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In Vivo Bioinsecticidal Activity toward *Ceratitis capitata* (Fruit Fly) and *Callosobruchus maculatus* (Cowpea Weevil) and in Vitro Bioinsecticidal Activity toward Different Orders of Insect Pests of a Trypsin Inhibitor Purified from Tamarind Tree (*Tamarindus indica*) Seeds

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A proteinaceous inhibitor with high activity against trypsin-like serine proteinases was purified from seeds of the tamarind tree (*Tamarindus indica*) by gel filtration on Shephacryl S-200 followed by a reverse-phase HPLC Vidac C18 TP. The inhibitor, called the tamarind trypsin inhibitor (TTI), showed a M_r of 21.42 kDa by mass spectrometry analysis. TTI was a noncompetitive inhibitor with a K_i value of 1.7×10^{-9} M. In vitro bioinsecticidal activity against insect digestive enzymes from different orders showed that TTI had remarkable activity against enzymes from coleopteran, *Anthonomus grandis* (29.6%), *Zabrotes subfasciatus* (51.6%), *Callosobruchus maculatus* (86.7%), *Rhyzopertha dominica* (88.2%), and lepidopteron, *Plodia interpuncptella* (26.7%), *Alabama argillacea* (53.8%), and *Spodoptera frugiperda* (75.5%). Also, digestive enzymes from Diptera, *Ceratitis capitata* (fruit fly), were inhibited (52.9%). In vivo bioinsecticidal assays toward *C. capitata* and *C. maculatus* larvae were developed. The concentration of TTI (w/w) in the artificial seed necessary to cause 50% mortality (LD_{50}) of larvae was 3.6%, and that to reduce mass larvae by 50.0% (ED_{50}) was 3.2%. Furthermore, the mass *C. capitata* larvae were affected at 53.2% and produced ∼34% mortality at a level of 4.0% (w/w) of TTI incorporated in artificial diets.

KEYWORDS: ***Tamarindus indica*; trypsin inhibitor; bioinsecticide activity; insect pests**

INTRODUCTION

Worldwide crop loss without the use of pesticides is estimated to be ∼70% of production (*1*) and, despite of the use of pesticides, preharvest losses due to insect pest attack are ∼15% of the total crop production (*2*). Beginning in 1980, new strategies of insect pest control, such as integrated pest management (IPM) control and the use of transgenic crops have been proposed and tested to avoid these crop production losses. The use of transgenic crops has been rapidly advancing during the past decade with the discovery of effective plant genes that can be transferred and offer resistance to crop plants against pests and pathogens. The use of proteinaceous inhibitors as candidates in control strategies of insects has good potential, because insect digestive proteinases are promising targets in the control of various insects, including lepidopteran, such as, *Manduca sexta* (L.) (*3*), *Heliothis zea* (Boddie) (*4*), *Spodoptera litura* (Boisd.) (*5*), and *Lucilia cuprina* (Wied.) (*6*), and also various coleopterans (*7*−*11*). Despite these several suggested physiological functions in plants (*12*−*14*), the inhibitors are known for their

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roles in response to abiotic stresses (*15*, *16*) and biotic stresses, especially in plant defense processes against insect pest attack (*9*), where they are effective against insect digestive enzymes (*17*−*20*). These proteinaceous inhibitors in general are small, stable, and abundant proteins (*21*) showing specificity for serine proteinase, cysteine proteinase, aspartic proteinase, or metalloproteinases (*17*). Serine proteinase inhibitors are found in plant storage tissues, such as seeds, tubers, leaves, and fruits (*13*, *22*). Most of these inhibitors bind to cognate enzymes according to a common substrate-like canonical mechanism (*17*). Among them, the Kunitz trypsin inhibitor superfamily (*23*) has gained particular attention for its specific activity against trypsin-like serine proteinases, with no inhibition of other proteinase classes (*24*). Furthermore, Kunitz trypsin inhibitors are capable of inhibiting the proteolytic activity of several lepidopterans, such as black cutworm (*Agrotis ipsilon* Hufnagel), corn earworm (*H. zea*), tobacco budworm (*Heliothis virensscens* Fab.), and western spruce budworm (*Choristoneura occidentalis*) (*25*), and coleopteran such as the cotton boll weevil (*Anthonomus grandis*) (*10*). Several plants have been screened to isolate and characterize such proteinase inhibitors, among them the species *Tamarindus indica* that belong to the family Caesalpiniaceae, which are tropical and subtropical trees and shrubs. In this study, we have reported purifications and characterization of a related Kunitz inhibitor from tamarind tree seeds. We have also tested its bioinsecticidal activity in vitro toward insect pests of different orders and in vivo during larval development of *Ceratitis capitata* (fruit fly) and *Callosobruchus maculatus* (cowpea weevil) with a bioassays model.

MATERIALS AND METHODS

Materials. The enzymes papain, bromelain, bovine chymotrypsin, bovine trypsin, and porcine elastase and the substrates *N*-benzoyl-DL-arginyl-*p*-nitroanilide (BAPNA) and azocasein were purchased from Sigma Chemical Co. (St. Louis, MO).

Purification of Tamarind Trypsin Inhibitor (TTI). Tamarind (*T. indica*) seeds were obtained from the IBAMA (Environmental-middle Brazilian Institute) seed bank of Natal, RN, Brazil. Tamarind seed meal finely ground was extracted (1:10, w/v) with 0.05 M Tris-HCl buffer, pH 7.5, for 30 min at room temperature. After centrifugation for 30 min at 8000*g* at 4 °C, the supernatant (crude extract) was precipitated with ammonium sulfate at concentrations of 0−30, 30−60, and 60−90%. These fractions (F_{0-30}, F_{30-60}, and F_{60-90}) were then dialyzed against distilled water, freezer-dried, and submitted to antitryptic assays. The fraction denominated F_{30-60}, which corresponds to a 30−60% saturation range, showed high inhibitory activity against bovine trypsin. This fraction (22 mg mL^{-1}) was applied to a size exclusion column, Sephacryl 200-SH (84 cm × 2.5 cm column), equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. Fractions of 1.5 mL were collected using a flow rate of 30 mL h^{-1}. The peak (ST2) obtained was pooled, dialyzed, lyophilized, and applied (1.0 mg/mL) to a reverse-phase HPLC column (Vydac C-18 TP 1022), equilibrated with 0.1% TFA solution at a flow of 1.0 mL min^{-1}. Samples were eluted with a linear gradient of 0−60% acetonitrile, and the chromatography was monitored at 216 nm. The peak with antitrypsin activity, denoted TTI, was pooled, concentrated, and subjected to further analysis.

