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Purification of a Kunitz-type Inhibitor from *Acacia polyphylla* DC Seeds: Characterization and Insecticidal Properties against *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae)

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ABSTRACT: *Anagasta kuehniella* is a polyphagous pest that causes economic losses worldwide. This species produces serine proteases as its major enzymes for protein digestion. In this study, a new serine-protease inhibitor was isolated from *Acacia polyphylla* seeds (AcKI). Further analysis revealed that AcKI is formed by two polypeptide chains with a relative molecular mass of ~20 kDa. The effects of AcKI on the development, survival, and enzymatic activity of *Anagasta kuehniella* larvae were evaluated, by incorporating AcKI in an artificial diet. Bioassays revealed a reduction in larval weight of ~50% with the lower concentration of AcKI used in the study (0.5%). Although additional assays showed an increase in endogenous trypsin and chymotrypsin activities, with a degree of AcKI-insensivity, AcKI produces an anti nutritional effect on *A. kuehniella*, indicating AcKI as a promising bioinsecticide protein for engineering plants that are resistant to insect pests.

KEYWORDS: *Acacia polyphylla, Kunitz-type inhibitor, Anagasta kuehniella, pest insect*

INTRODUCTION

The incidence of pest insect attacks in crops of economic importance is a serious problem for food production, since losses due these attacks can reach 20% in large cultivars.[1] It is known that some plants can decrease the damage caused by insects and pathogens through the synthesis of Protease Inhibitors (PIs), a group of plant proteins that act as a natural defense mechanism, triggered by herbivory.[2] This group of proteins is widely found in the Fabaceae subfamilies and these enzymes are present in various plant tissues, including seeds, where they constitute up to 10% of the total protein.[3]

The most representative families of PIs are the Bowman-Birk and Kunitz inhibitors, where the latter are characterized by a molecular mass of between 18 and 22 kDa, with four cysteine residues that form two disulfide bridges and typically inhibit trypsin and chymotrypsin enzymes.[4,5] As such, PIs have shown insecticide effects on different pests and transgenic plants expressing PIs have been assessed as an alternative strategy for protection against pest insects.[6] Thus, there is an increasing demand for the discovery of novel PIs with different biological activities. However, the choice of study model needs to take into account its digestive physiology, as part of the PIs' insecticide effects result from the direct inhibition of insect midgut enzymes. Since insects from the Lepidoptera order possess serine proteases, such as trypsin and chymotrypsin, as the most representative in their digestive physiology,[7] these insects constitute good candidates for bioassays.

Modern agriculture is defined by the incorporation of technological innovations to meet the growing demands of the world market in a sustainable manner; however, it is through basic research that novel tools are studied and finally applied in the field. *Anagasta kuehniella* (Zeller) (Lepidoptera, Pyralidae, Phycitinae), the Mediterranean flour moth, is a polyphagous pest that feeds on stored grains, fruits, nuts, jellies, cakes, and candies, causing economic losses worldwide.[8] Depending on the temperature and humidity, a single female may lay nearly 600 eggs.[9] It is, however, important to carry out a thorough study of physiological and biochemical aspects of potential insecticidal proteins, before suggesting alternative ways to control a specific pest, since adaptive responses may be triggered in pest insects, bypassing the insecticide effect expected, as reported for some pest insects.[10,11] As such, this study aimed to describe the purification and biochemical properties of a new inhibitor purified from *Acacia polyphylla* seeds (AcKI). In addition, a complete study evaluating the insecticidal effects of AcKI on the larval development of *A. kuehniella* was performed, utilizing bioassays, measurements of physiological and nutritional parameters and finally *in vitro* assays, to show the potential of AcKI for the control of this pest.

MATERIALS AND METHODS

Materials. *A. polyphylla* seeds were collected locally (Cerrado-Pantanal, Mato Grosso do Sul, MS, Brazil), packed in plastic bags and

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transported to the laboratory, where were washed and stored at −20 °C until use. Bovine serum albumin (BSA), bovine pancreatic trypsin, bovine pancreatic α-chymotrypsin, *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl- L-tyrosyl-*p*-nitroanilide (BTPNA), Succinyl-alanyl-alanyl-propyl-phenylalanyl-*p*-nitroanilide (SAAPPNA), *N*-*p*-tosyl-lysine chloromethyl ketone (TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK) and electrophoresis reagents were purchased from Sigma (St. Louis, MO). Chromatography supports were purchased from GE Healthcare. All other chemicals and reagents used were of analytical grade.

Insects. The flour mouth (*A. kuehniella*) insect was originally supplied by Dr. J.R.P. Parra (Laboratório de Biologia dos Insetos, Escola Superior de Agronomia "Luiz de Queiroz", Universidade São Paulo, Piracicaba, SP, Brazil) and were housed at standard conditions (28 ± 1 °C, RH of 70 ± 5% and 16 h photo phase) in incubator B.O.D. (SPLABOR, SP-500) and maintained on a diet constituted of wheat germ and whole meal flour in a proportion of 3:2 wheat germ/whole meal. The determination of larval instars were made according Mbata and Osuji.[12]

Purification of AcKI. *A. polyphylla* seeds that were free of tegument were ground in a coffee mill and subsequently defatted with hexane. Crude extract from seeds was obtained by extraction of this meal with 0.1 M phosphate buffer, pH 7.6 (1:10, w/v), for 2 h followed by centrifugation at 10000× *g* for 30 min. The supernatant was dialyzed against distilled water for three days at 4 °C and lyophilized. For purification of AcKI, the crude extract was dissolved in 0.1 M phosphate buffer, pH 7.6, containing 0.1 M NaCl and applied to a Sephadex G-50 column (2 × 50 cm) that was equilibrated with the same buffer. Fractions of 3 mL were collected with a flow of 36 mL/h. The fractions with inhibitory activity were pooled and dialyzed during 48 h at 4 °C and lyophilized. The peak that showed inhibitory activity was applied to a trypsin-Sepharose column (^{12} cm ×3 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.6, containing 0.1 M NaCl at a flow rate of 30 mL/h. The protein bound to the column was eluted with 50 mM HCl, dialyzed and lyophilized. Proteins were detected by monitoring the absorbance at 280 nm.

HPLC Analysis of Purified AcKI. The peak eluted from the trypsin-Sepharose column was then submitted to Reverse phase High Performance Liquid Chromatography (RP-HPLC). The sample was applied to a C5 column (Symmetric, Waters System) that had been previously equilibrated with 0.1% (v/v) TFA (solvent A), followed by a 60-min linear gradient from 0 to 100% (v/v) of 66% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (solvent B). Proteins were detected by monitoring absorbance at 280 nm.

