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Characterisation of the mid-gut digestive proteinase activity of the two-spot ladybird (*Adalia bipunctata* L.) and its sensitivity to proteinase inhibitors

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Abstract

Proteinase activities in the midguts of adult and larval two-spot ladybirds were investigated. Both extracts showed a slightly acidic pH optimum for proteolysis against azocasein (pH 6.0 and pH 5.5 for larvae and adults respectively), which correlated well with the physiological pH of the midgut. The proteolytic activity of the larval midguts, detected by hydrolysis of Z-phe-arg-pNA, was almost totally inhibited by the cysteine proteinase inhibitor E-64. Inhibitors diagnostic for the other mechanistic classes of proteinase had little or no effect on proteolysis. In-gel assays showed that the proteolytic activity was due to three major cysteine proteinases with indicated mol. wts. 23, 30 and 55 kDa. In addition to cysteine proteinases, adult ladybirds also possessed a metallo-proteinase activity. Plant protein inhibitors of serine proteases had little effect on ladybird proteolytic activity, but cystatins (cysteine protease inhibitors) from plant and animal sources showed significant inhibition (up to 90% at 2 μM). The results suggest that ladybird digestion may be affected by cysteine proteinase inhibitors expressed in transgenic plants for pest control, *via* the tritrophic interaction that occurs between ladybirds, aphids and crop plants.

Keywords: Coccinellidae; Cysteine proteinase; Cystatin; Crop protection; Transgenic plants

1. Introduction

Proteinase inhibitors occur in the reproductive organs, storage organs and vegetative tissues of most plant families (Ryan, 1990; Richardson, 1991; Shewry and Lucas, 1997) where they are thought to act as storage proteins, as regulators of endogenous enzymes and in defence against pest or pathogen attack. While the full details of the mechanisms by which these proteins are toxic to pests are not yet understood, they are known to interfere with the pests digestive process through specifically binding to the proteolytic enzymes present in the gut (Ryan, 1990).

Much research has recently been focused on exploiting this defensive mechanism for crop protection, by genetically engineering crop plants with genes encoding plant protein proteinase inhibitors, to provide enhanced resistance to insect pests. Studies using artificial diets containing plant derived proteinase inhibitors, targeted to the mechanistic class of proteinase(s) in the gut of the insect, have demonstrated the ability of these proteins to retard growth and development of a wide range of insect pests. For example, the serine proteinase inhibitors SKTI (soybean Kunitz trypsin inhibitor), and CpTI (cowpea trypsin inhibitor) have been shown to effect many lepidopteran insects (Broadway and Duffey, 1986; Gatehouse et al., 1993; Johnston et al., 1993; McManus and Burgess, 1995), while coleopterans have been shown to be affected by both the multicystatin from potato and oryzacystatins from rice seed, both of which are cysteine proteinase inhibitors (Chen et al., 1992; Orr et al., 1994; Kuroda et al., 1996; Edmonds et al., 1996). Furthermore, these antimetabolic effects have been shown in lepidopteran insects that have been feeding on transgenic plants containing serine proteinase inhibitors

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(Hilder et al., 1987; Johnson et al., 1989; McManus et al., 1994).

Given the potential usage of transgenic plants expressing proteinase inhibitors for crop protection, it is necessary to evaluate the possible effects that these proteins may have on non-target organisms, including beneficial insects. Recently, Burgess et al. (1996) began to address this need and showed that the trypsin inhibitors BPTI (bovine pancreatic trypsin inhibitor) and SKTI significantly reduced the longevity of adult honeybees when administered in a sugar syrup diet.

Ladybirds are of considerable economic importance since many eat plant pests such as aphids, coccids (scale insects) and adelgids (woolly aphids) (Majerus, 1994). They are therefore regarded as being highly beneficial. These carnivorous ladybirds are most likely to encounter proteinase inhibitors by feeding on aphids that have been feeding on transgenic plants expressing proteinase inhibitors. Proteins present in the phloem sap of plants are taken up by aphids, and transgenes whose expression is driven by phloem-specific or constitutive promoters will result in foreign proteins being delivered to aphids (Shi et al., 1994). Many aphidophagous ladybirds will also feed on aphid honeydew, pollen and nectar when aphid prey become scarce (Majerus, 1994) and could therefore be directly exposed to proteinase inhibitors as a result of foraging on floral parts.

