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Effects of soybean Kunitz trypsin inhibitor on the cotton boll weevil (*Anthonomus grandis*)

Octávio L. Franco[a,b,c], Simoni C. Dias[a,b], Claudio P. Magalhães[d], Ana C. S. Monteiro[a], Carlos Bloch Jr[a], Francislete R. Melo[b,e], Osmundo B. Oliveira-Neto[a,b], Rose G. Monnerat[a], Maria Fátima Grossi-de-Sá[a,*]

[a]*Embrapa Recursos Genéticos e Biotecnologia, S.A.I.N. Parque Rural, Final W5, Asa Norte, 70770-900, Brasilia-DF, Brazil*
[b]*Departamento de Biologia Celular, UnB, Brasília-DF, Brazil*
[c]*Universidade Católica de Brasília, Brasília-DF, Brazil*
[d]*Faculdade de Ciências Farmacêuticas, USP, São Paulo, Brazil*
[e]*União Pioneira de Integração Social, Brasília-DF, Brazil*

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Abstract

The cotton boll weevil, *Anthonomus grandis*, is an economically important pest of cotton in tropical and subtropical areas of several countries in the Americas, causing severe losses due to their damage in cotton floral buds. Enzymatic assays using gut extracts from larval and adult boll weevil have demonstrated the presence of digestive serine proteinase-like activities. Furthermore, in vitro assays showed that soybean Kunitz trypsin inhibitor (SKTI) was able to inhibit these enzymes. Previously, in vivo effects of black-eyed pea trypsin chymotrypsin inhibitor (BTCI) have been demonstrated towards the boll weevil pest. Here, when neonate larvae were reared on an artificial diet containing SKTI at three different concentrations, a reduction of larval weight of up to 64% was observed for highest SKTI concentration 500 μM. The presence of SKTI caused an increase in mortality and severe deformities of larvae, pupae and adult insects. This work therefore represents the first observation of a Kunitz trypsin inhibitor active in vivo and in vitro against *A. grandis*. Bioassays suggested that SKTI could be used as a tool in engineering crop plants, which might exhibit increased resistance against cotton boll weevil.

Keywords: Cotton; *Anthonomus grandis*; Kunitz trypsin inhibitor; Serine proteinases; Soybean

1. Introduction

The boll weevil, *Anthonomus grandis* (Boheman) is an important cotton pest in tropical and subtropical areas of the New World. This insect-pest shows a preference for fruits and floral buds in target plants (Haynes and Smith, 1992; Alves et al., 1993), using these tissues as alimentary sources and a developmental habitat, and thereby causing severe damage to crop cotton.

Like other insect-pests, the boll weevil obtains essential amino acids by using extra-cellular proteinases secreted from the midgut lumen to digest soluble and structural proteins. Insect digestive proteinases are promising targets in the control of various Lepidopteran insects, including *Manduca sexta* (L.) (Johnson et al., 1989), *Heliothis zea* (Boddie) (Broadway and Duffey, 1986), *Spodoptera litura* (Boisd.) (Yeh et al., 1997) and *Lucilia cuprina* (Wied.) (Reed et al., 1999). However, relatively few studies have been reported exploring the use of proteinase inhibitors for the control of coleoptera (Gatehouse and Boulter, 1983; Leplé et al., 1995; Gatehouse and Gatehouse, 1998; Franco et al., 2003). Numerous plant proteinase inhibitors effective against insect digestive enzymes are known (Bode and Huber, 2000; Franco et al., 2002; Oliveira et al. 2002) and their suggested functions include action as storage proteins (Xavier-Filho, 1992), regulators of endogenous proteolytic activity (Ryan, 1991) and participants in the mechanisms of programmed plant cell

* Corresponding author. Tel.: +55-61-448-4705; fax: +55-61-340-3624.
E-mail address: fatimasa@cenargen.embrapa.br (M.F. Grossi-de-Sá).

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Nomenclature

AgPL	*Anthonomus grandis* gut proteinases from larvae
AgPA	*Anthonomus grandis* gut proteinases from adult insects
BBI	Bowman–Birk inhibitor
BPT	Bovine pancreatic trypsin
BTCI	Black-eyed pea trypsin chymotrypsin inhibitor
OCI	Oryzacystatin inhibitor
PMSF	Phenylmethylsulfonyl fluoride
SKTI	Soybean Kunitz trypsin inhibitor
TLCK	Na-*p*-tosyl-L-lysine chloro-methyl ketone
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone

death (Solomon et al., 1999). Innumerous proteinase inhibitors are known for their function in response to abiotic stresses (Pernas et al., 2000; Franco and Melo, 2000) and in plant defense processes against insect pests attack (Gatehouse and Gatehouse, 1998). These inhibitors in general are small, stable and abundant proteins (Walker et al., 1998) showing specificity for serine, cysteine, aspartic or metallo-proteinases (Bode and Huber, 2000).

