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Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*

Ian C. Paterson, A. Keith Charnley, Richard M. Cooper and John M. Clarkson

Author for correspondence: John M. Clarkson. Tel: +44 225 826826. Fax: +44 225 826779.

Microbial Pathogenicity Group, School of Biological Sciences, University of Bath, Bath BA2 7AY, UK

The insect pathogenic fungus *Metarhizium anisopliae* produces several extracellular cuticle-degrading proteases and evidence is consistent with one of these, PR1, which is a chymoelastase, being a determinant of pathogenicity. We have shown previously that PR1 production is regulated by both carbon catabolite and nitrogen metabolite repression and also by specific induction under derepressed conditions by insect cuticle. In the present work we have established that an enzymically released proteinaceous component(s) of insect cuticle is capable of inducing PR1 (based on appearance of extracellular activity). Cuticle of the desert locust *Schistocerca gregaria* treated with KOH to remove protein failed to induce PR1 production, whereas cuticle treated with either chloroform or ether to remove lipids still induced PR1. Cuticle digested with either PR1 or the trypsin-like PR2 of *M. anisopliae* released peptides mainly in the range 150–2000 Da; addition of these peptides generated by PR1 or PR2 at 3 μg alanine equivalents ml^{-1} induced PR1 production to a level similar (75%) to that obtained with untreated insect cuticle. Several amino acids and peptides which are abundant in insect cuticular protein (Ala, Gly, Ala-Ala, Ala-Ala-Ala, Ala-Pro and Pro-Ala) were tested at a range of concentrations and in restricted cultures for their ability to induce PR1. None induced the protease to the levels seen with cuticle or peptides enzymically released from cuticle, although some dimers and notably the monomers Ala and Gly gave 2–2·7-fold enhanced PR1 activity above derepressed basal levels (up to 48–57% of that achieved with induced synthesis on cuticle). There was evidence for more efficient uptake and/or catabolism by *M. anisopliae* of alanine di- and tripeptides than of monomer amino acids.

Keywords: *Metarhizium anisopliae*, insect pathogen, protease regulation

INTRODUCTION

Infection of insects by the filamentous Deuteromycete fungus *Metarhizium anisopliae* occurs by penetration of the cuticle by a combination of mechanical pressure and enzymic degradation (Charnley, 1984). *M. anisopliae* produces a range of extracellular enzymes, namely proteases, chitinases and esterases, that are active against the major components of insect cuticle (Charnley & St Leger, 1991). Three endoproteases, chymoelastase (PR1), trypsin-like protease (PR2) and trypsin-like cysteine protease (PR4) have been characterized (St Leger *et al.*, 1987a; Cole *et al.*, 1993). Several lines of evidence indicate that PR1 is essential for cuticle penetration (St Leger *et al.*, 1987b, 1988a) but the roles of PR2 and PR4 are unclear.

As with many other fungal proteases, PR1 and PR2 are controlled by multiple regulatory circuits which include carbon and nitrogen derepression (St Leger *et al.*, 1988b) and induction (Paterson *et al.*, 1993, 1994). It has been demonstrated that the regulation of PR1 by derepression is achieved at the level of transcription (St Leger *et al.*, 1991). Under derepressed conditions, PR2 is induced by a range of proteinaceous substrates (Paterson *et al.*, 1993) whereas PR1 is specifically induced by a component of insect cuticle (Paterson *et al.*, 1994). This is the first example of specific induction of any microbial protease

Abbreviation: NA, nitroanilide.

and presumably reflects the adaptation of *M. anisopliae* to insect parasitism. Insect cuticle is composed of two layers, the outer epicuticle and the procuticle. The epicuticle is a very complex, thin composite structure which is devoid of chitin but contains phenol-stabilized protein (Andersen, 1979). The procuticle constitutes the majority of the cuticle and comprises chitin fibrils embedded in a protein matrix, together with lipids and quinones. In insects such as locusts, the procuticle may be further differentiated into an outer exocuticle, in which the protein is stabilized (sclerotized) by quinone cross-links, and an inner endocuticle which is unsclerotized. Protein can contribute as much as 70% of the cuticle, with chitin content usually between 20 and 50%. It is therefore likely that PR1 is induced by either the lipid, the protein or the chitin component of cuticle. The exact nature of the inducing molecule(s) has yet to be identified. We report here that an enzymically released proteinaceous component of insect cuticle is capable of inducing PR1.