TTI Inhibitory Assay. The trypsin inhibitory assay was performed using BAPNA as substrate. Ten microliters of trypsin (0.3 mg mL^{-1} in 0.0025 M HCl) solution was incubated for 15 min at 37 °C with 60 μL of inhibitor solution and 120 μL of 0.05 M Tris-HCl, pH 7.5. Reactions were started with the addition of 500 μL of 1.25 mM BAPNA solution, prepared in 0.05 M Tris-HCl, pH 7.5. After 15 min at 37 °C, the reaction was stopped by the addition of 150 μL of 30% acetic acid solution. The color developed was measured by absorbance at 405 nm. One unit of inhibitory activity was defined as the amount of inhibitor that decreased absorbance by 0.01 at 405 nm. All assays were made in triplicate. The results of each series were expressed as the mean value ± SD.

Protein Determination. Protein content was measured according to the procedure of Bradford (*26*) with bovine serum albumin (BSA) as protein standard.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) in the absence and presence of β-mercaptoethanol (0.1 M) was conducted as described by Laemmli (*27*) at 25 °C. Protein molecular weight markers (full-range rainbow molecular weight markers) were purchased from Amersham Pharmacia.

Matrix-Assisted Laser Desorption Time-of-Flight (MALDI-TOF) Analysis. Freeze-dried peak III eluted from HPLC was prepared for MALDI-TOF on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA). The samples were dissolved in 1.0% trifluoroacetic acid, and the 50% matrix sinapinic acid (a saturated solution dissolved in acetonitrile/0.1% TFA, 1:1, v/v) from Sigma Chemicals was added. The solution was then vortexed, and 1 μL was applied to the Voyager Bioworkstation sample plate. Samples were air-dried at room temperature. The spectrometer, equipped with a delayed-extraction system, was operated in linear mode. Sample ions were evaporated by irradiation with a N_2 laser at a wavelength of 337 nm and accelerated at 23 kV potential in the ion source with a delay of 150 ns. Samples were ionized with 100−200 shots of a 3 ns pulse width laser light. The signal was digitized at a rate of 500 MHz, and averaged data were presented to a standard Voyager data system for manipulation.

Kinetics of Inhibitory Activity from TTI. The constant of dissociation (K_i) was determined for bovine trypsin enzyme by preincubating the enzyme with increasing concentrations of purified inhibitor (0.06, 0.12, and 0.18 μM) in 50 mM Tris-HCl, pH 7.5, 37 °C, followed by measurement of the residual activity using the synthetic substrate BAPNA (0.1, 0.5, 1.0, 1.25, and 1.5 mM). The velocity of the reaction was expressed as $1/v$ $(OD_{440}/h/mL)^{-1}$, and the K_i value was determined using a double-reciprocal plot of data (*28*).

Specificity of TTI toward Serine and Cysteine Proteinases. The ability of TTI to inhibit other serine (bovine chymotrypsin and porcine elastase) proteinases and also cysteine (papain and bromelain) proteinases was assayed using azocasein as substrate, as described by Xavier-Filho et al. (*29*).

Preparation of Insect Gut Proteinases. *C. maculatus* and *Z. subfasciatus* were supplied by the Laboratório de Química e Função de Proteínas from Departamento de Bioquímica, UFRN, Brazil. *Rhyzopertha dominica* was obtained from Centro Nacional de Recursos Genéticos e Biotecnologia (CENARGEM/EMBRAPA), Brasília, Brazil. *S. frugiperda*, *A. argillacea*, and *A. grandis* were obtained from Centro Nacional de Pesquisa do Algodão (CNPA/EMBRAPA), Campina Grande, Brazil, and *C. capitata* was from Laboratório de Mosca das Frutas of the Departamento de Biologia Celular e Genética, UFRN, Brazil. Larvae and adult insect proteinases were obtained after dissection and extraction of the guts. The guts were surgically removed from the animal and placed into an iso-osmotic saline (0.15 M NaCl) solution. Gut tissue was stirred and centrifuged at 10000*g* at 4 °C for 10 min. The supernatants were then recovered and used for in vitro assays.

TTI Inhibitory Assay against Proteinase Extracts from Insect Pests. TTI effects on the proteolytical activity of whole gut extracts were measured by using BAPNA (1.25 mM) as substrate. The assays were run in 50 mM Tris-HCl, pH 7.5. TTI (0.2 μg mL^{-1}) was incubated with a 40 μL aliquot of gut extracts at 37 °C for 15 min before the substrate was added. Reactions were started with the addition of 500 μL of 1.25 mM BAPNA solution, prepared in 0.05 M Tris-HCl, pH 7.5. After 30 min at 37 °C, the reaction was stopped by the addition of 150 μL of 30% acetic acid solution. The color developed was measured by absorbance at 405 nm. Enzymatic assays were made using BAPNA (1.25 mM) as substrate. All assays were made in triplicate. The results of each series were expressed as the mean value ± standard deviation (SD).

Insect Bioassay. To examine the effects of TTI on *C. maculatus* development, the artificial seed system previously developed by Macedo et al. (*30*) was used. Artificial seeds (~400 mg each) were made from finely ground cowpea seeds (Epace 10 cultivar) using a cylindrical brass mal and a hand press. Artificial seeds containing TTI at concentrations of 1, 2, and 4% (w/w) were obtained by thoroughly mixing the TTI

with cowpea seed meal and pressing as described above. Each treatment had three artificial seeds and was replicated six times for each of the above concentrations. After a 48-h period for adjustment in the growth chamber, the seeds were offered to nine 2−3-day-old fertilized females. After 24 h had been allowed for oviposition, the number of eggs per seed was reduced to three ($n = 6 \times 9$). Following incubation for 20 days at 28 °C and 60% relative humidity, the seeds were opened and the mass and number of larvae (third instar) were determined. The experiments were carried out in six replicates and the mean [± standard error of the mean (SEM)] was calculated. Control artificial seeds were made with Epace 10 cultivar meal without TTI.

The performance of *C. capitata* in an artificial diet system was carried out. Artificial diets (~500 mg each) were prepared using 10.4% finely ground sugar cane fibers, 3% wheat germ, 6.5%wheat flour, 12% crystal sugar, 9.9% yeast, 0.3% sodium benzoate (VETEC), 0.9% HCl, and 57% H_2O. To this diet was added lyophilized TTI, at standard concentrations of 1.0, 2.0, and 4.0% (w/w), which were obtained by thoroughly mixing the TTI. After preparation, the diets were presented to seven neonate larvae per diet ($n = 6 \times 21$) in dark glass vials at a controlled temperature of 28 ± 1 °C and 60−70% relative humidity in the growth chamber. After 4 days, diets were opened and the mass and number of the larvae were taken. The experiments were carried out in six replicates, and the mean (±SEM) was calculated. Control artificial diets were made without TTI.

