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Insensitive trypsins are differentially transcribed during *Spodoptera frugiperda* adaptation against plant protease inhibitors

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ABSTRACT

0The fall armyworm (*Spodoptera frugiperda*) is an important pest insect due to high degree of polyphagia. In order to better understand its adaptation mechanism against plant protease inhibitors, bioassays were carried supplementing diet with the Kunitz trypsin inhibitor from *Entada acaciifolia* seeds (EATI). *In vitro* assays showed an increase of proteolytic activity in EATI-fed larvae midgut. Moreover, the trypsin enzymes showed insensitivity to inhibition with EATI. In order to understand what genes were overexpressed after chronic exposition to EATI, quantitative RT-PCR analyses were performed and revealed an increase in transcription of two trypsin genes, suggesting its participation in insensitivity of midgut trypsins. Another important result was the expression of one chymotrypsin gene, which is not expressed in control fed-larvae but induced in EATI-fed larvae. New regions of higher molecular weight showing proteolytic activity were visualized in inhibitor-fed larvae by zymography gel electrophoresis, proposing that the new enzymes expressed in response of inhibitor dietary would be formatting oligomers. This is a characteristic also observed in other pest insects that adapt to feed in plant protease inhibitors diet. Additional assays revealed that trypsins from EATI-fed larvae also became insensitive against Kunitz and Bowman-Birk inhibitors from soybean. This result suggests a possible involvement of the same *S. frugiperda* genes in adaptation against Kunitz and Bowman-Birk inhibitors in their host plants.

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1. Introduction

The possibility of utilizing plant proteins for crop protection against insect predation established a new promissory alternative research field, since pest insects lead to serious economic damages every year and most conventional insecticides are environmentally aggressive and ineffective (Carlini and Grossi-de-Sá, 2002). The interest in new insecticide molecules increased and the Protease Inhibitors (PIs) are among the most studied plant proteins with insecticide activity. Works showing the insecticide effects of PIs against pest insects affecting the larval development and adult emergency have been reported (Macedo et al., 2010, 2011), and transgenic plants expressing PIs has shown promising results (Falco and Silva-Filho, 2003; Senthilkumar et al., 2010). Simultaneously, several reports have showed that some insects are able to develop physiological responses to overcome the plant barrier and feed on plant tissues containing PIs (Bown et al., 1997; Gruden et al., 2004). It is known that these adaptations are activated in a timescale compatible with the imposed by plant defenses and involve the expression of digestive proteases (Bown et al., 2004).

Insects generally have a wide spectrum of digestive proteases that are spatially and temporally expressed in the midgut (Terra and Ferreira, 1994; Vinokurov et al., 2006). Thereby, for control strategies based on biological agents, such as PIs and other proteins, the knowledge of the relative composition, arrangement and functioning of all component proteases is essential (Jongsma et al., 1996; Hilder and Boulter, 1999; Vinokurov et al., 2006). Among the strategies observed from different Lepidoptera, the differential expression of proteases has been reported, especially serine-proteases. The inducible proteases, including trypsins and chymotrypsins, corroborate the development of insect resistance (Terra and Ferreira, 1994; Oliveira et al., 2005) and the most common reports include the overproduction of identical sensitive proteases (De Leo et al., 1998; Markwick et al.,

Abbreviations: BAPNA, N-Benzoyl-DL-arginine *p*-nitroanilide; EATI, *Entada acaciifolia* trypsin inhibitor; qRT-PCR, quantitative real time PCR; SPI, soybean Bowman-Birk inhibitor; SKTI, soybean Kunitz inhibitor; Suc-AAPF-PNA, N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide; TLCK, N-α-tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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1998) or the expression of new proteases insensitive to PIs (Bown et al., 1997; Brioschi et al., 2007). However, the mechanism that triggers the adaptation is still unknown.

Furthermore, there is no agreement if the evolution of multigene families of proteases in some insects may have developed intrinsic adaptive responses to inhibitor families present in host plants or even led to a single general effective response for some inhibitor families. Evidence of distinct strategies was observed by Bown et al. (2004) in studies with corn earworm (*Helicoverpa armigera*). Comparing the effects of Kunitz and Bowman-Birk inhibitors of soybean on larval enzymatic activity, they concluded that insensitive proteases were differentially induced by both inhibitors on artificial diet. However, due to the lack of similar comparative works analyzing the effect of inhibitors from different families on adaptive pattern of different polyphagous pests, no conclusive results were reported yet. In the absence of information about *Spodoptera frugiperda* response against Kunitz-type inhibitors and with the purpose of better understanding this physiological process, bioassays with the Kunitz-type inhibitor from *Entada acaciifolia* seeds (EATI) were performed. Neonate larvae were chronically fed with EATI and the effect of inhibitor on complete development of *S. frugiperda* was evaluated; the midguts of 6th instar larvae were used for analysis of gene expression by qRT-PCR and biochemical assays, in order to obtain a comprehensive approach.

2. Materials and methods

2.1. Reagents and plant material

BAPNA, Suc-AAPF-PNA, STI, SPI, TLCK, TPCK, and electrophoresis reagents were purchased from Sigma. Trizol and SuperScript® III Platinum® SYBR® Green were purchased from Fermentas as well as DNase and other molecular biology reagents. *E. acaciifolia* (Fabaceae: Mimosoideae) seeds were collected in Campinas, SP, Brazil and used for purification of EATI (Oliveira et al., 2012). Briefly, a crude extract, prepared from *E. acaciifolia* seeds was submitted to gel filtration, ion exchange and bio-affinity chromatographies for EATI purification. The inhibitor lyophilized was subsequently mixed in artificial diet for bioassay development.

