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Insecticide activity of a peptidase inhibitor isolated from *Anadenanthera macrocarpa* seeds against *Anagasta kuehniella*

Atividade inseticida de um inibidor de peptidase das sementes de *Anadenanthera macrocarpa* contra *Anagasta kuehniella*

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ABSTRACT

Protease inhibitors (PIs) are a part of the plant defense system and reduce the proteolytic activity of the digestive enzymes of insect pests. The current study aimed to isolate and characterize an inhibitor of trypsin (AmTI) within the seed of *Anadenanthera macrocarpa* (Benth) (Leguminosae-Mimosoideae). Moreover, we tried to assess the defense mechanism of the larvae of *Anagasta kuehniella* against this inhibitor. Protein seed extracts were purified using Sephadex G-50 and trypsin-Sepharose columns. Electrophoresis revealed the molecular weight of the inhibitor to be 25 kDa. The stability evaluation demonstrated that the inhibitor was not denatured at temperatures of up to 60 °C, pH 2–10, and concentrations of up to 100 mM dithiothreitol for one hour. The inhibitor reacted in a 1:1 ratio with bovine trypsin with an inhibition constant [Ki] = 2.517×10^{-8}. Incorporating the inhibitor in a 1 mg per 100 mg proportion of artificial diet offered to *A. kuehniella* larvae led to a significant difference in the weight and survival of larvae of the fourth instar compared to the control. AmTI acted on the enzymatic activities of trypsin and chymotrypsin, not allowing until the fourth larval instar, *A. kuehniella,* to create adaptations against the inhibitor, as it had a simultaneous effect on larval weight and mortality. Therefore, a new trypsin inhibitor showing inhibitory activity against the digestive enzymes trypsin and chymotrypsin from *A. kuehniella* was isolated, indicating that these activities are correlated with the deleterious effects of this insect.

Index terms: Bioinsecticide; insect pest; peptidase inhibitor; plant-insect interaction.

RESUMO

Os inibidores de protease (IPs) fazem parte do sistema de defesa da planta e reduzem a atividade proteolítica das enzimas digestivas de insetos-praga. O presente estudo teve como objetivo isolar e caracterizar um inibidor de tripsina (AmTI) na semente de *Anadenanthera macrocarpa* (Benth) (Leguminosae-Mimosoideae). Além disso, tentamos avaliar o mecanismo de defesa das larvas de *Anagasta kuehniella* contra esse inibidor. Os extratos de sementes de proteína foram purificados usando colunas de Sephadex G-50 e tripsina-Sepharose. A eletroforese revelou que o peso molecular do inibidor era de 25 kDa. A avaliação da estabilidade demonstrou que o inibidor não foi desnaturado em temperaturas de até 60 °C, pH 2-10 e concentrações de até 100 mM de ditiotreitol por uma hora. O inibidor reagiu na proporção de 1:1 com tripsina bovina com uma constante de inibição [Ki] = 2,517 x 10^{-8}. A incorporação do inibidor na proporção de 1 mg por 100 mg da dieta artificial oferecida às larvas de *A. kuehniella* levou a uma diferença significativa no peso e na sobrevivência das larvas do quarto ínstar em relação ao controle. O AmTI atuou sobre as atividades enzimáticas da tripsina e quimotripsina, não permitindo até o quarto ínstar larval, *A. kuehniella*, criar adaptações contra o inibidor, pois teve efeito simultâneo no peso e mortalidade larval. Portanto, um novo inibidor de tripsina apresentando atividade inibitória contra as enzimas digestivas tripsina e quimotripsina de *A. kuehniella* foi isolado, indicando que essas atividades estão correlacionadas com os efeitos deletérios desse inseto.

Termos para indexação: Bioinseticida; inseto-praga; inibidor de peptidase; interação planta-inseto.

