Purification and Characterization of a Digestive Cysteine Proteinase from the Field Slug (*Deroceras reticulatum*): A Potential Target for Slug Control

Anthony J. Walker,* David M. Glen, and Peter R. Shewry

IACR—Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, United Kingdom

Proteinase activities present in crop, digestive gland, and salivary gland extracts of the pest slug species, *Deroceras reticulatum* (Müller), were investigated. The digestive gland was found to be responsible for ≈80% of the total proteolytic activity against the plant protein rubisco. This activity, also detected by maximal hydrolysis of the synthetic substrate Z-Phe-Arg-*p*NA at pH 6.0, was activated by thiol compounds and inhibited by the cysteine-specific proteinase inhibitors E-64 and chicken egg white cystatin (azocasein as substrate). Furthermore, activity was largely unaffected by class-specific inhibitors diagnostic for aspartic acid proteinases, metalloproteinases, or serine proteinases. Similar studies demonstrated that the weak proteolytic activities in the crop and salivary glands were due to serine proteinases and metalloproteinases. Cation-exchange chromatography of digestive gland extracts showed that the activity in the digestive gland was due to a single protein of approximate M_r 40 000, which had kinetic properties similar to those of cathepsin L. Inhibition constants of phytocystatins tested against this purified proteinase ranged between 1.28×10^{-7} and 6.55×10^{-7} M. These results suggest that the expression of phytocystatins in transgenic plants may be an alternative method for controlling slug populations in the field.

Keywords: *Deroceras reticulatum; mollusc; protease inhibitors; crop protection; gastropod*

INTRODUCTION

Slugs are serious pests of agricultural and horticultural crops worldwide and are particularly troublesome in northern Europe, North and Central America, and New Zealand (Barker, 1979; South, 1992; Hammond, 1996). Furthermore, the increasing use of integrated arable farming systems is likely to result in increases in slug populations because the agronomic practices associated with these systems favor slug population growth (Glen, 1994). Current strategies for protecting crops from slug damage involve combinations of both cultural and chemical control methods [see, for example, Glen et al. (1992)]. However, this approach is often ineffective (Wareing and Bailey, 1989; Wiltshire and Glen, 1989) due to environmental conditions and lack of contact between slugs and the molluscicide. Because currently used molluscicides are also toxic to nontarget organisms (Martin and Forest, 1969; Longbottom and Gordon, 1979; Studdert, 1985; Bieri et al., 1989; Kennedy, 1990), both economic and environmental benefits would come from a more effective and specific method of slug control.

An attractive alternative to molluscicide usage is the inhibition of slug digestive proteinases with plant-derived proteinase inhibitors expressed in transgenic crop plants. Such proteinase inhibitors occur in the reproductive organs, storage organs, and vegetative tissues of most plant families (Richardson, 1991; Shewry and Lucas, 1997) and are considered to be involved in defense against pest or pathogen attack (Bowles, 1991). They are generally small, cysteine-rich proteins of M_r 3000−25000 (excluding some multimeric forms), which are usually specific for one class of proteinase (serine, cysteine, aspartic acid, or metallo-). The digestive proteinases of many phytophagous insect pests have been shown to be inhibited by proteinase inhibitors, and many studies have shown that these defensive proteins have antimetabolic effects when presented in artificial diets or transgenic plants [see, for example, Hilder et al. (1987), Hines et al. (1990), Gatehouse et al. (1993), Burgess et al. (1994), Broadway and Villani (1995), Irie et al. (1996), and Kuroda et al. (1996)].

Detailed studies of the proteolytic enzymes present in molluscan digestive systems are limited, with much of the available information [reviewed by Vonk and Western (1984)] coming from early publications. In slugs, proteinase activity has been demonstrated in both *Arion ater* (Evans and Jones, 1962) and *Deroceras reticulatum* (Walker, 1969; Johnston, 1989), but the proteinases responsible for these activities were not characterized. As a basis for developing transgenic strategies using proteinase inhibitor genes for the control of pest slug species, it is necessary to understand the basic biology of slug digestion. Surveys of slug populations have shown that most arable fields contain a mixture of pest slug species, but *D. reticulatum* is almost always present (Gratwick, 1992), indicating that this the most economically important species. We have therefore characterized the major proteolytic activity in the digestive system of this species with a view to identifying candidate proteinase inhibitor genes for transfer into crop plants susceptible to slug damage.

* Address correspondence to this author at the Vascular Tissue Laboratory, Department of Medicine, University of Manchester, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, U.K. [telephone (0161) 276 8670; fax (0161) 274 4833; e-mail Anthony.Walker@man.ac.uk].

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MATERIALS AND METHODS

Materials. NAP-5 desalting and Hi-Trap SP cation-exchange prepacked columns were purchased from Pharmacia Biotech (Uppsala, Sweden). The substrates ribulose bisphosphatecarboxylase/oxygenase (rubisco) and azocasein were purchased from Sigma-Aldrich Co. Ltd. (Poole, U.K.), whereas carbobenzoxy-L-arginyl-L-aginine *p*-nitroanalide (Z-Arg-Arg-*p*NA) and Arg-*p*NA were gifts from Dr. Mark Taylor (IFR Reading, U.K.) and Z-Phe-Arg-*p*NA was from Bachem Ltd. (Saffron Waldon, U.K.). The inhibitors *trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64) and leupeptin were from The Peptide Institute (Osaka, Japan), whereas pepstatin, 1,10-phenanthroline, antipain, aprotinin, iodoacetic acid (IAA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), chicken egg white (CEW) cystatin, phenylmethanesulfonyl flouride (PMSF), soybean and lima bean trypsin inhibitors (SBTI and LBTI), and ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich. Phytocystatins (plant-derived proteins that inhibit cysteine proteinases) were also used: oryzacystatin I (OC-I) (Abe et al., 1987) was a gift from Dr. Keiko Abe (University of Tokyo, Japan), OC-IΔD86 (Urwin et al., 1995) and cowpea cysteine proteinase inhibitor (CCPI) (Fernandes et al., 1993) were gifts from Prof. Howard Atkinson (University of Leeds, U.K.), and papaya cystatin (Song et al., 1995) was from Dr. Mark Taylor. Molecular weight markers (MWMs, Electran marker kit) were from BDH Ltd. (Poole, Dorset, U.K.). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and all other reagents were obtained from Sigma-Aldrich.

Slugs were trapped and collected from various arable sites at IACR—Long Ashton using upturned flower pot saucers baited with bran. They were then kept at 10 °C in plastic ventilated boxes lined with moist cotton wool and were fed a diet of Chinese cabbage leaves (*Brassica chinensis* L.).

Enzyme Preparation. Slugs were quickly killed by placing them in the freezer, after which the crop, digestive glands, and salivary glands were removed, frozen in liquid nitrogen, and stored at −25 °C. When required, extracts were homogenized at 4 °C in chilled double-distilled water (5 μL mg^{-1} of tissue for the digestive glands and salivary glands) using a centrifugal microhomogenizer system (Biomedix, Pinner, U.K.; Hearse, 1984) before being centrifuged at 20000*g* for 15 min at 4 °C. The supernatants were then transferred to microfuge tubes on ice, and the crop juice was diluted 1.5 times with chilled double-distilled water. All of the homogenates were then frozen in aliquots and stored at −25 °C. No change in total proteolytic activity was found during storage for several months.

