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A Kunitz-Type Inhibitor of Coleopteran Proteases, Isolated from *Adenanthera pavonina* L. Seeds and Its Effect on *Callosobruchus maculatus*

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The cowpea weevil *Callosobruchus maculatus* is one of the major pests of *Vigna unguiculata* cowpea. Digestion in the cowpea weevil is facilitated by high levels of cysteine and aspartic acid proteinases. Plants synthesize a variety of molecules, including proteinaceous proteinase inhibitors, to defend themselves against attack by insects. In this work, a trypsin inhibitor (*ApTI*) isolated from *Adenanthera pavonina* seeds showed activity against papain. The inhibition of papain by *ApTI* was of the noncompetitive type, with a K_i of 1 μM. *ApTI* was highly effective against digestive proteinases from *C. maculatus*, *Acanthoscelides obtectus* (bean weevil), and *Zabrotes subfasciatus* (Mexican bean weevil) and was moderately active against midgut proteinases from the boll weevil *Anthonomus grandis* and the mealworm *Tenebrio molitor*. In *C. maculates* fed an artificial diet containing 0.25% and 0.5% *ApTI* (w/w), the latter concentration caused 50% mortality and reduced larval weight gain by approximately 40%. The action of *ApTI* on *C. maculatus* larvae may involve the inhibition of *ApTI*-sensitive cysteine proteinases and binding to chitin components of the peritrophic membrane (or equivalent structures) in the weevil midgut.

KEYWORDS: ***Adenanthera pavonina*; *Callosobruchus maculatus*; Kunitz inhibitor; phytocystatin; pest proteases.**

1. INTRODUCTION

The seeds of the family Leguminosae contain high amounts of protein, a small proportion of which consists of inhibitors that suppress proteolytic activities in vivo and in vitro by forming stable stoichiometric complexes (*1*). Of these, serine proteinase inhibitors are the most studied and have been isolated from various Leguminosae seeds (*2*−*4*). Legume seeds contain various proteinase inhibitors (PIs) classified as Kunitz-type, Bowman-Birk-type, potato I, potato II, squash, cereal superfamily, thaumatin-like, and Ragi A1 inhibitors (*1*).

PIs from a variety of plant and animal sources reduce the growth and survival of a range of insects when added to their diet (*2*, *5*). Cysteine proteinases account for most of the proteolytic activity in the gut of many coleopteran species (*6*, *7*), whereas many lepidopterans have serine proteinases as the major digestive enzymes (*8*).

The transformation of plant genomes with PI genes is an attractive approach for the biological control of insect pests (*7*, *9*, *10*), and much research has focused on exploiting this defense mechanism for crop protection. When serine- and cysteine-proteinase inhibitors are included artificial diets fed to Lepidoptera and Coleoptera, respectively, a variety of deleterious affects are observed, including reduced fecundity and oviposition, decreased weight gain, increased mortality, and severe deformations (*11*−*13*). The ability of PIs to interfere with the growth and development of insects has been attributed to their capacity to bind to and inhibit the action of insect digestive proteinases (*14*). For this reason, the incorporation of genes encoding cysteine proteinase inhibitors into transgenic grain has been proposed as a method for preventing seed damage by coleopteran pests, while presenting few or no side effects in vertebrates (*15*). Insects may overcome the effect of PIs by proteolytically inactivating these inhibitors (*16*) or by expressing inhibitor-insensitive proteases (*17*).

Plant seeds contribute significantly to human and animal diets, with cereal/legume seeds being a major part of the human diet. Legumes, which include beans, are major crops in many tropical countries and serve as important sources of carbohydrates and proteins for poor populations in these regions (*18*). Substantial amounts of stored beans, such as the common bean (*Phaseolus*

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vulgaris) and cowpea (*Vigna unguiculata*), are lost through damage caused by three main storage pests, the bean weevil *Acanthoscelides obtectus* (Say), the Mexican bean weevil *Zabrotes subfasciatus* (Boh.), and the cowpea weevil *Callosobruchus maculatus* (F.). In periods of severe infestation, postharvest seed losses caused by bruchids can reach 100% within a period of 6 months. Controlling these Bruchidae generally requires the use of chemical insecticides that are toxic to humans and domestic animals and harmful to the environment (*19*).

Bruchids use mainly cysteine proteinases to digest proteins (*20*, *21*). Plant cysteine proteinase inhibitors (phytocystatins) have been implicated as defensive molecules against coleopteran and hemipteran insect pests. The defensive role of cystatins has been attributed to their inhibitory activity toward exogenous proteases from coleopteran insect pests and nematodes (*22*). Indeed, plant cystatins decrease coleopteran digestive cysteine proteinase activity in vitro (*23*, *24*). The expression of cystatins in transgenic plants to increase host-plant resistance has been successful. Thus, expression of rice cystatin in transgenic potatoes inhibited larval growth and caused death in the Colorado potato beetle (*7*).