METHODS

Culture conditions. The fungal isolate ME1, media and culture conditions, and the preparation of insect cuticle have been described previously (Paterson *et al.*, 1993, 1994). Carbon sources were supplied to cultures at 1% (w/v) unless otherwise stated. For certain experiments, amino acid monomers and dimers were also supplied at linear low rates in a restricted manner by means of diffusion capsules (Pirt, 1971; Cooper & Wood, 1975). Rates were controlled by altering the number of membranes (dialysis tubing) through which diffusion occurred. Capsules containing alanine (10%, w/v) and three membranes, or alanine dimers (5%, w/v) and one membrane, gave diffusion rates into 100 ml medium of approximately 20 $\mu g\ ml^{-1}\ h^{-1}$. Empty diffusion capsules were washed in absolute ethanol and then autoclaved with carbon sources before placing in cultures. Insoluble carbon and nitrogen sources were sterilized in an atmosphere of propylene oxide.

Processing of cuticle. Protein was removed from insect cuticle by hydrolysing powdered cuticle in 30% (w/v) KOH at 80 °C for 2 h. The chitinous cuticle, which is resistant to KOH treatment, was removed from the hydrolysate, then washed extensively in distilled water and dried (Grosscurt, 1978). Hydrocarbons, glycerides and sterol esters were removed from cuticle by stirring powdered cuticle in diethyl ether or chloroform for 2 h at room temperature. The remaining cuticle was then stirred as above in ethanol to extract phospholipids and fatty acids (Gilby, 1980), then washed in distilled water and dried. Chitin consisted of crab chitin (Sigma) which had been de-proteinated as described above for insect cuticle.

Transfer experiments. Complete medium (Paterson *et al.*, 1993), 100 ml in 250 ml Erlenmeyer flasks, was inoculated with 4×10^6 conidia and incubated at 27 °C in an orbital shaker (150 r.p.m.) for 3 d in order to establish extensive fungal growth (about 300 mg; Paterson *et al.*, 1994). Cultures developed as uniform submerged mycelial balls with no sporulation evident; the morphology of fungal cultures remained constant over the duration of the experiments. The culture was sieved through two layers of sterile muslin. Retained mycelium was washed with sterile basal salts medium (BSM) (Paterson *et al.*, 1993), transferred to 100 ml BSM, and then incubated for a further 24 h under the same conditions as above to ensure complete catabolite derepression. Potential inducers of PR1 were then added and PR1 activity in the culture supernatant was assayed for up to 24 h. The numbers of replicates and experiments are given in Tables 1–3.

Enzyme assays. PR1 and PR2 were assayed by monitoring the release of nitroanilide (NA) from the peptide substrates succinyl-Ala-Ala-Pro-Phe-NA and benzoyl-Phe-Val-Arg-NA, respectively, as previously described (Paterson *et al.*, 1993, 1994).

Chemicals. All chemicals, unless otherwise stated, were from Sigma. HPLC grade solvents were from Rathburns.

HPLC. All HPLC was performed as described by Paterson *et al.* (1994) except that the injection valve was fitted with either a 20 or 100 µl loop.

Enzyme purification. Prior to enzyme purification, culture filtrates were reduced in volume about 100-fold by ultrafiltration on YM5 membranes (Amicon; 5 kDa exclusion). PR1 was purified by cation-exchange HPLC. Separation was performed on a Dynamax SCX-i (Rainin) strong cation-exchange column (4·6 mm × 10 cm) equilibrated with 20 mM sodium acetate pH 5·5 (1 $ml\ min^{-1}$). Elution of basic proteins was achieved with a linear gradient (0–0·4 M NaCl) in similar buffer with detection at 280 nm. The majority of the proteins did not bind to the column; PR1 and its pro-enzyme (S. C. J. Cole, R. M. Cooper & A. K. Charnley, unpublished) were usually the only proteins eluted by the gradient.

PR2 was purified by anion-exchange chromatography on a column (10 × 1 cm) of Q-Sepharose equilibrated in 10 mM Tris/HCl pH 8. Prior to loading the sample, the column was washed with 30 ml equilibration buffer and the proteins then eluted with a linear 60 ml Tris/HCl gradient (0·01–0·75 M).

