Analysis of aminopeptidase and dipeptidylpeptidase IV from the entomopathogenic fungus *Metarhizium anisopliae*

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Analytical and preparative isoelectric focusing were used to separate extracellular isoenzymes of aminopeptidase (pI 4.51, *M_r* 45 000, pH optimum 7.0) and prolyl-dipeptidylpeptidase (pI 4.01, *M_r* 74 000, pH optimum 8.0) produced by the entomopathogenic fungus *Metarhizium anisopliae* during growth on locust cuticle. Production of both activities is repressed by readily utilized nitrogen sources, but unlike the aminopeptidase, the dipeptidylpeptidase was also excreted at high levels during growth on casein. Casein-grown cultures contained additional isoenzymes with activity against lysyl-alanyl-4-methoxy-2-naphthylamine indicating *M. anisopliae* possesses multiple peptidases as an adaptation to different nutrient conditions. The aminopeptidase hydrolysed alanyl-leucyl-alanine and showed a broad specificity versus monoaminoacyl β -naphthylamine (β NA) substrates with alanine β NA being the most rapidly hydrolysed. Inhibition by both bestatin and amastatin indicated similarities to the class of alanyl aminopeptidases (aminopeptidase M). Metal complexing agents also inhibited the aminopeptidase indicating a metal ion requirement. A specific inhibitor for serine proteases [diisopropyl fluorophosphate (DFP)] was without effect. The dipeptidylpeptidase showed a strong preference for substrates having a penultimate proline residue including alanyl-prolyl-glycine and aa-prolyl- β NA substrates. The enzyme showed a broad specificity at the N-terminal amino acid. Inhibition by diprotin A indicates similarities with mammalian prolyl-dipeptidylpeptidases. The enzyme was also inhibited by DFP, implying involvement of a serine residue in catalysis. The results are discussed in the context of cuticle degradation and the participation of exopeptidases as mediators in releasing amino acids necessary for pathogen growth.

Introduction

The proteolytic activity of the fungal entomopathogen *Metarhizium anisopliae* enables penetration of the proteinaceous cuticular barrier of insects (Goettel *et al.*, 1989; St Leger *et al.*, 1987*a*, 1988*a,b*). The first step in proteolysis has been shown to be catalysed by an extracellular chymoelastase protease (Pr1) that constitutes the major protein product synthesized by *M. anisopliae* infection structures (St Leger *et al.*, 1989, 1992). The action of Pr1 on the cuticle releases peptides

with a mean residue length of five (St Leger *et al.*, 1986*a,b*), which may be further degraded and rendered available for uptake and metabolism by exopeptidases produced during penetration. In this context, we detected high aminopeptidase activity on mature appressoria (St Leger *et al.*, 1987*b*). The preference of the enzyme for alanyl rather than leucyl histochemical substrates was considered noteworthy as alanine is the major amino acid component of insect cuticle and supports better growth of *M. anisopliae* than other amino acids (St Leger *et al.*, 1986*c*). More recently, we found that aminopeptidases were produced by several unrelated entomopathogenic fungi during growth on insect cuticle, implying a ubiquitous function for these enzymes (Samuels *et al.*, 1990). In view of the potential importance of aminopeptidases in the hydrolysis of cuticle peptides and the provision of nutrients, we have studied the properties of the enzyme from *M. anisopliae*.