Oliveira et al. (*25*) purified a Kunitz-type inhibitor from the seeds of *Prosopis juliflora* D. C. that was active against papain and showed a high homology (88%) with the Kunitz-trypsin inhibitor from *Adenanthera pavonina* seeds (*1*, *26*). In the present report, we examined the activity of the inhibitor from *A. pavonina* seeds against papain and against papain-like proteinases obtained from the larvae of *C. maculatus*, *Z. subfasciatus*, *A. obectus*, *Tenebrio molitor* (L.), and *Anthonomus grandis* (Boheman). We also examined the effect of this inhibitor on the development and survival of *C. maculatus* larvae.

2. MATERIALS AND METHODS

2.1. Materials. Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The following abbreviations are used: *ApTI*, *Adenanthera pavonina* trypsin inhibitor; EDTA, ethylenediamine tetraacetic acid; TCA, trichloroacetic acid; BAPNA, *N*-benzoyl-DL-arginyl-*p*-nitroanilide; BTPNA, *N*-benzoyl-L-tyrosyl-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMSO,dimethylsufoxide.

2.2. ApTI Purification. *ApTI* was purified according to Richardson et al. (*27*), with some modifications. *A. pavonina* seeds were collected locally in the city of Três Lagoas, in the State of Mato Grosso do Sul (Brazil). After removal of the testae, the cotyledons were defatted with hexane and ground in a coffee mill. A crude inhibitor preparation was obtained by extraction of this meal with 0.1 M phosphate buffer, pH 7.6 (1:10, w/v) for 2 h at 25 °C with subsequent centrifugation at 10 000*g* for 30 min. The supernatant was fractionated by ammonium sulfate precipitation into three fractions, corresponding to 40, 60, and 80% saturation. The three fractions were dialyzed against distilled water for 24 h at 4 °C and lyophilized. The fraction corresponding to 40−60% ammonium sulfate saturation (precipitate II or PII) was selected for further purification.

Lyophilized PII was dissolved in 0.1 M phosphate buffer, pH 7.6, containing 0.1 M NaCl, and applied to a Sephadex G-75 column (2.6- × 80-cm) equilibrated with the same buffer. The single peak of antitryptic activity obtained was collected and dialyzed against distilled water and lyophilized. This material was further fractionated by ion-exchange chromatography on a DEAE-Sepharose column (2- × 20-cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and eluted with a linear gradient of NaCl (0−0.5 M) in the same buffer. The fraction eluted with 0.2 M NaCl was applied to a trypsin-Sepharose column (2- × 10-cm) equilibrated with 0.1 M phosphate buffer, pH 7.6, containing 0.1 M NaCl. The absorbed *ApTI* was eluted with 0.1 M HCl.

2.3. Protein Quantification. Protein concentrations were determined by the dye-binding method of Bradford (*28*), with bovine serum albumin as the standard.

2.4. Polyacrylamide Gel Electrophoresis. SDS polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) was done as described by Laemmli (*29*). The proteins used as molecular mass standards were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). The proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

2.5. Papain Inhibition Assays. The inhibition of papain by *ApTI* was assayed using a 1% azocasein solution as substrate (25). Aliquots (30 μL) of papain (1 mg ml^{-1} in 0.1 M phosphate buffer, pH 7.8) were preincubated with 40 μL of an activation solution containing 0.02 M EDTA and 0.05 M cysteine, pH 8.0, and 40 μL of 50 mM Tris-HCl buffer, pH 7.5, for 10 min at 45 °C. Subsequently, 50 μL of *ApTI* (1.0 mg ml^{-1} in 50 mM Tris-HCl buffer, pH 7.5) and 340 μL of 50 mM Tris-HCl buffer, pH 7.5, were added and the incubation continued for a further 10 min. The reaction was started by adding 200 μL of 1% azocasein solution. After 10 min, the reaction was stopped by adding 300 μL of 10% TCA. The samples were centrifuged and the supernatants were alkalinized with 0.25 N NaOH solution. One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased the absorbance at 440 nm by 0.01. The mechanism of inhibition (competitive, noncompetitive, or uncompetitive) was determined using Lineweaver−Burk plots, in which the inverse of the initial rate was plotted against the inverse of the substrate concentration (0.07, 0.1, 0.2, 0.3, 0.4, 0.6, 0.7, 0.8, 0.9, and 1 mM) in the absence or presence of *ApTI* (3, 4.5, and 6 μM). The K_i of the reaction between *ApTI* and papain was determined essentially as described above. The velocity of the reaction was expressed as $1/v$ (OD_{440} nm/10 min/mL)$^{-1}$, and the K_i value was determined using a double-reciprocal plot of the data (*30*).

2.6. Detection of Papain Activity and Inhibitory Activity in SDS-PAGE Containing 0.1% Gelatin. For this assay, the control mixture contained 2.0 μL of papain (1 μg μL^{-1}), 3.0 μL of activation solution (0.02 M EDTA and 0.05 M L- cysteine, pH 8.0), and 10 μL of 0.05 M Tris-HCl buffer, pH 7.5. The treatment mixtures (T1 and T2) contained 2.0 μL of papain, 3.0 μL of activation solution, and 10 μL (T1) or 20 μL (T2) of *ApTI* (3 μg μL^{-1} in 0.05 M Tris-HCl, pH 7.5). The mixtures (T1 and T2) were preincubated for 10 min at 45 °C, after which 7.5 μL of nonreducing sample buffer was added. To prevent the irreversible inactivation of the proteinases, the samples were not boiled prior to electrophoresis in 10% polyacrylamide gels containing SDS and 0.1% gelatin (*31*). After electrophoresis at 5 °C, the gels were washed with 2.5% Triton X-100 solution for 2 h to remove the SDS and then incubated with activation solution (0.001 M EDTA and 0.0025 M L-cysteine, pH 8.0) for 60 min at 45°C. The gels were subsequently stained with Coomassie brilliant blue R-250.

