medium was recorded by taking 1.2 ml samples from triplicate 200 ml cultures in 1% soya peptone and mineral salts (see Section 2.2.1) at regular intervals. The cells were removed by centrifugation in a microfuge at 10,000 g for 5 min and 100 µl aliquots of the supernatant were assayed for protease activity using 400 µl 0.1 M Tris-HCl buffer (pH 7.9) and 500 µl Azocoll (10 mg ml⁻¹ in the same buffer). Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Mycelia of similar cultures were collected after 4 days on Whatman no. 1 filter paper in sterile conditions, washed with one volume of sterile distilled water, and transferred to 600 ml of fresh soya peptone medium. Samples (30 ml each) were taken from these cultures during the next 7 days, the mycelia collected, dried and

weighed, and the supernatants assayed for subtilisin-like activity after concentration by dialysis on solid sucrose at 4°C overnight.

6.2.2 Regulatory response to medium amendments

Verticillium chlamydosporium, isolate 10, was grown in a liquid medium containing 1% soya peptone and 0.5% D-glucose on a rotary shaker at 23°C for 4 d. The mycelium was collected by vacuum filtration and transferred aseptically, after washing with one volume of distilled water, to an equal volume of distilled water with mineral salts (see Section 2.2.1), in which incubation continued under the same conditions for 12 h. Aliquots of this starved mycelium were transferred to duplicate 50 ml erlenmeyer flasks with 10 ml distilled water and mineral salts (see Section 2.2.1), amended with one of the following: (a) freeze-dried eggs of *Meloidogyne incognita*, or (b) cysts of *Globodera rostochiensis*, collected and surface-sterilized as described in Section 5.2.1; (c) washed, finely comminuted roots from uninfested *Solanum melongena* plants were freeze-dried and also used as a medium amendment; (d) third-stage juveniles of the entomopathogenic nematode *Steinernema feltiae* were obtained from infested *Galleria melonella* larvae (kindly donated by Miss H. Menti, IACR-Rothamsted). These vermiform nematodes were freeze-dried and applied in the medium at 0.1% (w/v). (e) The effect of insects in the medium was studied using adult, freeze-dried and homogenized *Phaedon cochleariae* (0.1% [w/v]). (f) Cellulose was insoluble, medium fibrous (Sigma), and (g) chitin was practical grade from crab shells (Sigma). The chitin fraction that passed through a 30 mesh sieve was used in

the medium. (h) Collagen was insoluble, type I from bovine Achilles tendon (Sigma), whereas (i) gelatin was a soluble powder (Hopkin & Williams).

Eight hours after transfer to these and other media (see tables 6.1-6.3), the mycelium of all cultures was removed by centrifugation (10,000 g for 5 min), and the supernatant assayed for subtilisin-like activity using suc-(Ala)₂-Pro-Phe-pNA as the substrate, essentially as described in Section 2.2.8, except that 500 µl culture supernatant was added to the substrate, which was prepared in 0.2 M Tris-HCl, pH 7.9. Cellulase activity in selected samples was measured as the hydrolysis of cellulose azure (Sigma), using a procedure similar to that of Azocoll described in Section 2.2.8. Briefly, a cellulose azure suspension (10 mg ml⁻¹) in 0.2 M phosphate buffer, pH 5.8 (Dawson *et al.*, 1986) was stirred for 1 h to release unbound dye. The substrate was spun at 8,000 g for 5 min and then resuspended in the final volume of the same buffer, after decanting the unbound dye. All samples consisted of 500 µl substrate (final concentration, 10 mg ml⁻¹) and 500 µl culture filtrate from transfer experiments. Incubation was on a rotary shaker at 37°C until the substrate had visibly been hydrolyzed (*ca* 4 h). Absorbance of the released dye was measured at 570 nm. One unit of cellulase activity is the amount of enzyme that gave an increase in A₅₇₀ of 0.1 absorbance unit h⁻¹ under the conditions of the assay.

6.2.3 Effect of C/N ratio on growth and protease production

The impact of the C/N ratio of the medium on growth of *V. chlamydosporium* was studied in liquid media amended with various amounts of glucose and/or NH₄Cl. Triplicate Erlenmeyer flasks containing 100 ml of mineral salts solution (see Section

2.2.1) were amended with D-glucose and NH_4Cl , in all combinations of 1, 10 and 100 mM of each. Inoculation and incubation were as described in Section 2.2.1. After 6 days of incubation, the cultures were filtered by vacuum filtration through Whatman filter paper no. 1 on a Büchner funnel. The mycelial mat was oven-dried at 60°C for 12 h and weighed, while the filtrate was concentrated overnight by dialysis on crystalline sucrose at 4°C . Protease activity was measured using a standard assay using $\text{suc}-(\text{Ala})_2\text{-Pro-Phe-pNA}$ as the substrate.

6.2.4 Effect of C/N ratio on pathogenicity

Water agar (WA, 1 % [w/v], Fisons, Loughborough, England) plates were inoculated with *V. chlamydosporium* (isolate 10) using a suspension of 1×10^5 conidia and 3×10^3 chlamydospores in 0.2 ml sterile distilled water per 30 mm plate. The basic medium was amended with the same amounts of glucose and NH_4Cl as described in the previous Section, except that an additional treatment consisted of 1 mM glucose and 100 mM KNO_3 . The fungus was allowed to grow for 3 days before *M. incognita* eggs were added; incubation was at 18°C , in the dark. The nematode eggs (ca 300 per plate) were added fresh to the growing fungal culture, i.e. the day after collecting and surface-sterilizing as described in Section 5.2.1. The proportion of eggs infected by *V. chlamydosporium* was calculated after examining 50 eggs each on three replicate plates per treatment, using the fluorescence microscopy method described in Section 5.2.3. These assessments were made 8, 12 and 16 days after adding nematode eggs to the fungus lawn.

6.2.5 Detection of VCP1 during growth in the rhizosphere

Attempts were made to develop an immunological detection system to verify whether the protease VCP1 was produced by *V. chlamydosporium* during growth in the rhizosphere. Tomato seeds were surface sterilized by shaking them in 7% (w/v) CaOCl for 45 min, followed by four washes in distilled water. The seeds were germinated on a medium containing 10 g l⁻¹ glucose, 0.1 g l⁻¹ yeast extract, 0.1 g l⁻¹ peptone and 12 g l⁻¹ agar, and then transferred to sterile vermiculite. All but controls were inoculated with *M. incognita* eggs and/or a plug from a growing colony of *V. chlamydosporium*. Plants, nematodes and fungus developed for *ca* four weeks, when the vermiculite was carefully removed from the root system with forceps. A replica of the root system was made on nitrocellulose, sandwiched between filterpaper wetted with PBS. Gentle pressure was applied for 1 h. The nitrocellulose membranes were blocked with PBSTM for 2 h, washed 6 times with PBST, and incubated with the primary anti-VCP1 polyclonal antibody (1/25 in PBST) for 1.5 h. Unbound antibody was removed by 6 washes with PBST, followed by incubation with the secondary antibody (goat anti-rabbit - alkaline phosphatase conjugate 1/500) for 2 h. After final washes in PBST, the membranes were developed in BCIP/NBT.

6.3 RESULTS

6.3.1 *In vitro* response to C and N: growth and protease production

The time-curves of the secretion by *V. chlamydosporium* of total protein and protease in soya peptone medium did not follow the same pattern. Whereas the amount of

protein increased exponentially from time zero onwards, presumably reflecting growth, there was a significant lag phase in the secretion of protease (Fig. 6.1). No protease activity was observed during the first 2 days of the culture, followed by a very sharp rise over the next 2 days, after which a gradual decline in the protease titre was seen. The declining phase was characterised by relatively large variations between the replicates (Fig. 6.1).

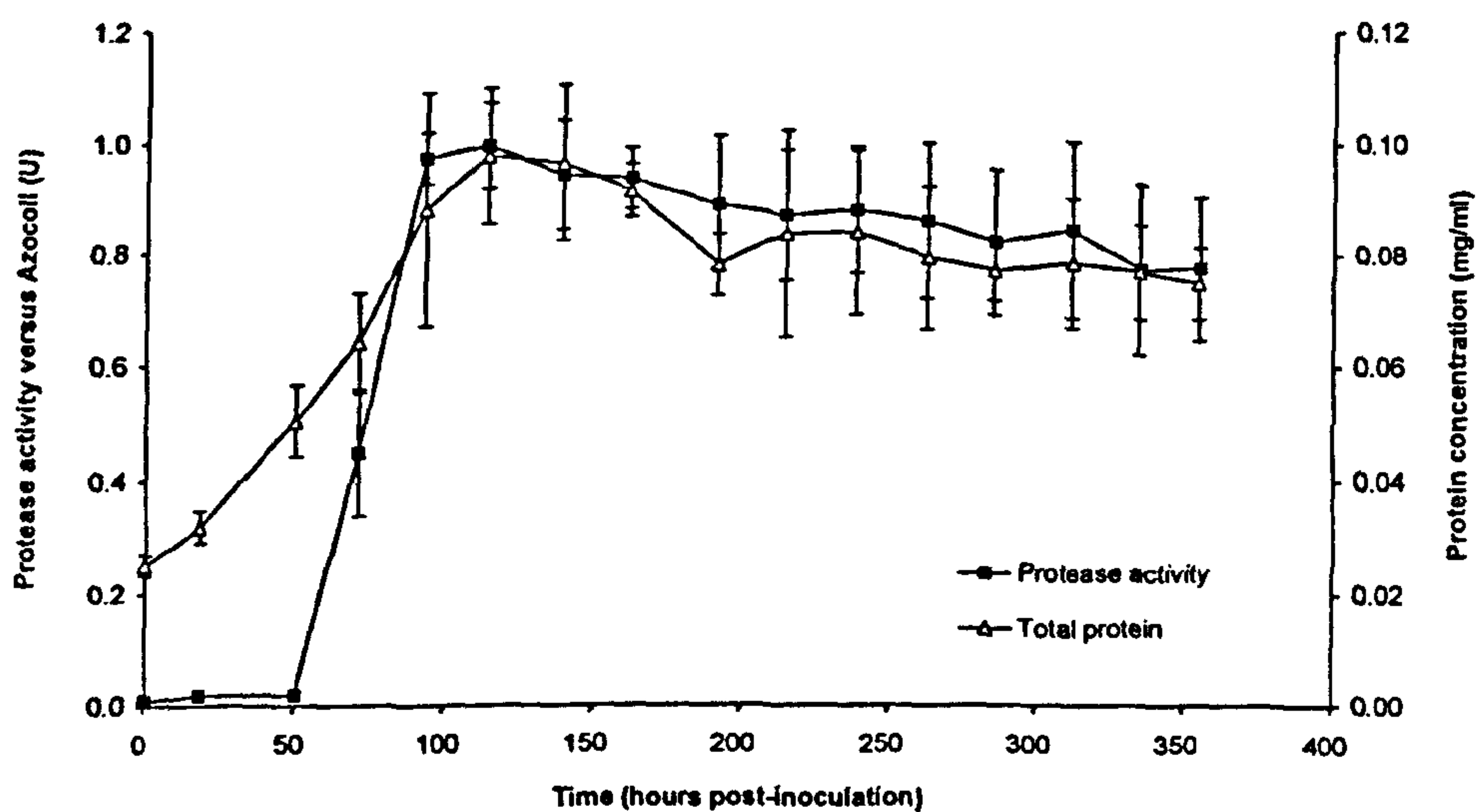


Fig. 6.1 Time-course of protease production by *V. chlamydosporium* isolate 10 in soya peptone medium. Enzyme production was measured as Azocoll-degrading activity, as described in Section 2.2.8. Each data point is the means of two replicate cultures \pm S.D.

Mycelium that was near the end of its exponential growth stage in soya peptone medium secreted proteases, but when it was transferred to fresh medium of the same type, no VCP1 was observed for another 24 h (Fig. 6.2). Although enzyme production was apparently repressed by the fresh nutrients, growth immediately resumed, and a similar pattern as before was seen, i.e. rapid VCP1 production when the rate of increase in biomass slowed down (Fig. 6.2).

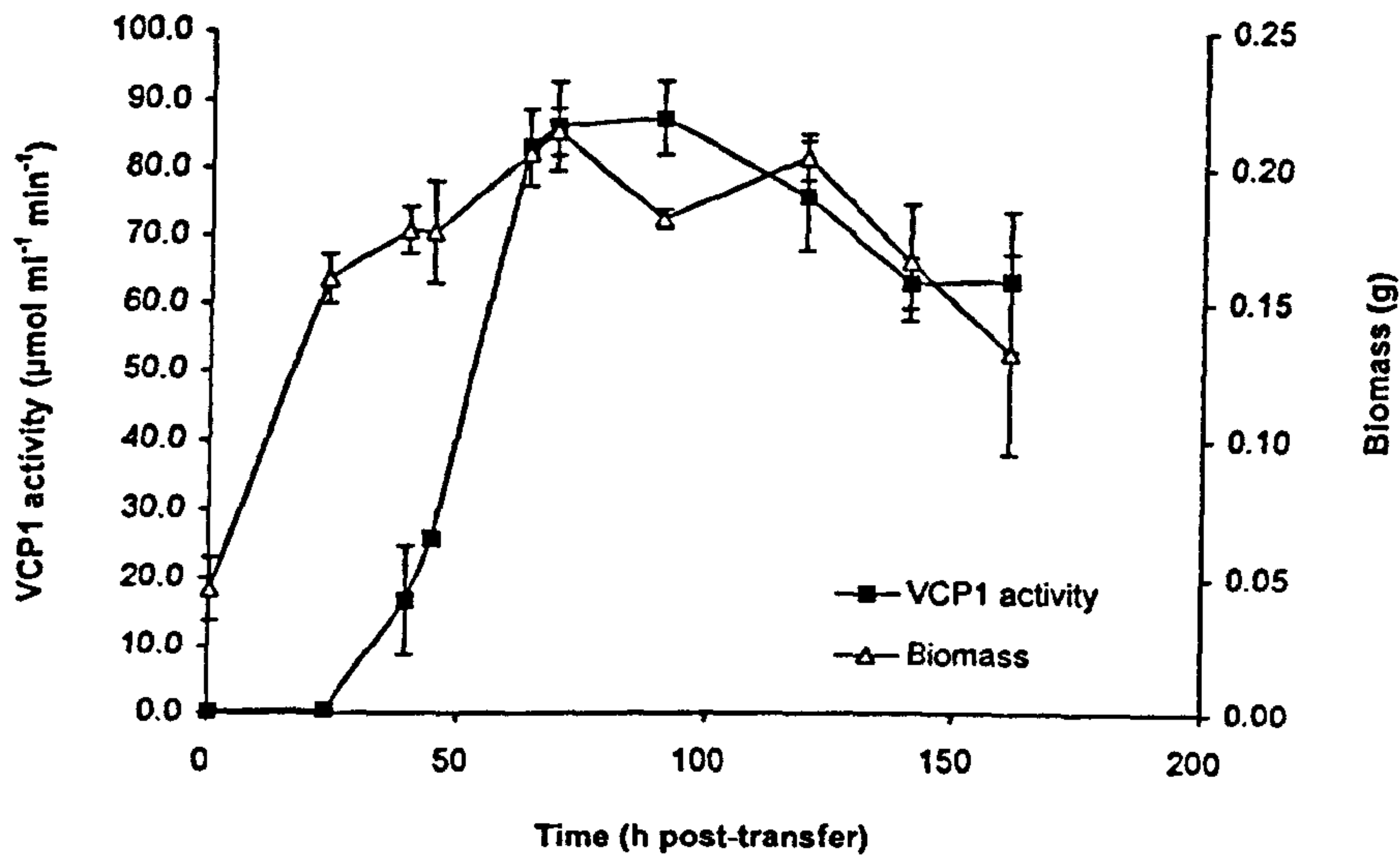


Fig. 6.2 The effect of mycelial transfer from 1% soya peptone broth to fresh medium on production of VCP1. Enzyme activity was measured as μmol nitroaniline released $\text{ml}^{-1} \text{min}^{-1}$ from $\text{suc}(\text{Ala})_2\text{Pro-Phe-pNA}$. Each data point is the means of two replicate cultures \pm S.D.

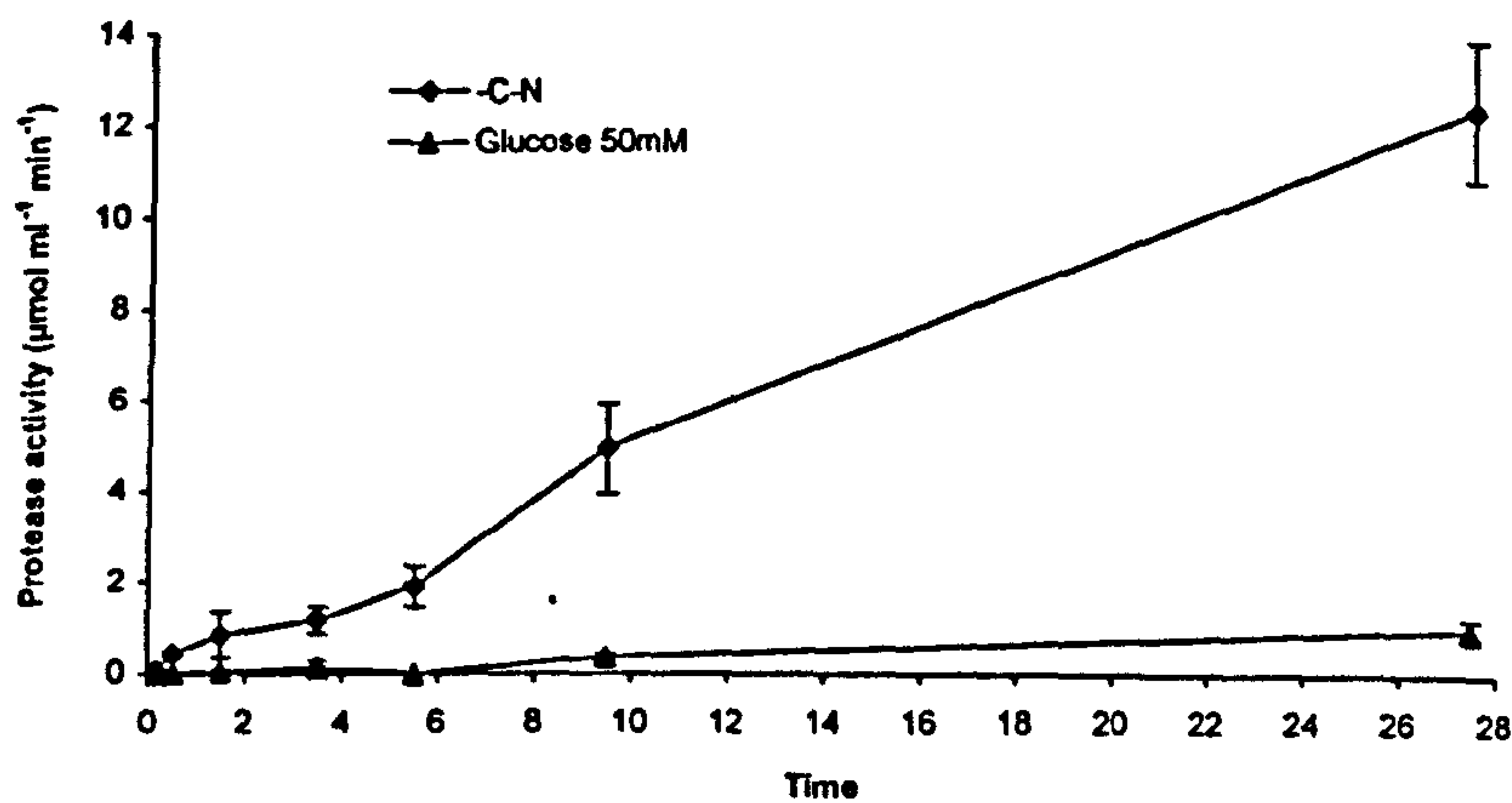


Fig. 6.3 The rate of derepression of VCP1 after transfer from soya peptone medium to nutrient-deprived conditions, or to 50 mM glucose. Enzyme activity was measured as μmol nitroaniline released $\text{ml}^{-1} \text{min}^{-1}$ from $\text{suc}(\text{Ala})_2\text{Pro-Phe-pNA}$. Time is h post-transfer. Each data point is the means of two replicate cultures \pm S.D.

To ascertain the link between nutrient content of the medium and VCP1 production, mycelium was also washed and transferred from 1% soya peptone plus 0.5% D-glucose to nutrient-deprived conditions (-C-N) and to 50 mM glucose after

4 days. In the medium devoid of carbon and nitrogen, the protease was detected as soon as 10 min after transfer, and its titre continued to increase for the duration of the sampling period, i.e. 27 h post-transfer (Fig. 6.3). On the other hand, no VCP1 was measured during the first few hours after mycelial transfer to glucose broth. At the end of the experiment, the amount of enzyme in the glucose medium was only approximately one tenth of that in nutrient-deprived conditions (Fig. 6.3).

