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ORIGINAL PAPER

Regulation of cuticle-degrading subtilisin proteases from the entomopathogenic fungi, *Lecanicillium* spp: implications for host specificity

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Abstract The ability to produce cuticle-degrading proteases to facilitate host penetration does not distinguish per se entomopathogenic fungi from saprophytes. However, adapted pathogens may produce host-protein specific enzymes in response to cues. This possibility prompted an investigation of the regulation of isoforms of the subtilisin Pr1-like proteases from five aphid-pathogenic isolates of *Lecanicillium* spp. Significant differences were found in substrate specificity and regulation of Pr1-like proteases between isoforms of the same isolate and between different isolates. For example, the pI 8.6 isoform from KV71 was considerably more active against aphid than locust cuticle and was induced specifically by *N*-acetylglucosamine (NAG). Isoform pI 9.1 from the same isolate was only produced on insect cuticle while most other isoforms were more prominent on chitin containing substrates but not induced by NAG. The ability to regulate isoforms independently may allow production at critical points in host penetration. Appearance of proteases (not subtilisins) with pI 4.2 and 4.4 only on aphid cuticle was a possible link with host specificity of KV71. The absence of C or N metabolite repression in subtilisins from KV42 is unusual for pathogen proteases and may help to account for differences in virulence strategy between aphid-pathogenic isolates of *Lecanicillium longisporum* (unpublished data).

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Abbreviations

dH_2O	Distilled water
Suc–Ala–Ala–Pro–Phe–NA	Succinyl–alanyl–alanyl–prolyl–phenylalanyl–*p*–nitroanilide
Bz–Phe–Val–Arg–NA	*N*–benzoyl–phenylalanyl–valyl–arginyl–*p*–nitroanilide
NAG	*N*-acetylglucosamine
BSA	Bovine serum albumen
IEF	Isoelectric focusing

Introduction

Entomopathogenic fungi invade their hosts through the external skeleton (cuticle). This is a complex, composite structure with a high-protein content. Thus, particular attention has been focused on the role of proteases in the penetration process (see Charnley 2003 for a recent review). The Ascomycotina fungus *Metarhizium anisopliae* produces multiple isoforms of a number of families of extracellular cuticle-degrading proteases viz. subtilisins (Pr1), trypsin-like proteases (Pr2), chymotrypsins, metalloendoproteases and several classes of exo-acting enzymes (Screen and St. Leger 2000; St. Leger et al. 1993, 1994a, b). The cDNAs and genes of representatives of several classes of these proteases have been cloned and sequenced (e.g. Smithson et al. 1995; St. Leger et al. 1992).

Pr1-like proteases have been found in all Deuteromycotina and Ascomycotina entomopathogenic fungi tested. Genes that are significantly similar to *Pr1* are present in

Aspergillus flavus and *Lecanicillium* (=*Verticillium*) *longisporum* (St. Leger et al. 1992) and *Pr1*-like genes from two isolates of *Beauveria bassiana* have been cloned and sequenced (Joshi et al. 1995; Kim et al. 1999). However, production of high levels of a subtilisin-like protease is not an indicator of entomopathogenicity because subtilisin-related proteases are also the principal broad-spectrum proteases of many saprophytes (Gunkle and Gassen 1989). The level of importance of Pr1 to pathogenicity has still not been established. *M. anisopliae* ARSEF 2575 has some 11 *Pr1* genes (Freimoser et al. 2003). Thus, it is not surprising that a null mutant of one of them, *Pr1A*, retained near normal pathogenicity under certain bioassay conditions (St. Leger 1995). However, the plethora of *Pr1s* in *M. anisopliae* sf. *anisopliae* (11 genes) compared with the scarcity of subtilisins in saprophytic filamentous fungi (2–3 genes) (Freimoser et al. 2003) certainly implies a key role in disease.

Insect cuticle contains many different proteins, which vary between types of insect (Andersen et al. 1995; Dombrovsky et al. 2003). Thus, while Pr1 enzymes may not be a prerequisite for pathogenicity, isolate virulence or specificity could depend in part on having proteases with high activity for host-specific cuticular proteins. Just a few strategic amino acid substitutions can affect cuticle binding and activity of Pr1 from *M. anisopliae* (St. Leger et al. 1986a, 1992). Since Leal et al. (1997) found significant variation in sequence between *Pr1s* from 57 isolates from seven species of fungi there may be sufficient variability in Pr1 substrate specificity to influence virulence and or/host specificity, particularly since multiple forms of Pr1 in the same isolate could improve efficiency against different families of host cuticular protein.

Pr1 from *M. anisopliae* ARSEF 2575 is controlled by multiple regulatory circuits which include carbon and nitrogen derepression (St. Leger et al. 1988), induction (Campos et al. 2005; Paterson et al. 1994b) and pH (St. Leger et al. 1998, 1999). Under derepressed conditions Pr1 is specifically induced by an as yet ill-defined proteinaceous component of insect cuticle; presumably an adaptation of *M. anisopliae* to insect parasitism (Paterson et al. 1994b). While secretion of proteases by *M. anisopliae* is enhanced during growth at pHs that are optimum for protease activity, a further threefold increase in production occurs in the presence of cuticle (St. Leger et al. 1998).

Isolate virulence or specificity could depend in part on the ability to produce a battery of proteolytic enzymes with specificity for the range of proteins in the cuticle of a particular insect, in response to particular host cues at the correct stage in penetration. The present work is a first attempt to explore the potential for this by comparing the regulation and activity of isoforms of subtilisin Pr1-like proteases within and between isolates of entomopathogenic fungi. *L. longisporum* and *L. muscarium* (Ascomycota: Incertae sedis) were chosen for this study because as pathogens in particular of plant sucking insects of the Order Hemiptera, they are more specific than the more widely studied *M. anisopliae* and *B. bassiana* (both Ascomycota: Hypocreales).

Methods

Organisms and growth conditions

The fungal isolates were kindly provided by Koppert B.V., The Netherlands: *L. longisporum* isolates KV71 (ex aphid, Aphididae and the active ingredient of the commercial mycoinsecticide Vertalec®), KV22 (ex unknown), KV42 (ex plum aphid, *Rhopalosiphum nymphaeae*, Aphididae, Homoptera); *L. muscarium* isolates KV01 (ex glasshouse whitefly *Trialeurodes vaporariorum*, Aleyrodidae, Homoptera and the active ingredient of the commercial mycoinsecticide Mycotal®) and KV54 (ex the bracken fern, *Pteridium aquilinum*, Pteridophyta). All the isolates were pathogenic for the peach potato aphid *Myzus persicae* though KV01 is only weakly so (Roditakis and Charnley, unpublished data).

The fungus was cultured routinely on malt agar (2% w/v malt extract, 2% agar) and malt agar supplemented with bacteriological peptone (5% w/v) at 23°C. Conidia were harvested from actively growing cultures (10-day old) in sterile distilled water (dH_2O) and filtering through two layers of sterile muslin.