After purification, both enzymes were pure as demonstrated by single bands on SDS-PAGE and had the same molecular mass and substrate specificities as described by St Leger *et al.* (1987a).

Hydrolysis and separation of cuticular peptides. One gram of cuticle was digested in 100 ml buffered BSM (Paterson *et al.*, 1993) containing 1 mg purified PR1 or PR2 ml^{-1} and incubated at 27 °C, 150 r.p.m. for 24 h. The same conditions were used to solubilize peptides with buffer without the addition of enzyme. The products were analysed by HPLC using an Anagel TSK 3000 SWXL gel filtration column (7·8 mm × 30 cm). The mobile phase was 0·1 M Na_2SO_4 in 0·1 M sodium phosphate buffer, pH 7·5, with a flow rate of 0·8 $ml\ min^{-1}$ and detection at 280 nm. The column was calibrated with L-phenylalanine, L-alanyl-L-proline, glutathione and insulin A (molecular mass 165, 186, 307 and 2531 Da, respectively).

RESULTS

Cuticle components as inducers of PR1 production

In view of the structure of insect cuticle it is likely that PR1 is induced by either the lipid, the protein or the chitin component. To test this hypothesis, cuticle, modified cuticle or cuticle components were added to cultures starved of C and N for 24 h; PR1 activity was then measured after 4 and 12 h (Table 1). The treatments were: deproteinated chitin; deproteinated (by KOH) cuticle; and cuticle extracted with ether or with chloroform (to remove the lipid components).

Ether- or chloroform-extracted cuticle induced PR1 production to a level similar to that with untreated cuticle,

Table 1. PR1 induction in *M. anisopliae* by components of insect cuticle

C/N source (1%, w/v)	Enzyme activity*	
	4 h	12 h
Control (−C, −N)	181·8±6·9	290·9±3·7
Cuticle	738·6±21·5	2551·6±58·9
Ether-extracted cuticle	545·5±19·4	2863·7±80·3
KOH-extracted cuticle	252·3±11·5	593·2±30·8
Chloroform-extracted cuticle	637·5±17·4	2687·7±61·7
Chitin	418·0±26·1	711·4±22·9

*Mean activity ±SD from three replicates 4 h and 12 h after the addition of potential inducers to cultures starved of C and N (−C, −N) for 24 h. Enzyme activity is expressed as nmol NA ml^{-1} min^{-1} released from succinyl-$(Ala)_2$-Pro-Phe-NA. The results are representative of three similar experiments.

suggesting that cuticular lipids do not affect PR1 production (Table 1). Activity with chitin was nearly 2·5 times that of controls lacking C and N (−C, −N) but activities after 12 h were only about 30% of those with cuticle. It is possible that chitin was utilized as a source of C and N, enabling slightly enhanced PR1 production without causing catabolite repression. Deproteinized cuticle also failed to induce PR1 and resulted in levels of activity similar to those with chitin. These results imply that the inducer of PR1 is some component of cuticular protein.

Table 2. Effect of peptides solubilized from insect cuticle on PR1 production by *M. anisopliae*

C/N source	Concn (μg ml^{-1})	Enzyme activity*	
		4 h	8 h
Controls −C, −N		185·4±16·9	268·3±23·4
Cuticle	10^4†	485·7±49·7	1297·0±64·5
PR1 products	1·5	375·6±25·4	607·4±39·1
	3·0	400·1±19·2	818·7±40·8
	4·5	214·4±11·5	482·2±17·6
PR2 products	1·5	354·6±17·4	849·9±25·9
	3·0	427·2±33·1	977·2±63·3
	4·5	271·6±22·6	662·3±29·7
Soluble products	1·5	118·2±16·4	374·4±21·2
	3·0	227·2±17·5	413·6±29·7
	4·5	97·1±6·8	428·1±27·9

*Mean activity ±SD from three replicates 4 h and 12 h after the addition of peptides generated from insect cuticle to cultures starved of C and N (−C, −N) for 24 h. Other details as for Table 1.

†1% (w/v).