The Pr1 enzymes of five species of entomopathogenic fungi show similar discrimination by their S₃, S₂, and S₁

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Abbreviations: aa, amino acid; Ben, benzoyl; Suc, succinyl; Z, N-benzyloxycarbonyl; MNA, 4-methoxy-2-naphthylamine; β NA, β -naphthylamine; pNA, *p*-nitroaniline; DFP, diisopropyl fluorophosphate; FBB, fast blue B (tetrazotized diorthoanisidine); IEF, isoelectric focusing; MM, 0.1% KH₂PO₄, 0.05% MgSO₄; Pr1, chymoelastase protease of *M. anisopliae*; Pr2, trypsin-like protease of *M. anisopliae*; SDB, Sabouraud dextrose broth.

subsites against proline (St Leger *et al.*, 1987*a, c*). Proline constitutes 10% (w/w) of total amino acid residues in locust cuticle (St Leger *et al.*, 1986*a*) suggesting that one or more peptidases capable of hydrolysing peptide bonds involving proline will be necessary to complement Pr1 for complete hydrolysis of cuticle proteins and maximum cell growth. One such enzyme is prolyl dipeptidylpeptidase (dipeptidylpeptidase IV; EC 3.4.14.5), which releases N-terminal dipeptides from polypeptides containing proline in the penultimate position. The enzyme has been characterized from mammalian systems (Imai *et al.*, 1983), several bacteria (Khalid & Marth, 1990), and *Saccharomyces cerevisiae* (Alvarez *et al.*, 1985). This communication reports the partial characterization of an extracellular prolyl dipeptidylpeptidase in culture filtrates of *M. anisopliae* and compares its activity with the aminopeptidase.

Methods

Organisms and growth. The fungal isolate (*M. anisopliae* ME1), culture media, and preparation of locust cuticle were described by St Leger *et al.* (1986*a*).

Chemicals. All amino acids used were L configuration unless otherwise stated. Ala-pNA, (Ala)₂-pNA, (Ala)₃-pNA, Suc-(Ala)₃-pNA, Gly-Pro-pNA, Suc-(Ala)₂-Pro-Phe-pNA, Ben-Phe-Val-Arg-pNA, Gly-Pro-MNA, Lys-Ala-MNA, Ala-MNA, Leu-MNA, Met-MNA, monoaminoacyl β NA substrates, Ala-Leu-Ala and Ala-Pro-Gly were from Sigma. Other peptide derivatives of β NA or MNA were supplied by Bachem. Pure grade FBB was from Serva, Sephadex G-100 fine and Ampholines were from Pharmacia. Other chemicals used were commercial products of analytical grade.

Analytical isoelectric focusing (IEF). Filtrates from four-day-old cultures grown on 1% casein/basal salts or 1% locust cuticle/basal salts media (St Leger *et al.*, 1987*a*) were dialysed (14 h at 4 °C with 200 vols distilled water, pH 6, M_r cut-off 6–8000) and concentrated ten-fold in a dialysis bag surrounded by polyethylene glycol (M_r 20000). Concentrated filtrates (20 μ l) were applied using paper strips to Ampholine PAG plates, pH 3.5–9.5 (LKB, Bromma, Sweden). The electrofocusing was performed using an LKB 2117 Multiphor apparatus for 90 min according to the manufacturer's instructions. Afterwards, gel plates were divided into the required portions, rinsed in 2 mM-potassium phosphate buffer (pH 7) for 15 s and incubated at 25 °C for up to 2 h in fresh buffer containing peptidase substrates (monoaminoacyl and peptide derivatives of MNA at 1.5 mM) and Fast Blue B (1 mg ml⁻¹). Enzymes were indicated by red bands. This procedure using MNA derivatives is a modification of the histochemical techniques we used previously to localize cuticle-degrading enzymes on infection structures (St Leger *et al.*, 1987*b*).

Enzyme purification. Filtrates from four-day-old cultures grown on 1% locust cuticle/basal salts containing aminopeptidase (vs Ala- β NA) and dipeptidylpeptidase (vs Gly-Pro- β NA) were dialysed and concentrated with polyethylene glycol before ammonium sulphate precipitation and fractionating by IEF [LKB 8101 110 ml column, pH range 3.5–10.0, as described by St Leger *et al.* (1986*a*)]. Enzyme activities in the eluate were determined after removal of ampholytes using Amicon (Danvers, MA) Centricon-10 ultrafiltration units. Fractions containing the highest levels of each activity were applied separately to a Sephadex G-100 fine column (60 $\times$ 2.5 cm) (Pharmacia) equilibrated with 0.15 M-potassium phosphate buffer at pH 6.5 (St