Desert locusts (*Schistocerca gregaria*) were reared as described by Gillespie et al. (1998) and the peach potato aphids (*M. persicae*) were a gift from Dr. Robert Lind (Syngenta, Jealott's Hill, UK). Liquid cultures were grown either in Modified Czapek Dox (Oxoid, Hampshire, UK) supplemented with 2 g l^{-1} each of casein hydrolysate, mycological peptone, yeast extract and malt extract [complete medium (CM)] or modified basal salts plus trace elements which contained per litre 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and trace elements at ppm: 0.2 $FeSO_4 \cdot 7H_2O$, 1.0 $ZnSO_4 \cdot 7H_2O$, 0.02 $NaMoO_4 \cdot 2H_2O$, 0.02 $CuSO_4 \cdot 5H_2O$ and 0.02 $MnCl_2 \cdot 4H_2O$, plus 50 mM 2-[*N*-morpholino] ethane sulphonic acid and buffered to pH 6. Carbon/nitrogen sources, were supplied at 1% (w/v) or as indicated. Cuticles from mature adult *S. gregaria* and *M. persicae* were prepared according to the method of Andersen (1980). Approximately 100 locusts were frozen at –20°C for 1 h and then homogenised in a Waring blender in 1 l of 1% w/v potassium tetraborate. The cuticle pieces were washed extensively in dH_2O, stirred overnight in 2 l of 1% (w/v) potassium tetraborate and then air dried at room temperature. The cuticle pieces were then milled to a fine powder using a Glen Creston Ball Mill (DEH48) using the

0.2 mm sieve. The powder was washed in 2 l of 1% potassium tetraborate and finally in dH_2O, allowed to settle and any floating material removed. The method was scaled for use with aphids. One locust is approximately equivalent in weight to 5,000 aphids. Practical grade chitin from crab shells was either used as purchased (and designated as "chitin") or hydrolysed in 30% (w/v) potassium hydroxide at 80°C for 2 h to remove residual protein (designated as deproteinised chitin or "KOH chitin"). The residual chitin was washed extensively in dH_2O and then allowed to air dry.

Transfer experiments

Hundred ml of CM in 250 ml conical flasks was inoculated with 4×10^6 conidia and incubated at 23°C in an orbital shaker (120 rpm) for 4 days to allow extensive fungal growth. The mycelial biomass was harvested by sieving through two layers of sterile muslin, washed with sterile basal salts medium, and transferred to 100 ml basal salts medium without nutrients for 24 h to achieve metabolite derepression (referred to as derepressed mycelia). All manipulations were conducted under aseptic conditions. The fungus was then supplied with a nutrient source [viz. cuticle, chitin, KOH chitin or Bovine Serum Albumen (BSA)] at 1% (w/v) and the culture supernatant was assayed for Pr1-like activity at 16 and 36 h. Due to the limited supply of aphid cuticle, transfer experiments comparing locust and aphid cuticle were performed in smaller cultures viz. 0.1 g cuticle in 10 ml medium in 25 ml flasks. For carbon and nitrogen repression experiments, the fungal biomass was transferred to buffered basal salts containing 1% cuticle but deficient in one or both of an additional soluble source of C (1% w/v sucrose) and N (0.4% w/v NH_4Cl).

Supplying *N*-acetylglucosamine, in a restricted manner, to established biomass

An established biomass was starved for 24 h and transferred to buffered basal salts. *N*-acetylglucosamine (NAG) was supplied to these cultures either from the outset or at linear flow rates in a restricted manner by means of diffusion capsules (Lab-line Instruments Inc., Melrose Park, IL, USA), in order to prevent sugar build up in the medium to repressive levels (Pirt 1971). Rates were controlled by altering the number of membranes (dialysis tubing) through which diffusion occurred and the concentration of NAG within the capsule. The optimum flow rate of 20–25 μg NAG ml^{-1} h (determined using Nelson–Somoygi reducing sugar assay Nelson 1944) was achieved with 5% NAG and one dialysis tube membrane. Controls contained capsules charged with water rather than NAG. Cultures were incubated at 23°C and 120 rpm. Samples were removed at 16 h, centrifuged and Pr1-like activity was determined in the supernatant.

Enzyme assays

Pr1-like activity was assayed by monitoring the release of nitroaniline (NA) at 405 nm from 2 mM solutions of the peptide substrate succinyl–alanyl–alanyl–prolyl–phenylalanyl–*p*–nitroanilide (Suc–Ala–Ala–Pro–Phe–NA) and Pr2 from the release of NA from 2 mM *N*–benzoyl–phenylalanyl–valyl–arginyl–*p*–nitroanilide (Bz–Phe–Val–Arg–NA) essentially as described by St. Leger et al. (1987a). Assays were performed in microtitre plates and measured on a Dynatech MR5000 plate reader. Activity was expressed as nkat NA released ml^{-1} min^{-1} or in nmol nitroalanine h^{-1}.

The use of ergosterol as a measure of fungal biomass

Determination of biomass in cultures grown on an insoluble nutrient source such as insect cuticle has to be done indirectly. Ergosterol is a fungal sterol found in few other organisms. There is a significant positive correlation between ergosterol content and fungal biomass for the isolates of *L. longisporum* and *L. muscarium* used in the present study (Graystone and Charnley, unpublished data) and thus ergosterol content was used as an indirect measure of fungal biomass. The procedure used for the extraction and quantification of ergosterol was Paterson et al. (1994b) modification of the method described by Seitz et al. (1979).

Flat-bed isoelectric focusing

Culture filtrates 20-h post-transfer into the test conditions were dialysed against several changes of 1% glycine for 24 h, followed by dH_2O for a further 24 h to eliminate salt then concentrated by lyophilisation. Fifty micrograms of protein was loaded into each lane of a broad range pH 3–10 Ampholine precast IEF gel (Pharmacia Biotech, Piscataway, NJ, USA). Samples were then focused for 1.5 h at 10°C at a constant voltage of 30 W, as per the manufacturer's instructions. Proteins on the IEF gels were identified as proteases by their ability to degrade gelatin. Immediately after focusing, gels were overlaid with X-ray film (pre-soaked in dH_2O). The overlay was photographed with a digital camera at regular intervals, to record the position of degradation bands as soon as they first became apparent. The film was left in contact with the gel until no further bands of degradation were observable. The position of the proteases was compared to the position of broad range pI markers,

fixed and stained in Coomassie Blue R-250. Subtilisin (Pr1-like) and trypsin (Pr2-like) enzymes were identified after focusing as follows. About 1 mm slices were excised from the gel from the direction of the cathode for each sample lane. These slices were transferred to wells of a microtitre plate containing 100 µl Tris–HCl (pH 8.0) buffer and homogenised using a multiple well homogeniser (Burkhard). About 50 µl of 2 mM *N*–Suc–Ala–Ala–Pro–Phe–NA (Pr1 substrate) or Bz–Phe–Val–Arg–NA (Pr2 substrate) was then added and the release of NA monitored over 1 h.

Protein determination

Hydrolysis of protein from locust or aphid cuticle by purified Pr1 isoforms was estimated by measuring the absorbance at 260 and 280 nm with a Cecil 2040 spectrophotometer.

$$\text{Amount of protein/mg protein ml}^{-1} = [1.55 \times A_{280}] - [0.76 \times A_{260}]$$

(Walker 1996).

Results

Effect of different substrates on protease production

Preliminary experiments established that cellulose, gelatin, urea and xylan did not promote subtilisin production by established biomass of KV71. Therefore work was concentrated on cuticle and its two major components protein and chitin. BSA was included as a soluble protein for comparison.

All isolates produced significantly higher subtilisin-like protease activity in cultures containing cuticle, than in control (basal salts alone) at 16 and 36 h (Table 1), which rules out regulation by derepression alone. KV71 and KV42 produced the highest levels of enzyme overall. The order of enzyme production for KV71 at 16 and 36 h was KOH chitin ≥ cuticle > BSA > chitin > control. KV54 displayed a similar pattern to KV71, however BSA did not support substantial enzyme activity and levels in control cultures were negligible.