INTRODUCTION

Increasing global food production is a significant challenge in ensuring food security. With population growth, changes have been made to ensure food production since agricultural emissions should increase by approximately 60% to supply quantities and qualities of food materials (Frona; Szenderák; Harangi-Rákos, 2019). Developing countries are attempting to increase the amount of arable land, which has resulted in using areas of native vegetation, thus, undermining the other forms of enhanced productivity (Riggs; Fields; Cross, 2018). Herbivores are responsible for 10 to 20% of the

total loss of agricultural products. Synthetic insecticides decrease losses in the field but affect human health (Mossa; Mohafrash; Chandrasekaran, 2018). The agricultural pests, such as *Anagasta kuehniella* (Zeller, 1879; Lepidoptera: Pyralidae), the Mediterranean flour moth, are found worldwide, particularly in stored grain, nuts, and fruits. It causes heavy losses in flour mills (Oliveira et al., 2017). The larvae of this pest move rapidly, decreasing the market value and reducing the consumption of these products. Serinopeptidase enzymes help the digestive process of Lepidopterans. Chymotrypsin and trypsin are the most important enzymes for digestion in insects and mediate up to 95% of the total proteolysis in Lepidopterans (Johnston et al., 1995).

Controlling these insects requires using chemical insecticides, including chlorpyrifos, deltamethrin, malathion, methoprene, pyrethrin, and piriformis. These insecticides reduce losses in the field but can be toxic to humans and household animals and harm the environment (Naggar; Giesy; Kholy, 2019). Thus, the biotechnological development of transgenic plants expressing protease inhibitors, enhancing their resistance against insects and other pathogens, as an alternative solution could be essential for biopesticides. It could improve agricultural production without significant dependence on chemical fertilizers and pesticides. (Subbanna et al., 2020). The proteinase inhibitors (PIs) have a sophisticated defense mechanism protecting plants from being attacked by herbivores and/or pathogens. These peptides or proteins are synthesized constitutively or after induction as a counter-attacking measure. They have specific and reversible abilities to interact with different proteolytic enzymes, competitively inhibiting them (Rodríguez-Sifuentes et al., 2020). Among the various proteins involved in plant defense mechanisms is the group of serine proteinase inhibitors. This group can bind to specific amino acids at the active sites of the target enzymes. It is composed of the Kunitz families (inhibits trypsin), Bowman-Birk (inhibits trypsin and chymotrypsin), the potato type I inhibitor (specific against chymotrypsin and subtilisin), and potato type II (specific against trypsin and chymotrypsin), barley trypsin and pumpkin inhibitors. (Kirar et al., 2022). These inhibitors are stable because they have disulfide bonds conferring heat resistance and stability against changes in temperature, pH, and proteolysis (Cotabarren et al., 2020). The complex formed between the protease inhibitor and the enzyme is temporary since it is released in its free form after hydrolysis (González-Castro; Gómez-Lim; Plisson, 2020). The leguminous seeds of taxonomic subfamilies Mimosoideae, Caesalpinioideae, and Papilionoideae facilitate Kunitz-type inhibitors that are widely investigated as tools for analyzing proteinases in physiological and pathological events (Bonturi et al., 2022). Bezerra-Silva et al. (2015) revealed that *Anadenanthera colubrina* (Leguminosae-Mimosoideae) extract could inhibit trypsin and lead to mortality in *Aedes aegypti*. De Oliveira et al. (2015) demonstrated that at least six active digestive enzymes from *Aedes aegypti* larvae were severely affected after the *Clitoria fairchildiana* inhibitor exposure, with an 87.93% reduction in enzyme activity. Therefore, the current study aimed to isolate and characterize the trypsin inhibitors from *A. macrocarpa* seeds and assess their insecticidal effects on 3rd, 4th and 5th-instar larvae of *A. kuehniella.*

MATERIAL AND METHODS

Material

We purchased N-benzoyl-L-arginine-p-nitroanilide (BApNA), N-succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (SAAPFpNA), trypsin from bovine pancreas, sodium dodecyl polyacrylamide molecular marker and Dalton Mark VI sulfate from Sigma- Aldrich (St. Louis, USA). Potassium phosphate, sodium chloride, and hexane were provided by Merck and Co. (Brazil). Sephadex and Sepharose gels were procured from Pharmacia Biotech (Uppsala, Sweden).