Leger *et al.*, 1986*a*). Molecular mass estimations were based on elution volumes (Andrews, 1964) of the following marker proteins: ribonuclease (M_r 13700), chymotrypsinogen A (M_r 25000), ovalbumin (M_r 43000), albumin (M_r 67000), and aldolase (M_r 158000). Removal of contaminating trypsin-activity (Pr2) from the dipeptidylpeptidase was achieved by passage through an affinity column of soybean trypsin inhibitor-Sepharose prepared and used as described previously (St Leger *et al.*, 1987*a*).

Enzyme assays. Except where stated, aminopeptidase and dipeptidylpeptidase were assayed at pH 7 and pH 8, respectively. Pr1 activity vs Suc-(Ala)₂-Pro-Phe-pNA or Pr2 activity vs Ben-Phe-Val-Arg-pNA were assayed as described by St Leger *et al.* (1987*a*). Likewise, activity against Suc-(Ala)₃-pNA, Ala-pNA, (Ala)₂-pNA, (Ala)₃-pNA and Gly-Pro-pNA were assayed in reaction mixtures containing 0.1 ml enzyme and 1.8 ml substrate (1 mM in Britton-Robinson Universal buffer). Results are expressed as nmol or μ mol pNA released ml⁻¹ min⁻¹. Aminopeptidase activity (vs monoaminoacyl β NA substrates) or dipeptidase activity (vs peptide β NA substrates) were assayed in reaction mixtures containing 0.1 ml enzyme and 1 ml of substrate (1 mM in Britton-Robinson Universal buffer), as described by St Leger *et al.* (1986*c*). Activity units are expressed as μ mol β NA released ml⁻¹ 10 min⁻¹. Enzyme activities towards peptides (Ala-Pro-Gly and Ala-Leu-Ala) were assayed in a reaction mixture containing 1.8 ml substrate (1 mM in 10 mM phosphate buffer), and 0.2 ml purified enzyme incubated at 30 °C, the α -amino groups released were measured with Cd-ninhydrin (Method C) (Doi *et al.*, 1981). The results are expressed as μ mol glycine equivalents released ml⁻¹ h⁻¹. N-acetylglucosaminidase activity (vs p-nitrophenyl-N-acetyl- β -D-glucosamine) was assayed as described by St Leger *et al.* (1986*c*). Activity units are expressed as nmol p-nitrophenol released ml⁻¹ h⁻¹.

All controls contained autoclaved enzyme (121 °C for 30 min) in place of unheated enzyme solutions.

Transfer experiments. Standardized mycelial inocula (5 g wet wt) from 48 h SDB cultures (St Leger *et al.*, 1991) were incubated with shaking (100 r.p.m.) for 8 h in 50 ml sterile (120 °C, 15 min) basal media (0.1% KH₂PO₄, 0.05% MgSO₄, pH 6) alone (MM) or supplemented with carbon and/or nitrogen sources at 0.2–1% (w/v). Cultures were buffered at pH 6 with MES (St Leger *et al.*, 1986*c*). For some experiments, cultures were inoculated with conidia (3×10^6) and incubated for up to 4 d.

Permeabilization experiments. Mycelial inocula (5 g wet wt) were transferred to fresh SDB media or 1% chitin/MM for 8 h. The mycelium was washed with 0.7 M-sorbitol (3 $\times$ 50 ml), collected by centrifugation (1000 g, 10 min) and made permeable by suspending in 10 ml 0.7 M-sorbitol, adding 1 ml toluene/ethanol (1:4 by vol.) and shaking the suspension vigorously for 5 min at room temperature (Hirsch *et al.*, 1988).