Serine proteinase inhibitors are found in plant storage tissues, such as seeds, tubers, leaves and fruits (Xavier-Filho, 1992; Melo et al., 2002). Most of these inhibitors bind to cognate enzymes according to a common substrate-like canonical mechanism (Bode and Huber, 2000). Among them, soybean Kunitz trypsin inhibitor (SKTI) has gained particular attention for its specific activity against trypsin-like serine proteinases, with no inhibition of other proteinase classes (Gatehouse et al., 1999). Furthermore, SKTI was capable of inhibiting the proteolytic activity from several lepidopterans, such as black cutworm (*Agrotis ipsilon* Hufnagel), corn earworm (*H. zea*), tobacco budworm (*Heliothis virenscens* Fab.), western spruce budworm, *Choristeneura occidentalis* (Purcell et al., 1992) and coleopteran such as the cotton boll weevil. The black-eyed pea trypsin chymotrypsin inhibitor BTCI demonstrated an in vivo effect only against *A. grandis* (Franco et al., 2003).

Here, we have investigated the presence of serine proteolytic activities in larvae and adult insect gut extracts of *A. grandis*. In vitro and in vivo inhibitory effects of SKTI on *A. grandis* were also reported.

2. Results and discussion

2.1. Detection of serine proteinases in A. grandis gut extract

Different inhibitors were used in order to test the sensitivity of the proteolytic classes in cotton boll weevil gut. By using synthetic substrates, the presence of large amounts of serine proteinases were detected in midgut extracts of *A. grandis*. Table 1 shows the proteolytic activity of larvae midguts determined by using fluorogenic substrates. Both chymotrypsin-like and trypsin-like activity in larval gut extracts were observed, as was indicated by an efficient hydrolysis of Ala-Ala-Pro-Phe-MCA and Phe-Arg-MCA. Inhibition by TPCK, TLCK and SKTI were also determined (Table 1). These results corroborate with those obtained by Purcell et al. (1992), which reported the presence of high serine proteinase activity in *A. grandis* midgut fluid. Although some coleopterans such as the bean weevil *Acanthoscelides obtectus* Say (Wieman and Nielsen et al., 1988) and the Colorado potato beetle *Leptinotarsa decemlineata* Say (Purcell et al., 1992) synthesize predominantly cysteine proteinases, a digestive trypsin-like activity has been reported in most insect species examined. Among them the yellow mealworm *Tenebrio molitor* (L.), three species of the genera *Caribus* and the larvae of *Costelytra zealandica* (White) have a proteolytic system based on trypsin-like serine proteinases (Levinsky et al., 1977; Vaje et al., 1984; Christeler et al., 1989). Important exceptions are Hemiptera species and species belonging to the series Cucujiformia of Coleoptera, in which no trypsin-like activity was observed (Terra and Ferreira, 1994).

Gelatin polyacrylamide gel electrophoresis of the midgut extracts showed many bands with proteolytic activity in a wide range of molecular masses (Fig. 1, lane I). The predominant bands at MW of 28 kDa and 12 kDa were inhibited by serine proteinase inhibitors, the

Table 1
Proteolytic assays of lumen digestive extract from *A. grandis* larvae fed with artificial diet (control) and with artificial diet containing SKTI. Each treatment was carried out in triplicate at a constant pH of 8.6 and at a constant temperature of 25 °C. Activity is presented as percentage of the control activity. Inhibition replicate values differed by no more than 10%

Treatment	Substrates	Digestive enzymes	Digestive enzymes and SKTI	Digestive enzymes and TLCK	Digestive enzymes and TPCK
Control larvae	Phe-Arg	100%	0.63%	0.92%	97.5%
	Ala-Ala-Pro-Phe	100%	99.3%	99.7%	0.5%
SKTI fed larvae	Phe-Arg	100%	100%	100%	100%
	Ala-Ala-Pro-Phe	100%	100%	100%	1.36%

