Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 63 (2003) 343–349
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Effects of black-eyed pea trypsin/chymotrypsin inhibitor on proteolytic activity and on development of *Anthonomus grandis*

Octávio L. Franco[a,b,*], Roseane C. dos Santos[a,d], João A.N. Batista[a], Ana Cristina M. Mendes[a], Marcus Aurélio M. de Araújo[c], Rose G. Monnerat[a], Maria Fátima Grossi-de-Sá[a], Sonia M. de Freitas[c]

[a]*EMBRAPA Recursos Geneticos e Biotecnologia, Brasília-DF 70770 900, Brazil*
[b]*Universidade Católica de Brasília, Brasília-DF 70770 900, Brazil*
[c]*Universidade de Brasília, Brasília-DF, 70910 900, Brazil*
[d]*EMBRAPA/Algodão, Campina Grande–PB, Brazil*

Received 4 September 2002; received in revised form 24 January 2003

Abstract

The cotton boll weevil *Anthonomus grandis* (Boheman) is one of the major pests of cotton (*Gossypium hirsutum* L.) in tropical and sub-tropical areas of the New World. This feeds on cotton floral fruits and buds causing severe crop losses. Digestion in the boll weevil is facilitated by high levels of serine proteinases, which are responsible for the almost all proteolytic activity. Aiming to reduce the proteolytic activity, the inhibitory effects of black-eyed pea trypsin/chymotrypsin inhibitor (BTCI), towards trypsin and chymotrypsin from bovine pancreas and from midguts of *A. grandis* larvae and adult insects were analyzed. BTCI, purified from *Vigna unguiculata* (L.) seeds, was highly active against different trypsin-like proteinases studied and moderately active against the digestive chymotrypsin of adult insects. Nevertheless, no inhibitory activity was observed against chymotrypsin from *A. grandis* larval guts. To test the BTCI efficiency in vivo, neonate larvae were reared on artificial diet containing BTCI at 10, 50 and 100 μM. A reduction of larval weight of up to approximately 54% at the highest BTCI concentration was observed. At this concentration, the insect mortality was 65%. This work constitutes the first observation of a Bowman–Birk type inhibitor active in vitro and in vivo toward the cotton boll weevil *A. grandis*. The results of bioassays strongly suggest that BTCI may have potential as a transgene protein for use in engineered crop plants modified for heightened resistance to the cotton boll weevil.

Keywords: *Gossypium hirsutum*; Plant defense; Cotton; *Anthonomus grandis*; Black-eyed pea trypsin/chymotrypsin inhibitor; Serine proteinases; Cowpea

1. Introduction

Cotton boll weevil, *Anthonomus grandis* (Boheman) is a pest in tropical and subtropical areas of the New World. This coleopteran feeds on the fruits and floral buds of cotton and uses these tissues as appropriate habitat, causing severe damages to cotton crops (Haynes and Smith, 1992). Most biopesticides and chemical insecticides are inefficient against the boll weevil due its endophytic larval development, which is totally isolated from insecticides (Alves et al., 1993).

Protein digestion in the boll weevil is facilitated mainly by serine proteinases in the midgut lumen (Purcell et al., 1992). The inhibition of serine proteinases provides a promising mechanism to control a range of pests, such as *Plutella xylostela* (L.), *Mamestra brassicae* (L.), *Spodoptera litorallis* (Boisd.) (De Leo et al., 2001) and *Lucilia cuprina* (Wied.) (Reed et al., 1999). Nevertheless, only a few studies have been explored the potential use of serine proteinase inhibitors in the control of coleopteran pests (Gatehouse and Boulter, 1983; Leplé et al., 1995).

Several serine proteinase inhibitors have been isolated and characterized from plant storage tissues such as

* Corresponding authors. Universidade Católica de Brasília, Pós-Graduação em Ciências Genômiças e Biotecnologia, 916N, Brasília, DF. Tel.: +55-61-448-4705; fax +55-61-340-3658.
E-mail addresses: ocfranco@cenargen.embrapa.br (O.L. Franco),

0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00108-0

Nomenclature

AgPL	*Anthonomus grandis* proteinases from larvae
AgPA	*Anthonomus grandis* proteinases from adult insects
MCA	amidomethylcoumarin
BPC	bovine pancreatic chymotrypsin
BPT	bovine pancreatic trypsin
BTCI	black-eyed pea trypsin chymotrypsin inhibitor
MALDI-TOF	Matrix assisted laser desorption ionizated time-of-flight
SKTI	soybean Kunitz trypsin inhibitor
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone
TLCK	Na-*p*-tosyl-L-lysine chloromethyl ketone