For KV42 and KV22 at 16 h activity was in the order cuticle = chitin = KOH chitin. KV22 produced significantly higher protease activity in control cultures than on BSA. Isolate KV42 exhibited a marked increase in enzyme activity from 16 to 36 h in all cultures, in contrast in KV54 cultures activity on cuticle, chitin and KOH chitin, dropped sharply from 16 to 36 h.

The response of KV01 subtilisins to the four substrates was in the order BSA > cuticle > chitin > KOH chitin viz. subtilisin activity was related to the proportion of protein in the substrate. Thus, KV01 appears to produce subtilisin in response to protein whereas the other four isolates appear to be more responsive to chitin. Consistent with this, restricted feeding of NAG (the monomer of chitin) to biomass of KV71 induced protease to 61% of that on cuticle (Table 2). In contrast NAG had little effect on subtilisin production by KV01 (11% of that on cuticle). For both isolates batch cultures with NAG as sole nutrient source were repressive for subtilisin.

Differences between treatments could not be accounted for in terms of fungal growth as there were no significant differences in biomass (estimated using content of the fungal sterol ergosterol) between any of the experimental treatments 24 h after transfer (data not shown). Also in all transfer experiments similar levels of enzyme activity were observed in culture supernatants before and after dialysis against water, showing that differences between isolates and between the same isolate on different media were not due to low-molecular weight inhibitors (data not shown).

Proteases from cuticle cultures of KV71, KV42 and KV01 were separated on IEF. Activity was determined by

Table 1 Subtilisin production by derepressed mycelia of five isolates of *Lecanicillium* spp on a number of polymeric substrates

Isolate	Cuticle		Chitin		KOH chitin		BSA		Control	
	16 h	36 h	16 h	36 h	16 h	36 h	16 h	36 h	16 h	36 h
KV42	11.6 ± 1.8	24.9 ± 5.7	9.6 ± 2.5	26.7 ± 4.4	9.6 ± 2.5	23.2 ± 11.8	3.9 ± 0.2	11.4 ± 0.4	2.8 ± 0.66	8.1 ± 1.05
KV22	7 ± 1.7	3.9 ± 0.4	7.7 ± 1	3.6 ± 0.8	7.9 ± 1.4	6.4 ± 0.5	2.8 ± 0.5	1.7 ± 0.3	4.4 ± 0.5	2.6 ± 0.7
KV	12.1 ± 2.1	13.6 ± 2.1	8.6 ± 1.1	10.7 ± 1.8	2.5 ± 0.4	7.9 ± 2.9	15.7 ± 2.5	20.3 ± 5.4	1.4 ± 0.1	1.1 ± 0.1
KV54	6.4 ± 0.8	2.8 ± 0.8	3.5 ± 0.3	0.5 ± 0.2	7.7 ± 1.2	1.8 ± 0.05	0.5 ± 0.5	1.6 ± 0.3	0.7 ± 0.2	0.6 ± 0.1
KV71	10.6 ± 0.7	14 ± 2.1	2.8	6.2 ± 1.2	13.6 ± 3.5	11.7 ± 2.5	8.1 ± 0.5	4.6 ± 0.2	3.5 ± 0.2	3.2 ± 0.09

Four-day-old fungus, grown in complete medium was starved for 24 h before transfer to cultures containing either 1% (w/v) locust cuticle, chitin, KOH treated chitin (deproteinised chitin), Bovine serum albumen (BSA) or just basal salts (control). Enzyme activity against *N*–Suc–$(Ala)_2$–Pro–Phe–NA was recorded for each of the five isolates on these substrates at 16 and 36-h post-transfer. Results are mean ± SD in nkat NA released min^{-1} ml^{-1}, $N = 4$. ANOVA and Fishers pairwise comparisons indicated that enzyme activities were significantly different from control at both time points apart from KV54 on BSA at 16 h

Table 2 The effect of *N*-acetylglucosamine on Pr1-like activity in two isolates of *Lecanicillium* spp

Substrate	Subtilisin activity in nkats ml^{-1} min^{-1}	
	Mean ± SD, *N* = 4	
	KV71	KV01
N-acetylglucosamine provided from diffusion capsules	13.9 ± 1.5	3.4 ± 0.9
50 µl 5% *N*-acetylglucosamine	0	0
100 µl 5% *N*-acetylglucosamine	0	0
1% w/v, locust cuticle	22.3 ± 1.7	32.2 ± 4.1
Activity in reaction when the substrate is *N*-acetylglucosamine expressed as % of the activity when cuticle is the substrate	62	11

Four days old established biomass of KV71 and KV01 was starved for 24 h then supplied with substrate as indicated. The supernatant was assayed for Pr1-like activity 16-h post-transfer. The activities are corrected for activity in control flasks which were supplied with dH_2O in an unrestricted or restricted fashion

X-ray film over lay (see Fig. 1) and excised bands from the gel were tested against *N*–Suc–Ala–Ala–Pro–Phe–NA and Bz–Phe–Val–Arg–NA to identify subtilisin (Pr1-like) and trypsin (Pr2-like) isoforms, respectively (see Fig. 2).

For all isolates tested, subtilisin activity was only detected in the basic section of the gel (pH 7–10) (Fig. 2). Four distinct degradation zones were seen on the gel with KV71, indicating proteases with pI values of 8.62, 8.83, 9.10 and ≥9.47 [the limit of the resolution of the gel (Fig. 1)]. All four proteases exhibited Pr1-like activity and a low level of Pr2-like activity was also observed at ≥9.47 (Fig. 2). Gelatin degrading activity was also observed at pI 6.48, 6.30 and 5.8 (not shown), these forms of protease had activity against the trypsin substrate (Fig. 2).

KV42 produced two very distinct couplets of protease activity at pI 8.0, 8.19 and 8.92, 8.98 (Fig. 1). Isoforms of pI 8.92 and 8.98 exhibited considerably higher levels of Pr1-like activity than those at pI 8.0 and 8.19 (Fig. 2). Pr2-like activity could also be detected at pI 8.92 and 8.98. KV01 had one gelatin-degrading enzyme at pI 8.95 and three forms close to one another at pI 7.7, 7.85 and 8.19. Both KV01 and KV42 produced high-subtilisin activity at the most basic section of the gel (>9.47), however little or no gelatin degrading activity was observed, compared to that of KV71. The Pr1-like activity of this very basic form from KV01, was approximately tenfold higher than that of pI 7.7 and 8.19, which were not highly active against the subtilisin substrate. KV01 and KV42 also produced a much less basic protease at *ca.* 6.6 which did not have an affinity for the subtilisin substrate, but had some Pr2-like activity for KV01.