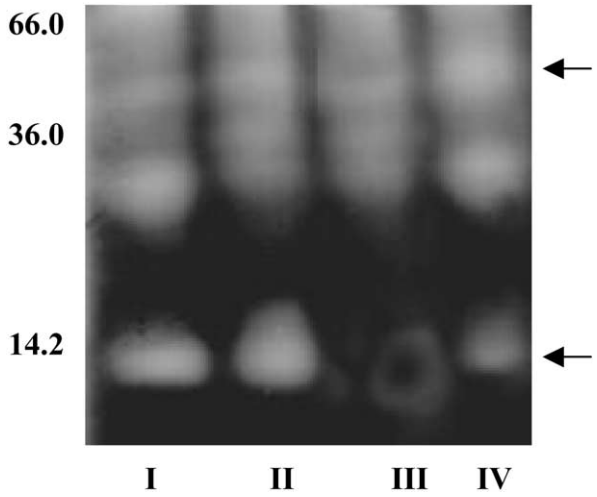

Fig. 1. Gelatin polyacrylamide gel electrophoretical analysis of proteolytic activity of gut extract from *Anthonomus grandis* larvae. I—crude extract midgut; II—crude extract midgut + TLCK (inhibitor of trypsin-like activities), III—crude extract midgut + TPCK (inhibitor of chymotrypsin-like activities) and IV—crude extract midgut from larvae fed on SKTI (trypsin inhibitor). The molecular weight was determined by a comparison from molecular mass migration of α-lactalbumin (14.2 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa) and bovine serum albumin (66.0 kDa). Black arrows demonstrate the enzymes modified in insect digestive tract due the presence of SKTI. Ten gel replicates were carried out.

28 kDa band protein with the trypsin inhibitor TLCK (Fig. 1, lane II) and the 12 kDa band with TPCK (a chymotrypsin inhibitor) (Fig. 1, lane III). Most trypsin molecular mass values are in the range 20–35 kDa, as in the case of 28 kDa protein (Fig. 1, lane II). On the other hand, the 12 kDa protein inhibited by TPCK (Fig. 1, lane III) do not correspond to common insect chymotrypsins, which are in the range 20–30 kDa (Terra and Ferreira, 1994).

2.2. Soybean Kunitz trypsin inhibitor purification

Lyophilized powder (Sigma Co.) was precipitated with ammonium sulfate (rich fraction- RF) and subjected to analytical reversed-phase HPLC, which yielded a number of peaks as shown in Fig. 2A. A major peak, denominated SKTI, showed high inhibitory activity against bovine pancreatic trypsin (BPT), while no inhibitory activity was found in the other peaks (data not shown). SDS-PAGE analysis of the rich fraction (RF) and SKTI fractions is shown in Fig. 2C. A major band with a molecular mass of approximately 20 kDa with minor contaminants was found in the fraction RF, while in the major peak, only the band of SKTI (20 kDa) was observed. Kim et al. (1985) isolated three isoforms of Kunitz trypsin inhibitor in soybean, denominated SKTI^a, SKTI^b and SKTI^c. Later, Gotor et al. (1995) identified a different unnamed isoform. In order to identify the SKTI isoform present in our fractions, mass spectral analysis was performed. The data