seeds, tubers, leaves and fruits. These inhibitors demonstrate several putative functions such as endogenous regulators of proteolytic activity (Ryan, 1991), as storage proteins (Xavier-Filho, 1992) and as an important factor in response to abiotic (Franco and Melo, 2000) and biotic stresses (Gatehouse and Gatehouse, 1998). Serine proteinase inhibitors in general are small, stable and abundant proteins showing specificity to trypsin and/or chymotrypsin (Bode and Huber, 2000). Most of these inhibitors bind to cognate enzymes according to a common substrate-like standard mechanism (Grutter et al., 1990; Melo et al., 2002). Bowman–Birk type inhibitors have a low molecular mass, high disulfide bond content (Richardson, 1981) and are capable of inhibiting both trypsin and chymotrypsin simultaneously using two independent active sites (Laskowski and Kato, 1980; Freitas et al., 1999). Bowman–Birk inhibitors are recognized as part of the arsenal defense mechanisms that plants use against microorganism and insect attack (Xavier-Filho, 1992) and although the activities of serine proteinase inhibitors are well characterized, the role of Bowman–Birk inhibitors has not been totally elucidated in this process (Ryan, 1991; Pernas et al., 2000). The black-eyed pea trypsin chymotrypsin inhibitor (BTCI) is a Bowman-Birk inhibitor purified from *Vigna unguiculata* (L.) seeds (Ventura et al., 1971; Morhy and Ventura, 1987), which is composed of a single peptide chain of 83 residues. This inhibitor shows high pH stability, from pH 3 to 12, and also a high degree of thermal stability (Silva et al., 2001).

In this work, we report the effects of BTCI against serine proteinases from the guts of larval and adult *A. grandis*, an economically important crop pest of the New World. In addition, in vivo effects were also investigated with respect to the development and growth of larvae and adult insects. Modifications in the organization pattern of midgut tissues caused by the presence of BTCI were also investigated. These effects were explored through the use of enzymatic assays, feeding tests and optical microscopy.

2. Results and discussion

2.1. Enzymatic inhibitory activity of BTCI

The purity of BTCI was analyzed by SDS–PAGE (data not shown), where a unique band of approximately 10 kDa was observed. The MALDI–TOF spectrum of BTCI previously described by Silva et al. (2001), showed a single peak corresponding to a molecular mass of 9084 Da, confirming the purity of the inhibitor.

The inhibitory activities of BTCI towards bovine pancreatic trypsin and serine proteinases from larvae (AgPL) and adult boll weevil insects (AgPA) were determined by in vitro enzyme assays and are shown in Fig. 1. The inhibitor was highly active against bovine pancreatic trypsin, inhibiting 100% of proteolytic activity at a concentration of 14 μg ml^{-1} (Fig. 1A). The inhibitory effects of BTCI against trypsin-like enzymes of *A. grandis* adults (AgPA) were similar to the inhibition of bovine trypsin. In this case, at the same concentration, the inhibition of trypsin-like enzymes by BTCI was almost 90% (Fig. 1C). However, to inhibit all trypsin-like activity from *A. grandis* larvae (AgPL), a BTCI concentration of 40 μg ml^{-1} (Fig. 1B) was required. These results suggest that BTCI is more active toward adult trypsins, when compared to its inhibitory activity against trypsin-like enzymes from boll weevil larvae gut. Several reports have demonstrated the inhibitory activity of plant serine proteinase inhibitors against insect proteinases (Reed et al., 1999; Valaitis et al., 1999). Bowman–Birk type inhibitors belong to this group of molecules and have been demonstrated to inhibit gut proteinases from pests such as *Phaedon cochleariae* (F.) (Girard et al., 1998), and boll weevil *A. grandis* (Purcell et al., 1992). The results obtained for the BTCI assay reflect those reported for a soybean trypsin inhibitor, which showed high levels of inhibitory activity against the digestive proteinases of *Helicoverpa armigera* (Hubner) and *Helicoverpa virenscens* (Ferreira et al., 1994; Johnston et al., 1995). The synthetic inhibitor TLCK was highly active against trypsin-like enzymes, inhibiting all activity at a concentration of 3.5 mg ml^{-1} (Fig. 1). The remaining activity observed in adult proteinases of *A. grandis* (Fig. 1C) could be explained by the presence of insensitive proteinases and/or minor digestive cysteine proteinases as observed by Franco et al. (data not shown).

Earlier studies on the gut proteinase activity of *A. grandis* have shown that it is predominantly trypsin-like, although the presence of chymotrypsin and other proteolytic enzymatic classes have also been demonstrated (Franco et al., data not shown). Fig. 2 shows the enzymatic assays of BTCI against bovine pancreatic chymotrypsin (BPC) and the probable chymotrypsin-like proteinases from the guts of *A. grandis* larvae and

