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Effects of a potato cysteine proteinase inhibitor on midgut proteolytic enzyme activity and growth of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae)

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Abstract

The major proteinase activity in extracts of larval midguts from the southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi*, was identified as a cysteine proteinase that prefers substrates containing an arginine residue in the P1 position. Gelatin-zymogram analysis of the midgut proteinases indicated that the artificial diet-fed SCR, corn root-fed SCR, and root-fed western corn rootworms (*Diabrotica virgifera virgifera*) possess a single major proteinase with an apparent molecular mass of 25 kDa and several minor proteinases. Similar proteinase activity pH profiles were exhibited by root-fed and diet-fed rootworms with the optimal activity being slightly acidic. Rootworm larvae reared on corn roots exhibited significantly less caseinolytic activity than those reared on the artificial diet. Midgut proteolytic activity from SCR was most sensitive to inhibition by inhibitors of cysteine proteinases. Furthermore, rootworm proteinase activity was particularly sensitive to inhibition by a commercial protein preparation from potato tubers (PIN-II). One of the proteins, potato cysteine proteinase inhibitor-10′, PCPI-10′, obtained from PIN-II by ion-exchange chromatography, was the major source of inhibitory activity against rootworm proteinase activity. PCPI-10′ and E-64 were of comparable potency as inhibitors of southern corn rootworm proteinase activity (IC_{50} =31 and 35 nM, respectively) and substantially more effective than chicken egg white cystatin (IC_{50} =121 nM). Incorporation of PCPI-10′ into the diet of SCR larvae in feeding trials resulted in a significant increase in mortality and growth inhibition. We suggest that expression of inhibitors such as PCPI-10′ by transgenic corn plants in the field is a potentially attractive method of host plant resistance to these *Diabrotica* species.

Keywords: Midgut; Rootworm; Cysteine proteinase; Potato inhibitor; Engineered host plant resistance

1. Introduction

The southern corn rootworm, *Diabrotica undecimpunctata howardi*, infests the roots of many grass crops and weeds as well as those of peanuts, alfalfa, and occasionally cucurbits (Anonymous, 1998). It is one of the most damaging pests of corn and peanuts and is widely distributed, occurring in most areas east of the Rocky Mountains, in southern Canada and in Mexico, but it is most abundant and destructive in the southern US. The beetles are general feeders on at least 280 plant species, including most cultivated crops.

Both cultural and chemical methods are used to control rootworms. For example, early plowing and/or disking at least 30 days before planting corn removes vegetation and discourages egg-laying. Also, early planting

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and seeding rates near the maximum for the variety ensure a good stand. Finally, carrying out recommended agronomic practices leads to vigorous plants with improved tolerance. Pre-emergence granular insecticides can be used for the control of larvae, but it is not economically feasible to control larvae by spraying of the adults.

Cultivars resistant to insect attack offer another strategy for rootworm control. Although some success in attaining resistance to rootworms has been obtained through conventional breeding programs, more resistance germplasm is needed. Transgenic plant technology is now producing resistant crops by introducing novel resistance genes into plant species (Estruch et al., 1997; Hilder and Boulter, 1999). Genes encoding inhibitors of insect proteinases are one example of insecticidal protein genes that have been isolated from various sources and transferred to crops by genetic engineering (Gatehouse and Gatehouse, 1998; Michaud and Vrain, 1998; Reeck et al., 1998). A number of transgenic plants, including tobacco, potato, rice, strawberry, oilseed rape, and poplar have been produced, which express foreign protease inhibitors, some of which exhibit enhanced resistance to their insect pests. Expression of cystatins, a family of proteinaceous inhibitors of cysteine proteinases, in transgenic plants to increase host-plant resistance has only been marginally successful. For example, transgenic potato expressing rice cystatin inhibited larval growth and exhibited mortality of the Colorado potato beetle (Lecardonnel et al., 1999). However, growth compensation and faster development of the same species feeding on potato foliage expressing rice cystatin has also been observed (Cloutier et al., 1999, 2000). In two varieties of transgenic poplar expressing cysteine proteinase inhibitors from rice (Leple et al., 1995) and *Arabidopsis* (Delledonne et al., 2001), substantial levels of resistance to two chrysomelid beetle species were achieved.

Use of genes that encode foreign proteinase inhibitors in transgenic plants for host plant resistance to rootworms has not yet been reported. In an artificial diet, soyacystatin N, a soybean cysteine proteinase inhibitor, inhibited both western corn rootworm gut proteolysis and larval growth (Kiowa et al., 2000). Apparently, one or more cathepsin L-like cysteine proteinases of the papain superfamily, present in the rootworm gut, are the targets of this inhibitor.

As a further step in identifying potential inhibitors that might be used for transgenic crop protection against rootworms, we have determined some biochemical characteristics of the midgut proteolytic enzymes of southern and western corn rootworms, and also evaluated numerous potential protease inhibitors in vitro for inhibitory activity toward midgut proteinases in the southern corn rootworm. The most abundant or active proteinase is a cysteine proteinase with an apparent preference for peptide bonds involving arginine residues. From a heterogeneous, commercial preparation potato tuber proteins, we have purified and sequenced a particularly effective inhibitor of this major proteinase. This protein is a member of a previously well-characterized group of closely related inhibitors of cysteine proteinases (Yamagishi et al., 1991; Krizaj et al., 1993; Gruden et al., 1997) and based on sequence similarity, we have assigned the name potato cysteine proteinase inhibitor-10′ (PCIP-10′) to this protein.

2. Materials and methods

2.1. Materials

Casein, ^{14}C-methylated casein, fluorescein isothiocyanate (FITC)-casein, buffer salts, trichloroacetic acid (TCA), and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol and scintillation cocktail were obtained from Fisher (Pittsburgh, PA). Inhibitors were from Sigma or Calbiochem (San Diego, CA). Calbiochem was the source of the PIN-II inhibitor preparation from potato (catalog number 230907), which was used as the starting material for our purification of PCPI-10′. The bicinchoninic acid protein assay kit was obtained from Pierce (Rockford, IL). Recombinant oryzacystatin (rOC) was prepared by the method of Chen et al. (1992).