Information on the digestive physiology of ladybirds is lacking, making it impossible to predict any effects that proteinase inhibitors may have on the digestive enzymes of these insects. We have therefore characterized the proteolytic activity present in the midgut of fourth instar larvae of the two-spot ladybird, *Adalia bipunctata*. This allows the inhibitory effect of a range of plant derived proteinase inhibitors on gut proteolysis to be determined.

2. Materials and methods

2.1. Materials

The substrates carbobenzoxy-L-phenylalanine-L-arginine-*p*-nitroanilide and carbobenzoxy-L-arginine-L-arginine-*p*-nitroanilide (Z-phe-arg-*p*NA and Z-arg-arg-*p*NA) were purchased from BACHEM (Saffron Waldon, UK) whereas azocasein was from Sigma-Aldrich Co Ltd (Poole, UK). The inhibitors phenylmethanesulfonyl fluoride (PMSF), Kunitz soybean trypsin inhibitor (SKTI), lima bean trypsin inhibitor (LBTI), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK), benzamidine, *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64), chicken egg-white cystatin (CEW cystatin), pepstatin, 1,10 phenanthroline and leupeptin were from Sigma-Aldrich, whereas cowpea cysteine proteinase inhibitor (CCPI) and papaya cystatin were gifts. Cowpea trypsin inhibitor (CpTI; purified from cowpea seeds by affinity chromatography on immobilised trypsin) and recombinant oryzacystatin (rOC-I; produced in *E. coli* containing the expression vector pHEVrozc, and purified by heat treatment and ion-exchange chromatography) were prepared as previously described (Gatehouse et al., 1980; Edmonds et al., 1996). Protein assay reagents and pre-stained molecular weight markers were from Bio-Rad Laboratories plc (Hemel Hempsted, UK) and reagents used for midgut pH determinations were from Merck Ltd (Poole, UK). All other chemicals came from Sigma-Aldrich and were of analytical quality.

2.2. Enzyme preparations

Cultures of two spot ladybird, *Adalia bipunctata*, were established in the laboratory of the authors. Both fourth instar larvae and adults were fed on either *Myzus persicae* (peach potato aphid) or *Acyrthosiphon pisum* (pea aphid) prior to dissection. Individuals were decapitated and their midguts dissected out over ice. The guts and their contents were then immediately placed in chilled 1 mM DTT (20 μl gut^{-1}) and were homogenized on ice prior to centrifuging at 14 000 ***g*** for 5 min at 4°C. The aqueous supernatants were then pooled, divided into aliquots, and stored frozen at −20°C until required.

2.3. Proteinase assays

General proteinase activity was determined using azocasein as the substrate protein. 5 μl of either larval or adult midgut extract was mixed with 20 μl of buffer (citrate phosphate, citrate phosphate + MES (2-(N-morpholino)ethanesulphonic acid), MES, MES + Bis-Tris Propane, Bis-Tris Propane and CAPS for pH 4.0–5.0, 5.5, 6.0, 6.5, 7.0–9.5 and 10 respectively; final buffer concentration 50 mM), 5 μl of water and 10 μl of azocasein solution (1% w/v) in 0.05% SDS). The reaction mixture was then incubated at 25°C for 2.5 hours. 30 μl of chilled 10% TCA was added to stop the reaction and the tubes were placed on ice for 30 min before centrifuging at 14 000 ***g*** for 5 min to remove precipitated protein. The supernatant (60 μl) was then withdrawn and was added to the well of a microtitre plate containing 40 μl of 1 M NaOH. All incubations were done in duplicate (and were repeated) and the absorbancies of the resulting solutions were read at 405 nm in a microtitre plate reader along with those for appropriate blanks and controls.

Cysteine proteinase activity in both larval and adult midgut extracts was measured using the substrates Z-phe-arg-*p*NA and Z-arg-arg-*p*NA. 2.5 μl of midgut extract was pre-incubated in 125 μl of buffer (buffers as for azocasein assay but containing DTT, EDTA and Brij 35 at final concentrations of 5 mM, 1 mM and 0.1% (w/v) respectively) and 110 μl of water for 15 min at

25°C. The reaction was then started by adding 12.5 μl of substrate (final concentration 0.5 mM) and the change in absorbance at 405 nm was recorded at 25°C in a microlitre plate reader against appropriate controls.