Protein Quantification. Protein contents were determined by Coomassie blue staining, dye-binding method,[13] using BSA (1 mg/mL) as standard. The assays were developed in microplate wells, where 5 μL of standard concentrations of BSA (from 0.003125 to 0.5 mg/mL) were used for construct the standard curve of known concentration. Subsequently, 5 μL of samples of unknown concentration were added to microplate and then 250 μL Bradford was added. The microplate was measured at 595 nm and based in standard curve the concentration of protein in known samples were determined.

Inhibitory Activity Assays. Inhibitory activity assays were determined by measuring the residual hydrolytic activity of bovine trypsin and chymotrypsin toward the substrates, BAPNA and BTPNA, respectively. Both of the enzymes were incubated with AcKI (1 mg/mL) for 10 min at 37 °C, in Tris-HCl buffer (50 mM), pH 8.0, prior to the reaction. To start the reaction, 1 mM BAPNA or 20 mM BTPNA were added to the assay. After 15 min, the reaction was stopped by the addition of 30% (v/v) acetic acid. The release of chromophore p-nitroanilide was measured at 405 nm for both substrates. The linearity of the relationship between the changes in absorbance with time was checked to ensure that the substrate concentrations were not limiting. Substrate and enzyme controls were run to ensure the validity of sample absorbance readings.

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE, 12.5%) in the absence and presence of Dithiothreitol (DTT, 100 mM) was carried out as described by Laemmli.[14] The proteins were detected by staining with 0.1% Coomassie brilliant blue R-250. For Native PAGE (15%), 2 μg of purified AcKI was used and the gel was submitted to APNE staining. The gel was fixed in a 12.5% TCA solution, incubated for 30 min in 0.1 mol/L sodium phosphate buffer, pH 7.4 containing 0.1 mg/mL trypsin and then transferred to a solution containing APNE (N-acetyl-DL-phenylalanine-β-naphthyl ester) (2.5 mg/mL in dimethylformamide) and tetrazotized o-dianisidine (0.55 mg/mL in 0.1 mol/L sodium phosphate buffer, pH 7.4) until the appearance of transparent bands against a pink background.

Isoelectric focusing was carried out on a flat bed apparatus (LKB). Ampholine solutions (40%, v/v) in the pH range of 3.5−9.5 were used with subsequent Coomassie brilliant blue G-250 staining, according to Westermeier.[15] The inhibitors were detected using the negative staining technique of Uriel and Berges.[16]

Temperature and pH Stability. An AcKI sample (1 mg/mL in 50 mM Tris−HCl buffer, pH 8.0) was heated for 30 min in a water bath at various temperatures (37−100 °C), and then cooled to 0 °C before testing for residual inhibitory activity. To measure pH stability, a solution of AcKI (1 mg/mL) was diluted with an equal volume of various buffers (100 mM): sodium citrate (pH 2−4), sodium acetate (pH 5), sodium phosphate (pH 6−7), Tris−HCl (pH 8) and sodium bicarbonate (pH 9−10). After incubation in each buffer for 1 h at 37 °C, the pH was adjusted to pH 8.0 and the inhibitory activity on trypsin was assayed as described above. All experiments were carried out in triplicate and the results reported are the mean of three assays.

Amino-Terminal Sequencing. The N-terminal amino acid sequence of AcKI was determined by direct sequencing using automated Edman degradation and a PROCISE amino acid sequencer (Applied Biosystems). The phenylthiohydantoine (PTH) amino acids were identified in a model 140C microgradient PTH amino acid analyzer, based on their retention times. The sequence was submitted to automatic alignment, which was performed using the NCBI-Blast search system.

Midgut Preparation. Proteases were obtained from the midgut larvae, according to a protocol by Macedo et al.[17] The larvae were cold-immobilized and dissected, and the midguts were surgically removed and placed in iso-osmotic saline solution; aliquots of 10 midguts were prepared. The *A. kuehniella* midguts were subsequently homogenized in 150 mM NaCl and centrifuged at 6000× *g* for 20 min at 4 °C, and supernatants were stored at −20 °C until use in enzymatic assays.

Activity Inhibitory of AcKI, TLCK and TPCK against Different Enzymes. Bovine trypsin and chymotrypsin were incubated with synthetic trypsin (TLCK) and chymotrypsin inhibitors (TPCK), respectively, as well as AcKI (2 μg) at 37 °C, in 50 mM Tris-HCl buffer, pH 8.0. The substrates, BAPNA and BTPNA, were used as described above. The same assay was performed for the midgut extract of fourth-instar *A. kuehniella* larvae (5 μg), using the substrates BAPNA and SAAPPNA, the latter being specific for chymotrypsin. All assays were performed in triplicate.

Effects of AcKI on *A. kuehniella* Development. To examine the effects of AcKI on *A. kuehniella* development, larvae were fed on a diet containing AcKI at concentrations of 0.5%, 1.0% and 2.0% (w/w) until the fourth-instar. For obtainment of eggs, adults were aspirated from colony into a 300 mL jar covered with a screen of mesh size of 0.8 mm and then the jar was inverted on to a Petri dish. The eggs were collected and the neonate larvae were transferred to a clean bottle containing artificial diet with a soft brush. The control diet was prepared without inhibitor addition and for each AcKI concentration evaluated, 4 neonate larvae were placed in a clean bottle and this process was repeated 10 times (n = 40). Following incubation for 22 days under standard conditions, the weight and number of larvae were determined. Linear regression analysis was used to evaluate the response of *A. kuehniella* to the concentrations of AcKI. Effective doses for the 50% response were defined as the concentration of AcKI that decreased the larval weight to 50% in relation to the control group. After the choice of the ideal concentration of AcKI (weight reduction of close to 50%), further bioassays were performed, in order to evaluate the weight and survival in third (16 days), fourth (22 days) and fifty-instar larvae (28 days), under the same conditions established in the first experiment. Diet intake and fecal output were measured

