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Research paper

Effect of trypsin inhibitor from *Crotalaria pallida* seeds on *Callosobruchus maculatus* (cowpea weevil) and *Ceratitis capitata* (fruit fly)

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Abstract

A proteinaceous trypsin inhibitor was purified from *Crotalaria pallida* seeds by ammonium sulfate precipitation, affinity chromatography on immobilized trypsin–Sepharose and TCA precipitation. The trypsin inhibitor, named CpaTI, had M_r of 32.5 kDa as determined by SDS-PAGE and was composed of two subunits with 27.7 and 5.6 kDa linked by disulfide bridges. CpaTI was stable at 50 °C and lost 40% of activity at 100 °C. CpaTI was also stable from pH 2 to 12 at 37 °C. CpaTI weakly inhibited chymotrypsin and elastase and its inhibition of papain, a cysteine proteinase, were indicative of its bi-functionality. CpaTI inhibited, in different degrees, digestive enzymes from *Spodoptera frugiperda*, *Alabama argillacea*, *Plodia interpunctella*, *Anthonomus grandis* and *Zabrotes subfasciatus* guts. In vitro and in vivo susceptibility of *Callosobruchus maculatus* and *Ceratitis capitata* to CpaTI was evaluated. *C. maculatus* and *C. capitata* enzymes were strongly susceptible, 74.4 ± 15.8% and 100.0 ± 7.3%, respectively, to CpaTI. When CpaTI was added to artificial diets and offered to both insect larvae, the results showed that *C. maculatus* was more susceptible to CpaTI with an LD_{50} of 3.0 and ED_{50} of 2.17%. *C. capitata* larvae were more resistant to CpaTI, in disagreement with the in vitro effects. The larvae were more affected at lower concentrations, causing 27% mortality and 44.4% mass decrease. The action was constant at 2–4% (w/w) with 15% mortality and 38% mass decrease.

Keywords: Trypsin inhibitor; *Crotalaria pallida*; *Ceratitis capitata*; *Callosobruchus maculatus*; Resistance

1. Introduction

Beginning in 1980, new strategies of insect pest control, such as integrated pest management (IPM), use of proteinaceous compounds as sprays and more recently the use of transgenic plants have been proposed and tested, to avoid losses in crop production [1,2]. The use of proteinaceous inhibitors in insect control strategies has good potential, because insect digestive proteinases are promising targets in the control of various insects, including lepidopterans such as *Manduca sexta* [3], *Heliothis zea* [4], *Spodoptera litura* [5], and *Lucilia cuprina* [6], and also various coleopterans [7–11]. Despite several suggested physiological functions in plants [12–14], the inhibitors are known for their role in response to abiotic [15,16] and

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biotic stresses, especially in plant defense processes against insect pest attack [9,17–20]. These plant proteinaceous inhibitors are generally small, stable and abundant proteins [21] showing specificity for serine proteinase, cysteine proteinase, aspartic proteinase or metallo-proteinases [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 super family [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 were capable of inhibiting the proteolytic activity of several lepidopterans, such as the black cutworm (*Agrotis ipsilon*), corn earworm (*H. zea*), tobacco budworm (*Heliothis virescens*), Western spruce budworm (*Choristoneura occidentalis*) [25] and coleopterans such as the cotton boll weevil (*A. grandis*) [10]. Several plants have been screened in order to isolate and characterize such proteinase inhibitors, among them the species *Crotalaria pallida,* which belongs to the Fabaceae family (Sub-family Faboideae), the members of which are herbs, shrubs and trees found in both temperate and tropical areas. In this study, we have reported the purification and characterization of a proteinaceous trypsin inhibitor from *Crotalaria pallida* seeds. We have also tested its activity in vitro and in vivo, during the larval development of *Ceratitis capitata* (fruit fly) and *Callosobruchus maculatus* (cowpea weevil).

2. Material and methods

2.1. Material

The 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).