6.3.2 Regulatory response to medium amendments

The question of which nutrients would result in repression, and whether there was also induction, i.e. protease production at a rate significantly higher than under derepressed conditions, was further investigated by transferring aliquots of pre-starved mycelium to liquid media amended with a wide range of carbon and/or nitrogen sources. The size of the experimental setup precluded recording a time-course as before, therefore all observations were made 8 h post-transfer.

The production of VCP1 was repressed by all simple carbon sources tested. Glucose (C6), arabinose (C5, abundant in plants) and lactose (dimer, normally absent in soil) were repressive to the same extent (43-44% of the control activity), but the strongly repressive effect of trehalose (dimer of D-glucose) was possibly the result of glucose, generated by trehalase activity (Table 6.1). The effect of inorganic nitrogen depended on the source. Whereas VCP1 was almost completely repressed by ammonium-nitrogen (7.9 and 0.9% of the activity of the control, in the case of 50 and 200 mM NH_4Cl , respectively), it was remarkable that the same molarities of nitrate-nitrogen did not repress, but rather enhanced the amount of VCP1, in comparison with basal, derepressed activity.

Table 6.1 Protease production by *V. chlamydosporium* isolate 10, 8 h after transfer of mycelium to media with or without C and N source. Activities are expressed as nmol nitroaniline released ml⁻¹ min⁻¹ from suc-(Ala)₂-Pro-Phe-pNA and are the means of two replicate cultures \pm S.D.

	Protease activity	Percentage of control
Control (-C -N)	109.0 \pm 10.2	100.0
<i>Meloidogyne incognita</i> eggs 0.125%	415.6 \pm 52.8	381.5
<i>Globodera rostochiensis</i> cysts 0.1%	108.6 \pm 67.9	99.7
<i>G. rostochiensis</i> cysts 0.5%	202.5 \pm 6.8	185.9
<i>Steinernema feltiae</i> 0.1%	213.8 \pm 14.0	196.2
Beetle 0.1%	91.4 \pm 3.0	83.9
Roots 0.1%	147.3 \pm 18.5	135.1
Glucose 50 mM	48.1 \pm 1.7	44.1
Arabinose 50 mM	47.4 \pm 5.4	43.5
Lactose 50 mM	47.0 \pm 1.1	43.1
Trehalose 50 mM	13.3 \pm 18.1	12.2
NH ₄ Cl 50 mM	8.6 \pm 8.7	7.9
NH ₄ Cl 200 mM	1.0 \pm 0.5	0.9
KNO ₃ 50 mM	200.5 \pm 19.3	184.0
KNO ₃ 200 mM	213.2 \pm 10.2	195.7
KNO ₃ + Glucose	25.8 \pm 1.8	23.7
Glycine 50 mM	36.9 \pm 4.7	33.9
Cellulose 1%	106.9 \pm 33.0	98.1
Chitin 1%	338.1 \pm 8.9	310.3
Soya peptone 0.1%	91.6 \pm 40.3	84.1
Soya peptone 1%	7.9 \pm 1.7	7.3
Albumin 1%	103.7 \pm 18.7	95.2
Gelatin 1%	4.3 \pm 2.6	3.9
Collagen 1%	239.8 \pm 47.4	220.1
Collagen 0.5% + chitin 0.5%	333.1 \pm 48.2	305.8

The combination of nitrate and glucose, however, repressed VCP1, more than glucose alone. Glycine (50 mM) was applied both as carbon and nitrogen sources, and found to repress the enzyme to about a third of its basal level (Table 6.1). Whereas 0.1% soya peptone had little effect on the protease production, 1% strongly repressed it after 8 h. Soya peptone presumably contains high levels of small peptides, amino acids and other readily utilisable carbon and/or nitrogen sources, resulting in repression of the protease. Cellulose is a carbon source that the fungus is likely to encounter in the rhizosphere, but its effect on VCP1 production, even at a concentration as high as 1%, was neutral. High VCP1 titres were obtained in liquid

media containing chitin and collagen (3.1 and 2.2 times the amount produced by the derepressed fungus, respectively). The combined effect of these inducers was slightly more than additive (Table 6.1), possibly suggesting different signalling pathways for the inductive components as any repressive effect is avoided by using each of them at only 0.5% (w/v). Remarkably, insoluble, fibrous collagen enhanced protease production, while the same concentration of soluble gelatin strongly repressed it. The inductive effect of proteins was clearly not a generic response, as albumin was another protein amendment that did not induce VCP1 (Table 6.1). The clearest induction, however, was seen when *V. chlamydosporium* was exposed to *M. incognita* eggs (0.125% [w/v]), resulting in almost four times the amount of VCP1 that was produced by derepression only. Similar concentrations of *G. rostochiensis* cysts had no effect, while a small but measurable increase in the amount of VCP1 was recorded with much higher concentrations of cysts, i.e. 0.5% (w/v). Another collagen-containing amendment was the vermiform nematode *Steinernema feltiae*, which also resulted in increased protease activity in the medium. On the other hand, the complex nutrient source provided by homogenized beetles had no effect on VCP1. If the protease is induced by a chitinous elicitor, then this result suggests that the particular organisation of the chitin fibrils, or the complex matrix in which it is embedded in insect cuticle (St. Leger, 1991) may have precluded induction of the *V. chlamydosporium* protease.

Derepression and induction are the two distinct types of enzyme regulation that were observed with VCP1. Attempts were made to identify some components of the regulatory pathways involved. Looking at the cascade of events, from the

protease gene down to secretion of active enzyme, several stages could be the regulatory bottleneck.

Table 6.2 Protease production by *V. chlamydosporium* isolate 10, 8 h after transfer of mycelium to media without C and N source, but including a metabolic inhibitor or stimulant. Activities are expressed as nmol nitroaniline released ml⁻¹ min⁻¹ from suc-(Ala)₂-Pro-Phe-pNA and are the means of two replicate cultures ± S.D.

Effector	Mode of action	Protease activity	Percentage of control
None (control)	-	109.0 ± 10.2	100.0
Ethidium bromide (50 µg/ml)	Inhibits DNA synthesis ^a	111.4 ± 14.9	102.2
Actinomycin D (50 µg/ml)	Inhibits DNA-primed RNA polymerase ^a	25.4 ± 4.4	21.5
Cycloheximide (10 µg/ml)	Inhibits initiation, elongation and termination of proteins ^a	10.7 ± 0.7	9.8
cAMP 5 mM	various ^b	65.4 ± 2.0	60.0
cAMP 5 mM + Glucose 50 mM		60.6 ± 14.9	55.6
Cyclosporin 5 µM	Inhibits calmodulin-binding kinases ^c	17.0 ± 2.6	15.6
Cyclosporin 5 µM + Glucose 50 mM		21.9 ± 6.0	20.1
Cyclosporin 5 µM + NH ₄ Cl 200 µM		0	0
Lysocellin 5 µM	Inhibits IP3-dependent kinases ^d	27.3 ± 15.5	25.1
Lysocellin 5 µM + Glucose 50 mM		33.8 ± 16.6	31.0
Lysocellin 5 µM + NH ₄ Cl 200 µM		0	0

^a Dawson et al. (1986); ^b Pall (1981); ^c Borel et al. (1994); ^d Imoto et al. (1990)

Table 6.3 Protease production by *V. chlamydosporium* isolate 10, 8 h after transfer of mycelium to inductive media, with 1 % (w/v) collagen, and a metabolic inhibitor or stimulant. Activities are expressed as nmol nitroaniline released ml⁻¹ min⁻¹ from suc-(Ala)₂-Pro-Phe-pNA and are the means of two replicate cultures ± S.D.

Effector	Mode of action	Protease activity	Percentage of control
None (collagen 1%)	-	239.8 ± 47.4	100.0
Hydroxyurea (100 µg/ml)	Inhibits DNA synthesis ^a	249.2 ± 24.7	103.9
Actinomycin D (50 µg/ml)	Inhibits DNA-primed RNA polymerase ^a	56.1 ± 7.4	23.4
Cycloheximide (10 µg/ml)	Inhibits initiation, elongation and termination of proteins ^a	20.0 ± 4.2	8.3
Adenosine 5' O-thio-monophosphate (100 µg/ml)	Inhibits cAMP dependent kinases ^a	155.0 ± 41.8	64.6
Glucose 5 mM		216.8 ± 32.7	90.4
Glucose 50 mM		74.2 ± 15.0	30.9
NH ₄ Cl 50 mM		110.7 ± 15.6	46.2

^a Dawson et al. (1986)

The protease that was secreted as the result of derepression or induction, was not the product of newly formed DNA, as the DNA synthesis inhibitors ethidium bromide

or hydroxyurea did not have any effect (Tables 6.2 and 6.3). Actinomycin D, an inhibitor of DNA-primed RNA polymerase (Dawson *et al.*, 1986), significantly reduced the protease titre, suggesting that regulation was controlled at the level of transcription. It was no surprise that inhibitory events, downstream of transcription, also interfered with protease production. Inhibition of translation by cycloheximide reduced the amount of VCP1 produced in conditions of derepression or induction. In some systems, notably enteric bacteria, catabolite repression is relieved by cAMP (Pall, 1981). That was apparently not the case in *V. chlamydosporium*, because the addition of 5mM of cAMP to 50 mM glucose (Table 6.2) had approximately the same effect on VCP1 as glucose by itself (Table 6.1). On its own, cAMP had some inhibitory effect as it reduced the VCP1 activity to 60% of that of the control. The indirect involvement of the second messenger calcium in protein phosphorylation events was verified by using cyclosporin, an inhibitor of calmodulin-binding protein kinases (Borel *et al.*, 1994). Cyclosporin reduced the protease activity to 15.6% of that of the control, and including the inhibitor with glucose or NH_4Cl reduced the remaining amount of protease, normally produced in those conditions, even further. This suggests that calmodulin-binding kinases are required for expression of VCP1, in both repressed and derepressed conditions. Lysocellin, an inhibitor of inositol 1,4,5-triphosphate (IP_3) (Imoto *et al.*, 1990) had similar effects as cyclosporin, also suggesting the importance of phosphorylation events that are linked to IP_3 , in the production of VCP1. A third type of phosphorylation was detected in inductive conditions (i.e. transfer to collagen). Adenosine 5' O-thiomonophosphate, which

inhibits cAMP-dependent kinases (Dawson *et al.*, 1986), reduced VCP1 activity to 64.6% of that of the control.

Cellulose is a polymer that is not found in the nematode host, but it is abundant in the rhizosphere habitat. Therefore, cellulose hydrolysis would occur when the fungus grows saprotrophically. Some degree of adaptation was suggested by the significant induction of cellulolytic activity ($P < 0.001$, ANOVA) after transfer to cellulose-containing medium (Table 6.4). The *V. chlamydosporium* cellulase(s) did not respond to chitin, collagen or nematode eggs, as approximately the same basal activity as in the -C-N medium was observed.

Table 6.4 Cellulase production by *V. chlamydosporium* isolate 10 after transfer from nutrient-rich medium to the media indicated. Enzyme activity was measured as the hydrolysis of cellulose azure (10 mg ml⁻¹) at pH 5.8. One unit of cellulase activity is the amount of enzyme that gave an increase in A_{570} of 0.1 absorbance unit h⁻¹ under the conditions of the assay. Values were corrected for autolysis of the substrate and are the means of two separate cultures \pm S.D.

Transfer medium	Cellulase activity
Control (-C -N)	1.50 \pm 0.16
<i>Meloidogyne incognita</i> eggs 0.125%	1.49 \pm 0.01
Cellulose 1%	4.23 \pm 0.04
Chitin 1%	1.54 \pm 0.06
Collagen 1%	1.30 \pm 0.01

6.3.3 Effect of C and N proportions on growth and pathogenicity *in vitro*

Before the impact of the amount and proportion of carbon and nitrogen in the medium on fungal infectivity was assessed, some aspects of the C and N dependence of the growth of *V. chlamydosporium* were determined. The submerged culture system was used because biomass and VCP1 production could easily be measured.

In the following, "C/N ratio" is expressed as mM glucose/mM NH₄Cl. Not unexpectedly, there was a dependence of biomass generated, on the C/N ratio of the medium. Whereas the absence of C and N resulted in 5.8 mg dry biomass, supplying as little as 1 mM glucose and 1 mM NH₄Cl, doubled that amount ($P < 0.05$, one-tailed t-test), and a C/N ratio of 100/100 gave a biomass that was almost twenty times higher ($P < 0.001$) (Table 6.5).

Table 6.5 Characteristics of submerged cultures of *V. chlamydosporium* isolate 10 grown in media with varying "C/N ratios", expressed as mM glucose/mM NH₄Cl. The culture supernatants were concentrated by dialysis on crystalline sucrose, and subtilisin-like activity expressed as nmol nitroaniline released ml⁻¹ min⁻¹ from suc-(Ala)₂-Pro-Phe-pNA. All values are means of three replicate cultures \pm S.D. Concentrate colour "Y" indicates yellow and viscous culture concentrate.

"C/N ratio"	Biomass (mg)	Supernatant			
		Colour	Protein (μ g/ml)	Protease activity (nmol ml ⁻¹ min ⁻¹)	Specific activity (nmol μ g ⁻¹ min ⁻¹)
0/0	5.8 \pm 1.7		4.7 \pm 1.5	0	0
1/1	11 \pm 0.4		25.7 \pm 5.9	444.9 \pm 76.5	17.9 \pm 4.3
1/10	15.2 \pm 1.6		42.3 \pm 7	392.5 \pm 74.2	9.3 \pm 1.3
1/100	15.6 \pm 1.2		55.3 \pm 9.9	505.0 \pm 67.4	8.3 \pm 7.1
10/1	55.1 \pm 5.5	Y	47.7 \pm 9.1	0	0
10/10	59.2 \pm 18		57.3 \pm 2.1	217.8 \pm 44.0	3.8 \pm 0.9
10/100	66.9 \pm 15		63.7 \pm 9.7	0	0
100/1	76.6 \pm 18	Y	38.3 \pm 1.5	9.3 \pm 16.2	0.2 \pm 0.4
100/10	96.6 \pm 7.6	Y	72 \pm 11	36.4 \pm 53.7	0.4 \pm 0.6
100/100	104.2 \pm 7.9	Y	58 \pm 9.2	0	0

The main determinant of growth was the amount of carbon in the medium ($P < 0.0001$, ANOVA), rather than nitrogen ($P < 0.05$), with relatively small increases when more ammonium was added ($P > 0.05$) (Table 6.5). The amount of protein secreted in the medium followed a similar pattern, as it increased with the carbon concentration ($P < 0.01$), but increasing the nitrogen content of the medium enhanced protein secretion significantly ($P < 0.0001$). The enzyme activity, however, depended on the C/N ratio in a different way. There was no protease detected in the control, in

the samples with a C/N ratio of 10/1, presumably through carbon repression, and in those with C/N ratio of 10/100, possibly through repression by ammonium. Little or no protease was secreted in all media with 100 mM glucose, and on the whole, the carbon content of the medium was the main determinant of protease production ($P < 0.0001$). Expressing the enzyme production as specific activity ($\text{nmol nitroaniline } \mu\text{g}^{-1} \text{ min}^{-1}$), however, revealed a significant interaction between the carbon and nitrogen content of the media ($P < 0.01$). A yellow, very viscous concentrate was obtained in those media where the molarity of glucose numerically exceeded that of ammonium (Table 6.5). The fungus may have secreted mucilage and a pigment as a way of excreting excess carbon.

The bioassay system, as described in Chapter 5, was modified to examine the effect of carbon and nitrogen in the medium on pathogenicity of *V. chlamydosporium*. The results (Table 6.6) revealed that almost no *M. incognita* eggs were infected in the media that contained 10 mM glucose or more, independent of the nitrogen status. There was some similarity with the dependence of VCP1 activity on the C/N ratio of the medium, since the carbon content of the medium was the main effect, although the impact of ammonium concentration was also important. As in the case of the specific activity of VCP1, the interaction between carbon and ammonium was significant ($P < 0.01$ after 8, and $P < 0.001$ after 16 days post-inoculation). The medium that resulted in the greatest number of eggs infected, was the one amended with 1 mM glucose and 1 mM NH_4^+ . Although the infection rate on that medium appeared much greater than that on the control medium where no carbon or nitrogen had been added, a significant difference ($P < 0.05$, one-tailed t-test) between C/N 0/0

and 1/1 was seen only 12 days post-inoculation. Remarkably, when nitrate was used as a nitrogen source, rather than ammonium, very low infection rates were seen, at all carbon concentrations tested (Table 6.6). This effect on pathogenicity did not relate to the effect of nitrate on the regulation of VCP1 (Table 6.1).

Table 6.6 Pathogenicity of *V. chlamydosporium* isolate 10 against *M. incognita* eggs on water agar media with varying "C/N ratios". Pathogenicity is expressed as the percentage of eggs infected by the fungus, 8, 12 or 16 days after placing nematode eggs on growing fungal colonies. The nutrient status of the medium is expressed as mM glucose/mM NH₄Cl added, except in *, where it is mM glucose/mM KNO₃.