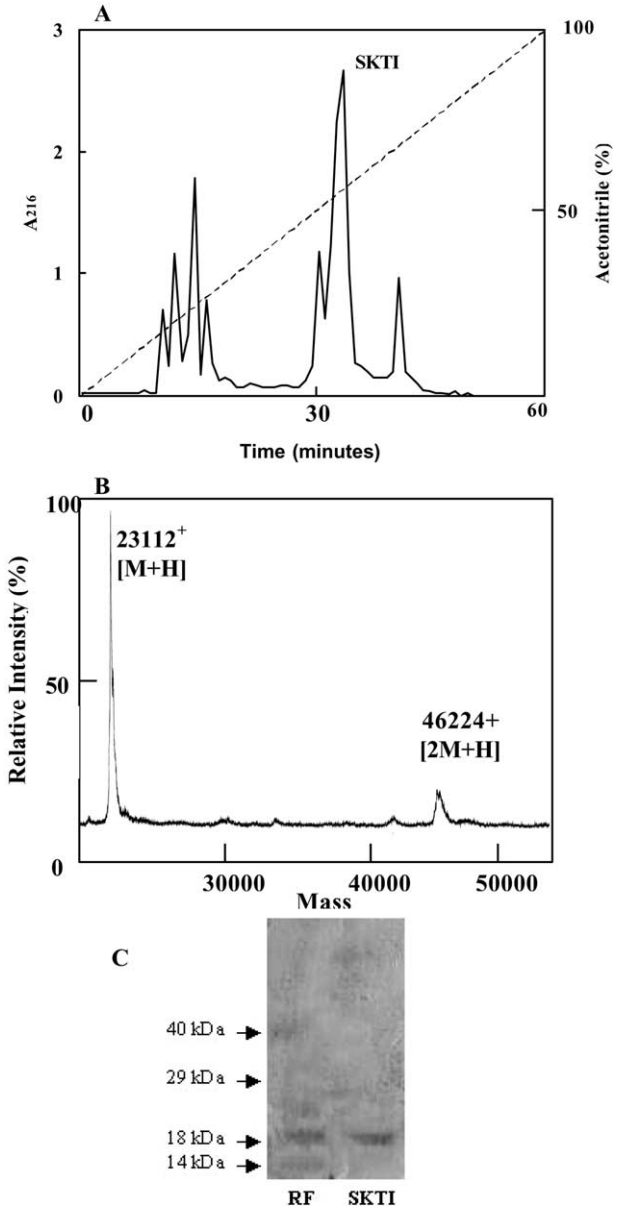

Fig. 2. (A) Analytical reversed-phase HPLC of soybean trypsin inhibitor ammonium sulfate fraction F_{0-100}. Separation was carried out on a Vydac 218TP analytical column using a flow rate of 1.0 ml/min. TFA (0.1%) was used as ion-pairing agent and the dashed line indicates the acetonitrile gradient. The sample contained 1.0 mg of protein. (B) MALDI-TOF spectrum of SKTI purified from reversed phase HPLC. (C) SDS–PAGE analysis of the rich fraction (RF) of commercial inhibitors and purified SKTI, stained with Coomassie Blue.

showed a monomeric protein with molecular mass of 23,112 Da and a dimeric protein with 46,224 Da (Fig. 2B). The molecular mass obtained and a peptide mass fingerprint (data not shown) showed that this inhibitor is different from the three isoforms observed by Kim et al. (1985) but it is in agreement with the theoretical protein mass observed by Gotor et al. (1995) indicating that the soybean inhibitor purified here is the same inhibitor isolated by Gotor et al. (1995).

2.3. In vitro inhibitory activity of SKTI

The inhibitory activity of SKTI isolate towards bovine pancreatic trypsin, proteinases from larvae (AgPL) and adult boll weevil insects (AgPA) were determined in vitro as shown in Fig. 3. For this assay, 10 μM of N-CBZ-Phe-Arg was used as a substrate at standard conditions described in Experimental, in order to test trypsin-like activity. SKTI, tested at a standard concentration of 50 μg ml^{-1}, showed similar results to those observed by Purcell et al. (1992) with their inhibitory activity of 64.7% against AgPL (in white) and 95% against BPT (in black) (Fig. 3). However when SKTI was tested with AgPA, a decrease of inhibition by 32.0% (in gray) was observed. Franco et al. (2003) had observed different results, in which BTCI showed a higher activity against AgPA in comparison to AgPL. The obtained results could be explained by a reduction of trypsin-like serine-proteinases in guts from adult insects. We have once observed an increased level of other classes of proteinases in adult midguts (data not shown). In this case the remaining activity could be explained due the presence of other proteolytic enzymes classes (Table 1, data not shown). It is also possible that the soybean trypsin inhibitor has less affinity for AgPA trypsins than for AgPL trypsins.

Several reports have demonstrated the inhibitory activity of plant proteinase inhibitors against insect proteinases (Reed et al., 1999; Valaitis et al., 1999). Girard et al. (1998) have observed that oryzacystatin (OCI) and Bowman–Birk inhibitors (BBI) showed a potent in vitro inhibitory activity against gut proteinases from *Phaedon cochleariae* (F.). Similar observations were found when experiments were carried out with serine proteinases from larvae of tomato moth, *L. oleracea* that were inhibited by SKTI and CpTI (Cowpea Trypsin Inhibitor) (Gatehouse et al., 1999). These observations are also in agreement with those reported for *H. armigera*, *H. virenscens* and other lepidopterans, in which SKTI was the most effective among the inhibitors tested (Ahamad et al., 1980; Johnston et al., 1991; Ferreira et al., 1994; Jonhston et al., 1995). Other studies have also demonstrated that these inhibitors are highly active both in vitro and in vivo, when the insects were fed with an artificial diet containing inhibitors (Gatehouse et al., 1999; Franco et al., 2003). Nevertheless, in some cases, the inhibitors have low or no in vivo activity (Purcell et al., 1992). This fact could be explained by the presence of proteinases and amino peptidases, which could degrade the inhibitors, as observed in case of BBI and OCI (Girard et al., 1998). Some shifts in the composition of proteinase complement (Jongsma et al., 1995; Bown et al., 1997) have also been observed when some insects were fed with proteinase inhibitors. These studies suggested that an alteration of secreted proteinases is a way for larvae to avoid the antimetabolic effects of inhibitors. This could also be an explanation for the reduction of SKTI inhibitory activity against AgPA (Fig. 3) since *A. grandis* adult insects are able to modify their digestive proteolytic composition in response to presence of SKTI (Oliveira-Neto et al., unpublished). To demonstrate the effectiveness of SKTI as a defense factor toward this weevil, in vivo assays were carried out.