The effects of proteinase inhibitors on the proteolytic activity of larval midguts were determined using both azocasein and Z-phe-arg-*p*NA. Chemical inhibitors were made up as aqueous solutions (benzamidine, cysteine, DTT, E-64, leupeptin, pepstatin, 1,10 phenanthroline) or solutions in ethanol (TPCK), methanol (PMSF) or DMSO (pepstatin) and were used fresh, or stored in solution at −20°C where appropriate. PMSF, TPCK and E-64 were checked for activity against enzymes known to be sensitive to inhibition. Inhibitors were used at concentrations determined from preliminary assays; for irreversible inhibitors these were at least 10-fold in excess over total protein in the gut extract, and for other inhibitors levels were used which gave > 90% of maximal inhibition. Inhibitors were pre-incubated with the enzyme at 25°C for 15 min prior to the addition of the substrate.

2.4. Assay of proteinase activity by electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in discontinuous vertical slab gels (Laemmli, 1970) with 0.25% (w/v) azocasein co-polymerized with the 12.5% (v/v) acrylamide resolving gel. Samples were not reduced or heat treated and the pre-stained molecular weight markers were used at three times the manufacturers recommended loading. Electrophoresis was conducted at 4°C and then proteolytic activity in midgut extracts was detected using a modification of the method described by Sarath et al. (1989). Gels were washed for 30 min in 1% (v/v) Triton X-100 to remove SDS before being incubated overnight at 37°C in 50 mM MES buffer (pH 6.0) containing 1 mM DTT. To detect the mechanistic class(es) of the active proteinases, inhibitors were included in the Triton X-100 solution and incubation buffer. EDTA was used as an inhibitor of metalloproteinases instead of 1,10 phenanthroline, as recommended by the reference cited. Gels were then stained with 40% methanol, 7% glacial acetic acid and 0.05% Coomassie Brilliant Blue R and briefly destained. Proteolytic activity was then seen as clear zones against a dark blue background.

2.5. Determination of midgut pH

Midguts were dissected out of adults and fourth-instar larvae and the pH of the gut contents estimated using narrow range pH papers (pH 4.0–7.0). Measurements were done in triplicate and the results compared against pH values obtained using universal indicator solution.

2.6. Protein determination

Protein contents of enzyme solutions were determined by the method of Bradford (1976) using the Bio-Rad microassay with BSA as the protein standard.

3. Results

The pH dependence of the proteolytic activity in midgut extracts of *A. bipunctata* was determined using the protein substrate azocasein, in a mixed buffer system. Midgut extracts from fourth-instar larvae showed a pH optimum for proteolysis at pH 6.0 ($P < 0.001$; Fig. 1A), with activity decreasing sharply at pH values below optimum, and more gradually at higher pH values. Studies with adult midgut extracts showed that the pH optimum for proteolysis (pH 5.5) was slightly more acidic than that for fourth-instar larvae. The activity of adult extracts was significantly reduced at pH 6.0 ($P < 0.05$) and decreased more rapidly with increasing pH; whereas larval extracts showed rates of proteolysis approx. 90% of optimal at pH 7.0, in the adult extract the rate had fallen to less than 40% of optimal at this pH. On the other hand, the adult extracts showed higher relative activities (when compared to optimal rates) than larval extracts at pH 4.5–5.0. Although results with the mixed buffer system suggested that the pH optima for proteolysis were quite sharp, data obtained from overlapping buffer systems (results not presented) showed that protease activity was greater than 50% of the maxima over the pH range 4.0–7.0, and did not indicate a significant difference in the optima of larval and adult protease activity.