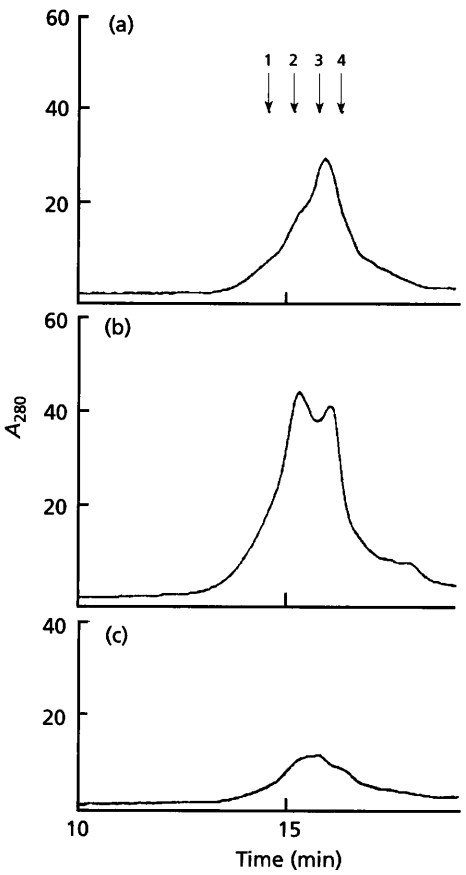

Fig. 1. High performance gel filtration of peptides released by digestion of cuticle by PR1 (a) or PR2 (b), or solubilized by buffer (c). Locust cuticle (1 g) was incubated with purified PR1 or PR2 (1 mg ml^{-1}) in 100 ml buffered BSM at 27 °C, 150 r.p.m. for 24 h. The same conditions were used to solubilize peptides with buffered BSM only. Peptide products were detected by absorbance at 280 nm after separation by gel filtration. Molecular mass markers (numbered arrows) are (1) 2531, (2) 307, (3) 186 and (4) 165 Da.

Cuticular protein as an inducer of PR1 production

Attempts were made to obtain by HPLC the peptides hydrolysed by the two proteases which could function as inducers of PR1 production. PR1 released peptides mainly in the range 150–2000 Da, with a predominant peak at approximately 200 Da (Fig. 1a). PR2 generated peptides of a similar size range to PR1 but with a different distribution; peaks were at approximately 300 and 160 Da (Fig. 1b). Some peptides were solubilized in buffer alone; these were in the range 100–300 Da and were released in smaller quantities than that released by enzymic hydrolysis (Fig. 1c). The PR1-, PR2- and buffer-solubilized products contained 24, 17·5 and 9·5 μg alanine equivalents ml^{-1}, respectively (assayed by the method of Moore & Stein, 1948).

To test the ability of the peptides to induce PR1, mycelium starved of C and N as described above was transferred to 100 ml BSM containing 1·5, 3 and 4·5 μg alanine equivalents ml^{-1} of either the PR1-, PR2- or buffer-solubilized peptides. PR1 activity was assayed after 4 and 8 h (Table 2).

Addition of peptides generated by PR1 and PR2 at 3 μg alanine equivalents ml^{-1} induced PR1 production to a level similar to that seen with insect cuticle after 4 h exposure. After 12 h incubation, the PR2- and PR1-generated peptides induced PR1 production to about 75 % and 63 %, respectively, of that in cultures with insect cuticle. Addition of PR1- or PR2-generated peptides at 4·5 μg alanine equivalents ml^{-1} resulted in lower activities compared to those detected at 3 μg alanine equivalents ml^{-1}, presumably as a result of catabolite repression.

Addition of buffer-solubilized peptides caused a slight increase in PR1 production which was investigated further. Mycelia starved of C and N were transferred to flasks containing 90 ml BSM plus 10 ml of the digestion products released after 24 h either by enzyme or by buffer. PR1 induction occurred in flasks containing the PR1 products (85 % of that seen with cuticle after 4 h) or PR2 products (81 % of that seen with cuticle after 4 h) but with the buffer-solubilized peptides there was only 9 % of the activity seen with cuticle after 4 h (data not shown). This suggests that PR1 induction is not due to passive release of solubilized peptides, but occurs as a result of protease (PR1 or PR2) action on cuticle proteins.