RESULTS

Inhibitor Isolation and Purification of TTI. Crude soluble protein extract obtained from the mature seeds from tamarind trees was initially precipitated at 30, 60, and 90% of saturation with ammonium sulfate, and three protein fractions (F_{0-30}, F_{30-60}, and F_{60-90}) were obtained. The F_{30-60} protein fraction obtained showed a strongly inhibitory activity against trypsin, whereas the other fractions presented low inhibitory activities. The F_{30-60} was then applied on Sephacryl 200-SH, and the peaks were assayed against trypsin. Three peaks (ST1, ST2, and ST3) revealed the presence of three peak inhibitor activities with 50.6, 84.3, and 28.5% of trypsin inhibition, respectively (**Figure 1A**). The ST2 fraction was then applied to reverse-phase HPLC (**Figure 1B**). The elution of the ST2 fraction revealed the presence of several peaks (I−VI). These peaks were assayed against trypsin, and only peak III showed activity toward trypsin. This purification procedure to trypsin inhibitor from *T. indica* seeds is shown in **Table 1**.

Electrophoretic and MALDI-TOF Analysis of TTI. Electrophoretic analysis, in the presence and absence of a reducing agent (β-mercaptoethanol), of peak III showed one protein with a molecular mass of ~20 kDa (**Figure 2A**). This fraction III or inhibitor TTI was applied in a MALDI-TOF showing a monomeric molecular mass of 21 420 Da (**Figure 2B**).

Kinetics of Inhibition and Specificity of TTI toward Other Proteinases. To determine the inhibition mechanism of inhibitor TTI against trypsin, the inhibition kinetic data were analyzed by Lineweaver−Burk double-reciprocal plots (**Figure 3**). The analysis showed noncompetitive type kinetics of inhibition, characterized by lack of change of the K_m value and decrease of the V_{max} when compared with the reaction in the absence of the inhibitor. The K_i value of TTI was determined from a double-reciprocal plot of the data, where the K_i value was 1.7×10^{-9} M.

Specificity of TTI to serine proteinases showed that it was not active to chymotrypsin and affected weakly pancreatic elastase (3.1% of inhibition). Cysteine proteinases (papain and bromelain) were not inhibited by TTI (**Table 2**).

TTI Inhibitory Assay against Proteinase Extracts from Insect Pests. TTI was assayed against different insect gut proteinases from coleopteran, lepidopteran, and dipteran pests

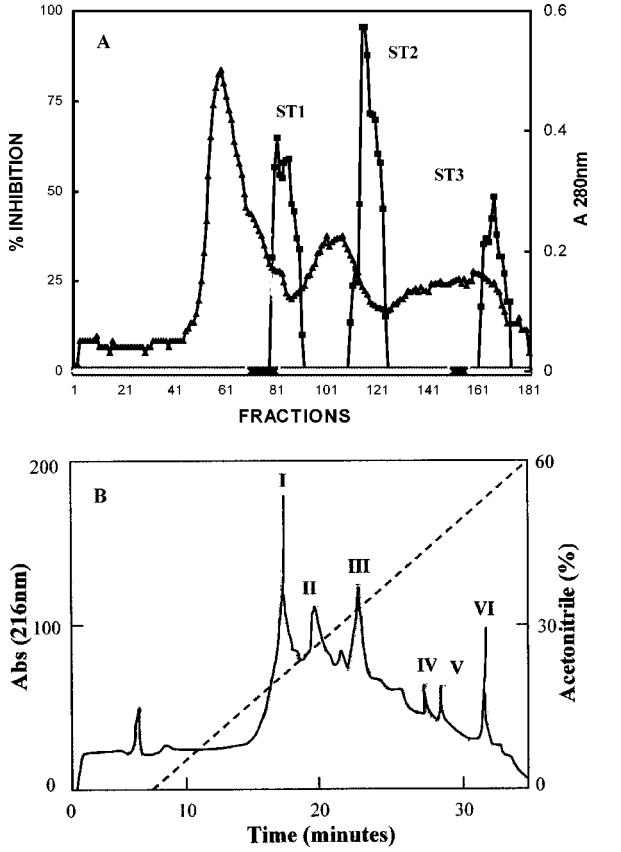

Figure 1. (**A**) Elution profile on Sephacryl 200-SH of F_{30-60}, from *T. indica* seeds. Approximately 22 mg of protein was applied in the column equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, and fractions were eluted and monitored at 280 nm (●) and assayed against trypsin. Active fractions ST1, ST2, and ST3 were obtained (■). (**B**) Analytical reverse-phase HPLC of the ST2 peak. The separation was carried out on a Vydac 218TP analytical column using a flow rate of 1 mL/min. TFA (0.1%) was used as an ion-pairing agent, and the dashed line indicates the acetonitrile gradient. The sample contained 1.0 mg of protein.

Table 1. Purification of Trypsin Proteinase Inhibitor from Tamarind Seeds

protein fraction	total inhibitory activity (IU)	total protein (mg)	specific activity (IU mg^{-1})	purification (*X*)	yield (%)
CE	175000	1140.0	153.5	1.00	100
F_{30-60}	24990	96.0	260.3	1.69	14.3
ST2	7208.5	1.95	3696.7	24.08	4.1
TTI	3920.0	0.2	19600	127.7	2.2

[a] One trypsin proteinase inhibitor unit (1 UI) was defined as inhibitor amount that decreased the absorbance at 405 nm by 0.1 OD in the trypsin assay conditions.

(**Table 2**). Among the different gut proteinases tested, TTI showed high in vitro inhibition to *R. dominica* (lesser grain borer), cowpea weevil (*C. maculatus*), and *S. frugiperda* (armyworm) proteinases. Moderate inhibitory activity toward gut proteinases from *A. argillacea* (cotton leafworm), *C. capitata* (fruit fly), and *Z. subfasciatus* (bean weevil) was also observed, and low inhibitory activity was seen against gut proteinase from *A. grandis* (boll weevil) and *P. interpuncptella* (Indian meal moth).