Hydrolysis of the synthetic substrates Z-phe-arg-*p*NA and Z-arg-arg-*p*NA by larval and adult midgut preparations was used to further investigate the activities present (Fig. 1B). The larval midgut extract contained a higher level of proteinase activity than the adult ladybird midgut extract (on a protein basis), when estimated by hydrolysis of Z-phe-arg-*p*NA, across the whole pH range studied (pH 3.5–7.5). Optimal proteolysis of Z-phe-arg-*p*NA, at pH 5.5, by larval midgut extracts was 5.5 times that of the adult gut extracts (optimal at pH 5.0). Z-phe-arg-*p*NA is very sensitive to cathepsin L [EC 3.4.22.15] (Barrett and Kirschke, 1981) and larval midgut extracts optimally hydrolysed this substrate approximately fifteen times faster than Z-arg-arg-*p*NA, a substrate often used for cathepsin B [EC 3.4.22.1] (substrate concentrations 5×10^{-4} M). Assays conducted with larval midgut extract and varying Z-phe-arg-*p*NA concentrations gave a linear Michaelis-Menten plot, with $K_m = 3.7 \times 10^{-4}$ M and $V_{max} = 70$ nmoles min^{-1} mg^{-1} protein. The adult gut extract showed little change in rate of hydrolysis of Z-phe-arg-*p*NA over the pH range 3.5–7.5, whereas the larval extract showed a distinct peak at pH 5.5; the two extracts also differed in their relative activities towards