2.7. Assay of ApTI Activity Against Serine Proteinase. Trypsin-like enzymes were assayed using BAPNA as substrate (*32*). For routine assays, BAPNA was used at a final concentration of 1 mM in 1% (v/v) DMSO in a final volume of 1.5 mL and pH 8. Buffer and enzyme were preincubated to at 37 °C for 10 min before adding substrate to start the reaction, which was allowed to proceed for 20 min and then stopped with 200 μL of 30% (v/v) acetic acid. The resulting absorbance was read at 410 nm. Trypsin inhibitory activity was determined by measuring the remaining enzymatic activity toward BAPNA at pH 8.0 after preincubation with *ApTI* (1 mg/20 mL). One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased the absorbance at 410 nm by 0.01.

Chymotrypsin-like enzymes were assayed using BTPNA as substrate. For routine assays, BTPNA was used at a final concentration of 0.1 mM in 1% (v/v) DMSO in a final volume of 750 μL and pH 8. Buffer and enzyme were preincubated to 37 °C for 10 min before adding substrate to start the reaction, which was allowed to proceed for 5 min and then stopped with 200 μL of 30% (v/v) acetic acid. The resulting absorbance was read at 410 nm. Chymotrypsin inhibitory activity was determined by measuring the remaining activity toward BTPNA at pH 8.0 after preincubation with *ApTI* (1 mg/20 mL). One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased the absorbance at 410 nm by 0.01.

2.8. Insects. The colonies of *C. maculatus*, *Z. subfasciatus*, and *A. obectus*, all of the family Bruchidae, were maintained at 28 ± 1° C, at a relative humidity of 65−75% (16 h photophase). *C. maculatus*

was fed with seeds of a susceptible cowpea cultivar (Epace-10), and *Z. subfasciatus* and *A. obectus* were fed with seeds of *Phaseolus vulgaris*. These colonies were maintained in the Laboratório de Purificação de Proteínas e suas Funções Biológicas (LPPFB), Departamento de Ciências Naturais, Universidade Federal de Mato Grosso do Sul, Três Lagoas (MS), Brazil. The eggs and larvae of *T. molitor* were obtained from the Laboratório de Entomologia of Universidade Estadual de Campinas, SP, Brazil; larvae of *A. grandis* were provided by Dr. J. R. P. Parra (Escola Superior de Agronomia "Luiz de Queiroz", Universidade São Paulo, Piracicaba, SP, Brazil; and all were maintained in LPPFB.

2.9. Midgut Preparation. Proteinases were obtained from the midguts of fourth-instar larvae according to Macedo et al. (*33*). The insects were cold immobilized, dissected, and the midguts were surgically removed from the larvae and placed in iso-osmotic saline (150 mM NaCl solution). The guts and contents were stored at −20 °C until used. When required, the guts were homogenized in 150 mM NaCl, centrifuged at 10 000*g* for 10 min at 4 °C, and the supernatants pooled and kept on ice for the enzymatic assays.

2.10. Enzyme Assay Against Proteinase Extracts from Coleoptera Larvae. The total proteolytic activity was determined using a 1% azocasein solution, pH 5.6. Midgut larval extracts (50 μg of protein) were incubated with 40 μL of an activation solution containing 0.02 M EDTA and 0.05 M L-cysteine, pH 8.0, and 40 μL of 0.1 M citrate/phosphate, pH 5.6, for 10 min at 45°C, in a final volume of 0.1 mL for 10 min. Subsequently, 300 μL of 0.1 M citrate/phosphate, pH 5.6, was added, and the incubation continued for a further 10 min before the addition of 200 μL of azocasein solution. The reaction was allowed to proceed for 10 min at 45 °C and then stopped by adding 0.3 mL of 10% (v/v) TCA. The samples were centrifuged and 800 μL of supernatants were alkalinized with 800 μL of 0.1 N NaOH solution. The absorbance was read at 440 nm. Proteinase activity was expressed as ABS/mg/min relative to the total protein of the homogenate, which was measured using the Bradford method. Incubations were done for at least three periods, unless stated otherwise, and the initial rates of hydrolysis were calculated. The assays were done under conditions in which the enzyme activity was proportional to protein concentration and to the length of incubation.

The inhibition of cysteine-type protease activity was determined by measuring the remaining activity toward azocasein at pH 5.6, after preincubation with *ApTI*. One UI was defined as the amount of inhibitor that decreased the absorbance at 440 nm by 0.01.