The profile of hydrolysis products generated from cuticle by PR2 showed two peaks, at approximately 160 and 300 Da. A sample of these peptides, equivalent to 87·5 μg alanine, was separated into two fractions, < 200 Da and > 200 Da, and added separately to derepressed mycelium. The fraction of < 200 Da completely repressed PR1 production whilst the fraction > 200 Da induced PR1 to a level similar to that seen with insect cuticle (data not shown).

Potential peptide inducers from insect cuticle

Abundant amino acids and/or peptide sequences that are unique to and common in host insect cuticle are possible candidates to effect PR1 induction. Alanine is the predominant (about 35 %) amino acid in locust cuticle (St Leger *et al.*, 1986), but comparatively little information is available on the primary structure of cuticular proteins. The N-terminal sequences of eight exocuticle proteins and the entire sequences of seven other exocuticle proteins from *Locusta migratoria* have been determined (Hojrup *et al.*, 1986a, b; Klarskov *et al.*, 1989; Andersen *et al.*, 1993). In general, the sequences of these proteins are characterized by three types of region: (i) regions enriched in glycine, leucine or tyrosine, (ii) hydrophobic regions with repeats of an Ala-Ala-Pro-Ala/Val motif often preceded by tyrosine, and (iii) hydrophilic regions, dominated by amino acids with relatively large side chains. Only one adult locust endocuticular protein has been sequenced (Talbo *et al.*, 1991); it has sequence similarity with a number of proteins from soft larval cuticles from Lepidoptera (Rebers & Riddiford, 1988; Willis, 1987) and Diptera (Snyder *et al.*, 1982; Henzel *et al.*, 1985) but is very different from adult locust exocuticle proteins. In particular there are no repeat motifs of Ala-Ala-Pro-Ala/Val. Since it is most likely that the inducer(s) is a frequently occurring peptide motif, the hypothesis was made that the exocuticle is the source of the inducer(s). We investigated the possibility that either alanine, glycine or one of the repeat peptide motifs (complete or partial) could induce PR1 production.

Table 3. Effect of potential inducers on Pr1 production by *M. anisopliae*

C/N source	Concn (%, w/v)	Enzyme activity*	
		4 h	12 h
Control (−C, −N)		172·8 ± 11·4	440·6 ± 19·7
Cuticle		620·1 ± 37·6	2560·9 ± 69·7
Ala	†	240·4 ± 28·6	726·7 ± 35·4
Ala	0·1	221·5 ± 15·2	894·7 ± 41·6
Ala	0·01	243·6 ± 33·7	897·3 ± 59·6
Gly	†	309·0 ± 25·3	1082·8 ± 75·1
Gly	0·1	314·4 ± 26·7	914·3 ± 41·8
Gly	0·01	356·1 ± 31·9	1178·4 ± 86·2
Ala_2	†	218·2 ± 19·6	650·5 ± 28·5
Ala_2	0·1	0	327·5 ± 22·1
Ala_2	0·01	239·3 ± 24·8	761·4 ± 31·8
Ala_3	0·1	0	456·9 ± 39·7
Ala_3	0·01	169·9 ± 15·6	579·7 ± 23·7
Ala-Pro	†	277·2 ± 29·1	711·3 ± 51·7
Ala-Pro	0·1	0	295·6 ± 17·9
Ala-Pro	0·01	354·5 ± 28·4	776·1 ± 44·4
Pro-Ala	†	316·8 ± 32·2	652·6 ± 41·4
Pro-Ala	0·1	0	314·6 ± 14·6
Pro-Ala	0·01	272·7 ± 21·9	698·4 ± 36·9

* Details as for Table 1.

† Restricted supply from diffusion capsules as described in the text.

Mycelia starved of C and N for 24 h as described in Methods were supplied with the potential inducers alanine, glycine, Ala-Ala, Ala-Pro and Pro-Ala at 0·1 and 0·01 %, and by means of diffusion capsules. Ala-Ala-Ala was supplied at 0·1 and 0·01 % only. The concentrations of amino acids and peptides were kept low in an attempt to avoid C and N repression. The concentration of amino acids detected in culture fluids from cultures containing diffusion capsules was 2·9 μg ml^{-1} (equivalent to 0·0029 %) and 2·3 μg ml^{-1} (equivalent to 0·0023 %) for alanine and its dimer, respectively, indicating that they were being utilized by the fungus at approximately the rate of diffusion, and were not rising to levels likely to cause catabolic repression. Biomass determinations were not performed because the maximum possible increase with inducers at 0·01 % would represent about 3 %

increase in dry weight in 4 h and with diffusion capsules < 3% in 4 h.