Insect Bioassay. On the basis of in vitro enzymatic studies, standard feeding trials were carried out to assess the potential bioinsecticidal effects of TTI toward two pests, *C. capitata* and

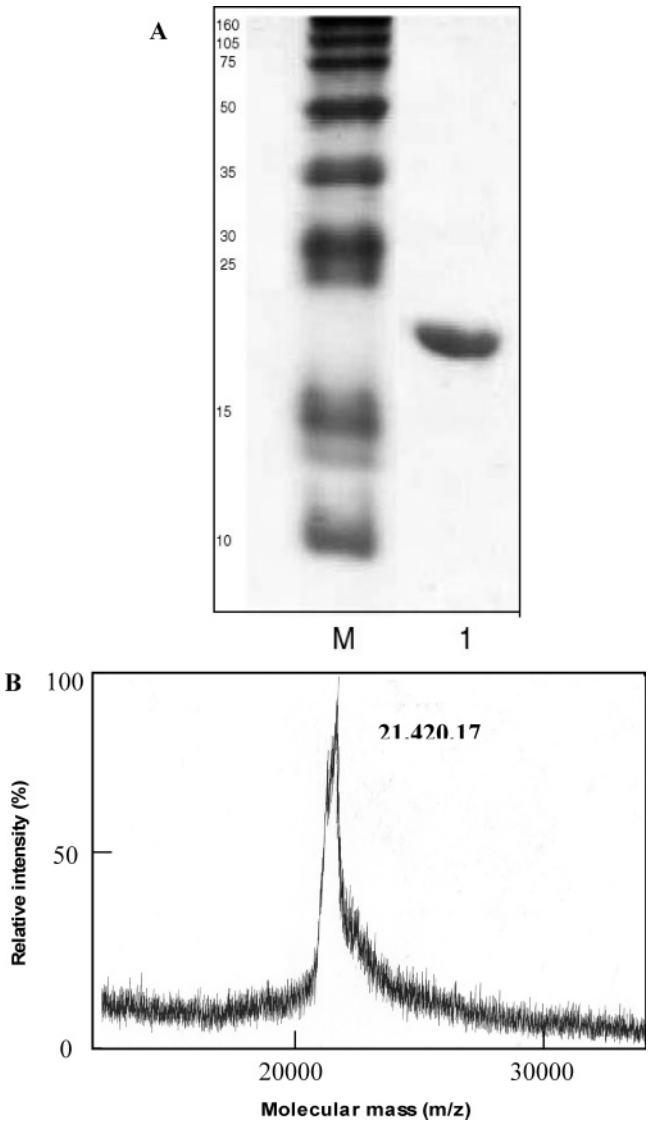

Figure 2. (**A**) SDS-PAGE analysis of purified TTI, stained with Coomassie Blue: (lane 1) TTI; (lane M) protein molecular weight markers (full-range rainbow molecular weight markers); (**B**) MALDI-TOF spectrum of TTI purified from reversed phase HPLC.

C. maculatus, which were used as models. Panels **A** and **B** of **Figure 4** show the influence of TTI on the weight and number of survivors of *C. capitata* and *C. maculatus*, respectively, during larval developmental when larvae were fed with a diet containing different concentrations of TTI. The inhibitor added to the diet of these pests in artificial seeds was effective against *C. maculatus* and produced 50% mortality to this bruchid at the level of 3.6% (LD_{50}, lethal dose). It also affected the mass of larvae at 50.0% with an ED_{50} (effective dose) at the level of 3.2%. In artificial diets to *C. capitata*, the mass of larvae was affected at 53.2% and larvae mortality was 34% at the level of 4% of TTI.

DISCUSSION

Serine proteinase inhibitors such as related Kunitz family trypsin inhibitors have been purified and characterized from a variety of plant sources (*31*−*36*). The role of Kunitz-type inhibitors as defensive compounds against predators was studied as early as 1947 when Mickel and Standish (*37*) observed that larvae of certain insects were unable to develop on soybean

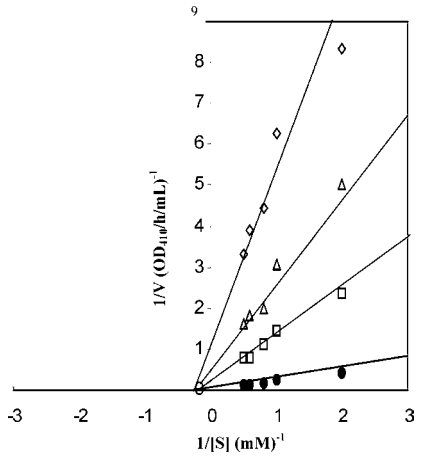

Figure 3. Inhibition of trypsin activity by inhibitor TTI. Kinetic mechanism data are illustrated by Lineweaver–Burk double-reciprocal plots. TTI activity was evaluated using several concentrations of BAPNA (0.1, 0.5, 1.0, 1.25, and 1.5 mM) as substrate in the absence (◆) or in the presence (□, △, ◇) of the crescent concentration (0.06, 0.12, or 0.18 μM) of TTI.

Table 2. Inhibitory Activity of Inhibitor TTI toward Proteinases and Gut Insect Proteinases

enzyme[a]	inhibition[b] (%)
serine proteinases	
porcine elastase	3.1 ± 0.86
bovine chymotrypsin	ND
cysteine proteinases	
papain	ND
bromelain	ND
coleopteran	
RdP (lesser grain borer)	88.2 ± 0.45
CmP (cowpea weevil)	86.7 ± 0.34
ZsP (bean weevil	51.6 ± 1.23
AgP (boll weevil)	29.6 ± 0.23
lepidopteran	
SfP (armyworm)	75.5 ± 2.30
AaP (cotton leafworm)	53.8 ± 0.43
PiP (Indian meal moth)	26.7 ± 1.54
dipteran	
CcP (fruit fly)	52.9 ± 0.78

[a] Colepteran proteinases from *R. dominica* (RdP), *C. maculatus* (CmP), *Z. subfasciatus* (ZsP), and *A. grandis* (AgP). Lepidopteran proteinases from *S. frugiperda* (SfP), *A. argillacea* (AaP), and *P. interpunctella* (PiP). Dipteran proteinase from *C. capitata* (CcP). [b] Values are means ± standard error. ND, not detectable.

products. A later report showed that trypsin inhibitors were toxic to *Tribolium confusum* (flour beetle) larvae (*38*). Following these studies several research groups have investigated this protein family as candidates in the development of new strategies to pest control.