2.11. Insect Biossays. To examine the effects of *ApTI* on *C. maculatus* development, the artificial seed system previously developed by Macedo et al. (*2*) was used. Artificial seeds (ca. 400 mg each) were prepared from finely ground cowpea seeds (cultivar Epace-10) using a cylindrical brass mal and a hand press. Artificial seeds containing *ApTI* at concentrations of 0.25 and 0.5% (w/w) were obtained by thoroughly mixing the inhibitor with cowpea seed meal and pressing as described above. Artificial seeds ($n = 10$) were prepared for each of the above concentrations and were repeated twice on different dates. Following a 48 h period to allow the insects to adapt to the growth chamber, the seeds were offered to 2−3-day-old fertilized females. After allowing 24 h for oviposition, the number of eggs per seed was reduced to 4 ($n = 40 \times 2$). Following incubation for 20 days at 28 °C and 70−75% relative humidity, the seeds were opened, and the weight and number of larvae were determined. Control artificial seeds were prepared with Epace-10 cultivar meal containing no added *ApTI*. Linear regression analysis was used to describe the response of *C. maculatus* to different amounts of *ApTI*. The effective doses for 50% response (ED_{50}) are the concentrations fractions that decrease the mass of the larvae to 50% of that of control larval. Lethal doses (LD_{50}) are the concentrations of the fractions that reduce the number of larvae to 50% of the number found in control seeds. Because of the wide range in mortality expressed as percentage (20−50% for *C. maculatus*), the percentages were not transformed prior to regression analysis.

2.12. Proteinase Activity of Insect Extracts in Polyacrylamide Gels Containing 0.1% Gelatin. Proteins extracted from the midguts of larvae fed in diets with or without *ApTI* were run on SDS-PAGE as described above, with some modifications. Midgut proteins (4 μg) from larvae fed on artificial diet and fed with *ApTI* at 0.5% were preincubated with solutions of *ApTI* (80 μg) for 10 min at 45 °C. These mixtures were then run on SDS-PAGE in gels containing 0.1% gelatin. Controls samples were not treated with *ApTI*. Following electrophoresis at 5 °C, the gels were washed with 2.5% Triton X-100 solution for 30 min with shaking to remove the SDS, after which the gels were incubated with 100 mL of activating solution (100 mM Tris-HCl, pH 6.5, containing 40 μl of β-mercaptoethanol and 200 μl of 95% Tritron X-100) for 2−3 h. The gels were subsequently stained with Coomassie brilliant blue R-250.

2.13. Inhibition of Larval Protease Activity after Ingestion of ApTI. The effect of *ApTI* on the enzyme activity of gut extracts from larvae fed a diet containing 0.5% *ApTI* was measured using azocasein as substrate (see above).

2.14. Chitin-Column Chromatography. To examine the possibility of *ApTI* interaction with chitin, the inhibitor was chromatographed on a chitin column (2- × 6-cm; 20-mL bed volume) equilibrated with 50 mM phosphate buffer, pH 7.6. After adsorption of the proteins (5 mg of *ApTI* in 1 mL of phosphate buffer), the column was washed with the same buffer until the absorbance at 280 nm returned to zero, after which the adsorbed *ApTI* was eluted with 0.1 M HCl. Fractions (2 mL) were collected, and proteins were estimated based on the absorbance at 280 nm, after which the inhibitory activity against trypsin was assayed using BAPNA as substrate.

2.15. Statistical Analysis. All data were examined using one-way analysis of variance (ANOVA) (general linear models, GLM). The Student-Neuman-Keul's test was used to identify the means which differed when ANOVA test indicated significance. A p value < 0.05 was considered to be significant.

3. RESULTS

3.1. Inhibition of Papain by ApTI. Figure 1 shows the Lineweaver−Burk double reciprocal plots for the inhibition of papain by *ApTI* (**Figure 1A**). The inhibition was of the noncompetitive type, in which there was a decrease in V_{max} with no change in Km compared to the reaction in the absence of inhibitor. The K_i value for *ApTI* was estimated to be 10 μM. The inhibitory activity of *ApTI* against papain involved a slow-tight binding. A linear extrapolation to 100% inhibition indicated that *ApTI* bound papain in a 1:1 molar ratio (**Figure 1B**). Polyacrylamide gels containing 0.1% gelatin were used to confirm the action of *ApTI* on papain activity. Proteolytic activity appeared as a clear zone against a dark blue background. In **Figure 1C**, the papain proteolytic activity (control) was visualized in lane C, and lanes T1 and T2 show that the papain proteolytic activity was inhibited by *ApTI*.

3.2. Effects of ApTI on Cysteine- and Serine-Protease Activity. As shown in **Table 1**, *ApTI* had high activity toward trypsin and chymotrypsin and moderate activity toward papain.

3.3. Effects of ApTI on Endogenous Proteolytic Activity. The larval digestive enzymes extracted from five coleopteran pests of economic importance contained cysteine-like enzymes (**Figure 2**). **Figure 3** shows the specificity of *ApTI* against these insect enzymes, all of which were strongly inhibited by *ApTI*, except for *T. molitor* and A. *grandis*, for which there was only weak inhibition. The inhibitory activity of *ApTI* against insect proteases, expressed as a percentage of that against cysteine-like proteases, was 60, 64, and 84% for *Z. subfasciatus*, *A. obtectus*, and *C. maculatus*, respectively.