PR1 production under these conditions is summarized in Table 3. Basal synthesis was detected in derepressed (−C, −N) controls, with induced levels on cuticle 3·6- and 5·8-fold greater after 4 h and 12 h, respectively, as reported previously (Paterson *et al.*, 1994). The most notable effects of the potential inducers were seen with glycine and Ala-Pro. After 4 h, activity was doubled with glycine and Ala-Pro (both at 0·01%); after 12 h, Ala-Pro was still stimulatory but the monomers alanine and glycine gave the greatest increased production, which was 2–2·7-fold over controls.

Absence of even basal PR1 activity after 4 h with Ala-Ala, Ala-Ala-Ala, Ala-Pro and Pro-Ala when supplied at 0·1% probably resulted from catabolite repression because (i) basal synthesis of PR1 is regulated by derepression; (ii) the effect was no longer evident by 12 h; (iii) lower concentrations (restricted supply or 0·01%) gave activities similar to controls; and (iv) the peptides were not acting as competitive inhibitors of PR1, as revealed by a 30 min preincubation of pure PR1 with these peptides at 0·1% and 0·01% before assay (data not shown).

DISCUSSION

We have shown previously that PR1 is specifically induced by insect cuticle (Paterson *et al.*, 1994). The component of insect cuticle which induces PR1 appears to be cuticular protein (or peptides thereof), as shown by (i) reduced PR1 levels in cultures grown on deproteinized cuticle, (ii) other cuticle components (i.e. chitin and lipids) having no effect on induction, and (iii) the induction of PR1 by peptides released from cuticle by pure preparations of PR1 and PR2.

The products of cuticle digestion released by pure preparations of either PR1 or PR2 were capable of inducing PR1 production when added to derepressed mycelium, but compounds solubilized by buffer were relatively ineffective, which demonstrates that an enzymically released peptide(s) of cuticular protein is the inducing molecule(s). Induction is therefore likely to result from the action of PR1 and PR2 produced at basal levels, which convert cuticle proteins to oligopeptide inducers.

The first proteinaceous barrier that the invading fungus encounters is the epicuticle. The nature of the proteins in this region is not known for any insect. However, epicuticular protein constitutes only a very small fraction of the total cuticular protein and if induction plays a significant role in regulating PR1 during infection it seems likely that peptide components of proteins in the main body of the cuticle are the effectors. The extreme N-terminal regions of all but one of the 15 *Locusta migratoria* cuticle proteins which have been sequenced (Hojrup *et al.*, 1986a, b; Klarskov *et al.*, 1989; Andersen *et al.*, 1993) are rich in glycine and large stretches of the remainder of the cuticle contain repetitive sequences dominated by alanine. Thus, for example in 'protein 38' from *L. migratoria* Ala-Ala-Ala is found six times, Ala-Ala-Pro-Ala ten times and Ala-Ala-Pro-Val six times (Hojrup *et al.*, 1986b); these repeated sequences constitute about 70% of the entire sequence.

It is possible that the inducer(s) of PR1 released from cuticle is in some way related to the preferred cleavage sites of either PR1 or PR2. The best substrate of PR1 is succinyl-$(Ala)_3$-Phe-NA, although the substrates succinyl-$(Ala)_2$-Pro-Ala-NA, succinyl-$(Ala)_2$-Pro-Phe-NA and acetyl-$(Ala)_3$-NA are also readily hydrolysed (St Leger *et al.*, 1987a). These substrates bear a striking resemblance to the repetitive peptides seen in locust cuticle, which would be excellent substrates for PR1, containing many cleavage sites. The action of basal levels of PR1 on cuticle would therefore release many peptides rich in alanine which could hypothetically induce further PR1 production. Whereas many fungal proteases have a broad specificity, PR2 has a primary specificity for arginine and lysine comparable to that of bovine trypsin (St Leger *et al.*, 1987a). There are no obvious cleavage sites for PR2 in the repetitive sequences in locust cuticle, which contains only about 5% lysine and arginine (St Leger *et al.*, 1986). However, presumably cuticle degradation by PR2 would also release peptides rich in alanine because alanine is the predominant amino acid in locust cuticle (St Leger *et al.*, 1986).