2.2. Purification of Crotalaria trypsin inhibitor (CpaTI)

Crotalaria pallida (Fabaceae) seeds were obtained from the IBAMA (Brazilian Environmental Institute) seed bank in Natal/RN-Brazil. Finely ground *Crotalaria* seed meal was extracted (1:10, w/v) with 0.05M Tris–HCl buffer pH 7.5, for 3 hours at room temperature. After centrifugation for 30 min at 12,000 × *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 anti-tryptic assays. The $F_{30–60}$ fraction, which corresponds to a 30–60% saturation range, showed a high level of inhibitory activity against trypsin. This fraction, denominated F_2, was applied to a trypsin–Sepharose affinity column (1 5 × 1.5 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.5. The retained proteins were eluted with 1 mM HCl solution at flow rate of 30 ml h^{-1}. The anti-tryptic peak was pooled and precipitated with 20% TCA solution to a final concentration of 2.5%. After 30 min of centrifugation at 15,000 × *g* at 4 °C, the supernatant was collected and dialyzed against water and freezer dried. This sample, with anti-tryptic activity, denoted CpaTI, was subjected to further analysis.

2.3. CpaTI 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 were incubated for 15 min at 37 °C with 100 ml of inhibitor solution and 120 ml 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 1% (v/v) DMSO and 0.05 M Tris–HCl, pH 7.5. After 15 min at 37 °C, the reaction was stopped by adding 150 μl of 30% acetic acid solution. The color developed was measured by absorbance at 405 nm. Assays without inhibitor were made and trypsin inhibition activity was determined by measuring the remaining enzymatic activity at pH 7.5 after preincubation with CpaTI (0.1 μg $μl^{-1}$). One unit of inhibitory activity was defined as the amount of inhibitor that decreased absorbance by 0.01 at 405 nm. All assays were performed in triplicate. The results of each series were expressed as the mean value ± S.D.

2.4. Protein determination

Protein content was measured according to the procedure of Bradford [26] with bovine serum albumin as protein standard.

2.5. Polyacrylamide gel electrophoresis

SDS polyacrylamide (12.5% and 25%) 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. The proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

2.6. Specificity of CpaTI for serine and cysteine proteinases

The ability of CpaTI to inhibit other serine proteinases (bovine chymotrypsin and porcine elastase) and cysteine proteinases (papain and bromelain) was assayed using azocasein as substrate, as described by Xavier-Filho et al. [28]. All assays were performed in triplicate. The results of each series were expressed as the mean value ± S.D.

2.7. Thermal and pH stability of CpaTI

Thermal stability of CpaTI (0.2 μg $μl^{-1}$) was tested by incubating the protein at different temperatures (37, 40, 50, 60, 70, 80, 90, and 100 °C) for 30 min. After cooling the samples at 4 °C for 10 min, the inhibitory assays against trypsin were performed. The stability in a broad range of pH was also

checked. Samples of CpaTI (0.2 μg μl^{-1}) solution were prepared with: 100 mM glycine-HCl (pH 2–4), 100 mM sodium phosphate (pH 5–8) and 100 mM glycine-NaOH (pH 9–12). After 1 hour of incubation at 37 °C, the samples were dialyzed against 50 mM Tris–HCl buffer, pH 7.5 and inhibitory assays against trypsin were performed. All assays were done in triplicate. The results of each series were expressed as the mean value ± S.D.

2.8. Preparation of the 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* 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 10,000 × *g* at 4 °C, for 10 min. The supernatants were then recovered and used for in vitro assays.

2.9. CpaTI inhibitory assay against proteinase extracts from insect pests

CpaTI effects on the proteolytic 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. CpaTI (0.1 μg ml^{-1}) was incubated with a 40 μl aliquot of gut extracts at 37 °C for 15 min before adding the substrate. 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 adding 150 μl of 30% acetic acid solution. The resulting color developed was measured by absorbance at 405 nm. Enzymatic assays were performed using BAPNA (1.25 mM) as substrate. All assays were done in triplicate. The results of each series were expressed as the mean value ± S.D.

2.10. Binding of CpaTI to chitin-column

To examine the possibility of CpaTI interaction with chitin, the inhibitor was chromatographed on a chitin column (5 ml bed volume) equilibrated with 50 mM Tris–HCl, pH 7.5. After adsorption of the proteins (10 mg ml^{-1}), the column was washed with the same buffer until the absorbance at 280 nm returned to zero, after which the adsorbed protein was eluted with 0.1 M HCl. Fractions (2 ml) were collected, and inhibitory activity against trypsin was assayed using BAPNA as substrate.