Insects

In this experiment, we used *Anagasta kuehniella* larvae from the colonies at the insectary of the Laboratory of Protein Purification and its Biological Functions (UFMS). Later, they were kept in an environmental chamber (27 ±1 °C, relative humidity 65% - 70%, 16 h photoperiod).

Purification of *Anadenanthera macrocarpa* trypsin inhibitor (AmTI)

We milled and defatted the *Anadenanthera macrocarpa* seeds using hexane at room temperature for 24h. The soluble proteins were extracted in potassium phosphate buffer (100 mM, pH 7.6) using constant stirring at room temperature for 2 h. The suspension was centrifuged (3,000 × *g*), the supernatant was dialyzed against distilled water (12 kDa cut off), lyophilized, and then stored at - 20 °C and denominated crude extract.

After applying the crude extract onto a Sephadex G-50 column (2.0 cm × 42 cm) by previously equilibrating

using potassium phosphate buffer (100 mM, pH 7.6) and sodium chloride (100 mM), size-exclusion chromatography was performed. The elution was carried out at a flow rate of 60 mL h^{-1} through fractions of 3 mL. The antitrypsin activity was monitored from the fractions eluted by an *in vitro* assay with the BApNA substrate (Oliveira et al., 2012). The active fractions were reserved and dialyzed using distilled water and denominated as AmTI after lyophilization.

The AmTI was loaded onto a trypsin-Sepharose column (1.0 cm × 6.2 cm) to obtain a higher purity material. The column was equilibrated using potassium phosphate buffer (100 mM, pH 7.6) and sodium chloride (10 mM). The sample was subjected to a flow rate of 20 mL h^{-1}, 1 mL $fraction^{-1}$, until it became undetectable at 280 nm. The active proteins were eluted in 10 mM hydrochloric acid, dialyzed, lyophilized, and stored at - 20 °C.

SDS-PAGE

We determined the purification efficacy and molecular weight pattern of the sample using electrophoresis in a polyacrylamide gel with 12.5% sodium dodecyl sulfate (SDS- PAGE), based on Laemmli (1970), without reducing proteins. Dalton Mark VI was used as the molecular marker (Sigma-Aldrich).

Protein quantification

The soluble proteins in the fraction were quantified based on Bradford's (1976) method, using bovine serum albumin as the standard. Readings were obtained at 595 nm.

Characterization of the thermal inhibitor stability

The physicochemical characterization of the inhibitor stability was conducted according to Macedo et al. (2011). The AmTI was incubated for 30 min at 37, 40, 50, 60, 70, 80, 90, and 100 °C to determine the thermal stability, and the samples were immediately frozen.

pH stability

We evaluated the stability of the AmTI against different pH values by diluting the AmTI in sodium citrate buffer (pH 2, 3, 4, and 5), sodium phosphate (pH 6, 7, and 8), and sodium bicarbonate (pH 9 and 10). The samples were incubated at 37 °C for 60 minutes, and the solution was frozen.

Dithiothreitol (DTT) denaturation

AmTI was incubated with DTT at concentrations of 10 and 100 mM at 37 °C, at intervals of 30, 60, and 120 min for each concentration to check the stability against a reducing agent. The incubation was stopped by adding iodoacetamide in an amount equal to twice the DTT molar concentration.

The residual inhibitory activity was checked by incubating the inhibitor with bovine trypsin and BApNA at the end of each procedure.