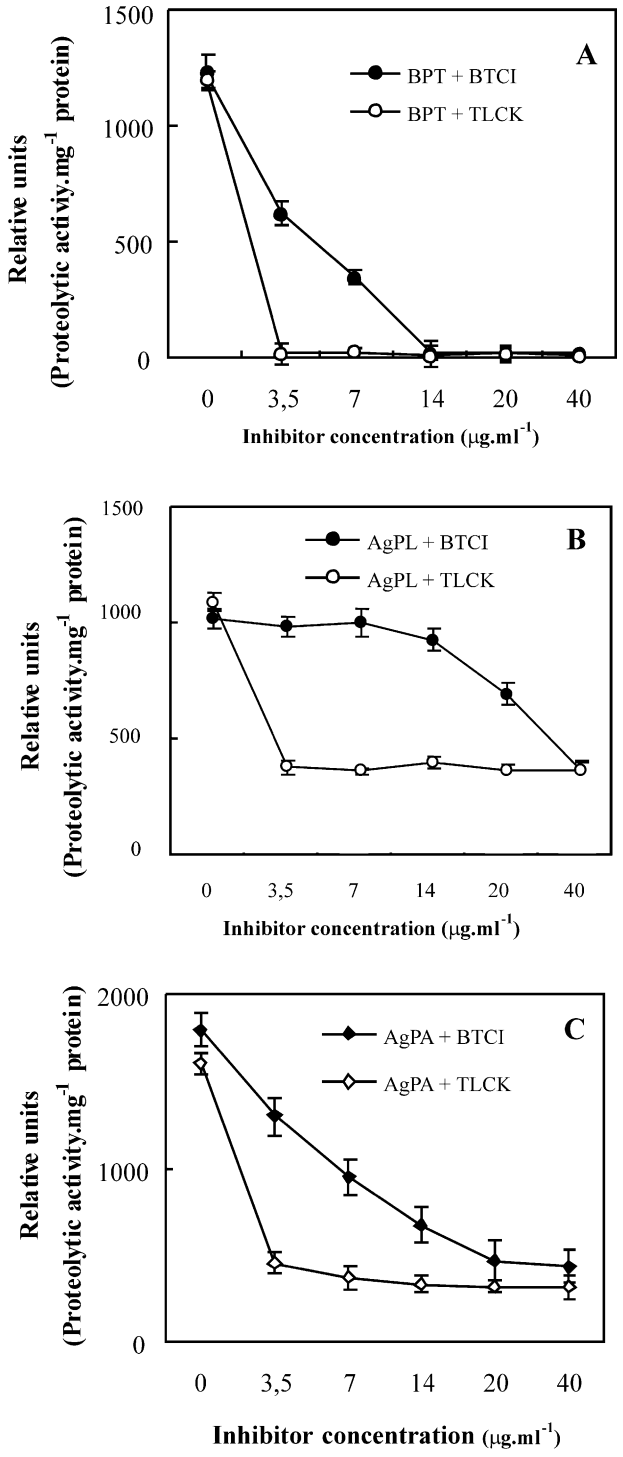

Fig. 1. (A) Inhibitory activities of BTCI and TLCK towards bovine pancreatic trypsin (BPT), (B) proteinases from *A. grandis* larvae (AgPL) and (C) adult insects (AgPA). Phe-Arg-MCA was used as substrate in this enzymatic assay. Each measurement was done in triplicate. The error bars represent the standard deviation.