3.4. Effect of ApTI on the Development of *C. maculatus* Larvae. The effect of *ApTI* on the development of *C. maculatus* was assessed by determing the number and weight of surviving fourth-instar larvae fed a diet containing increasing amounts of *ApTI*. The dose−response effect of *ApTI* on the growth and mortality of the insect larvae is shown in **Figure 4**. The survival (**Figure 4A**) and weight (**Figure 4B**) of cowpea weevil larvae fed on control seeds (represented by the *y*-intercept value) were

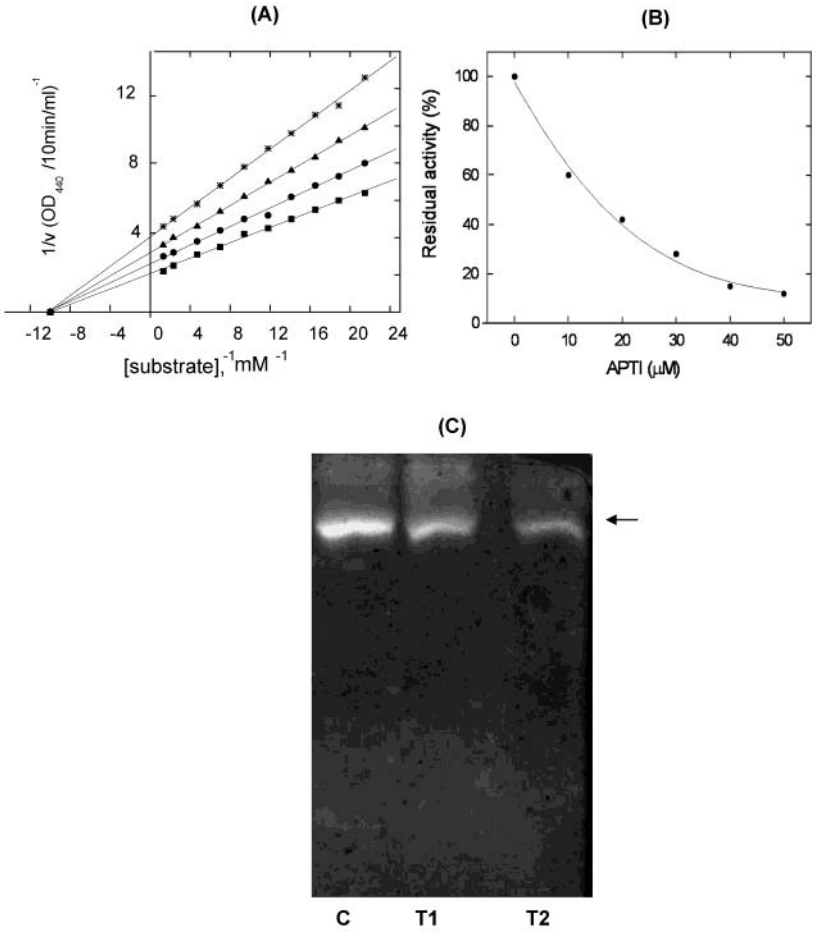

Figure 1. Inhibition of papain activity by *ApTI*. (**A**) Cystatin activity was evaluated using several concentrations of azocasein as substrate in the absence (■) or in the presence of 3 μM (●), 4 μM (▲), and 6 μM (*) *ApTI*. (**B**) Titration curve of papain (43 μM) inhibition by *ApTI*. (**C**) SDS-PAGE (10%) containing 0.1% gelatin: C, positive control (2 μg papain); T1 (2 μg papain + 30 μg *ApTI*); and T2 (2 μg papain + 60 μg *ApTI*). Arrow indicates the papain band.

Table 1. Inhibitory Activity of APTI toward Proteinases

enzymes	inhibition (%)[a]
papain	51.8 ± 1.2
trypsin	63.4 ± 0.5
chymotrypsin	62.0 ± 1.7

[a] The values are the mean ± standard error of determinations.

about 80% and 7.4 mg, respectively, whereas seeds containing 0.5% of *ApTI* caused 50% mortality (LD_{50}) and a 39% decrease in weight. Regression analysis showed that for each 0.1% increase in the *ApTI* dose, there was an 8% increase in mortality ($r^2 = 0.99$). For each increase of 0.1% in *ApTI* content, there was a 0.56 mg decrease ($r^2 = 0.98$).

3.5. Proteinase Activity by SDS-PAGE and Inhibition of Larval Activity after Ingestion of ApTI. General proteinase activity was significantly enhanced in larvae reared on a diet containing 0.5% *ApTI* A 2-fold increase was observed for cysteine proteinase activity (**Figure 5A**). Electrophoretic analysis confirmed this increase in proteinase activity (**Figure 5B**, lanes 1 and 2). This method would not enable novel proteinases of the same molecular mass to be detected. However, the cysteine proteinase activity in 0.5% *ApTI*-larvae was sensitive to *ApTI* (**Figure 5B**, lanes 3 and 4, and **Figure 6**).

3.6. Chitin-Column Chromatography. When *ApTI* was chromatographed on a chitin column, three peaks were obtained, two eluting with the equilibrium buffer (50 mM phosphate buffer, pH 7.6) and the other with 100 mM HCl (**Figure 7**). Inhibitory activity was detected in the first nonretained peak and in the retained peak eluted with HCl.