In cases of dual activity the pattern of inhibition by the protease inhibitors chymostatin (subtilisin/chymotrypsin inhibitor), leupeptin (trypsin inhibitor) and turkey egg

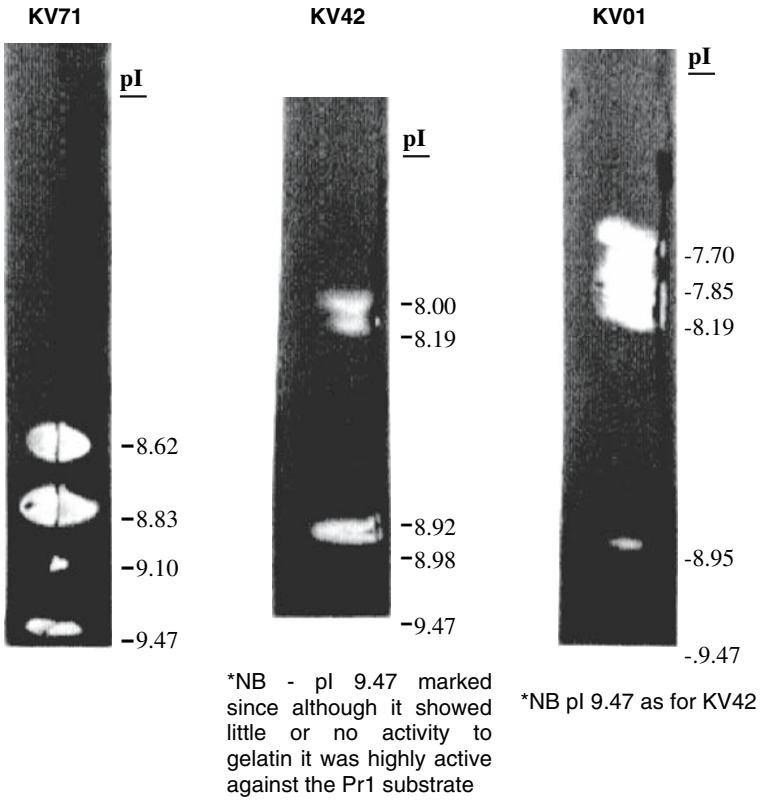

Fig. 1 Gelatin overlay indicating proteases produced in locust cuticle cultures of *Lecanicillium* spp. Fifty micrograms of protein from KV71, KV42 and KV01 cuticle cultures, 20-h post-transfer, was focused on a broad range iso-electric focusing gel (Ampholine PAGplate™) at a constant wattage of 30 W. Immediately after focusing, the gel was overlaid with moist X-ray film. The position of degradation zones were noted and the pI of the proteases estimated by comparison with broad range pI markers run on the same gel and stained with Coomassie Blue. The experiment was repeated three times with similar results

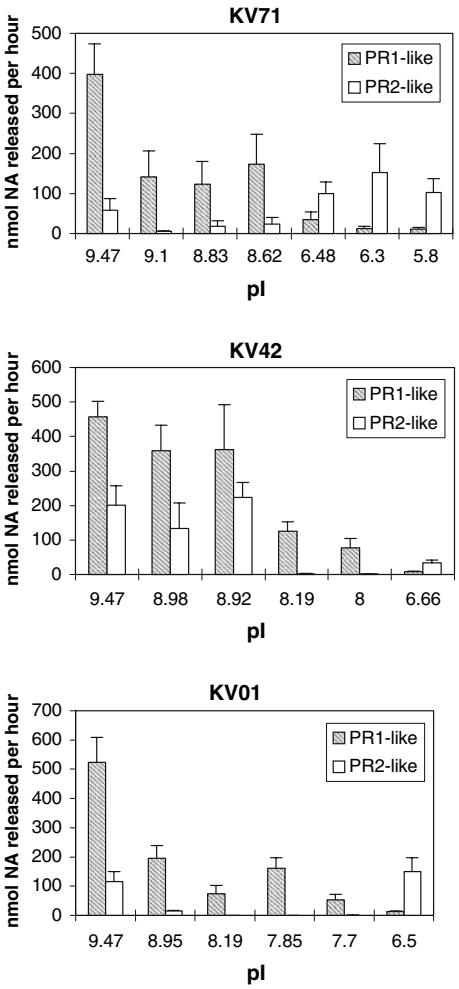

Fig. 2 Pr1-like and Pr2-like activity at each iso-electric point for three isolates of *Lecanicillium* spp grown on locust cuticle. Fifty micrograms of protein from each cuticle culture 20-h post-transfer was focused on a broad range iso-electric focusing gel as described. One millimeter slices were excised from the gel and incubated with *N*–Suc–Ala–Ala–Pro–Phe–NA and Bz–Phe–Val–Arg–NA for Pr1 and Pr2-like activity, respectively. The estimated iso-electric point (*pI*) where enzyme activity was found is given. Activity given in nmol nitroalanine h^{-1}, mean ± SD, $N = 4$

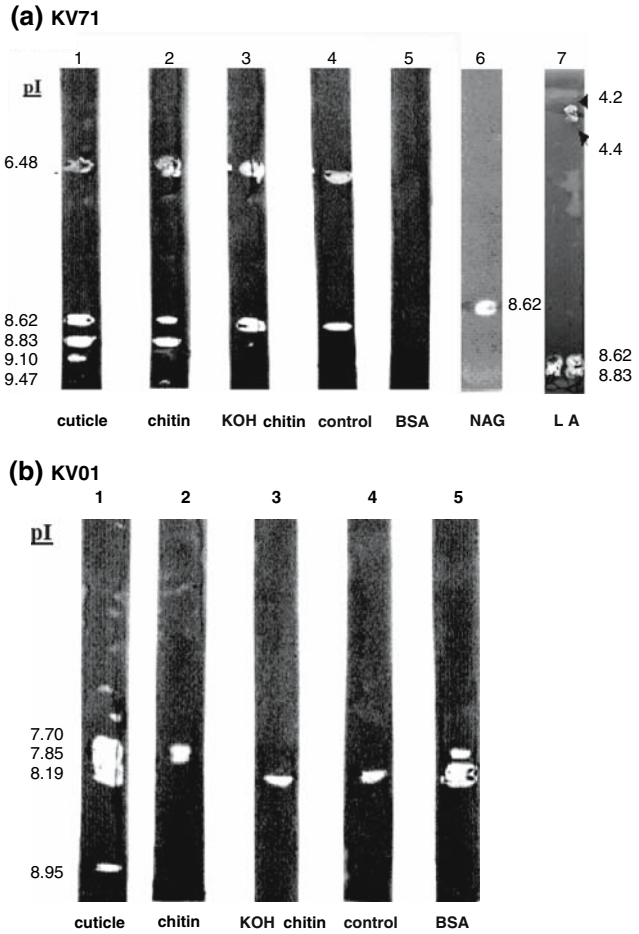

Fig. 3 IEF gels were overlaid with moist X-ray film and degradation zones were noted and photographed as they developed. Figure shows the situation after 40 min. Representative of three separate experiments. **a** KV71, Lanes 1–5 from cuticle (locust cuticle), chitin (from crab shells), KOH chitin (deproteinised crab chitin) and control (basal salts alone), BSA; Lane 6 from restricted feed NAG culture; Lane 7 from cultures on locust (*L*) or aphid cuticles (*A*) **b** KV01, Lanes 1–5 as per **a** KV71

white inhibitor (subtilisin/chymotrypsin and trypsin inhibitor) was similar when Pr1 and Pr2 substrates were used suggesting that a single enzyme and not two forms was present in each case (data not shown). Up to 2 mM 1, 10 phenanthroline (a metalloprotease inhibitor) did not affect activity of any of the enzymes against the Pr1 substrate, confirming that the activity was due to a subtilisin and not a metalloendoprotease. Inhibitors used were those identified by St. Leger et al. (1987b, 1994b) as active against comparable proteases from insect Ascomycotina pathogens.