General Proteinase Assays. Proteinase activity against rubisco was measured by mixing 10 μL of crop, digestive gland, or salivary gland extract with 70 μL of buffer [MES (pH 6.0) and Bis-Tris propane (pH 7.0) for digestive gland and crop and salivary gland extracts, respectively; final buffer concentrations 50 mM] and 10 μL of rubisco [1% (w/v) in 0.06% (w/v) SDS] and incubating at 25 °C for 1 h. The reaction was then stopped by adding 30 μL of 10% (w/v) ice-cold TCA, and the tubes were placed on ice for 30 min before centrifugation at 8000*g* for 4 min to remove precipitated protein. The supernatant (90 μL) was then added to 300 μL of TNBS reagent [1 volume of 0.3% (w/v) TNBS in water mixed with 9 volumes of 4% (w/v) sodium tetraborate in 0.15 M NaOH (pH 9.9)], and the mixture was incubated for 1 h at 25 °C before 150 μL of 0.5 M HCl was added. The absorbance of the resulting solution was then read at 340 nm (Habeeb, 1966) in a spectrophotometer against appropriate blanks and controls. All incubations were done in triplicate, and the activities were derived using the equation describing the line of best fit from a standard curve of L-leucine in MES buffer.

Proteinase activity against azocasein was measured by mixing 10 μL of crop, digestive gland, or salivary gland homogenate with 20 μL of 100 mM buffer [citrate phosphate (pH 5.0) and Bis-Tris propane (pH 7.0) (final assay concentrations 50 mM) for digestive gland and crop and salivary gland extracts, respectively] and 10 μL of azocasein solution [1% (w/v) in 0.06% (w/v) SDS] and incubating at 25 °C for 1 h before the process was stopped with 30 μL of ice-cold TCA [10% (w/v)]. Following precipitation on ice, samples were centrifuged at 8000*g* for 4 min and the supernatants (60 μL) added to wells of a microtiter plate (Nunc Maxisorp immunoplate, Life Sciences, Glasgow, U.K.) containing 40 μL of 1 M NaOH. All incubations were done in triplicate, and the absorbances of the resulting solutions were read at 405 nm in a microtiter plate reader, together with those for appropriate blanks and controls. Azocasein hydrolysis by column fractions was allowed to proceed for 3 h.

Cysteine Proteinase Assays. Digestive gland cysteine proteinase activity was measured using Z-Phe-Arg-*p*NA. Crude digestive gland homogenate or homogenate (2.5 μL) desalted on a Nap-25 column was preincubated for 20 min at 25 °C in 235 μL of MES buffer (pH 6.0, final assay concentration 50 mM) containing cysteine, EDTA, and Brij 35 [final concentrations 5 mM, 1 mM, and 0.1% (w/v), respectively]. The reaction was then started by adding 12.5 μL of Z-Phe-Arg-*p*NA (final assay concentration 0.5 mM), and the change in absorbance at 405 nm (Sarath et al., 1989) was recorded for 30 min at 25 °C in a microtiter plate reader. Absorbances of reagent and enzyme controls were also measured, and all incubations were done in triplicate unless otherwise stated. Column fraction and purified enzyme activities were measured with 10 μL of sample, 227.5 μL of buffer, and a final substrate concentration of 0.25 mM for 3 h and 90 min, respectively. The cysteine proteinase activity of the purified proteinase was also measured against the synthetic substrates Z-Arg-Arg-*p*NA and Arg-*p*NA. Velocities were calculated using $\epsilon = 8800$ M^{-1} cm^{-1} (Erlanger et al., 1961).

Optimal pH and Temperature. The effects of pH on the proteolytic activities of crude homogenates against rubisco were determined between pH 3.0 and 10.0 (using citrate phosphate, citrate phosphate + MES, MES, MES + Bis-Tris propane, Bis-Tris propane, and CAPS buffers for the pH ranges 3.0−5.0, 5.5, 6.0, 6.5, 7.0−9.5, and 10, respectively; final buffer concentrations 50 mM). The activities of the digestive gland crude homogenate against azocasein and of both the digestive gland crude homogenate and purified enzyme against Z-Phe-Arg-*p*NA were determined between pH 3.0 and 7.5.

The crude digestive gland homogenate and purified enzyme were incubated with Z-Phe-Arg-*p*NA at various temperatures (5−55 °C, in 5 °C increments) for 5 min and their proteolytic activities measured.

Thermal Stability. Both digestive gland crude homogenate and purified enzyme were preincubated in MES buffer (pH 6.0, final assay concentration 50 mM) containing cysteine, EDTA, and Brij 35 [final concentrations 5 mM, 1 mM, and 0.1% (w/v), respectively] at various temperatures (5−55 °C, in 5 °C increments) for 30 min. These samples were then equilibrated at 25 °C and their remaining activities determined by adding Z-Phe-Arg-*p*NA.

Effect of Reductants and Inhibitors. Crude digestive gland proteinase (10 μL) was preincubated in 20 μL citrate−phosphate buffer (100 mM, pH 5.0) containing inhibitor [pepstatin (0.007 mM), IAA (1 mM), CEW cystatin (0.02 mM), DTNB (1 mM), NEM (1 mM), E-64 (0.02 mM), PMSF (5 mM), SBTI (0.05 mM), LBTI (0.022 mM), EDTA (10 mM), 1,10-phenanthroline (10 mM), antipain (0.1 mM), and leupeptin (0.1 mM)] or 1 mM reducing agent (cysteine, DTT, or 2-Me), which activates cysteine proteinases, for 20 min at 25 °C before the addition of azocasein. The effects of the inhibitors [pepstatin (0.007 mM), E-64 (0.02 mM), PMSF (5 mM), and EDTA (10 mM)] and a reducing agent [cysteine (1 mM)] on the proteolytic activities of crop and salivary gland crude homogenates were determined using 25 μL of crude homogenate and 50 μL of 100 mM Bis-Tris propane buffer (pH 7.0). The activity was reported as the percentage of the control without the inhibitor. The specificities of the inhibitors (Tables 1 and 2) have been reviewed by Storey and Wagner (1986).

The effects of various concentrations (1−5 mM) of the reducing agents cysteine, DTT, and 2-Me (cysteine proteinase activators) on the Z-Phe-Arg-*p*NAase activity of crude digestive gland homogenate were also determined. Crude digestive

Table 1. Effects of Inhibitors and Activators on the Proteolytic Activity of *D. reticulatum* Digestive Gland Extracts

proteinase class	inhibitor/activator	concn (mM)	relative activity[c] (%)
none			100
cysteine	IAA	1.0	52***
	CEW cystatin	0.02	20***
	DTNB	1.0	75*
	NEM	1.0	81[ns]
	E-64	0.02	3***
	cysteine[a]	1.0	125***
	DTT[a]	1.0	122*
	2-mercaptoethanol[a]	1.0	116[ns]
serine	PMSF[b]	5.0	102[ns]
	SBTI	0.05	101[ns]
	LBTI	0.022	113[ns]
aspartic	pepstatin	0.007	116[ns]
metallo	EDTA	10.0	113[ns]
	1,10-phenathroline	10.0	78*
serine/cysteine	antipain	0.1	3***
	leupeptin	0.1	7***

[a] Stimulates activity of cysteine proteinases. [b] Serine proteinase inhibitor but may also inactivate cysteine proteinases. [c] Values are means relative to control activity (no inhibitor). Relative activities that are significantly different from the controls are indicated as follows: *, $P < 0.05$; ***, $P < 0.001$ (analysis of variance); [ns], not significant.

Table 2. Effects of Inhibitors and an Activator on the Proteolytic Activity of Crop and Salivary Gland Extracts from *D. reticulatum*

proteinase class	inhibitor/activator	concn (mM)	relative activity[c] (%) C	relative activity[c] (%) SG
none			100	100
cysteine	E-64	0.02	88[ns]	89[ns]
	cysteine[a]	1.0	85[ns]	35***
serine	PMSF[b]	5.0	78[ns]	35***
aspartic	pepstatin	0.007	98[ns]	89[ns]
metallo	EDTA	10.0	52**	8***

[a] Stimulates activity of cysteine proteinases. [b] Serine proteinase inhibitor but may also inactivate cysteine proteinases. [c] Values are means relative to control activity. C, crop; SG, salivary gland extracts. Relative activities that are significantly different from the controls are indicated as follows: **, $P < 0.01$; ***, $P < 0.001$ (analysis of variance); [ns], not significant.

gland extract (2.5 μL) was mixed with 235 μL of MES buffer (pH 6.0, final assay concentration 50 mM) containing EDTA, Brij 35 [final concentrations 1 mM and 0.1% (w/v), respectively], and the reducing agent. The samples were then preincubated for 20 min at 25 °C before the addition of Z-Phe-Arg-*p*NA.