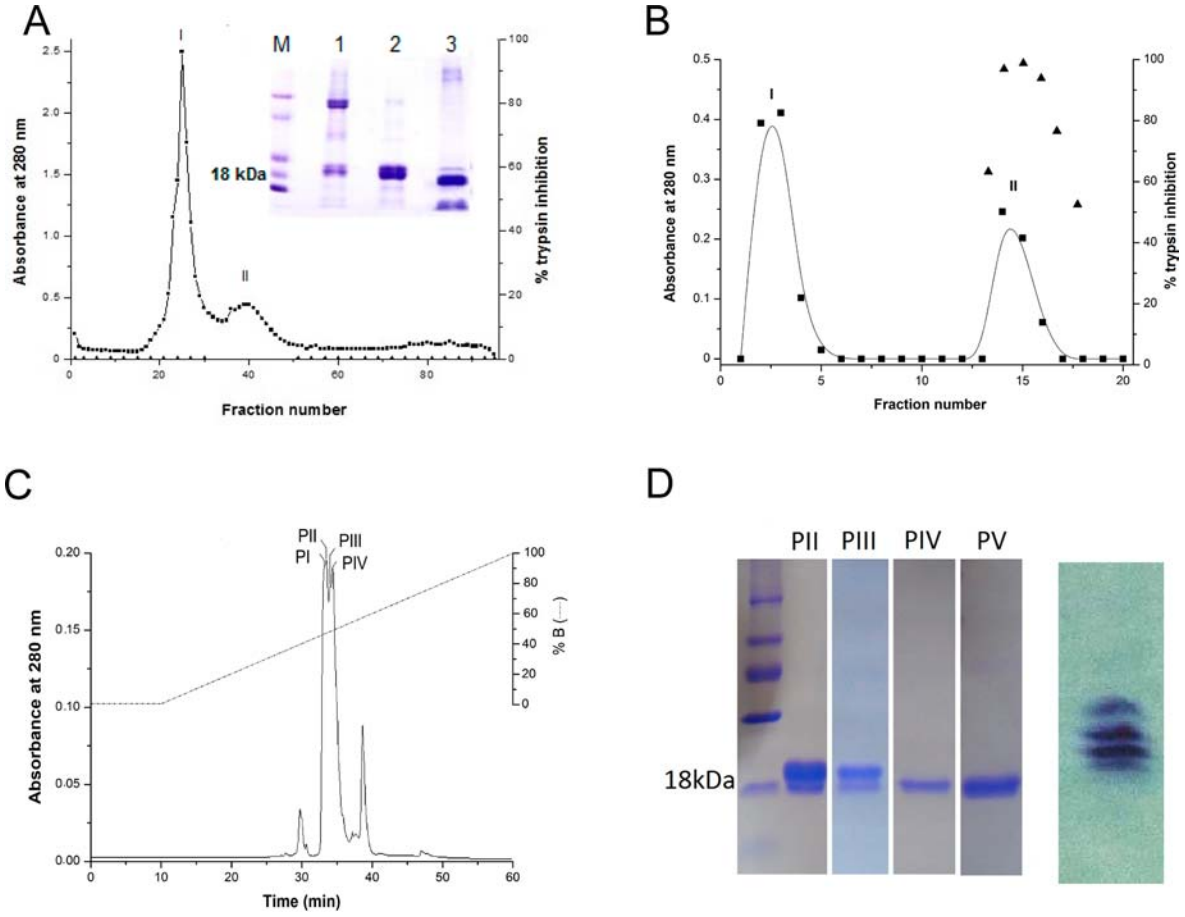

Figure 1. (A) Gel filtration (Sephadex G-50) of extract from *A. polyphylla* seeds, with antitryptic activity in peak II. Inset: SDS-PAGE analysis of crude extract (1), peak with antitryptic activity from Sephadex G-50 (2) and partially purified AcKI, obtained from the second peak of Sephadex G-50, reduced with 0.1 M DTT (3), (M) Molecular markers. (B) Sepharose-Trypsin Affinity chromatography of peak II from Sephadex G-50; the peak II from Sepharose-Trypsin column present antitryptic activity. (C) Elution profile for the RP-HPLC fractionation (hydrophobicity column C-5) of AcKI and (D) SDS-PAGE analysis of the four major peaks (I, II, III and IV) from RP-HPLC and isoeletric focusing of AcKI.

and used for the elaboration of Nutritional Parameters. Biochemical assays with the major enzymes present in the *A. kuehniella* midgut were also performed.

Nutritional Parameters. The nutritional parameters were compared among third, fourth and fifth-instar larvae exposed to either the AcKI-fed diet or the control diet. The larvae, feces, and uneaten food were separated using a microscope stereoscope, dried and weighed. Nutritional parameters of consumption, digestion and utilization of food were calculated, as described by Waldbauer [18] and Farrar et al.[19] The nutritional index, denominated efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD) and approximate digestibility (AD) were calculated as follows:

$$\mathrm{ECI} = (\Delta B/I) \times 100$$

$$\mathrm{ECD} = [\Delta B/(I - F)] \times 100$$

and

$$\mathrm{AD} = [(I - F)/I] \times 100$$

where I is weight of food consumed, ΔB is change in body weight, and F is weight of feces produced during the feeding period. Metabolic cost (CM) was calculated as 100 − ECD.

Protease Activity of Midgut Extracts in Zymography Gel Electrophoresis. Proteins extracted from the midgut extracts of *A. kuehniella* larvae were run on SDS−PAGE (12.5%) containing 0.1% gelatin.[20] Following electrophoresis at 4 °C, the gels were washed with 2.5% Triton X-100 solution for 2 h with shaking to remove the SDS, after which the gels were incubated with 0.1 M Tris−HCl, pH 8.0, for 2−3 h. The gels were subsequently stained with Coomassie brilliant blue R-250. Bands of proteolytic activity appeared as clear zones against a blue background.

Effect of Ingested AcKI on *A. kuehniella* Midgut Proteases. Trypsin and chymotrypsin enzymes of midgut extracts from *A. kuehniella* larvae (both AcKI-fed and control-fed larvae) were assayed using the substrate BAPNA and SAAPPNA. A sample of midgut extract (5 μg) was incubated at 37 °C for 10 min before adding the substrate to start the reaction, which was allowed to proceed for 15 min for BAPNA and 5 min for SAAPPNA. The reaction was then stopped by adding 30% (v/v) acetic acid.

To analyze the ability of AcKI to inhibit the enzymatic activity of *A. kuehniella* midgut enzymes, midgut samples were incubated with increasing concentrations of AcKI (0−2 μg). Inhibition assays for trypsin and chymotrypsin were then performed. All assays were performed in triplicate.

AcKI Digestion. To verify the possibility that AcKI may be digested by digestive enzymes of *A. kuehniella*, AcKI was incubated with 50 mM Tris buffer pH 8.0 and digestive enzymes of the midgut extract from fourth-instar larvae, dissected and processed as described previously, for 72 h at 30 °C (same protein concentration for the midgut extract and AcKI). The digestion was stopped by immersing the tubes in an ice bath. The remaining inhibitory activity of AcKI was analyzed by inhibition of trypsin activity, using BAPNA as substrate.