2.2. Purification of PCPI-10′

PIN-II, a heterogeneous protein preparation from potato purchased from Calbiochem, was fractionated to identify the major component that inhibited the major proteinase in rootworm extracts. The PIN-II preparation was dissolved in 25 mM sodium acetate, pH 5.5, and subjected to anion exchange chromatography on an HR 10/16 Sepharose FF column equilibrated with the same buffer. A gradient was developed from 0 to 0.5 M NaCl in sodium acetate, pH 5.5, over 170 min at a flow rate of 1 ml/min. Five distinct peaks of protein were eluted. The last peak to elute contained strong inhibitory activity and was essentially homogeneous as judged by SDS gel electrophoresis and capillary electrophoresis. It was subjected to amino acid sequence determination (see Section 2.5) and was named PCPI-10′ on the basis of its sequence.

2.3. Insects and gut extracts

Southern corn rootworm larvae were routinely reared using Stoneville artificial media as described by Czapla and Lang (1990). In two experiments (see Figs. 1 and 2), southern corn rootworm larvae were grown on roots of corn plants, as were larvae of the western corn rootworm. Midguts of the larvae were dissected into liquid

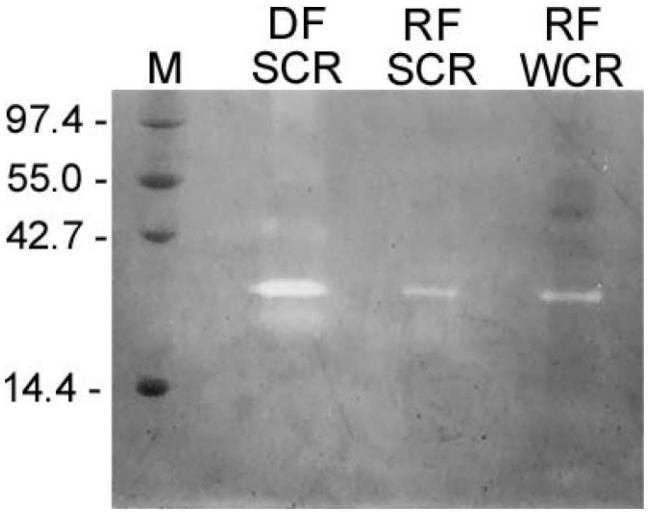

Fig. 1. Substrate-gel electrophoresis of caseinolytic activity in midgut extracts of, from left to right: artificial diet-fed southern corn rootworm (DF SCR); corn root-fed southern corn rootworm (RF SCR); corn root-fed western corn rootworm (RF WCR). Each sample-well contains approximately 10 μg of protein from each gut extract. Separation was conducted under denaturing/non-reducing conditions using 12% SDS-PAGE gels copolymerized with bovine gelatin. Following electrophoresis, the gel was allowed to incubate in a solution of Triton X-100 to remove SDS and allow subsequent recovery of proteolytic activity. Proteolysis of the gelatin substrate within the gel occurred during incubation in a pH 6.2 buffered solution. The dark blue background was obtained by staining the gel in a Coomassie Brilliant Blue R-250 solution and the clear bands of proteolytic activity were visualized following incubation in destaining solution. The apparent molecular masses of the proteolytic activity bands were estimated by comparison with the protein molecular weight marker (M).

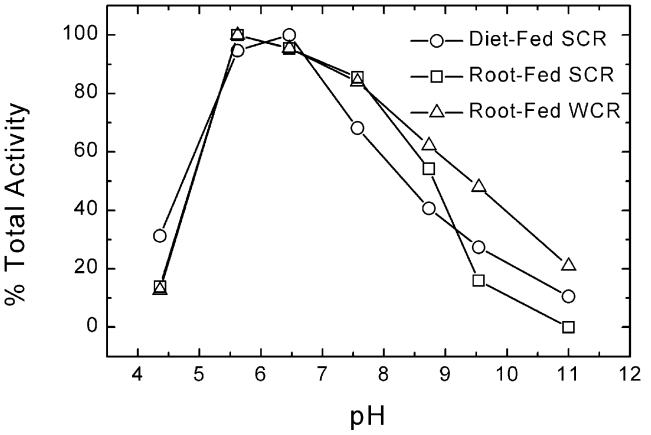

Fig. 2. pH dependence of the caseinolytic activity in midgut extracts of artificial diet-fed southern corn rootworm, corn root-fed southern corn rootworm, and corn root-fed western corn rootworm. In a total volume of 200 μl, 1 μg of diluted gut extract protein was incubated in universal buffer at pH 4.4, 5.6, 6.5, 7.6, 8.8, 9.5, or 11.0 with 25 mM DTT for 20 min at 25°C. The assays were initiated by addition of FITC-casein followed by incubation at 37°C. The reactions were stopped by the addition of TCA and allowed to further incubate at 37°C for 5 min. Unhydrolyzed FITC-casein was precipitated on ice for 15 min followed by centrifugation. Four-tenths ml of the supernatants was added to 1.6 ml of 0.5 mM Tris pH 8.8 and the fluorescence intensity was measured at an excitation wavelength of 492 nm and an emission wavelength of 525 nm.

nitrogen at Pioneer Hi-Bred International and shipped on dry ice to Kansas State University. The guts, approximately 50 mg tissue per ml, were homogenized in a cooled glass mortar with a teflon pestle, using 10–20 strokes, in chilled distilled water. The homogenate was centrifuged for 5 min at 10,000*g* at 4°C. The supernatant was divided into aliquots and stored at −80°C. Microscopic examination of the pellet revealed that the midgut cells were lysed by the homogenization procedure.

2.4. Protein concentration and assays for protease activity

Protein concentration in homogenates was determined using the Pierce BCA protein determination kit as directed by Pierce, using bovine serum albumin as a standard.

Proteolytic activity was determined using the procedure of Gillikin et al. (1992) with minor modifications as explained in Section 3. Aliquots of homogenate, inhibitors, and buffer were combined in a volume of 25 μl and 25 μl of the substrate was added. Between 0.08 and 0.15 μg of extract protein was used per assay. Substrate consisted of 0.5 μl of ^{14}C-casein (5 nCi/μl or 2.5 nCi/assay) or FITC-casein (2.5 μg), 0.6 μl of casein (5 mg/ml), 2.5 μl 20 mM DTT (or buffer), and 21.4 μl buffer. Universal buffer (Frugoni, 1957) was used for all assays. Final concentrations in the assay were ^{14}C-casein (50 nCi/ml), casein (60 μg/ml), and DTT (1 mM). The mixture of substrate and sample was incubated for 5 min at 37°C. The reaction was stopped by adding 50 μl of 20% TCA. The mixtures were placed on ice for 15 min and then centrifuged for 5 min at 13,000*g* at room temperature. The supernatant (75 μl) was added to 4 ml of scintillation fluid, vortexed for 10 s, and counted in a Packard 1600CA scintillation analyzer. One unit of activity was defined as the amount that would liberate 1% of the radioactivity in a sample in 1 min. Specific activities are expressed as units/μg protein.