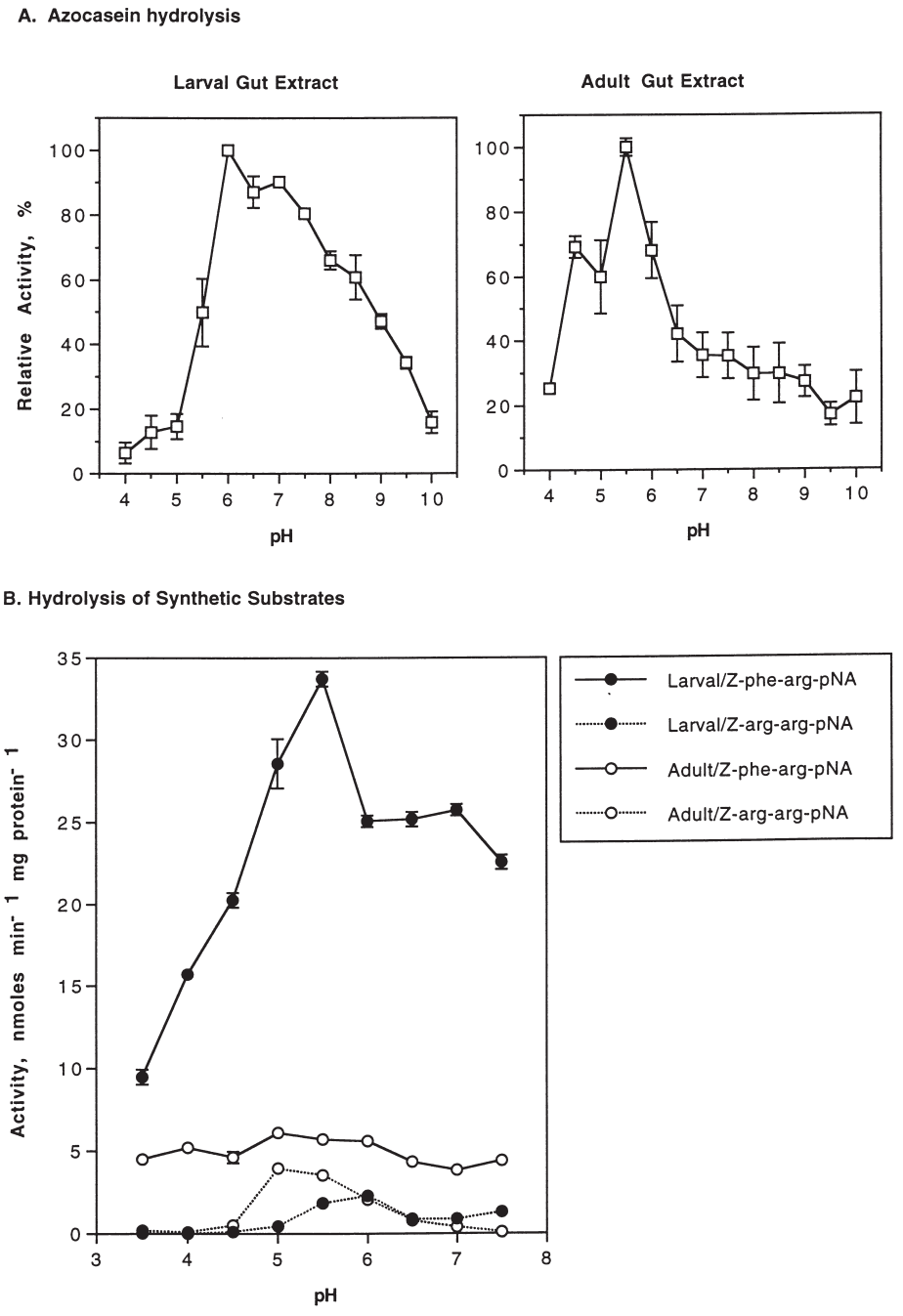

Fig. 1. (A) The effect of pH on the proteolytic activities against azocasein of fourth-instar larval and adult midgut extracts of the two-spot ladybird. Points and error bars represent the means and standard errors of at least four independent assays. (B) The effect of pH on the proteolytic activities of fourth-instar larval (●) and adult (○) midgut extracts against the synthetic substrates Z-phe-arg-*p*NA (—) and Z-arg-arg-*p*NA (- - - -). Points and error bars represent the means and standard errors of at least four independent assays.

Z-phe-arg-*p*NA and Z-arg-arg-*p*NA, in that the adult extract showed comparable optimal activities towards the two substrates. Neither adult or larval midgut extracts hydrolysed N-benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA) or N-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanalide (SAAPF*p*NA), substrates used for typsin- and chymotrypsin-like proteinases respectively (data not shown).

The physiological gut pH, as determined using narrow range pH papers and universal indicator solution, was pH 6.0–6.5 in both fed fourth-instar larvae and fed adults. This slightly acidic physiological pH correlates well with the activity range of the midgut proteinases (Fig. 1).

Inhibitors and activators diagnostic for the four mechanistic classes of proteinase were used to characterise proteinases which are present in the midgut of fourth-instar ladybird larvae (Table 1). Chemical inhibitors of

Table 1
The effect of inhibitors on the proteolytic activity of fourth instar ladybird larvae against azocasein and Z-phe-arg-*p*NA

Inhibitor	Specificity	Concn.	Relative activity (%)	
			Azocasein	Z-phe-arg-*p*NA
None			100	100
Chemical Inhibitors				
PMSF	Serine proteases	5 mM	96	113
Pepstatin	Aspartic proteinases	1 μM	90**	108
1,10 phenanthroline	Metallo-proteinases	10 mM	101	100
E-64	Cysteine proteinases	20 μM	3***	1***
Leupeptin	Ser/cys proteinases	100 μM	4***	2***
TPCK	Ser/cys proteinases	100 μM	33***	55***
Benzamidine	Arg binding pocket	10 mM	77***	125**
Protein Inhibitors				
SKTI	Trypsin	1.2 μM	88**	121**
LBTI	Trypsin	1.2 μM	97	122**
CpTI	Trypsin/chymotrypsin	1.2 μM	93*	105
CEW cystatin	Cysteine proteinases	2 μM	8***	2***
CCPI	Cysteine proteinases	2 μM	28***	53***
Papaya cystatin	Cysteine proteinases	2 μM	67***	82***
rOC-I	Cysteine proteinases	2 μM	13***	36***

Values are means of four replicates relative to the control activity (no inhibitor). Relative activities that are significantly different from the controls are shown as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Other differences are not significant.