2.11. Insect bioassay

To examine the effects of CpaTI on *C. maculatus* development, the artificial seed system previously developed by Macedo et al. [29] was used. Artificial seeds (ca. 400 mg each) were made from finely ground cowpea seeds (Epace 10 cultivar) using a cylindrical brass mill and a hand press. Artificial seeds containing CpaTI at concentrations of 0.5%, 1%, 2% and 4% (w/w) were obtained by thoroughly mixing the CpaTI 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 adjustment period in the growth chamber, the seeds were offered to nine 2–3-day-old fertilized females. After allowing 24 h 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 (4th instar) were determined. The experiments were carried out with six replicates and the mean (± S.E.M.) was calculated. Artificial control seeds were made with Epace-10 cultivar meal without CpaTI.

The performance of *C. capitata* in an artificial diet system was assessed. Artificial diets (ca. 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. Lyophilized CpaTI at standard concentrations of 0.5%, 1.0%, 2.0% and 4.0% w/w was added to the diet. The diets were presented to seven neonate larvae per diet ($n = 6 \times 21$) in a dark glass vial 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 larvae were recorded. The experiments were carried out with six replicates and the mean (± S.E.M.) was calculated. Artificial Control diets were made without CpaTI.

3. Results

3.1. Inhibitor isolation and CpaTI purification

Crude soluble protein extract obtained from the mature *Crotalaria* seeds was initially precipitated at 30%, 60% and 90% saturation with ammonium sulfate and three protein fractions (F_1, F_2 and F_3) were obtained. The F_2 protein fraction obtained showed strong inhibitory activity against trypsin, while the other fractions exhibited low inhibitory activity. The F_2 was then applied to a trypsin–Sepharose affinity column and the retained peak was assayed against trypsin. (Fig. 1A). The anti-tryptic peak was pooled and precipitated with 20% TCA solution to final concentration of 2.5%. This purification procedure to trypsin inhibitor from *C. pallida* seeds was observed by SDS-PAGE (Fig. 1B) and resulted in a high purification of 180.4-fold with a 0.5% yield (Table 1).

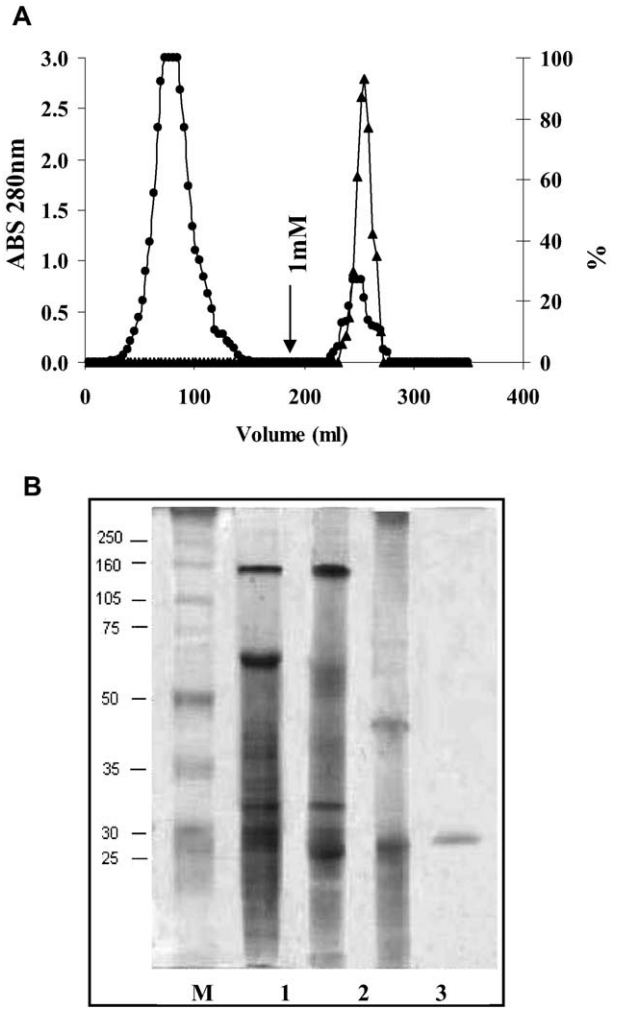

Fig. 1. (**A**) Elution profile on trypsin–Sepharose 4B of F_2, from *C. pallida* seeds. The column equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, and retained peak was eluted with 1 mM HCl solution, monitored at 280 nm (●) and assayed against trypsin (▲). (**B**) SDS-PAGE at 12% of purified CTI, stained with Coomassie Blue. (M) Protein molecular weight markers (Full-Range Rainbow Molecular Weight Markers); (CE).Crude extract; (F_2) $F_{30–60}$; (F_2/AF) Peak retained in trypsin–Sepharose 4B; (CTI) *Crotalaria* trypsin inhibitor.