2.2. Rearing insects and in vivo assays

The colony of *S. frugiperda* (J.E. Smith, 1797) (Lepidoptera, Noctuoidea) was maintained in standard conditions (27 ± 1 °C, 60–70% relative humidity and a L14:D10 photoperiod). The artificial diet used in fall armyworm feeding was composed of soybean meal (*Glycine max*), sucrose, wheat germ, agar, a vitamin complex, ascorbic acid, Wesson salts, choline chloride, agar and microbial inhibitors (tetracycline, sorbic acid and nipagin). To evaluate the effects of EATI on *S. frugiperda* development, neonate larvae were selected and individually transferred to artificial diet supplemented with purified inhibitor at a concentration of 0.5% w/w. A second group of larvae also was fed in artificial diet without EATI addition, constituting the control group. Each treatment was composed of twenty larvae, and the experimental results are the average of three independent bioassays. When the larvae reached 6th instar at standard conditions, the effect on larval weight gain and survival was determined. Pupal weight and adult emergence were determined on the first day following pupation and upon adult eclosion, respectively.

2.3. Preparation of midguts

For enzymatic activity assays, the larval midguts were prepared according to Macedo et al. (2003). Sixth instar larvae were cold-immobilized and dissected in cold 150 mM NaCl. The midguts were surgically removed using tweezers. After removing all extraneous tissues by rinsing in 150 mM NaCl, the midgut tissues were homogenized in a hand-held Potter-Elvehjem homogenizer immersed in ice at a final volume of 10 mL. Midgut homogenates were centrifuged at 14,000 *g* for 20 min at 4 °C, and the supernatants were collected, quantified and used as enzyme sources.

For total RNA obtainment, the 6th instar larvae were cold-immobilized and ten midguts were removed in an RNase free workplace in two replicates. Midguts were immediately frozen in liquid nitrogen, stored at −80 °C and used for total RNA extraction.

2.4. Sample quantification

Protein quantification was determined by the dye-binding method of Bradford (1976) with bovine serum albumin (1 mg/mL) as the standard.

2.5. Enzymatic activity assays

In vitro trypsin activity (EC 3.4.21.4) assay was carried out in microplates according to Oliveira et al. (2011). Trypsin-like and chymotrypsin-like activities from gut extract samples of *S. frugiperda* were determined using the chromogenic substrates BAPNA (Erlanger et al., 1961) and Suc-AAPF-PNA (DelMar et al., 1979). For trypsin-like activity assay, a stock solution of BAPNA (100 mM) was firstly prepared in DMSO and then diluted in a work solution (1 mM) in 50 mM Tris–HCl buffer, pH 8.0. Samples of 5 μg from midgut larvae were incubated with assay buffer, and then 200 μL of BAPNA was added, thus completing a final volume of 270 μL. The assay was carried for 30 min at 37 °C. For chymotrypsin-like activity (EC 3.4.21.1) assay, a stock solution of Suc-AAPF-PNA (100 mM) was prepared in a solution of 10% dimethylformamide diluted in 50 mM Tris–HCl buffer, pH 8.0 and then diluted in a final concentration of 1 mM in 50 mM Tris–HCl buffer, pH 8.0. Samples of 3 μg from midgut larvae were incubated with assay buffer, and then 20 μL of Suc-AAPF-PNA was added, thus completing a final volume of 120 μL. The assay was carried for 5 min at 37 °C. In both assays the releases of chromophore *p*-nitroaniline were determined by monitoring the absorbance change at 410 nm (VersaMax Microplate Reader, Molecular Devices, US).

For the evaluation of the sensitivity of trypsin midgut proteases to inhibition by EATI, an inhibition curve was made with crescent EATI concentrations (0–0.5 μg), and the trypsin activity assay was carried out as described above. Other assay evaluated the sensitivity of trypsin midgut enzymes for different inhibitors. The plant inhibitors EATI, SKTI and SPI were used in a single concentration (0.2 μg) and the synthetic inhibitor TLCK was used in a fixed volume and concentration (6 μL of 10 μM TLCK). The inhibitors were incubated with midgut samples, and the assays were carried in the same conditions described above.

2.6. Quantitative real-time PCR

Total RNA was extracted from frozen midguts using 1 mL of TRIzol™ (Invitrogen). The samples were treated with 3 units of DNase I (Fermentas) of 37 °C for 1 h. Synthesis of the cDNAs was primed by oligo d(T) using an ImProm-II™ Reverse Transcriptase (Promega) according to the manufacturer's recommendations.

The cDNA was diluted in a work solution and 4 μL was used in qRT-PCR. The reactions were carried out in a thermocycler StepOne™ Real-Time PCR System (Applied Biosystems) using Maxima® SYBR Green/ROX qPCR Master Mix (2×) (Fermentas). The master mix prepared for analysis of each gene was composed of 0.3 μL of forward primer (0.12 μM), 0.3 μL of reverse primer (0.12 μM), 12.5 μL of SYBR®, and 4 μL of cDNA in a total volume of 25 μL. The amplification reaction conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. A negative control without a cDNA template was run with each analysis to evaluate the overall specificity. The ribosomal protein S30 was used to normalize the data (NCBI locus AF400225). Efficiency and specificity reaction of

each primer set were evaluated by a standard curve and a melting curve (Table 1). Relative quantification was carried out using REST® model with efficiency correction (Pfaffl, 2001). The experiments were repeated twice for validation of results.

2.7. In vitro digestion of EATI by S. frugiperda proteases

EATI was incubated with *S. frugiperda* midgut proteases of both treatments in 50 mM Tris–HCl buffer, pH 8.0 with an EATI/midgut protein ratio of 1:5 (w/w). The samples were incubated at 37 °C, and a total of 10 digestion intervals were used (1, 3, 6, 9, 12, 15, 18, 24, 48 and 72 h). The digestion was stopped by immersing the tubes in boiling water for 2 min and in cold water immediately after. The degradation of BSA was used as a positive control for proteolytic activity. The proteins were separated by 12.5% SDS-PAGE as described by Laemmli (1970), and molecular weight standards, low range (BIO-RAD) were used. The gels were subsequently stained with Coomassie brilliant blue R-250.