K_i Determination. K_i values of various plant-derived proteinase inhibitors (OC-I, OC-IΔD86, papaya cystatin, and CCPI) against purified digestive gland proteinase were determined using the method of Salvesen and Nagase (1989). Briefly, hydrolysis of Z-Phe-Arg-*p*NA was continuously monitored for 5 min at 405 nm to establish the uninhibited rate of substrate hydrolysis (v_0). Inhibitor (10 μL) was then added and the reaction allowed to proceed until the rate of hydrolysis had stabilized at a new steady state, the inhibited rate (v_i). Duplicate assays were carried out for each inhibitor. $K_{i(app)}$ (i.e., K_i in the presence of substrate) was then calculated from the relationship $v_0/v_i = 1 + [I]/K_{i(app)}$ (where [I] is the concentration of inhibitor).

Effect of Metal Ions. Purified proteinase was preincubated with 1 mM concentrations of various metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}) for 20 min at 25 °C in MES buffer (pH 6.0) containing cysteine and Brij 35 [final concentrations 5 mM and 0.1% (w/v), respectively]. Residual activities were then measured by adding the substrate Z-Phe-Arg-*p*NA to the solutions and measuring the absorbance change.

Protein Determinations. Protein contents of enzyme solutions were determined according to the method of Bradford (1976) using Bio-Rad protein assay reagents with BSA fraction V as the protein standard.

Fractionation on SP-Sepharose High Performance. Digestive gland extract was desalted on a 1 mL Pharmacia NAP-25 column at 4 °C using citrate−phosphate buffer (pH 5.0) containing 5 mM cysteine and 1 mM EDTA as the elution buffer. The eluted active fraction (1 mL) was then chromatographed on a 1 mL Pharmacia Hi-Trap SP cation-exchange column equilibrated in 20 mM citrate−phosphate buffer (pH 5.0) containing 5 mM cysteine and 1 mM EDTA (pH 5.0) at a flow rate of 1 mL min^{-1}. The column was washed for 5 min before a linear gradient of 0−500 mM NaCl was applied at 1 mL min^{-1} for 15 min; 500 μL fractions were collected and assayed against azocasein and Z-Phe-Arg-*p*NA.

Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out at 200 V using the Bio-Rad Mini Protean system with discontinuous vertical slab gels (Laemmli, 1970) containing 12.5% (v/v) acrylamide in the resolving gel.

Statistical Analyses. Analysis of variance (ANOVA) was done on data using the statistical software package GENSTAT 5 (Payne et al., 1987).

RESULTS

Classes of Proteinase Present in the Crop, Digestive Gland, and Salivary Gland. Preliminary experiments showed that slugs which were administered antibiotics in an artificial diet (to reduce the total and proteolytic bacterial populations in the crop, digestive gland, and salivary gland) had total proteinase activities and pH profiles similar to those of control slugs fed artificial diet without antibiotic (Walker, 1997). There did not, therefore, appear to be any major proteinases produced by the bacterial microflora of these regions (data not shown).

The pH optimum of the proteinase activity of the digestive gland homogenate (pH 5.0) against rubisco was considerably lower than those of the crop and salivary gland homogenates, which shared a similar pH optimum (pH 7.5) (Figure 1). Furthermore, analysis of variance revealed significant differences between the proteolytic activities of these three regions ($P < 0.001$). Whereas the digestive gland was responsible for 82% of the total proteolytic activity within the slug gut, the crop (13%) and salivary glands (5%) were less important. Further studies were therefore focused on characterizing the proteinase enzymes in the digestive gland.

Inhibitors and activators diagnostic for the four mechanistic classes of proteinase were used to identify the classes of proteinase present in the digestive gland of *D. reticulatum* (Table 1), using azocasein at pH 5.0, the optimum pH for proteolytic activity against this substrate (data not shown). The cysteine proteinase inhibitors DTNB and NEM were not very potent inhibitors of *D. reticulatum* digestive gland proteinase activity, but IAA, CEW cystatin, and the highly specific cysteine proteinase inhibitor, E-64, significantly reduced total proteinase activity to 52, 20, and 3% of the control, respectively ($P < 0.001$). This activity was also significantly enhanced by the presence of 1 mM cysteine or 1 mM DTT ($P < 0.001$ and $P < 0.05$, respectively), both of which activate cysteine proteinases. Both antipain and leupeptin (which inhibit serine and cysteine proteinases) inhibited proteolysis to a similar extent to E-64, which was probably due to inhibition of cysteine proteinases since several inhibitors of serine proteinases (PMSF, SBTI, and LBTI) were ineffective. While pro-

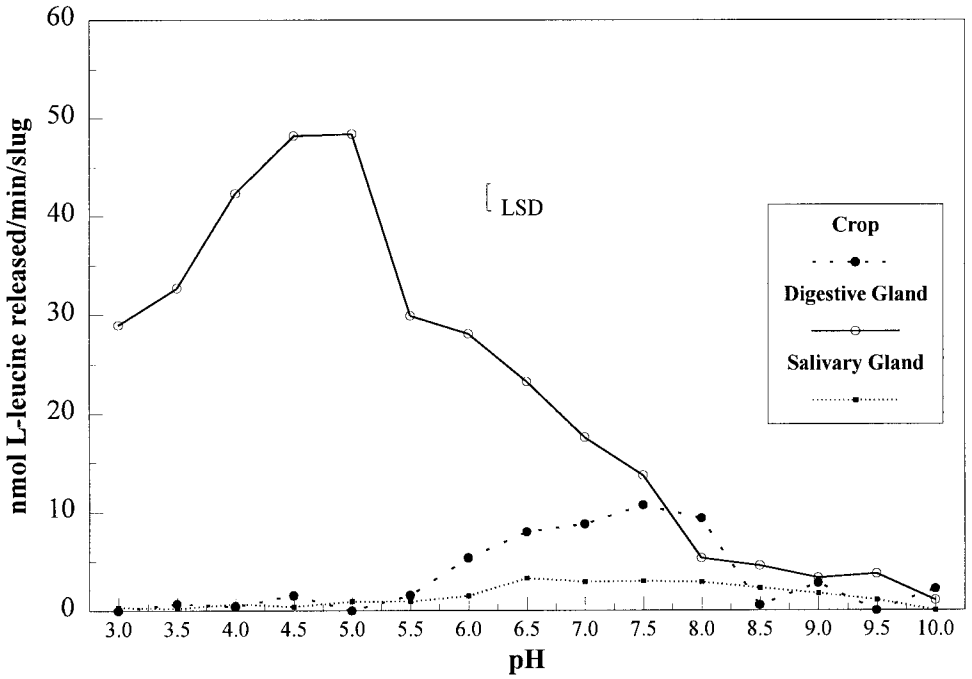

Figure 1. Proteolytic activities of crop, digestive gland, and salivary gland extracts of *D. reticulatum* against rubisco. Activities are expressed per slug. (LSD for comparing between gut regions, $P = 0.05$, 90 df.)

teolytic activity was slightly reduced by the inclusion of 1,10-phenanthroline, it was not affected by EDTA. Since both of these inhibitors react with metalloproteinases by chelating divalent metal ions and almost all of the proteolytic activity was inhibited using E-64 and antipain, it is probable that 1,10-phenathroline cross-reacted with the cysteine proteinases. Pepstatin did not reduce proteolysis of azocasein, indicating that aspartic proteinases are not present in *D. reticulatum* digestive glands.