Table 1
Purification steps of Kunitz trypsin inhibitor from *C. pallida* seeds

Steps	Volume (ml)	Total protein (mg)	Total IU [a]	Specific activity (UI/mg)	Purification (×)	Recovery (%)
CE	640	3584	10048	2.8	1.0	100
F2	375	787.5	6828	8.7	3.1	67.9
F2-AF	9.0	1.8	201.6	112	40.0	2.0
CTI	1.0	0.1	50.5	505	180.4	0.5

[a] One Inhibition Unit is defined as the amount of the inhibitor that decreased the absorbance at 410 nm by 0.01 O.D. under the trypsin inhibition assay.

3.2. Electrophoretic analysis of CpaTI

Electrophoretic analysis of *CpaTI* in the presence and absence of a reducing agent (β-mercaptoethanol), showed one protein band with molecular mass of approximately 32.5 kDa

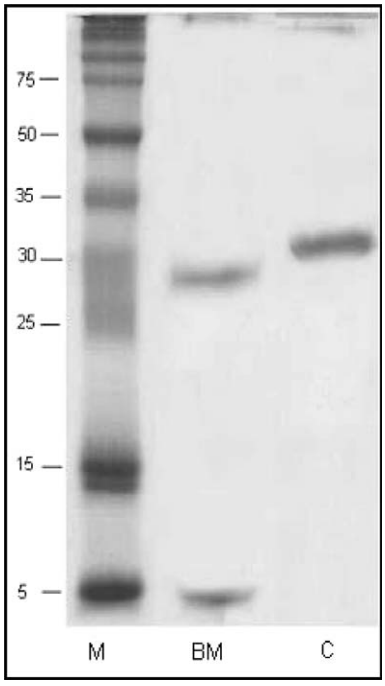

Fig. 2. SDS-PAGE at 25% of CTI after treatment with β-mercaptoethanol. (M) Protein molecular weight markers (Full-Range Rainbow Molecular Weight Markers); (BM) CTI treated with β-mercaptoethanol; (C) CTI without treatment.

which after treatment with β-mercaptoethanol decomposed to two subunits of 27.6 and 5.6 kDa (Fig. 2).

3.3. Thermal and pH stabilities of CpaTI

The study of the temperature effect on CpaTI showed that the inhibitory activity was stable at 80 °C, losing only 40% of activity at 100 °C. (Fig. 3A). Preincubation of the inhibitor in the pH range (2.0–12.0) did not affect trypsin inhibitory activity (Fig. 3B).

3.4. Specificity of CpaTI to serine and cysteine proteinases

CpaTI weakly inhibited elastase (15.9 ± 7.4%) and chymotrypsin (36.2 ± 7.9%). The inhibition of papain (43.9 ± 8.6% of inhibition), a cysteine proteinase was indicative of the bi-functionality of CpaTI. Inhibition against bromelain was not detected (Table 2).

3.5. In vitro activity of CpaTI for proteinase from different orders of insect pests

The digestive proteinases from Coleopteran and Lepidopteran pests were tested (Table 2). Among the different gut proteinases tested, CpaTI showed high in vitro inhibitory effect on *S. frugiperda* (100.0 ± 18.4%), *A. argillacea* (97.9 ± 17.2%). Moderate inhibitory activity was also observed for gut protei-

nases of *P. interpunctella* (66.8 ± 7.4%), *A. grandis* (52.1 ± 10.1%); and low inhibitory activity against gut proteinase from. *Z. subfasciatus* (36.5 ± 13.7%).