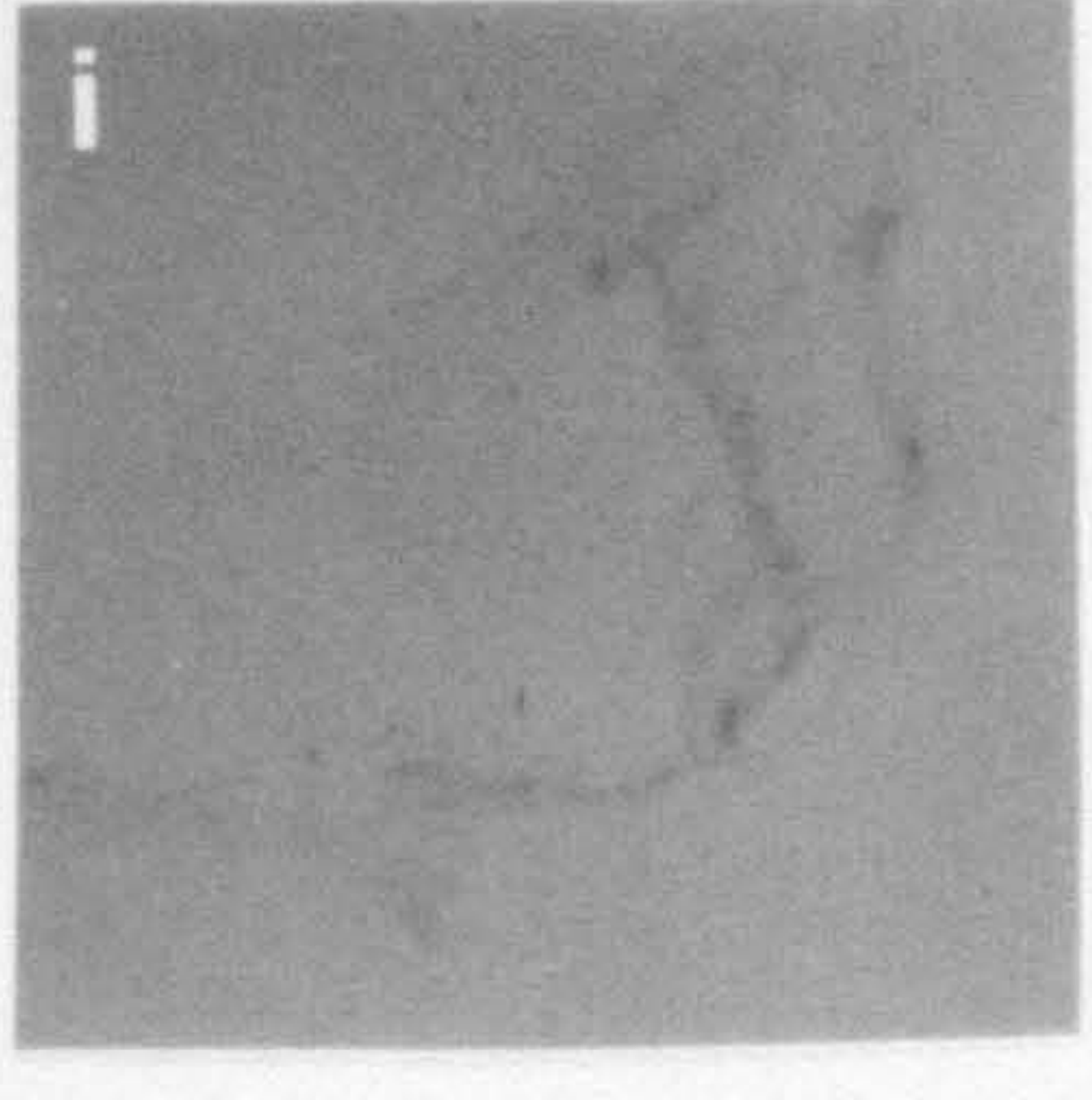
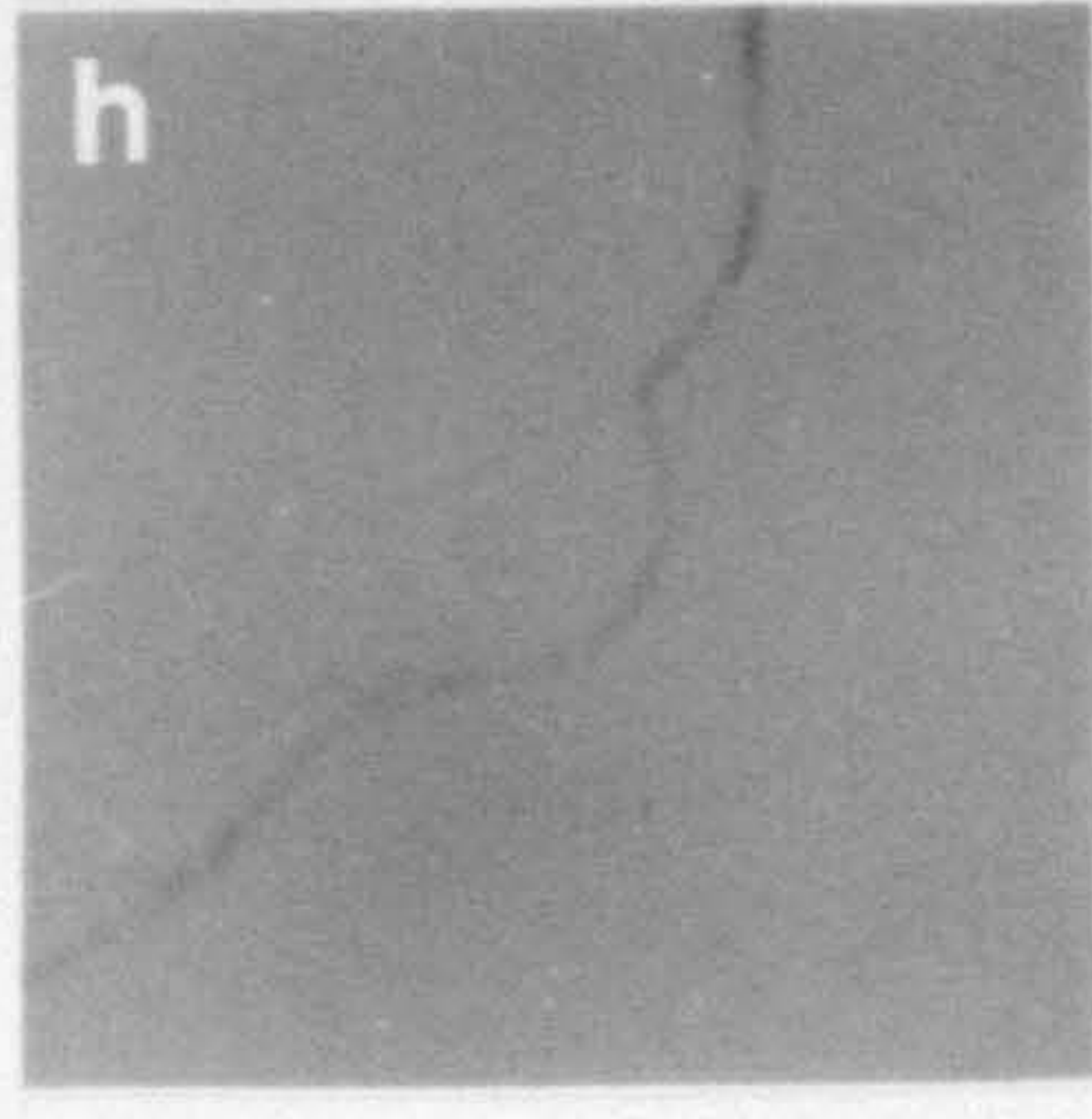
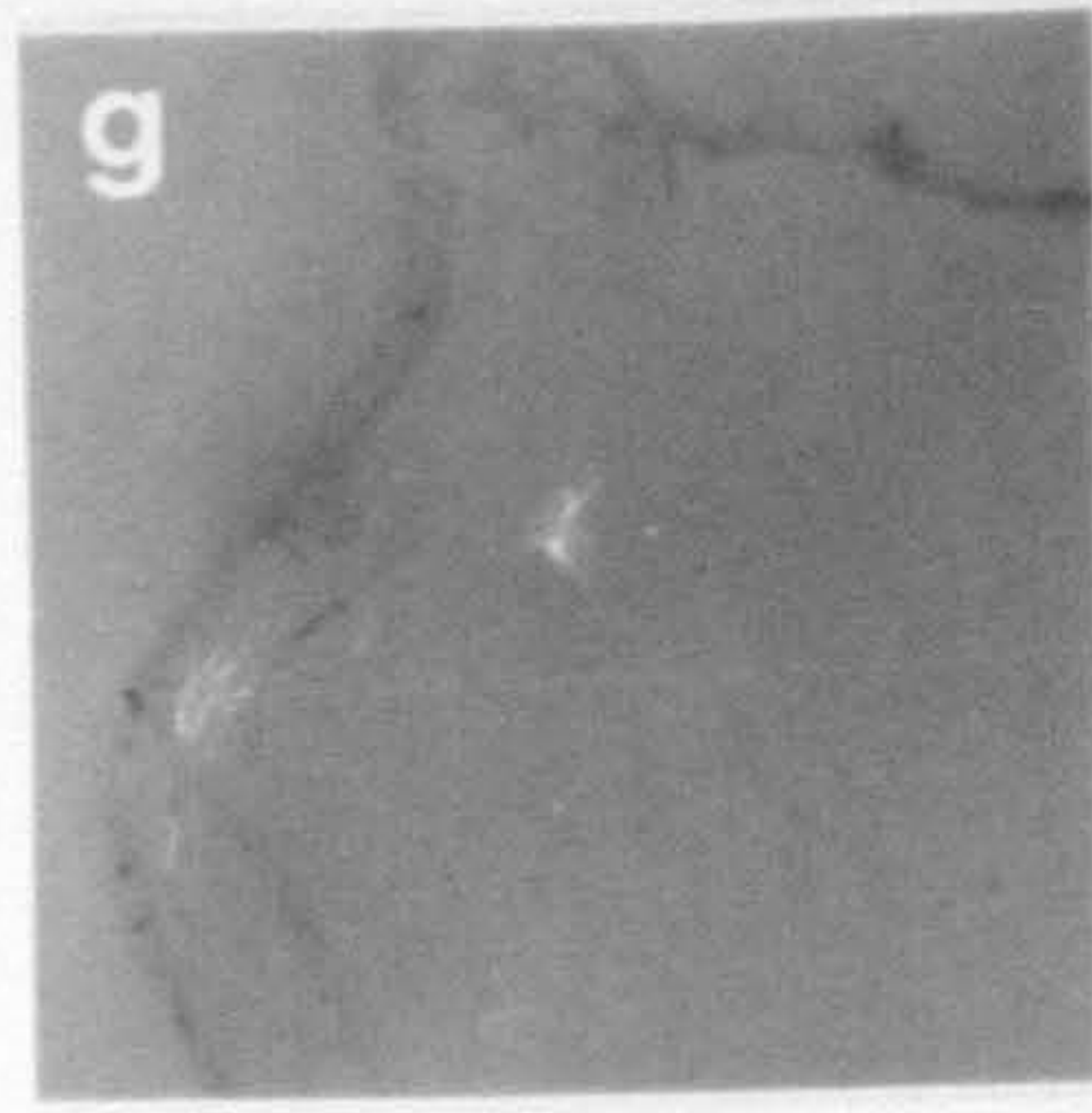
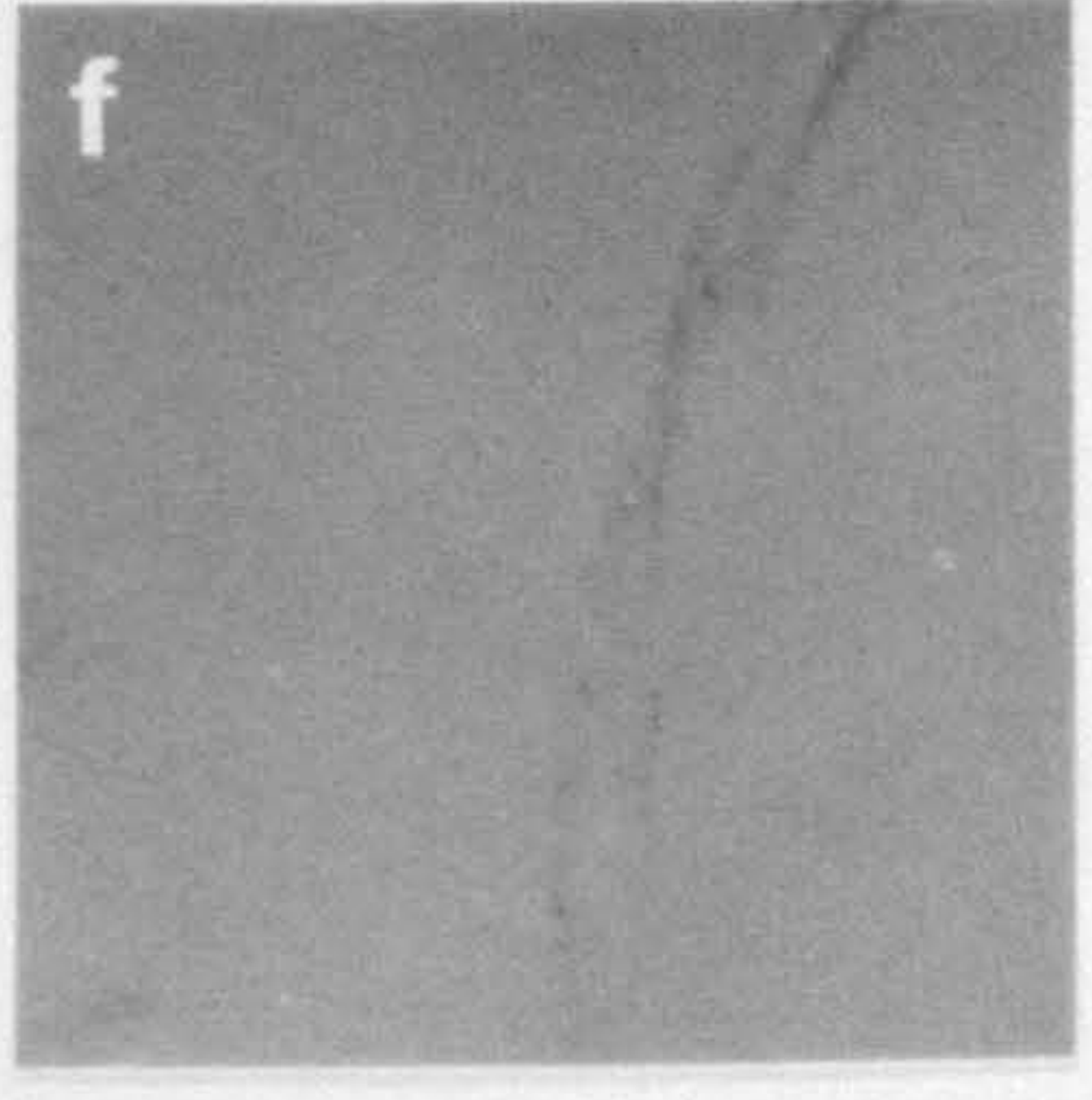
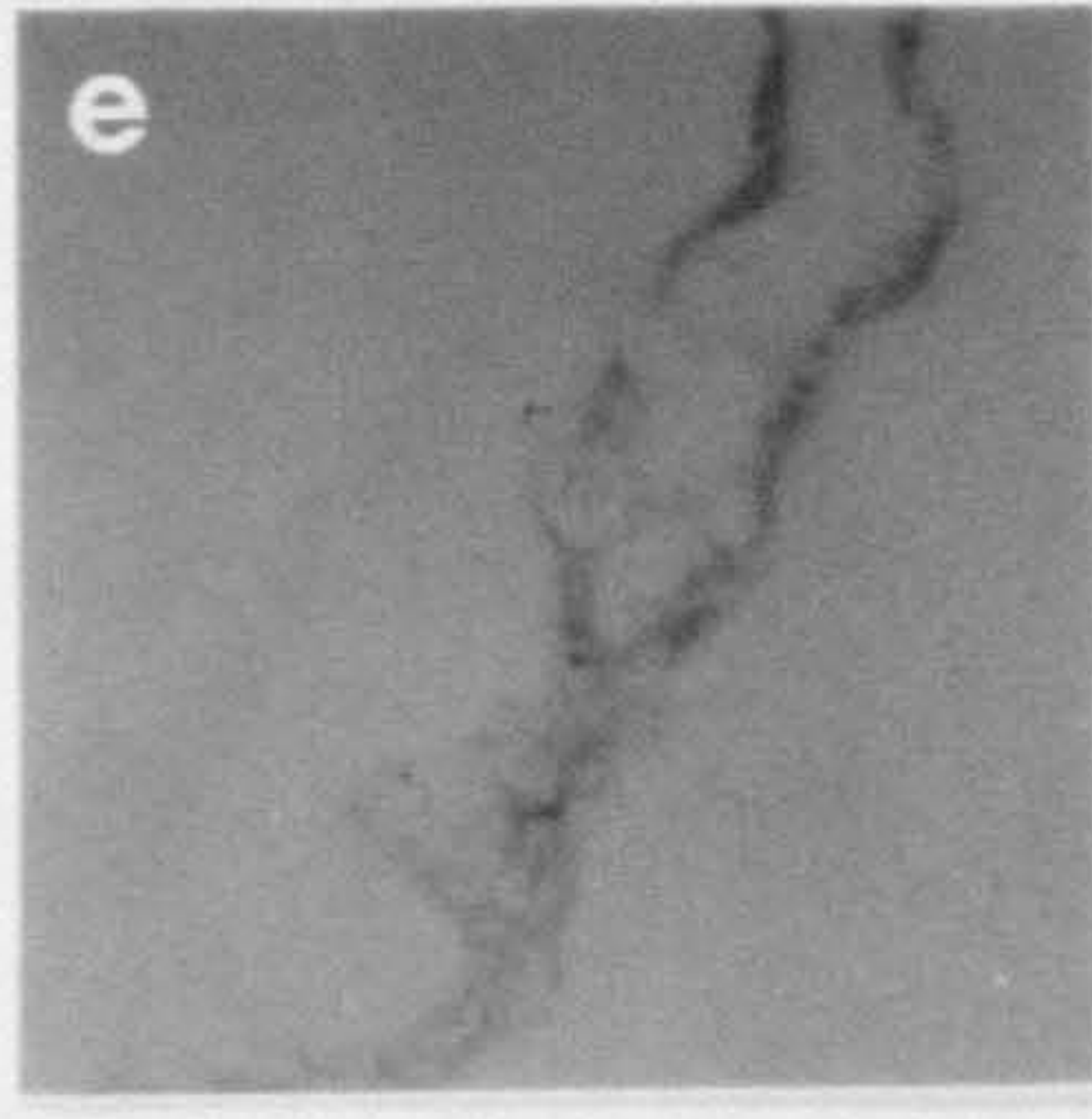
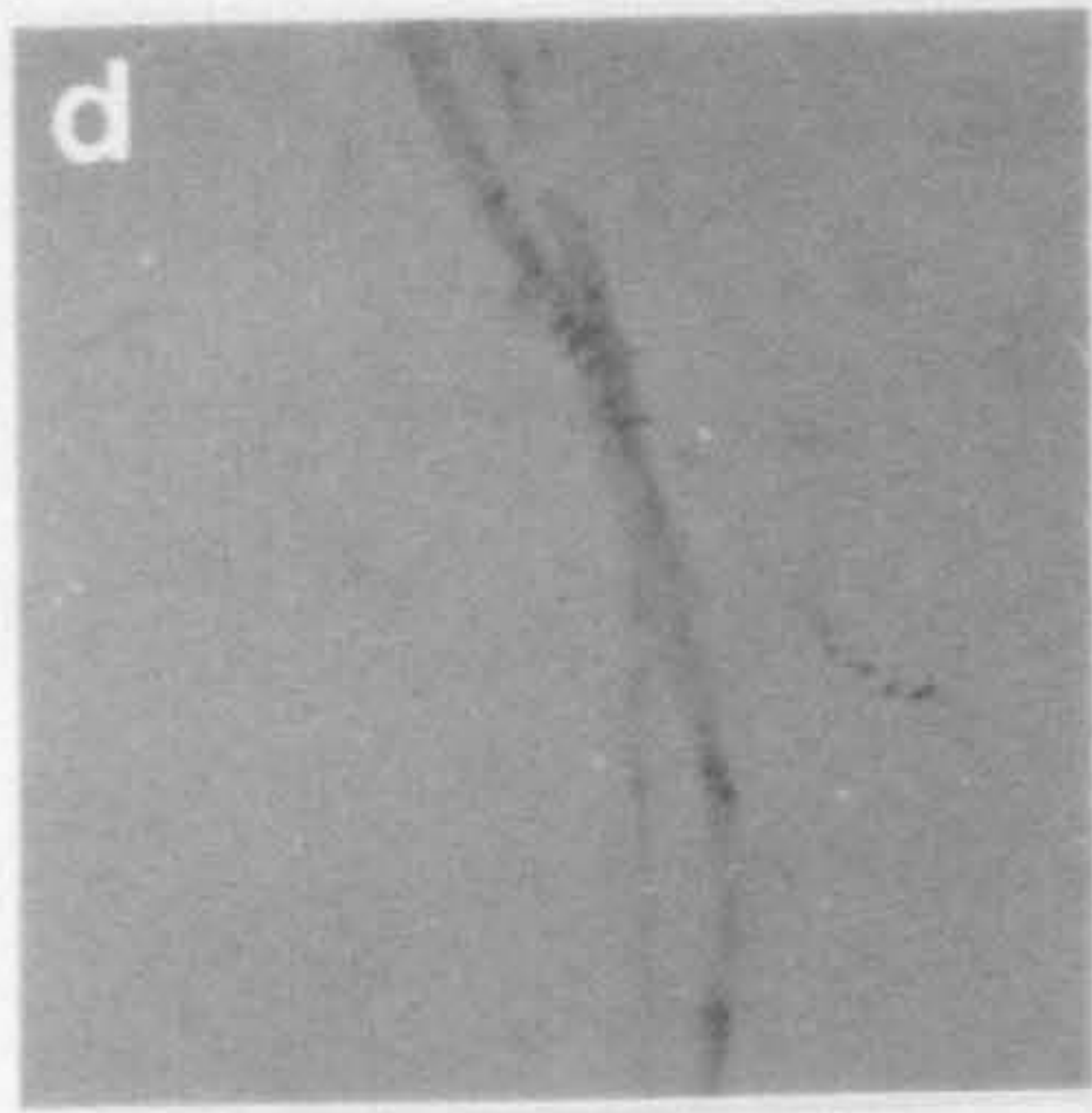
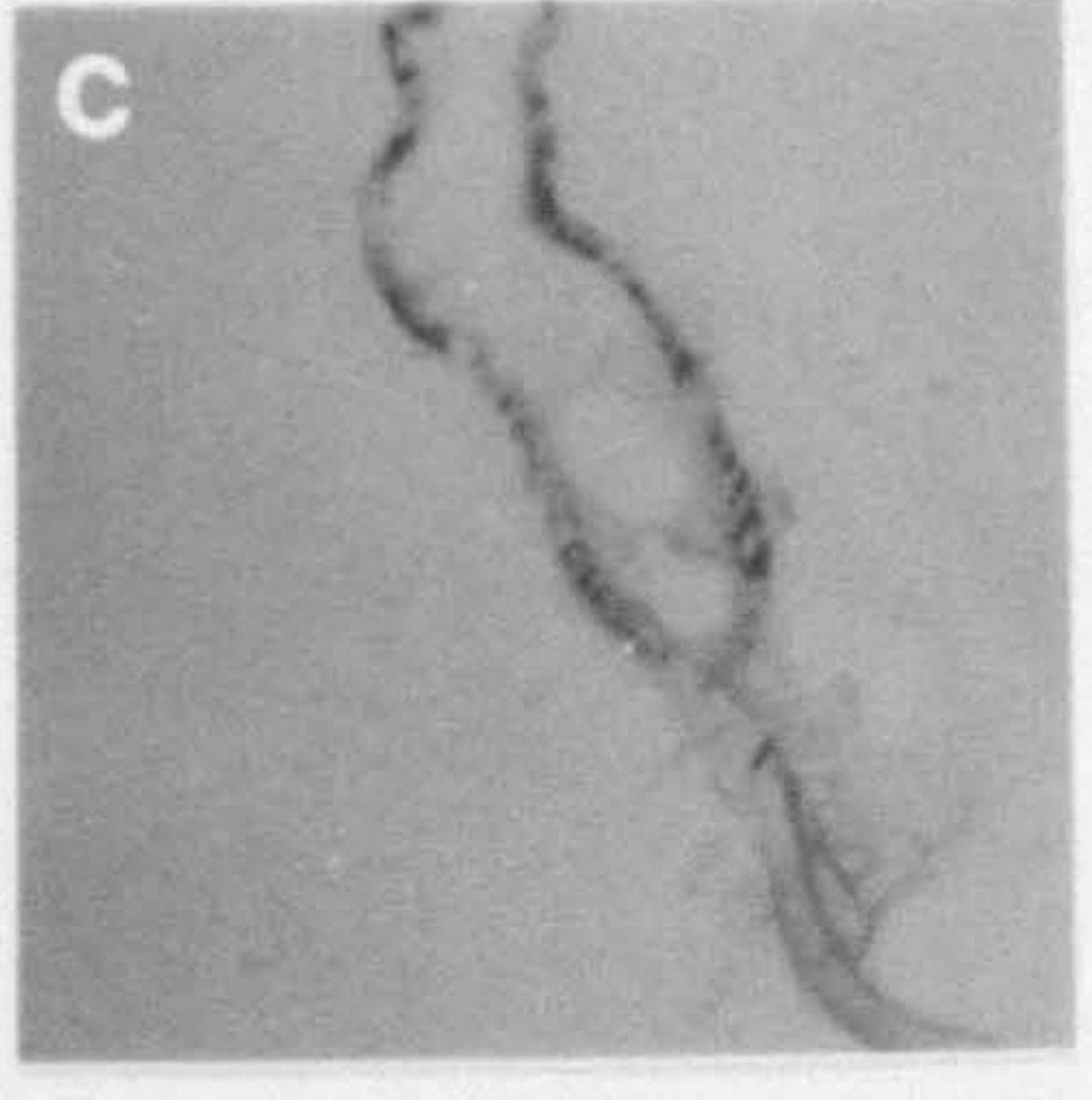
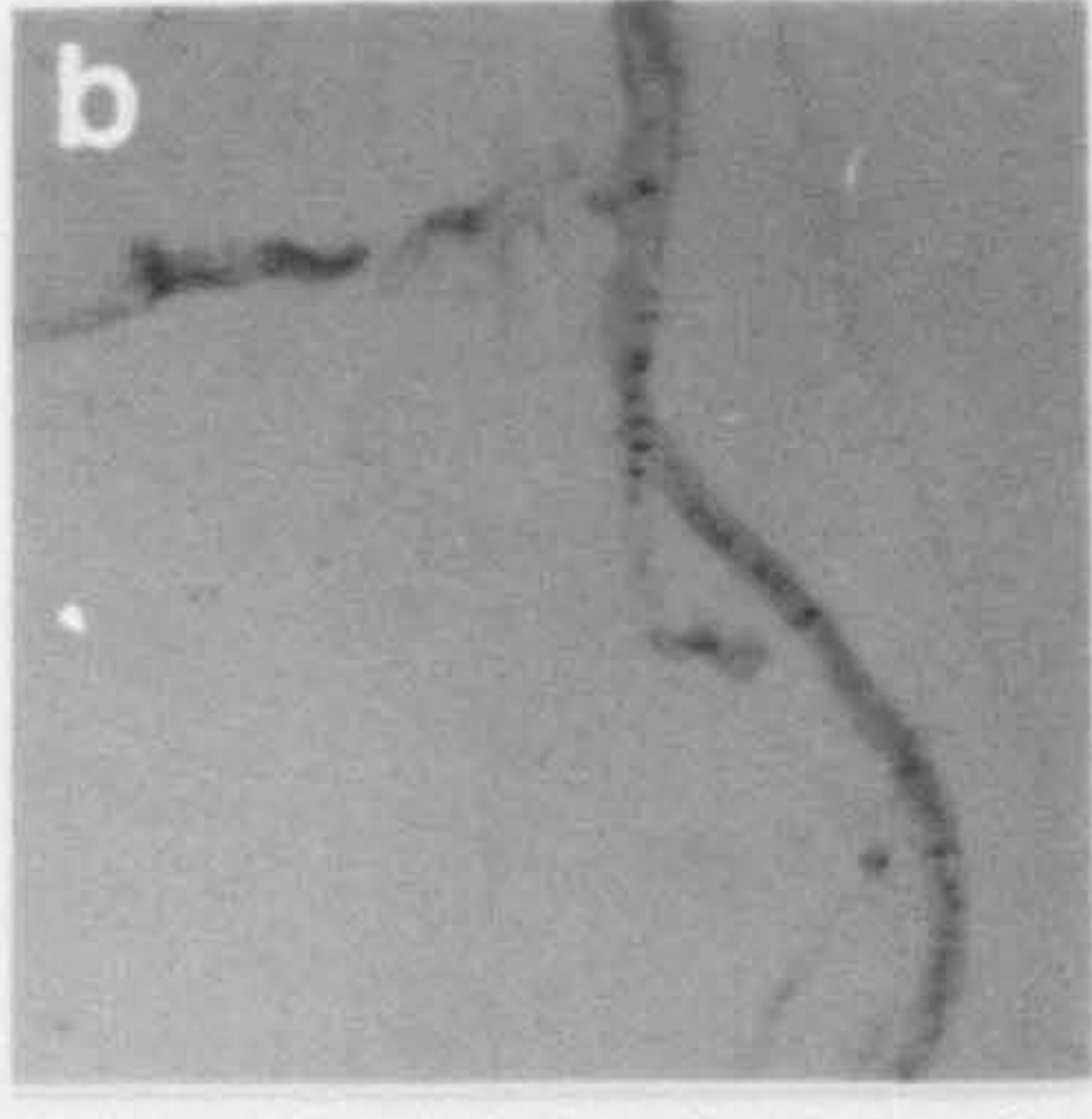
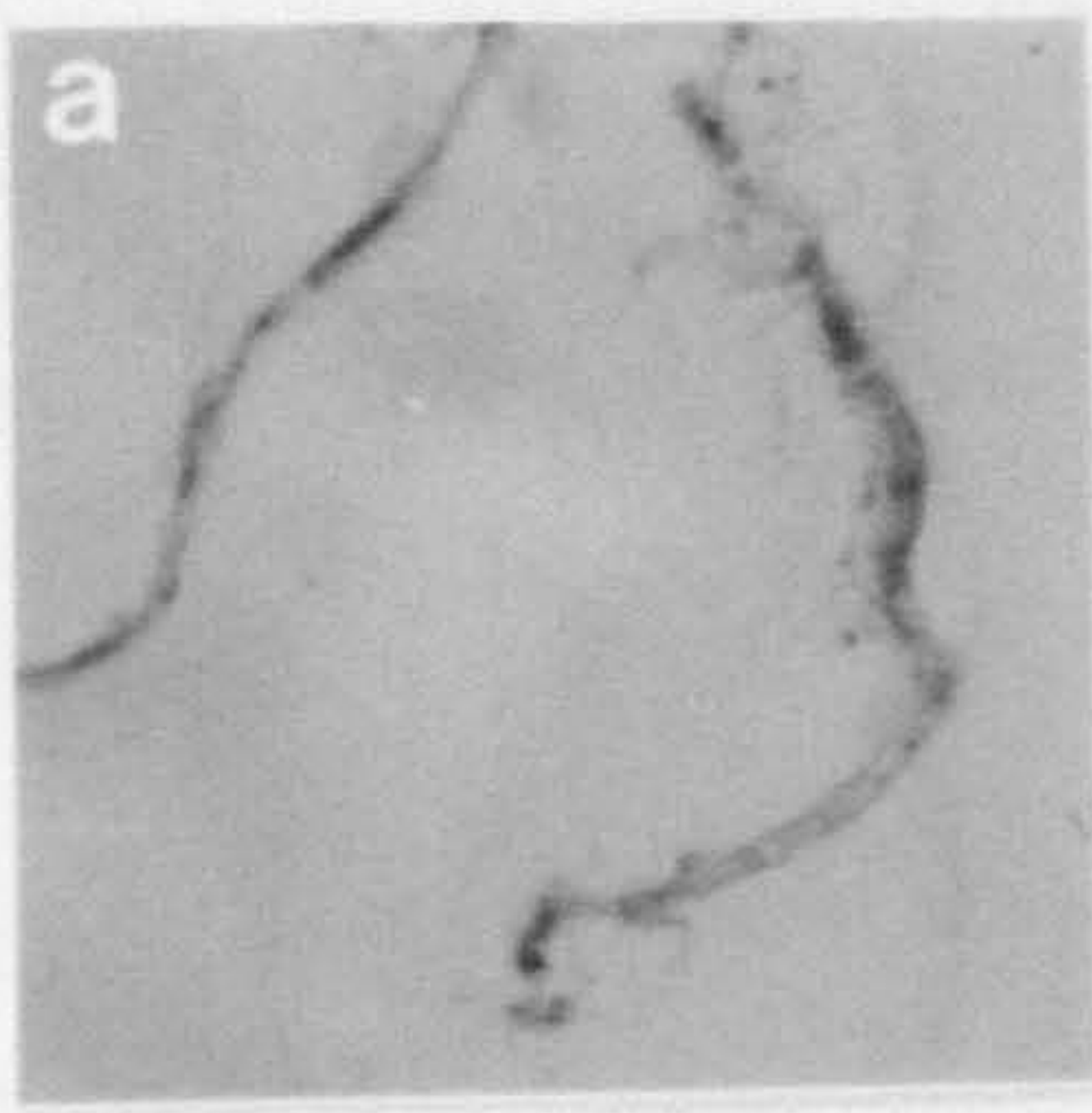
"C/N ratio"	Infection rate (% eggs infected)		
	8 days	12 days	16 days
No fungus	0.7	0.7	0.7
0/0	13.3	14.0	34.0
1/1	26.0	17.3	50.6
1/10	16.7	24.0	47.4
1/100	5.3	27.3	24.0
1/100 NO ₃ ⁻ *	4.7	6.7	4.7
10/1	0.7	0.7	2.7
10/10	0.7	0.7	0.7
10/100	0	0	0
10/100 NO ₃ ⁻ *	0	0.7	2.0
100/1	0	0.7	0.7
100/10	0	0	0
100/100	0	0	0
100/100 NO ₃ ⁻ *	2	3.3	2.7