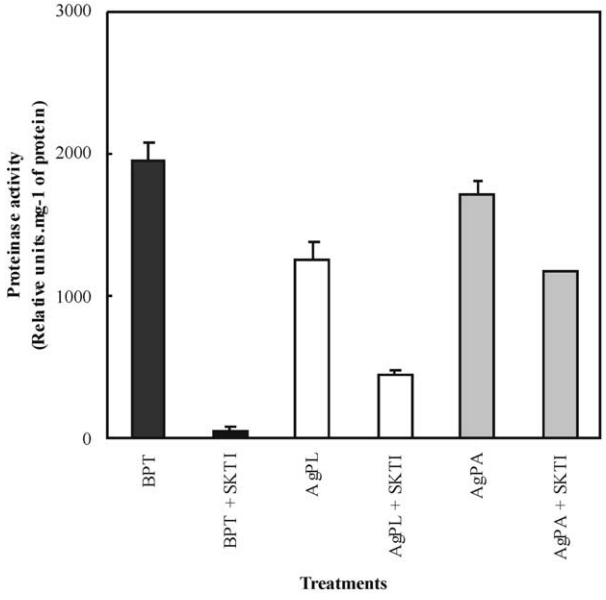

Fig. 3. Inhibitory activities of SKTI towards bovine pancreatic trypsin (BPT) (in black) and proteinases from larvae AgPL (in white) and adult insect AgPA (in grey) of boll weevil *A. grandis*. Each measurement was done in triplicate (±S.D.).

When the gut extracts of surviving larvae were analyzed by gelatin/gel electrophoresis some proteinase profiles were modified. The activity band around 12 kDa that was observed to be the strongest in the control larvae (Fig. 1, lane I), show reduced intensity in the larvae fed on SKTI (Fig. 1, lane IV). However, the active protein around 40 kDa was weaker in the control in comparison to the larvae fed on SKTI extract. Our data suggests that the *A. grandis* larvae adapt their enzymatic complement in order to cope up with the anti-nutritional activity of SKTI, by over expressing proteinases resistant to this inhibitor. This fact was also observed by Oliveira-Neto et al. (unpublished) when mRNAs encoding a trypsin-like serine proteinase insensitive to SKTI was over expressed in boll weevils fed on SKTI conditions. None of the specific serine proteinase inhibitors was able to inhibit the 40 kDA serine proteinase in our gel (Fig. 1, lane II and III). An over expression of proteinases resistant to the presence of inhibitors was observed in other pests (Broadway and Duffey, 1986).

2.4. In vivo inhibitory activity of SKTI

Based on in vitro enzymatic studies, standard feeding trials were carried out to assess the potential insecticidal effects of SKTI toward cotton boll weevil (Monnerat et al., 1999). Fig. 4A shows the influence of SKTI on the weight of boll weevils at different developmental stages, after 4, 10 and 21 days when fed a diet containing three different concentrations of SKTI. At low concentration of SKTI (10 μM), no effect was observed at any analyzed stage. At higher concentrations (100 μM and 500 μM), a remarkable reduction of weight (around 23% and 60%, respectively) was observed for neonate larvae (Fig. 4A). These results are extremely important since the control of cotton boll weevils must occur at the beginning of their life cycle, in order to preserve the cotton fibers. Furthermore, a continuous exposure to the inhibitor is necessary to increase the harmful effects of SKTI. Adult insects that are able to move to different hosts could avoid or reduce the SKTI effects. Nevertheless, studies using transgenic plants must be conducted in order to answer this question. The presence of 500 μM of SKTI in the diet caused several deleterious effects at all stages of *A. grandis* development, including pupae (Fig. 4A) and adult insects (Fig. 4B). A decrease of 75% in larval weight was observed at the highest SKTI concentration. These results are in contrast to those found by Purcell et al. (1992) in which no in vivo activity of SKTI toward cotton boll weevil was found. Such difference could be due to the amount of inhibitor (approximately 40 μM) used by Purcell et al. (1992), approximately 12 times less than the concentration used in this work. Conversely, the results presented here agree with those observed in *L. cuprina* when SKTI was incorporated in artificial diet. At 500 μM SKTI, the reduction in the weight of larvae of *L. cuprina* was around 80% (Casu et al., 1994). SKTI also showed an in vivo activity against *L. oleracea*, both in artificial diet and in transgenic plants (Gatehouse et al., 1999). Bioassay results also corroborate with Franco et al. (2003), using BTCI toward *A. grandis* and with De Leo et al. (2001), who showed that *Plutella xylostella* (L.), *Mamestra brassicae* (L.) and *Spodoptera littoralis* (Boisd.) were sensitive to trypsin inhibitor from mustard. The interaction between proteinase inhibitors in plants and the digestive physiology and biochemistry of their insect predators is clearly more complex than the original concept of simple inhibition of a fixed complement of digestive proteinases. In other insects, such as *Baris coerulescens* (Olivier), some physiological adaptations were observed. However, in larvae of this insect pest, the first stage of development was insensitive to the OCI (Bonadé-Bottino et al., 1999) whereas SKTI were found to have severe effects on growth and development of boll weevil insect.