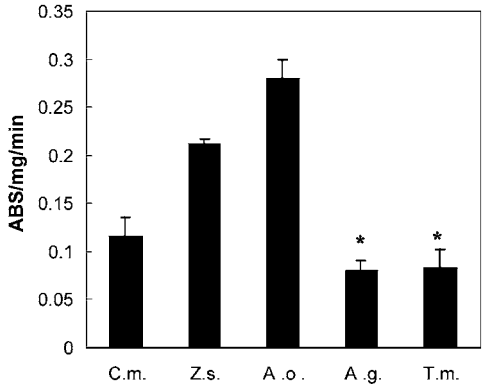

Figure 2. Cysteine-like activities from fourth-instar larvae reared on control artificial diet. (C.m), *C. maculatus*; (Z.s.), *Z. subfasciatus*; (A.o.), *A. obtectus*; (A.g.), *A. grandis*; (T.m), *T. molitor*. Data with asterisk were not significantly different ($p < 0.05$). Azocasein were used for total protease.

4. DISCUSSION

Various studies have shown that protease inhibitors are active against the enzymes of different insect species both in vitro and in vivo bioassays (*34*, *35*). In this work, the action of a trypsin

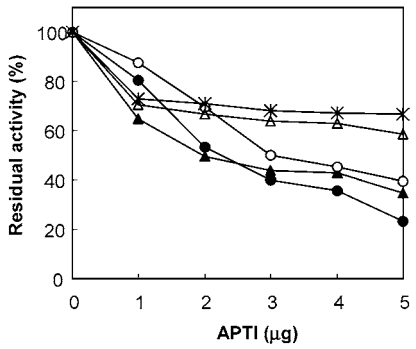

Figure 3. Inhibition by *ApTI* of the midgut proteolytic activity (assayed with azocasein) of fourth-instar larvae. (●), *C. maculatus*; (▲), *A. obtectus*; (○), *Z. subfasciatus*; (*), *T. mollitor*.

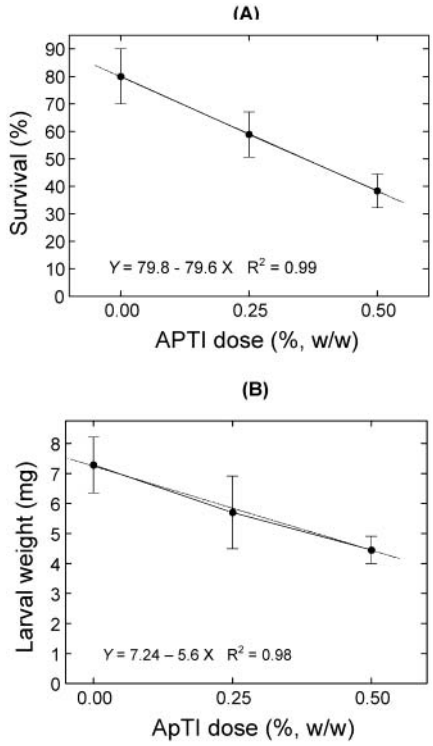

Figure 4. Effect of dietary *ApT*I on *C. maculatus* within-diet artificial. Intercept in (**A**) survival and (**B**) weight, using an artificial diet bioassay. Each point has an $n > 80$. Error bars indicate standard error of the mean.

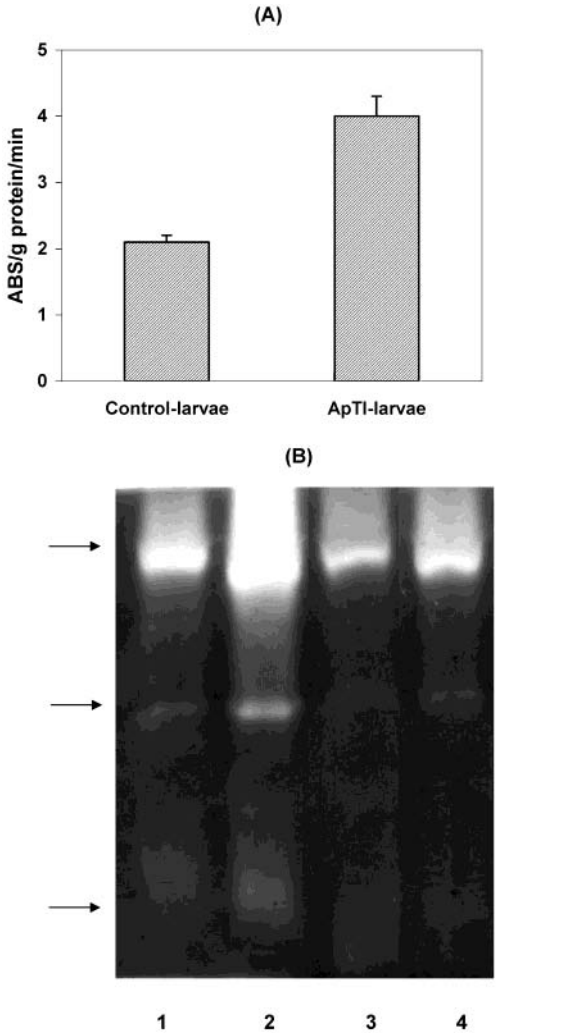

Figure 5. Response of *C. maculatus* proteinase to *ApTI* ingestion. (**A**) Azocaseinase activities after 20 days development on control diet or diet containing 0.5% *ApTI*. (**B**) Proteinase assays after SDS-PAGE: (1) control diet-fed larvae, (2) 0.5% *ApTI*-fed larvae, (3) control diet-fed larvae and their inhibition by *ApTI*, and (4) 0.5% *ApTI*-fed larvae and their inhibition by *ApTI*. Values correspond to the activity of these extracts of larvae ± standard deviations.