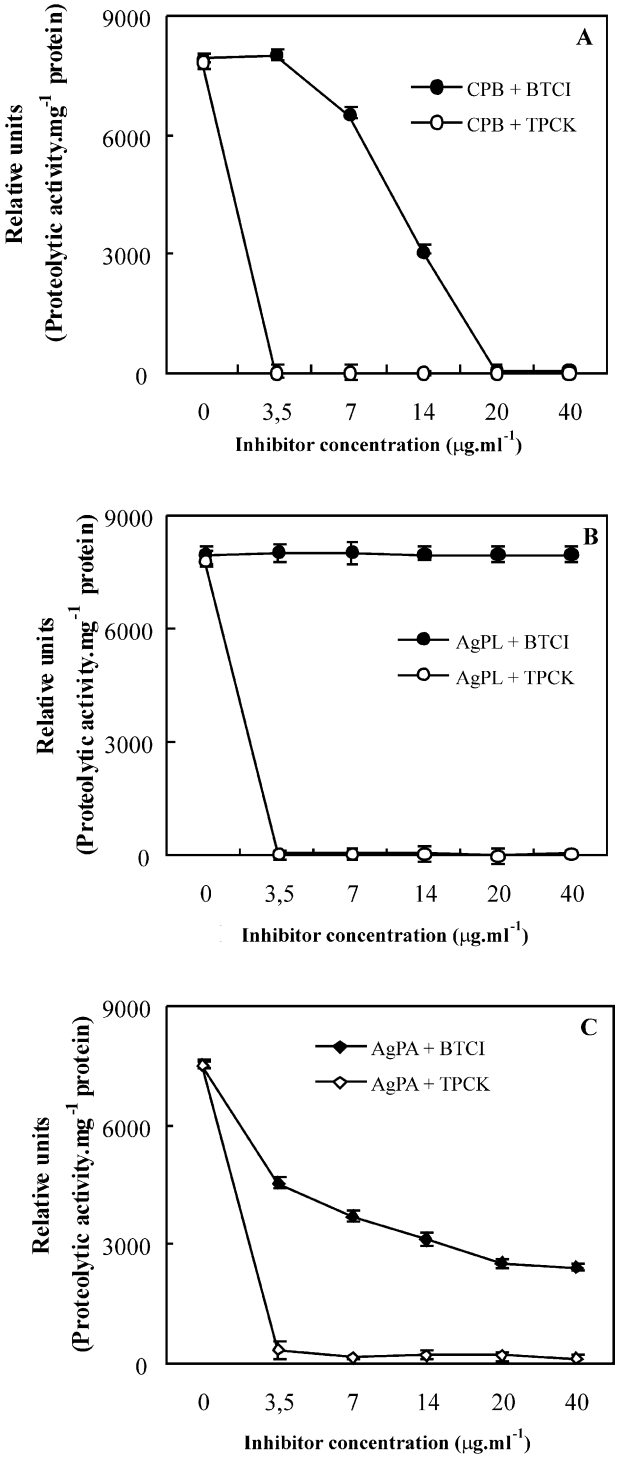

Fig. 2. (A) BTCI and TPCK inhibitory activities toward bovine pancreatic chymotrypsin (BPC), (B) proteinases from *A. grandis* larvae (AgPL) and (C) adult insects (AgPA). Ala-Ala-Pro-Phe-MCA was used as substrate in this enzymatic assay. Each measurement was done in triplicate. The error bars represent the standard deviation.

adults. To assay the activity of chymotrypsin-like serine proteinases, the synthetic substrate Ala-Ala-Pro-Phe-MCA was used. The synthetic inhibitor TPCK was highly active against chymotrypsin-like proteinases, inhibiting totally the activity at concentration of 3.5 μg ml^{-1}. BTCI was highly active against bovine chymotrypsin, inhibiting 100% of proteolytic activity at a concentration of 20 μg ml^{-1} (Fig. 2A). However, BTCI did not inhibit the chymotrypsin-like enzymes from the guts of *A. grandis* larvae (Fig. 2B). In addition, its affinity for adult chymotrypsin-like proteinases was lower than the affinity for bovine pancreatic chymotrypsin. To inhibit approximately 70% of AgPA, a BTCI concentration of 40 μg ml^{-1} was required (Fig. 2C). Considering that trypsin is essential for insect development, the ability of BTCI to inhibit chymotrypsin activity in adult boll weevils indicate that it is a promising candidate for expression in transgenic plants engineered for pest resistance. The insensitivity of larval chymotrypsin-like enzymes to BTCI and its low inhibitory activity against the enzymes of adult insects guts may be explained by the presence of insensitive serine proteinases and/or leucine amino peptidases that could cleave the toxic inhibitors, as previously reported (Girard et al., 1998; Harsulkar et al., 1999; Patankar et al., 2001). Therefore, it is evident that insects have complex regulatory mechanisms of gut proteinases in response to the presence of a toxic protein and future studies will elucidate these mechanisms in *A. grandis*.

2.2. Feeding tests

In vivo feeding tests were carried out to investigate the potential role of BTCI as a defense factor against the cotton boll weevil. Several insecticidal proteins, such as lectins, α-amylase inhibitors and proteinase inhibitors do not cause acute mortality, but may retard pest growth and development (Boulter, 1993; Morton et al., 2000; Franco et al., 2002). For this reason, the reduction in the larval growth caused for BTCI was investigated. Fig. 3B shows the influence of BTCI at larval weight at three different inhibitor concentrations. At the lowest BTCI concentration (10 μM), no effect was observed when compared to control larvae. In the presence of an intermediate concentration (50 μM), a reduction of 12% in larval weight was observed. On the other hand, a larval weight reduction of 46% was observed at the highest BTCI concentration (100 μM). These results are similar to those presented by Gatehouse et al. (1999), in which the soybean Kunitz trypsin inhibitor (SKTI), in artificial diet and expressed in transgenic plants, caused a high mortality to *Lacanobia oleracea* (L.). Furthermore, De Leo et al. (2001) using trypsin inhibitors from mustard expressed in tobacco obtained a substantial reduction in the development of *P. xylostella*, *M. brassicae* and *S. littoralis*.

The effects of BTCI on insect mortality were also investigated. Fig. 3A shows the weevil mortality as a function of increased BTCI concentration. BTCI concentrations of 10, 50 and 100 μM caused 17, 40 and 62% of mortality, respectively. These results indicated a LC50 of 78 μM. These results could be compared to the advanced results obtained by Yeh et al. (1997), where sweet potato trypsin inhibitors expressed by transgenic tobacco plants conferred resistance to *Spodoptera litura* (F.), causing high mortality. SKTI and mustard trypsin inhibitors also conferred resistance to lepidopterans, when expressed in transgenic tobacco plants (Gatehouse et al., 1999; De Leo et al., 2001). These results strongly suggest that serine proteinase inhibitors could be effective in the control of the cotton boll weevil, retarding their development and reducing the insect population.