6.3.4 *In situ* detection of VCP1 in the rhizosphere of tomato

At the initial antibody titre of 1/25, the "Western" type of detection of VCP1 production by *V. chlamydosporium* on the surface of tomato roots, was prone to false positives. All root systems, including those of the control, gave a positive result (not shown). An additional blocking step with pre-immune serum (1/10 in PBST) was to no avail. To verify that the plant roots did not secrete subtilisin-like proteases, that could be antigenically similar to the fungal enzyme, a protease assay was done on homogenized roots. No enzyme was detected with the common VCP1 substrate suc-

Fig. 6.4 "Western" detection of VCP1 on root replica's of tomato. Titration of the pre-immune serum to eliminate non-specific binding of the anti-VCP1 polyclonal antibody to the root surface, using tomato plants not inoculated with nematodes or fungus. Antiserum dilutions were a) 1/20, b) 1/50, c) 1/100, d) 1/150, e) 1/200, f) 1/300, g) 1/500, h) 1/800, and i) 1/1000.

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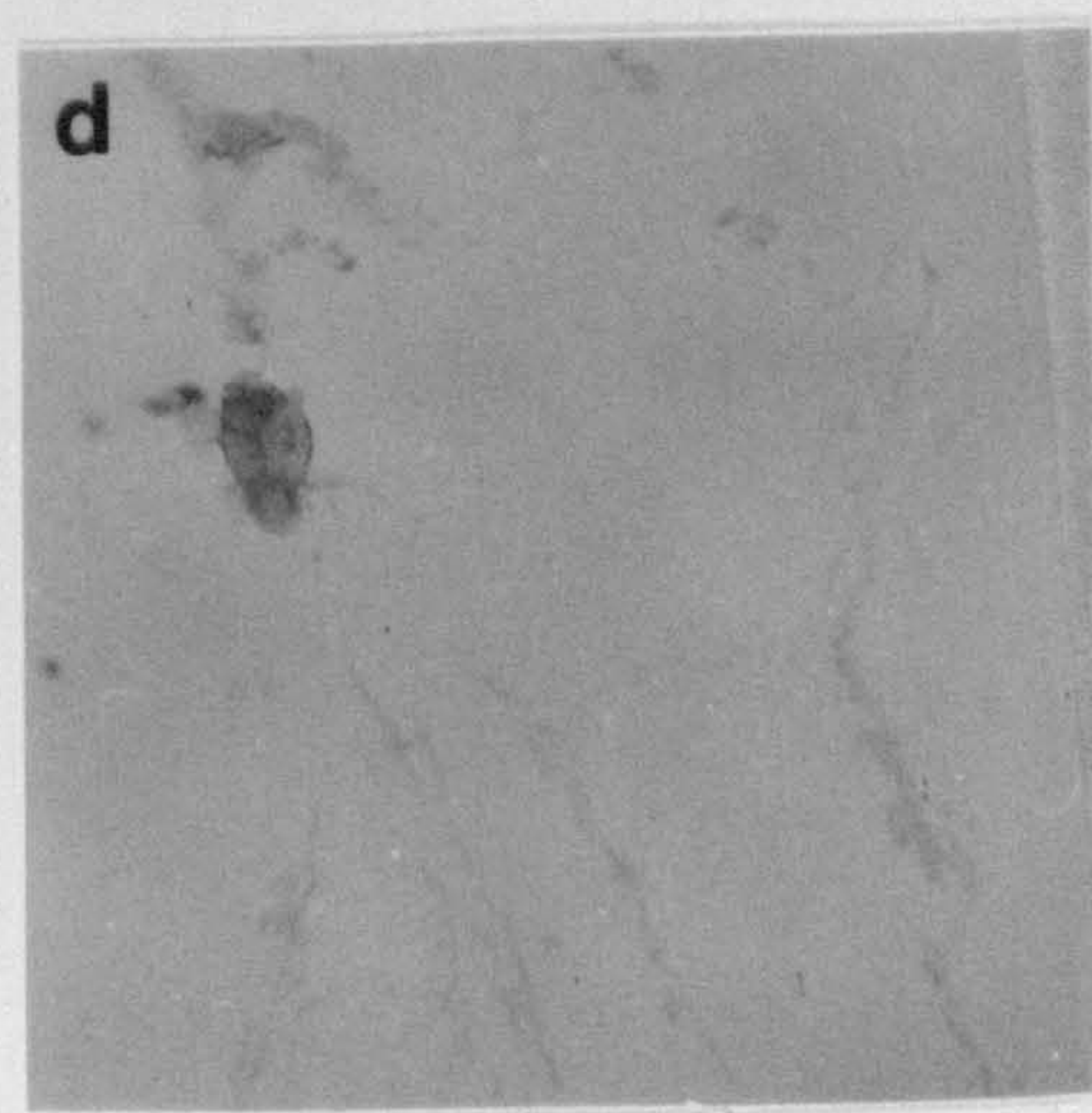
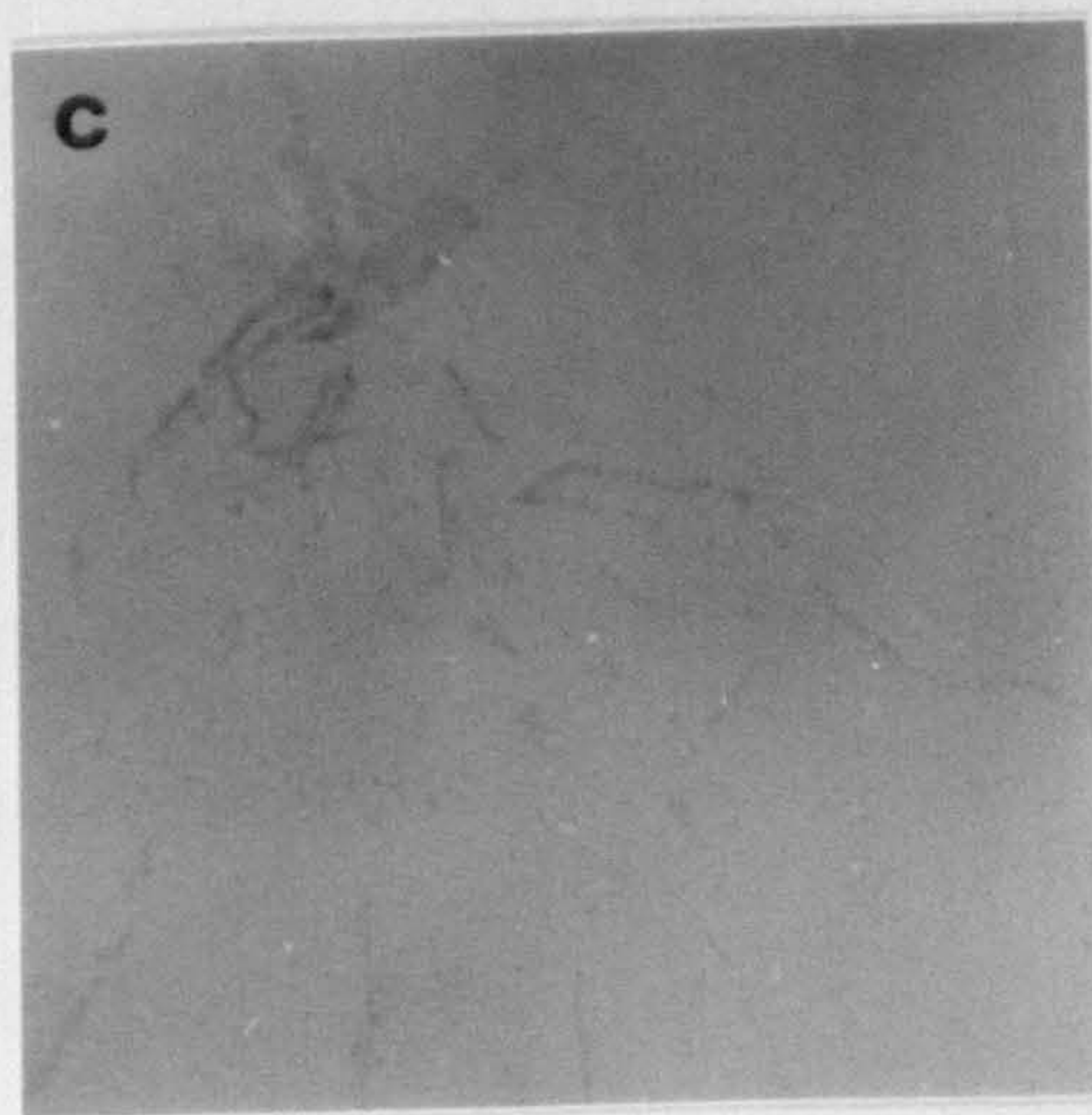
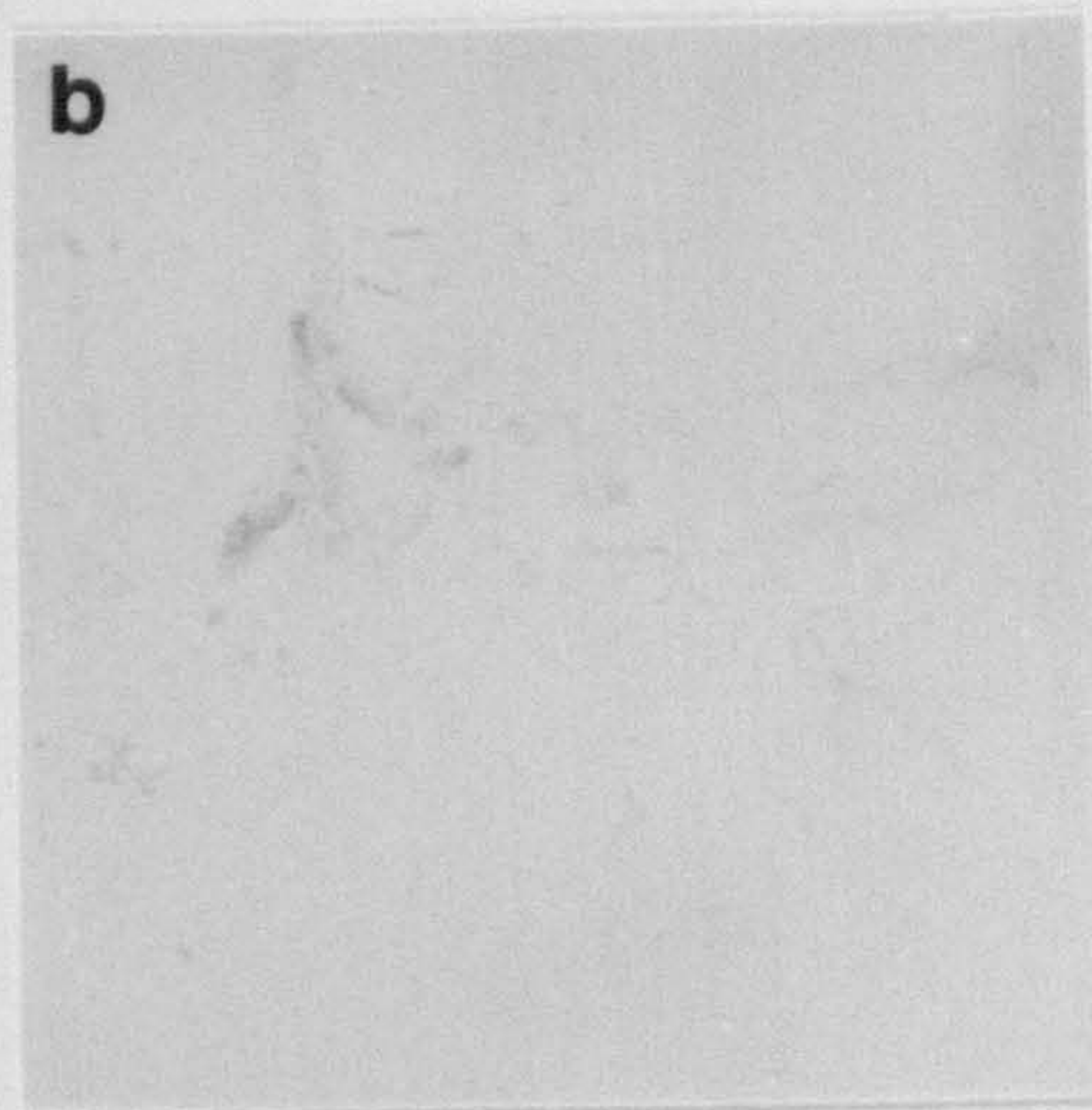
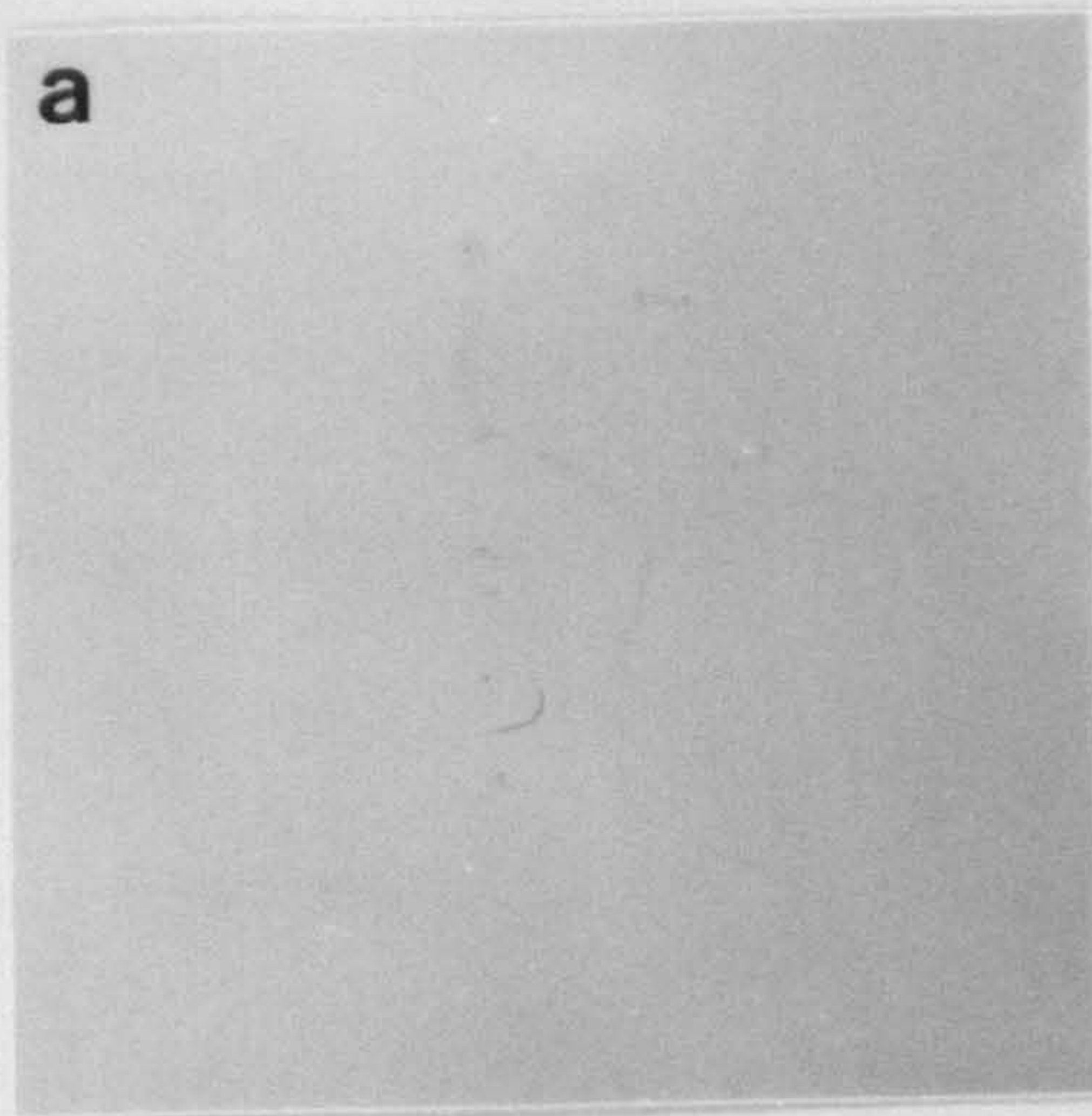
(Ala)₂-Pro-Phe-pNA. A titration was made by developing blots of root fragments with dilutions of the polyclonal, from 1/20 to 1/1000. A trace of the whole root system was visible at all dilutions, although stronger at the lower dilutions (Fig. 6.4). Subsequent blots were developed with the antibody at a dilution of 1/1000. A faint outline of all root systems was visible in the control and in all treatments. There appeared to be little difference between control plants and those with nematodes and/or fungus (Fig. 6.5). Switching to a different secondary antibody, conjugated with horseradish peroxidase, gave no improvement. Since the antibody concentration, required to get a positive signal in Western blots (see Chapter 3) was much greater than the concentration used here, it seems likely that all positives obtained with root replica's were the result of non-specific binding of IgG's to a component on tomato roots. Similar results (not shown) were obtained with kale (*Brassica oleracea*), that also supports growth of *V. chlamydosporium* in its rhizosphere (Bourne *et al.*, 1994).

