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Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds

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Abstract

Cotton (*Gossypium hirsutum* L.) is an important agricultural commodity, which is attacked by several pests such as the cotton boll weevil *Anthonomus grandis*. Adult *A. grandis* feed on fruits and leaf petioles, reducing drastically the crop production. The predominance of boll weevil digestive serine proteinases has motivated inhibitor screenings in order to discover new ones with the capability to reduce the digestion process. The present study describes a novel proteinase inhibitor from chickpea seeds (*Cicer arietinum* L.) and its effects against *A. grandis*. This inhibitor, named CaTI, was purified by using affinity Red-Sepharose Cl-6B chromatography, followed by reversed-phase HPLC (Vydac C18-TP). SDS-PAGE and MALDI-TOF analyses, showed a unique monomeric protein with a mass of 12,877 Da. Purified CaTI showed significant inhibitory activity against larval cotton boll weevil serine proteinases (78%) and against bovine pancreatic trypsin (73%), when analyzed by fluorimetric assays. Although the molecular mass of CaTI corresponded to α-amylase/trypsin bifunctional inhibitors masses, no inhibitory activity against insect and mammalian α-amylases was observed. In order to observe CaTI in vivo effects, an inhibitor rich fraction was added to an artificial diet at different concentrations. At 1.5% (w/w), CaTI caused severe development delay, several deformities and a mortality rate of approximately 45%. These results suggested that CaTI could be useful in the production of transgenic cotton plants with enhanced resistance toward cotton boll weevil.
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Keywords: Serine proteinase inhibitor; *Cicer arietinum*; *Anthonomus grandis*; Plant defense; Cotton; Mass spectrometry; Trypsin; Digestive enzymes

1. Introduction

Cotton crops (*Gossypium hirsutum* L.) are extremely important in providing income to millions of farmers in tropical and subtropical areas of the New World. Cotton serves as an engine of economic growth and thus contributes to food security worldwide. Cotton boll weevil *Anthonomus grandis* is an important insect pest responsible for severe cotton crop damage in several countries of America. Adults feed on fruit and leaf petioles, reducing crop production and damaging cotton fibers (Haynes and Smith, 1992; Alves et al., 1993). The larval phase is endophytic and is not efficiently controlled by pesticides and adult insects are

Abbreviations: AgPL, *Anthonomus grandis* proteinase larvae; AoPL, *Acanthoscelides obtectus* proteinase larvae; BBI, Bowman-Birk inhibitor; BPC, Bovine pancreatic chymotrypsin; BPT, Bovine pancreatic trypsin; BTCI, Black-eyed pea trypsin chymotrypsin inhibitor; CaRP, *Cicer arietinum* Red-sepharose retained peak; CaTI, *Cicer arietinum* trypsin inhibitor; CmPL, *Callosobruchus maculatus* proteinase larvae; CpTI, Cowpea trypsin inhibitor; HPLC, High performance liquid chromatography; HPT, Human pancreatic trypsin; MALDI-TOF, Matrix assisted laser desorption ionizated-time of flight; OCI, Oryzacystatin inhibitor; PPA, porcine pancreatic α-amylase; RASI, Rice α-amylase/subtilisin inhibitor; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SKTI, soybean Kunitz trypsin inhibitor; SfPL, *Spodoptera frugiperda* proteinase larvae.

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able to move to safe areas, escaping from toxic chemicals. Under favorable conditions, insects move back to the crop area to complete their life cycle (França, 1993).

Insect digestive proteinases are promising targets in the control of several insect pests since insects obtain essential amino acids by using extra-cellular proteinases secreted into the midgut lumen, digesting soluble and structural proteins. Despite the predominance of digestive cysteine proteinase-like activity in some coleopterans as the bean bruchids *A. obtectus*, *Z. subfasciatus* and *C. maculatus* (Wielman and Nielsen, 1988; Zhu-Salzman et al., 2003; Melo et al., 2003), the Colorado potato beetle *Leptinotarsa decemlineata*, the black vine weevil *Otiorynchus sulcatus* (Michaud et al., 1996) and phytophagous beetle, *Phaedon cochleariae* (Girard and Jouanin, 1999); the cotton boll weevil *A. grandis* and others pests including the coleopteran *Tenebrio molitor* (Lee et al., 2002), *Holotrichia diomphalia* (Kwon et al., 2000), the lepidopterans *Manduca sexta* (Johnson et al., 1989), *Heliothis zea* (Broadway and Duffey, 1986), *Spodoptera litura* (Yeh et al., 1997) and the dipteran *Lucilia cuprina* (Reed et al., 1999) have large quantities of digestive serine proteinases. For this reason several plant serine proteinase inhibitor screenings have been undertaken in order to identify inhibitors highly effective against digestive enzymes (Leplé et al., 1995; Gatehouse and Gatehouse, 1998; Bode and Huber, 2000; Franco et al., 2004).