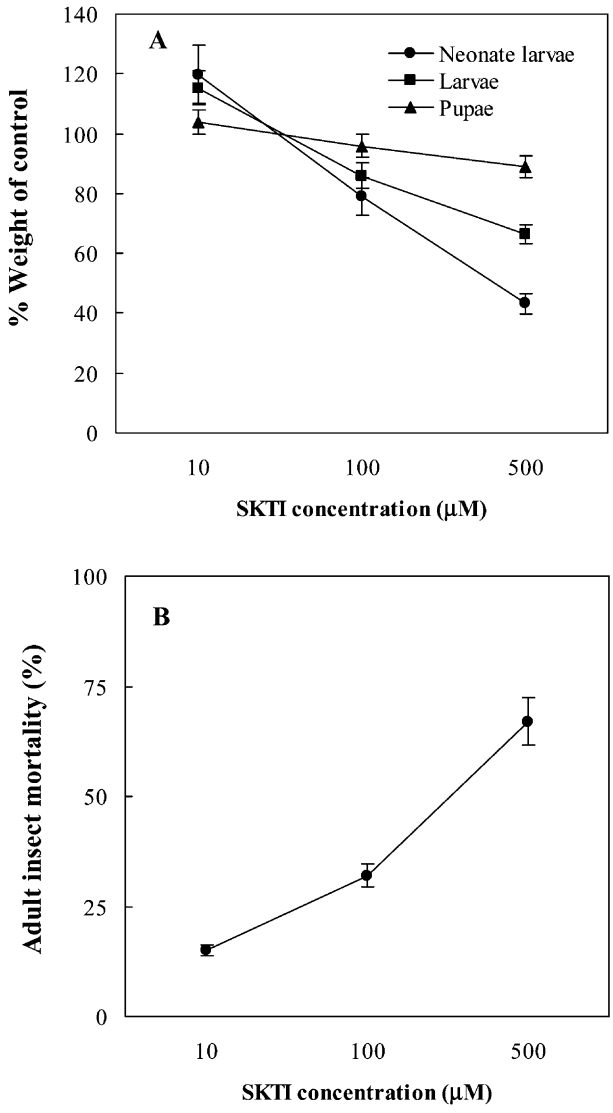

Fig. 4. Effects of SKTI on (A) neonate larvae, larvae and pupae relative weights of control and (B) on adult insect mortality. Each value represents the weight of 15 larvae. Each mean represent 4 replicates (±S.E.).

Another interesting observation was that the inhibitor caused a high ratio of deformities in pupae and adult insects (Fig. 5A and B). At 500 μM of SKTI we observed deformities of around 50% in pupae and 81% in adult insects (Fig. 5A), suggesting that SKTI probably delayed metamorphosis. Some deformations, such as the absence of wings and thorax, were observed at adult stages (Fig. 5B). These deleterious effects could be caused by the absence of certain proteins necessary for metamorphosis (Chapman, 1982). Some proteins, localized in insect cuticle (Missios et al., 2000) are necessary for the production of new adult tissues and enzyme systems. These proteins are synthesized primarily from free amino acids following a re-organization of peptides from larval proteins without total degradation of amino acids (Hopkins et al., 2000). Proteolytic activity is necessary for this re-organization, including serine and