Results

Analytical electrofocusing

One major band and four minor bands could be distinguished when aminopeptidase activity in cuticle grown cultures was tested with Ala-MNA (Fig. 1). The substrates Leu-MNA and Met-MNA gave similar zymograms with regard to the number and focusing of the bands, but bands obtained with Ala-MNA were far more intense. Incubation of gels with any two of these substrates simultaneously resulted in the same pattern of bands, confirming that activities vs these monoaminocyl substrates were due to the same enzymes. No bands were

Fig. 1. Isoelectric focusing of cultures grown on 1% (w/v) locust cuticle (lanes 1, 3, and 5) or 1% (w/v) casein (lanes 2, 4, and 6) and band development with the dipeptidylpeptidase IV substrate Gly-Pro-MNA (lanes 1 and 2), the aminopeptidase M substrate Ala-MNA (lanes 3 and 4) and the dipeptidylpeptidase II substrate Lys-Ala-MNA (lanes 5 and 6). Additional bands vs Lys-Ala MNA are indicated by arrowheads. The results are typical of at least three similar experiments using different enzyme preparations.

Fig. 2. Isoelectric focusing (pH 3–10) of peptidases in cuticle culture filtrate. The experiment was performed in a 110 ml column for 48 h at 300–1000 V with the cathode at the top of the column. Fractions (4 ml) were collected, pH was determined (○) and activity vs Ala-βNA (□) and Gly-Pro-βNA (△) was assayed in each fraction.

detected using Pro-MNA as substrate. Zymograms of filtrates from casein grown cultures revealed no bands with Ala-MNA (Fig. 1).

Zymograms stained with the dipeptidylpeptidase IV substrate Gly-Pro-MNA (Gossrau, 1985) revealed two closely spaced bands (Fig. 1) which also stained with Phe-Pro-MNA (not shown). Both bands focused separately from the major aminopeptidase activity and were

Fig. 3. Determination of pH optimum of aminopeptidase (■, vs Ala-βNA) and dipeptidylpeptidase (●, vs Gly-Pro-βNA) using Britton–Robinson buffer (pH range 4.5–9.5).

Table 1. Specificity of aminopeptidase and dipeptidylpeptidase against βNA substrates

M. anisopliae peptidases were partially purified and assayed as described in the text. The maximum aminopeptidase and dipeptidylpeptidase activities corresponding to 100% were 22.8 ± 0.32 units (mg protein)⁻¹ and 34.5 ± 0.34 units (mg protein)⁻¹, respectively, for 3 replicates $\pm$ SD.

Substrate	Relative activity (%)	
	Aminopeptidase	Dipeptidylpeptidase
L-Ala-βNA	100	<0.1
D-Ala-βNA	0	0
β-Ala-βNA	0	0
Ser-βNA	51	<0.1
Val-βNA	41	<0.1
Gly-βNA	34	<0.1
Arg-βNA	19	<0.1
Phe-βNA	18	<0.1
Leu-βNA	15	<0.1
Asp-βNA	3	<0.1
Pro-βNA	1	<0.1
Gly-Pro-βNA	0.3	100
Z-Gly-Pro-βNA	0	0
Phe-Pro-βNA	0.2	57
Gly-Phe-βNA	0.5	3
Arg-Arg-βNA	0.1	5
Lys-Ala-βNA	1	2

of equal intensity in filtrates from cuticle and casein grown cultures. By contrast, staining of gels with the dipeptidylpeptidase II substrate Lys-Ala-MNA (Lojda, 1985) revealed weak co-reaction with the aminopeptidase and dipeptidylpeptidase IV bands. However, two more basic bands were observed in casein grown culture filtrates (Fig. 1).