Statistical Analysis. The effects of AcKI on thermal and pH stabilities, enzymatic activities, weight gain, survival and nutritional parameters were examined using one-way analysis of variance (ANOVA) with post-test using Tukey as multiple comparison in

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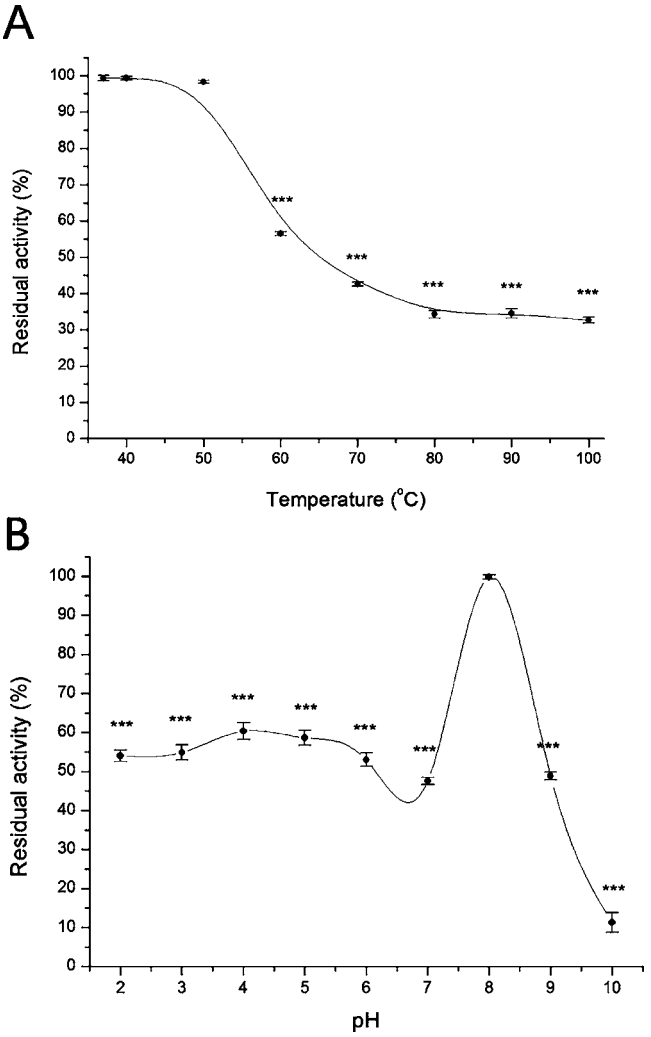

Figure 2. Stability of AcKI. (A) Thermal stability of AcKI after incubation for 30 min at the indicated temperatures. (B) pH Stability of AcKI after incubation at the indicated pH at 37 °C. The residual trypsin inhibitory activity was measured using BAPNA as substrate. The statistical analysis was made using one-way ANOVA with post test Tukey as multiple comparison. Significant differences between the treatments are showed as * if $P < 0.05$, ** ($P < 0.01$) and *** ($P < 0.001$).

order to identify the means that differed if the ANOVA test indicated significance. The p value <0.05 was considered to be significant.

RESULTS AND DISCUSSION

AcKI Purification. Different chromatography techniques were used for the purification of AcKI. In the first step, the gel filtration separated the crude extract into two major groups, where only the second peak showed strong inhibitory activity for trypsin (Figure 1A). In the second step, the second peak from the gel filtration was submitted to bio affinity chromatography and the peak eluted from the column exhibited trypsin activity inhibition (Figure 1B). The inhibitor purified by the affinity column was subjected to RP-HPLC. Using a linear gradient of buffer B, the presence of 4 major peaks were observed, with a similar retention time (Figure 1C), suggesting the presence of isoinhibitors, as reported for other Kunitz inhibitors.[5]

SDS-PAGE (12.5%) analysis revealed that a single protein had been purified, showing a relative mass of approximately 20 kDa under nonreducing conditions. Thus, this protein was named AcKI and used for further characterization. Even though AcKI presented a single band under nonreducing conditions, the treatment of AcKI with DTT demonstrated the presence of two bands of distinct molecular masses, indicating that AcKI is composed of two polypeptide chains (Figure 1A, inset). Other PIs isolated from the *Acacia* genus have a range of masses of 18−21 kDa: *A. elata* ≈ 20 kDa,[21] *A. siberiana* ≈ 19 kDa,[22] *A. confusa* ≈ 21 kDa,[23] *A. victoriae* ≈ 18.3 kDa,[4] *A. plumosa* ≈ 20 kDa,[24] and *A. Senegal*.[25] Moreover, the presence of two polypeptide chains also appears to be another characteristic shared among inhibitors from the *Acacia* genus, as for a number of other inhibitors from the Mimosoideae subfamily.

Isoelectric focusing analysis of AcKI showed the presence of four bands with p*I*s ranging from 6.5 to 5.85 (Figure 1D), indicating the occurrence of isoinhibitors. Protease inhibitors may present various molecular forms and differ in their p*I* values;[3] however, most kunitz-type inhibitors are acidic.[14,26] The existence of isoforms in nature is not completely elucidated, although the hypothesis that these isoforms and the synergism among them ensure the plant's survival [5] is recognized.

Thermal and pH Stability. Kunitz inhibitors often demonstrate characteristics of tolerance at high temperatures and over a wide variation of pH.[27−30] *In vitro* assays revealed that AcKI has its inhibitory activity affected by increase in temperature. A significant reduction of AcKI biological activity is noticed from 60 °C up to higher temperatures analyzed ($P < 0.001$) (Figure 2A). Similar thermal stability has been reported for other Kunitz inhibitors.[23,26,28] In relation to pH stability, AcKI showed a narrow range of optimum activity around pH 8, losing on average 45% of inhibitory activity from neutral to lower pH values ($P < 0.001$). At pH 10, the highest decrease in inhibitory activity was observed, about 90% ($P < 0.001$) (Figure 2B). This is an unusual characteristic shared among Kunitz inhibitors. Possibly, alterations of pH provoked modifications in reactive site, hindering the complexation with trypsin. An advantage in use of an enzymatic inhibitor with these

Table 1. Comparative N-Terminal Sequence Alignment of Known Kunitz Type Inhibitors[a]

																								Identities (%)
AcKI	Q	V	F	D	T	E	G	N	G	I	R	N	G	G	T	Y	Y	I	L	P	D	R	W	G	K	G	
TI p20		V	F	D	T	E	G	N	P	I	R	N	G	G	T	Y	Y	V	L	P	V	I	R	G	K	G	80
KTI		V	F	D	T	E	G	N	P	I	R	N	G	G	T	Y	Y	V	L	P	V	I	R	G	K	G	80
p20 1		V	F	D	T	E	G	X	S	I	R	N	G	G	T	Y	Y	V	L	P	V	I	R	G	K	G	76
WTI 1A			D	S	E	G	E	L	V	R	N	G	G	T	Y	Y	L	L	P	D	R	W					75
WTI 1B			D	S	E	G	E	L	V	R	N	G	G	T	Y	Y	L	L	P	D	R	W					75

[a]TI p20, KTI and p20 1, *Glycine max* Trypsin Inhibitors; WTI 1A and WTI 1B, *Psophocarpus tetragonolobus* Trypsin Inhibitors. Identical residues are in shaded blocks. The alignment of sequences was made with the help of MegAlign program.

characteristic is that this protein was readily inactivated during the process of cooking food, with no side effects on consumers, emphasizing its benefical effects on agriculture and crop protection with minimal or no side effects.