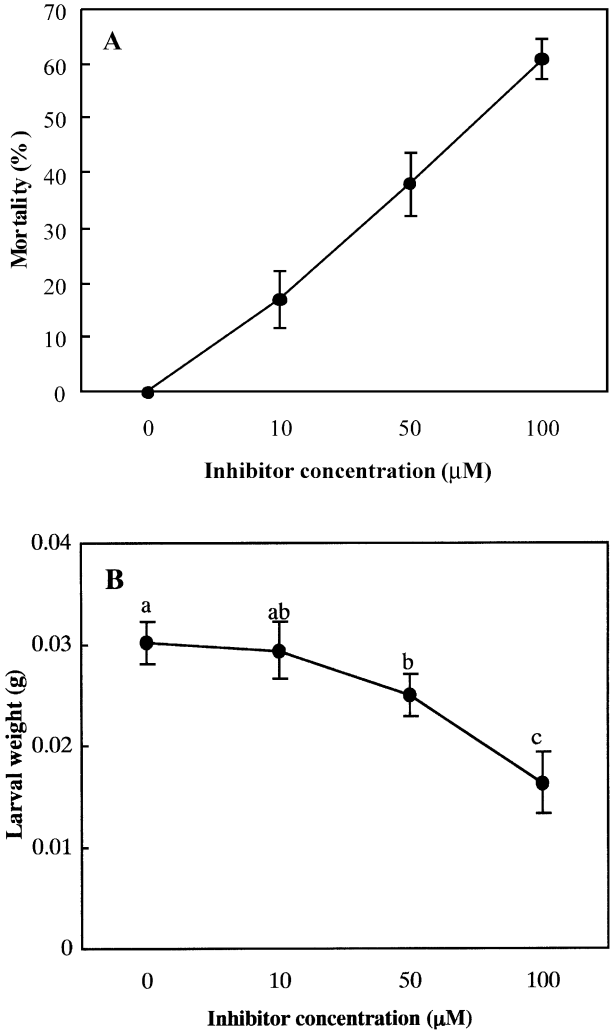

Fig. 3. Feeding tests with BTCI. The inhibitory effects of BTCI in the larval weight (A) and in the mortality (B). Mean values followed by the same letter were not statistically different ($P<0.05$) by this test. Each measurement was done in four replicates. The error bars represent the standard deviation.

2.3. Midgut cell analysis

Optical microscopic analysis of midguts from control and larvae previously treated with BTCI showed a well-developed striated border in the apical part of the cells and a conserved basal membrane. However, highly vacuolated regions in the epithelial cells were observed in the BTCI treated-samples (Fig. 4), suggesting it is implicated in cell degeneration. Several authors have reported that cell degeneration is characterized by an increase in the number and volume of authophagous vacuoles, which generally appear in cells, which are near death (Fain-Maurel et al., 1973; Martoja and Ballan-Dufrançais, 1984). Vacuoles have also been observed in the synthesis of digestive enzymes such as α-amylases and proteinases (Cristofoletti et al., 2001). In the yellow mealworm *Tenebrio molitor* (L.), trypsin-like proteins are synthesized in midgut cells and packed in the Golgi area into secretory vesicles that undergo fusion as they migrate to the cell apex. At the same time, the cell apex undergoes structural disorganization with the disappearance of cell organelles. This was observed in the midgut tissues treated with BTCI (Fig. 4B). Eventually, the apical cytoplasm with the large proteinase-containing membranous structure is discharged into the midgut lumen. After extruding the apical cytoplasm, the cell apparently remains functional, since cells are found to lack the cell apex, but have all the other normal ultra structural features. These observations suggest that the observed vacuoles in the tissues treated with BTCI could be due to an over-expression of digestive proteinases in response to the presence of the toxic inhibitor (Cristofoletti et al., 2001).

3. Experimental

3.1. Isolation of midgut fluid

Anthonomus grandis larvae were obtained from Biological Control Department of EMBRAPA/Cenargen (Brasília-DF, Brazil). Larvae were reared on an artificial diet at 25 °C and 55% relative humidity in dark regimen (Monnerat et al., 1999). The guts were surgically removed over ice from larvae (7 days) and adult insects (21 days) and placed into an iso-osmotic saline (0.15 M NaCl). Midgut tissues were homogenized and centrifuged for 10 min at 10,000 *g* at 4 °C and the supernatant was removed and used totally fresh for enzymatic assays.