The serine proteinase inhibitor family is composed of abundant, small and stable molecules found in plant storage tissues such as seeds, tubers, leaves and fruits (Richardson, 1977, 1991; Haq and Khan, 2003). Various functions have been suggested for these plant proteinase inhibitors, including their action as storage proteins (Xavier-Filho, 1992), regulators of endogenous proteolytic activity (Ryan, 1991) and components of programmed plant cell death mechanisms (Solomon et al., 1999). Mostly serine proteinase inhibitors are known for their function in response to abiotic stresses (Franco and Melo, 2000) and in plant defense processes against pests and pathogens (Gatehouse and Gatehouse, 1998) reacting with cognate enzymes, binding to catalytic sites in a canonical fashion (Laskowski and Kato, 1980; Grütter et al., 1990). All canonical inhibitors possess an exposed loop that simulates the substrate, with a rapid binding and a slow dissociation mechanism (Laskowski and Kato, 1980; Grütter et al., 1990; Richardson, 1992). Despite strong efforts dedicated to understanding the structure and specificity of canonical serine proteinase inhibitors, there are still unanswered questions awaiting detailed investigation concerning mechanistic aspects of inhibitors in plant defense. Due to the complexity of enzyme/inhibitor interactions, the choice of an efficient inhibitor will determine the success of pest a control strategy. Our main goals in this study were the purification and characterization of a novel serine-proteinase inhibitor (CaTI) from chickpea seeds (*C. arietinum*), and evaluation of the in vitro and in vivo activity of this inhibitor against the cotton boll weevil *A. grandis*.

2. Materials and methods

2.1. Purification of C. arietinum trypsin inhibitor (CaTI)

Chickpea seeds (*C. arietinum*) were triturated into a fine textured flour. The flour was defatted with acetone 50% and was extracted with a solution of 0.5 M Tris–HCl pH 7.0 containing 100 mM NaCl in the proportion of 1:3 (w/v) for 4 h under agitation at 4 °C. This extract was centrifuged at 5000×*g* for 40 min at 4 °C. Precipitate was discarded and the supernatant was dialyzed against distilled water. Crude extract was precipitated with ammonium sulphate (100%), following further centrifugation. Precipitate was dissolved and dialyzed against distilled water. This fraction was applied onto a Red-Sepharose Cl-6B affinity column equilibrated to 0.1 M Tris–HCl buffer, pH 7.0 containing 5.0 mM $CaCl_2$. The retained peak (CaRP) was eluted using a single step of 0.1 M Tris–HCl buffer, pH 7.0 containing 3.0 M NaCl. Fractions of 3.0 mL were collected and the optical density was measured at wavelength of 280 nm. After dialysis and lyophilization, 4.0 mg of CaRP fraction was dissolved in 250 μL of 0.1% trifluoroacetic acid and applied onto a semi-preparative reversed phase HPLC column (Vydac C18-TP) and eluted with a liner acetonitrile gradient (0–100%) generating several peaks. The purified *Cicer arietnum* trypsin inhibitor, which was named CaTI, composed one of them.

2.2. Isolation of fluid from larval pest midgut

A. grandis and *Spodoptera frugiperda* larvae were obtained from the Biological Control Department of EMBRAPA/Cenargen (Brasília-DF, Brasil). Larvae were reared on an artificial diet (Monnerat et al., 1999) at 25 °C and 55% relative humidity. Furthermore, *Callosobruchus maculatus* and *Acanthoscelides obtectus* were reared in cowpea and common bean seeds, respectively. The guts were dissected from larvae and placed into an iso-osmotic saline (0.15 M NaCl). Midgut tissues were homogenized and centrifuged for 10 min at 16,000×*g* at 4 °C. The supernatant was removed and used for enzymatic assays.

2.3. Proteinase inhibitory assays

Bovine and human pancreatic trypsin (BPT and HPT), as well bovine pancreatic chymotrypsin were purchased from Sigma, St. Louis, MO, USA, and proteinase extracted from larvae of *A. grandis* (AgPL), *S. frugiperda* (SfPL), *A. obtectus* (AoPL) and *C. maculatus* (CmPL) were used for enzymatic assays. Proteolytic inhibitory activities were tested against AgPL, SfPL, CmPL, AoPL and BPT using 10 mM of fluorogenic peptide Z-CBZ-Phe-Arg-7-MCA (Sigma) and against bovine pancreatic chymotrypsin (BPC) using 10 mM of fluorogenic peptide Ala-Ala-Pro-Phe-MCA. Assays were performed in 25 mM Tris–HCl, pH 6.5 and 20 mM DMSO according to Solomon et al. (1999).

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