Partial purification of *M. anisopliae* peptidases

Peptidase activity in culture filtrates was resolved into aminopeptidase (pI 4.31) and dipeptidylpeptidase (pI 4.01) components. These enzymes could be distinguished by their activities towards substrates Ala-βNA and Gly-

Table 2. *Effect of inhibitors on aminopeptidase and dipeptidylpeptidase activities*

The rate of hydrolysis of Ala- β NA and Gly-Pro- β NA in the absence of any inhibitor (0.5 μ mol β NA ml⁻¹ 10 min⁻¹) was taken as 100%. Values for the actual residual activities, in μ mol β NA ml⁻¹ 10 min⁻¹, are given in parentheses (3 replicates $\pm$ SD).

Reagent	Preincubation time (h)	Residual activities (%)	
		Aminopeptidase	Dipeptidylpeptidase
Buffer alone	1.0	100	100
1,10-phenanthroline (1 mM)	1.0	0	94
DFP (1 mM)	0.5	100	7 (0.035 $\pm$ 0.005)
PMSF (0.2 mM)	1.0	100	74 (0.37 $\pm$ 0.02)
Amastatin (50 μ g ml ⁻¹)	1.0	68 (0.34 $\pm$ 0.03)	100
Bestatin (50 μ g ml ⁻¹)	1.0	73 (0.37 $\pm$ 0.03)	100
Diprotin A (50 μ g ml ⁻¹)	1.0	96	22 (0.11 $\pm$ 0.02)

Table 3. *Peptidase production by M. anisopliae*

Peptidase production was assayed 8 h after transfer of growing mycelium to cultures containing either a carbon and nitrogen source or nutrient limiting conditions. Mean aminopeptidase activity (vs Ala- p NA), dipeptidase activity (vs Gly-Pro- p NA) and Pr1 activity (vs Suc-(Ala)₂-Pro-Phe- p NA) are given for 3 replicates $\pm$ SD. The results are representative of two similar experiments.

Addition	Enzyme activity (nmol nitroanilide ml ⁻¹ mm ⁻¹)		
	Aminopeptidase	Dipeptidylpeptidase	Pr1
None	0.2 $\pm$ 0.01	0	10.2 $\pm$ 2.3
NH ₄ Cl (0.4 %)	0	0	0.2 $\pm$ 0
Glucose (1 %)	0.1 $\pm$ 0	0.01 $\pm$ 0	2.2 $\pm$ 0.3
Cellulose (1 %)*	0.2 $\pm$ 0.01	0.02 $\pm$ 0.01	18.7 $\pm$ 2.24
Locust cuticle (1 %)	8.3 $\pm$ 0.5	0.62 $\pm$ 0.04	38.7 $\pm$ 3.8
Elastin (1 %)	11 $\pm$ 0.8	0.34 $\pm$ 0.03	22.4 $\pm$ 1.9
Casein (1 %)	0.5 $\pm$ 0.07	0.36 $\pm$ 0.03	23.6 $\pm$ 2.0
Ala (1 %)	0.2 $\pm$ 0.03	0.07 $\pm$ 0.013	5.3 $\pm$ 0.23
Leu (1 %)	0.15 $\pm$ 0.02	0.07 $\pm$ 0.015	6.8 $\pm$ 0.24
Gly-Pro (1 %)	0.1 $\pm$ 0.01	0.15 $\pm$ 0.012	7.1 $\pm$ 0.8
Locust cuticle (1 %) + cycloheximide (10 μ g ml ⁻¹)	0.1 $\pm$ 0	0.06 $\pm$ 0.01	0.1 $\pm$ 0

* Insoluble crystalline cellulose.

Pro- β NA (Fig. 2). The molecular masses of the enzymes were estimated by gel filtration as M_r 45000 (aminopeptidase) and 74000 (dipeptidylpeptidase). The resulting preparations of aminopeptidase and dipeptidylpeptidase used in our subsequent experiments were free of co-reactivity (vs Ala- β NA or Gly-Pro- β NA) and were inactive against substrates for Pr1 (vs Suc-(Ala)₂-Pro-Phe- p NA) and Pr2 (vs Ben-Phe-Val-Arg- p NA).