Stoichiometry and Inhibition Constant [Ki] determination

We conducted a titration to determine the existence of a stoichiometric relationship between the AmTI and trypsin. Increasing amounts of a 1:1000 of AmTI solution were introduced to 10 µL of trypsin (0.125 mg mL^{-1}). BApNA (200 µL, 1 mM) was added after a 10 min incubation at 37 °C, and the mixture was incubated again for 30 min, followed by spectrophotometric reading at 410 nm. The amount of residual activity was defined with the titration curve where the x-axis referred to the molar ratio of the inhibitor: enzyme and the y-axis represented the residual enzyme activity. Close to 10% residual activity demonstrates that most trypsin inhibitors complexed, suggesting a stoichiometric relationship between the inhibitor and enzyme. The [Ki] was defined by the inhibition curve developed using the Enziftter software (Biosoft).

Evaluation of activity against *Anagasta kuehniella* larvae

A standard diet (three parts whole-wheat flour and two parts wheat germ) containing 1% AmTI was fed to the Neonate larvae of *A. kuehniella*. Each larvae group (n = 4) was given 120 mg of diet. Upon reaching the 3rd, 4th, and 5th-instar, the larvae were weighed and dissected to remove the midguts. Then, these were homogenized and centrifuged (12,000 × *g*, 15 min, 4 °C), collected, and stored at - 20 °C for further biochemical analysis of the recovered supernatant.

Nutritional parameters

The changes in energy utilization were determined by calculating the larval parameters for each replicate, as previously described by Panizzi and Parra (1991). The efficiency of the conversion of ingested food (ECI), the efficiency of the conversion of digested food (ECD), metabolic cost (MC) and approximate digestibility (AD), were evaluated as follows: ECI = (ΔB/I) x 100; ECD = [(ΔB/I-F)] x 100; MC = 100 x ECD and AD = [(I-F)/I] x 100 where ΔB = change in body weight, I = weight of

the food ingested, and F = weight of the feces produced during the feeding period.

Evaluation of trypsin and chymotrypsin activity

We incubated the homogenate of larval midguts with BApNA and SAAPFpNA as chromogenic substrates in microplates to compare the trypsin and chymotrypsin activity in the control group and the group fed with AmTI. Samples of 2 µg of midgut larvae were incubated using the assay buffer before adding the chromogenic substrates. Both assays assessed chromophore p-nitroanilide release by monitoring the absorbance at 410 nm.

Statistics evaluation

Statistical analysis was performed using Statistica software. The data were subjected to analysis of variance (ANOVA) followed by Tukey tests for multiple mean comparisons at a 5% probability level.

RESULTS AND DISCUSSION

Extraction and purification of the inhibitor from seeds of *Anadenanthera macrocarpa:*

We purified a trypsin inhibitor from *Anadenanthera macrocarpa* seeds and analyzed its properties. A total of 3.81 g of soluble protein was obtained from 50 g of seeds after Sephadex G-50 chromatography. This chromatographic step showed two prominent peaks at 280 nm, with antitrypsin activity identified toward the end of the first peak (Figure 1 A).

The pool of active fractions purified using the trypsin-Sepharose column led to an HCl-eluted retained peak active against trypsin (Figure 1 B).

This process yielded a trypsin inhibitor having a 10.67 - fold purification (Table 1). SDS-PAGE demonstrated that this inhibitor was 25 kDa in size, consistent with the molecular mass of the Kunitz inhibitors. However, three bands remained in the eluate of the purified inhibitor from affinity chromatography. These isoforms could adhere to bovine trypsin.

The plant seeds store high protein amounts protecting against microorganisms and/or invertebrates by inhibiting the proteolytic activities within these organisms. This characteristic provides an insecticidal biotechnological potential for these inhibitors of proteolytic enzymes. Thus, our study purified a new trypsin inhibitor from *Anadenanthera macrocarpa* (AmTI), consistent with Kunitz- type inhibitor.

Properties of inhibitor activity

The effects of heat, pH, and DTT on the stability of the AmTI are represented in Figures 2 (A, B, and C), respectively.