6.4 DISCUSSION

Without the presence of inducing molecules, or in those systems that are not prone to induction, the production of extracellular protease is often observed at the end of active growth and during the stationary phase in batch cultures. This occurs widely in bacteria (Allison & MacFarlane, 1990; Bascarán *et al.*, 1990) and also in fungi (Ebeling *et al.*, 1974; St. Leger *et al.*, 1991c; Micales, 1992). Proteases whose production is concurrent with exponential growth usually appear not to be prone to catabolite repression (Hellmich & Schauz, 1988; Zhu *et al.*, 1994; Hoffert *et al.*,

Fig. 6.5 "Western" detection of VCP1 on root replica's of tomato. Treatments were a) control plant, b) tomato plant with *M. incognita* galls, c) tomato plant with *V. chlamydosporium*, and d) combination treatment with nematodes and fungus.

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1995). In *V. chlamydosporium*, in the absence of inducer, protease production was also incompatible with high growth rates. The cue for protease production seemed to be the suboptimal growth conditions at the end of exponential growth in batch culture, or after transfer to nutrient-poor medium. It is likely that the actual cue for enhanced protease production is nutrient deprivation. However, it cannot be ruled out from this experiment that protease was possibly only secreted when the initial repressive effect of simple nitrogen and carbon sources in the complex soya peptone medium was alleviated, followed by induction by the protein component of soya peptone. This scenario may not be very likely as the induction of VCP1 seemed not to be a general response to protein.

The lower the growth rate supported by the transfer medium, the higher the protease titre that is generally obtained (Sessoms & Lilly, 1986; Bascarán *et al.*, 1990). Although the only growth measurements that were made in the transfer experiments with *V. chlamydosporium* were those in soya peptone medium, this appears a plausible correlation in case of this fungus, too. The absence of a measurable lag phase in fungal growth after transfer to 1% soya peptone is in accord with data from *V. albo-atrum* which has a growth lag that becomes significant only when the medium contains more than 1% (w/v) carbon (Malca *et al.*, 1966).

Transfer experiments are increasingly being used to study basic enzyme regulation, as many early reports that claimed enzyme induction are methodologically flawed in the sense that no distinction can be made between the effects of derepression and induction (Cohen, 1980). According to this type of experiment, the protease VCP1 was regulated by derepression, as transfer of young mycelium from

nutrient-rich to nutrient-deprived conditions resulted in a rapid increase in protease titre, but transfer to 50 mM glucose caused long-term repression (Fig. 6.3). Such a speed of fungal response is not without precedent. Messenger RNA from the subtilisin Pr1 from *M. anisopliae* was absent in rapidly growing cells in nutrient-rich cultures, but was produced rapidly (<2 h) when cells were deprived of nutrients (St. Leger *et al.*, 1992a). Moreover, it only takes 7 min to process amino acids into secreted active enzyme, during inductive conditions (St. Leger *et al.*, 1991c). Cutinase gene transcripts could be detected within 15 minutes after addition of inducers to *Fusarium solani* f.sp. *pisi* (Woloshuk & Kolattukudy, 1986).

The physiological status of the mycelium determined the impact that carbon sources had on repression of VCP1. When transfer experiments included a starvation step between the initial "inoculation medium" and the ultimate "treatment medium" with a nutrient source under investigation, then repression by glucose resulted in protease activity that was 44.1% of the control (Table 6.1). However, when that intermediate starvation step was omitted, then the activity after transfer was only *ca* 6% (5.6-6.7% in three experiments). The reason for stronger repression in non-starved mycelium is not clear. However, it is feasible that mycelium that was transferred from 1% soya peptone plus 0.5% glucose without a starvation step may have contained more intracellular carbon, and possibly residual carbon sources from the medium in the cell wall. The 50 mM glucose in the transfer medium would have amounted to such a carbon load for the fungus in those conditions that strong repression was the result.

VCP1 was also regulated by induction. Small amounts of *M. incognita* eggs (0.125% [w/v]) resulted in the largest single increase in protease production. Although it would be spurious at this stage to pinpoint the actual inducer, the media with collagen or chitin had a similar effect to that of the nematode eggs. Chitin is a component of nematode eggshells, it is assembled in microfibrils consisting of a chitin core embedded in a protein core which is proline-rich (Clarke *et al.*, 1967; Bird & McClure, 1976; Bird & Bird, 1991). However, since the chitin in this experiment was a relatively impure preparation, it cannot be ruled out that the actual effector may have been of a proteinaceous nature. Collagen-type proteins include about 80% of the proteins present in the nematode cuticle (Kingston, 1991). Although collagen as such has not been reported in nematode eggshells, its presence in this structure, too, is suggested by the amino acid composition of eggshell proteins (Clarke *et al.*, 1967). It is feasible that a protease inducer is a repeated sequence motif (Paterson *et al.*, 1994a), which is provided by collagen, since it has a highly repetitive peptide motif $(\text{Gly-X-Y})_n$, in which X and Y can be any amino acid but they are frequently proline and hydroxyproline (Eyre, 1980). The proposal of a collagenous inducer is supported by the enhancing effect of small concentrations of the vermiform nematode, *Steinernema feltiae*, which introduced cuticular collagen in the medium.

It is interesting to note that *G. rostochiensis* cysts have much less effect on VCP1 than the eggs of the susceptible host *M. incognita*. It has to be borne in mind that the *G. rostochiensis* eggs were not artificially released from the cysts prior to medium preparation. Therefore, it could be argued that these eggs, in contrast to those of *M. incognita*, were less available to the fungus. This cannot entirely be

refuted. If that was the case, then the current data reflect the regulatory response of the *V. chlamydosporium* protease to the cyst surface only. However, mature cysts are not sealed, and the fungus can be expected to gain access relatively easily through natural openings (mouth, anus, vulva) in the cyst wall.

Extracellular proteases that are regulated by repression/derepression only and not by induction are those of the saprotrophs *Aspergillus nidulans* (Cohen, 1981) and *Rhizopus oligosporus* (Farley & Ikasari, 1992). Other proteases are induced as a non-specific response to protein, e.g. that of *Mucor miehei* (Lasure, 1980), *Hebeloma crustuliniforme* (Zhu *et al.*, 1994) and the trypsin Pr2 from *M. anisopliae* (Paterson *et al.*, 1993). It is tempting to interpret specific induction by components of the host as an adaptation to a pathogenic lifestyle. However, the actual inducer of VCP1 has not yet been identified, and testing more carbon and/or nitrogen sources may reveal a response less specific than apparent at this stage. Moreover, all media were heat-sterilized, and therefore destruction of the higher-level structure of proteinaceous components can be expected. However, the differences in primary protein structure should not be ignored, as gelatin strongly repressed VCP1 production, while type I collagen, despite being heat-treated, was inductive. There is only one other case of specific induction of a microbial protease, and that is the example of the subtilisin Pr1 from the entomogenous fungus *M. anisopliae* (Paterson *et al.*, 1994a,b). The same authors demonstrated that hydrolytic products, generated from insect cuticle by Pr1, are able to induce it, which fits in the established hypothesis that Pr1 has a role in the infection of insects by *M. anisopliae* (St. Leger, 1995). Several dimeric

peptides and single amino acids that are probably abundant in insect cuticle were pinpointed as putative inducers (Paterson *et al.*, 1994a).

Repression overrides induction of VCP1. There are few reports of inducible proteases that are not repressed, e.g. protease production by the basidiomycetes *Agaricus bisporus*, *Coprinus cinereus*, *Hebeloma crustuliniforma* and *Volvariella volvacea* is not repressed by glucose or ammonium (Kalisz *et al.*, 1987; Zhu *et al.*, 1994). This is thought to be important to these litter-degraders as proteins or peptides are expected to be the nitrogen source with the greatest availability in their natural habitats, and simple carbon sources may be abundant. These fungi probably cannot afford to have a repressed proteolytic system. Other protein-inducible enzymes that are not prone to repression by glucose, are the serine protease from the plant pathogen *Ustilago maydis* (but repressed by ammonium, Hellmich & Schauz, 1988) and the aspartyl protease from *Mucor miehei* (not repressed by ammonium, Lasure, 1980).

The repressive effect of glucose is better documented than that of any other metabolite. Other simple carbon sources may also cause repression, but in general, carbon sources which support a rapid growth rate are most effective in producing catabolite repression. The main control appears to be at the level of glycolysis, as those substrates that feed rapidly into the early reactions of glycolysis are usually the strongest repressors (Paigen & Williams, 1970). However, the way in which an increased glycolytic flux triggers repression is unclear (Ronne, 1995).

When exposed to high ammonium concentrations, *S. cerevisiae* limits metabolism of ammonia by partly shutting down the main aminating pathway. The

synthesis of glutamate dehydrogenase, which forms glutamate from α -ketoglutarate, is repressed (Bogonez *et al.*, 1985). However, the effects of high nitrogen concentrations can stretch beyond that, as the expression of a wide range of genes can be affected (reviewed by Marzluf, 1981). VCP1 provides another example, as its production by starved mycelium was very sensitive to NH_4^+ . Ammonium nitrogen has been found to repress extracellular proteases in numerous other fungal systems (e.g. Cohen & Drucker, 1977; Hellmich & Schauz, 1988; St. Leger *et al.*, 1988b).

It is unclear why nitrate was not repressive in *V. chlamydosporium*. The fungus is likely to be able to absorb nitrate (at least in the absence of ammonium) and subsequently convert it to ammonium, since these capabilities are very common among fungi (Pateman & Kinghorn, 1976). Although all regulation studies cited so far use ammonium as the nitrogen source of choice, it is known for both *Neurospora* and *Aspergillus* that there are regulatory genes (called *nit-2* and *areA* in the two fungi, respectively), the expression of which is controlled by nitrate. The products of these genes govern nitrate and nitrite reductase, but also mediate nitrogen catabolite repression (reviews by Marzluf, 1981, 1993). If a similar circuit exists in *V. chlamydosporium*, then nitrate should theoretically be able to cause repression. The reason why it did not, is unclear.

VCP1 was regulated at the level of transcription. That appears to be usually the case with hydrolytic enzymes, e.g. the subtilisin Pr1 from *M. anisopliae* (St. Leger *et al.*, 1988b), and cutinase from *Fusarium solani* (Podila *et al.*, 1988). This is indeed the most common level of gene regulation in eukaryotes, probably because it is a very flexible and economical system, avoiding stores of mRNA or other

intermediates that need to be sufficiently stable for storage. It is, however, not the only possible level at which control over gene expression can be exerted. For example, several proteins have been identified in yeast that are enhanced after starvation, achieved entirely through stimulation of mRNA translation (Altman & Trachsel, 1993). Still further down transcription and translation pathways, secretion of proteins can also be regulated (Burgess & Kelly, 1987). One of the characteristics of regulated, as opposed to constitutive, secretion is the storage of product-filled secretory granules in the cytoplasm for long periods of time. This allows rapid discharge of the respective protein following the relevant stimulus, without *de novo* protein synthesis (Burgess & Kelly, 1987). Although no intracellular localization of VCP1 was carried out in this study, regulation of this protease at the level of secretion may not be very likely, in view of the results obtained with the inhibitors of transcription and translation.

Much early work on catabolite repression, mainly in enteric bacteria, gave cAMP a central role in this regulatory process (reviewed by Pall, 1981). Increasing glucose concentrations cause a drop in the amount of cAMP in the cell, which disables the cAMP receptor protein that is the activator of catabolite-repressed genes. In this scenario, exogenous cAMP could reverse catabolite repression. Where that indeed is possible in enteric bacteria (Pall, 1981; Hueck & Hillen, 1995), *V. chlamydosporium* reacted as most eukaryotes, in that cAMP does not appear to have that central role in repression (Ronne, 1995). Despite claims by Klapper *et al.* (1973) and Zonneveld (1980), fungal catabolite repression is generally not associated with a drop in cAMP levels, but it involves direct negative control of target promoters by

a DNA-binding repressor protein (Ronne, 1995). In this study, cAMP did not relieve glucose repression, but cAMP on its own inhibited the production of VCP1. Similar observations have been made in other systems (Long *et al.*, 1981; Priest, 1984). It is unclear why cAMP should be repressive, but a similar effect has been reported in fission yeast (Ronne, 1995). Clearly, few generalisations can be made about the role of cAMP. It would be imprudent to say that cAMP plays no part in the regulation of protease production of *V. chlamydosporium*, as there appeared to be kinase activity dependent on cAMP in the induction response to collagen. St. Leger *et al.* (1989b), using another inhibitor, did not find any involvement of cAMP-dependent kinase in Pr1 production by *M. anisopliae*.

The production of VCP1 in derepressed conditions seemed to depend to some extent on IP₃- and calmodulin-binding kinases. These inhibitors, however, did not completely inhibit protease production. This could be the result of a concentration-dependent effect of the inhibitor, or alternatively, this could suggest the involvement of more than one signal transduction pathway in derepression. This hypothesis awaits further investigation. Despite the many elements of the IP₃ pathway that have been identified in fungi, there is apparently no record of it actually functioning in response to any stimulus (Prior *et al.*, 1994). If the observations of this study genuinely reflect the utilisation of the pathway, then *V. chlamydosporium* has provided the first example of effective IP₃-mediated signal transduction in fungi, the stimulus being derepression.

The pathogenicity of *V. chlamydosporium*, in an *in vitro* bioassay system, depended significantly on the carbon and nitrogen content of the medium. On water

agar with 10 mM glucose or more, the fungus grew profusely over the agar surface, but stopped interacting with *M. incognita* eggs. *Verticillium chlamydosporium* became effectively a saprotroph in nutrient-rich conditions (see General Discussion). This result can hardly be reconciled with earlier data from Irving & Kerry (1986), who found that nutrient concentration is a negligible factor in their *in vitro* virulence assay. The virulence of *V. chlamydosporium* was not affected by diluting corn meal agar medium (Irving & Kerry, 1986). That study did not use isolate 10, but it is not clear to what extent that explains the difference. A possible factor is the physiological state of the mycelium, as in the study by Irving & Kerry (1986) the fungus had colonized the agar plates from a central plug for several weeks before nematode eggs were added.

There was a correlation between the decline of virulence on media with high C and N concentrations, and the repression of the protease VCP1 in those conditions. This correlation, however, has to be interpreted with caution. It has been indicated before that carbon and nitrogen repression can have a pleiotropic physiological impact on micro-organisms, and suggesting a direct causal link between repression of VCP1 and of pathogenicity, might be an oversimplification. Nevertheless, these observations are commensurate with the suggested role of VCP1 in the infection of nematodes.

The morphology of fungal colonies on agar media was altered significantly by varying the proportions of carbon and nitrogen in the medium. Colonies grown on media with small amounts of nutrients produced a less dense mycelium, but resulted in greater radial growth rates. Radial growth rate was deemed an unsuitable estimate

of fungal biomass produced under these nutritional conditions, and its measurement was abandoned. However, it may be ecologically significant that the fungus would cover a greater area in nutrient-poor conditions.

It is difficult to make general statements about carbon or nitrogen concentrations in the rhizosphere, since the quality and quantity of root exudates and nutrients from microbial sources are variable and dependent on a complex array of factors (Bowen & Rovira, 1976; Stirling, 1991; Shepherd, 1994). It is unfortunate that the attempts to demonstrate VCP1 production, during growth in a simplified rhizosphere situation, have failed. As yet, it remains a vexed question which of the regulatory patterns, observed in the *in vitro* context of the regulation experiments reported here, would occur in a more realistic plant-nematode-fungus-soil interaction.