Since the proteolytic activities of crop and salivary gland extracts were very low, only a limited range of inhibitors and activators were used to attempt to identify which class(es) of proteinase was (were) present (Table 2). EDTA significantly reduced proteolysis to 52 and 8% of the control levels with crop ($P < 0.01$) and salivary gland ($P < 0.001$) extracts, respectively, indicating the presence of metalloproteinases. Furthermore, cysteine, which enhances the activity of cysteine proteinases and is also known to inhibit metalloproteinases (Storey and Wagner, 1986), reduced the proteolytic activities in these extracts. E-64 reduced the proteolytic activities of both extracts slightly (although not statistically significantly), indicating that some cysteine proteinase may have been present. However, any enhancement of cysteine proteinase activity by cysteine may have been masked by its inhibitory effect on the metalloproteinases present. Serine proteinases may also be present in the salivary gland of *D. reticulatum* since PMSF significantly reduced proteolysis by salivary gland extracts ($P < 0.001$).

The effects of cysteine proteinase activators on the proteolytic activity of crude digestive gland homogenates were determined using the cysteine proteinase substrate Z-Phe-Arg-*p*NA (Figure 2) at pH 6.0, the optimum for Z-Phe-Arg-*p*NA hydrolysis (Figure 5). The profiles of concentration-dependent activation were similar for both cysteine and DTT over the concentration range studied (0.5−5.0 mM), with optimal proteinase activation at 4 mM (4.8 times) and 2 mM (4.6 times),

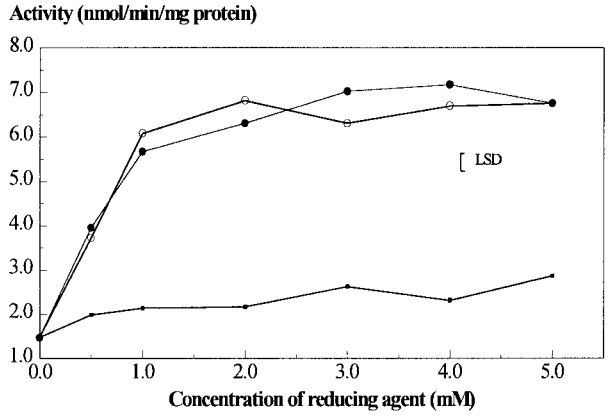

Figure 2. Effect of increasing concentrations of cysteine (●), DTT (○), and 2-mercaptoethanol (■) on the proteolytic activity of crude digestive gland homogenate against Z-Phe-Arg-*p*NA. (LSD for comparing activities, $P = 0.05$, 42 df.)

respectively. Although 2-mercaptoethanol significantly enhanced the digestive gland cysteine proteinase activity at 0.5 mM ($P < 0.01$), it was not as effective as cysteine or DTT at any of the concentrations studied.

Purification and Characterization of the Major Digestive Gland Proteinase. Digestive gland proteinase was isolated by desalting crude homogenate on a Pharmacia Nap-25 column and applying the active eluted fraction to a Pharmacia Hi-Trap SP cation-exchange column. The proteinase adsorbed to the cation-exchange column at pH 5.0, and a major peak of proteolytic activity against both azocasein and Z-Phe-Arg-*p*NA was eluted at a salt concentration of ≈0.17 M NaCl (Figure 3). The corresponding fractions consistently contained a single protein of approximate M_r 40 000 when analyzed by SDS−PAGE under reducing conditions (Figure 4). Furthermore, the amount of this protein in the peak fractions, observed by staining with Coomassie Brilliant Blue R250, corresponded to the proteolytic activities of the fractions against both sub-

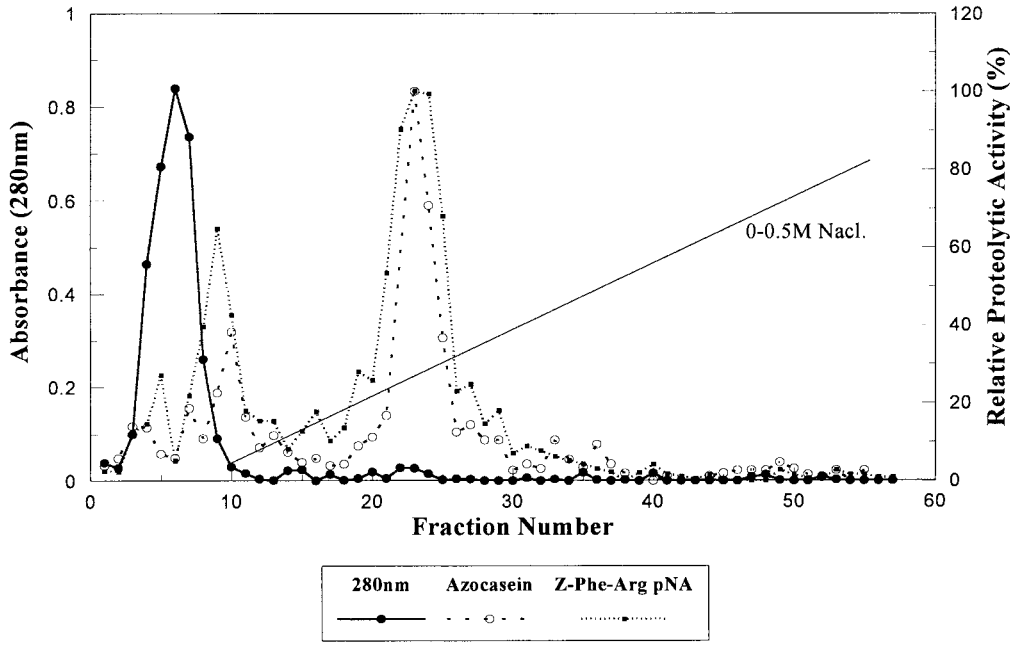

Figure 3. SP-Sepharose high-performance ion-exchange chromatography of *D. reticulatum* digestive gland extract.

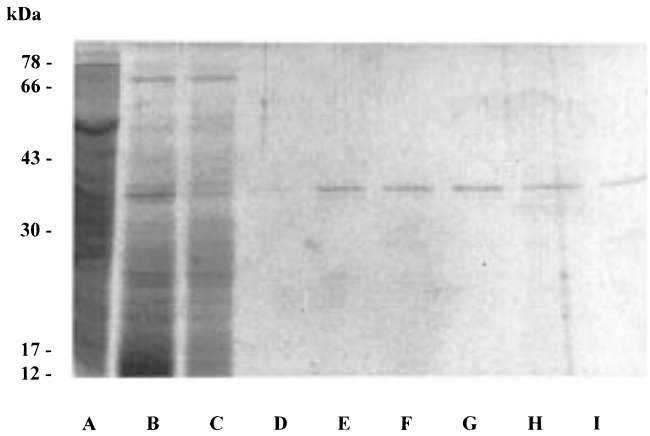

Figure 4. SDS−PAGE of stages in the isolation of digestive gland proteinase: (lanes A−I from left to right) (A) crude digestive gland homogenate; (B) active fraction recovered from Nap-25 column; (C and D−I) fraction 9 and fractions 20, 22, 23, 24, 25, and 27 eluted from the Hi-Trap cation-exchange column, respectively. Equal volumes (20 μL) were applied to the gel for all cation-exchange fractions. The molecular weight markers were ovotransferin (M_r 78 000), BSA (M_r 66 250), ovalbumin (M_r 42 700), carbonic anhydrase (M_r 30 000), myoglobin (M_r 17 200), and cytochrome *c* (M_r 12 300) (Electran calibration kit).