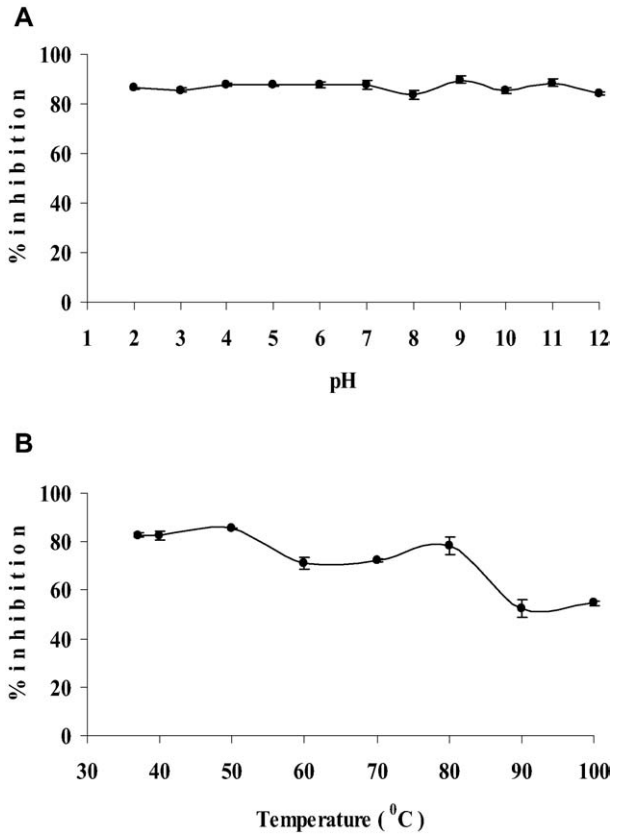

Fig. 3. Stability of CTI. (**A**) pH stability of CTI after incubation at the indicated pH at 37 °C; (**B**) Temperature stability of CTI inhibitory activity after incubation for 30 min at the indicated temperature. The residual trypsin inhibitory activity was assayed by using BAPNA in 50 mM Tris–HCl, pH 7.5. Each mean represent three replicates (±S.E.).

Table 2
Inhibitory activity of Inhibitor CpaTI towards proteinases and gut insect proteinases

Enzymes [a]	Inhibition (%) [b]
Serine proteinases	
Porcine elastase	15.9 ± 7.4
Bovine chymotrypsin	36.2 ± 7.9
Cysteine proteinases	
Papain	43.9 ± 8.6
Bromelain	ND
Coleopteran	
CmP (cowpea weevil)	74.4 ± 15.8
ZsP (bean weevil	36.5 ± 13.7
AgP (boll weevil)	52.1 ± 10.1
Lepidopteran	
SfP (armyworm)	100.0 ± 18.4
AaP (cotton leafworm)	97.9 ± 17.2
PiP (Indian meal moth)	66.7 ± 7.4
Dipteran	
CcP (Fruit Fly)	100.0 ± 7.3

[a] Coleopteran 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). *ND (not detectable).

[b] Values are mean ± standard error.

3.6. In vitro and in vivo effect of CpaTI on C. maculatus and C. capitata

Enzymes from *C. maculatus and C. capitata* guts were strongly susceptible to CpaTI in the in vitro assays, producing inhibition of 74.4 ± 15.8% and 100.0 ± 7.3%, respectively. Based on these in vitro enzymatic studies, standard feeding trials were carried out to assess the potential effects of CpaTI on *C. capitata* and *C. maculates,* which were used as models. Panels A and B of Fig. 4 show the influence of CpaTI on the mass and number of *C. capitata* and *C. maculates* survivors during larval development when the larvae were fed with a diet containing different CpaTI concentrations The inhibitor added to the diet of these pests in artificial seeds was moderately effective against *C. maculatus* and caused mortality to this bruchid (LD_{50}: 2.1%) and reduced the mass of larvae (ED_{50}: 3.2%). In artificial diets fed to *Ceratitis capitata*, the larvae were more affected in initial concentrations, causing 27% mortality and 44.4% mass decrease. The action was constant at 2–4% (w/w) with 15% mortality and 38% mass decrease.