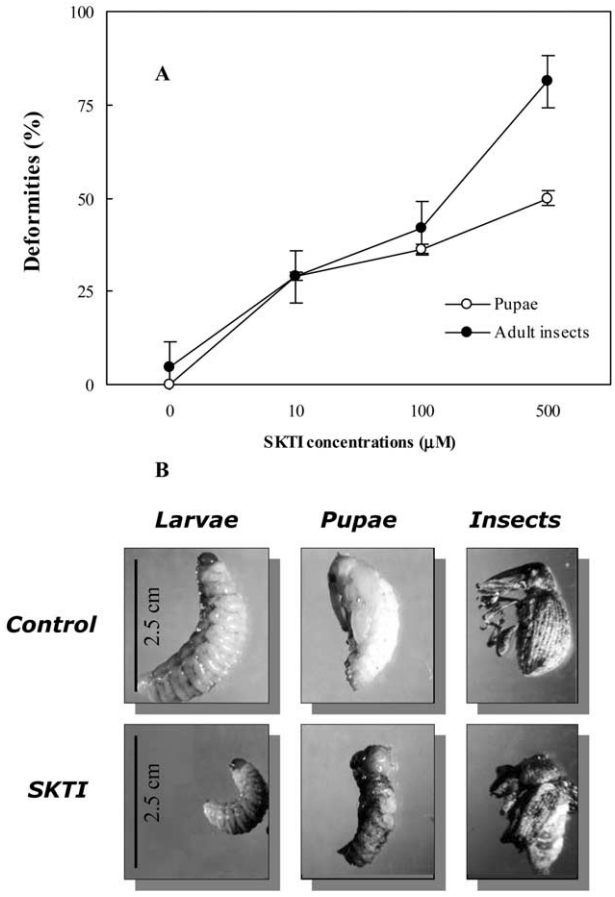

Fig. 5. (A) Deformity percentage observed in pupae and adult insects of *A. grandis* fed on SKTI and (B) larvae, pupae and adult insects reared in artificial diet with distilled water (control) and in artificial diet containing SKTI 500 μM.

cysteine proteinases. The mode of action of these inhibitors is still under debate, and it remains unclear whether the deleterious effects of proteinase inhibitors is due an anti-digestive effect, through proteolysis inhibition (Jongsma et al., 1995), or due to toxic effect by inducing hyper production of proteinase, leading to a shortage in amino acids (Broadway and Duffey, 1986). The deformity and retardation of metamorphoses caused by SKTI indicates a different mode of action of inhibitors against insects on plant defense area.

The discovery of an efficacious biological agent, which avoids the upset in natural control associated with broad-spectrum insecticides, would be an important component of the Integrated Pest Management (IPM) programs for cotton throughout the Americas (Wolfenbarger et al., 1997). One strategy that was suggested for the control of this weevil pest is the biological control with the exotic parasitoid *Catalaccus grandis* (L.) (Legaspi et al., 1998). However, transgenic plant technology can provide a major contribution to the production of crop varieties that are tolerant to insect pests. Inhibitors of proteinases and α-amylases have both been used successfully for protection of engineered plants. When expressed in transgenic plants at levels from tenths to several percent of total soluble protein, proteinase inhibitors are able to confer some protection to plants and trees against pests (Schroeder et al., 1995; Jouanin et al., 1998) with no effects on mammals (Pusztai et al., 1999). In many cases, transgenic plants containing genes encoding serine proteinase inhibitors have shown enhanced resistance toward insect pests (Johnson et al., 1989; Xu et al., 1996). However, despite SKTI being an effective inhibitor of the insect digestive proteinases of many species of insects in vitro (Johnston et al., 1995) only few studies show that this inhibitor can be efficacious in transgenic plants (Gatehouse et al., 1999; De Leo et al., 2001). This work shows for the first time a Kunitz proteinase inhibitor active in vivo against the cotton boll weevil *A. grandis*. These results indicate that transgenic cotton plants expressing SKTI gene could probably present an enhanced resistance against this weevil pest.

3. Experimental

3.1. Isolation of midgut fluid

Boll weevil larvae were obtained from the Biological Control Department of CENARGEN/EMBRAPA (Brasília-DF, Brazil). Larvae were reared on artificial diet at temperature of 28 °C and 55% relative humidity according to Oliveira-Neto et al. (2003). The guts were surgically removed from larvae and adult insects 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 fresh supernatant was removed and used for enzymatic assays.

3.2. Purification of soybean Kunitz trypsin inhibitor

The fraction rich in trypsin inhibitors (70% p/p) was purchased from Sigma Co., St. Louis, USA (Code T9003). Proteins were precipitated in the presence of 100% ammonium sulfate. Precipitation was followed by analysis in an HPLC reversed-phase analytical column (Vydac 218 TP 1022 C-18) at a flow rate of 1.0 $ml.min^{-1}$. SKTI samples were dissolved in 0.1% aqueous trifluoroacetic acid and were eluted with a linear gradient of acetonitrile (0–100%). Fractions were collected, lyophilized and stored at −20 °C.

3.3. Polyacrylamide gel electrophoresis

A discontinuous buffer system of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), using a 4% stacking gel and a 15% resolving gel, was carried out as described by Laemmli (1970), at room temperature. Bromophenol Blue was used as the tracking dye.

3.4. MALDI-TOF analysis

Freeze-dried samples of the peaks from HPLC were prepared for Matrix-Assisted Laser Desorption Time of Flight Analysis (MALDI-TOF) on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA, USA). Samples were dissolved in 1.0% aqueous trifluoroacetic acid and the matrix sinapinic acid added (a saturated solution dissolved in acetonitrile/0.1% TFA 1:1, v/v). The solution was then vortex mixed and aliquots of 1.0 ml were applied onto 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 was presented to a standard Voyager data system for manipulation. MALDI-TOF was calibrated using a Saquazyme calibration mixture (Applied Biosystems) consisting of bovine insulin (5,734 Da), *E. coli* thioredoxin (11,674 Da) and horse apomyoglobin (16,952 Da).