Table 3. Purification of the M_r 40 000 Cysteine Proteinase from the Digestive Gland of *D. reticulatum*

purifn stage	total protein[a] (μg)	activity[b] (nmol min^{-1})	spec activity [nmol min^{-1} (mg of protein)$^{-1}$]	purifn (-fold)
crude extract	49.75	0.47	9.5	1.0
Nap-25	18.48	0.27	14.3	1.5
Hi-Trap SP[c]	0.51	0.26	509.4	53.6

[a] Relates to amount of protein in enzyme assay. [b] Rate of hydrolysis of peptide substrate Z-Phe-Arg-*p*NA. [c] Activity of fraction 23.

strates (Figure 4). A purification of ≈50-fold compared to the original crude extract was obtained (Table 3).

The purified proteinase hydrolyzed Z-Phe-Arg-*p*NA at pH 6.0 (Figure 5) with a specific activity of 511 nmol min^{-1} (mg of protein)$^{-1}$, whereas its activity against

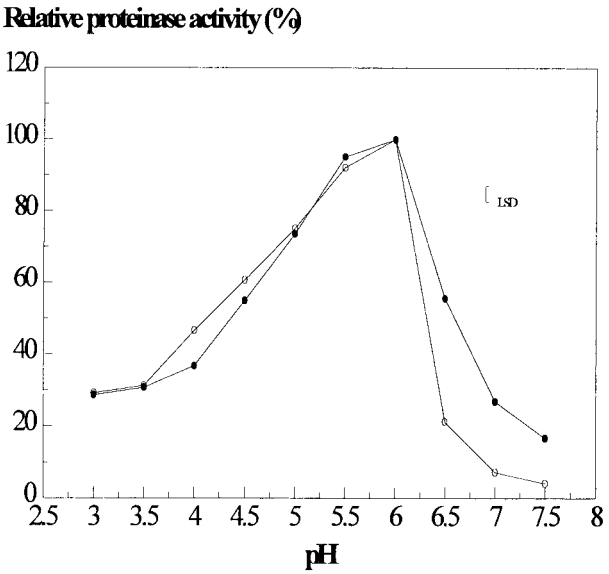

Figure 5. Effect of pH on the mean proteolytic activities of digestive gland crude homogenate (●) and purified proteinase (○) against the synthetic substrate Z-Phe-Arg-*p*NA. (LSD for comparing activities between crude homogenate and purified proteinase, $P = 0.05$, 40 df.)

Z-Arg-Arg-*p*NA was significantly lower [82 nmol min^{-1} (mg of protein)$^{-1}$, $P < 0.001$]. Z-Phe-Arg-*p*NA is a good substrate for cathepsin L (EC 3.4.22.15) and Z-Arg-Arg-*p*NA for cathepsin B (EC 3.4.22.1) (Barrett and Kirschke, 1981). The digestive gland proteinase did not hydrolyze Arg-*p*NA, a substrate for cathepsin H (EC 3.4.22.16).

Assays conducted with the purified digestive gland proteinase and various concentrations of Z-Phe-Arg-*p*NA gave a linear Michaelis−Menten plot, with $K_m = 3.55 \pm 0.51 \times 10^{-5}$ M and $V_{max} = 1.62$ μmol of *p*NA produced min^{-1} (mg of protein)$^{-1}$. The use of the active-site titrant, E-64, allowed the concentration of active enzyme to be determined and the catalytic constant ($K_{cat} = V_{max}$/[enzyme]) to be calculated as 1.73 ± 0.05 s^{-1}. The catalytic efficiency (K_{cat}/K_m) of the proteinase was therefore estimated to be 48 870 M^{-1} s^{-1}.

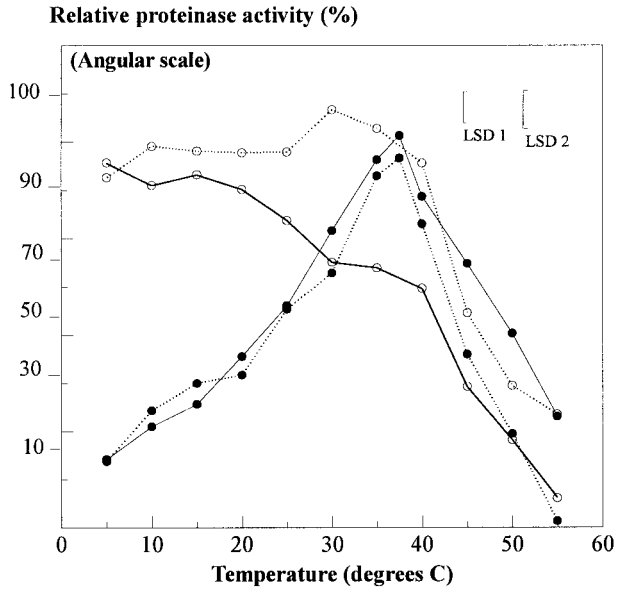

Figure 6. Effect of temperature on Z-Phe-Arg-*p*NA hydrolyzing activity of the crude homogenate (—) and purified proteinase (· · ·): (●) profile of temperature-dependent activity; (○) profile of thermal stability. (LSD 1 for comparing thermal stability and LSD 2 for comparing temperature-dependent activity between crude homogenate and purified proteinase, $P = 0.05$, df LSD 1, 39; df LSD 2, 46).

Table 4. Effect of Metal Ions on the Proteolytic Activity of Digestive Gland Purified Proteinase against Z-Phe-Arg-*p*NA

metal ion[a] (1 mM)	relative activity[b] (%)	metal ion[a] (1 mM)	relative activity[b] (%)
none	100.0	Mn^{2+} **	88.1
K^{+} ns	93.7	Co^{2+} ns	105.3
Na^{+} ns	104.4	Ni^{2+} ***	64.2
Mg^{2+} *	107.0	Cu^{2+} ***	17.7
Zn^{2+} ***	8.5	Hg^{2+} ***	7.5
Ca^{2+} ***	84.3	Fe^{2+} ***	88.0

[a] Chloride was the counterion in all cases. [b] Values are means relative to control activity (no metal ion). Relative activities significantly different from the controls are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (analysis of variance); ns, not significant, 24 df.

Analysis of variance (with angular transformation) revealed significant differences between the crude homogenate and the purified proteinase for both the thermal stability and temperature-dependent proteolytic activity ($P < 0.001$) (Figure 6). Both preparations had an optimum temperature for proteolytic activity of 37.5 °C, and the purified proteinase was significantly less active than the crude homogenate at 45 °C and above ($P < 0.001$). The T_m values (temperature for 50% inactivation after 30 min of preincubation) (Jiang et al., 1994) for the crude homogenate and purified proteinase were approximately 41 and 45 °C, respectively.

Since various metal chelates have been shown to be toxic to slugs and snails [see, for example, Young (1996)] and metal ions are known to affect the catalytic activities of enzymes (Malmström and Rosenberg, 1960), the effects of various metal ions on the proteolytic activity of the purified proteinase were determined (Table 4). In the presence of 5 mM cysteine, both $Zn^{2+}$and Hg^{2+} inhibited the proteolytic activity of the purified proteinase by >90% ($P < 0.001$). While Cu^{2+} was also a potent inhibitor, it was not as effective as either Zn^{2+} or Hg^{2+}. Ni^{2+} also had an inhibitory effect since it reduced proteolysis to 64% of the control ($P < 0.001$). In contrast, Ca^{2+}, Fe^{2+}, and Mn^{2+} gave only slight inhibition, and no significant inhibition was measured in the presence of K^{+}, Na^{+}, Mg^{2+}, or Co^{2+}. The inhibitory activity of some metal ions could be due to chelation of amino acids: Cu^{2+} and Ni^{2+} with histidine, Hg^{2+} with cysteine, and Zn^{2+} with either histidine or cysteine (Malmström and Rosenberg, 1960).