Amino-Terminal Sequence. The Peak IV (AcKI), obtained by RP-HPLC (Figure 1D), was subjected to amino-terminal sequence analysis and showed homology to inhibitors of the Kunitz family. Table 1 shows the sequence comparison between AcKI and some kunitz inhibitors with greater than 75% identity, such as the *Glycine max* [31] and *Psophocarpus tetragonolobus* [32] inhibitors.

Inhibitory Activity of AcKI. The inhibitory activity of AcKI against trypsin and chymotrypsin from *A. kuehniella* and from a commercial source were assayed. AcKI demonstrated a stronger inhibition of trypsin than chymotrypsin (Figure 3). This profile

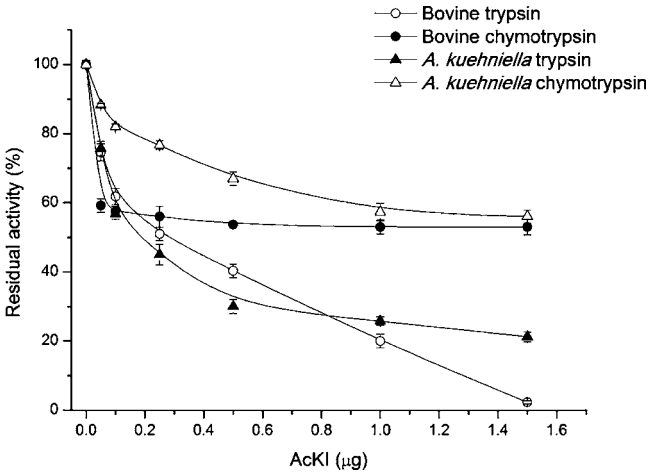

Figure 3. Inhibition by AcKI of digestive enzymes of *A. kuehniella* and bovine enzymes using the synthetic substrate BAPNA for trypsins, BTPNA for bovine chymotrypsin and SAAPPNA for *A. kuehniella* chymotrypsin. All values represent the means of triplicates.

is characteristic of some kunitz-type inhibitors, which present inhibition against trypsin and chymotrypsin, although to a lesser degree for the latter enzyme. Such enzymes include the *Acacia elata* trypsin inhibitor;[21] *Acacia confusa*, ACTI;[23] *Enterolobium contortisiliquum*, ECTI,[26] *Peltophorum dubium*, PDTI;[28] *Archidendron ellipticum*, AeTI;[33] *Acacia plumosa*, ApTI.[24] AcKI was more effective at inhibiting commercial trypsin than *A. kuehniella* trypsin, since 1.5 μg AcKI inhibited about 98% of the commercial trypsin's activity, while an equal amount of the protein inhibited approximately 80% of *A. kuehniella* trypsin's activity. This preference of kunitz-type inhibitors for vertebrate trypsin, rather than insect trypsin, was also reported by Macedo et al.[28] Another trypsin inhibitor, isolated from *Pithecelobium dumosum* seeds, inhibited approximately 98% of bovine trypsin, while inhibiting approximately 90% of *A. kuehniella* trypsin activity at the same concentration.[34] Studies with AeTI and SKTI also showed a lower degree of inhibition of *Spodoptera litura* trypsin, compared to bovine trypsin.[33] This behavior has been studied by different authors and an overall understanding is that during the coevolution of insects and plants the trypsins of insects have developed an increased hydrophobicity their reactive sites, making their interaction with the hydrophilic reactive sites of PIs weaker, an adaptation mechanism involved in the resistance of insects to PIs.[35]

The inhibition of digestive enzymes of *A. kuehniella* by synthetic inhibitors of trypsin (TLCK) and chymotrypsin (TPCK) were also assayed in fourth-instar larvae midgut enzymes (Figure 4). When added *in vitro* to the *A. kuehniella*

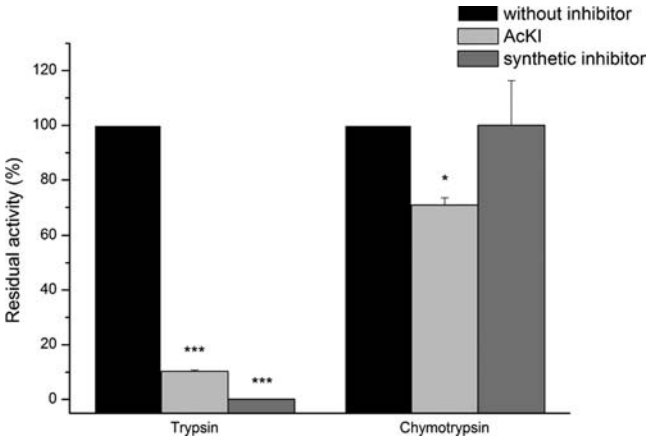

Figure 4. Inhibition of crude extract *A. kuehniella* midgut trypsin and chymotrypsin. In this assay the inhibitor AcKI as well the synthetic inhibitors, TLCK (trypsin inhibitor) and TPCK (chymotrypsin inhibitor) were used. The statistical analysis was made using one-way ANOVA with post test Tukey as multiple comparison. Significant differences between the treatments are showed as * if $P < 0.05$, ** ($P < 0.01$) and *** ($P < 0.001$).

midgut extract, TLCK fully inhibited trypsin activity, while TPCK did not inhibit chymotrypsin activity. Under the same conditions, AcKI inhibited about 90% and 30% of trypsin and chymotrypsin activity, respectively, reaffirming that AcKI is able to inhibit chymotrypsin to a lesser degree. Previous studies have reported the inactivation of insect chymotrypsin by TPCK in *Galleria mellonella* [36] and *Ostrinia nubilalis*;[37] however no effect was observed in *Heliothis virescens*,[38] *Spodoptera littoralis*,[39] *Manduca sexta*,[40] *Bombyx mori*,[41] *Helicoverpa zea*,[42] *Spodoptera frugiperda*.[43] According to Lopes et al.,[43] chymotrypsin from insects pertaining to orders other than Lepidoptera are readily inactivated, whereas those from Lepidoptera vary. Most insects of this order have distinct chymotrypsin enzyme characteristics, with amino acid replacements that may cause differences that result in the reduction of the access of substrate and inhibitors to the active site due to steric hindrance. Another possibility is that changes in the vicinity of His 57 in lepidopteran chymotrypsin may affect the pK_a value, thus decreasing the reactivity of His with ketones and other reactants.[43] This possible replacement of amino acids may be an adaptation to selective pressure due a generalist food habit, as is the case of *A. kuehniella*.