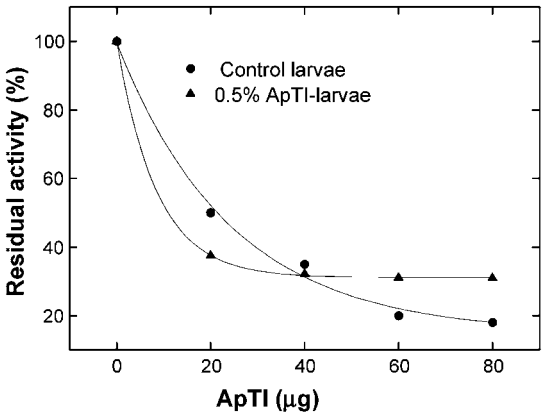

Figure 6. Interaction, in vitro, with a range *ApTI* concentration of a cysteine-like (azocasein-hidrolizing) activity from the digestive tract of fourth-instar of *C. maculatus.* (●), larvae reared on the control diet; (▲), larvae reared on the diet cointaining 0.5% *ApTI*.

inhibitor against proteases of different coleopteran were compared, and the mode of action in *C. maculatus* larvae was examined. Prabhu et al. (*26*) reported that *ApTI* inhibited trypsin and chymotrypsin noncompetitively, with K_i of 2.92×10^{-10} M and 4.46×10^{-10} M, respectively. As shown here, *ApTI* can also inhibit papain ($K_i = 10\ \mu$M), and this was confirmed by inhibition of the gelatinase activity of papain. *ApTI* is the second example of a Kunitz-type proteinase inhibitor capable of inhibiting papain noncompetitively (*25*). The molecular mass of *ApTI* (21 kDa) is consistent with that of other cystatins. Phystocystatins have a wide range of molecular masses (from 10 to 87 kDa), as seen with *Enterolobium contortisiliqum* (∼60 kDa) (36), soybean seeds (∼26 kDa) (*37*), and rice (∼12 kDa) (*38*).

In the Coleoptera, cysteine proteinases have been best characterized in the Bruchidae (*39*−*41*), it also occurs in the Tenebrionidae, Chrysomelidae, Coccinelidae, Curculionoideae, Meloidae, and Silphidae (*20*, *42*). Our results suggested that *A. obtectus* had higher levels of cysteine proteinases than the other species examined in this work. *Z. subfasciatus* has ∼2-fold more azocasein hydrolyzing activity than does *C. maculatus*, in

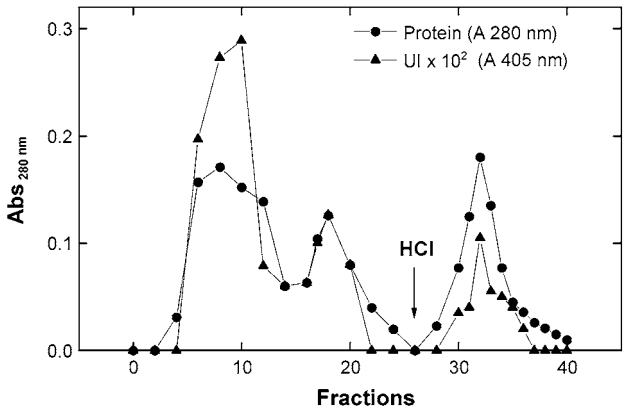

Figure 7. Chromatography of *ApTI* on a chitin column. The column (6- × 2-cm) was equilibrated with 50 mM phosphate buffer (pH 7.6) and eluted with 100 mM HCl. (●), $A_{280\ nm}$; (▲), inhibitory activity ($A_{405\ nm}$).

agreement with Silva and Xavier-Filho (*21*). *A. grandis* and *T. molitor* apparently has the same level of cysteine proteinase activity. The data for *A. grandis* agreed with the results by Murdock et al. (*20*), but it differed from these of Purcell et al. (*43*), which did not detect this type of proteolitic activity in this insect. This apparent discrepancy may reflect the manner in which the samples were prepared. In our studies, the midguts were homogenized and centrifuged, and the supernatants were pooled for enzymatic assays, whereas the other authors used only the midgut fluid without homogenizing the tissue. The presence of cysteine proteinase activity in *T. molitor* confirmed the data of Thie and Houseman (*44*) regarding the presence of serine and cysteine proteinases in the larval midgut of this species. Our data reinforce the hypothesis of Terra and Cristofoletti (*45*) that the Cucujiformia ancestor was a beetle that ingested seeds rich in naturally occurring serine proteinase inhibitors and therefore used cysteine proteinases for protein digestion in addition to or instead of serine proteinases.