7.1 What does the protease reveal about the fungus ?

Verticillium chlamyosporium, a fungal pathogen of nematode eggs and females, produced a subtilisin-like protease in liquid culture and, during infection, on the surface of nematode eggs. The fungus has no morphological adaptations, such as traps or adhesive spores, that would pre-adapt it to a pathogenic lifestyle. Several aspects of the enzyme, viz its regulation, substrate utilization, isoforms and coding genes, are reviewed here in an attempted assessment of the degree of specialization of the fungus. What information that sheds light on the life-history of *V. chlamyosporium* can be derived from VCP1 ?

VCP1 was induced by host components, associated with collagen and chitin (Chapter 6). The nature of the inducer appeared important, as no induction was observed with other protein sources, such as albumin, suggesting that the induction may be a rather specific response. Specific induction may be an indication of a specialized host-pathogen interaction, as protease induction in most fungi is insensitive to the type of protein provided, e.g. *Neurospora crassa* (Cohen & Drucker, 1977). Protein-inducible activities have been demonstrated for a number of pathogenic species, including the clinically important opportunistic pathogens *Trichophyton rubrum* Castellani (Meevootisom & Niederpruem, 1979) and *Candida albicans* (Lerner & Goldman, 1993), and the entomogenous fungus *M. anisopliae* (St. Leger *et al.*, 1988b; Paterson *et al.*, 1994b). Are proteases, for which there is reasonable evidence of involvement in the infection process, always induced by a

small set of host-derived molecules? Such a scenario would suggest a high degree of specialization in a pathogenic lifestyle. There are only a few fungi that have proteases, considered determinants of pathogenicity, for which there is also sufficient knowledge of regulatory mechanisms. *Metarhizium anisopliae* (St. Leger *et al.*, 1988b; St. Leger, 1995) has to breach the insect cuticle, and its subtilisin, Pr1, is induced by cuticular components (St. Leger *et al.*, 1988b; Paterson *et al.*, 1994a). *Candida albicans* (Calderone, 1994; Hensel *et al.*, 1995) is a human commensal that occasionally causes systemic mycosis (Ghannoum & Abu Elteen, 1986; Hostetter, 1994). Its aspartyl proteases are induced by a wide range of proteins and peptides (Lerner & Goldman, 1993). Clearly, it is difficult to make a case with such a limited range of examples. Axiomatically linking specific protease induction with a pathogenic lifestyle may be an unwarranted simplification of the complexity of, and variety in, pathogenic life-histories. As yet, the only examples of specifically host-induced proteases appear to be Pr1 from *M. anisopliae* (St. Leger *et al.*, 1988b, Paterson *et al.*, 1994a,b) and the subtilisin VCP1 from the nematophagous fungus *V. chlamydosporium* (this study).

Repression overrode induction of VCP1. This is the most frequently observed pattern; a rare exception is the protease production by some higher basidiomycetes, not repressed by glucose or ammonium (Kalisz *et al.*, 1987; Zhu *et al.*, 1994). This could be of importance to these litter-degraders as proteins or peptides are probably the most abundant nitrogen sources available to these organisms in their natural habitats. Repression of proteases by simple carbon sources, of which there may be a plentiful supply, might result in nitrogen deficits in the fungus. If this type of

regulation is a general pattern, then *V. chlamydosporium* clearly falls in an ecological category different from these obligately saprotrophic basidiomycetes.

Lewis (1973) proposed that biotrophy arose from necrotrophy by tighter regulation of hydrolytic enzymes. Opportunistic necrotrophs, such as *Botrytis cinerea*, often produce large amounts of derepressible pectolytic enzymes, causing extended tissue destruction. Hemibiotrophs, such as *Magnaporthe grisea*, produce these enzymes in smaller amounts, and more localized. Mechanical pressure becomes more important in penetration (Deising & Mendgen, 1992; Oliver & Osbourn, 1995; Talbot, 1995). Unfortunately, there are insufficient data on infection processes to validate these ideas in pathogens of nematodes. However, while enzyme induction by components of the host can be considered a fairly specialized, "tight" form of control, a regulatory system consisting only of repression/derepression would be a less specific, "looser" type of control. In Lewis' (1973) view, *V. chlamydosporium* would probably be considered more specialized than *B. cinerea*, by virtue of its infection structures, secreting a host-induced protease, but it would be considered less specialized than the hemibiotroph *M. grisea*. Care has to be taken when using the epithet "more specialized", as that is not synonymous with "more specialized pathogen". The presence of a protease, inducible by nematode eggs, does not make *V. chlamydosporium* a specialized pathogen; such an inducible system could serve in the saprotrophic degradation of nematode cadavers in soil. Similarly, several obligate saprotrophs with cutin-inducible cutinases allow these fungi to degrade cutin in plant debris (Köller, 1991; Stahl & Schäfer, 1992), rather than innately conferring

phytopathogenicity. The nematode-inducible proteolytic system in *V. chlamydosporium* could indicate that the fungus is merely a specialized saprotroph.

Confusingly, several saprotrophs produce “pathogenicity-related enzymes” similar to pathogens. The saprotroph, *Aspergillus nidulans*, produces a set of cell wall-degrading enzymes similar to that of some phytopathogens but that, apparently, is insufficient to make it a pathogen. Dean & Timberlake (1989) postulated that the pathogen’s enzyme systems, unlike those from the saprotroph, would not be repressed by the ample nutrient supply in wound tissue. One example of such a protease system of a pathogen, not prone to catabolite repression, is that of *Ustilago maydis* (Hellmich & Schauz, 1988). Dean & Timberlake (1989) tried to make *A. nidulans* plant pathogenic by creating a mutant strain that is not prone to catabolite repression. However, *A. nidulans* was not non-pathogenic because of its sensitivity to catabolite repression, as the pathogenicity of the mutant was not enhanced. Similar to *A. nidulans*, *V. chlamydosporium* has proteolytic activity prone to carbon and nitrogen catabolite repression. There was evidence for the involvement, at least in the early stages of infection, of this enzyme in the infection of nematodes. It would be interesting to investigate whether the broad-spectrum protease, VCP1, is involved in later stages of infection as well, since the necrotrophic maceration of the egg content, most likely, requires proteases of some kind. It is unclear whether the nematode egg, during degradation, would provide concentrations of catabolites, repressive to the protease.

A second example of a saprotroph, producing “pathogenicity-related enzymes” is *Paecilomyces farinosus* (Holm ex S.F. Grey) Brown and Smith, some

isolates of which are insect-pathogenic, whereas others are saprotrophic. Harney & Widden (1991) demonstrate that, although there is no evidence for distinct populations in leaf litter and insects, the proteolytic activity of insect-attacking isolates was generally greater, while cellulolytic activity was generally greater in saprotrophic isolates. The authors conclude that *P. farinosus* is a versatile saprotroph that can attack insects opportunistically. Isolates parasitizing insects would be selected from a pool of isolates that essentially have all enzymatic abilities for saprophytic survival, as well as pathogenicity. In many ways, *V. chlamydosporium* appears to be similar to *P. farinosus*. Its subtilisin, VCP1, was not a useful marker for the change in trophic phase *in vitro*, as it was produced when the fungus was cultivated saprotrophically, as well as during infection. However, it is possible that the isoforms that were detected in several isolates of *V. chlamydosporium* have different roles, which could be reflected by differences in substrate utilization and regulatory patterns. The expression of different cutinase isozymes during the saprotrophic and parasitic stages of *Alternaria brassicicola* has been demonstrated (Köller *et al.*, 1995), and there is also some evidence for differential regulation of Pr1-isoforms of *M. anisopliae* (St. Leger *et al.*, 1994). Clearly, the role of isoforms is potentially an important issue that needs investigation in *V. chlamydosporium*.

All pathogenicity-related alkaline subtilisins from mycopathogens of nematodes, insects, and fungi, reported to date, are related (St. Leger *et al.*, 1992b; Geremia *et al.*, 1993; Bonants *et al.*, 1995; Joshi *et al.*, 1995; this study). It has been proposed that these enzymes would be more similar to each other than to other subtilisins because they play a conserved role, with perhaps a conserved substrate,

in pathogenesis (J.M. Clarkson, Bath, personal communication). The sequence comparison presented in Table 3.1 did indeed indicate that the majority of fungi that produce proteases homologous with VCP1 have strains, pathogenic to nematodes (*Acremonium* sp., *Fusarium* sp., *P. lilacinus*, *V. chlamydosporium*), insects (*A. flavus*, *B. bassiana*, *M. anisopliae*, *P. lilacinus*), humans (*A. fumigatus*) or fungi (*T. harzianum*). *Tritirachium album*, which produces proteinases K (Swiss-Prot accession no. P06873), R (P23653) and T (P20015), appeared not to be in accord with this hypothesis, as the fungus is an obligate saprotroph. However, *T. album* is synonymous with *B. alba* (de Hoog, 1972), which suggests close entomopathogenic affiliations. Moreover, Limber (1940) separated the genus *Tritirachium* from *Verticillium*, which has a number of subtilisin producing nematode-, insect-, and fungus-pathogenic taxa. However, not all homologous subtilisins are, even remotely, produced by pathogens (Table 3.1). Examples are the enzymes of *Saccharomyces cerevisiae* (Accession no. P09232) and *Agaricus bisporus* (Burton *et al.*, 1993). The “conserved-subtilisin theory” may be based on a false premise, in that fungal subtilisins may have been looked for mainly in pathogenic fungi. Furthermore, the homology of the subtilisins of fungal pathogens need not be a surprise, since all subtilisin-like proteases from fungi are related, irrespective of their life-history (Siezen *et al.*, 1991). This widespread homology might reflect the fact that relatively little time has elapsed for the ancestral subtilisin gene of higher fungi to diverge (Ragan & Chapman, 1978). Alternatively, it could illustrate the versatility of these proteases, similar enzymes being functional in saprotrophy and pathogenesis. Several of the “pathogens” mentioned, are econutritionally close to saprotrophy, e.g. the

nematophagous *Fusarium* and *Acremonium* sp., the entomogenous *A. flavus*, and the human pathogen *A. fumigatus* are all opportunistic necrotrophs. It is tempting to speculate that saprotrophic species have either slightly modified their proteolytic activity, or acquired other pathogenicity-related traits that allowed them to become infectious in certain conditions. There are insufficient sequence data to confirm the conserved nature of these enzymes in invertebrate and fungicolous mycopathogens.

Econutritionally, the soil-borne fungus *V. chlamydosporium* has several attributes of a saprotroph, including a derepressible, broad-spectrum protease, and inducible cellulolytic activity. Although live *M. incognita* eggs were infected, dead eggs were colonized to a greater extent, suggesting that a pathogenic lifestyle may be optional. The pathogenic mode of *V. chlamydosporium* could not be identified by any of the enzymatic or morphological events associated with the colonization of nematode eggs. Both VCP1 production, and the differentiation of appressoria and the typically bulbous mycelium inside eggs, could be seen during the infection and colonisation of live and dead eggs. The environment could ultimately determine the trophic mode of the fungus, as in high-nutrient conditions *V. chlamydosporium* effectively became a saprotroph, ignoring the presence of nematode eggs. Competition in the rhizosphere is an environmental interaction, the effects of which on growth and pathogenicity of *V. chlamydosporium* are largely undocumented. The fungus is probably not highly competitive as there is often poor recovery of *V. chlamydosporium* following soil inoculation with nutrient-rich formulations (Godoy *et al.*, 1983; de Leij *et al.*, 1993b). Presumably, the fungus is outcompeted in these conditions. Competition for nutrients, particularly nitrogen, is usually thought to be

fierce in the rhizosphere, making it unlikely that conditions repressive for the protease would generally prevail. The utilization of nematodes as an additional food source is thought to give nematophagous fungi a competitive advantage (Cooke, 1962a,b; Barron, 1991). If VCP1 is genuinely a pathogenicity-related enzyme, required for infection, then its repressibility by carbon, but particularly nitrogen, is revealing. It would, once more, indicate that the fungus is not dependent on nematodes as a nitrogen source and that a supply of inorganic nitrogen would be a suitable, possibly energetically more favourable, alternative. The hypothesis was confirmed in a bioassay with increased availability of ammonium-nitrogen, which reduced pathogenicity.

If nematophagous fungi have a competitive advantage in soil by attacking nematodes, it is likely that they will enjoy the greatest advantage when nematode populations are dense, and other nutrients are sparse. That is most likely the case when there are heavy infestations of plant-parasitic nematodes on crops growing on sandy soils (Schenk & Pramer, 1976). However, those are not necessarily the conditions under which *V. chlamydosporium* performs best. Heavy root-knot infestations give relatively poor control, presumably because many egg-masses remain unexposed in the galls (de Leij *et al.*, 1992b). Although the bioassay in Chapter 6 confirmed the relation between nutrient sparsity and infectivity, there was no absolute linearity. Greatest infectivity was not achieved on pure water agar, but on water agar amended with 1 mM each of glucose and ammonium, while higher concentrations of either nutrient reduced the infection rate. Presumably, a limited amount of carbon and nitrogen enhanced fungal growth, and therefore the chance of

encountering nematode eggs, whilst not impeding infection by repressing the protease.

The question what makes *V. chlamydosporium* a nematophagous fungus cannot conclusively be answered on the basis of the current data. The biochemistry and molecular biology of virulence-determinants in this pathogen are too immature. However, it is possible that i) its protease(s) and other hydrolytic enzymes are particularly well suited for degradation of the nematode substrate. ii) Although there is little or no evidence of host defense responses from the part of the nematode, protease inhibitors of various kinds have been identified in a number of nematodes (Hawley *et al.*, 1994), although there are no reports of protease inhibitors in plant-parasitic nematodes. Although purely speculative, the enzyme systems of successful fungal isolates may be less prone to inhibition by components of the nematode. iii) Whereas other proteases, e.g. Pr1 from *M. anisopliae*, may be nearly as efficient at damaging, or removing, the vitelline layer of *M. incognita*, the delivery system may make all the difference. While *V. chlamydosporium* grows in the rhizosphere, potentially near nematode females and egg masses, *M. anisopliae* does not (Peixoto de Oliveira *et al.*, 1980; Glare & Milner, 1991). All results suggest that *V. chlamydosporium* is intrinsically poorly specialized towards infection of nematodes, but it may be its rhizosphere competence that makes the system work. In this view, the rhizosphere competence traits of *V. chlamydosporium* urgently deserve an increased research effort.

Adamson & Caira (1994) reviewed the nature of specificity in animal and human parasites and conclude that "parasite specificities are, in part, a legacy of the

habits of their free-living progenitors". If extrapolation to fungal pathogens would be warranted, then *V. chlamydosporium* would be foremost a rhizosphere fungus, its pathogenicity against sedentary stages of nematodes having developed as a secondary trait. This scenario is not unlikely, in view of the soil-borne nature of some of the other *Verticillium* species, e.g. *V. tricorpus* and *V. nubilum*. In the same, speculative scheme, *V. albo-atrum* and *V. dahliae* may have developed from a rhizosphere-competent ancestor towards becoming increasingly specialized plant-pathogens, losing their rhizosphere competence along the way. However tempting such simple, theoretical scheme may be, it has to be emphasized that there are currently no data to support, or refute it. In this context, the attempts at developing *V. lecanii* as a nematophagous fungus are also interesting (Uziel & Sikora, 1992; Meyer, 1994). This fungus has subtilisin-like activity, coded by a gene that is homologous with that of *V. chlamydosporium* (Chapter 4). That is, apparently, insufficient to make it promising as a biological control agent against nematode pests, as witnessed by the poor success obtained in soil trials (Meyer, 1994). Recovery of the fungus after application was negligible (Meyer & Meyer, 1995), suggesting that the problem with the nematophagous fungus *V. lecanii* may not be its proteolytic system, but rather its poor adaptation to the soil and/or rhizosphere environment.

It has been suggested by workers on plant disease that certain criteria, with obvious parallels to Koch's postulates, should be satisfied before assigning a role in plant disease to a putative pathogenicity determinant. These are: the determinant, at physiological concentrations, should induce symptoms in the plant, the presence of the determinant *in planta*, a correlation between determinant production and

virulence, and breakdown products of the host, should all be demonstrated (Yoder, 1980; Cooper, 1984). As yet, VCP1 has not satisfied all criteria of this restrictive definition. The presence of VCP1 during infection was demonstrated by immunofluorescence, while egg shell breakdown was shown by protein assays and fluorescence microscopy. However, the relation between the enzyme production of different isolates and virulence was not straightforward (Section 4.3.1). It has to be borne in mind that such correlation is not necessarily meaningful. The proposed dual role of VCP1 in pathogenesis and in saprotrophy may well confound the attempted correlation, in that enzyme yields after saprotrophic cultivation need not reflect the productivity of the fungus during pathogenesis. The literature of entomogenous fungi, in particular, abounds with reports that endeavour to correlate the virulence of a set of isolates with their production, *in vitro*, of proteolytic enzymes. The association was confirmed in some cases (Bidochka & Khachatourians, 1990; Gupta *et al.*, 1994), but refuted in at least as many other (Champlin *et al.*, 1981; Sosa Gómez & Alves, 1983; Latgé *et al.*, 1984; Jackson *et al.*, 1985; Samuels *et al.*, 1989), suggesting that simplistic generalizations concerning the role of proteases in pathogenesis should be avoided. Furthermore, the infection process is likely to involve more than merely a blast of protease. It may well consist of a sequential production of lytic enzymes and other fungal products, making it difficult to quantitatively correlate virulence with any one individual component, and impossible *in vitro*. Verifying such correlation in realistic conditions, if possible, would certainly be a technically demanding task.

However, within a single isolate, it may be possible to demonstrate a valid correlation between determinant production and virulence. Targeted disruption of the gene encoding a putative pathogenicity-determinant can be expected to abolish pathogenicity. This is currently the best method of investigating the possible involvement of a gene in pathogenesis (Schäfer, 1994), and results have often been revealing, although not conforming with preconceived ideas. Disruption of those hydrolase genes, anticipated to encode pathogenicity-determinants, has not always decreased pathogenicity to the expected extent, discounting the involvement of the enzyme in question. *Aspergillus fumigatus* produces a subtilisin-like protease that was thought to be involved in animal and human aspergillosis (Monod *et al.*, 1991). Although its disruption leads to a large reduction in protease activity, the mutant was equally pathogenic as the wild-type parent (Monod *et al.*, 1993b). The residual protease activity was subsequently identified as a metalloprotease with a substrate specificity similar to that of the subtilisin (Monod *et al.*, 1993a). Even a double mutant with its subtilisin and metalloprotease disrupted, remains pathogenic (Jaton-Ogay *et al.*, 1994). Markaryan *et al.* (1994) recently reported yet another similar metalloprotease from *A. fumigatus*, further illustrating that seemingly parallel enzyme systems can exist, and invalidating the simplistic notion of "one" pathogenicity-determinant. Remarkably, a nearly identical scenario can be seen in *M. anisopliae*, in that a Pr1⁻ mutant, produced by targeted gene disruption, demonstrates only a partial loss of virulence. It was demonstrated that another Pr1 isoform, and a metalloprotease with similar substrate specificity are secreted at unusually high levels by the mutant (St. Leger, 1995), suggesting the presence of "backup systems" in

these fungi. Disruption of the *VCP1* gene will not necessarily provide evidence for its involvement in the pathogenesis of *V. chlamydosporium*, since in most isolates tested, several isozymes have been identified and it seems unlikely that these relate to the same gene. If a gene family is confirmed for *V. chlamydosporium*, then it is certainly a moot question whether stepwise inactivation of all secreted proteases is a worthwhile or even practicable objective. Gene disruption remains valuable, if only to demonstrate the presence and importance of such "backup systems" in *V. chlamydosporium*.

In an enlightening review, Groisman & Ochman (1994) suggest several scenarios to explain the pathogenicity of *Salmonella* spp., which also share many of their virulence-determinants with closely related, non-pathogenic, Gram-negative bacteria. Three scenarios may be relevant for fungi.

i) Virulence may be attributable to specific genes, that are absent altogether in non-pathogens. This is unlikely to explain the nematophagous character of *V. chlamydosporium*, as the presence of genes, highly homologous with the putative pathogenicity-related enzyme VCP1, was demonstrated, by Southern analysis, in a range of fungi occupying different niches.

ii) Virulence results from mutational differences between essentially homologous genes. The presence of isolate-dependent isoforms in *V. chlamydosporium* and the Southern analysis of subtilisin-homologous genes suggested that there was considerable molecular variation in these enzymes. The exact way in which such molecular variation would result in pathogenic variation is unclear, but one mechanism is suggested by Bidochka & Khachatourians (1994), who showed that the

protease from *B. bassiana* differed in its adsorption to the host surface from those of *V. lecanii* and *M. anisopliae*. The enzymes adsorb electrostatically onto host cuticle (St. Leger *et al.*, 1986a). It is possible that homologous genes, with products that differ in charge, as the isozymes seen in *V. chlamydosporium*, differ in their adsorption onto nematode egg shells, with consequences for pathogenicity.

iii) In a third scenario (Groisman & Ochman, 1994), virulence results from the differential regulation of the same complement of genes. Although theoretically possible (Lewis, 1973), this scenario could not be verified experimentally (Dean & Timberlake, 1989), as discussed before.