2.8. Protease activity of midgut extracts in electrophoresis gels

Zymography gel electrophoresis was made according to Schmidt et al. (1988) with some modifications. The samples were incubated with non-denaturing sample buffer for 30 min on ice. A second group of samples were also incubated with 6 μL 10 mM TLCK to inhibit the trypsin-like activity. Afterwards, the samples were separated on 9% SDS-PAGE at 200 V at 4 °C. Then, the gel was electro-blotted to a second 9% polyacrylamide gel containing 0.1% (w/v) gelatin at 60 V for 20 min, followed by washing with 2.5% Triton X-100 solution for 2 h with shaking to remove the SDS. After the washing, the gelatin gel was incubated at 37 °C with 50 mM Tris–HCl buffer, pH 8.0, for 30 min. The gels were subsequently stained with Coomassie brilliant blue R-250. The white bands visualized against the blue background indicate proteolytic activity.

2.9. Statistical analysis

The data was examined using one-way analysis of variance (ANOVA) with post-test using Tukey as multiple comparisons. The p-value of <0.05 was considered significant. The analysis was performed with the GraphPad InStat program (GraphPad software).

Table 1
Sequence of primers designed for RT-PCR studies.

SfTry3	5′-ACGTCCATTGACAAGTTCCC-3′ (F)
	5′-AGCTTGGGTCGTAGAAGATG-3′ (R)
SfTry4	5′-AACTTATGGCGGCTAGCGTA-3′ (F)
	5′-AAGGGACCTCCGAAGTCACC-3′ (R)
SfTry5	5′-CAGAGGATTGTGGGTGGTTC-3′ (F)
	5′-TGGGCAGCAGTAAGGATAGC-3′ (R)
SfTry7	5′-TCGAGAATTACCCCAGCATC-3′ (F)
	5′-AGGTACCGGCTCTGATACGA-3′ (R)
SfChy1	5′-AAGTCATCATGCACCCACAA-3′ (F)
	5′-GACACGTGGCTTACCACAGA-3′ (R)
SfChy2	5′-CGTCTCAGGAAAACCCAGTC-3′ (F)
	5′-AGGGACGTTGGAAATGTCAG-3′ (R)
SfChy3	5′-TTCCTTCCCCCTTATCGTTC-3′ (F)
	5′-CAAGAGAGGGCTGTTGTTCC-3′ (R)
SfChy5	5′-TTACCGCTAACCGTGTGCTT-3′ (F)
	5′-CGATGACATCTGACAAGGCG-3′ (R)
SfChy7	5′-TTCCTGAGCCACGTTAGCTT-3′ (F)
	5′-CAAGACAGGCTCGTTGTTGA-3′ (R)
SfChy9	5′-GGATCTCAGCTCGGAGAAAA-3′ (F)
	5′-GCAAGAGGACCACCAGAGTC-3′ (R)
SfChy11	5′-GGCTCCACCACTCTCTTCAC-3′ (F)
	5′-GCAATGGGTTGGATGTTAGC-3′ (R)

3. Results and discussion

3.1. Effects of EATI on S. frugiperda development

The evaluation of the chronic effect of EATI on *S. frugiperda* took into account all development stages. The EATI-fed 6th instar larvae showed a 20% decrease in average mass compared with control-fed larvae (Fig. 1). Along with a decrease in larval mass, we also noticed a significant extension of larval stage by 2 days (Table 2). Nevertheless, the following measurements in the next stages, such as pupal mass, adult emergence rate and insect life-cycle did not show distinction between the both groups, demonstrating that *S. frugiperda* larvae adapted to the dietary EATI. The extension of larval stage combined with the reduction of larval mass may be a signal of the use of essential amino acids in synthesis of midgut proteases (Broadway and Duffey, 1986) and other proteins related to metabolism, detoxification and stress tolerance (Petek et al., 2012). This way, the extension of larval stage would be a compensatory effect, because during the adaptive process there is a considerable energetic expense, therefore, the larvae should accumulate the energy necessary to complete the next stages of development. Similar reduction on larval weight without effects on larval mortality has been reported for other polyphagous insects known to adapt to the plant defense compounds (Broadway, 1996; Petek et al., 2012).

3.2. Real-time PCR

Once determined the occurrence of adaptive response, the modifications in enzymatic activity and in expression of serine-protease genes in *S. frugiperda* were analyzed. Through qRT-PCR analyses four trypsin genes were studied: SfTry3, SfTry4, SfTry5 and SfTry7 (Fig. 2A). In comparison with control group, the trypsin genes in EATI-fed larvae showed two distinct patterns. The first one was characterized by an increase in transcription of trypsin genes, where SfTry5 and SfTry7 reached approximately a 2-fold increase in relation to EATI-fed larvae. On the other hand, the second group of trypsin genes did not show a significant increase in transcription, such as SfTry4 ($p = 0.073$), or which had a slight decrease in expression level, such as SfTry3, compared with control level.