Table 5. Comparison of K_i Values for Inhibition of the Purified Digestive Gland Proteinase by Plant-Derived Proteinase Inhibitors

inhibitor/pro-region	K_i (M)
OC-IΔD86	$(6.55 \pm 0.37) \times 10^{-7}$
OC-I	$(1.76 \pm 0.27) \times 10^{-7}$
papaya cystatin	$(4.97 \pm 0.06) \times 10^{-7}$
CCPI	$(1.28 \pm 0.15) \times 10^{-7}$

Analysis of variance revealed significant differences among the plant-derived proteinase inhibitors in their ability to inhibit the purified digestive gland proteinase (Table 5) ($P < 0.001$). Whereas there was no significant difference between the equilibrium constants for inhibition (K_i) by OC-I and CCPI, both were significantly more potent than papaya cystatin ($P < 0.001$), which was in turn a better inhibitor than OC-IΔD86 ($P < 0.01$). Moreover, OC-I showed a 3−4-fold higher level of inhibition against the digestive gland proteinase than its engineered variant OC-IΔD86.

DISCUSSION

Proteolysis in Relation to pH. In all three gut regions, proteolysis occurred at pH values corresponding to their physiological pH as determined using ion-selective microelectrodes [pH 5.99, 6.52, and 6.94 for the crop, digestive glands, and salivary glands, respectively (Walker et al., 1996)]. The slightly acid pH optima for hydrolysis of rubisco (pH 5.0), azocasein (pH 5.0), and Z-Phe-Arg-*p*NA (pH 6.0) by crude digestive gland extracts of *D. reticulatum* are typical for cysteine proteinases and are close to the physiological pH of this organ. However, they are far removed from the optimum (pH 1.7) reported by Johnston (1989) for proteolysis of hemoglobin by whole gut extracts of *D. reticulatum*. The large difference in pH optima between that reported here and by Johnston (1989) is unlikely to be due to the use of different substrates and assay conditions. The data presented here, however, are in agreement with a study carried out by Walker (1969), who reported that proteolysis of gelatin by *D. reticulatum* gut extract occurred between pH 4.0 and 6.5.

Crop and Salivary Gland Proteinases. Inhibition studies with crop and salivary gland extracts indicate that the low activities in these organs are partly due to serine and metalloproteinases. Serine proteinase activity has been reported in molluscan salivary glands by Walker (1970), but the species of origin was not clear (*D. reticulatum* or *Lymnaea stagnalis*). While crop juice proteinases may be produced by the crop wall, they may also originate from the salivary glands since salivary secretions enter the crop with food material. The limited inhibition of crop extract by E-64 demonstrates that the digestive glands are not the major source of the extracellular proteinases present in the crop juice, disproving earlier assumptions made by Evans and Jones (1962) and Walker (1969).

Digestive Gland Proteinase: General Properties. The decreases in proteolytic activity following preincubation of crude digestive gland extract with IAA, CEW cystatin, E-64, antipain, and leupeptin, the enhancement of activity by thiol reagents, and the lack of

inhibition by inhibitors diagnostic for other mechanistic classes of proteinase suggest the dominance of cysteine proteinases in the digestive gland of *D. reticulatum*. Furthermore, hydrolysis of Z-Phe-Arg-*p*NA by digestive gland extracts also indicates that this class of enzyme is responsible for the major proteinase activity in the gut.

Separation of the digestive gland extract by SP-Sepharose high-performance chromatography and subsequent analysis by SDS−PAGE indicate that the digestive gland proteolytic activity is due to one major protein. The activity profiles for pH, temperature dependence, and thermal stability of the purified proteinase against Z-Phe-Arg-*p*NA are similar to those of the crude extract, which further supports this finding. The specificity of the purified M_r 40 000 cysteine proteinase toward synthetic peptide substrates, its pH optimum, and its sensitivity to leupeptin [which does not effectively inhibit cathepsin H (Barrett and Kirschke, 1981)] suggest that it has cathepsin L-like activity. Furthermore, whereas OC-1 was an effective inhibitor of *D. reticulatum* digestive gland proteinase [$K_i = (1.76 \pm 0.27) \times 10^{-7}$], it does not exhibit appreciable inhibitory activity against cathepsin B (Abe et al., 1994). Cathepsins B, H, and L are cysteine proteinases present in the lysosomes of cells of higher animals and are responsible for much of the bulk turnover of proteins in the cell (Barrett, 1987). The cathepsin L-like proteinase present in the digestive gland of *D. reticulatum* is likely to be responsible for the observed degradation of endocytosed food material as it migrates basally in the digestive cells (Walker, 1969).

Digestive Gland Proteinase: Inhibition by Metal Ions. The inhibitory effects of Zn^{2+}, Hg^{2+}, Cu^{2+}, and Ni^{2+} on the proteolytic activity of purified digestive gland proteinase against Z-Phe-Arg-*p*NA indicate the presence of histidine and cysteine (Malmström and Rosenberg, 1960) in the active site. These residues are common to all cysteine proteinases (Dunn, 1989). Magnesium, zinc, cadmium, and phosphate have all been shown to accumulate in the digestive gland of slugs (Ireland, 1979; Recio et al., 1988). Furthermore, various metal salts have been shown to possess molluscicidal activities and may act as contact poisons [reviewed by South (1992)]. The effects of these metal salts as stomach poisons are limited because molluscs fail to consume sufficient amounts to be lethal, but chelation of the metal can increase efficacy (Young, 1996). Our results indicate that both zinc and copper may be effective at disrupting digestion in *D. reticulatum* in vivo. Clearly, further studies into the effects of these metals on slug digestion would be worthwhile in relation to the development of metal-based molluscicides with both contact poison and enzyme inhibition properties.

Digestive Gland Proteinase: Inhibition by Phytocystatins. Of the plant-derived cysteine proteinase inhibitors (phytocystatins) tested, OC-I and CCPI had the strongest inhibitory activities against the purified proteinase, followed by papaya cystatin and OC-IΔD86. The K_i for OC-I was ≈3.5 times less than that of its engineered variant OC-IΔD86, which has Asp86 deleted. Deletion of this residue from OC-I led to a 13−14-fold improvement in the K_i against both papain and the nematode *Caenorhabditis elegans* cysteine proteinase gcp-1 (Urwin et al., 1995). Whereas OC-IΔD86 was less effective against the proteinase isolated from the digestive gland of *D. reticulatum* than OC-I, the results against papain and gcp-1 (Urwin et al., 1995) demonstrate that protein engineering can be used to enhance the interaction between pest proteinases and phytocystatins. Furthermore, corn cystatin may be more effective at reducing the activity of *D. reticulatum* digestive gland proteinase than the phytocystatins tested in this study. This is because corn cystatin has a wider spectrum of activity and is more effective than OC-I against cathepsins H and L and papain (Irie et al., 1996).

Comparison with Other Slugs. Whereas proteinases from other pest slug species have not yet been characterized, the pH optimum for proteolysis in *Arion ater* was found to be pH 5.6 (Evans and Jones, 1962). Furthermore, the physiological pH of the digestive systems of fed *Arion intermedius* and *Tandonia budapestensis* were found to range from 5.8 to 6.7 and from 6.1 to 6.7, respectively (Walker et al., 1996). These pH values suggest the presence of cysteine proteinases in the digestive systems of these slug species, and phytocystatins may therefore inhibit proteolysis in these slugs in a way similar to that of *D. reticulatum*.

Comparison with Cysteine Proteinases from Other Invertebrates. Few detailed studies of the digestive cysteine proteinases of invertebrate species have been reported. A cysteine proteinase with kinetic properties very similar to those of the digestive gland proteinase of *D. reticulatum* has been identified in the coleopteran insect *Callosobruchus chinensis* (Azuki bean weevil) (Kuroda et al., 1996). This cathepsin L-like proteinase was also strongly inhibited by OC-I. Furthermore, Matsumoto et al. (1995) isolated a gene encoding a similar proteinase from the midgut of the "model insect", *Drosophila melanogastor*. The amino acid sequence deduced from this *Drosophila* CP1 gene showed very high (67%) similarity to that of a cathepsin L (CP3) isolated from the digestive gland of the American lobster (*Homarus americanus*) (Laycock et al., 1991). Cathepsin L-like proteinases have therefore been shown to possess a digestive function in a range of invertebrate species, including the slug, *D. reticulatum*.