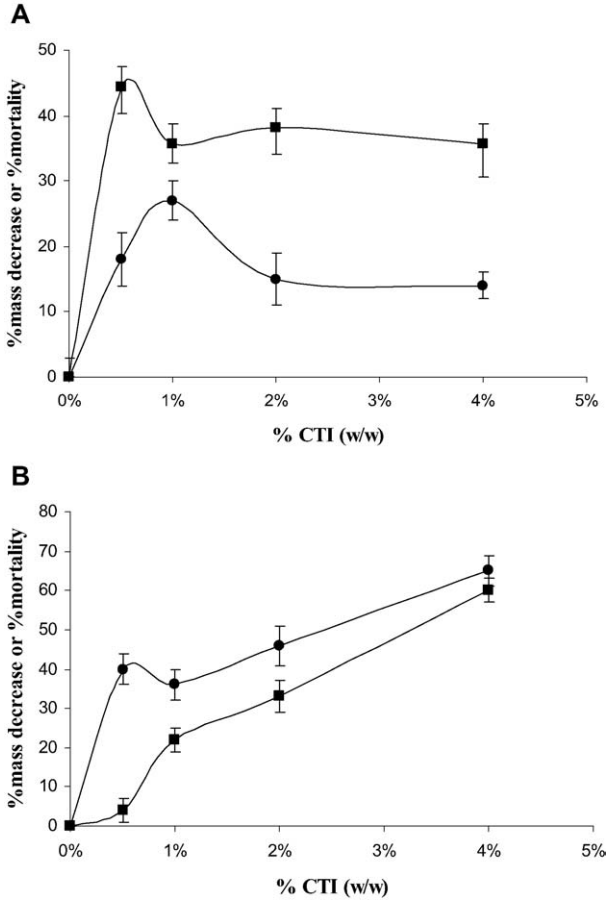

Fig. 4. In vivo bioinsecticidal activities of CTI (**A**) on *C. capitata* larvae and (**B**) on *C. maculatus larvae*: (■) % larvae mass; (♦) % survival larvae. Each mean represent six replicates (±S.E.).

4. Discussion

Proteinaceous inhibitors have been purified and characterized from a variety of plant sources [30–35]. The role of these inhibitors as defensive compounds against predators was studied as early as 1947 when Mickel and Standish [36] observed that larvae of certain insects were unable to develop on soybean products. A later report showed that trypsin inhibitors were toxic to *Tribolium confusum* (flour beetle) larvae [37]. Following these studies several research groups have investigated these proteins as candidates for developing new pest control strategies.

In this study, a related trypsin inhibitor from *C. pallida* seeds was purified, characterized and its deleterious effects against insect pests were examined in vitro and in vivo. The CpaTI purified here is a protein with two polypeptide chains of molecular mass of 27.6 and 5.6 kDa, as observed by SDS-PAGE (reducing and non-reducing conditions) analysis, which is similar to the molecular mass of other trypsin inhibitors [38–44]. The specificity of the inhibitory activity of the Fabaceae family serine proteinase inhibitors varies. Some Kunitz-type inhibitors isolated from the most primitive species of the Caesalpinioideae subfamily have only activity for trypsin [45]. Others from the Papilionoideae or Faboideae subfamily contain special chymotrypsin inhibitors and are known as Bowman–Birk-type inhibitors. However, Kunitz-type inhibitors are also found, suggesting that there is some relationship between the inhibitor families found in Fabaceae seeds and the evolution of these plants [46]. Here, CpaTI, isolated from *Crotalaria pallida* seeds, belonging to the Papilionoideae or Faboideae subfamily, weakly inhibited other serine proteinases, such as elastase and chymotrypsin, and was active against papain, a cysteine proteinase. So far, only three related Kunitz-type inhibitors, all belonging to the Mimosoideae sub-family, were found to be active against papain-like enzymes, and were strongly active against enzymes from the midgut of bruchid larvae [19,20,47], suggesting that Kunitz-type inhibitors from both subfamilies are bifunctional inhibitors.

Intramolecular disulfide bridges are presumably responsible for the functional stability of Kunitz-type inhibitors in the presence of physical and chemical denaturants such as temperature, pH and reducing agents. Thermal inactivation of CpaTI at different temperatures resulted in a progressive loss of trypsin-inhibiting activity at temperatures >80 °C and ~40% decrease at 100 °C. These results indicate that, in its native state, CpaTI has high intrinsic stability due to the presence of disulfide bridges. Its high degree of thermal stability was also similar to other trypsin inhibitors [40,45,482]. For the same reasons, the inhibitory activity of CpaTI was not sensitive to pH over the range 2–12; a similar result was reported for ACTI [39], DMTI-II [33] and PDTI [45].