Studies have shown that proteinase inhibitors enriched in cysteine residues are generally stable and often resistant to extreme pH ranges, heat, and proteolysis by proteinases (Cotabarren et al., 2020). AmTI inhibitory activity was at 60% even after heating up to 60 °C for 30 min (Figure 2A). However, we did not observe significant changes in inhibitory activity when pH values were adjusted between 2 and 10 (Figure 2B). Similar results were obtained from the Kunitz family inhibitors present in other Mimosoideae subfamily species, including *Entada acaciifolia* (Macedo et al., 2011) and *Inga vera* (Bezerra et al., 2016).

The inhibitory activity of AmTI was stable due to a reducing agent, DTT (Figure 2C). It demonstrated an inhibitory activity of above 50% when subjected to 100 mM for 30 and 60 min. However, DTT (10 mM) did not affect the activity or stability of PPTI, *Poecilanthe parviflora* trypsin inhibitor (Garcia et al., 2004). Macedo et al. (2003) found that the inhibitory activity of PDTI, a Kunitz type trypsin inhibitor from *Peltophorum dubium*, was unaffected by exposure to 1 mM DTT for up to 120 min, with a 40% to 60% loss of activity at 10 mM DTT after 45 and 120 min, respectively. Up to 10 mM of DTT did not affect the activity or stability of ILTI, a Kunitz-type trypsin inhibitor obtained from *Inga laurina* (Macedo et al., 2007). Zhou et al. (2008) depict that AKTI, a Kunitz inhibitor from *Albizzia kalkora,* was not affected by 2 mM DTTl in the reductive process for up to 60 min. In contrast, its activity was quickly lost over 60 min, with only 8% of activity remaining after 240 min. Lehle et al. (1996) observed that ETI, a Kunitz-type trypsin inhibitor obtained from *Erythrina caffra*, retained its inhibitory activity after DTT reduction. The stability of these trypsin inhibitors is not associated with the presence of disulfide bridges. This is because reports have described inhibitors isolated from Bauhinia sp. seeds without disulfide bridges and cysteine residues (Oliva et al., 2001; Mello et al., 2002).

Our data indicate stability, partly by the high degree of cross-linking through disulfide bridges. However, other non-covalent interactions could contribute to the structure-functional stability of the inhibitors (Oliva et al., 2011; Bateman; James, 2011; Bezerra et al., 2016). Inhibitors against *Putranjiva roxburghii* (Chaudhary et al., 2008) and *Erythrina velutina* (Lucena et al., 2022) seeds showed similar behaviors.