Partial characterization of M. anisopliae peptidases

The effect of pH on the activity of aminopeptidase and dipeptidylpeptidase was determined using Britton–Robinson buffer over the pH range 4.5–9.5. The optimum pHs of aminopeptidase (vs Ala- β NA) and dipeptidylpeptidase (vs Gly-Pro- β NA) were 7.0 and 8.0, respectively (Fig. 3).

The range of activities of aminopeptidase and dipeptidylpeptidase were assessed from the relative rates of hydrolysis of β NA substrates (Table 1). Although possessing a very broad specificity, the aminopeptidase had a preference for apolar residues. The best substrate in the series was L-Ala- β NA; replacement of the methyl group in alanine by an isopropyl group (valine) or by an H atom (glycine) substantially decreased hydrolysis. The enzyme was inactive against substrates containing D- or β -alanine and possessed only trace activity vs Pro- β NA and Asp- β NA.

The best substrates for the dipeptidylpeptidase contained proline residues in the position penultimate to the free amino acid termini (Gly-Pro- β NA and Phe-Pro- β NA) but a blocked peptide Z-Gly-Pro- β NA was not hydrolysed indicating that the enzyme lacks endopeptidase activity.

Fig. 4. Production of (a) aminopeptidase (vs Ala- β NA) and (b) dipeptidylpeptidase (vs Gly-Pro- β NA) in cultures containing 1% (w/v) locust cuticle (○), or 1% (w/v) locust cuticle supplemented with 1% (w/v) glucose (▽), 1% (w/v) alanine (△) or 0.5% NH_4Cl (□). Each result represents the mean of 3 replicates $\pm$ SD. The results are representative of two similar experiments.

Both peptidases were tested for their activity against Ala-Leu-Ala and Ala-Pro-Gly to ensure they were true peptidases and not arylamidases (Prescott & Wilkes, 1966). The aminopeptidase hydrolysed Ala-Leu-Ala and Ala-Pro-Gly at the rate of 3 and 0.1 μmol amino acids $\text{ml}^{-1} \text{h}^{-1}$, respectively. The dipeptidylpeptidase lacked activity against Ala-Leu-Ala but hydrolysed Ala-Pro-

Gly (4 μmol amino acids $\text{ml}^{-1} \text{h}^{-1}$). Aminopeptidase activity vs Ala- p NA, (Ala) $_2$ - p NA and (Ala) $_3$ - p NA were 195, 39 (20%) and 5 (2.6%) $\text{nmol } p\text{NA } \text{ml}^{-1} \text{min}^{-1}$, respectively. Suc-(Ala) $_3$ - p NA was not hydrolysed indicating that the release of p NA from (Ala) $_3$ - p NA occurs via processive hydrolysis of Ala residues.

Effect of inhibitors

The results of inhibition studies are shown in Table 2. The metal complexing agent 1,10-phenanthroline greatly decreased aminopeptidase activity, indicating that there was a metal ion requirement. Amastatin, an inhibitor of aminopeptidase A and M, and bestatin against aminopeptidase B and M (Umezawa & Aoyagi, 1983) also had some inhibitory effect on the *M. anisopliae* enzyme. PMSF and DFP, specific inhibitors for serine proteases, were without effect on the aminopeptidase. By contrast, DFP substantially inhibited the dipeptidylpeptidase. Diprotin A, an inhibitor of dipeptidylpeptidase IV (Umezawa & Aoyagi, 1983) was also a potent inhibitor of the *M. anisopliae* dipeptidylpeptidase. Phenanthroline, amastatin, and bestatin were without effect on the dipeptidylpeptidase.