Proteolytic activity using FITC-casein was monitored following the method adapted from Orr et al. (1994). About 1 μg of gut extract was allowed to incubate in universal buffer pH 6.2 containing 25 mM DTT for 20 min at 25°C. The reaction was then started by adding 5 μg of FITC-casein to each sample. The samples were allowed to incubate at 37°C for 30 min. The reactions were stopped by adding TCA to a final concentration of 3.5%. The quenched samples were allowed to further incubate at 37°C for another 5 min. The unhydrolyzed FITC-casein was precipitated on ice for 15 min followed by centrifugation at 10,000*g* for 5 min. Then 400 μl of the supernatants were added to 1.6 ml of 0.5 M Tris pH 8.8 in a four-sided cuvette and the fluorescence intensity was determined using an excitation wavelength of

492 nm and an emission wavelength of 525 nm in a Hitachi F-4010 Fluorescence Spectrophotometer.

Three fluorescent peptides were also used as substrates for analysis of proteolytic activity using a method adapted from Sasaki et al. (1984). The three synthetic peptide substrates were *N-t*-BOC-Gln-Ala-Arg-7-amido-4-methylcoumarin, *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin, and *N*-succinyl-Ala-Pro-Ala-7-amido-4-methylcoumarin (Sigma, St. Louis, MO). Prior to addition of the substrate, 5 μl of gut extract was incubated in 10 mM DTT for 5 min at 25°C. Then 1 μl of the enzyme mixture was pipetted into a four-sided cuvette with 1.985 ml of universal buffer pH 6.2 and 0.5 ml of 25 mM DTT. This mixture was allowed to incubate for 10 min at 25°C. The reaction was then started by adding 10.0 μl of 2 mM substrate in 40% DMSO with immediate mixing in the cuvette. The fluorescence intensity was determined using an excitation wavelength of 380 nm and an emission wavelength of 440 nm for 30 min with data collected every 5 min.

2.5. Amino acid sequence determination of PCPI-10′

The inhibitor, purified by ion-exchange chromatography as described above, was subjected to amino acid sequencing by Edman degradation at the Protein Structure Laboratory of Iowa State University. This was accomplished using a combination of enzymatic and chemical cleavage methods resulting in peptide fragments. These peptide fragments were purified by reversed phase chromatography. An explanation of the peptide mapping is given in the legend to Fig. 6.

2.6. Inhibitor assay and pH dependence of the protease activity

Potential inhibitors were incubated for 5 min with the extract before adding the substrate. The compounds and proteins as well as their sources and concentration in the assays are shown in Table 1. Potential inhibitors were dissolved in water, ethanol, or 1:1 water/ethanol, as appropriate, at 20 times the assay concentration, and 2.5 μl were used per 50 μl assay. For dose-response assays (shown in Fig. 7), varying concentrations of inhibitor were incubated with the extract for 5 min prior to assay. IC_{50} values were estimated graphically from the plots of percent inhibition versus inhibitor concentration.

The gut extract was assayed at pH 4.4, 5.6, 6.5, 7.6, 8.8, 9.5 and 11.0 in universal buffer at a constant ionic strength of 0.1 μ (Frugoni, 1957). The standard assay used 0.4 μg protein in a 30 min assay.

2.7. Zymogram analysis

Proteolytic activity was visualized by modification of the procedure used by Heussen and Dowdle (1980) and Gillikin et al. (1992), in which 12% SDS-polyacrylamide gels containing 0.3% gelatin were used. About 10 μl of gut extract were diluted 1:1 with SDS-PAGE sample buffer [4.6% SDS, 20% glycerol, 132 mM Tris–HCl (pH 6.8), 0.1% bromophenol blue]. Then 10 μl samples (0.05 gut equivalents) were loaded directly into wells of the stacking gel. Ten microliters of 1:10 diluted Mid-Range Protein Molecular Weight Markers (Promega, Madison, WI) were loaded to estimate the electrophoretic mobility and apparent molecular masses of the proteolytic activity bands. Electrophoresis was conducted at a constant current of 12 mA at 25°C for approximately 1 h. Following electrophoresis, the gel was placed in 2.5% Triton X-100 (w/v) in water for at least 2 h with shaking to remove SDS. Following the Triton wash, the gel was washed with water until no detergent foaming was visible. The gel was then placed into universal buffer, pH 6.2 for approximately 12 h at 25°C. The gel was placed into a stain solution (50% methanol and 12% acetic acid containing 0.5% Coomassie Brilliant Blue R-250) until a dark-blue background developed. Visualization of the clear proteolytic bands was achieved by placing the gel into destaining solution (20% methanol and 10% acetic

Table 1
Concentrations of inhibitors used in protease assays (source of inhibitor, if not obtained from Sigma, given in parentheses, as are standard abbreviations)

Inhibitor	Assay concentration (mM)
E-64[a]	0.020
Egg white cystatin	0.001
Recombinant oryzacystatin (rOC)	0.002
Calpain inhibitor II (Calbiochem)	0.026
Human calpastatin (Calbiochem)	0.004
Antipain	0.132
Leupeptin	0.152
Phenyl methyl sulfonyl fluoride (PMSF)	4.5
Diisopropyl fluoronyl phosphate (DFP)	1.0
Tosyl-L-lysine chloromethyl ketone (TLCK)	0.030
Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	0.030
Bovine pancreatic trypsin inhibitor (BPTI)	0.0001
Soybean Bowman–Birk trypsin inhibitor	0.0001
Soybean Kunitz trypsin inhibitor (STI)	0.00005
PIN-I preparation, potato (Calbiochem)	0.002
PIN-II preparation, potato (Calbiochem)	0.00075
Chymostatin (Calbiochem)	0.124
Elastase inhibitor (Calbiochem)	0.0002
Elastatinal (Calbiochem)	0.098
Carboxypeptidase inhibitor (Calbiochem)	0.018
Amastatin	0.158
EDTA	10.0
Iodoacetic acid (IAA)	2.0
Potato cystatin	0.001
Rice bran trypsin inhibitor (RBTI)	0.0005

[a] L-*trans*-Epoxysuccinyl-leucylamino-4-guanidinobutane, an irreversible inhibitor.

acid) for 1 h with shaking. Gel-scan analysis was performed on a Macintosh computer using the public domain NIH Image program developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image. Six separate scanning measurements were made for each sample lane to obtain an average integrated density for the major proteinase band/peak.