On the basis of the enzyme inhibition studies, feeding trials were done to assess the potential insecticidal effects of *ApTI*. When incorporated into artificial diet to a level of 0.5% (~0.3 mM), *ApTI* decreased the weight gain by ~40% and reduced the larval survival to 50%. The concentration of *ApTI* used (0.25−0.5%, w/w) corresponded to the levels in legume seeds and were similar to those used by other workers (*14*).

Many coleopterans, such as western corn rootworm, Colorado potato beetle, and cowpea bruchids, use cysteine proteases as their major digestive enzymes (*20, 46, 47*). These digestive proteases can also degrade plant defense proteins, as seen in *Z. subfasciatus*, which possesses a protease capable of degrading the α-amylase inhibitor of the common bean (*48*). The inhibition of insect enzymatic activities interferes with the insects nutrient uptake and growth. Oryzacystatin I repressed the growth of *Trilobium castaneum* (*6*), whereas potato multicystatin inhibited larval growth in the western corn rootworm (*49*). The soybean CPI soyacystatin N (scN) suppressed the digestive enzyme activity of the western corn rootworm and Colorado potato beetle, as well as the growth and development of these pests (*23, 47*). These results indicate that plant cystatins could be good candidates for environmentally friendly alternatives for controlling coleopteran pests.

The use of protease inhibitors to protect plant against insect pests is, however, complicated by the ability of insects to circumvent plant defenses. Available biochemical and molecular evidence indicates that some insects, such as *Spodoptera littoralis*, adapt to the presence of protease inhibitors by overproducing existing digestive proteases (*5*), whereas *S. frugiperda* adapts to soybean proteinase inhibitor by changing the expression of trypsin and chymotrypsin activities (*50*). Still others selectively induce inhibitor-insensitive proteases, as observed in Colorado potato beetles, *S. exigua*, and other insect species (*17, 51, 52*). Digestive proteolysis mediated by direct proteolytic fragmentation is another strategy used by insects to minimize the adverse effects of plant defense proteins on food digestion and nutrient uptake (*6, 53, 54*). Recently, Zhu-Salzaman et al. (*54*) suggested that the cowpea bruchid adapts to the challenge of scN by qualitatively and quantitatively remodeling its digestive protease complement and by activating scN-degrading proteases.

Recent work suggest that insects can overcome the deleterious effects of protease inhibitors by synthesizing different proteases that are insensitive to particular inhibitors (*50, 55, 56*) Our results suggest that *C. maculatus* does not adapt to the inhibitor, at least up to the fourth instar of the first generation. The fourth instar larvae reared on a diet containing *ApTI* showed a 2-fold increase in cysteine proteinase activities, as confirmed by azocasein as substrate (**Figure 5A**) and by activity in gels (**Figure 5B**). In addition, all of the cysteine proteinase activity in *ApTI*-fed larvae was sensitive to *ApTI*, indicating that no novel proteolytic form resistant to *ApTI* was induced in larvae reared on a diet containing this inhibitor (**Figure 5B**).

Ishimoto and Chrispeels (*48*) suggested the existence of a minor serine proteinase in the midgut of *Zabrotes subfasciatus* larvae. The detection of this protein was based mainly on the degradation of α-amylase. Silva et al. (*57*) and Macedo et al. (*2*) reinvestigated the specificity of intestinal proteinases in *C. maculatus* larvae using synthetic substrates. These authors observed that intestinal homogenates of larvae contained serine proteinases activities. Thus, *C. maculatus* probably still retains the capacity to express and use serine proteinases for digestion.

Oliveira et al. (*25*) isolated a proteinaceous inhibitor from *Prosopis juliflora* (*Pj*) with activity against papain, trypsin, and chymotrypsin. *ApTI* is a Kunitz-type inhibitor that noncompetitively inhibits trypsin, chymotrypsin, and papain. This *ApTI* characteristic can be related to the insecticide effects on *C. maculatus* larvae once this inhibitor can block the enzymes involved in this insect digestion.

Sales et al. (*58*) showed that chitin or chitinous structure is present in the midgut structures of *C. maculatus* larvae, particularly in the apical region of microvilli in the midgut epithelium. As shown here, *ApTI* bound to a chitin column, although not completely. Macedo et al. (*2*) isolated a trypsin inhibitor (DMTI−II) from *Dimorphandra mollis* seeds that is toxic to *C. maculatus* larvae. DMTI−II also bound to a chitin column and to chitinous structures in the midgut of this insect, as demonstrated by immunoblotting. Like DMTI−II, the possible association of *ApTI* to *C. maculatus* midgut may hinder the transport of enzymes and their hydrolysis products, which in combination with a reduced proteolysis, may limit the availability of amino acids for larval growth, leading to poor development and high mortality (*59*).

In conclusion, the action of *ApTI* on *C. maculatus* larvae may involve (1) massive overproduction of *ApTI*-sensitive cysteine proteinases, leading to a shortage of essential amino acids needed for the production of other proteins and (2) possible binding to chitin components of the peritrophic membrane (or equivalent structures) in the weevil midgut.

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