3.5. Proteinase and proteinase inhibitory assays

Semi-purified bovine pancreatic trypsin (BPT) and the proteinases from larval (AgPL) and adult insects (AgPA) of *A. grandis* were used for enzymatic assays. Bovine trypsin was purchased from Sigma Co., St. Louis, USA.

The hydrolysis of synthetic peptide substrates conjugated to aminomethylcoumarin (MCA) was measured using an Hitachi F4500 fluorimeter. The cleavage products were measured in fluorescence of liberated MCA at an excitation wavelength of 340 nm and emission monitored at 480 nm. Assays were performed by continuously monitoring enzyme activity against the fluorogenic substrates before and after inhibitor addition. Steady-state velocities prior to and following addition of each inhibitor were obtained by linear regression analysis. All determinations were based on assays with less than 2% substrate hydrolysis. For each individual assay, 100 ng ml^{-1} of protein extract were used.

Serine proteinase proteolytic activities were assayed using *N*-succinyl-Ala-Ala-Pro-Phe-MCA and N-CBZ-Phe-Arg-MCA (Sigma Co.) as substrates at a concentration of 10 μM. The enzymatic assays were performed at 25 °C in Tris–HCl buffer pH 8.6. The concentrations of different inhibitors used were: 50 μg ml^{-1} (135 μM) of TLCK, 100 μg ml^{-1} (284 μM) of TPCK and 170 μg ml^{-1} (1 mM) of PMSF. Soybean Kunitz trypsin inhibitor was tested at a standard concentration of 50 μg ml^{-1} and was assayed against AgPA and AgPL only using N-CBZ-Phe-Arg (trypsin-like activity). Assays were carried out in triplicate and inhibition replicate values differed by no more than 10%.

3.6. In vivo activity of SKTI against the boll weevil

Bioassays were conducted in a volume of 40 ml of artificial diet (Monnerat et al., 1999; Oliveira-Neto et al., 2003) sterilized without ascorbic and sorbic acids. SKTI was incorporated into the diet at concentrations of 10, 100 and 500 μM (or 0.023, 0.23 and 1.15% w/v) and protein concentration was calculated according to Bradford (1976). The diet was added to Petri dishes and larvae were placed, 48 h after hatching, in pits in the artificial diet without ascorbic and sorbic acids. Dead neonated larvae were counted after four days and the weight of the surviving neonate larvae was measured. Surviving larvae were transferred to dishes with artificial diet containing ascorbic and sorbic acids (Monnerat et al., 1999). Weights were measured for larvae at 10th day and of pupae at 21st day. Surviving adult insects were counted after 30 days in order to obtain the mortality rate. In negative controls, distilled water was added to the artificial diet. Each treatment was conducted with 4 repetitions, each replicate containing 15 larvae. The bioassay was maintained under controlled conditions at 28 °C and 55% stable humidity.

3.7. Proteinase assay in gelatin/polyacrylamide gel electrophoresis

The gelatin/polyacrylamide gel electrophoresis was adapted from Michaud et al. (1993) method. Midgut protein extracts were pre-incubated with specific inhibitors to discriminate among trypsin and chymotrypsin proteinases before electrophoresis. In order to inhibit trypsin, 5 μl was incubated with 10 μl of activation buffer (100 mM sodium phosphate pH 7.8, 8 mM EDTA, 10 mM cysteine and 0.2% Triton X-100) containing TLCK at a standard concentration of 8 μg μl^{-1} for 30 min at 37 °C. To inhibit chymotrypsin-like proteinases, TPCK was used instead of TLCK at the same standard concentration. Extracts pre-incubated with inhibitors were applied to a 10% SDS (w/v) polyacrylamide slab gel, containing 0.1% w/v of gelatin. To avoid migration of gelatin out of the resolving gel, the ratio of acrylamide to bis-acrylamide was adjusted to 30:1.2, and the migration was performed at 4 °C. Samples containing a standard concentration of 150 μg ml^{-1} of protein were diluted in the electrophoresis sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sucrose and 0.001% of Bromophenol Blue), loaded onto wells, and subject to electrophoresis using a Bio-Rad Mini-Protein Kit II™ system. After complete migration of Bromophenol Blue, electrophoresis was continued for an additional hour.

To renature the proteinases the gel was placed in a solution of 2.5% Triton X-100 for 1 h, followed by a proteinase assay with the activation buffer at 37 °C for 4 h. Proteolysis was stopped by transferring the gel to a staining solution (0.1% w/v Coomassie Brilliant Blue dissolved in 25% v/v MeOH and 10% v/v HOAc). The molecular weight was determined by the molecular mass migration of α-lactalbumin (14.2 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa) and bovine albumin (66.0 kDa).

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