2.8. Adsorption of protease to inhibitor-bound Sepharose beads

Cystatin, rOC, and the commercial PIN-II preparation were covalently bound to Affi-Gel 102 (Bio Rad, Hercules, CA) beads using the coupling method provided by the manufacturer. Then 100 μl of the Affi-Gel 102 beads were rinsed with 25 mM MES pH 6.0 until a pH of 6.0 was achieved. Rinsed beads were centrifuged at low speed and the supernatant was discarded. Three-fourths ml of 25 mM MES pH 6.0 and the following protein amounts were added to the beads with gentle stirring: 1.0, 1.8, and 2.0 mg of cystatin, PIN-II preparation, and rOC, respectively. The pH was adjusted to 4.7–5.0 with 1 N HCl. About 3 mg of the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling reagent was added to the beads with continuous stirring. The pH was again adjusted to 4.7–5.0 with 1 N HCl and the coupling reaction was allowed to proceed overnight at 4°C with gentle end-over-end stirring. Inhibitor-bound beads, 100 μl, were allowed to incubate with 10 μl of extract for 30 min at 25°C with gentle stirring. Following the incubation period, the slurry was centrifuged for 10 min at 10,000*g*. The supernatants were collected and assayed for proteolytic activity using SDS-PAGE zymogram analysis as outlined above.

2.9. Bioassays

The purified PCPI-10′ as well as the proteins of an unfractionated aqueous extract of potato were tested for growth inhibitory activity against the southern corn rootworm. Bioassay was performed using the Stoneville diet that was prepared in the standard fashion and allowed to cool to 38°C. Lyophilized inhibitor preparations were solubilized in PBS at pH 7.4 and vortexed thoroughly. Following sufficient cooling, 0.4 ml of the test compound or substance was added to 1.6 ml diet in solution and mixed vigorously. Negative controls were similarly prepared and consisted of PBS buffer pH 7.4 applied to the diet. The diet was then dispensed into 14-well acrylic microtrays and allowed to cool and solidify for several hours. Each well with solid diet containing the test compound or substance was then infested with a newly hatched southern corn rootworm larvae and covered with vented mylar strips to prevent escape while allowing proper air exchange. Fourteen larvae were used in each of the two replicates of each treatment. The larvae were incubated on the diet for seven days at 28°C and 60% relative humidity. Mortality and weights of surviving insects were recorded. Significant differences between diet treatments were determined using the Tukey HSD multiple comparison test from the software program SYSTAT (Wilkinson, 1989).

3. Results

3.1. Extraction and stability of proteinase activity

Because much of the proteolytic activity in midgut extracts of corn rootworms is apparently from cysteine proteinases (Gillikin et al., 1992 and see later), we included a reducing agent (1 mM dithiothreitol) in all enzyme assays. In addition we examined the potential for enhanced release of proteolytic activity by including Triton X-100 in the extraction buffer. This nonionic detergent has been successfully used to enhance the extraction of proteolytic activity from other insect tissues, presumably by releasing membrane-bound proteases. After an extraction using our conditions but lacking Triton-X-100, we carried out a second extraction of the tissue residue under the same conditions that included Triton X-100 at 0.1% (v/v). The second extract had only about 5% of the caseinolytic activity of the first extract. We concluded that approximately 95% of the caseinolytic activity in rootworm midguts was soluble and that only as much as 5% was membrane bound. We therefore omitted Triton-X-100 from subsequent extractions and utilized the soluble activity for subsequent analyses.

Extracts made under our standard conditions could be stored at −70°C for several weeks without loss in caseinolytic activity or change in the pattern of protease bands observed after substrate-containing polyacrylamide gel electrophoresis (data not shown).

3.2. Substrate-gel electrophoretic analysis of proteases in midgut extracts

Fig. 1 shows the results of a gelatin substrate-gel electrophoretic analysis of gut extracts from laboratory diet-fed and corn root-fed southern corn rootworms and corn root-fed western corn rootworms. All three of the extracts exhibited only one major gelatin-digesting protease that has an apparent molecular mass of approximately 25 kDa. Numerous less intense bands of proteolysis were also visible, with the laboratory diet-fed southern corn rootworms displaying the highest levels. The apparent molecular masses of the minor protease-activity bands were about 20, 40, 70, and >100 kDa.

When we stained gels with Coomassie brilliant blue, more than 20 proteins bands were visualized in gels of

midgut extracts from laboratory diet-fed and corn root-fed southern corn rootworms and laboratory diet-fed western corn rootworms (data not shown). These included several protein bands in the 25 kDa region of the gel.

3.3. Effect of pH on caseinolytic activity

Two types of modified casein were used as substrates to examine the effect of pH on proteolytic activity. Fig. 2 shows that the pH activity profiles for gut extracts from laboratory diet-fed and corn root-fed southern corn rootworms and corn root-fed western corn rootworms were comparable, with optimum pH values of 5.5–6.5 when FITC-casein was used as the substrate. A similar profile was obtained when gut extracts from laboratory diet-fed southern corn rootworms were assayed using radiolabeled casein as the substrate (data not shown). Similarly, Gillikin et al. (1992) found the pH optimum for *Diabrotica virgifera* against a similar substrate to be between pH 5 and 6.

3.4. Substrate specificity

The time course for hydrolysis of a low molecular weight fluorescent peptide, *N*-*t*-BOC-Gln-Ala-Arg-7-amido-4-methylcoumarin (P1-Arg substrate), by the three midgut extracts is shown in Fig. 3. Using the same midgut equivalents, relative hydrolytic activities for laboratory diet-fed southern corn rootworms, corn root-fed western corn rootworms, and corn root-fed southern corn rootworms were 1.0/0.2/0.1. Midgut extracts from the laboratory diet-fed southern corn rootworms were used for further analyses because this preparation exhibited the highest activity, all of the gut extracts from laboratory diet-fed and root-fed insects were similar in both electrophoretic patterns and pH activity profiles, and the laboratory diet-fed southern corn rootworms were most readily available as a tissue source.