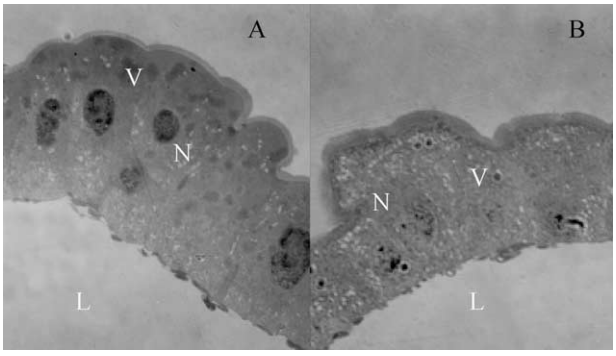

Fig. 4. Microscopical analysis of *A. grandis* midgut tissues (N—nucleus, V—vesicular region, L—lumen) fed on artificial diet (A) and fed on artificial diet containing 100 μM BTCI (B).

3.2. Molecular masses and purity analysis

SDS–PAGE (15%) was carried out as described by Laemmli (1970), at room temperature. Protein molecular weight markers (Gibco), in a range of 14–96 kDa, were used, with bromophenol blue as the tracking dye.

3.3. Proteinase inhibitor assays

Bovine pancreatic trypsin (BPT) and bovine pancreatic chymotrypsin (BPC), were purchased from Sigma Co., St. Louis, MO, USA, and midgut were used for enzymatic assays. BTCI was purified from *V. unguiculata* seeds cv. Seridó, grown in a greenhouse, by following the procedure previously described (Ventura et al., 1971). Proteolytic inhibitory activities were tested against AgPL, AgPA, BPT and BPC using 10 μM fluorogenic peptides Z-CBZ-Phe-Arg-7-MCA (Sigma Company) and *N*-succinyl-Ala-Ala-Pro-Phe-MCA, respectively. The assays were performed in 25 mM Tris–HCl, pH 6.5 and 20 mM DMSO according to Solomon et al. (1999). The reaction was stopped with 1.9 ml of 0.2 M Na_2CO_3. The endpoint reaction was measured after 30 min in a DyNA Quant 500 fluorescence reader (Pharmacia-Biotech), with excitation at 365 nm and emission at 460 nm. BTCI was tested at several concentrations. The inhibitory activities were calculated using the fluorescence reduction. The inhibitory activities were direct compared to free MCA produced by trypsin and chymotrypsin-like enzymes. One relative unit corresponds to 0.5 mM of free MCA. The blank fluorescence readings (minus substrate) were subtracted. Assays were carried out in triplicate, with variability in endpoint fluorescence values not exceeding 10%.

3.4. Feeding tests of BTCI against the boll weevil

Bioassays were performed in 40 ml sterilized artificial diet (Monnerat et al., 1999). BTCI was incorporated in the diet at concentrations of 10, 50 and 100 μM. The protein concentration was calculated according to Bradford (1976). The diet was added to Petri dishes and larvae 48 h post-hatch were placed in pits created in the artificial diet. After 7 days, the dead larvae were counted and the weight of surviving larvae was measured. In the control treatment, distilled water or BSA in the same inhibitors concentrations was added to the

artificial diet. Each treatment was repeated four times, each replicate using 15 larvae. The bioassay was maintained under controlled conditions at 28 °C and 55% stable humidity. A completely random design was used and the comparisons of the means of the larval weight treatments were made by the Tukey's test at a 5% level of probability.

3.5. Midgut histology

Midguts from larvae fed on artificial diet containing BTCI were dissected after 6 days in 0.1 M cacodylate buffer (pH 7.3). The tissue was fixed in 0.1 mM sodium cacodylate buffer (pH 7.3) containing 2% glutaraldehyde, 4% paraformaldehyde and 5 mM calcium chloride and post fixed in a solution containing 2% osmium tetroxide and 1.6% potassium ferricyanide. Samples were dehydrated with an acetone gradient and embedded in Spurr solution. Semi-thin 500 nm sections, obtained with Leica Ultracut microtome, were stained with toluidine blue and observed under a light microscope.

4. Concluding remarks

The relation between plant proteinase inhibitors and insect proteinases is more complex than the concept of a simple inhibition of fixed digestive proteinases. To plan a proteinase inhibitor based strategy for insect resistance, it is necessary to understand the insect's capability to alter gut composition and reduce the inhibitors' effects. It is an apparent paradox that insects feed on plants despite the fact that proteinase inhibitors are ubiquitous, especially in the case of legumes. Nevertheless, it is well know that insects can adapt to plant proteinase inhibitors by producing inhibitor-insensitive, inhibitor-resistant and/or inhibitor degrading proteinases in their midgut to compensate for the effects of the inhibitors (Girard et al., 1998; Patankar et al., 2001). Additionally, it is important to understand the inhibitor's mode of action. Proteinase inhibitors can cause a starvation effect, due the inhibition of proteolysis (Jongsma et al., 1995), a toxic effect due to the induction of hyper production of proteinases (Broadway and Duffey, 1986) and increased levels of deformity, due to the potential inhibition of proteinases involved in metamorphosis (Franco et al., data not shown).