In this study a related trypsin inhibitor from *T. indica* seeds was purified and characterized, and its in vitro and in vivo potential insecticidal activity against different insect pests from Lepidoptera, Coleoptera, and Diptera orders was examined. The TTI purified here is a protein with a single polypeptide chain of a molecular mass of 21 420 Da, as observed by MALDI-TOF and SDS-PAGE (reducing and nonreducing conditions) analyses, which is consistent with the molecular mass of other Kunitz trypsin inhibitors (*39*−*44*). TTI is a noncompetitive inhibitor like ApTI [(*Adenanthera pavonina* trypsin inhibitor) (*20*) and *Pj* (*Prosopis juliflora* Kunitz-type trypsin inhibitor) (*19*). This is in opposition to the findings of Laskowsky and Kato (*45*), who proposed that serine proteinase inhibitors were

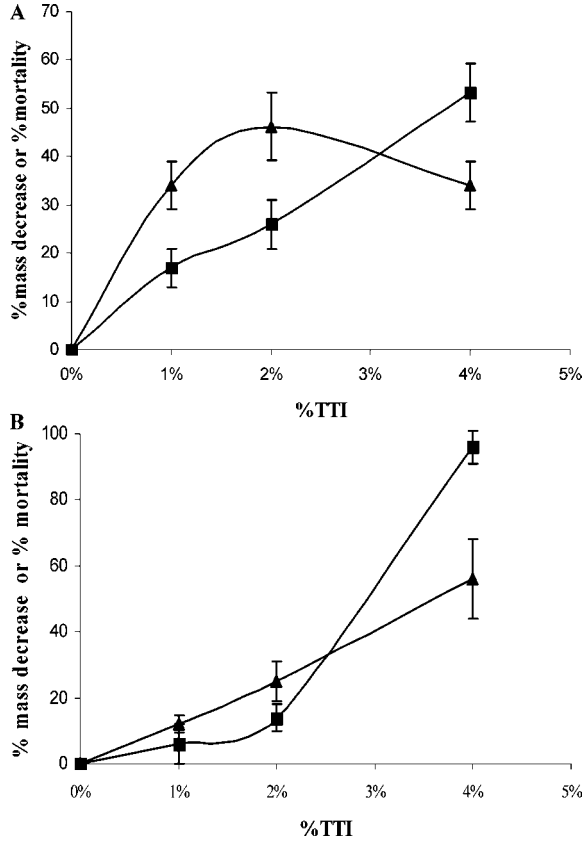

Figure 4. Effects of TTI (%, w/w) on larva development in artificial diet system: (**A**) *C. capitata* larvae, $n = 6 \times 21$; (**B**) *C. maculatus* larvae, $n = 6 \times 9$; (■) % larva mass decrease; (▲) % mortality larvae. Each mean represent six replicates (±SE).

competitive inhibitors. The K_i value of TTI was 1.7×10^{-9} M to trypsin, in agreement with those reported for Bowman−Birk and Kunitz-type inhibitors (*20*, *46*−*48*), but TTI did not inhibit chymotrypsin and other cysteine proteinases, such as papain and bromelain. The inhibitory activity of Kunitz-type proteinase inhibitors is varied, and few inhibitors of this family are specific for chymotrypsin (*20*, *49*). Until now only three related Kunitz-type inhibitors were found to be active toward papain-like enzymes and were also strongly active to enzymes from the midgut of bruchid larvae (*19*, *20*, *48*).

For an efficient establishment of a novel insect control strategy based on proteinaceous inhibitors, two initial steps are necessary: they are the inhibitor purification and knowledge of the digestive system from target insects. The digestive system from phytophagous pests is based principally on serine and cysteine proteinase classes; serine proteinases are the major enzymes found in Lepidoptera/Diptera orders, and acid and cysteine proteinases are predominant in the coleopteran of the Bruchidae family (*50*−*53*), but serine proteinases also were present (*54*). To verify the activity of purified TTI, digestive proteinases from coleopteran, lepidopteran, and dipteran pests were extracted and assayed against BAPNA, a specific trypsin substrate. TTI showed the highest inhibition to trypsin-like enzymes from *C. maculatus* (86.7 ± 0.34), *R. dominica* (88.2 ± 0.45), and *S. frugiperda* (75.5 ± 2.30). It was also observed to have moderate inhibitory activity toward gut proteinases of *A. argillacea* (53.8 ± 0.43) *C. capitata* (52.9 ± 0.78), and *Z. subfasciatus* (51.6 ± 1.23). A number of Kunitz related inhibitors from plants were evaluated in vitro for the potential to decrease the activity of digestive enzyme from lepidopteran/coleopteran larvae. Soybean Kunitz trypsin inhibitor (SKTI) showed a potent in vitro inhibitory activity against gut proteinases from *A. grandis* (*11*), SKTI, and cowpea trypsin inhibitor (CpTI) and to serine proteinases from larvae of the tomato moth (*L. oleracea*) (*24*) and others reported for *H. armigera*, *H. virenscen*, and *L. cuprina*, in which SKTI was the most effective among the inhibitors tested (*55*−*60*). *Adenanthera pavonina* trypsin inhibitor(ApTI) and *Dimorphandra mollis* trypsin inhibitor (DMTI-II) inhibited 84% (*20*) and 80% (*48*) of the digestive trypsin-like enzymes of *C. maculatus*, a Coleptera: Bruchidae, which are results similar to those for TTI.

On the basis of the concentration of proteinaceous inhibitors that occurs naturally in legume seeds, 1−10% of their total proteins (*61*, *62*), the potential bioinsecticidal effects of TTI at 1−4% doses (w/w) were tested in feeding trial models to *C. capitata* and *C. maculatus* larvae, two important and economical pests for agribusinesses of tropical and subtropical regions. Results showed different deleterious effects of TTI on the mass and survival of *C. capitata* and *C. maculatus* during larval developmental when larvae were fed with a diet containing different concentrations of the inhibitor. For *C. capitata*, the larval mass was decreased by 53.2%, and mortality was 34% at the level of 4% (w/w). This minor effect of TTI on the mortality of *C. capitata* larvae was probably due to the fact that its digestive system is based on both chymotrypsin- and trypsin-like serine proteinases (*63*). TTI is specific to trypsin-like enzymes and could explain high *C. capitata* survival, because chymotrypsin would be active in its digestive processes. However, these survivor larvae had delayed development with mass similar to those larvae of 2 days of age. This could be important due to the seasonality of this pest. Bioassays against dipteran insect pests are rare in the literature. For example, when SKTI was added at a level of 1.15% in artificial diets and offered to *Lucilia cuprina*, a strong reduction in the weight of larvae was observed of ∼80%. This susceptibility can be explained because the major proteinase of these larvae is a trypsin-like serine proteinase (*59*).