Two other fluorescent peptides, *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (P1-Tyr substrate) and *N*-succinyl-Ala-Pro-Ala-7-amido-4-methylcoumarin (P1-Ala substrate), were used to further examine the substrate specificities of the southern corn rootworm proteases. Fig. 3 shows the time course for hydrolysis of the P1-Arg substrate and, for comparison, those of the P1-Ala and P1-Tyr peptides. While the P1-Arg compound was a good substrate for the midgut enzymes, little hydrolysis occurred with the latter two substrates.

3.5. Effects of potential inhibitors

About 25 potential inhibitors of southern corn rootworm proteolytic activity were surveyed using ^{14}C-casein as the substrate (Fig. 4). The most inhibitory were a mixture of E-64 with PMSF (95%) or antipain (94%).

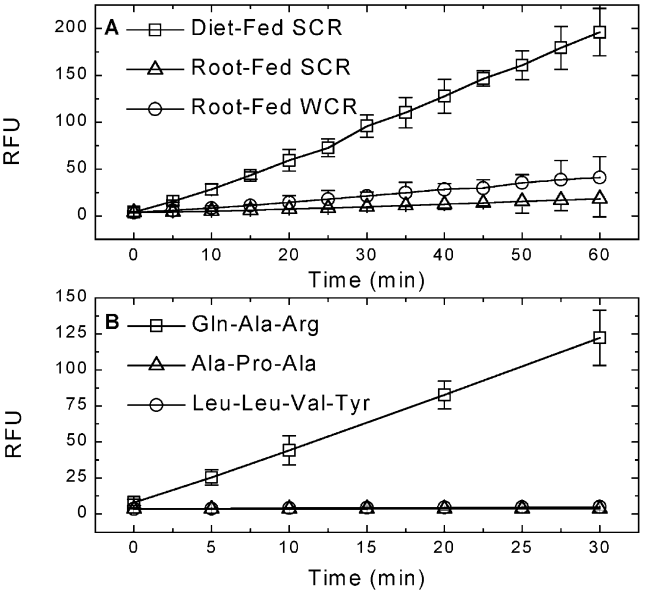

Fig. 3. Hydrolysis of P1-Arg-fluorescent substrate by midgut extracts of artificial diet-fed southern corn rootworm, corn root-fed southern corn rootworm, and corn root-fed western corn rootworm. (A) Gut extract protein (2.5 μg) from diet-fed southern corn rootworm, root-fed southern corn rootworm, and root-fed western corn rootworm were assayed against the fluorescent substrate *N*-*t*-BOC-Gln-Ala-Arg-7-amido-4-methylcoumarin in pH 6.2 Universal buffer with 10 mM DTT. (B) Diet-fed southern corn rootworm gut extract protein (2.5 μg) was assayed against *N*-*t*-BOC-Gln-Ala-Arg-7-amido-4-methylcoumarin, *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin, and *N*-succinyl-Ala-Pro-Ala-7-amido-4-methylcoumarin in pH 6.2 universal buffer containing 10 mM DTT for 30 min at 25°C. All values were corrected for spontaneous hydrolysis by running identical samples without enzyme. Samples were assayed in duplicate with mean relative fluorescence units and their corresponding 0.5 range values given.

These were followed in effectiveness by calpain inhibitor (90%), chymostatin (90%), a mixture of PMSF and DFP (85%), the commercial PIN-II preparation (80%), a commercial PIN-I preparation (78%), PMSF (75%), potato cystatin (74%), E-64 (71%), hen's egg white cystatin (68%), and recombinant oryzacystatin (64%). Other materials ranged from 0–60% in inhibitory activity. Based on these data, it appeared that most of the proteolytic activity in the rootworm midgut was due to a mixture of both cysteine and serine proteinases with the former type being more predominant.

3.6. Identification of a PCPI-10′ as a strong inhibitor of rootworm proteinases

As described in Section 2, PIN-II, a commercially available, heterogeneous preparation from potato tubers was fractionated by ion-exchange chromatography. The last of the five major peaks of protein to elute was a potent inhibitor of proteinase activity in rootworm gut extracts (Fig. 5). Amino acid sequence determination of the protein in this peak (Fig. 6) revealed a sequence that is very similar to previously sequenced inhibitors from