Multiple trypsin and chymotrypsin isoenzymes have been recognized and the sequencing of multifamily genes for insect trypsins is

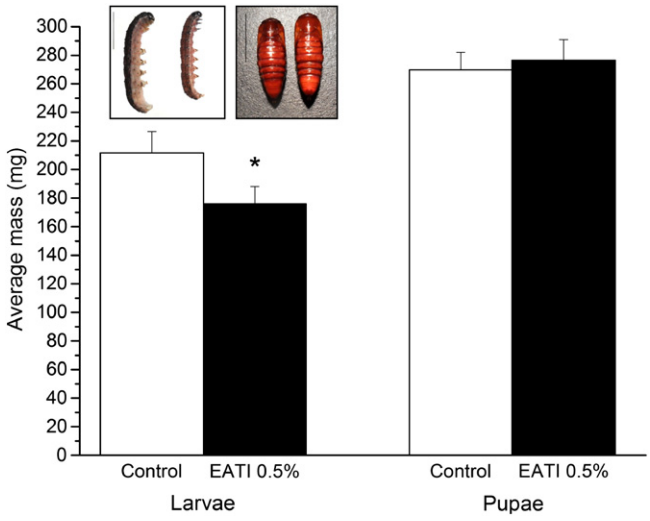

Fig. 1. Effect of EATI dietary on the mass of *S. frugiperda* larvae. Inset: variation in size of sixth instar fed on control diet (left) or diet containing 0.5% EATI (right). Variation in size of pupae from control group (left) or 0.5% EATI group (right). Bar = 1 cm. The * means that the difference between the treatments is significant (ANOVA, $p < 0.05$), based in Tukey post-hoc test. These data represent the average of three different experiments.

Table 2
Physiological parameters analyzed during *S. frugiperda* development.

	Control group	EATI 0.5%
Larval mass (mg)	211.6 ± 15.0a	176.0 ± 12.2b
Pupal mass (mg)	269.7 ± 12.4a	276.5 ± 14.5a
Larval stage (days)	12.85 ± 0.69a	14.66 ± 0.86b
Adult emergence (%)	100.0a	100.0a
Insect life-cycle (days)	31.16 ± 0.4a	31.0 ± 0a

Different letters indicates that the difference between the treatments is significant (ANOVA, $p < 0.05$).

a recent focus of attention (Bown et al., 1997; Mazumdar-Leighton and Broadway, 2001a; Oliveira et al., 2005; Brioschi et al., 2007). The genes analyzed in this work are a representative part of total trypsin genes in *S. frugiperda* genome, but we cannot rule out the possibility of sequencing more serine-protease genes, with the development of additional transcriptome studies. Based in our results, it is interesting to emphasize that there was no down-regulation of trypsin genes. Hence, changes in trypsin activity and sensitivity are related to an increase in transcription of SfTry5 and SfTry7 trypsin genes. Furthermore, the data corroborates with the pattern of general increase of transcription, described by Brioschi et al. (2007) for *S. frugiperda* larvae fed with soybean Bowman-Birk inhibitor in an artificial diet. A similar adaptation strategy is known for *H. armigera* larvae, another polyphagous pest insect, exposed to dietary SKTI (Bown et al., 2004). SKTI-fed larvae decrease the transcription of HaTC16, mRNA corresponding to a sensitive trypsin, while the other serine-protease mRNAs are up-regulated. Additional studies have been made in order to sequence more serine-protease genes and to discover if other *S. frugiperda* genes are down-regulated after exposition to PIs.

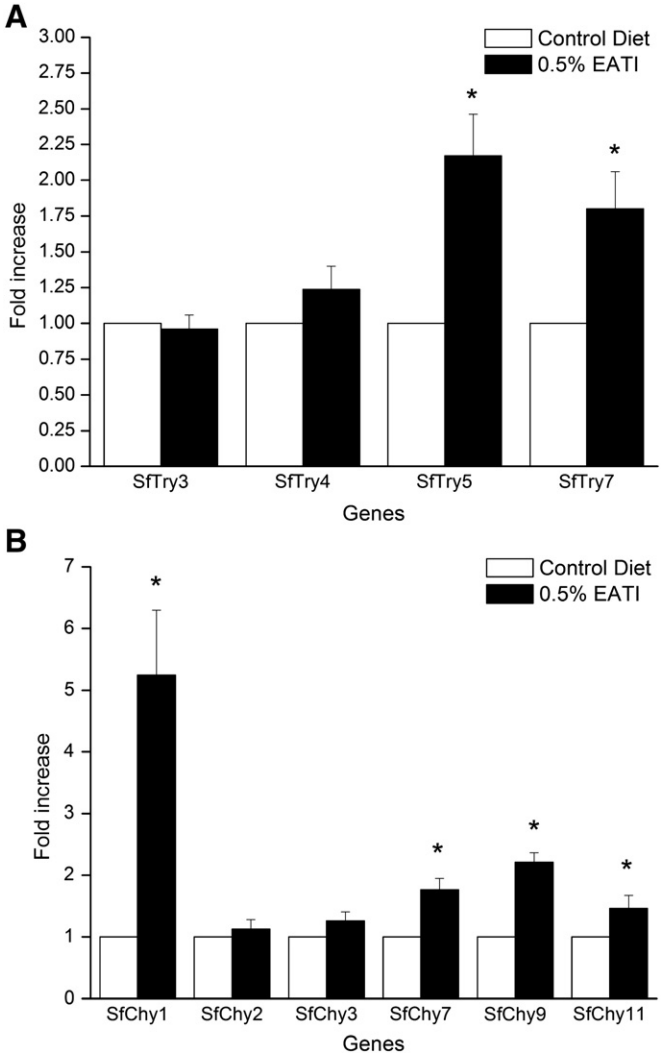

Fig. 2. (A) qRT-PCR gene expression analyses of *S. frugiperda* trypsin genes and (B) qRT-PCR gene expression analyses of *S. frugiperda* chymotrypsin genes. The * means that the difference between the treatments is significant (ANOVA, $p < 0.05$), based in Tukey post-hoc test. These data represent the average of two different experiments.