Effect of AcKI on the Development of *A. kuehniella* Larvae. The effect of AcKI on larval development was monitored by feeding the larvae on a diet containing AcKI (from 0 to 2%) and then determining the number and mass of survival of the fourth-instar larvae. The average weight of control-fed larvae was 9 mg and larvae fed on a diet containing 0.5% AcKI weighed 4.4 mg, showing a reduction of about 50% (data not shown). As the concentration of 0.5% AcKI was defined as optimal for further tests, measurements of weight and survival were taken in third, fourth and fifth-instars (Figure 5A). AcKI-fed larvae showed significant differences in weight gain, 48.5% in forth-instar ($P < 0.001$) and 43% in fifth-instar larvae ($P < 0.001$), when compared with respective control-fed larvae. However, at third-instar a nonsignificant difference in average

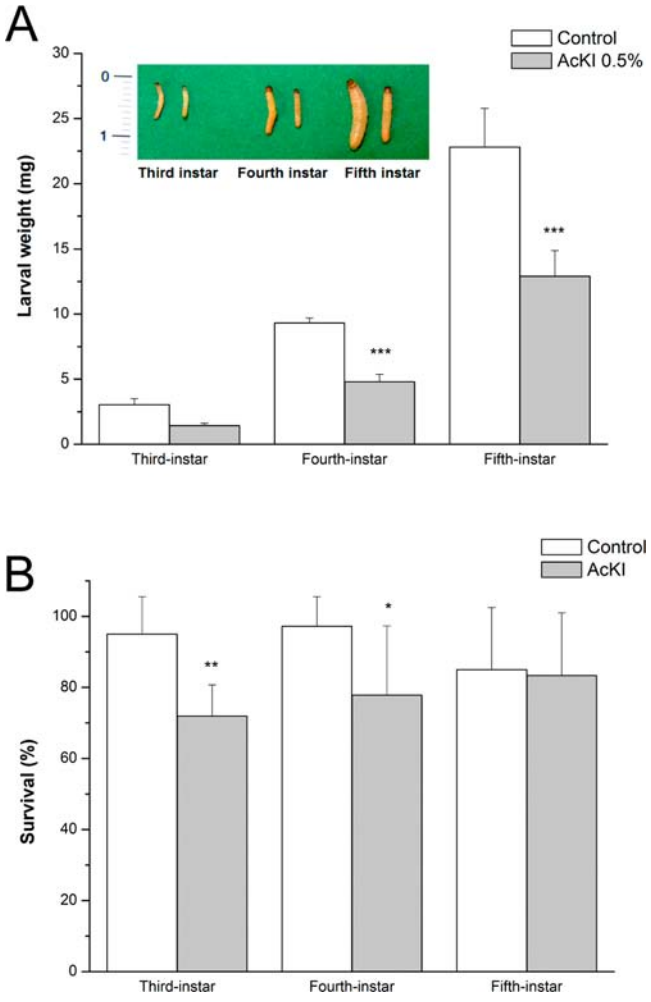

Figure 5. Effect of artificial diet containing 0.5% AcKI on the development of *A. kuehniella* larvae in third, fourth and fifth-instar larvae. (C) Larval weight. Insert: Photograph of *A. kuehniella* larvae (D) Survival. The statistical analysis was made using one-way ANOVA with post test Tukey as multiple comparison. Significant differences between the treatments are showed as * if $P < 0.05$, ** ($P < 0.01$) and *** ($P < 0.001$).

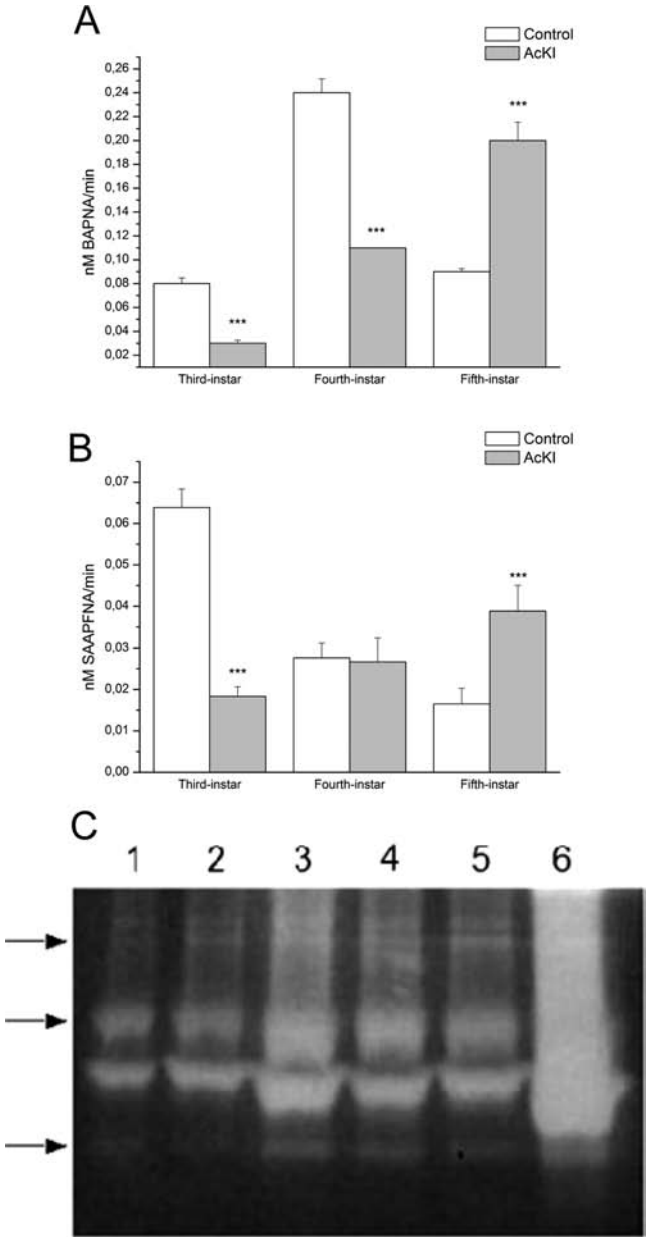

Figure 6. (A) Trypsin activity of the midgut extract in third, fourth and fifth-instar larvae of control-fed larvae and AcKI-fed larvae, using BAPNA as substrate. (B) Chymotrypsin activity of the midgut extract in third, fourth and fifth-instar larvae of control-fed larvae and AcKI-fed larvae, using SAAPPNA as substrate. The statistical analysis was made using one-way ANOVA with post test Tukey as multiple comparison. Significant differences between the treatments are showed as * if $P < 0.05$, ** ($P < 0.01$) and *** ($P < 0.001$). (C) Zymography gel electrophoresis. (1) midgut of third-instar larvae fed on a control diet; (2) midgut of third-instar larvae fed on a diet containing 0.5% AcKI; (3) midgut of fourth-instar larvae fed on a control diet; (4) midgut of fourth-instar larvae fed on a diet containing 0.5% AcKI; (5) midgut of fifth-instar larvae fed on a control diet; (6) midgut of fifth-instar larvae fed on a diet containing 0.5% AcKI. Proteolytic activity appears as a clear zone against a dark blue background. Arrows pointing upward represent the approximate molecular weights of 18 kDa, 34 kDa and 66 kDa, respectively.

weight was noticed. Regarding survival rate, the two first instars analyzed showed that AcKI affect this parameter significantly, decreasing an average of 25% de survival in third ($P < 0.01$) and fourth-instar ($P < 0.05$) (Figure 5B).