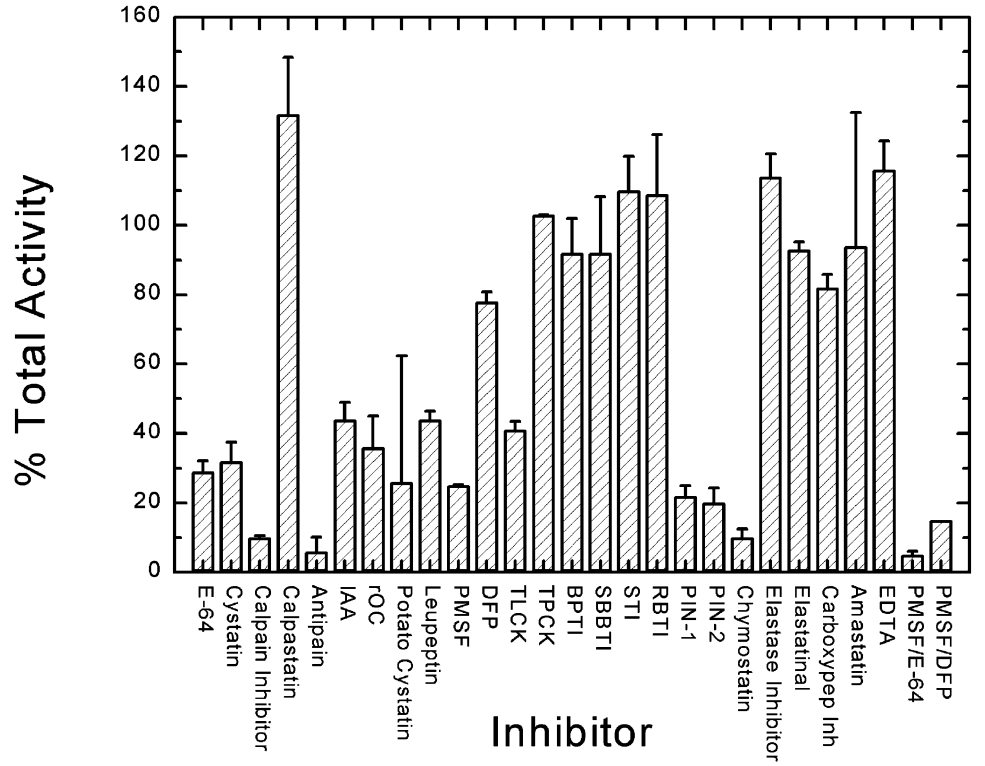

Fig. 4. Effects of 25 potential inhibitors on the caseinolytic activity in midgut extracts of artificial diet-fed southern corn rootworm. See Table 1 for abbreviations, source, and concentrations of potential inhibitors. Gut extract protein from diet-fed southern corn rootworm was assayed for ^{14}C-casein hydrolysis in Frugoni's universal buffer, pH 6.2 in the presence or absence of 25 potential inhibitors. Activity is expressed as percent inhibition of total activity without inhibitor. All samples were run in duplicate and corrected for spontaneous hydrolysis by running identical samples without enzyme. Error bars represent 0.5 range values for duplicate samples.

potato (for the first of these, see Yamagishi et al., 1991; Krizaj et al., 1993). Numerous sequences are now known for proteins in this family or variants of this protein. The sequence of the inhibitor as purified from the commercial PIN-II preparation is most similar to PCPI-10 (Gruden et al., 1997) and hence we have called our protein PCPI-10′. PCPI-10′ is identical at 169 of the 179 corresponding positions in the translated amino acid sequence of PCPI-10 (Gruden et al., 1997). The molecular mass and pI of PCPI-10′ are 20,162 Da and pH 8.62 for the form of PCPI-10′ with Arg at position 131, where Pro is also found.

3.7. Comparison of inhibitor–titration curves

Fig. 7 shows dose–response curves for three of the proteinase inhibitors, E-64, hen's egg white cystatin, and our purified PCPI-10′, on southern corn rootworm proteolytic activity using ^{14}C-casein as the substrate. The low-molecular-weight epoxy irreversible inhibitor, E-64, eliminated approximately 80% of the activity at about 1 μM concentration. Hen's egg cystatin was approximately four-fold less effective on a molar basis as an inhibitor. PCPI-10′ was the most potent, with an IC_{50} of 31 nM, followed the irreversible inhibitor, E-64 (35 nM), and chicken egg white cystatin (121 nM).

3.8. Selective batch-adsorption of major proteinase in southern corn rootworm gut extract

Three proteinaceous inhibitors, the commercial PIN-II preparation (containing PCPI-10′), recombinant oryzacystatin, and hen's egg cystatin, were covalently attached to AffiGel 107 beads to determine whether they might be used for selective adsorption of the proteases in southern corn rootworm midgut extracts. Fig. 8 shows a densitometric gel scan analysis of a zymogram and demonstrates that, under these conditions, a component of PIN-II, presumably PCPI-10′, selectively adsorbed the 25 kDa proteinase from the gut extract, whereas hen's egg cystatin and rOC adsorbed little or none of the proteinase. Apparently, only the enzyme complex with PCPI-10′ was tight enough for effective adsorption from solution.

3.9. Feeding studies using PCPI-10′

Incorporation of PCPI-10′ into artificial diets for southern corn rootworm showed marked effects on mortality and weight loss (Table 2). Rootworm larvae reared on diet containing 500-, 750-, and 1000-ppm PCPI-10′ exhibited significantly higher mortality ($p<0.030$) and weight loss ($p<0.000$) compared to larvae reared on

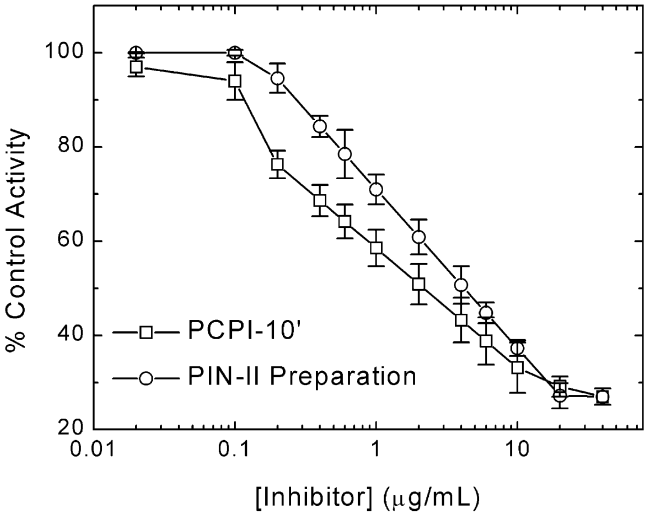

Fig. 5. Inhibitory potencies of the potato PIN-II preparation and a purified component, PCPI-10′, of that preparation. The dose effectiveness of the crude PIN-II preparation and a purified component of PIN-II, PCPI-10′, as inhibitors against artificial diet-fed southern corn rootworm gut proteolytic activity was monitored using FITC-labeled casein as a substrate. Then 1 μg of gut extract protein was incubated with appropriately diluted PIN-II or PCPI-10′ in pH 6.2 Universal buffer containing DTT at a final concentration of 12.5 mM for 15 min at 25°C. Final inhibitor concentrations were 0.02, 0.1, 0.2, 0.4, 0.6, 1, 2, 4, 6, 10, 20, and 40 μg/ml. The reactions were initiated by the addition of 25 μg FITC-casein to the reaction mixture. Reactions were carried out for 30 min at 37°C. The reactions were stopped by the addition of TCA to precipitate unhydrolyzed FITC-casein and assayed for proteolytic activity as outlined in Section 2. Control activity was determined by running simultaneous reactions without inhibitor. All samples were run in duplicate and corrected for spontaneous hydrolysis by running identical samples without enzyme. Error bars represent 0.5 range values for duplicate samples.