The understanding of VCP1 may help in the selection of fungal isolates, as the protease has some potential as a molecular marker. The five isolates tested in Chapter 4 all showed distinct isozyme patterns after isoelectric focusing and enzymoblotting, using suc-(Ala)₂-Pro-Phe-pNA as the substrate (Section 4.3.2), indicating the potential value of these isozymes in distinguishing isolates. It may also be possible to generate antibodies, specific for a particular, unique isoform. These should preferably be monoclonal antibodies, as the supply of polyclonal antibodies is never infinite, and subsequent versions may have different epitope affinities, as exemplified in the polyclonals generated for Chapters 3 and 4. Evidently, many isolates need to be tested to validate the assumption that they can be identified by such isozyme or immunological techniques. The identification of virulent isolates based on protease isozymes has proven to be a viable technology in the case of the ovine footrot pathogen *Bacteriodes nodosus*, as current laboratory diagnostic tests for ovine footrot are based on protease zymogram patterns, distinctive for virulent

isolates (Every, 1982). The problem with secreted enzyme markers is that cultural conditions may have great impact on their production, and therefore, standardization is a likely obstacle in their development and use. This is, in general, a disadvantage of isozymes (Micales *et al.*, 1992). Genomic markers do not suffer from this drawback. Recently, fungal protease genes, have been selected as target DNA for PCR-based detection of *Candida albicans*, using the sequence of its aspartyl protease (Sugita *et al.*, 1993), and *Aspergillus fumigatus* and *A. flavus*, using their alkaline protease genes (Tang *et al.*, 1993). DNA markers, in a PCR-based approach, are often based on repetitive sequences (Mitchell *et al.*, 1994), but in the cited cases the selected sequences, which are low copy number, successfully recognized only the species indicated. This may also be within reach for *V. chlamydosporium*, as each isolate tested had isoforms with a unique sequence. Interestingly, isolate-specific RFLPs could be obtained in *M. anisopliae* by digestion with restriction enzymes of PCR products generated with primers based on the sequence of *Pr1* (S.C.M. Leal, IACR-Rothamsted, personal communication). Also in this species there is apparently sufficient intraspecific variation in the subtilisin-coding genes to make them a potentially useful marker.

7.2 Future prospects: towards a molecular dissection of pathogenicity

Some more poorly explored areas in the pathogenicity of nematophagous fungi, not yet mentioned, are indicated here as suggestions for further research.

The identification of the genes encoding pathogenicity determinants is an essential step towards a better understanding of fungal pathogenicity. The increasing

availability of, and improvements in the technology involved, has resulted in a surge in interest, witnessed by a lengthening series of reviews discussing techniques and results (Garber, 1991; Oliver *et al.*, 1991; Clarkson, 1992; Goldman *et al.*, 1994; Schäfer, 1994; Tudzynski *et al.*, 1994; Bouchara *et al.*, 1995; Oliver & Osbourn, 1995). Essentially, two methods can be used to identify the genes for pathogenicity determinants. They are either based on prior, biochemical knowledge of a pathogenicity-determining gene product (cloning by heterologous hybridization, cloning with oligonucleotide probes, cDNA cloning by antibody screening), or are based on a black-box approach (cloning by transformation, cDNA cloning by differential hybridization, differential display). Although the first group of techniques has proved to be extremely useful in the study of pathogenicity of many fungi, a comprehensive understanding of pathogenicity in any one system is likely to be achieved only with those methods that do not require *a priori* knowledge of the pathogenicity-determinants that are being looked for.

Irrespective of the methodology used, a gene cloning programme will allow targeted gene disruption studies, confirming or refuting the involvement of the gene of interest. Site-directed mutagenesis can deliver an understanding, and possibly manipulation, of substrate utilization and regulation. It may be possible to tailor proteases, making them more effective against a nematode pest of interest, e.g. *Globodera* spp. Constitutive expression, which has been achieved with *Pr1* in *M. anisopliae* (R.J. St. Leger, Ithaca, USA, personal communication), may result in faster pathogenesis. Control of the regulatory circuits governing carbon and nitrogen

metabolism may lead to a better understanding of rhizosphere competence, and ultimately its control.

Following the identification of pathogenicity-determining genes, their genetic manipulation can lead to strain improvement, with specific aims speed of pathogenesis, or altered host range. Introduction of multiple copies of a gene can be expected to lead to an increase in the amount of corresponding protein. However, successful manipulation requires a thorough understanding of the regulatory systems involved. If the genes coding for regulatory proteins are not concomitantly amplified, then shortage of the regulatory products could result, resulting in amounts of gene product that differ from the expectations (Punt & van den Hondel, 1992). The manipulation of gene copy number will stand a good chance of being successful if and when the transcriptional regulation of the gene involved is understood.

Current technology allows not only to manipulate the copy number of homologous genes, but the introduction of heterologous genes as well. Several remarkable studies have attempted to genetically alter the trophic mode, effectively “making” a pathogen from a saprotroph, or changing the host range of a fungal pathogen by inserting heterologous genes encoding pathogenicity-related enzymes. Cutinase from the hemibiotroph *Nectria haematococca* (*Fusarium solani* f.sp. *pisi*) was inserted in the opportunistic necrotroph *Mycosphaerella* sp., enabling the transformant to infect intact papaya fruit (Dickman *et al.*, 1989). Pisatin demethylase, a detoxifying enzyme required by *Nectria haematococca* to be pathogenic on peas, was inserted in *Cochliobolus heterostrophus* (a fungal pathogen of maize, but not of pea), which then became pathogenic on pea. The saprotroph *Aspergillus nidulans*,

however, did not infect peas after this transformation (Schäfer *et al.*, 1989), suggesting that this enzyme contributes to host specificity, but by itself is insufficient to make a saprotroph pathogenic. The third example is the subtilisin-like protease gene, *Pr1*, from *M. anisopliae*, that was introduced in another entomogenous deuteromycete, *Ascherschonia aleyrodis*, which consequently became a pathogen of late instar whitefly (R.J. St. Leger, Ithaca, personal communication). These transformations not only provide evidence for the involvement of the respective enzymes in pathogenesis, but also indicate that they can be involved in host specificity, as suggested for VCP1 from *V. chlamydosporium*.

Any transformation-based method requires a transformation system. Needed are a selectable marker and a method to make fungal cells take up foreign DNA. Uptake of DNA can be stimulated by PEG/CaCl₂ treatment of protoplasts, electroporation, or by bombardment of DNA-coated micro-particles by gene gun (Fincham, 1989; Goldman *et al.*, 1995). *Verticillium chlamydosporium*, isolate 10, was resistant to a wide range of potential dominant markers, including ampicillin, hygromycin, kanamycin, tetracyclin, phleomycin, with limited susceptibility to benomyl (R. Segers, unpublished observations). Preliminary attempts at transforming *V. chlamydosporium* with the benomyl resistance gene have failed, because insufficient sensitivity of the wild-type fungus to benomyl impeded the detection of transformants. Alternatively, an auxotrophic selection system can be used, but this method relies on the availability of auxotrophic mutants. In the case of *V. chlamydosporium*, these are not yet available.

Whereas manipulation of the fungus could, to a certain extent, lead to improved performance, fungal pathogens can be considered as largely unexplored pools of potentially interesting genes. These could be isolated and either formulated as a product, or used in a plant transformation programme. While VCP1 is probably too non-specific to be useful in such a scheme, toxins may be better candidates for this approach (Thorn & Barron, 1984; Anke *et al.*, 1995).

Two morphological events were associated with the infection of nematode eggs by *V. chlamydosporium*, and are worth investigating further. The first is appressorium formation. An understanding of its regulation could provide the means for an improved biological control agent, as it may lead to enhanced infection. Since slightly different appressorium populations were observed on the susceptible host, *M. incognita*, compared to the poor host, *G. rostochiensis*, fungal virulence against the latter might be increased through the manipulation of appressorium formation. The second morphological event was the typical post-penetration, absorptive mycelium. The increased surface offered by these hyphae potentially enhances nutrient uptake. It would be interesting to see whether this morphology is merely the result of the osmolarity of the egg content, or a specific adaptation by the fungus, that causes enhanced nutrient-uptake during infection. The modified nature of the post-penetration hyphae, being pathogenicity-related, could be studied by immunological methods. Monoclonal antibodies raised against biotrophic, intracellular hyphae formed in the *Colletotrichum lindemuthianum*-bean interaction allowed the identification of glycoproteins, specific for the pathogenic phase of this fungus (Pain

et al., 1994). The pathogenic mode of *V. chlamydosporium* is so poorly characterized that such an approach is likely to be informative.

There is obviously a long way to go towards understanding pathogenicity in *V. chlamydosporium*, but it is hoped that the experimental work on the fungal protease, VCP1, reported here, provides a useful stepping stone.

APPENDIX

Characterization of a cDNA-clone from *Verticillium chlamydosporium*

A.1 INTRODUCTION

Numerous protease genes have been cloned, from a wide range of organisms. Some of these enzymes are economically very important, e.g. some bacterial subtilisins, and have been extensively engineered (Wells & Estell, 1988; Siezen *et al.*, 1991). In other cases, cloning has been the stepping stone to a better understanding of pathogenicity. Gene cloning can be used as a prelude to gene disruption, allowing the assessment of the involvement of the gene product in pathogenesis. Furthermore, it can take the study of regulation to the molecular level, while transformation with isolated genes could yield improved strains. The importance of gene cloning, and some current techniques have been illustrated in the General Discussion.

No genes have been cloned from *V. chlamydosporium*, and there is only one report of a cloned protease from a nematophagous fungus, *Paecilomyces lilacinus* (Bonants *et al.*, 1995). However, that is an interesting record, as *P. lilacinus* and *V. chlamydosporium* have similar modes of action, being parasites of nematode eggs and females. The N-terminal amino acid sequence of VCP1 is homologous with the *P. lilacinus* protease (Section 3.3.3). Comparing the nucleotide sequences of the proteases of these two fungi is likely to generate interesting information.

Unfortunately, no subtilisin-like clone was identified in this experiment. Since the result obtained did not seem to have any bearing on the thrust of the thesis, it is presented here, briefly, as an appendix.

A.2 MATERIALS AND METHODS

A.2.1 Fungal growth conditions and mRNA isolation

Verticillium chlamyosporium, isolate 10, was grown in modified Sabouraud dextrose broth (tryptone, 10 g l⁻¹; glucose, 40 g l⁻¹) on a rotary shaker at 23°C for 4 days. The fungus was collected under vacuum on Whatman No. 1 filter paper on a Büchner funnel, washed extensively with sterile distilled water, transferred to minimal medium (mineral salt solution, see Section 2.2.1), in which incubation continued for 9 h. During that period VCP1-like activity increased significantly from 1.0 ± 0.5 nmol nitroaniline released ml⁻¹ min⁻¹ in the Sabouraud dextrose broth to 21.1 ± 0.6 nmol nitroaniline released ml⁻¹ min⁻¹ (± S.E., n = 3) after transfer to nutrient-poor conditions. The mycelium was washed with sterile distilled water, and finely ground in liquid nitrogen. Poly (A)⁺ RNA was extracted and affinity-purified on biotinylated oligo(dT) probe using the PolyATtract[®] System 1000 (Promega). Streptavidin-conjugated paramagnetic particles allowed quick purification of mRNA.

A.2.2 cDNA Synthesis and transformation of *E. coli*

Five µg of mRNA was used in the ZAP Express[™] cDNA synthesis kit (Stratagene). The resulting cDNA was size-fractionated on Sephacryl S-500 spin columns according to the manufacturer's instructions and ligated into the ZAP Express[™] vector (Stratagene). Lambda phage was packaged with Gigapack[®] II Gold packaging extract (Stratagene). Petri dishes containing NZY agar (5 g l⁻¹ NaCl, 2 g l⁻¹ MgSO₄·7H₂O, 5 g l⁻¹ yeast extract, 10 g l⁻¹ Select peptone 140 [Gibco BRL], 15 g l⁻¹ agar, pH 7.50) were overlaid with a thin layer of NZY top agar (as NZY agar, but

agar replaced with 0.7% agarose) that was mixed with the *Escherichia coli* strain XL1-Blue MRF' and 1 μ l, or a serial dilution, of phage library.

A.2.3 Titration and amplification of the library

The library was titered, i.e. the concentration of clones with and without cDNA insert determined, by including 15 μ l of 0.5 M isopropyl-1-thio- β -D-galactopyranoside (IPTG) and 50 μ l of 250 mg ml⁻¹ of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) in 3 ml of NZY top agar and plating several hundred plaques per plate. Background plaques, without insert, were blue, while recombinant plaques were white. The library was amplified by mixing aliquots of ca. 50,000 plaque-forming bacteriophage with XL1-Blue MRF' cells and plating on 150-mm plates of NZY agar. These plates were incubated at 37°C until plaques with a diameter of 1 mm appeared, when the plates were overlaid with 8 ml of SM buffer (50 mM Tris-HCl pH 7.5, 5.8 g l⁻¹ NaCl, 2.0 g l⁻¹ MgSO₄·7H₂O, 0.01% gelatin) and placed on a rotary shaker at 4°C overnight. The next morning, cell debris was removed by adding 5% (v/v) chloroform to the bacteriophage suspension, mixing and centrifuging for 10 min at 500 g. The amplified library was titered as above.

A.2.4 Replica blotting of DNA from plaques

Library aliquots containing ca. 10,000 plaque-forming units were incubated with XL1-Blue MRF' cells and grown overnight on 150 mm NZY agar plates at 37°C. In the morning, plates were cooled at 4°C for 1 h and overlaid with a Magnagraph nylon membrane (Genetic Research Instrumentation, Dunmow, Essex) for 1 min. Bacterial

and phage DNA on filters was denatured (1.5 M NaCl, 0.5 M NaOH) on a sheet of Whatman 3MM paper for 5 min. Filters were neutralized in 1.5 M NaCl and 0.2 M Tris-HCl, pH 8.0, and rinsed briefly in 0.2 M Tris-HCl, pH 7.5 and 2 x SSC solution (20 x SSC is 175.3 g l⁻¹ NaCl, 88.2 g l⁻¹ sodium citrate, pH 7.0). After air-drying, the DNA was cross-linked on a UV trans-illuminator for 3 min. Filters were rewetted in 2 x SSC, rolled in nylon mesh and placed in hybridisation bottles with 10 ml of freshly prepared pre-hybridisation solution (6 x SSC, 1% SDS, 5 x Denhardt's solution¹, and 100 µg ml⁻¹ denatured salmon sperm DNA) at 65°C for 4 h.

A.2.5 Probing of library and recovery of cloned insert

Two probes were used to identify *VCP1* from *V. chlamydosporium*. The first one was the cDNA of *Pr1* from *M. anisopliae* (St. Leger *et al.*, 1992a). This choice was based on the homology of the N-terminal amino acid sequences of these two subtilisins (see Section 3.3.3). *Escherichia coli* containing the Bluescript vector with this insert (kindly donated by Dr. R.J. St. Leger, Ithaca, USA) was grown overnight in Luria-Bertoni medium (LB medium: 10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl, pH 7.5) with 50 µg ml⁻¹ ampicillin. Plasmid DNA was purified from the cells using standard procedures (Sambrook *et al.*, 1989). The *Pr1*-cDNA was excised by digestion of the plasmid with *EcoRI*, and recovered by cutting the 1.2 kbp band from low-melting point agarose with a clean scalpel blade. The second probe (kindly donated by Miss S.C.M. Leal, IACR-Rothamsted) was a nested-PCR product, generated from total DNA of *V. chlamydosporium*, isolate 10, using primer sets

¹ 100 x Denhardt's solution contains bovine serum albumin, polyvinyl-pyrrolidone and Ficoll™, each present at 2% (w/v).

METPR1 (5' CAC TCT TCT CCC AGC CGT TC 3') plus METPR4 (5' GTA GCT CAA CTT CTG CAC TC 3'), and subsequently METPR2 (5' AGG TAG GCA GCC AGA CCG GC 3') plus METPR5 (5' TGC CAC TAT TGG CCG GCG CG 3'), which were based on the sequence of *Pr1* of *M. anisopliae* (St. Leger *et al.*, 1992a). Both probes were radio-labelled with α -³²P dATP (3000 μ Ci mmol⁻¹) using the Megaprime™ DNA labelling system (Amersham), denatured by boiling for 5 min and added to the hybridisation solution. Hybridisation continued at 65°C overnight.

In the morning, membranes were washed at 65°C in 6 x SSC and 0.1% SDS, 2 x SSC and 0.1% SDS, and twice in 0.2 x SSC and 0.1% SDS. They were placed on Fuji X-ray film between intensifying screens and stored at -70°C. After developing the film (usually the next morning), positive plaques were identified and picked from the corresponding agar plate with sterile Pasteur pipettes. These plaques were suspended in 1 ml of SM buffer, vortexed and used as phage stock for the second round of screening, which used only the PCR product as probe.

Cloned inserts that reacted positively were *in vivo* excised from the ZAP Express vector and recircularized as phagemids containing the insert, according to the manufacturer's instructions. Single colonies containing the pBK-CMV double-stranded phagemid vector with the cloned insert were maintained on LB-kanamycin agar, while glycerol stocks were stored at -80°C. Insert size was determined on a 0.8% (w/v) agarose gel, following digestion of the positive clones with *EcoRI* and *XhoI*.

A.2.6 Sequencing

Single colonies of positive clones were grown overnight in LB-kanamycin broth at 37°C. The plasmid was purified using the SpinBind® Mini-Prep System (FMC BioProducts, Rockland, USA) and used, as double-stranded template, in a cycle-sequencing reaction with dye-labelled terminators (ABI Prism™, Perkin-Elmer, Warrington). The initial primers, used with all positive clones, were pUC/M13 forward and reverse primers (Promega), while subsequent primers were custom-synthesized (Cruachem, Glasgow). Sequencing data were collected in an ABI 373 automated sequencer, and analyzed using the GCG software package, version 8 (Genetics Computer Group, 1991).

A.3 RESULTS AND DISCUSSION

A cDNA library was constructed that originally consisted of 3.4×10^5 recombinants, and 6.7×10^3 clones without insert (background). Amplification increased the titre *ca* 1,000-fold, resulting in an amplified library of 2,800 plaque forming units μl^{-1} .

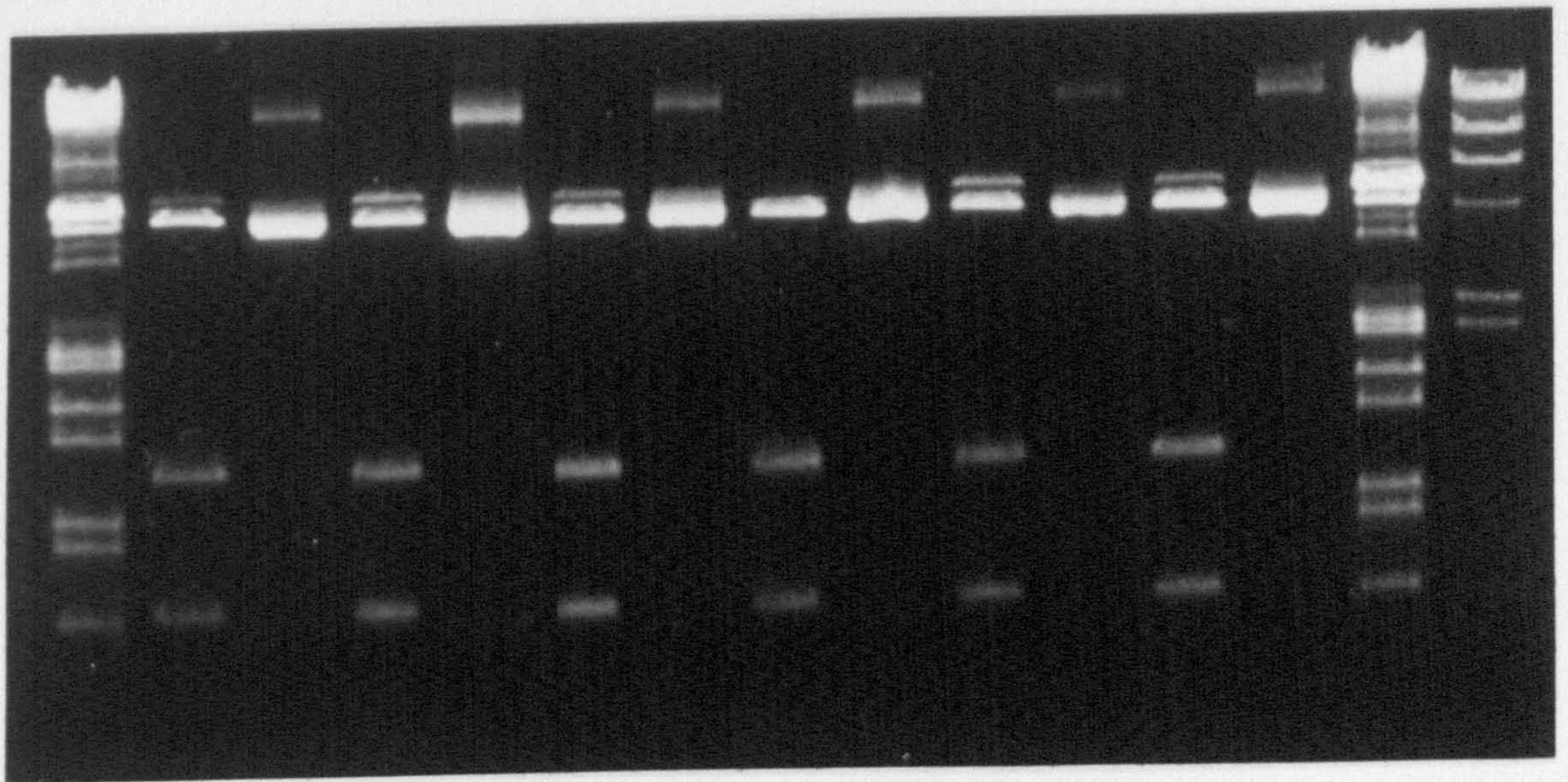
Heavy background on the autoradiographs of the filters hybridised with the *Pr1* cDNA precluded the identification of positive clones with this probe. Hybridisation with fewer filters in plastic bags, rather than in hybridisation bottles, gave the same result. Since a positive result was obtained with the parallel hybridisation to the homologous PCR product, probing with the *Pr1*-cDNA probe was abandoned.