The integrated pest management (IPM) programs for cotton throughout the Americas aim to discover effective biological agents or defense mechanisms, which increase the resistance of cotton plants to pest attack. Some strategies that have been suggested for the control of the boll weevil, involve the case of exotic parasitoids as *Catalaccus grandis* (L.) (Legaspi et al., 1998). Nevertheless, the expression in transgenic plants of digestive enzymes inhibitors has been shown to be an effective strategy in crop protection (Gatehouse et al., 1999; Morton et al., 2000). Due to the insecticidal nature of their action, it has an advantage of delaying the development of resistance to the proteinase inhibitor within the insect population, as well as retaining the anti-insect effect for a much longer period (Yeh et al., 1997). Some proteinase inhibitors are more effective than others. For this reason, in assays to characterize potential molecules in biorational control, it is very important to combine different activities in the same molecule, increasing the insecticide potential. This property was observed for the inhibitor purified from millet finger endosperm, which inhibits α-amylase and trypsin, simultaneously (Strobl et al., 1998). The inhibitor from coix (*Coix lachrima-jobi* L.) exhibits inhibitory activity against both α-amylase and chitinase enzymes (Ary et al., 1989) and Bowman–Birk type inhibitors can act on trypsin and chymotrypsin from insects.

Here, we present a study of BTCI activity in vitro against two serine proteinases from *A. grandis* adult insects and against serine trypsin-like proteinases from *A. grandis* larvae. This work also describes an effective in vivo effect of BTCI against *A. grandis* larvae, causing morphological modifications that demonstrate that this inhibitor could be used as an important tool in the engineering of transgenic cotton plants with increased resistance to insect pests.

Acknowledgements

We thank to Dra. Francislete Rodrigues Melo, Dra. Ana Carolina S. Monteiro, Dr. Carlos Bloch Jr. and Charles Dayler to technical assistance. Capes, CNPq, FACUAL and Common Found for Commodities/International Cotton Advisory Committee supported this work.

References

Alves, P.M.P., Ramalho, A.R., Teixeira, C.A.D., 1993. Monitoramento do algodão (*Anthonomus grandis*, Coleóptera, Curculionidae) em Rondônia. Reunião Nacional do Algodão/EMBRAPA-CNPA, Cuiabá-MT, Brasil, 139.

Ary, M.B., Richardson, M., Shewry, P.R., 1989. Purification and characterization of an insect α-amylase inhibitor/endochitinase from seeds of Job's Tears (*Coix lachryma-jobi*). Biochem. Biophys. Acta 913, 260–266.

Bode, W., Huber, R., 2000. Structural basis of the endoproteinase-protein inhibitor interaction. Biochim. Biophys. Acta. 1477, 241–252.

Boulter, D., 1993. Insect pest control by copying nature using genetically engineered crops. Phytochemistry 34 (6), 1453–1466.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. 72, 248–254.

Broadway, R.M., Duffey, S.A., 1986. 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. 34, 1111–1117.

Cristofoletti, P.T., Ribeiro, A.F., Terra, W.R., 2001. Apocrine secretion of amylase and exocytosis of trypsin along the midgut of *Tenebrio molitor* larvae. J. Insect Physiol. 47, 143–155.

De Leo, F., Bonadé-Bottino, M., Ceci, L.R., Gallerani, R., Jouanin, L., 2001. Effects of a mustard trypsin expressed in different plants on three lepidopteran pests. Insect. Biochem. Mol. Biol. 31, 593–602.

Fain-Maurel, M.A., Cassier, P., Alibert, J., 1973. Étude infrastructurale et cytochimique de l'intestin moyen de *Petrobius maritimus* Leach en rapport avec ses fonctions excrétrices et digestives. Tissue Cell 5, 603–631.

Ferreira, C., Capella, A.N., Sitnik, R., Terra, W.R., 1994. Properties of the digestive enzymes and the permeability of the peritrophic membrane of *Spodoptera frugiperda* (Lepidoptera) larvae. Comp. Biochem. Physiol. A 107, 631–640.

Franco, O.L., Melo, F.R., 2000. Osmoprotectants, a plant strategy in response to osmotic stress. Russ. J. Plant Physiol. 47 (1), 137–144.

Franco, O.L., Rigden, D.J., Melo, F.R., Grossi-De-Sa, M.F., 2002. Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases. Eur. J. Biochem. 269 (2), 397–412.

Freitas, S.M., Ikemoto, H., Ventura, M.M., 1999. Thermodynamics of the binding of chymotrypsin with the black-eyed pea trypsin and chimotrypsin inhibitor (BTCI). J. Prot. Chem. 18 (3), 307–313.

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

Gatehouse, A.M.R., Gatehouse, J.A., 1998. Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. Pest. Sci. 52, 165–175.

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

Girard, C., Le-Metayer, M., Bonadé-Bottino, M., Pham-Delegue, M-H., Jouanin, L., 1998. High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. Insect Biochem. Mol. Biol. 28, 229–237.

Grutter, M.G., Priestle, J.P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., Stone, S.R., 1990. Crystal structure of the thrombin-hirudin complex: a novel model of serine proteinase inhibition. EMBO J. 9, 2361–2365.