the different protease classes showed that the cysteine proteases made up almost all the detectable activity. PMSF, an inhibitor diagnostic for serine proteases, did not inhibit significantly. The inhibitors TPCK and leupeptin, which are sometimes considered diagnostic for serine proteases, both inhibited significantly. However, leupeptin is known to be effective against cysteine proteases as well as serine proteases, and the strong inhibitory effect of TPCK, which irreversibly inhibits chymotrypsin, is almost certainly due to its poor specificity since it is also known to inhibit many other serine and cysteine proteinases (Salvesen and Nagase, 1989). Similarly, inhibition of azocasein proteolysis by benzamidine is not diagnostic for serine proteases since this arginine analogue will act as a competitive inhibitor for substrates which are cleaved C-terminally to this amio acid; it did not inhibit hydrolysis of Z-phe-arg-*p*NA, which presumably binds more strongly to the enzyme active site, and is present at a higher concentration. The failure to cleave either BA*p*NA or SAAPF*p*NA suggests that serine proteases of the typsin or chymotrypsin type are not present. The highly specific cysteine proteinase inhibitor E-64 caused inhibition of $> 95\%$ of activity against azocasein and Z-phe-arg-*p*NA; the presence of cysteine proteases was supported by slight enhancement (5–10%) of the larval midgut proteinase activity in the presence of 1 mM cysteine or DTT (data not presented). 1,10-Phenanthroline, an inhibitor of metalloproteinases, did not reduce proteolysis against azocasein or activity towards Z-phe-arg-*p*NA; similar results were obtained with EDTA (results not presented). Pepstatin (inhibitor of aspartic proteinases) significantly reduced proteolysis, its effect was small (10% reduction) ($P < 0.01$), and only shown in decreasing proteolysis of azocasein (Z-phe-arg-*p*NA is unlikely to be a good substrate for an aspartic proteinase).

Results obtained with the chemical inhibitors were supported when protein proteinase inhibitors were used. Although the cysteine proteinase inhibitor papaya cystatin was not a very potent inhibitor of larval midgut extracts, CEW cystatin, and the plant derived cysteine proteinase inhibitors CCPI and rOC-I, significantly reduced total proteinase activities to 8%, 28% and 13% of the controls respectively ($P < 0.001$). Similar levels of inhibition were recorded for these inhibitors when using the cysteine proteinase substrate Z-phe-arg-*p*NA. With both azocasein and Z-phe-arg-*p*NA as substrates, rOC-I, was significantly better at inhibiting larval midgut proteinases than any other plant derived cysteine proteinase inhibitor ($P < 0.001$). On the other hand, several inhibitors of serine proteinases (PMSF, LBTI, SKTI and CpTI) were not very effective in decreasing proteolysis of azocasein (all less than 12% inhibition), and caused higher rates of hydrolysis of Z-phe-arg-*p*NA (activation by approx. 20%).

The electrophoretic separation of larval and adult gut extracts in polyacrylamide gels containing azocasein revealed multiple zones of proteinase activity (Fig. 2). Although this method does not give accurate estimates for protease molecular weights, since the proteins are not heat-treated in the presence of reducing agents before electrophoresis, and migration may be affected by interactions with the substrate in the gel, it is a useful indication of the protease species present. In the larval midguts three major bands of activity were present at indicated molecular weights of approximately 23, 30 and