After the first round of screening, four putative positive clones were identified with the PCR-derived homologous probe. Three of those were retained after the

Fig. A.1 Size and restriction pattern, with *EcoRI* and *XhoI*, of cDNA inserts from ZAP Express clones, identified with a PCR-derived homologous probe in a *V. chlamydosporium* cDNA library. Plasmid was isolated from 2 separate colonies, from each of 3 positive clones. Markers were λ DNA, digested with *HindIII* and *EcoRI* (lanes 1 and 14), or with *HindIII* only (lane 15). Plasmid cut with *EcoRI* and *XhoI* (lanes 2 and 4; 6 and 8; 10 and 12), or uncut (lanes 3 and 5; 7 and 9; 11 and 13) from clones 1, 2 and 3, respectively.

190a

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



second screening round, and excised as phagemid from the ZAP Express vector. All three contained the same insert, indicated by identical size, and restriction pattern of the insert (Fig. A.1). Two restriction fragments (560 and 1,200 bp) were obtained after reaction of the inserts with *EcoRI* and *XhoI*, suggesting a total insert size of *ca* 1.7 kbp, and the presence of either an *EcoRI* or *XhoI* site, at the same position in each insert.

```

1   TTCGGCACGA GCGGCACGAG CGGCACGAGC AAACATTACA TTGCTCTACT
51  CGCTATTCAC ATTACAATCA CAAACACATC AGTCATCACA AACATCATGA
101 TGGGTTCCCTC ACAGTCCACC ACCAAATCCG ACCAGGAGCC CAACAAGTCC
151 AAGTCCCTAT ATCAGAAATA CCAAGAGAAA AAGGGCCCCA AGCCCATCAG
201 CGACGAAGAC ATCCTCAAGT ACACCGGCAA AACGAGGGCC GAGATCGACG
251 ACTGGGCAGA CACCCAGCCC GGCCTGGGCA AGAACCAGCT CGCTGGCAAA
301 ATCTCCATCG GCAACGCCAG CGGCCTGGGA GGAGCAGCTG TCGCAGGAGG
351 ATTTGGAGGT TGGGGACCGA GTGCTGAGCC TCAGGGCCCG AATCGGGGCA
401 TGAAGTTTCC GCCAAAGGAT CTGGAGGGGA AGCCTAAGCT TGTGGACGAG
451 TAGATTGGAT ATGAAGGGAG GGTGCCTTTA TGGAGCAGTT GCAGATGGAC
501 GGGATATTGA GCGTGTGAGA GCGGATTATT TTTGGTCTTG AAAAAAAAAA
551 AAAAAAAAAAC TGACTAGTTC TCGTGCCGAA TTCGGCACGA GACATTTTGC
601 GCAAATATCC TGGCTACGAG GGTGTGATTC CTACCTTGTG TGAGCATATC
651 GATGAACTTG ATGAGCCTGA AGCACGGGGG TCTCTCATTT GGATCGTTGG
701 CGAGTACGCC GAGAAGATTA GCAACGCCGA TGAAATATTG GAGAGCTTTG
751 TCGAAGGATT TATGGAAGAG TTTACTCAA CGCAATTGCA AATATTAACG
801 GCAGTGGTTA AGCTCTTCCT GAAGAAGCCC GGCAACACTC AAGGACTTGT
851 GCAAAAGGTA TTGCAGGCTG CGACTGCGGA GAATGACAAC CCGGACATCC
901 GGGACAGAGC CTATGTCTAC TGGCGTTTGC TATCTGGTGA TTTAGATGTT
951 GCGAAGAATA TTGTCTTTTC CAAAAGCCT ACCATCTCCA CCACTATGAC
1001 CAGCTTGCCG CCTTCACTAC TGGAGCAGCT GCTTTCAGAG CTGTCGACCC
1051 TAGCTTCAGT GTACCACAAA CCTCCGGAGT CATTGTTGG TAAGGGACGT
1101 TTCGGAGCGG ACGAAATTC ACGAGCAGCC ATCCAGGAGC AAAGACAAA
1151 CGCCGCCGAC AACCCCATCG CTGCGTCCGT GGCAGCAGCG GCCGCCAATG
1201 GTACTGGCTC TGGCGCTTCG CAAAACAACA TTGAAAACCTT GCTTGACATC
1251 GACTTTGATG GCGCCGCTCC CGCATCTCAC GAGCAACAGA GCGCGACAGC
1301 AACGCCCGAC CGAGTGGCCA GCCCGGCCGG TGGCGCCGCA TCTGGCGGTA
1351 TGGCAGATAT GATGAGCATG TTCGATGCCC CTCCAATGGA GTCAAACAAC
1401 AACGGGGCTG CAGCAGGACC TAGCTCTGGC AGTGGTATGA ATGATTTGAT
1451 GAGCGGGTTT GACGGGTTGA ACTTTGGCAA TACCAATTCG GGCCAGCCCC
1501 TGCCAGCGGC AATGCAGCTT AATAGTGCGC AGGGCGGAAG TACCACACAG
1551 ACGCAGCAGA AGGACAGTGA TGACTTGTTG GGTGCTGTG AAGGGGGTAG
1601 TGGTTTCTAT CTACAAAAG AGGTCTGGCT GAATAGGAAA ATAGTAGGCG
1651 CACCGTTTAT AGACCAGGCA GTGTCAATGG AATAAGAAGC ATGTCGATTT
1701 GGAAGCAGAA AAAAAAAAAA AAAAAAAAAA AACTCGAG

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Fig. A.2 Nucleotide sequence of VC_cDNA, a cDNA clone from *V. chlamydosporium*. The insert is shown without flanking ZAP Express vector sequences. The *EcoRI* site in the insert is in bold.

Preliminary sequencing reactions confirmed that the three positive clones related to the same cDNA. Although the second strand was not completely sequenced, reactions were repeated until all ambiguities were resolved. Since 3 positive recombinants were identified from *ca* 50,000 clones, the corresponding mRNA was rare, as it constituted *ca* 0.006% of total mRNA (Bertioli *et al.*, 1995). Further sequencing confirmed the insert size as 1,739 bp (Fig. A.2). The restriction site was identified as an *EcoRI* site, splitting the insert in two fragments, sized 578 and 1161 bp, respectively, which confirmed the restriction pattern observed.

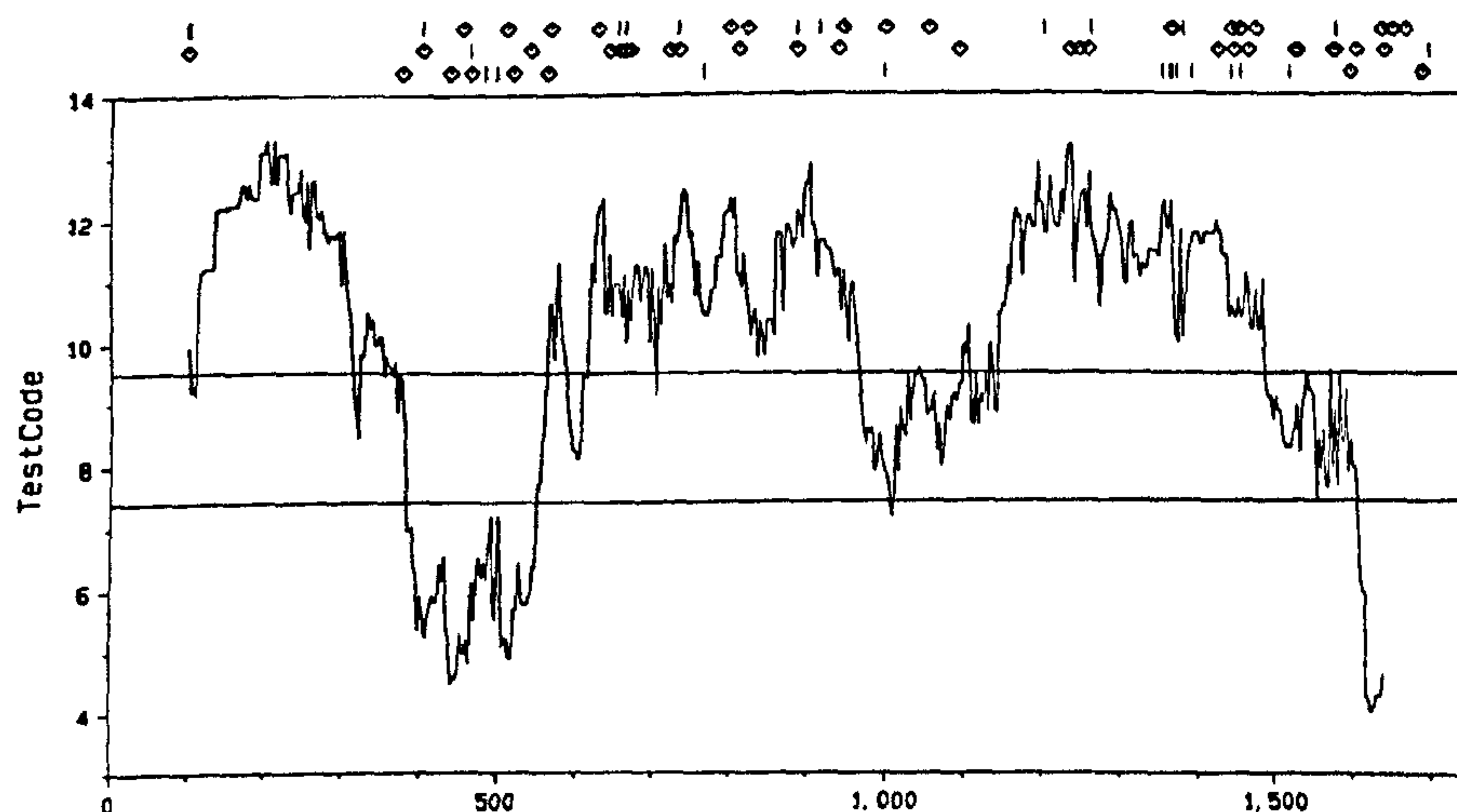


Fig. A.3 Open reading frames in VC_cDNA, and the probability of being transcribed, calculated by the GCG program TESTCODE (Genetics Computer Group, 1991). The probability is plotted in three zones; upper, intermediate and lower, corresponding to high, intermediate and low probabilities, respectively. The window size used in the calculation was 200 bp. Potential start and stop codons in the three forward frames are indicated above the diagram as bars (start) or diamonds (stop codons).

Open reading frames (ORFs) were identified with the program TESTCODE from the GCG package. This program calculates the probability that a region of a nucleotide sequence is transcribed, based on the codon bias that is typical of transcribed sequences (Genetics Computer Group, 1991). The region from 1 up to

VC_cDNA	-58	-----RDILRKYPG-YEGVIPTLCEHIDELDEPE-----ARG	-28
CLAP_MAMMAL		TLLDLIQTKVNYVVQEAIVVIRDIRKYPNKYESI IATLCENLDSLDEPD-----ARA	
CLAP_YEAST1		ILLELLE-RQDTIKDDCCISLCDLLRHCPGN-DKMAKQVCAVFNWTSNPEVLLQSDIAKC	
CLAP_YEAST2		VLLDLLEFGVDYVVQEIISVFRNILRKYPNNFKANVTELVKHTEVVQEP-----SKN	
		... * * . . . * . . .	
VC_cDNA	-27	SLIWIVGEYA EKISNADEILES FVEGFMEEF TQTQLQILTAVVKLFLKPKG-NTQGLVQK	32
CLAP_MAMMAL		AMIWIVGEYAERIDNADELLESFLEGFHDESTQVQLTLLTAIVKLFKPKS-ETQELVQQ	
CLAP_YEAST1		NYVWLLGQHPNNSDLESKINIFIENFVQEEAL TQMSLLMTIVRL---HAT-LTGSMLOS	
CLAP_YEAST2		AMIWIITQYSDVIPNYLELFRVFSNMFS ETLVQFSILNSAIKFFIRSPTKETEELCMD	
		. * * * * * . . . * . . .	
VC_cDNA	33	VLQAATAENDNPDIRDRAVYWRLLS--GDL DVA-----KNIVLSQKPTISTTMTSL	82
CLAP_MAMMAL		VLSLATQSDNPDRLDRGYIWRLLS--TDPVTA-----KEVVLSEKPLISEETDLI	
CLAP_YEAST1		VLELATQOTHELDVRDMAMMYWRCLSM PN NESLV-----NDLCQNKLP MISNTLEKF	
CLAP_YEAST2		LLKGCIDHENNPDRLDKTLMYWRLLS LTKTSRISNAITFESLKSVDGELPLI-EMNTKL	
		. * . . . * . * * . * * * * . . . * * .	
VC_cDNA	83	PPSLLEQLLSELSTLASVYHKPPESFVGKGRFGA DEI QRA--AIQEQRQNAADNP IAASV	140
CLAP_MAMMAL		EPTLLDELICHIGSLASVYHKPPNAFVE---GSHGIHRKHLPIHHGSTDAGDSPVGT TTT	
CLAP_YEAST1		SPEVLEKLLMELGTISSIYFKPDSNRRKGK KYVQNI VKGKHI---EELESMAKNEISSKA	
CLAP_YEAST2		DPTVLEELELNIGTIVSIYLPKVS-----HIFRLNKTKLLPQSPILNPN	
		* . * . * * . * * *	
VC_cDNA	141	AAAAANGTGSGASQNNIENLLDIDFDGAAPASHEQ QSATATPDRVA-----	186
CLAP_MAMMAL		ATNLEQPQVIPSQGDLLGDLNLDLGPVNV PQVSSMQMGAVDLLGGGLDSL VGQSFIPS	
CLAP_YEAST1		N-----DDVL-LDFDERDDVTNTNAGMLN TLTTL-GDLDDLFD-----	
CLAP_YEAST2		K-----DLLPV-----VGNSFPPT	
		. . * . .	
VC_cDNA	187	-----SPAGGAASGGMADMSMFDAPPMESNNNGAAAG----PSSGSG-----	225
CLAP_MAMMAL		SVPATFAPSPTPAVVSSGLNDFELSTGIGMAPGGYVAPKAVWLPVAKAGLEISGTFTH	
CLAP_YEAST1		-----FGPSEDATQINTN-----	
CLAP_YEAST2		GANRDRQNSSESQSSTKSRKTAMMD-----	
		*	
VC_cDNA	226	-----MNDLMSGFDGLNFGNTNSG-----QPLPAAMQLNS-	255
CLAP_MAMMAL		RQGHYMEMNFTNKALQHMTDFAIQFNKNSFGVIPSTPLAIHTPLMPNQSIDVSLPLNTL	
CLAP_YEAST1		-----DTKAVQGLKELKLGGSNGISS-----	
CLAP_YEAST2		-----DYDK-----	
VC_cDNA	256	-----AQQGS-----	260
CLAP_MAMMAL		GPVMKMEPLNQLQVAVKNNIDVFYFSC LIPLNVLFVEDGKMERQVFLATWKDIPNENELQ	
CLAP_YEAST1		-----GGKNNPDV-----SGGNIVSQDLLD-----	
CLAP_YEAST2		-PAEKINQLKGRKSSSNP-----	
VC_cDNA	261	-----TTQTQOKDSDDL-----	273
CLAP_MAMMAL		FQIKECHLNADTVSSKLQNNVYTI AKRNV EGQDMLYQSLKLTNGIWI LAELRIQPGNPN	
CLAP_YEAST1		-----LF-----	
CLAP_YEAST2		-----SKLSRK-----PSTLLRKL SMKRPF-----	
		*	
VC_cDNA	274	-----GLL--	276
CLAP_MAMMAL		YTL SLK CRAPEVSQYIYQVYDSILKN	
CLAP_YEAST1		-----	
CLAP_YEAST2		-----S	

Fig. A.4 Translated open reading frame (ORF) of VC_cDNA, aligned with homologous amino acid sequences. CLAP_MAMMAL: Accession no. P21851 (Beta adaptin; clathrin assembly protein complex 2, beta large chain, from rat, human and bovine); CLAP_YEAST1 and CLAP_YEAST2: Accession nos. P36000 and P27351, respectively (probable beta adaptin; clathrin assembly protein complex 2, beta large chain, from *Saccharomyces cerevisiae*). Numbering corresponds to the ORF of VC_cDNA. Residues, identical in all three sequences are marked with "*", while conserved substitutions are ". ". The first methionine of the ORF is marked as "M". Alignment by GCLUSTALV, integrated in the GCG package (Genetics Computer Group, 1991).

400 bp was a possible, incomplete, ORF (Fig. A.3), but no significant homologies were found in nucleotide and protein databases. A second ORF, with high probability of being transcribed, was identified between 762 and 1590 bp, corresponding to a peptide of 276 amino acids. This region was homologous, both at nucleotide and peptide level, with several clathrin-associated proteins. With the first methionine residue of the ORF given number 1, there was considerable homology in the region from amino acid residue number -58 to 104. This homology was particularly clear with the mammalian clathrin-associated protein, beta-adaptin, and less so with *Saccharomyces cerevisiae* beta-adaptin (Fig. A.4).

Clathrin is an evolutionary highly conserved protein complex, that occurs at specialized regions of the inner surface of the plasma membrane, called coated pits, where endocytotic vesicles are formed. The plasma membrane at these sites can invaginate into the cell and pinch off to form coated vesicles. The invagination of a coated pit is believed to be driven by forces generated by the assembly of clathrin and other coat proteins associated with it (Alberts *et al.*, 1989). Clathrin-coated pits and coated vesicles are associated at the plasma membrane with the early stages of endocytosis, and in the Golgi region with exocytotic vesicles (Kirchhausen *et al.*, 1989). The clathrin-associated proteins are complexes made up of several kinds of polypeptide chains, small (17-20 kDa), medium (45-50 kDa), and large (100-115 kDa). The mammalian and yeast clathrin-associated proteins with which the *V. chlamydosporium* clone was homologous, belong to the group of the large polypeptides. They have a complex role both in the assembly of the clathrin coat and collection of cargo into nascent vesicles (Kirchhausen *et al.*, 1989).

Despite the high degree of homology, in particular with the mammalian protein, the true identity of the clone identified remained uncertain. The clathrin-associated proteins in question are all in the range of 100-115 kDa, while the *V. chlamydosporium* ORF in question was only 276 amino acid residues long. Moreover, the homology stretched from residue -26, upstream of the start of the ORF, and not beyond residue 104, which is less than halfway in the ORF. Over the whole of the translated ORF, VC_cDNA had 57.4% similarity with the mammalian clathrin-associated protein, while the identity of the corresponding nucleotide sequences was 45.6%.

The sequence was also remarkable because of the presence of a poly-A region (541-559 bp, see Fig. A.2), closely followed by an *EcoRI* site (at 578 bp). As cDNA is cloned into the ZAP Express vector with a 5' *EcoRI* end, its presence in this position raised the suspicion of the clone actually consisting of two different cDNA's that artefactually merged during the cloning procedure. Although not impossible in a single cDNA, the combination of the poly-A stretch, at the 3' end of a cDNA, and the neighbouring restriction site, possibly indicating the 5' start of the second cDNA, would be most coincidental. However, no vector sequence was recognized in this area.

This experiment failed to correctly identify a subtilisin-like gene in *V. chlamydosporium* with a PCR-derived homologous probe. At the point where the true nature of the identified clone was realized, time constraints precluded reprobng the library. Although the reason for the failure could not exactly be pinpointed, it is likely that the problem was probe-related. Despite the nested procedure in the PCR to

generate the probe, using two sets of *Pr1*-derived primers (S.C.M. Leal, IACR-Rothamsted, personal communication), the reaction may have inadvertently amplified a non-target sequence from *V. chlamydosporium*. It was unfortunate that the initial screening with the heterologous *Pr1*-cDNA, the identity of which was certain, resulted in too strong a signal and background to identify genuine positives. This problem may have been as simple as overloading the plaque lifts with an excess of heavily labelled probe, but could also not be corrected, due to time constraints.

The ZAP Express cDNA library that was constructed, from starvation-induced *V. chlamydosporium*, is believed to remain a valuable resource, that could still harbour *VCP1* and other co-regulated cDNAs.

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