Harsulkar, A.M., Giri, A.P., Patankar, A.G., Gupta, V.S., Sainani, M.N., Ranjekar, P.K., Deshpande, V.V., 1999. Successive use of non-host plant proteinase inhibitors required for effective inhibition of *Helicoverpa armigera* gut proteinases and larval growth. Plant Physiol. 121, 597–606.

Haynes, J.W., Smith, J.W., 1992. Longevity of laboratory reared boll weevils (Coleóptera: Curculionidae) offered honey bee-collected pólen and plants unrelated to cotton. J. Entom. Sci. 27 (4), 366–374.

Johnston, K.A., Lee, M., Brough, C., Hilder, V.A., Gatehouse, A.M.R., Gatehouse, J.A., 1995. Proteinases activity in the larval midgut of *Heliothis virenscens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochem. Mol. Biol. 25, 375–383.

Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D., Stiekma, W.J., 1995. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proc. Nat. Acad. Sci. USA 92, 8041–8045.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Laskowski Jr., M., Kato, I., 1980. Protein inhibitors of proteinases. Annu. Rev. Biochem. 49, 593–626.

Legaspi Jr., B.C., Allen, J.C., Brewster, C.C., Morales-Ramos, J.A., King, E.G., 1998. Area wide management of the cotton boll weevil: use of a spatio-temporal model in augmentative biological control. Ecol. Model. 110, 151–164.

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

Martoja, R., Ballan-Dufrançais, C., 1984i. The ultrastructure of the digestive and excretory organs. In: King, R.C., Akai, H. (Eds.), Insect Ultrastructure, Chapter 6. Plenum Press, New York, p. 624.

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

Morhy, L., Ventura, M.M., 1987. The complete amino acid sequence of the *Vigna unguiculata* (L.) Walp. seed trypsin and chymotrypsin inhibitor. An. Acad. Brasil. Ciênc. 59, 71–81.

Morton, R.L., Schroeder, H.E., Bateman, K.S., Chrispeels, M.J., Armstrong, E., Higgins, T.J.V., 2000. Bean alpha-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. Proc. Nat. Acad. Sci. USA 97 (8), 3820–3825.

Monnerat, R., Dias, S.C., Oliveira-Neto, B., Nobre, S.D., Grossi de Sá, M.F., 1999. Criação de bicudo do algodoeiro *Anthonomus grandis* em dieta artificial e estabelecimento para bioensaios com *Bacillus thuringiensis*. IV Congresso Brasileiro de Algodão, Ribeirão Preto-SP, pp. 214–216.

Patankar, A.G., Giri, A.P., Harsulkar, A.M., Sainani, M.N., Deshpande, V.V., Ranjekar, P.K., Gupta, V.S., 2001. Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. Insect. Biochem. Mol. Biol. 31 (4-5), 453–464.

Pernas, M., Sanchez-Ramos, I., Sanchez-Monge, R., Lombardero, M., Arteaga, C., Castañera, P., Salcedo, G., 2000. Derp1 and Derf 1, the higly related and major allergens from house dust mites, are differentially affected by a plant cystatin. Clin. Exp. All. 30, 972–978.

Purcell, J.P., Greenplate, J.T., Sammons, R.D., 1992. Examination of midgut luminal proteinase activities in six economically important insects. Insect Biochem. Mol. Biol. 22 (1), 41–47.

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

Richardson, M., 1981. Protein inhibitors of enzymes. Food Chem. 6, 235–253.

Ryan, C.A., 1991. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. Ann. Rev. Phytol. 28, 425–449.

Silva, L.P., Leite, J.R.S.A., Bloch Jr., C., Freitas, S.M., 2001. Stability of a black eyed pea trypsin/chymotrypsin inhibitor (BTCI). Prot. Pept. Lett. 8 (1), 33–38.

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

Strobl, S., Maskos, K., Wiegand, G., Huber, R., Gomis-Ruth, F.X., Glockshuber, R., 1998. A novel strategy for inhibition of α-amylases: yellow mealworm α-amylase in complex with the *ragi* bifunctional inhibitor at 2.5 Å resolution. Structure 6, 911–921.

Valaitis, A.P., Augustin, S., Clancy, K.M., 1999. Purification and characterization of the western spruce budworm larval midgut proteinases and comparison of gut activities of laboratory-reared and field-collected insects. Insect Biochem. Mol. Biol. 29, 405–415.

Ventura, M.M., Xavier-Filho, J., Moreira, R.A., Aquino, A.M., Pinheiro, P.A., 1971. A trypsin and chymotrypsin inhibitor from black-eyed pea (*Vigna sinensis* L.). II. Further studies on its characterization and a reevaluation of earlier results. An. Acad. Bras. Cienc. 43 (1), 233–242.

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

Xavier-Filho, J., 1992. The biological roles of serine and cysteine proteinase inhibitors in plants. Braz. J. Plant Physiol. 4 (1), 1–6.