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1   LVLPEVYDQD GNPLRIGERY IIKNPLLGAG   30
31  AVYLDNIGNL QCPNAVLQHM SIPQFLGDGT   60
61  PVVFVRKSES DYGDVVRLMT AVYIKFFVKT   90
91  TKLCVDQTVW KVNDEQLVVT GGKVGNENDI  120
121 FKIRKIDLVT RGMKNVYKLL HCPSHLQCKN  150
151 IGSNFKNGYP RLVTVNDDKD FIPFVFIKA   179
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Fig. 6. Amino acid sequence of the inhibitory component of PCPI-10′. The following regions of the sequence of the indicated fragments were sequenced (numbers refer to the positions in the protein, not in the fragments): 1–49, the intact protein; 1–20, a GluC fragment; 25–53, a LysC fragment; 51–67, a cyanogen bromide fragment; 59–66, a GluC fragment; 64–71, a secondary fragment released by CNBr cleavage; 68–84, a GluC fragment; 80–93, a cyanogen bromide fragment; 92–101, a LysC fragment; 101–137, a BNPS-skatole fragment; 123–131, a LysC fragment; 132–161, an ArgC fragment; 133–169, a cyanogen bromide fragment; 138–156, a LysC fragment; 157–170, a LysC fragment; 171–179, a LysC fragment. At position 131, proline is found in addition to Arg. An exhaustive set of overlaps was not obtained since the sequence exhibited such high sequence similarity to PCPI-10 and other members of that family. Differences with the PCPI-10 sequence are at positions 58 (D in PCPI-10′/K in PCPI-10), 65 (V/I), 97(Q/E), 104(D/N), 113(K/N), 124(R/K), 126(I/T), 130(T/I), 147(Q/E), and 168(D/E). The predicted molecular weight of the sequence we determined (20,182) agrees well with the molecular weight of the protein itself as determined by mass spectroscopy (20,130).

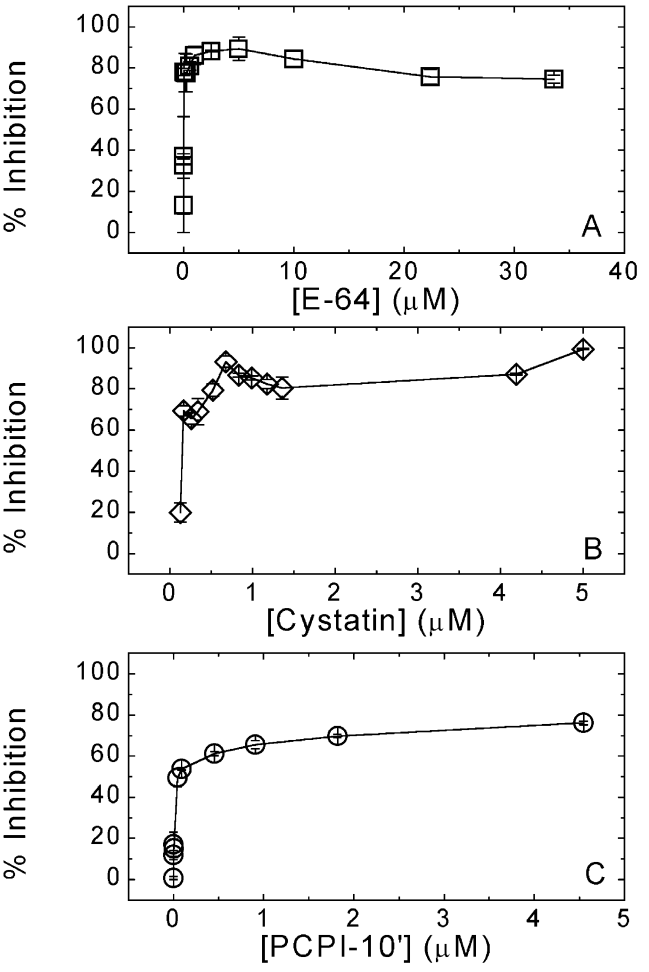

Fig. 7. Titration of ^{14}C-casein hydrolyzing activity in midgut extracts of artificial diet-fed southern corn rootworm by E-64 (A), chicken egg-white cystatin (B), and PCPI-10′ (C). About 80–150 ng of total gut extract protein was allowed to incubate with different inhibitor concentrations at 25°C for 5 min before adding the ^{14}C-casein substrate. Assays were conducted as previously outlined in Section 2. The concentration range of inhibitors is as follows: 0–33.6 μM E-64, 0–5 μM hen's egg cystatin, and 0–4.5 μM PCPI-10′ from this study. All samples were run in duplicate and corrected for spontaneous hydrolysis. Error bars represent 0.5 range values for duplicate samples.

control media. PCPI-10′ was considerably more potent on a mass basis than an unfractionated protein extract from potato, an effect most evident at the lowest levels examined in the diet (500 ppm, p=0.002). The protein extract fraction, which was also assayed and was more complex in composition than PIN-II, exhibited a somewhat unexpectedly high potency in this assay and may contain inhibitors other than PCPI-10′.

4. Discussion

Midgut proteinases are potentially good targets for genetically engineered enhanced resistance of plant species towards insects (for reviews see Jongsma et al.,

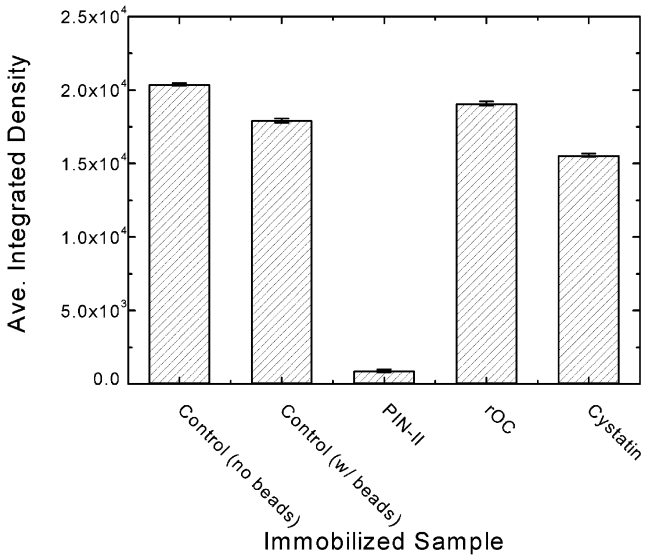

Fig. 8. Depletion of proteolytic activity in southern corn rootworm gut extracts by immobilized PIN-II, recombinant oryzacystatin and hen's egg cystatin. Then 10 μg of diet-fed southern corn rootworm gut extract was allowed to incubate with the inhibitor-bound beads for 30 min at 25°C. Unbound proteins were collected by centrifugation of resin. About 10 μl of the supernatants was diluted in SDS sample buffer and loaded onto a 12% SDS-PAGE zymogram gel. Electrophoresis and development of activity bands was conducted as outlined in Section 2. Each bar represents the mean of six gel scans.