The pattern of protease isoform production in KV71 cultures supplied with chitin was very similar to that on cuticle, apart from the absence of a band at pI 9.1 (Fig. 3a). In cultures with KOH chitin isoform 9.1 was absent, 8.83 very much reduced and 8.62 enhanced with respect to cuticle cultures. Interestingly IEF of proteases from a restricted feed NAG culture (see above) produced a single band at pI 8.62 (Fig. 3a). The reduction/loss of pI 9.1 and 8.83 also occurred in control cultures (Fig. 3a). All isoforms were absent from BSA cultures. Enzyme assays performed using gel slices and chromogenic substrate showed the presence of a subtilisin at pI 9.47, even if the band on gelatin film was weak or absent (as on BSA). Interestingly the pI 9.47 activity was high in all treatments except for BSA viz. cuticle, 398 ± 76 nmol NA h^{-1}; chitin 399 ± 99 nmol NA h^{-1}; KOH-treated chitin 462 ± 108 nmol NA h^{-1}; BSA, 50 ± 8 nmol NA h^{-1}, Control,

486 ± 106 nmol NA h^{-1} (mean ± SD, $N = 3$, in each case).

For KV01 the five isoforms produced on cuticle (see Figs. 1, 2, 3b) were variously produced on other substrates. The most basic isoform pI 9.47 was produced on them all, though only shown in gel slice assays viz. cuticle, 524 ± 102 nmol NA h^{-1}; chitin 459 ± 96 nmol NA h^{-1}; KOH-treated chitin 463 ± 93 nmol NA h^{-1}; BSA, 415 ± 102 nmol NA h^{-1}, Control, 283 ± 72 nmol NA h^{-1} (mean ± SD, $N = 3$, in each case). pI 8.95 and 8.19 were absent from chitin cultures. pI 8.95, 7.85 and 7.7 were missing from KOH chitin and control cultures. Interestingly only pI 8.95 was missing from the BSA culture.

Pr1-like activity on host and non-host cuticle

The subtilisin activities in cultures supplied with either locust or aphid cuticle are shown in Fig. 4. In KV71 cultures, a similar level of Pr1-like activity was observed at 16 and 24 h on both cuticles. In KV01 cultures, at 16 h, again similar Pr1-like activity was recorded on both aphid and locust cuticle, however at 24 h, significantly more protease activity was observed on locust cuticle than on aphid cuticle ($P < 0.05$, Student's t-test).

Subtilisin isoform production by KV71 was the same on aphid and locust cuticles. Two acidic proteases pI 4.2 and 4.4 were observed in aphid cuticle but not in locust cuticle cultures (Fig. 3a). These proteases did not have activity against the Pr1 or Pr2 substrates.

Filtrates from KV71 and KV42 cultures, that contained cocktails of cuticle-degrading enzymes, hydrolysed three to fourfold more protein from aphid than locust cuticle. This was not the case with KV01 cultures (data not shown). The cuticle hydrolysing abilities of subtilisin isoforms, semi-purified by elution from IEF gels, were determined for KV71, KV42 and KV01. Experiments were carried out in two ways. Cuticle digestion was determined when the same amount of enzyme protein (20 μg) was added to the reaction mixture, or when the same amount of Pr1-like activity was added to the cuticle (that which released 10 nkat NA min^{-1} ml^{-1} from *N*–Suc–Ala–Ala–Pro–Phe–NA) (Fig. 5). Although the relative activities of isolates differed between the two experimental protocols, essentially the same pattern emerged with both. For KV71 and KV42 activities of most isoforms were significantly greater against aphid than locust cuticle, whilst with KV01 either there was no difference between the two or activity against locust cuticle was greater than aphid cuticle. It is interesting to note the particular preference of the KV71 pI 8.62 isoform for aphid cuticle.

Metabolite repression of subtilisins from isolates of *Lecanicillium* spp

The effects on enzyme activity of supplementing insect cuticle with more readily available, low-molecular weight sources of carbon (C) and nitrogen (N) in the form of sucrose (1% w/v) and ammonium chloride (0.4%), respectively, are shown in Table 3. At 16 and 36 h maximum production of Pr1 was generally found for all isolates on cuticle alone (–C–N). Supplementing cultures with N (–C+N) significantly reduced protease activity of KV01 and KV71 at both time points, and KV22 and KV54 at 16 h only. Interestingly, at 36 h, N significantly enhanced protease production in KV22 cultures. However, protease production in N supplemented cultures of KV42 was similar to that of cuticle alone at both 16 and 36 h.

When C was supplied to cuticle cultures (+C–N), almost total repression of subtilisin activity was observed at both time points for most isolates, KV54 and KV71 were the most sensitive. Subtilisin production by KV01 was repressed equally under conditions of additional C or N, at 16 and 36 h. KV42 was the only isolate to continue to produce subtilisin in C containing cultures at 16 and 36 h to the same level as cuticle only cultures. It appeared to be unaffected by N or C.

All isolates exhibited almost total repression of subtilisin activity when both N and C (+C+N) were supplied to cuticle cultures at 16 and 36 h; the two nutrients appeared to act synergistically to inhibit enzyme production.

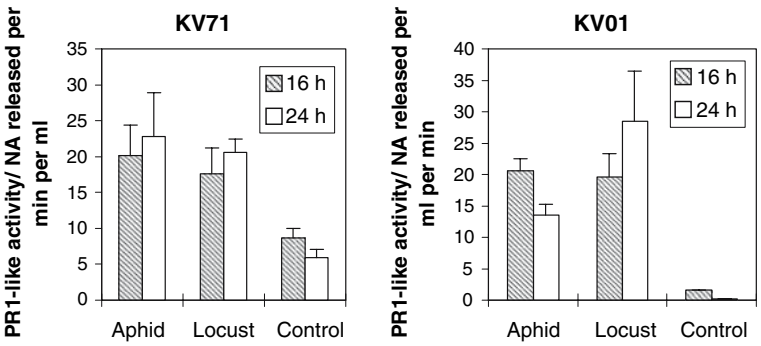

Fig. 4 Pr1-like activity 16 and 24 h following transfer of a starved biomass of KV71 and KV01 into basal salts containing aphid cuticle, locust cuticle or just basal salts (control). Activity is expressed as nkats NA released ml^{-1} min^{-1}, mean ± SD, $N = 4$