We also studied the transcription level of 7 chymotrypsin genes, and as reported by Brioschi et al. (2007) it was possible to divide the chymotrypsin genes in two groups: one constitutively expressed, represented by SfChy1, SfChy2, SfChy3, SfChy7, SfChy9 and SfChy11, and another gene transcribed only in inhibitor-fed larvae, represented by SfChy5. The analysis of the first group of chymotrypsin genes revealed that among the 6 genes within the group, only SfChy2 and SfChy3 showed a non-significant increase in transcription level ($p = 0.63$), and that the other genes were up-regulated (Fig. 2B). Another particularity is that with the exception of SfChy1, which increased the transcription level by 5-fold in EATI-fed larvae, all chymotrypsin genes constitutively expressed showed a small increase after the chronic exposition to inhibitor (Fig. 2B). Moreover, the SfChy5 gene, transcribed only by EATI-fed larvae, suggests the involvement of this chymotrypsin gene in adaptive response against PIs, but until this moment its role continues unknown. A plausible hypothesis would be that the increase of other class of proteases, like chymotrypsins, would compensate the main class of protease inhibited. This is a strategy adopted by other polyphagous pests (Wu et al., 1997; Gatehouse et al., 1998).

In a discussion about the complex response against PIs related to the timescale, Bown et al. (2004) divided the response to ingestion of PIs in three phases: an initial phase where all protease-encoding mRNAs are increased, which occurs during the first 12 h in contact with inhibitor. A second phase involves the down-regulation of sensitive protease sequences, reaching a steady-state level that occurs between 12 and 24 h, and is followed by a third phase, in which the differences in mRNA levels are either maintained or decreased in the relation control-fed larvae. The results obtained by Brioschi et al. (2007) in 48 h in feeding a diet containing SPI correspond to the third phase of response and is valid for a comparative analysis with our results of chronic exposition, since the differences in mRNAs levels are established in this phase. As a consequence, the comparison of pattern of expression of *S. frugiperda* serine-protease genes in response to exposition to EATI, a Kunitz inhibitor, and SPI, a Bowman-Birk inhibitor, provide evidences that may suggest a single and efficient adaptation by *S. frugiperda* against serine-protease inhibitors from different families.

Through a comprehensive alignment study of known insect trypsin sequences, Lopes et al. (2004) analyzed the insect trypsin interactions with Kunitz and Bowman-Birk inhibitors. After analyzing the interaction of different insect trypsin residues with Kunitz and Bowman-Birk inhibitors, they concluded that both inhibitor families share a high number of interactions in common and others are specific for each one. In these circumstances, the residues of common interaction could have been selected by *S. frugiperda* and evolved a response pattern efficient for both families of inhibitors. In part, this hypothesis could withstand the high degree of polyphagia presented by this pest, because the existence of a single response was able to overcome the effects of different PIs. This adaptation would allow the fall armyworm to feed on different crops with distinct compositions of Kunitz and Bowman-Birk inhibitors, the most representative PIs among the crops attacked by this pest.

3.3. In vitro assays

It is well known that some polyphagous insects produce PI-resistant trypsins and chymotrypsins in response to the consumption of PIs

(Jongsma et al., 1995; Bown et al., 1997; Mazumdar-Leighton and Broadway, 2001a,b; Volpicella et al., 2003; Brioschi et al., 2007), but the molecular features that contribute to PI resistance have not been completely characterized (Dunse et al., 2010). The EATI-fed larvae showed an increase in trypsin and chymotrypsin-like activities by 40% and 30%, respectively, corroborating the increase in transcription rate of serine-protease genes. Furthermore, sensitivity of trypsin-like enzymes present in midguts from EATI-fed larvae was pronouncedly altered, as observed by inhibition curve showed in Fig. 3A. We choose a point in curve where there was no inhibitor excess (0.2 μg) to analyze the sensitivity of *S. frugiperda* trypsins against other PIs, using the synthetic inhibitor TLCK as positive control. The three plant inhibitors analyzed, EATI and STI, Kunitz inhibitors, and SPI, Bowman-Birk inhibitor, showed a similar percentage of 65% for the inhibition of trypsin activity in control-fed larvae. However, the inhibition of trypsin activity in EATI-fed larvae decreased to 31.5% for the three inhibitors assayed, thus showing that the larvae exposed to EATI triggered an adaptation that switched its sensitivity for other plant inhibitors (Fig. 3B). Based in our data, we have indicators to suggest that the insensitivity of trypsins observed through *in vitro* assays would be related to the up-regulation of SfTry5 and SfTry7 genes. The positive control showed that the trypsins from both groups were strongly inhibited by TLCK, showing a residual enzymatic activity of 15%. Further *in vitro* assays showed that neither EATI nor TPCK, a synthetic chymotrypsin inhibitor, was able to inhibit the *S. frugiperda* chymotrypsins (data not shown).

TLCK and TPCK are irreversible inhibitors that alkylate the His-46 and His-57 residues that are present in the active site of trypsins and chymotrypsins, respectively. The trypsin enzymes synthetized in response to EATI were inhibited by TLCK at the same degree as the trypsins from control fed-larvae, thus suggesting that the two sets of trypsins contain the His-46 residue in their active sites. The low reactivity of *S. frugiperda* chymotrypsins toward TPCK was investigated by Lopes et al. (2009), who proposed the occurrence of replacements in the neighborhood of catalytic His-57 residue, thus affecting its pKa value and decreasing the reactivity of His57 residue for chloromethyl ketones. Johnston et al. (1995) observed that the chymotrypsins of *Heliothis virescens* were also not inactivated by TPCK. These differences would result in the reduction of the access of substrates and inhibitors to the active site (Lopes et al., 2009), thus conferring an evolutionary advantage to the insects against plant defenses.

A qualitative analysis of proteolytic activity in *S. frugiperda* midgut was performed by zymography gel electrophoresis. Three regions in the gel showed the most evident alterations and were marked as B1, B2 and B3 (Fig. 4). The increase in number of bands with proteolytic activity close to 45 kDa in EATI-fed larvae (B1 region) can be observed and is better visualized with a small addition of TLCK. Additionally, there are no reports of insect trypsins with this pattern of mass, once that the most trypsins described possess molecular mass ranging from 20 to 35 kDa (Terra and Ferreira, 1994). Moreover, the presence of two new bands with approximately 16 and 23 kDa (B2 and B3 regions, respectively) was observed. This is the first time that the visualization of stable protease oligomers is reported for *S. frugiperda*. Replacements of amino acid residues might alter the hydrophobicity of resistant-proteases, and the hydrophobic trypsins possess a higher tendency to form oligomers, thus hindering the access of plant PIs to enzyme active site (Brito et al., 2001). During *in vivo* studies, Brito et al. (2001) compared the enzymatic profile of *H. virescens* midgut fed in artificial diet, without protease inhibitor, or on tobacco leaf discs, with the presence of tobacco leaf inhibitors.