Among the *Acacia* seed trypsin inhibitors that have been previously studied, AsPI (*A. senegal* protease inhibitor) showed insecticidal activity on *Helicoverpa armigera* (Lepidoptera), affecting the larval development of this insect.[25] The concentration of AcKI used in our study (0.5%), corresponds to the level in legume seeds and was similar to those used by other authors.[44,45] Nutritional parameters are illustrated in Table 2 and show that AcKI fed-larvae demonstrated a decrease in the efficiency of conversion of ingested food (ECI), justifying the lower larval weight. The metabolic cost and approximate digestibility in AcKI-fed larvae were higher, indicating that a considerable percentage of food metabolized is used for the maintenance of vital processes and a minor portion is used in weight gain. *A. kuehniella* larvae that chronically ingested 0.7% PFTI (*Plathymenia foliolosa* trypsin inhibitor) had the same behavior.[45]

Effect of AcKI on Digestive Enzymes. The proteolytic activities of the trypsin and chymotrypsin enzymes of *A. kuehniella* larvae that were chronically fed on AcKI were measured utilizing BAPNA and SAAPPNA as substrates, respectively (Figure 6). In relation to trypsin activity, there was a peak of

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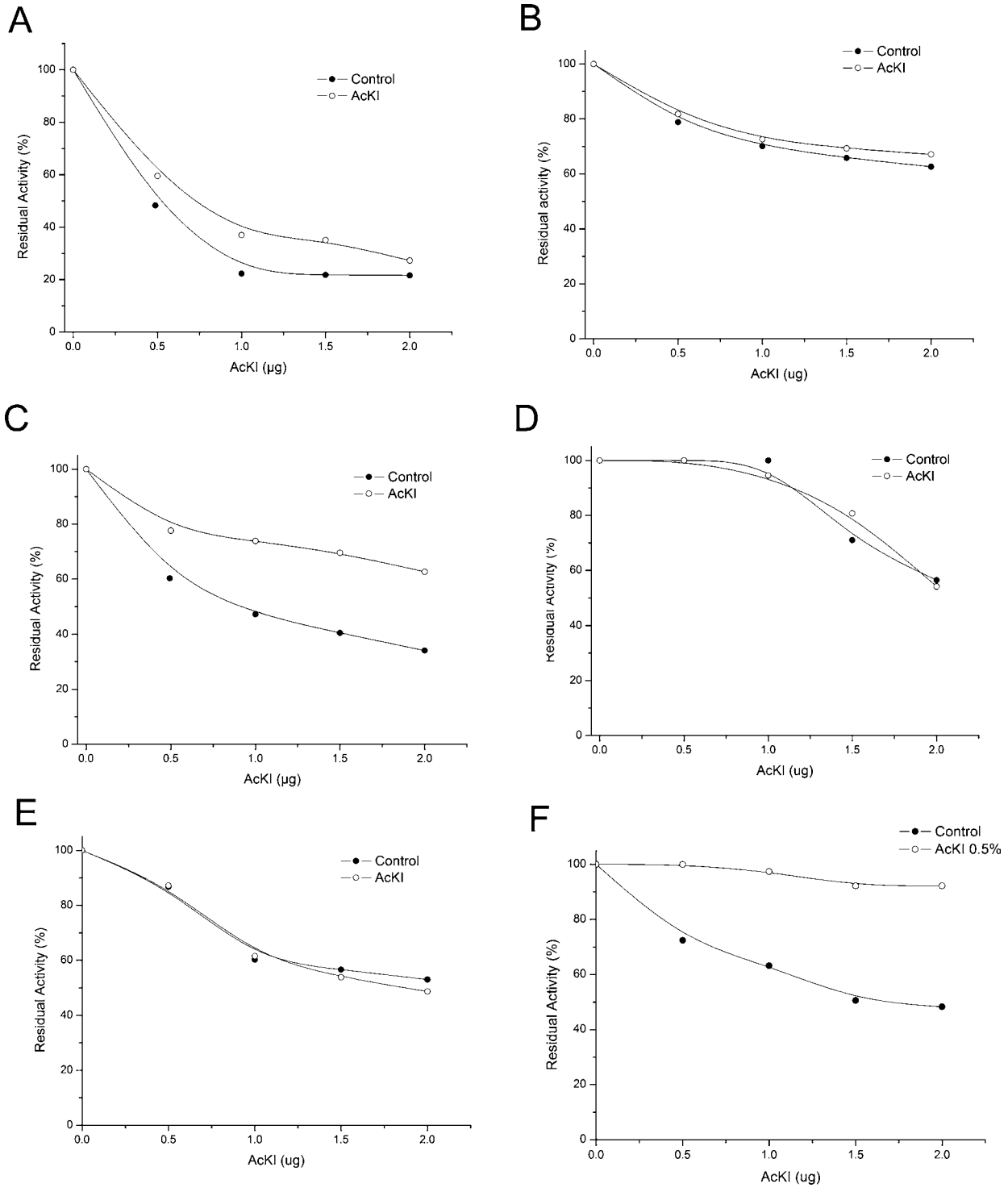

Figure 7. Inhibition by AcKI of the trypsin activity of midgut extract in third (A), fourth (B) and fifth-instar (C) and representation of inhibition of chymotrypsin activity of midgut extract in third (D), fourth (E) and fifth-instar (F) larvae reared on an artificial diet containing 0.5% AcKI or control diet. The proteolytic activity of trypsin was assayed, using BAPNA as substrate, and of chymotrypsin, using SAAPPNA as substrate. Experiments were carried out in triplicate.

trypsin activity in the fourth-instar, a decreased activity in the fifth-instar (Figure 6A), while for chymotrypsin, a high chymotrypsin activity began in the third-instar and then decreased in subsequent instars (Figure 6B). The fifth-instar is the last before the pupal stage, and a plausible explanation for reduction in proteolytic activity observed in control-fed larvae. However, this profile of reduction did not occur in AcKI-fed larvae. At the fifth-instar, both trypsin and chymotrypsin in AcKI-fed larvae presented the highest activities (Figure 6A and B), suggesting that more enzymes are being synthesized by *A. kuehniella* larvae in response to the AcKI diet. Biochemical evaluations and molecular evidence indicate that some insects try to adapt to PIs by overproduction of native proteases [11] or by the synthesis of isoenzymes,[46−48] in agreement with the