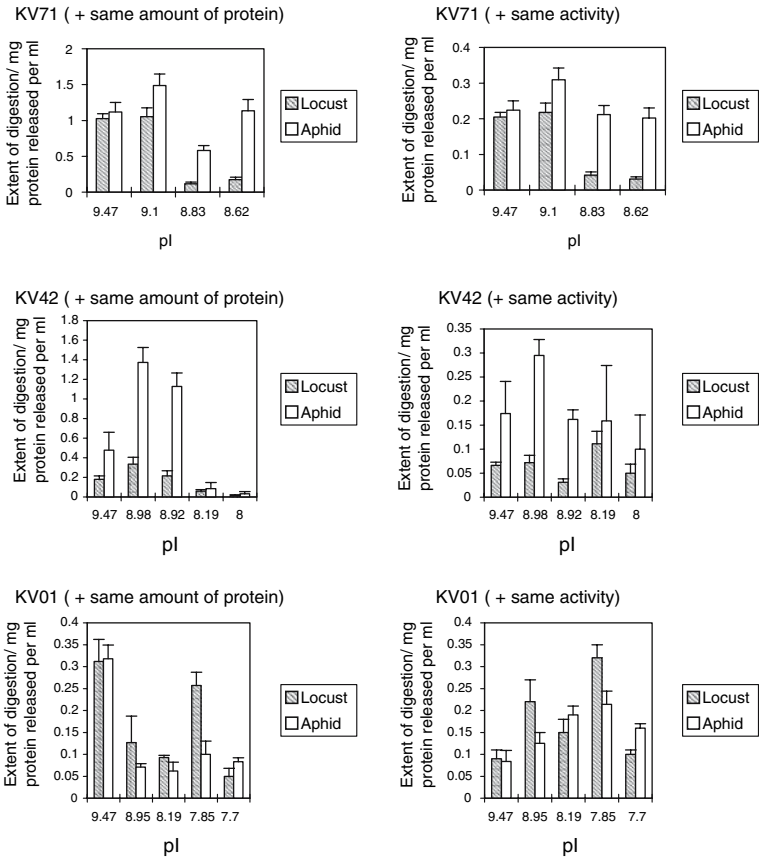

Fig. 5 A comparison of the amount of protein released from locust and aphid cuticle after an overnight incubation with isoforms of Pr1-like enzyme as semi-purified from locust-cuticle cultures. *Same amounts of protein*: 20 μg protein at the iso-electric points shown *same enzyme activity*: equal amounts of Pr1-like activity (that which causes the release of 10 nkats NA per min per ml) of each isoform. In each case enzyme was incubated with 5 mg cuticle in buffer overnight at 28°C. The amount of protein released is shown as the mean ± SD, $N = 3$

Culture filtrates from these experiments were subject to IEF to determine whether isoforms were affected similarly by metabolite repression. In the case of KV 71 added nitrogen had particular impact on pI 9.10 (Table 4). Carbon or carbon *and* nitrogen, repressed all four isoforms. For KV42, however, all five proteases with Pr1-like activity observed on cuticle as the sole source of carbon and nitrogen, were present when cuticle was supplemented with either soluble nitrogen or carbon. No observable differences in the pattern of proteases was observed between these three cultures. As with KV71, no subtilisin activity was observed when both soluble carbon and nitrogen were supplied together.

Discussion

A number of lines of evidence from this study suggest that the Pr1-like subtilisin enzymes of *Lecanicillium* are induced by insect cuticle. For KV71 cellulose, xylan, urea and gelatin generated no subtilisin activity and elastin only to a limited extent, though all substrates supported growth

Table 3 Effect of carbon and nitrogen repression on subtilisin production by five isolates of *Lecanicillium* spp

Isolate	–C–N		+C+N		+C–N		–C+N	
	16 h	36 h	16 h	36 h	16 h	36 h	16 h	36 h
KV42	10.5 ± 1.8	24.4 ± 5.5	0.6	2.5 ± 1	14.4 ± 4.6	24.8 ± 9.2	11.8 ± 8.4	29.4 ± 1.8
KV22	7.1 ± 1.6	4.1 ± 0.5	0.5	0.1	2.7 ± 1	1.8	2 ± 0.1	14.2 ± 2.3
KV01	12.1 ± 3.2	13.1 ± 2.4	0.4	0	3.7	4 ± 0.1	5 ± 0.7	4.8 ± 1.7
KV54	6.3 ± 0.7	2.6 ± 0.8	0.2	0.2	0	0.5	1.9 ± 0.3	2.5 ± 0.4
KV71	10.2 ± 0.7	14.1 ± 2.1	0	0	0.4	0.8	7.5 ± 0.8	8.4 ± 0.9

Four days old fungus, grown in complete medium was starved for 24 h before transfer to cultures containing 1% (w/v) locust cuticle [–C–N], or 1% locust cuticle in the presence of additional, more readily available forms of carbon and/or nitrogen: [+C+N; +C–N; –C+N]. Enzyme activity against *N*–Suc–$(Ala)_2$–Pro–Phe–NA was recorded for each of the five isolates shown on these substrates at 16 and 36-h post-transfer. Results are mean ± SD in nkat NA released min^{-1} ml^{-1}, $N = 4$. ANOVA and Fishers pairwise comparisons indicated that enzyme activities were significantly different from cultures without additional nutrient source [–C–N] at both time points apart from KV54 (–C+N) at 36 h and KV42 (+C–N and –C+N) at both time points

Table 4 Effect of carbon and nitrogen repression on the pattern of isoforms of subtilisins produced by two isolates of *Lecanicillium* spp

Estimated pI			
–C–N	–C+N	+C–N	+C+N
KV71			
≥9.47	≥9.47	No Pr1-like	No Pr1-like
9.10	(9.10)[a]	Activity detected	Activity detected
8.83	8.83	In lane	In lane
8.62	8.62		
KV42			
≥9.47	≥9.47	≥9.47	No Pr1-like activity
8.98	8.98	8.98	Detected in lane
8.92	8.92	8.92	
8.19	8.19	8.19	
8.00	8.00	8.00	

Isoforms of proteases from culture supernatants were separated on an isoeletric focusing system. The gels were overlaid with moist X-ray film and degradation zones were noted and photographed as they developed and compared to the position of Coomassie stained markers. Gel slices were excised from replicate lanes and assayed for Pr1-like activity. The Pr1-like enzymes identified are shown for KV71 and KV42 on cuticle alone (–C–N), cuticle with soluble nitrogen (–C+N), cuticle with soluble carbon (–N+C) or cuticle with both carbon and nitrogen (+C+N). The experiment was repeated three times with similar results

[a] Isoform 9.10 was slower to degrade the gelatin overlay, and exhibited a fourfold reduction in Pr1-like activity compared to the form produced in the absence of additional nitrogen

in batch cultures. All five isolates of *Lecanicillium* produced considerable subtilisin when grown on locust cuticle as the sole source of carbon and nitrogen, and significantly more than derepressed mycelia on basal salts alone. There were no consistent significant differences in subtilisin production between aphid and locust cuticles and thus the inducing agents are present to equal extent in the two cuticles. Since all isolates are to varying extents pathogenic for aphids but not locusts induction of subtilisin activity is not host-related. Furthermore unlike *M. ansiopliae* ARSEF 2575 (Paterson et al. 1994a), the inducer is not unique to insect cuticle and differs between isolates. The subtilisins of the virulent aphid-pathogenic *L. longisporum* isolates KV71, KV42 and KV22 and *L. muscarium* KV54 appeared strongly on KOH chitin and cuticle, identifying chitin as an inducer. The reduced importance of protein in the regulation of subtilisin is particularly apparent with KV54 and KV71 for which chitin (crab chitin with residual protein) supported significantly less enzyme activity than KOH chitin (crab chitin without residual protein). In contrast to the other isolates, although subtilisins of *L. muscarium* KV01 (ex whitefly and a weak pathogen of aphids) were supported to a limited extent by chitin, activity increased in direct proportion to the protein content of the substrate. It would seem that in common with Pr2 from *M. anisopliae* ARSEF 2575 (Paterson et al. 1993), subtilisin from KV01 is induced primarily non-specifically by protein.