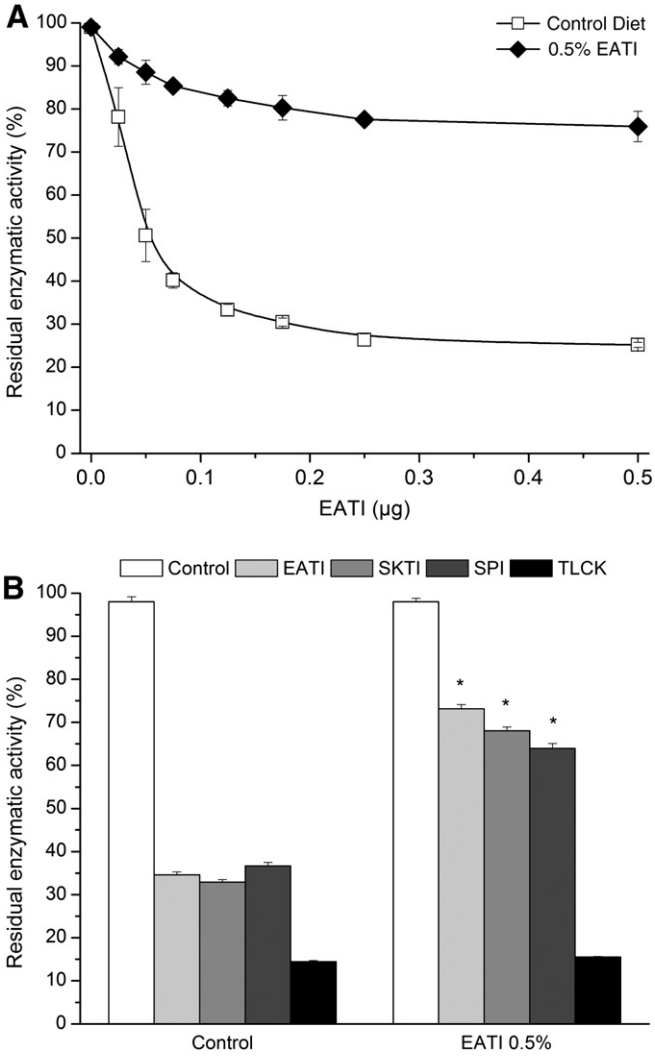

Fig. 3. (A) Inhibition of proteolytic activity of midgut trypsins of *S. frugiperda* fed on control diet (open square) and diet containing 0.5% EATI (filled diamond). (B) Inhibition of proteolytic activity with a unique concentration of EATI, STI and SPI (0.25 μg) or 5 μL 10 mM TLCK. The * means that the difference between the treatments is significant (ANOVA, $p < 0.05$), based in Tukey post-hoc test. The assays were made in triplicate.

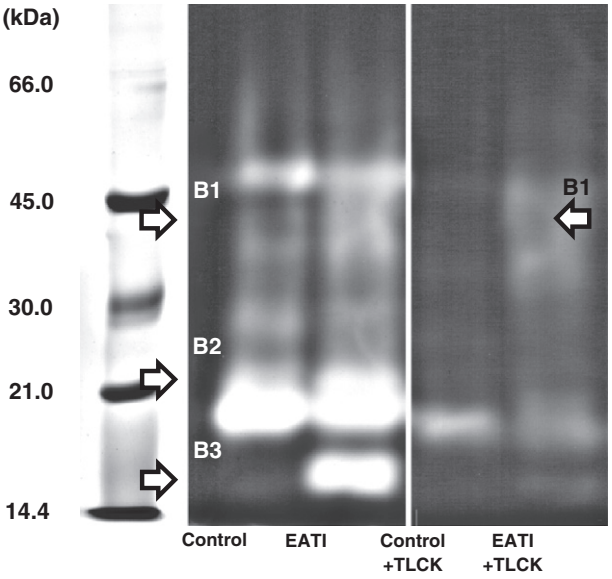

Fig. 4. Protease activity of midgut proteases in zymography gel electrophoresis. Samples fed with control diet or diet containing 0.5% EATI were used for the determination of enzyme profile. The samples were also treated with TLCK for the inhibition of trypsin activity. The B1, B2 and B3 regions are shown as arrows.

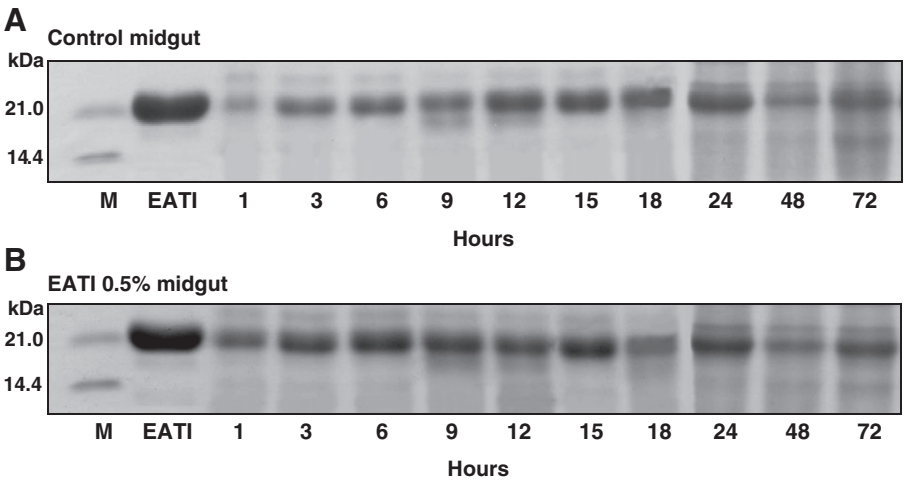

Fig. 5. SDS-PAGE of EATI *in vitro* digestion by *S. frugiperda* midgut proteases. The EATI was incubated at ratio 1:5 (inhibitor: midgut extract). The digestion occurred at different time intervals indicated. (A) Digestion with midgut from control-fed larvae and (B) midgut from EATI-fed larvae. M: The molecular mass markers of 20 and 14 kDa are shown.

