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Plant Physiology and Biochemistry 43 (2005) 1095–1102
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research paper

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

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Received 12 June 2005
Available online 07 December 2005

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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0981-9428/$ - see front matter © 2006 Elsevier SAS. All rights reserved.
doi:10.1016/j.plaphy.2005.11.004

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