At first sight, it seems counter intuitive that proteases should be induced by chitin. However, given that the cuticle consists primarily of chitin fibrils embedded in a protein matrix (Neville 1984) and that fungal chitinases are also usually induced by chitin (Smith and Grula 1983; St. Leger et al. 1986b), co-ordinated regulation of enzymes that hydrolyse the two main constituents of cuticle may prove most efficient under some circumstances. The subtilisin PRB1 from the mushroom pathogen *Trichoderma harzianum* is induced also by chitin in fungal cell wall preparations and is repressed in the presence of casein or BSA (Geremia et al. 1993). Since chitin is an insoluble polymer the most likely inducer is the monomer NAG which plays a similar role for PRB1 (Geremia et al. 1993) and *Metarhizium* endochitinase (St. Leger et al. 1986b). For KV71, Pr1 activity was produced to 61% of that on cuticle when the fungus was supplied with NAG at a rate that did not exceed demand (restricted culture); high concentrations of NAG in unrestricted cultures were repressive. In contrast KV01 did not produce subtilisin when NAG was supplied in an unrestricted manner and only to 11% of that on cuticle when supplied in a restricted fashion. It is unfortunate that there is no comparative information on the cuticle composition of aphids and whiteflies. This might otherwise account for the different regulation of proteases from isolates of *Lecanicillium* that are adapted to these groups of insects.

In common with *M. anisopliae* ARSEF 2575 the Pr1 from the five isolates of *Lecanicillium* tested here were all metabolite repressed under nutrient rich conditions. Pr1 from *M. anisopliae* was repressed by a soluble source of C, but predominantly by low-molecular weight N (St. Leger et al. 1988). In contrast, Pr1-like enzymes of KV71, KV54 and KV22, showed a level of repression by N but were predominantly repressed by C. KV01 appears to exhibit control of subtilisin production equally by C and N repression. Dual control also occurs with KV22, though in this case nitrogen repression is short lived.

Interestingly, and in contrast to the other isolates, Pr1-like enzymes from KV42, were insensitive to the presence of either low M_r C or N. Fungal subtilisins play a part in invasion of host cuticle but are rarely produced during fungal growth in the haemolymph, probably because of metabolite repression. The absence of independent C and N repression of subtilisins from KV42 may account for the early appearance of fungal subtilisins during invasion of *M. persicae* by this isolate (Bye and Charnley, unpublished data). This reflects a different virulence strategy adopted by KV42 in comparison to KV71. The latter grows prolifically in the haemolymph; its subtilisins, subject to C and N

repression, are not produced during early infection; widespread early host tissue invasion/degradation leads to host death. In contrast KV42 has minimum growth in the haemolymph prior to death; its subtilisins, not subject to C or N repression, are produced early in infection; there is no tissue invasion prior to death which is probably as a consequence of toxins (Bye, Graystone and Charnley, unpublished data).

Isoforms of subtilisins produced by the isolates of *Lecanicillium* studied were not co-coordinately regulated. Therefore the overall activity depends on the individual responses of each isoform. Most previous studies on protease regulation by *M. anisopliae* and *B. bassiana* have not examined differences between isoforms, e.g. (St. Leger et al. 1988, 1994b). St. Leger et al. (1994b) found the largest number of Pr1 and Pr2 isoforms produced by *M. anisopliae* on insect cuticle and the number of Pr1 isoforms on cuticle diminished with time. No further forms were observed on any other substrate tested. Furthermore only two forms were produced, albeit at diminished levels, on elastin or cellulose. They attributed these results to differing sensitivities of genes to carbon and nitrogen derepression, rather than differential induction.

For *Lecanicillium* isolates also the most Pr1-like isoforms was produced on cuticle as the sole source of carbon and nitrogen, no further forms were found on other substrates. The predominant isoform produced by KV01, KV42 and KV71 was a very basic form, pI ≥9.47 at the limit of the resolution of the gel. High-subtilisin activity was recovered from this section of the gel for all three isolates. However, for KV42 and KV0l, this protease was unable to degrade gelatin—indicating a difference from the isoform produced by KV71. The protease extracted from the extreme basic end of the gel also had some activity against the Pr2 substrate though inhibitor studies and Coomassie staining were consistent with the presence of a single enzyme. Samuels et al. (1990) similarly found proteases produced by a number of *Erynia* spp. that had mixed chymotrypsin/trypsin activity.

For KV01 and KV71, the very basic isoform was produced on all test substrates including basal salts, aphid and locust cuticles. This isoform was repressed, as were all other forms, when additional low M_r source of carbon was supplied to cuticle cultures of KV71. The equivalent KV42 isoform, however, was unaffected by the addition of a freely available source of carbon. Thus, although these two isolates have a Pr1-like enzyme with similar pI, these isoforms appear to be regulated differently. With the exception of this basic form of the enzyme, each isolate studied, produced its own distinctive pattern of Pr1-like isoforms on insect cuticle.

In addition to these inter-isolate differences, isoforms of the same isolate were regulated differently also. For KV71, on BSA, the low levels of subtilisin were attributable to the most basic form (≥9.47), all others forms were repressed. However, KV01 produced the same isoforms on BSA as it did on locust cuticle. KV71 produced one Pr1-like isoform of pI 9.10 only on insect cuticle. It was not derepressed by starvation nor was it induced by chitin or protein. It appears to be induced specifically by a component of the insect cuticle itself. Furthermore, this form was particularly sensitive to both carbon and nitrogen repression. It was completely repressed in the presence of low M_r carbon, but unlike the other forms, was sensitive to nitrogen. The repression of this isoform accounts for the overall loss of subtilisin activity in cuticle cultures supplemented with nitrogen.

The whitefly isolate, KV01, did not produce any isoforms exclusively on cuticle, however one form, pI 8.95 was only produced on cuticle and BSA. Isoforms of pI 7.7 and 7.85 were reduced on deproteinised chitin. Furthermore, the low levels of activity found in control cultures, were predominantly due to that at pI 9.47. The common denominator for these KV01 isoforms appears to be non-specific protein induction.

KV42, in addition to pI ≥9.47, produced two distinct couplets of protease with subtilisin activity on insect cuticle. Unlike KV71 where one form was repressed by nitrogen, and all forms were repressed by carbon, all the KV42 isoforms were unaffected when more accessible sources of carbon *or* nitrogen were supplied to cuticle cultures; though all forms were repressed when the two were supplied together. Thus with respect to carbon and nitrogen derepression, all isoforms of Pr1-like enzyme produced by KV42 are co-ordinately regulated.

KV71 produced two proteases with a low pI on aphid cuticle that were not apparent on locust cuticle. These proteases showed no Pr1 or Pr2-like activity. It seems likely that they were induced by some component specific to host aphid cuticle. Interestingly we extracted a pI 4.4 protease from aphids infected with this isolate also (Bye and Charnley, unpublished data). These enzymes could be aspartyl proteases, similar to those found in cultures of *M. anisopliae* (St. Leger et al. 1998).

Host (aphid) cuticle did not induce more Pr1-like activity than non-host (locust) cuticle and the pattern of isoforms was also the same. However, a protease cocktail from ex-aphid isolates, KV71 and KV42, digested 3.5-fold more host cuticle than non-host cuticle and in most cases individual isoforms showed similar preference. Dombrovsky et al. (2003) established the first sequences of aphid cuticle proteins from *M. persicae* cDNA libraries. Similar genes were found in five other aphid species and they appear to be unique to this group of insects. If aphid-adapted isolates of *L. longisporum* prove to have host specific proteases this would be the

first example of such adaptation amongst entomopathogenic fungi.