The authors observed that in both treatments there was the formation of oligomers, but in tobacco-fed larvae the oligomers were more stable, since that remain the oligomers even in the presence of SDS. Thus, the authors suggested that the difference in trypsin monomer's constitution in both groups is responsible for this effect. A quite similar pattern was observed in our study, shown as B1 region (Fig. 4). As a differential expression of proteases occurred in EATI-fed larvae, the presence of these oligomers may be the result of interaction of new proteases with molecular mass between 16 and 23 kDa, differentially expressed in response inhibitor dietary. These proteases are represented by B2 and B3 regions in Fig. 4, and we suggest that they be trypsin enzymes, since the incubation with TLCK decreased the proteolytic activity in B1 region. Further assays have been made in order to confirm and characterize the existence of these oligomers.

3.4. Digestion of EATI by S. frugiperda midgut proteases

In order to analyze if the increase in proteolytic activity observed in EATI-fed larvae is responsible for the digestion of EATI, the susceptibility of EATI degradation by proteases of *S. frugiperda* was carried through the incubation of EATI with midgut proteases in a 1:5 ratio. Interestingly, even with the considerable increase of general proteolytic activity in EATI-fed larvae, the time of digestion of EATI by midgut proteases was not altered. The appearance of a band with approximated 15 kDa, the result of inhibitor digestion, occurred at the same interval in both treatments, between 48 and 72 h of incubation (Fig. 5). In the positive control, the digestion of BSA occurred within 3 h (data not shown). There are two possible explanations for this fact: first, the new synthetized *S. frugiperda* trypsins are insensitive and the inactivation of PIs ceases to be an important adaptation, thus PIs would represent no more risk for insect digestive process. And second, PIs are known for their resistance to digestion by midgut protease for some insects, and this would be another characteristic that needs to be studied with special attention. Moreover, the inactivation of PIs by digestion is commonly reported for coleopteran insects (Girard et al., 1998), being an adaptation strategy well known for this order of insects.

4. Conclusions

S. frugiperda responds to the ingestion of PIs with the increase of proteolytic activity, and *in vitro* assays showed that the trypsins became insensitive to both Kunitz and Bowman-Birk inhibitors. In this way, it is possible that the transcription of SfTry5, SfTry7 and SfChy5 genes confers resistance against Kunitz and Bowman-Birk inhibitors, an evolutionary mechanism that would favor the high degree of polyphagia observed for the fall armyworm, a new way to approach the subject. Additionally, the adaptation strategy of *S. frugiperda* involves the formation of trypsin oligomers, hindering the interaction of PIs with their target enzymes. The formation of oligomers would alter the enzymatic activity, as active sites may be blocked, and the increase of proteolytic activity observed in our study would be a consequence of the formation of oligomers, in order to maintain a standard rate of enzymatic activity. More details about this mechanism needs to be studied in order to find conclusive results for comparisons with other polyphagous pest models, contributing to the design of better strategies of pest control.

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References

Bown, D.P., Wilkinson, H.S., Gatehouse, J.A., 1997. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochem. Mol. Biol. 27, 625–638.

Bown, D.P., Wilkinson, H.S., Gatehouse, J.A., 2004. Regulation of expression of genes encoding digestive proteases in the gut of a polyphagous lepidopteran larva in response to dietary protease inhibitors. Physiol. Entomol. 29, 278–290.

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

Brioschi, D., Nadalini, L.D., Bengtson, M.H., Sogayar, M.C., Moura, D.S., Silva-Filho, M.C., 2007. General up regulation of *Spodoptera frugiperda* trypsins and chymotrypsins allows its adaptation to soybean proteinase inhibitor. Insect Biochem. Mol. Biol. 37, 1283–1290.

Brito, L.O., Lopes, A.R., Parra, J.R.P., Terra, W.R., Silva-Filho, M.C., 2001. Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitors may be mediated by the synthesis of new proteinases. Comp. Biochem. Physiol. 128, 365–375.

Broadway, R.M., 1996. Dietary proteinase inhibitors alter complement of midgut proteases. Arch. Insect Biochem. Physiol. 32, 39–53.

Broadway, R.M., Duffey, S.S., 1986. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. J. Insect Physiol. 32, 827–833.

Carlini, C., Grossi-de-Sá, M., 2002. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon 40, 1515–1539.

De Leo, F., Bonadé-Bottino, M.A., Ceci, L.R., Gallerani, R., Jouanin, L., 1998. Opposite effects on *Spodoptera littoralis* larvae of a low and high expression level of a trypsin proteinase inhibitor in transgenic plants. Plant Physiol. 119, 997–1004.

DelMar, E.G., Largman, C., Brodrick, J.W., Geokas, M.C., 1979. A sensitive new substrate for chymotrypsin. Anal. Biochem. 99, 316–320.

Dunse, K.M., Kaas, Q., Guarino, R.F., Barton, P.A., Craik, D.J., Anderson, M.A., 2010. Molecular basis for the resistance of an insect chymotrypsin to a potato type II proteinase inhibitor. Proc. Natl. Acad. Sci. U. S. A. 107, 15016–15021.

Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

Falco, M.C., Silva-Filho, M.C., 2003. Expression of soybean proteinase inhibitors in transgenic sugarcane plants: effects on natural defense against *Diatraea saccharalis*. Plant Physiol. Biochem. 41, 761–766.

Gatehouse, L.N., Shannon, A.L., Burgess, E.P.J., Christeller, J.T., 1998. Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in response to four proteinase inhibitors in the diet. Insect Biochem. Mol. Biol. 27, 929–944.

Girard, C., Le Métayer, M., Bonadé-Bottino, M., Pham-Delègue, 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.

Gruden, K., Kuipers, A.G.J., Gunčar, G., Slapar, N., Štrukelj, B., Jongsma, M.A., 2004. Molecular basis of Colorado potato beetle adaptation to potato plant defence at the level of digestive cysteine proteinases. Insect Biochem. Mol. Biol. 34, 365–375.

Hilder, V.A., Boulter, D., 1999. Genetic engineering of crop plants for insect resistance — a critical review. Crop. Prot. 18, 177–191.

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

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

Jongsma, M.A., Stiekema, W.J., Bosch, D., 1996. Combatting inhibitor-insensitive proteases of insect pests. Trends Biotechnol. 14, 331–333.

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

Lopes, A.R., Juliano, M.A., Juliano, L., Terra, W.R., 2004. Coevolution of insect trypsins and inhibitors. Arch. Insect Biochem. Physiol. 55, 140–152.

Lopes, A.R., Sato, P.M., Terra, W.R., 2009. Insect chymotrypsins: chloromethyl ketone inactivation and substrate specificity relative to possible coevolutional adaptation of insects and plants. Arch. Insect Biochem. Physiol. 70, 188–203.

Macedo, M.L.R., Freire, M.G.M., Cabrini, E.C., Toyama, M.H., Novello, J.C., Marangoni, S., 2003. A trypsin inhibitor from *Peltophorum dubium* seeds active against pest proteases and its effect on the survival of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Biochim. Biophys. Acta 1621, 170–182.

Macedo, M.L.R., Durigan, R.A., Silva, D.S., Marangoni, S., Freire, M.G.M., Parra, J.R.P., 2010. *Adenanthera pavonina* trypsin inhibitor retard growth of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Arch. Insect Biochem. Physiol. 73, 213–231.

Macedo, M.L.R., Freire, M.G.M., Franco, O.L., Migliolo, L., Oliveira, C.F.R., 2011. Practical and theoretical characterization of *Inga laurina Kunitz* inhibitor on the control of *Homalinotus coriaceus*. Comp. Biochem. Physiol. 158, 164–172.

Markwick, N.P., Laing, W.A., Christeller, J.T., McHenry, J.Z., Newton, M.R., 1998. Overproduction of digestive enzymes compensates for inhibitory effects of protease and alpha-amylase inhibitors fed to three species of leafrollers (Lepidoptera: Tortricidae). J. Econ. Entomol. 91, 1265–1276.

Mazumdar-Leighton, S., Broadway, R.M., 2001a. Identification of six chymotrypsin cDNAs from larval midguts of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. Insect Biochem. Mol. Biol. 31, 633–644.

Mazumdar-Leighton, S., Broadway, R.M., 2001b. Transcriptional induction of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. Insect Biochem. Mol. Biol. 31, 645–657.

Oliveira, M.G.A., De Simone, S.G., Xavier, L.P., Guedes, R.N.C., 2005. Partial purification and characterization of digestive trypsin-like proteases from the velvet bean caterpillar, *Anticarsia gemmatalis*. Comp. Biochem. Physiol. 140, 369–380.

Oliveira, C.F.R., Luz, L.A., Paiva, P.M.G., Coelho, L.C.B.B., Marangoni, S., Macedo, M.L.R., 2011. Evaluation of seed coagulant *Moringa oleifera* lectin (cMoL) as a bioinsecticidal tool with potential for the control of insects. Process. Biochem. 46, 498–504.

Oliveira, C.F.R., Vasconcelos, I.M., Aparicio, R., Freire, M.d.G.M., Baldasso, P.A., Marangoni, S., Macedo, M.L.R., 2012. Purification and biochemical properties of a Kunitz-type trypsin inhibitor from *Entada acaciifolia* (Benth.) seeds. Process. Biochem. 47, 929–935.

Petek, M., Turnšek, N., Gašparič, M.B., Novak, M.P., Gruden, K., Slapar, N., Popovič, T., Štrukelj, B., Jongsma, M.A., 2012. A Complex of genes involved in adaptation of *Leptinotarsa decemlineata* larvae to induced potato defense. Arch. Insect Biochem. Physiol. 79, 153–181.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29.

Schmidt, T.M., Bleakley, B., Nealson, K.H., 1988. Characterization of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. Appl. Environ. Microbiol. 54, 2793–2797.

Senthilkumar, R., Cheng, C.-P., Yeh, K.-W., 2010. Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. Plant Biotechnol. J. 8, 65–75.

Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. B 109, 1–62.

Vinokurov, K.S., Elpidina, E.N., Oppert, B., Prabhakar, S., Zhuzhikov, D.P., Dunaevsky, Y.E., Belozersky, M.A., 2006. Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. Comp. Biochem. Physiol. B 145, 126–137.

Volpicella, M., Ceci, L.R., Cordewener, J., America, T., Gallerani, R., Bode, W., Jongsma, M.A., Beekwilder, J., 2003. Properties of purified gut trypsin from *Helicoverpa zea*, adapted to proteinase inhibitors. Eur. J. Biochem. 270, 10–19.

Wu, Y., Llewellyn, D., Mathews, A., Dennis, E.S., 1997. Adaptation of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to a proteinase inhibitor expressed in transgenic tobacco. Mol. Breed. 3, 371–380.