In Vivo Effects of Cysteine Proteinase Inhibitors on Insect Pests. The cysteine proteinase inhibitor E-64 has been shown to affect growth and development in the coleopterans *Acanthoscelides obtectus* (common bean weevil), *Callasobruchus maculatus* (cowpea weevil), and *Epilachna varivestis* (Mexican bean beetle), when administered in an artificial diet (Murdock et al., 1988; Hines et al., 1990; Wolfson and Murdock, 1995). Furthermore, the multicystatin from potato tubers has deleterious effects on the growth of *Diabrotica* (corn rootworm, Coleoptera) larvae, and oryzacystatins are inhibitory to growth of *Tribolium castaneum* (red flour beetle, Coleoptera), *Callasobruchus chinensis* (Azuki bean weevil, Coleoptera), and *Riptortus clavatus* (Hemiptera), a pest of soybeans and other field-cultured beans (Chen et al., 1992; Orr et al., 1994; Kuroda et al., 1996). Oyzacystatin I has been expressed in transgenic tobacco and potato plants under control of the CaMV 35S promoter (Masoud et al., 1993; Benchekroun et al., 1995), and extracts of the potato leaves have been shown to partially inhibit *Leptinotarsa decemlineata* (Colorado potato beetle) proteinases. Higher expression levels were obtained when corn cystatin, which inhibits cysteine proteinase more effectively than oryzacystatin, was expressed in transgenic rice (Irie et al., 1996). This

corn cystatin showed strong inhibitory activity against *Sitophilus zeamais* (maize weevil) in vitro.

Summary and Relevance for Slug Control. The strong inhibition of the major proteinase found in the digestive system of *D. reticulatum* by phytocystatins suggests that the expression of these inhibitors in transgenic crop plants may reduce their susceptibility to slug pests. We are therefore currently studying the effects of feeding slugs on artificial diets containing cysteine proteinase inhibitors and on transgenic plants expressing a phytocystatin. The deployment of such plants in cropping systems may lead to reductions in molluscicide usage and a reduction in the growth rates of slug populations in the field.

LITERATURE CITED

Abe, K.; Emori, Y.; Kondo, H.; Suzuki, K.; Arai, S. Molecular cloning of a cysteine proteinase inhibitor of rice (Oryzacystatin). *J. Biol. Chem.* **1987**, *262*, 16793−16797.

Abe, M.; Abe, K.; Iwabuchi, K.; Domoto, C.; Arai, S. Corn cystatin I expressed in *Escherichia coli*: investigation of its inhibitory profile and occurrence in corn kernels. *J. Biochem.* **1994**, *116*, 488−492.

Barker, G. M. The introduced slugs of New Zealand (Gastropoda: Pulmonata). *N. Z. J. Zool.* **1979**, *6*, 411−437.

Barrett, A. J. The cystatins: a new class of peptidase inhibitors. *Trends Biochem. Sci.* **1987**, *12*, 193−196.

Barrett, A. J.; Kirschke, H. Cathepsin B, cathepsin H, and cathepsin L. *Methods Enzymol.* **1981**, *80*, 535−560.

Benchekroun, A.; Michaud, D.; Nguyen-Quoc, B.; Overney, S.; Desjardins, Y.; Yelle, S. Synthesis of active oryzacystatin I in transgenic potato plants. *Plant Cell Rep.* **1995**, *14*, 585−588.

Bieri, M.; Schweizer, H.; Christensen, K.; Daniel, O. The effect of metaldehyde and methiocarb slug pellets on *Lumbricus terrestris* Linne. In *Slugs and Snails in World Agriculture*; Henderson, I., Ed.; BCPC Monograph 41; British Crop Protection Council: Bracknell, U.K., 1989; pp 237−244.

Bowles, D. The wound response of plants. *Curr. Biol.* **1991**, *1*, 165−167.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Broadway, R. M.; Villani, M. G. Does host range influence susceptibility of herbivorous insects to nonhost plant proteinase inhibitors? *Entomol. Exp. Appl.* **1995**, *76*, 301−312.

Burgess, E. P. J.; Main, C. A.; Stevens, P. S.; Christeller, J. T.; Gatehouse, A. M. R.; Laing, W. A. Effects of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Teleogryllus commodus*. *J. Insect Physiol.* **1994**, *40*, 803−811.

Chen, M.-S.; Johnson, B.; Wen, L.; Muthukrishnan, S.; Kramer, K. J.; Morgan, T. D.; Reeck, G. R. Rice cystatin: bacterial expression, purification, cysteine proteinase inhibitory activity, and insect growth suppressing activity of a truncated form of the protein. *Protein Expression Purif.* **1992**, *3*, 41−49.

Dunn, B. M. Determination of protease mechanism. In *Proteolytic Enzymes, A Practical Approach*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, U.K., 1989.

Erlanger, B. F.; Kokowsky, N.; Cohen, W. The preparation and properties of two new chromogenic substrates for trypsin. *Arch. Biochem. Biophys.* **1961**, *95*, 271−278.

Evans, W. A. L.; Jones, E. G. A note on the proteinase activity in the alimentary canal of the slug *Arion ater* L. *Comp. Biochem. Physiol.* **1962**, *5*, 223−225.

Fernandes, K. V. S.; Sabelli, P. A.; Barratt, D. H. P.; Richardson, M.; Xavier-Filho, J.; Shewry, P. R. The resistance of cowpea seeds to bruchid beetles is not related to levels of cysteine proteinase inhibitors. *Plant Mol. Biol.* **1993**, *23*, 215−219.

Gatehouse, A. M. R.; Shi, Y.; Powell, K. S.; Brough, C.; Hilder, V. A.; Hamilton, W. D. O.; Newell, C. A.; Merryweather, A.; Boulter, D.; Gatehouse, J. A. Approaches to insect resistance using transgenic plants. *Philos. Trans. R. Soc. London B* **1993**, *342*, 279−286.

Glen, D. M. Ecology of slugs in cereals in relation to crop damage and control. In *Individuals, Populations and Patterns in Ecology*; Leather, S. R., Walters, K. F. A., Mills, N. J., Watt, A. D., Eds.; Intercept: Andover (Hants), U.K., 1994.

Glen, D. M.; Wiltshire, C. W.; Langdon, C. J. Influence of seed depth and molluscicide pellet placement and timing on slug damage, activity and survival in winter wheat. *Crop Prot.* **1992**, *11*, 555−560.

Gratwick, M., Ed. Slugs and Snails. *Crop Pests of the UK, Collected Edition of MAFF Leaflets*; Chapman and Hall: London, 1992.

Habeeb, A. F. S. A. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* **1966**, *14*, 328−336.

Hammond, R. B. Conservation tillage and slugs in the U.S. corn belt. In *Slugs and Snail Pests in Agriculture*; Henderson, I., Ed.; BCPC Monograph 66; British Crop Protection Council: Bracknell, U.K., 1996; pp 31−38.

Hearse, D. J. Microbiopsy metabolite and paired flow analysis: a new rapid procedure for homogenisation, extraction and analysis of high energy phosphates and other intermediates without any errors from tissue loss. *Cardiovasc. Res.* **1984**, *18*, 384−390.

Hilder, V. A.; Gatehouse, A. M. R.; Sheerman, S. E.; Barker, R. F.; Boulter, D. A novel mechanism of insect resistance engineered into tobacco. *Nature* **1987**, *300*, 160−163.

Hines, M. E.; Nielsen, S. S.; Shade, R. E.; Pomeroy, M. A. The effect of two proteinase inhibitors, E-64 and the Bowman-Birk inhibitor, on the developmental time and mortality of *Acanthoscelides obtectus*. *Entomol. Exp. Appl.* **1990**, *57*, 201−207.