Transfer experiments

Exponentially growing mycelium was transferred from SDB to MM (0.1% KH_2PO_4 , 0.05% MgSO_4) supplemented with various nutrients (Table 3). Little of the Pr1, aminopeptidase and dipeptidylpeptidase activities were excreted to the medium over 8 h in media containing NH_4Cl or glucose. As described previously (St Leger *et al.*, 1988b) limitation of carbon and nitrogen (MM) derepressed Pr1 secretion. By contrast, addition of

Table 4. Specific enzyme activities in growth media and permeabilized mycelia

Activities were measured 8 h after transfer of growing mycelium to medium containing either SDB or 1% chitin/MM. The activity of Pr1 (vs Suc-(Ala) $_2$ -Pro-Phe- p NA), aminopeptidase (vs Ala- p NA) and dipeptidylpeptidase (vs Gly-Pro- p NA) is given in $\text{nmol } p\text{NA } \text{ml}^{-1} \text{min}^{-1}$, that of *N*-acetylglucosaminidase is given in $\text{nmol } p\text{-nitrophenol } \text{ml}^{-1} \text{h}^{-1}$.

Substrate	Specific activities [U (mg protein) $^{-1}$]				Activity ratio	
	Culture filtrate		Permeabilized cells		filtrate/permeabilized cells	
	SDB cultures	Chitin cultures	SDB cultures	Chitin cultures	SDB cultures	Chitin cultures
Ala- p NA*	0.116	20.9 $\pm$ 1.10*	< 0.1	1.67 $\pm$ 0.06	—	12.5
Gly-Pro- p NA	0.12	1.35 $\pm$ 0.04	0.16	0.17	0.71	7.94
Suc-(Ala) $_2$ -Pro-Phe- p NA	< 0.1	94.5 $\pm$ 4.72	< 0.1	17.6 $\pm$ 1.87	—	5.37
<i>p</i> -Nitrophenyl- <i>N</i> -acetyl- β -D-glucosamine	2.23 $\pm$ 0.05	11.6 $\pm$ 1.31	11.8 $\pm$ 1.52	14.1 $\pm$ 1.04	0.16	0.82

* Mean of three replicates $\pm$ SD.

insoluble non-repressing protein substrates to MM was required for secretion of dipeptidylpeptidase. High aminopeptidase activity was only secreted in media containing locust cuticle which was also the optimum substrate for production of dipeptidylpeptidase and Pr1. Secretion of aminopeptidase and dipeptidylpeptidase was repressed when alanine or NH_4Cl were added to media containing cuticle showing that nitrogen repression overrides the enhancing effect of polymeric substrates (Fig. 4). Glucose was a comparatively ineffective repressing catabolite, indicating that nitrogen sources independently repress production even in the absence of carbon sources. The secretion of extracellular peptidases by mycelium provided with cuticle was probably not due to the release of preformed enzyme molecules since cycloheximide almost completely inhibited enzyme release (Table 3).

Effects of permeabilization

Evidence that active peptidases are principally extracellular was provided by permeabilization experiments (Table 4). Treatment of mycelia with toluene released only low levels of peptidases as compared with secreted activities detected in growth media. This response was similar to Pr1, an enzyme which is rapidly secreted from intact cells (St Leger *et al.*, 1988b). By contrast, toluene treatment released high levels of *N*-acetylglucosaminidase which is only partially secreted and provides a vesicle marker (St Leger *et al.*, 1988b).

Discussion

Substrate specificity is an important concern in studies on the biochemistry of peptidases: one substrate can be attacked by more than one enzyme and one enzyme can cleave several substrates (Lojda *et al.*, 1983). Therefore, we initially used analytical electrofocusing to resolve the spectrum of *M. anisopliae* peptidases and to investigate individual peptidases without the need for enzyme purification. The two prolyl-dipeptidylpeptidase bands were clearly distinct from the major aminopeptidase bands. The results reported here also provide evidence that the enzymes are regulated in different ways; prolyl-dipeptidylpeptidase production was similar in media containing cuticle and casein while aminopeptidase activity was only high in cuticle media. Additional bands in casein cultures which stained with the dipeptidylpeptidase II substrate Lys-Ala-MNA indicate that *M. anisopliae* possesses multiple peptidases as an adaptation to different nutrient conditions.