Cocktails of proteases in culture filtrates from KV01, ex whitefly, digested aphid and locust cuticles equally well. Individual isoforms hydrolysed both types of cuticle with equal facility, apart from pI 8.95 and 7.85 that had greater activity against locust than aphid cuticle. This could be a contributory factor to the low aphid-pathogenicity of this isolate (Hall 1984; Roditakis and Charnley, unpublished data).

In conclusion, the results presented here show significant differences in substrate specificity and regulation of subtilisin Pr1-like proteases between isoforms of the same isolate and between different isolates of *Lecanicillium* spp. For example, the pI 8.6 isoform from KV71 is considerably more active against aphid than locust cuticle and is induced specifically by NAG. Isoform pI 9.1 from the same isolate was only produced on insect cuticle while most other isoforms were more prominent on chitin containing substrates but not induced by NAG; pI 9.47 was strongly produced on all media and was the only one in evidence on BSA (but to a reduced extent). This ability to regulate isoforms independently may allow production at particularly critical points in the penetration process. Appearance of proteases with pI 4.2 and 4.4 only on host aphid cuticle is a possible link with host specificity of KV71. The absence of C or N metabolite repression in subtilisins from KV42 is unusual for pathogen proteases and helps to account for differences in virulence strategy between aphid-pathogenic isolates of *L. longisporum*.

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References

Andersen SO (1980) Cuticular sclerotization. In: Miller TA (ed) Cuticle techniques in arthropods. Springer, New York, pp 185–217

Andersen SO, Hojrup P, Roepstorff P (1995) Insect cuticular proteins. Insect Biochem Mol Biol 25:153–176

Campos RA, Arruda W, Boldo JT, da Silva MV, de Barros NM, de Azevedo JL, Schrank A, Vainstein MH (2005) *Boophilus microplus* infection by *Beauveria amorpha* and *Beauveria bassiana*: SEM analysis and regulation of subtilisin-like proteases and chitinases. Curr Microbiol 50:257–261

Charnley AK (2003) Fungal pathogens of insects: cuticle degrading enzymes and toxins. Adv Bot Res 40:241–321

Dombrovsky A, Huet H, Zhang H, Chejanovsky N, Raccah B (2003) Comparison of newly isolated cuticular protein genes from six aphid species. Insect Biochem Mol Biol 33:709–715

Freimoser FM, Screen S, Bagga S, Hu G, St. Leger RJ (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiol-Sgm 149:239–247

Geremia RA, Goldman GH, Jacobs D, Ardiles W, Vila SB, Vanmontagu M, Herreraestrella A (1993) Molecular characterization of the proteinase-encoding gene, Prb1, related to mycoparasitism by *Trichoderma harzianum*. Mol Microbiol 8:603–613

Gillespie JP, Bateman R, Charnley AK (1998) Role of cuticle-degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. J Invertebr Pathol 71:128–137

Gunkle FA, Gassen HJ (1989) Proteinase K from *Tritorachium album*. Eur J Biochem 179:185–194

Hall RA (1984) Epizootic potential for aphids of different isolates of the fungus *Verticillium lecanii*. Entomophaga 29:311–321

Joshi L, St. Leger RJ, Bidochka MJ (1995) Cloning of a cuticle-degrading protease from the entomopathogenic fungus, *Beauveria bassiana*. FEMS Microbiol Lett 125:211–217

Kim HK, Hoe HS, Suh DS, Kang SC, Hwang C, Kwon ST (1999) Gene structure and expression of the gene from *Beauveria bassiana* encoding bassiasin I, an insect cuticle-degrading serine protease. Biotechnol Lett 21:777–783

Leal SCM, Bertioli DJ, Butt TM, Carder JH, Burrows PR, Peberdy JF (1997) Amplification and restriction endonuclease digestion of the Pr1 gene for the detection and characterization of *Metarhizium* strains. Mycol Res 101:257–265

Nelson N (1944) A photometric adaptation of the Somoygi method for the determination of glucose. J Biol Chem 153:375–380

Neville AC (1984) Cuticle: organisation. In: Hahn BJ, Maroksy AG, Richards KS (eds) Biology of the integument. Springer, Berlin, pp 611–625

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1993) Regulation of production of a trypsin-like protease by the insect pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiol Lett 109:323–327

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994a) Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. Microbiology 140:3153–3159

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994b) Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. Microbiology 140:185–189

Pirt SJ (1971) The diffusion capsule device for the addition of a solute at a constant rate to a liquid medium. J Biochem 121:293–297

Samuels RI, Charnley AK, St. Leger RJ (1990) The partial characterization of endoproteases and exoproteases from 3 species of entomopathogenic entomophthorales and 2 species of deuteromycetes. Mycopathologia 110:145–152

Screen SE, St. Leger RJ (2000) Cloning, expression, and substrate specificity of a fungal chymotrypsin—evidence for lateral gene transfer from an actinomycete bacterium. J Biol Chem 275:6689–6694

Seitz LM, Sauer DB, Burroughs R, Mohr HE, Hubbard JD (1979) Ergosterol as a measure of fungal growth. Phytopathology 69:1202–1203

Smith RJ, Grula EA (1983) Chitinase is an inducible enzyme in *Beauveria bassiana*. J Invertebr Pathol 42:319–326

Smithson SL, Paterson IC, Bailey AM, Screen SE, Hunt BA, Cobb BD, Cooper RM, Charnley AK, Clarkson JM (1995) Cloning and characterization of a gene encoding a cuticle- degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. Gene 166:161–165

St. Leger RJ (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73:S1119–S1125

St. Leger RJ, Bidochka MJ, Roberts DW (1994a) Characterization of a novel carboxypeptidase produced by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Biochem Biophys 314:392–398

St. Leger RJ, Charnley AK, Cooper RM (1986a) Cuticle-degrading enzymes of entomopathogenic fungi mechanisms of interaction between pathogen enzymes and insect cuticle. J Invertebr Pathol 47:295–302

St. Leger RJ, Charnley AK, Cooper RM (1987a) Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253:221–232

St. Leger RJ, Cooper RM, Charnley AK (1987b) Distribution of chymoelastases and trypsin-like enzymes in five species of entomopathogenic deuteromycetes. Arch Biochem Biophys 258:123–131

St. Leger RJ, Cooper RM, Charnley AK (1986b) Cuticle-degrading enzymes of entomopathogenic fungi—regulation of production of chitinolytic enzymes. J Gen Microbiol 132:1509–1517

St. Leger RJ, Cooper RM, Charnley AK (1993) Analysis of aminopeptidase and dipeptidylpeptidase iv from the entomopathogenic fungus *Metarhizium anisopliae*. J Gen Microbiol 139:237–243

St. Leger RJ, Durrands PK, Cooper RM, Charnley AK (1988) Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Microbiol 150:413–416

St. Leger RJ, Frank DC, Roberts DW, Staples RC (1992) Molecular-cloning and regulatory analysis of the cuticle- degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. Eur J Biochem 204:991–1001

St. Leger RJ, Joshi L, Roberts D (1998) Ambient pH is a major determinant in the expression of cuticle- degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl Environ Microbiol 64:709–713

St. Leger RJ, Nelson JO, Screen SE (1999) The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. Microbiology 145:2691–2699

St. Leger RJ, Bidochka MJ, Roberts DW (1994b) Isoforms of the cuticle-degrading Pr1 protease and production of a metalloprotease by *Metarhizium anisopliae*. Arch Biochem Biophys 313:1–7

Walker JM (ed) (1996) The protein protocols handbook. Humana Press, Totowa, NJ, 807pp