Alanine di- and tripeptides, but not alanine or glycine, caused severe metabolite/catabolite repression of PR1 production (Table 3), which indicates a system of uptake and/or catabolism of these peptides which is more efficient than that for the constituent monomeric amino acids; this would seem to reveal a further adaptation by this insect pathogen to host cuticular proteins, but even at low levels these peptides did not induce PR1. Analogously, facilitated utilization of the disaccharide cellobiose compared with glucose was reported for the cellulolytic plant pathogen *Verticillium albo-atrum* by Gupta & Heale (1971), but in this case cellobiose is the inducer of cellulase.

Some peptides enhanced PR1 levels substantially (up to 2-fold) above basal synthesis but the greatest effect (up to 2·7-fold) was observed with monomeric alanine and glycine (Table 3). However, these activities were always less than that achieved on cuticle, where about 6–9-fold induction occurs after 12 h (Table 3 and Paterson *et al.*, 1994). Nevertheless in most studies on induction of depolymerases it has been difficult to achieve enzyme production as high as that obtained using a polymeric C source by supplying a pulse of a putative inducer (Cooper & Wood, 1975; Lerner & Goldman, 1993; Woloshuk & Kolattukudy, 1986); presumably this reflects the difficulty of achieving balance between nutrition, induction and derepression.

Cuticle digests were, however, effective in inducing high PR1 production when added at levels of amino acids similar to those in cultures where potential inducers were supplied from diffusion capsules. It would appear that we have yet to identify the true effector(s). The stimulatory effect on PR1 production of some of these cuticular peptides and amino acids remain unexplained.

Induction of depolymerases by monomers is known for many polysaccharidases (Cooper, 1977) but it is difficult to reconcile the enhancement of PR1 by monomeric glycine and alanine (Table 3) with the specificity of PR1 induction by insect cuticle but not by other proteins which also contain these amino acids. Also, preliminary data from size fractionations of enzyme-digested cuticular peptides suggested that the inducer(s) is larger than 200 Da. Recent evidence from *Candida albicans* indicates that induction of an extracellular aspartyl protease is effected by peptides of more than eight residues even though its permeases do not efficiently transport peptides > 6–7 residues (Lerner & Goldman, 1993). It is possible that a similar mechanism of peptide-initiated signal transduction occurs in *M. anisopliae*.

Whilst we have observed induction of PR1 by locust cuticle and enzymically solubilized peptides from locust cuticle, we have not yet determined if induction occurs by exposure to cuticle from other insects. All isolates of *M. anisopliae* that we have investigated (about 40) produce a PR1-like enzyme (unpublished results; St Leger *et al.*, 1987c) and isolates of *Metarhizium* are pathogenic for insects from a number of orders including Orthoptera, Lepidoptera, Coleoptera and Diptera. Some sequences are available for cuticular proteins from two species of Lepidoptera [*Manduca sexta* (Rebers & Riddiford, 1988) and *Hyalophora cercopia* (Willis, 1987)] and two species of Diptera [*Drosophila melanogaster* (Snyder *et al.*, 1982) and *Sarcophaga bullata* (Henzel *et al.*, 1985)]. While there is some sequence homology between these proteins and the one protein sequenced from adult locust endocuticle (Talbo *et al.*, 1991) they differ markedly from the locust exocuticle proteins described above. It remains possible, therefore, that PR1 induction only occurs during infection of certain insect hosts, namely those whose cuticular protein(s) contain the inducer molecule(s), and that another of the several proteases of *M. anisopliae* plays the key role in infection of different hosts. Alternatively, it is possible that induction by locust cuticle is some property of endocuticular rather than exocuticular protein.

It is remarkable that the effector molecule(s) responsible for induction of any microbial protease has yet to be identified. This may be because induction is effected by many different proteins, as in the case of the trypsin-like serine protease, PR2, from *M. anisopliae* (Paterson *et al.*, 1993) and the aspartic protease from *Mucor miehei* (Lasure, 1980). In contrast, the specificity of PR1 induction by insect cuticular protein should facilitate our ongoing search for the inducers.

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