To effectively establish a novel insect control strategy based on proteinaceous inhibitors, two initial steps are necessary: inhibitor purification and knowledge of the digestive system of target insects. The digestive system of phytophagous pests is based mainly on serine and cysteine proteinase classes; serine proteinases are the major enzymes found in Lepidoptera and Diptera orders. Acid and cysteine proteinases are predominant in Coleopterans of the Bruchidae family [49–52], but serine proteinases are also present [53]. In order to verify the activity of purified CpaTI, the digestive proteinases of Coleopteran, Lepdopteran and Dipteran pests were extracted and assayed against BApNA, a specific trypsin substrate. CpaTI showed high in vitro inhibitory effect on *S. frugiperda* and *A. argillacea*. Moderate inhibitory activity was observed for *P. interpunctella* and *A. grandis* gut proteinases; and low inhibitory activity against *Z. subfasciatus* (Bean weevil) gut proteinase. Enzymes from *C. maculatus and C. capitata* guts were strongly susceptible to CpaTI in the in vitro assays, producing inhibition of 74.4 ± 15.8% and 100.0 ± 7.3%, respectively. A number of Kunitz-related inhibitors from plants were evaluated in vitro for their potential of decreasing the activity of Lepdopteran/Coleopteran larvae digestive enzymes. SKTI (soybean Kunitz trypsin inhibitor) showed a potent in vitro inhibitory activity against *A. grandis* gut proteinases [11]. SKTI and CpTI (cowpea trypsin inhibitor) were shown to be active against tomato moth larvae (*L. oleracea*). Serine proteinases [24] acted on *H. armigera*, *H. virenscens* and *L. cuprina,* in which SKTI was the most effective among the inhibitors tested [54–59]. ApTI (*Adenanthera pavonina* trypsin inhibitor) and DMTI-II (*Dimorphandra mollis* trypsin inhibitor) inhibited 84% [20] and 80% [47] of the digestive trypsin-like enzymes of *C. maculatus,* a Colepteran (Bruchidae). These results are similar to those found for CpaTI.

On the basis of the concentration (1–10% of total protein) of proteinaceous inhibitors that occur naturally in legume seeds [60,61], the potential bioinsecticidal effects of CpaTI at 1–4% doses (w/w) were tested in feeding trial models for *C. capitata* and *C. maculatus* larvae, two important and economical pests in tropical and subtropical regions. Results showed deleterious effects of CpaTI on the growth and survival of *C. capitata* and *C. maculatus* during larval development. CpaTI was more active at low concentrations causing mortality (27%) and mass decrease (44.4%) at 0.55 (w/w) to *C. capitata*. The larval mass was decreased by 38%, and the mortality was 15% at 4% (w/w) of CpaTI, which are in disagreement with results obtained in vitro, wherein the inhibitor was strongly active causing 100% inhibition of the trypsin-like enzymes from the larval guts. CpaTI weakly inhibited chymotrypsin and this could be responsible for the minor effect of CpaTI on the mortality of *C. capitata* larvae, since the digestive system of these larvae is based on both chymotrypsin- and trypsin-like serine proteinases [62]. The activation of chymotrypsin-like enzymes could explain the high rate of survival of *C. capitata*. However, these surviving larvae had delayed development with a mass similar to the 3-day-old larvae. This could be important due to the seasonality of this pest. Bioassays against Dipteran insect pests are sparse in the literature. For example, when SKTI was added at a concentration of 1.15% in artificial diets and offered to *Lucilia cuprina*, a strong reduction in larval weight of ~80% was observed. This susceptibility can be explained by the fact that the major proteinase of these larvae is a trypsin-like serine proteinase [58].

The effect of CpaTI on the development of *C. maculatus* larvae was examined in bioassays based on an artificial seed system. CpaTI had a lethal dose (LD_{50}) of 3.0% and affected the larval mass by 50.0% at an effective dose (ED_{50}) of 2.17%. Two recent studies have shown the effect of a trypsin inhibitor in feeding trial assays for *C. maculatus*. DMTI-II and ApTI from *D. mollis* [47] and *A. pavonina* seeds [20], respectively, were strongly active in the 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, since these bruchid larvae possess chitin components of the peritrophic membrane (or equivalent structures) in the midgut [63]. Here, the occurrence of a high inhibition on CpaTI in vitro activity, similar to that of ApTI and DMTI-II, indicates that it may be possible to use CpaTI as an insecticidal agent. However, the in vivo CpaTI assay had higher LD_{50} and ED_{50} values when compared to those of ApTI and DMTI-II, despite the fact that the CpaTI doses found here are included in the concentration range of natural proteinaceous inhibitor occurrence in seeds. CpaTI and SKTI [64] are not chitin-binding inhibitors, unlike ApTI and DMTI-II. This could explain the minor effect of CpaTI on *C. maculatus* larvae. Insect resistance to transgenic plants is a possibility and proteinase inhibitors along with other toxins/inhibitors possessing diverse properties may be used for future pest control by targeting enzymes in a concerted manner.

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