The effect of TTI on the development of *C. maculatus* larvae was examined in bioassays based on artificial seeds system. TTI produced ∼55.6% mortality of this bruchid when incorporated at level of 4%, with a lethal dose (LD_{50}) of 3.6%, and affected the larval mass by 50.0% at an effective dose (ED_{50}) of 3.2%. Only two recent studies have shown the effect of a trypsin inhibitor in feeding trial assays to *C. maculatus*. DMTI-II and ApTI from *D. mollis* (*48*) and *A. pavonina* seeds (*20*), respectively, were strongly active in in vivo bioassays with LD_{50} values between 0.5 and 1%. These Kunitz-type inhibitors also showed an unusual property of interaction with a chitin matrix column. This chitin binding behavior could explain the greater deleterious effect on *C. maculatus* larvae, because these bruchid larvae possess chitin components of the peritrophic membrane (or equivalent structures) in the midgut (*64*). Here, the occurrence of a high inhibition on the in vitro activity of TTI, similar to ApTI and DMTI-II, was an indicator that it may be possible to use TTI as an insecticidal agent. However, the in vivo assay of TTI had higher LD_{50} and ED_{50} values when compared to those of ApTI and DMTI-II, despite the fact that the TTI doses found here are included in the concentration range of natural occurrence of proteinaceous inhibitors in seeds. Probably, TTI and SKTI (*65*) are not chitin-binding proteins, such as ApTI and DMTI-II. This could explain the minor effect of TTI on *C. maculatus* larvae. Insect resistance to transgenic plants is happening, and using proteinase inhibitors for pest control, in

the future, can be assigned in association of inhibitors with diverse properties to target enzymes in a concerted manner.

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LITERATURE CITED

(1) Lawrence, P. K.; Koundal, K. R. Plant protease inhibitors in control of phytophagous insects. *Eletronic J. Biotechnol.* **1968**, *5* (1), 93−109.

(2) Krattiger, A. F. Insect resistance in crops: a case study of *Bacillus thunringiensis* (bt) and its transfer to developing countries. *ISAAA Briefs* **1997**, *2*, 42.

(3) Johnson, R.; Narvaez, J.; An, G.; Ryan, C. A. Expression of proteinases inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9871−9875.

(4) Broadway, R. M.; Duffey, S. A. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of the larvae *Heliothis zea* and *Spodoptera exigua*. *J. Insect Physiol.* **1986**, *34*, 1111−1117.

(5) Yeh, K. W.; Lin, M. I.; Tuan, S. J.; Chen, Y. M.; Lin, C. Y.; Kao, S. S. Sweet potato (*Ipomoea batatas*) trypsin inhibitors expressed in transgenic tobacco plants confer resistance against *Spodoptera litura*. *Plant Cell Rep*. **1997**, *16*, 686−699.

(6) Reed, B. J.; Chandler, D. S.; Sandeman, R. M. Aminopeptidases as potential targets for the control of the Australian sheep blowfly, *Lucilia cuprina*. *Int. J. Parasitol.* **1999**, *29*, 839−850.

(7) Gatehouse, A. M. R.; Boulter, D. Assessment of the antimetabolic effects of trypsin inhibitors on the development of the bruchid beetle, *Callosobruchus maculatus*. *J. Sci. Food Agric.* **1983**, *34*, 345−350.

(8) Leplé, J. C.; Bonadé-Bottino, M.; Augustin, S.; Pilate, G. Toxicity to *Chrysomela tremulae* (Coleoptera: Chrysomelidae) of transgenic poplars expressing a cysteine proteinase inhibitor. *Mol. Breed.* **1995**, *1*, 319−328.

(9) Gatehouse, A. M. R.;, Gatehouse, J. A. Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. *Pestic. Sci.* **1998**, *52*, 165−175.

(10) Franco, O. L.; Santos, R. C.; Araújo, M. A. M.; Mendes, A. C. M.; Batista, J. A. N.; Monnerat, R. G.; Grossi-de-Sá, M. F.; Freitas, S. M. Effects of black-eyed pea trypsin/chymotrypsin inhibitor on proteolytic activity and on development of *Anthonomus grandis. Phytochemistry* **2003**, *63*, 343−349.

(11) Franco, O. L.; Dias, S. C.; Magalhães, C. P.; Monteiro, A. C. S.; Bloch Jr., C.; Melo, F. R.; Oliveira-Neto, O. B.; Monnerat, R. G.; Grossi-de-Sá, M. F. Effects of Kunitz trypsin inhibitor on the cotton boll weevil (*Anthonomus grandis*). *Phytochemistry* **2004**, *65*, 81−89.

(12) Ryan, C. A. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu. Rev. Phytol.* **1991**, *28*, 425−449.

(13) Xavier-Filho, J. The biological roles of serine and cysteine proteinase inhibitors in plants. *Rev. Bras. Fisiol. Veg.* **1992**, *4* (1), 1−6.

(14) Solomon, M.; Belenghi, B.; Delledone, M.; Menachem, E.; Levine, A. The involvement of cysteine proteinase and proteinase inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell* **1999**, *11*, 431−443.

(15) Pernas, M.; Sanchez-Monge, R.; Salcedo, G. Biotic and abiotic stress can induce cystatin expression in chestnut. *FEBS Lett.* **2000**, *467*, 206−210.

(16) Franco, O. L.; Melo, F. R. Osmoprotectants, a plant strategy in response to osmotic stress. *Russ. J. Plant Physiol*. **2000**, *47*, 137−144.

(17) Bode, W.; Huber, R. Structural basis of the endoproteinase protein inhibitor interaction. *Biochim. Biophys. Acta* **2000**, *1477*, 241−252.

(18) Franco, O. L.; Grossi-de-Sá, M. F.; Sales, M. P.; Mello, L. V.; Oliveira, A. S.; Rigden, D. J. Overlapping binding sites for trypsin and papain on a Kunitz-type proteinase inhibitor from *Prosopis juliflora*. *Proteins* **2002**, *49*, 335−341.

(19) Oliveira, A. S.; Pereira, R. A.; Lima, L. M.; Morais, A. A. H.; Melo, F. R.; Franco, O. L.; Bloch Jr., C.; Grossi-de-Sá, M. F.; Sales, M. P. Activity toward bruchid pest of a Kunitz-type inhibitor from seeds of the algaroba tree (*Prosopis juliflora* D.C.). *Pest Biochem. Physiol.* **2002**, *72*, 122−132.