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Table 2. Nutritional Parameters of *A. kuehniella* Larvae Fed on an Artificial Diet Containing 0.5% AcKI or a Control Diet, without AcKI[a]

	third-instar		fourth-instar		fifth-instar	
nutritional parameters	control	AcKI 0.5%	control	AcKI 0.5%	control	AcKI 0.5%
ECI (%)	6.8 ± 0.5 a	4.4 ± 0.5 b	6.2 ± 0.3 a	4.3 ± 0.5 b	7.6 ± 0.2 c	4.9 ± 0.9 b
ECD (%)	8.7 ± 1.1 a	4.8 ± 0.7 b	7.4 ± 0.4 a	5.0 ± 0.7 b	9.7 ± 0.3 c	5.7 ± 1.0 b
AD (%)	78.2 ± 5.1 a	93.3 ± 4.7 b	84.3 ± 0.9 c	87.7 ± 2.5 bd	77.6 ± 1.1 a	85.9 ± 1.0 d
MC (%)	91.3 ± 1.1 a	95.2 ± 0.7 b	92.6 ± 0.4 a	95.1 ± 0.7 b	90.3 ± 0.3 a	94.3 ± 1.0 b

[a]ECI, efficiency of conversion of ingested food; ECD, efficiency of conversion of digested food; AD, approximate digestibility; MC, metabolic cost. The statistical analysis was made using one-way ANOVA with post test Tukey as multiple comparison. Significant differences between the treatments are showed as different letters.

increase in trypsin and chymotrypsin activities observed in AcKI-fed larvae. Apparently, there was no change in enzymatic profile and, visually, there was no enzyme variant form, as analyzed by zymography gel electrophoresis (Figure 6C).

To verify this finding, an additional assay of proteolytic activity from both groups was measured after incubation with increasing concentrations of AcKI. At the third instar, the inhibition of trypsin (Figure 7A) and chymotrypsin (Figure 7D) enzymes was proportional to the addition of AcKI. A similar inhibition profile was observed in fourth-instar larvae for trypsin (Figure 7B) and chymotrypsin (Figure 7E). However, in the fifth-instar larvae it was possible to observe a decrease in inhibitory activity in AcKI-fed larvae for both classes of enzymes (Figure 7C and F). This alteration in inhibition profile indicates that the pool of *A. kuehniella* enzymes has acquired some insensitivity to the inhibition by AcKI. Clearly, the insects responded to the diet by attempting to adapt to the negative effects exerted by AcKI. Previous studies have shown that *A. kuehniella* larvae do not acquire insensitivity when exposed to PFTI.[45] However, it should be noted that a pest insect may respond to the presence of different PIs in different manners. Another possibility is that the change in sensitivity in the midgut enzymes only occurs in the final instar.

Insects also adapt to PIs by inducing the production of isoenzymes that are insensitive to PIs.[47,48] Studies have reported that, in response to the consumption of the trypsin/chymotrypsin inhibitor, some Lepidoptera insects, such as *Helicoverpa zea, Agrotis ipsilon, Spodoptera exigua* and *Spodoptera frugiperda*, synthesize new isoenzymes that are insensitive to tested PIs.[11,48]

AcKI Digestion. After the incubation for up 72 h, of AcKI with *A. kuehniella* midgut extracts from control-fed larvae or AcKI fed-larvae, *in vitro* digestibility was monitored by measuring the residual activity of trypsin. AcKI was resistant to digestion for up to 24 h of incubation and maintained inhibition of the trypsin enzymes in both midgut extracts (Figure 8). However, after 24 h of incubation at 30 °C, the midgut controls without the addition of AcKI (negative control) naturally lost enzymatic activity; thus, we evaluated the digestion times until this time point. On the basis of this result, we concluded that the enzymes present in both treatments were unable to digest the AcKI during the first 24 h. Resistance to digestion is an important prerequisite for a protein to exert an insecticidal effect.[49] Several toxic proteins with insecticidal activity are resistant to degradation by insect digestive enzymes, such as Talisin, an insecticidal protein with lectin-like activities and protease inhibitor properties of *Talisia sculenta* seeds.[50]

In conclusion, in this study a new protease inhibitor was purified, with characteristics shared among Kunitz inhibitors. Its insecticidal potential was evaluated through bioassays that revealed an evident effect on *A. kuehbiella* larvae weight at low

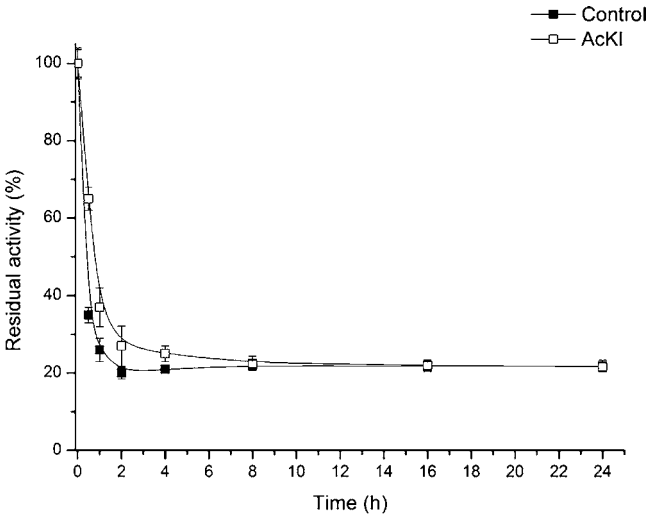

Figure 8. Inhibition of trypsin-like residual activity after the incubation with AcKI for 24 h at 30 °C. Control-fed and AcKI-fed midguts were used in this assay. Experiments were carried out in triplicate.

concentrations. Other direct effects on larval physiology were observed; including negative effects on nutritional parameters, possibly due to the increase in enzyme production in response to dietary AcKI. *A. kuehniella* midgut enzymes were unable to digest AcKI. Our hypothesis is that the hyper production of midgut enzymes in response to AcKI has a high energetic demand and uses part of the essential amino acids needed for larval development, provoking the inhibition of the growth and the mortality of the larvae, observed in our study. Taking into account all results showed in this work, we suggest that AcKI is a protein that has antinutritional effects on *A. kuehniella* beyond to exert a marked insecticide effect in higher concentrations in artificial diet. Further bioassays with other pest insects will be performed, as the effects of PIs vary between different pests.

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Notes

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ABBREVIATIONS

BAPNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; BTPNA, *N*-benzoyl- L-tyrosyl-*p*-nitroanilide; SAAPPNA, Succinyl-alanyl-alanyl-propyl-phenylalanyl-*p*-nitroanilide; TLCK, *N*-*p*-tosyl-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; DTT, dithiothreitol; BSA, bovine serum albumin

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