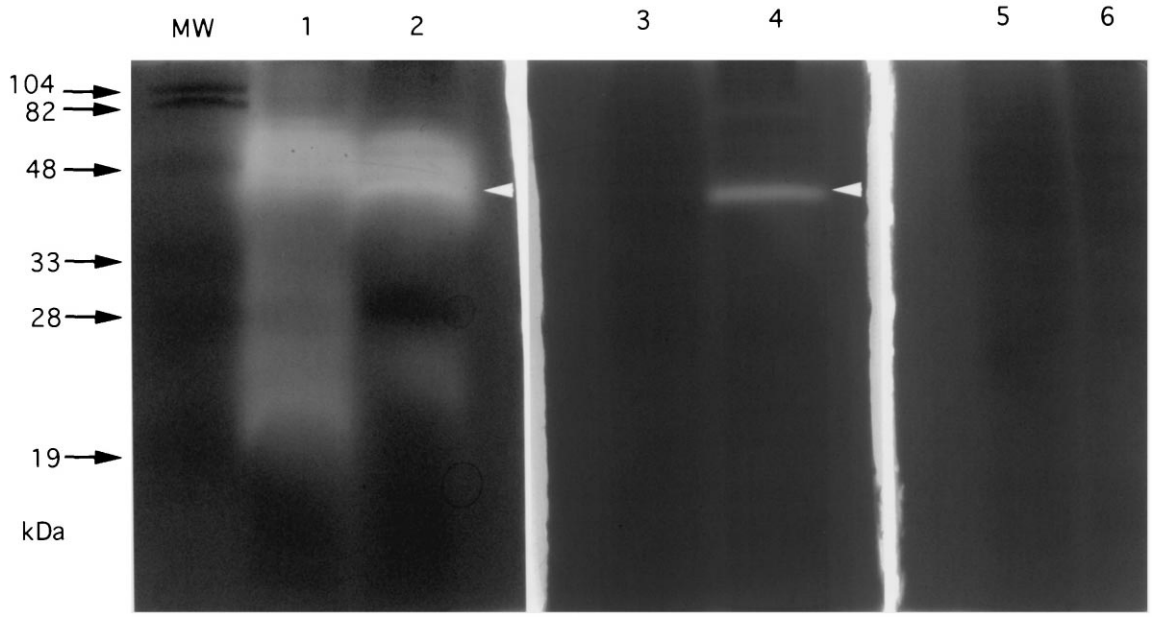

Fig. 2. Identification of the proteolytic activities of fourth-instar larval (lanes 1, 3, and 5; approx. 25 μg protein per lane) and adult (lanes 2, 4, and 6; approx. 13 μg protein per lane) midgut extracts in polyacrylamide gels containing azocasein. Gel slices 1 to 3 from left to right: slice 1 was incubated in 50 mM MES buffer (pH 6.0) containing 1 mM DTT, whereas slices 2 and 3 were incubated in buffer containing 20 μM E-64 and 20 μM E-64 + 10 mM EDTA. The pre-stained molecular weight markers were phosphorylase B (*M*r 104 000), bovine serum albumin (*M*r 82 000), ovalbumin (*M*r 48 300), carbonic anhydrase (*M*r 33 400), soybean trypsin inhibitor (*M*r 28 000) and lysozyme (*M*r 19 400).

55 kDa and all of the proteolytic activity was inhibited by incubating the gel in buffer containing the cysteine proteinase inhibitor, E-64. This finding is in agreement with the results of the inhibition study (Table 1) described above. Midgut extracts from adult ladybirds also had three major bands of activity (indicated molecular weights approximately 26, 45 and 55 kDa) but unlike the proteinases present in the larval midgut extracts they were not all inhibited by E-64. The addition of both E-64 and EDTA, an inhibitor of metalloproteinases (substitute for 1,10 phenanthroline) into the incubation buffer did, however, completely inhibit the proteolytic activity of adult midgut samples suggesting the presence of cysteine and metallo-proteinases in the midgut of adult *A. bipunctata*. This metallo-proteinase (shown by the white arrow in Fig. 2) has an approximate indicated molecular weight of 45 kDa.

The sensitivity of larval midgut proteinases to various concentrations of rOC-I was determined Fig. 3. This cysteine proteinase inhibitor reduced proteolysis of larval midgut extracts against azocasein to 11% of the control activity and its IC_{50} value was estimated to be 1.7×10^{-6} M (against 0.25 gut equivalents). The lack of sensitivity to serine protease inhibitors was confirmed by assaying inhibition by CpTI over a similar concentration range (Fig. 3); no significant increase in inhibition with increasing CpTI was apparent, and higher levels of the protein stimulated proteolysis slightly.

4. Discussion

In ladybirds, the principal function of the larval stage is growth and to achieve this *A. bipunctata* feeds on large quantities of aphids. These two-spot ladybird larvae grow rapidly and become pupae in three to four weeks, with adults emerging from these pupae approximately one week later (Majerus, 1994). Adults continue to principally feed on aphids throughout the summer and during later summer and autumn build up their energy reserves to enable them to overwinter. Since the life history of the two-spot ladybird is strongly dependent on the nutritional quality of its diet, natural control of aphid populations may be effected if 'foreign' anti-nutritional compounds are ingested by ladybirds.

The physiological pH of the midgut, the pH optimum for proteolytic activity of azocasein by midgut extracts and the sensitivity of these proteinases to proteinase inhibitors and enhancers, suggests the dominance of cysteine proteinases in the midgut of larval two-spot ladybirds. Moreover, this activity appears to be largely due to cathepsin-L like proteinase(s) since Z-phe-arg-*p*NA was hydrolysed more readily than Z-arg-arg-*p*NA and OC-I inhibits cathepsin-L better than cathepsin B (Abe et al., 1994). Proteolytic activities in the midgut of adult ladybirds are broadly similar, but do show some distinct differences, in that cathepsin-L like proteinases are less abundant, since Z-phe-arg-*p*NA is less favoured