(20) Macedo, M. L. R.; Sá, C. M.; Freire, M. G. M.; Parra, J. R. P. A Kunitz-type inhibitor from *Adenanthera pavonina* L. seeds active against coleopteran pest proteases and its effect on the development of *Callosobruchus maculatus* (Coleoptera: Bruchidae). *J. Agric. Food Chem.* **2004**, *52*, 2533−2540.

(21) Walker, A. J.; Ford, L.; Majerus, M. E. N.; Geoghegan, A. E.; Birch, N.; Gatehouse, J. A.; Gatehouse, A. M. R. Characterization of the midgut digestive proteinase activity of the two-spot ladybird (*Adalia bipunctata*) and its sensitivity to proteinase inhibitors. *Insect Biochem. Mol. Biol*. **1998**, *28*, 173−180.

(22) Melo, F. R.; Rigden, D. J.; Franco, O. L.; Mello, L. V.; Ary, M. B.; Grossi-de-Sá, M. F.; Bloch Jr., C. Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking. *Proteins* **2002**, *48*, 311−319.

(23) Bode, W.; Huber, R. Natural protein proteinase inhibitor and their interaction with proteinases. *Eur. J. Biochem.* **1992**, *204*, 433−451.

(24) Gatehouse, A. M. R.; Norton, E.; Davison, G. M.; Babbe, S. M.; Newell, C. A.; Gatehouse, J. A. Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea;* effects of plant proteinase inhibitor in vitro and in vivo. *J. Insect Physiol.* **1999**, *45*, 545−558.

(25) Purcell, J. P.; Greenplate, J. T.; Sammons, R. D. Examination of midgut luminal proteinase activities in six economically important insects. *Insect Biochem. Mol. Biol.* **1992**, *22*, 41−47.

(26) Bradford, M. M. A rapid and sensitive method from quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(27) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(28) Dixon, M.; Webber, E. C.; Therone, C. J. R.; Tripton, K. F. Inhibition and activation. In *Enzymes*; Academic Press: New York, 1979; Vol. 1, pp 332−381.

(29) Xavier-Filho, J.; Campos, F. A. P.; Ary, M, B,; Silva, C. P.; Carvalho, M. M. M.; Macedo, M. L. R.; Lemos, F. J. A.; Grant, G. Poor correlation between the levels of proteinase inhibitors found in seeds of different cultivars of cowpea (*Vigna unguiculata*) and resistance/susceptibility to predation by *Callosobruchus maculatus*. *J. Agric. Food Chem.* **1989**, *37*, 1139−1143.

(30) Macedo, M. L. R.; Andrade, L. B. S.; Moraes, R. A.; Xavier-Filho, J. Vicilin variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). *Comp. Biochem. Physiol.* **1993**, *105C*, 89−94

(31) Odani, S.; Koide, T.; Ono, T. The complete amino acid sequence of barley trypsin inhibitor. *J. Biol. Chem.* **1983**, *258* (13), 7998−8003.

(32) Hilder, V. A.; Barker, R. F.; Samour, R. A.; Gatehouse, A. M. R.; Gatehouse, J. A.; Boulter, D. Protein and cDNA sequences of Bowman-Birk protease inhibitors from the cowpea (*Vigna unguiculata* Walp.). *Plant Mol. Biol.* **1989**, *13* (6), 701−710.

(33) Macedo, M. L. R.; Xavier-Filho, J. Purification and partial characterization of trypsin inhibitors from seeds of *Clitoria ternatea. J. Sci. Food Agric*. **1992**, *58*, 55−58.

(34) Mello, G. C.; Oliva, M. L. V.; Sumikawa, J. T.; Machado, O. L. T.; Marangoni, S.; Novello, J. C.; Macedo, M. L. R. Purification and characterization of a new trypsin inhibitor from *Dimorphandra mollis*. *J. Protein Chem.* **2002**, *20*, 625−632.

(35) Negreiros, A. N. M.; Carvalho, M. M.; Xavier-Filho, J.; Blanco-Labra, A.; Shewry, P. R.; Richardson, M. The complete amino acid sequence of the janor Kunitz trypsin inhibitor from the seeds of *Prosopis juliflora*. *Phytochemistry* **1991**, *30* (9), 2829−2833.