Ireland, M. P. Distribution of essential and toxic metals in the terrestrial slug *Arion ater*. *Comp. Biochem. Physiol.* **1979**, *68A*, 37−42.

Irie, K.; Hosoyama, H.; Takeuchi, T.; Iwabuchi, K.; Watanabe, H.; Abe, M.; Abe, K.; Arai, S. Transgenic rice established to express corn cystatin exhibits strong inhibitory activity against insect gut proteinases. *Plant Mol. Biol.* **1996**, *30*, 149−157.

Jiang, S.; Lee, J.; Chen, H. Purification and characterisation of a novel cysteine proteinase from mackerel (*Scomber australasicus*). *J. Agric. Food Chem.* **1994**, *42*, 1639−1646.

Johnston, K. A. Biochemical Mechanisms of Resistance of Potato Cultivars to Slug Attack. Ph.D. Thesis, University of Newcastle Upon Tyne, 1989.

Kennedy, P. J. The Effects of Molluscicides on the Abundance and Distribution of Ground Beetles (Coleoptera, Carabidae) and Other Invertebrates. Ph.D. Thesis, University of Bristol, 1990.

Kuroda, M.; Ishimoto, M.; Suzuki, K.; Kondo, H.; Abe, K.; Kitamura, K.; Arai, S. Oryzacystatins exhibit growth-inhibitory and lethal effects on different species of bean insect pests, *Callosobruchus chinensis* (Coleoptera) and *Riptortus clavatus* (Hemiptera). *Biosci., Biotechnol., Biochem.* **1996**, *60*, 209−212.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **1970**, *277*, 680−685.

Laycock, M. V.; MacKay, R. M.; Fruscio, M. D.; Gallant, J. W. Molecular cloning of three cDNAs that encode cysteine proteinases in the digestive gland of the American lobster (*Homarus americanus*). *FEBS Lett.* **1991**, *292*, 115−120.

Longbottom, G. M.; Gordon, A. S. M. Metaldehyde poisoning in a dairy herd. *Vet. Rec.* **1979**, *104*, 454−455.

Malmström, B. G.; Rosenberg, A. Mechanism of metal ion activation of enzymes. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1960**, 131−167.

Martin, T. J.; Forrest, J. P. Development of DRAZA in Great Britain. *Pflanzenschutz−Nachr. Bayer* **1969**, *22*, 205−243.

Masoud, S. A.; Johnson, L. B.; White, F. F.; Reeck, G. R. Expression of a cysteine proteinase inhibitor (oryzacystatin-I) in transgenic tobacco plants. *Plant Mol. Biol.* **1993**, *21*, 655−663.

Matsumoto, I.; Watanabe, H.; Abe, K.; Arai, S.; Emori, Y. A putative digestive cysteine proteinase from *Drosophila melanogaster* is predominantly expressed in the embryonic and larval midgut. *Eur. J. Biochem.* **1995**, *227*, 582−587.

Murdock, L. L.; Shade, R. E.; Pomeroy, M. A. Effects of E−64, a cysteine proteinase inhibitor, on cowpea weevil growth, development, and fecundity. *Environ. Entomol.* **1988**, *17*, 467−469.

Orr, G. L.; Strickland, J. A.; Walsh, T. A. Inhibition of *Diabrotica* larval growth by a multicystatin from potato tubers. *J. Insect Physiol.* **1994**, *40*, 893−900.

Payne, R. W.; Lane, P. W.; Ainsley, A. E.; Bicknell, K. E.; Digby, P. G. N.; Harding, S. A.; Leech, P. K.; Simpson, H. R.; Todd, A. D.; Verriers, P. J.; White, P. R.; Gower, J. C.; Tuncliffe-Wilson, G.; Paterson, L. J. *GENSTAT 5 Reference Manual*; Oxford University Press: Oxford, U.K., 1987.

Recio, A.; Marigomez, J. A.; Angulo, E.; Moya J. Zinc treatment of the digestive gland of the slug *Arion ater* L. 1. Cellular distribution of zinc and calcium. *Bull. Environ. Contam. Toxicol.* **1988**, *41*, 858−864.

Richardson, M. Seed storage proteins: the enzyme inhibitors. In *Methods in Plant Biochemistry*; Academic Press: New York, 1991; Vol. 5.

Salvesen, G.; Nagase, H. Inhibition of proteolytic enzymes. In *Proteolytic Enzymes, A Practical Approach*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, U.K., 1989.

Sarath, G.; De La Motte, R.; Wagner, F. Protease assay methods. In *Proteolytic Enzymes, A Practical Approach*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, U.K., 1989.

Shewry, P. R.; Lucas, J. A. Plant proteins that confer resistance to pests and pathogens. In *Advances in Botanical Research*; Callow, J., Ed.; Academic Press: London, 1997.

Song, I.; Taylor, M.; Baker, K.; Bateman, R. C. Inhibition of cysteine proteinases by *Carica papaya* cystatin produced in *Escherichia coli*. *Gene* **1995**, *162*, 221−224.

South, A. *Terrestrial Slugs. Biology, Ecology and Control*; Chapman and Hall: London, 1992.

Storey, R.; Wagner, F. W. Plant proteases: a need for uniformity. *Phytochemistry* **1986**, *25*, 2701−2709.

Studdert, V. P. Epidemiological features of snail and slug bait poisoning in dogs and cats. *Aust. Vet. J.* **1985**, *62*, 269−271.

Urwin, P. E.; Atkinson, H. J.; Waller, D. A.; McPherson, M. J. Engineered oryzacystatin-I expressed in transgenic hairy roots confers resistance to *Globodera pallida*. *Plant J.* **1995**, *8*, 121−131.

Vonk, J. H.; Western, J. R. H. Invertebrate proteinases. In *Comparative Biochemistry and Physiology of Enzymatic Digestion*; Vonk, J. H., Western, J. R. H., Eds.; Academic Press: London, 1984.

Walker, A. J. Characterisation of the Proteolytic Activity in the Digestive system of the Field Slug *Deroceras reticulatum* as a Target for Novel Methods of Control. Ph.D. Thesis, University of Bristol, 1997.

Walker, A. J.; Miller, A. J.; Glen, D. M.; Shewry, P. R. Determination of pH in the digestive system of the slug *Deroceras reticulatum* (Müller) using ion-selective microelectrodes. *J. Moll. Stud.* **1996**, *62*, 387−390.

Walker, G. Studies on Digestion of the Slug, *Agriolimax reticulatus* (Müller) (Mollusca, Pulmonata, Limacidae). Ph.D. Thesis, University of Wales, Bangor, 1969.

Walker, G. Light and electron microscope investigation on the salivary glands of the slug, *Agriolimax reticulatus* (Müller). *Protoplasma* **1970**, *71*, 111−126.

Wareing, D. R.; Bailey, S. E. R. Factors affecting slug damage and its control in potato crops. In *Slugs and Snails in World Agriculture*; Henderson, I., Ed.; BCPC Monograph 41; British Crop Protection Council: Bracknell, U.K., 1989; pp 133−120.

Wiltshire, C. W.; Glen, D. M. Effects of molluscicides on slugs and soil arthropods in winter cereal crops. In *Slugs and Snails in World Agriculture*; Henderson, I., Ed.; BCPC Monograph 41; British Crop Protection Council: Bracknell, U.K., 1989; pp 399−406.

Wolfson, J. L.; Murdock, L. L. Potential use of protease inhibitors for host plant resistance: a test case. *Environ. Entomol.* **1995**, *24*, 52−57.

Young, C. Metal chelates as stomach poison molluscicides for introduced pests, *Helix aspersa*, *Theba pisana*, *Cernuella virgata* and *Deroceras reticulatum* in Australia. In *Slugs and Snail Pests in Agriculture*; Henderson, I., Ed.; BCPC Monograph 66; British Crop Protection Council: Bracknell, U.K., 1996; pp 237−243.

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