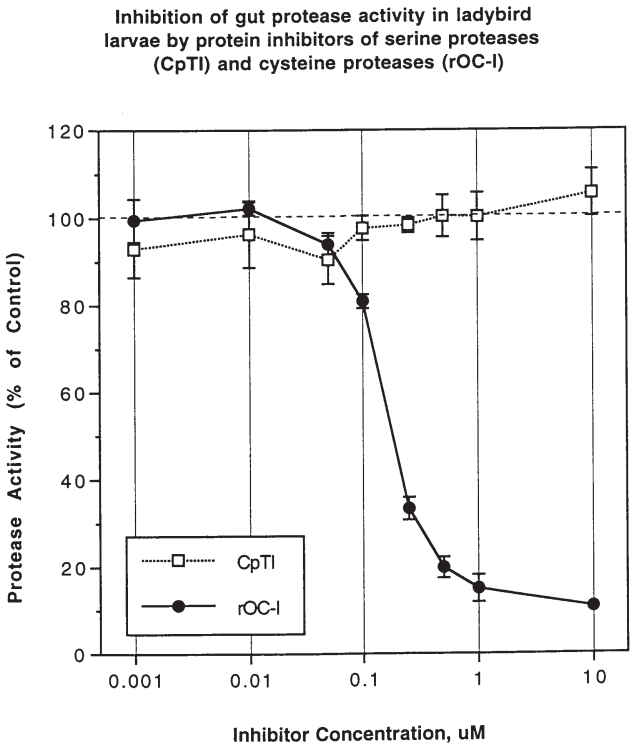

Fig. 3. Inhibition of the midgut proteolytic activity from fourth-instar ladybird larvae by plant-derived protein proteinase inhibitors over the concentration range 10^{-8} to 10^{-5} M. Representative inhibitors of serine proteases (CpTI) and cysteine proteases (rOC-I) are shown. 5 μl of midgut extract (representing approx. 25% of the total proteinase activity present per larva) was preincubated with various concentrations of CpTI and rOC-I and the remaining activity against azocasein was expressed as a percentage of the control activity (no inhibitor). Points and error bars represent the means and standard errors of at least 3 independent assays.

as a substrate over Z-arg-arg-*p*NA, and a metallo-proteinase activity is also present, as shown by the in-gel assay. While the in-gel assay is restricted to the detection of proteinases which are capable of renaturing after SDS-treatment and electrophoresis, results for the inhibition of larval midgut extracts correspond well with those of the spectrophotometric assay.

Cysteine proteinases have been found in many Coleoptera (for review see Terra and Ferreira, 1994) but studies on Coccinellidae are limited. The pH optimum for proteolytic activity of midgut extracts from the phytophagous Mexican bean beetle (*Epilachna varievestis*, Coccinellidae) was found to be in the range pH 5.0–6.5 and this correlated well with the physiological pH (5.8) (Murdock et al., 1987). This cysteine proteinase activity resembles that found in *A. bipunctata* even though the two-spot ladybird is aphidophagous.

Of the plant derived cysteine proteinase inhibitors tested rOC-I had the greatest inhibitory activity against larval ladybird midgut proteinases followed by papaya cystatin and CCPI. The differing abilities of these inhibitors to reduce proteolysis is probably a result of them possessing differing specificities towards the cysteine proteinases present. Our results do however show that phytocystatins inhibit the proteolytic activity of two-spot ladybird larvae and that adult proteinases are also likely to be susceptible to these proteinase inhibitors (although to a lesser extent due to the presence of the metallo-proteinase). Exposure to proteinase inhibitors may occur through the tritrophic interaction between plants, ladybirds and aphids (or coccids and adelgids), but it may also occur directly since the two-spot ladybird will also consume pollen and nectar when aphid prey become scarce (Majerus, 1994). Furthermore, dispersal of ladybird larvae is more limited than that of adults since they are less mobile. Therefore in agricultural ecosystems where the aphid host range is limited, oryzacystatins expressed in transgenic crops, may affect ladybird larvae more than the adult. Ultimately, the possible tritrophic effects of genetically engineered cysteine proteinase inhibitors on ladybirds should be determined *in vivo* where the transport of proteinase inhibitors can be monitored through each trophic level.

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