(36) Pearce, G.; Sy, L.; Russell, C.; Ryan, C. A.; Hass, G. M. Isolation and characterization from potato tubers of two polypeptide inhibitors of serine proteinases. *Arch. Biochem. Biophys.* **1982**, *213* (2), 456−462.
(37) Mickel, C. E.; Standish, J. Susceptibility of processed soy flour and soy grits in storage to attack by *Tribolium castaneum*. *Univ. Minn. Agric. Exp. Stn. Tech. Bull.* **1947**, *No. 178*, 1−20.
(38) Lipke, H.; Fraenkel, G. S.; Liener, I. E. Effects of soybean inhibitors on growth of *Tribolium confusum*. *J. Sci. Food Agric.* **1954**, *2*, 410−415.
(39) Lin, J. Y.; Chu, S. C.; Wu, H. C.; Hsieh, Y. S. Trypsin inhibitor from the seeds of *Acacia confusa*. *J. Biochem.* **1991**, *110*, 879−883.
(40) Batista, I. F. C.; Oliva, M. L. V.; Araújo, M. S.; Sampaio, M. U.; Richardson, M.; Fritz, H.; Sampaio, C. A. M. Primary structure of a Kunitz-type trypsin inhibitor from *Enterolobium contortisiliquum* seeds. *Phytochemistry* **1996**, *41* (4), 1017−1022.
(41) Macedo, M. L. R.; Matos, D. G. G.; Machado, O. L. T.; Marangoni, S.; Novello, J. C. Trypsin inhibitor from *Dimorphandra mollis* seeds: purification and properties. *Phytochemistry* **2000**, *54*, 553−558.
(42) Pando, S. C.; Oliva, M. L. V.; Sampaio, C. A. M.; Di Ciero, L.; Novello, J. C.; Marangoni, S. Primary sequence determination of a Kunitz inhibitor isolated from *Delonix regia* seeds. *Phytochemistry* **2001**, *57*, 625−631.
(43) Cavalcanti, M. S. M.; Oliva, M. L. V.; Fritz, H.; Jochum, M.; Mentele, R.; Sampaio, M.; Coelho, L. C. B. B.; Batista, I. F. C.; Sampaio, C. A. M. Characterization of a Kunitz trypsin inhibitor with one disulfide bridge purified from *Swartzia pickellii*. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 635−639.
(44) Teles, R. C. L.; Souza, E. M. T.; Calderon, L. A.; Freitas, S. M. Purification and pH stability characterization of a chymotrypsin inhibitor from *Schizolobium parahyba* seeds. *Phytochemistry* **2004**, *65*, 793−799.
(45) Laskowski, M., Jr.; Kato, I. Protein inhibitors of proteinases. *Annu Rev. Biochem.* **1980**, *49*, 593−626.
(46) Birk, Y. Chemistry and nutritional significance of proteinase inhibitors from plant sources. *Ann. N. Y. Acad. Sci.* **1968**, *146*, 388−399.
(47) Terada, S.; Fujimura, S.; Kino, S.; Kimoto, E. Purification and characterization of three proteinase inhibitors from *Canavalia lineata* seeds. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 371−375.
(48) Macedo, M. L. R.; Mello, G. C.; Freire, M. G. M.; Novello, J. C.; Marangoni, S.; Matos, D. G. G. Effect of a trypsin inhibitor from *Dimorphandra mollis* seeds on the development of *Callosobruchus maculatus*. *Plant Physiol. Biochem.* **2002**, *40*, 891−898.
(49) Garcia, V. A.; Freire, M. G. M.; Novello, J. C.; Marangoni, S.; Macedo, M. L. R. Trypsin inhibitor from *Poecilanthe parviflora* seeds: purification, characterization, and activity against pest proteases. *Protein J.* **2004**, *23* (5), 343−350.
(50) Lemos, F. J. A.; Campos, F. A. P.; Silva, C. P.; Xavier-Filho, J. Proteinases and amylases of larval midgut of *Zabrotes subfasciatus* eared on cowpea (*Vigna unguiculata*) seeds. *J. Appl. Entomol.* **1990**, *56*, 219−227.
(51) Silva, C. P.; Xavier-Filho, J. Comparison between the levels of aspartic and cysteine proteinases of the larval midgut of *Callosobruchus maculatus* (F.) and *Zabrotes subfascitus* (Boh.) (Coleoptera: Bruchidae). *Comp. Biochem. Physiol.* **1991**, *99B*, 529−533.
(52) Koiwa, H.; Shade, R. E.; Zhu-Salzman, K.; D'urzo, M. P.; Murdock, L. L.; Bressan, R. A.; Hasegawa, P. M. A plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases (DvCALs) in the larval midgut of western corn rootworm (*Diabrotica virgifera virgifera*). *FEBS Lett.* **2000**, *471*, 67−70.
(53) Girard, C.; Le Metayer, M.; Bonadé-Bottino, M.; Pham-Delegue, M.-H.; Jouanin, L. High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. *Insect Biochem. Mol. Biol.* **1998**, *28*, 229−237.
(54) Silva, C. P.; Terra, W. R.; Lima, R. M. Differences in midgut serine proteinases from larvae of the bruchid beetles *Callosobruchus maculatus* and *Zabrotes subfasciatus*. *Arch. Insect Biochem. Physiol.* **2001**, *47*, 18−28.
(55) Ahamad, Z.; Saleemuddin, M.; Siddi, M. Purification and characterization of three alkaline proteinases from the midgut of the larvae of army worm, *Spodoptera litura*. *Insect Biochem.* **1980**, *10*, 667−673.
(56) Johnston, K. A.; Lee, M.; Gatehouse, J. A.; Anstee, J. H. The partial purification and characterization of serine proteinase activity in midgut of larval *Helicoverpa armigera*. *Insect Biochem.* **1991**, *21*, 389−397.
(57) Ferreira, C.; Capella, A. N.; Sitnik, R.; Terra, W. R. Properties of the digestive enzymes and the permeability of the peritrophic membrane of *Spodoptera frugiperda* (Lepidoptera) larvae. *Comp. Biochem. Physiol.* **1994**, *107A*, 631−640.
(58) Johnston, K. A.; Lee, M.; Brough, C.; Hilder, V. A.; Gatehouse, A. M. R.; Gatehouse, J. A. Proteinases activity in the larval midgut of *Heliothis virenscens*: evidence for trypsin and chymotrypsin-like enzymes. *Insect Biochem. Mol. Biol.* **1995**, *25*, 375−383.
(59) Casu, R. E.; Jarmey, J. M.; Elvin, C. M.; Eisemann, C. H. Isolation of a trypsin-like serine proteinase gene family from the sheep blowfly *Lucilia cuprina*. *Insect Mol. Biol.* **1994**, *3* (3), 159−170.
(60) De Leo, F.; Bonadé-Botino, M.; Ceci, R. L.; Galerani, R.; Jouanin, L. Effects of mustard trypsin inhibitors expressed in different plants on three different lepidopteran pests. *Insect Biochem. Mol. Biol.* **2001**, *31*, 593−602.
(61) Ryan, C. A. Proteinase inhibitors. In *Biochemistry of Plants*; Marcus, A., Ed.; Academic Press: New York, 1981; Vol. 6, pp 351−370.
(62) Pearce, G.; Sy, L.; Russel, C.; Ryan, C. A.; Hass, G. M. Isolation and characterization from potato tubers of two polypeptide inhibitors of serine proteinases. *Arch. Biochem. Biophys.* **1982**, *213*, 456−462.
(63) Silva, F. C. B. L.; Macedo, L. L. P.; Açcazar, A.; Macedo, F. P.; Chavante, S. F.; Santos, E. A.; Sales, M. P. Identification and characterization of fruit fly (*Ceratitis capitata*) digestive enzymes during insect development. In *Abstracts of XXXII Meeting of the Brazilian Biochemistry and Molecular Biology Society*; Sao Paulo, Brazil, 2003; p 35.
(64) Sales, M. P.; Pimenta, P. P.; Paes, N. S.; Grossi-de-Sá, M. F.; Xavier-Filho, J. Vicilins (7S storage globulins) of cowpea (*Vigna unguiculata*) seeds bind to chitinous structures of the midgut of *Callosobruchus maculatus* (Coleoptera: Bruchidae) larvae. *Braz. J. Med. Biol. Res.* **2001**, *34*, 27−34.
(65) Sales, M. P.; Fernandes, K. V. S.; Gomes, V. M.; Xavier-Filho, J. Chitin-binding proteins from cowpea (*Vigna unguiculata*) seeds. *Braz. J. Med. Biol. Res.* **1996**, *29*, 319−326.

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