Table 2
Effect of potato extract and PCPI-10′ on southern corn rootworm growth and mortality

Additive	Treatment level (ppm)	Mortality (%)[a]	Weight loss (%)[a]
PCPI-10′	1000	57.0±0.0 a	86.0±3.0 a
	750	39.5±10.5 a,b	78.0±5.0 a,b
	500	28.5±7.5 b,c,d	61.5±0.5 b,c
Protein extract	1000	43.0±0.0 a,c	67.0±1.0 a,c
	750	17.5±3.5 b,d,e	62.0±8.0 b,c
	500	14.0±0.0 d,e	32.0±1.5 d
Buffer control	–	3.5±3.5 e	19.0±2.0 d

[a] Larval mortality and weight loss determined seven days after placement on test diets. Mean values±SE based on two replicates of n=14 insects per treatment. Data with the same letter were not significantly different (α=0.05) as determined by the Tukey HSD multiple comparison test.

1997; Reeck et al., 1998; Gatehouse and Gatehouse, 1998). Insects typically contain complex mixtures of classes and families of enzymes in their midguts. A well-informed attempt at genetic engineering of plant-resistance would include, as a first step, the identification of major proteases in the insect of interest and of inhibitors of them. It is with that perspective in mind that we have undertaken studies of midgut proteinases of the southern and western corn rootworms.

Murdock et al. (1987) first demonstrated the presence of cysteine proteinase activity in the southern corn rootworm. Subsequently, two other groups evaluated the potency of several kinds of proteinase inhibitors towards the midgut fluid proteinases from the southern (Purcell et al., 1992) and western (Gillikin et al., 1992) corn rootworms and identified inhibitors of cysteine proteinases (particularly E-64 and cystatins) as the most potent inhibitors. Edmonds et al. (1996) showed that not only E-64 but also chicken egg-white cystatin and oryzacystatin were strong inhibitors in vitro, and that the latter protein, when incorporated into an artificial diet at concentrations of ≥10 mM, caused significant decreases in larval survival and weight gain of *D. undecimpunctata*. Zhao et al. (1996) reported that the two wound-inducible soybean cysteine proteinase inhibitors were more effective in vitro against gut cysteine proteinases of western corn rootworms than either a constitutive homologue or E-64. Our data are consistent with those earlier results in that we too have found that cysteine proteinase activity is the major class of proteolytic activity in southern corn rootworm midgut extracts. In our case, much of that activity appears to reside in a single enzyme and that enzyme has specificity for positively charged side chains in its substrates. Beyond that, we have identified a particularly strong inhibitor of this cysteine proteinase in the form of a potato protein, PCPI-10′.

In the work reported here, we have found that a single proteinase or at least a single proteinase activity band is responsible for most of the protease activity against casein and a synthetic substrate that has arginine at the P1 position. This proteinase has an apparent mass of about 25 kDa. Based on the compounds and proteins that inhibit it, the enzyme can be classified as a cysteine proteinase with a preference for basic amino acid residues at the P1 site. It would appear to be a good target for genetically engineered increased resistance in corn towards attack by corn rootworms.

We have also identified a rather potent inhibitor of this enzyme. This inhibitor from potato has been purified and its amino acid sequence determined. It is a member of a closely related group of inhibitors called PCPI-10′. There are several members of this family or variants of the protein, and we have worked with only the variant or form as isolated from a commercial preparation of the potato proteins. The form we have isolated, which we have called PCPI-10′ based on its strong similarity in sequence to PCPI-10, is a good candidate to be used for enhancing the resistance of corn to rootworm attack.

Besides its very strong sequence similarity to other PCPIs, the PCPI-10′ sequence also exhibits sequence similarity to several functionally distinct inhibitors (Krizaj et al., 1993; Gruden et al., 1997) and to storage proteins. Homology (co-ancestry) with these proteins is a reasonable postulate or presumption, and it can be assumed that PCPI-10′ has a three-dimensional structure similar to two of these presumed homologues, Kunitz soybean trypsin inhibitor (Song and Suh, 1998) and a

bifunctional α-amylase/subtilisin inhibitor from barley seeds (Vallee et al., 1998), for which three-dimensional structures are available. Other homologues, including cathepsin D (an aspartyl proteinase) inhibitors from potato (Maganja et al., 1992) and sporamin, a storage protein that accounts for up to 90% of the protein mass in potato (Hattori and Nakamura, 1988), can similarly be presumed to adopt a similar folding pattern. It is quite remarkable that a single protein architecture or scaffold has found application in the course of evolution as both a storage protein and as an inhibitor of no less than five different families of hydrolytic enzymes (two families of serine proteinases as well as cysteinyl proteinases, aspartyl proteinases and α-amylases). The binding sites on the scaffold for α-amylase and subtilisin are, not surprisingly, distinct (Vallee et al., 1998). It will be most interesting to extend that sort of comparison to the binding sites on the scaffold for both aspartyl proteinases and cysteinyl proteinases. Given the rather different catalytic mechanisms of these enzymes, it would not be surprising for their inhibitors' binding sites to be at still different locations on the scaffold although that is by no means necessary. But even with what is known at this point, it appears that nature has been very resourceful in developing a variety of functions based on this single protein fold or scaffold.

Many other coleopteran species are also known to utilize the cysteine proteases rather than serine enzymes as the major class of midgut proteinase activity (Murdock et al., 1987; Thie and Houseman, 1990; Michaud et al., 1996). The majority of gut proteolytic activity for southern corn rootworm second instar larvae appears to be cysteine proteases (Orr et al., 1994). Both proteinaceous and nonproteinaceous inhibitors of cysteine proteinase retard the growth and development of several coleopterans, including rootworm species (Murdock et al., 1987; Hines et al., 1990; Liang et al., 1991; Oppert et al., 1993; Orr et al., 1994). Our results are consistent with these earlier studies and begin to more thoroughly characterize perhaps the major individual cysteine proteinase and a potent proteinaceous inhibitor of it.

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