Transfer experiments confirmed that, compared with other protein substrates, cuticle induced a greater percentage increase in production of aminopeptidase

than of the other proteases. Production of aminopeptidase consequently appears more inductive to some factor in insect cuticle than does the more constitutive production of Pr1. Enhanced production of all three enzymes on insect cuticle is consistent with the idea that a major function of extracellular proteases is to make nutrients available from the cuticle. However, our results also suggest that catabolite repression could operate if the release of degradation products from cuticle exceeded fungal requirements. The Pr1 gene has been cloned and is regulated by repression at the level of mRNA accumulation (St Leger *et al.*, 1992). This implies that the similar repression phenomenon observed here for other peptidases may also be regulated at the level of transcription.

We purified the peptidases to an extent which allowed their partial characterization. In several of its properties, *viz.* neutral pH optimum, molecular mass and inhibition by a metal chelating agent, the aminopeptidase activity approximates to the leucine aminopeptidases of some *Aspergillus* spp. (Lehmann & Uhlig, 1969; Nakadai *et al.*, 1973). However, the *M. anisopliae* enzyme showed optimum activity against Ala-MNA, indicative of an alanyl aminopeptidase (aminopeptidase M; McDonald, 1985), a classification supported by the partial inhibition of the enzyme by both amastatin and bestatin (Umezawa & Aoyagi, 1983).

The dipeptidylpeptidase of *M. anisopliae* had a M_r 74000 and a pH optimum of 8. The 40 kDa membrane bound prolyl-dipeptidylpeptidase (dipeptidyl aminopeptidase ysc IV) of *Saccharomyces cerevisiae* has a similar pH optimum of 7–7.5 (Alvarez *et al.*, 1985). Both of the fungal enzymes and most of the bacterial prolyl-dipeptidylpeptidases are sensitive to DFP, implying involvement of a serine residue in catalysis (Khalid & Marth, 1990). The *M. anisopliae* enzyme was classified as a prolyl-dipeptidylpeptidase (dipeptidylpeptidase IV) because of its strong preference for substrates having a penultimate proline residue rather than phenylalanine, arginine, or alanine at this position. The yeast enzyme is also inactive against substrates lacking proline, e.g. Ala-Ala-pNA (Alvarez *et al.*, 1985) although bacterial enzymes retain considerable activity against this substrate (Khalid & Marth, 1990). The *M. anisopliae* enzyme did not degrade Gly- β NA or Pro- β NA, precluding the possibility of a stepwise cleavage of Gly-Pro- β NA and confirming the importance of the free N-terminal group. This N-terminal position could be occupied by a very small amino acid (glycine) or a residue with a large aromatic side group (phenylalanine) indicating a broad specificity at this subsite. Inhibition of the *M. anisopliae* enzyme by diprotin A, a specific inhibitor of mammalian dipeptidylpeptidase IV (Umezawa & Aoyagi, 1983), indicates functional similarities with these enzymes.

The results reported here do not define the precise physiological roles of the exo-peptidases from *M. anisopliae*. In other systems, these enzymes function to release essential amino acids from peptide chains; prolyl-dipeptidylpeptidases participate in release of proline from proline-rich proteins by further hydrolysing the N-terminal X-Pro-Y-bond (McDonald *et al.*, 1971). The proteolytic and peptidase enzyme systems of *M. anisopliae* [Pr1, Pr2 (St Leger *et al.*, 1987a) aminopeptidase and dipeptidylpeptidase] complement each other in splitting most, if not all, types of peptide bonds. In the absence of the dipeptidylpeptidase, further degradation of Pr1 and Pr2 derived cuticle peptides by aminopeptidase would stop at peptide bonds involving proline, thus preventing further hydrolysis by aminopeptidase. Consequently, the aminopeptidase and dipeptidylpeptidase could participate in cuticle degradation as mediators in releasing amino acids which can be utilized as nutrients.

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