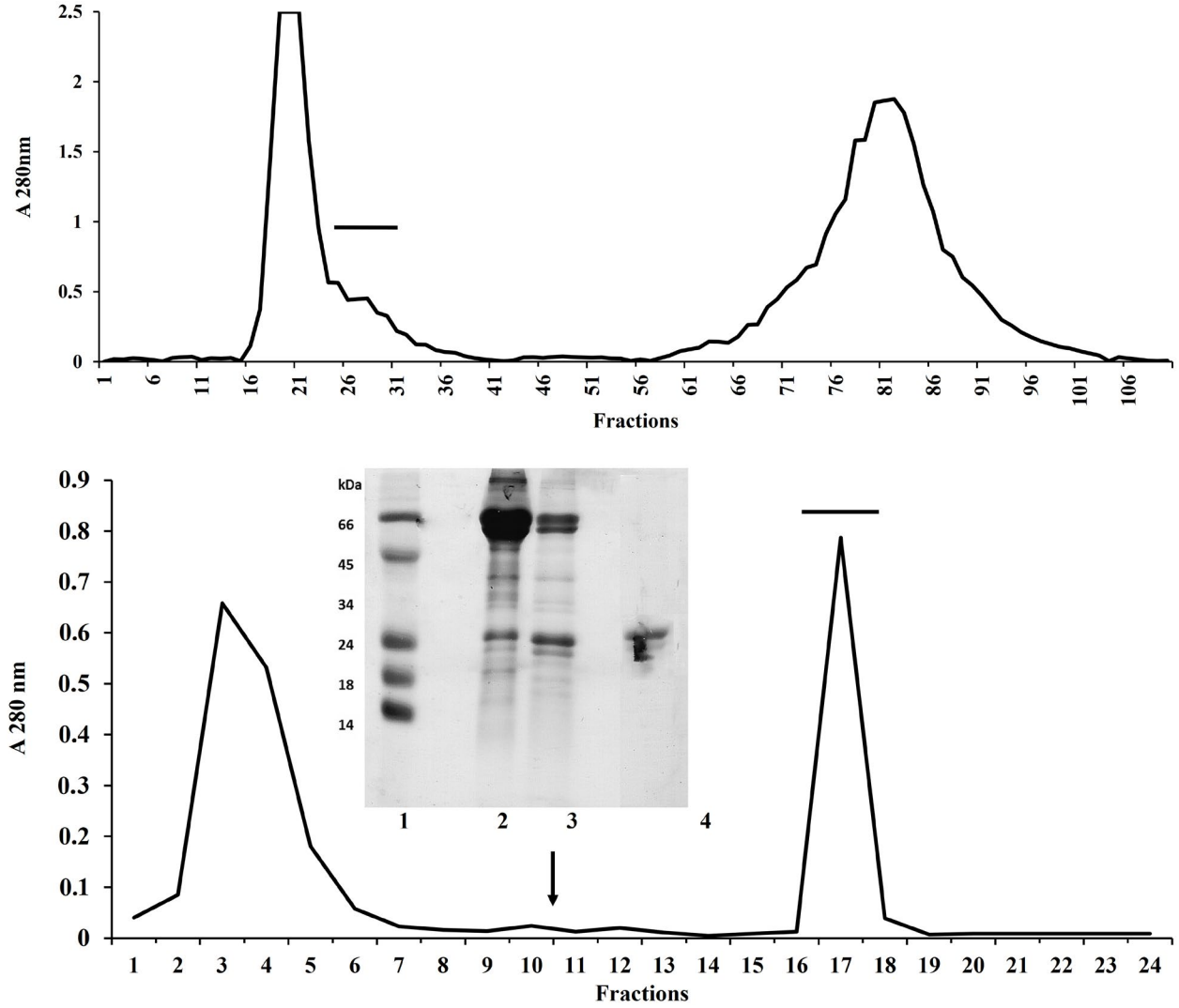

Figure 1: The trypsin inhibitor purification from the *Anadenanthera macrocarpa* seeds (AmTI): (A) Results of the Sephadex G-50 (2.0 cm × 42 cm) chromatography of the crude extract of *A. macrocarpa* seeds. Fractions having antitrypsin activity were loaded onto the Trypsin-Sepharose 4B affinity chromatography (B). Inhibitor elution was performed using 0.01 M HCl. (−) antitrypsin activity. *Inset:* SDS–PAGE describes the fractions showing antitrypsin activity through purification: *lane 1:* The molecular weight marker. *lane 2:* the crude extract. *lane 3:* Sephadex G-50. *lane 4:* The trypsin-Sepharose 4B.

Table 1: *Anadenanthera macrocarpa* trypsin inhibitor purification.

Steps Purification	Total protein (mg)	Total activity (TIU)	Specificity activity (TIU/mg)	Yield (%)	Purification (fold)
Crude extract	430.0	1032.0	2.4	100	1
Sephadex G-50	65.0	696.8	10.72	15.11	4.46
Trypsin-Sepharose 4B	10.4	266.4	25.61	2.42	10.67

TIU (Trypsin Inhibitor Unit) = Inhibitory amount decreasing absorbance at 410nm.

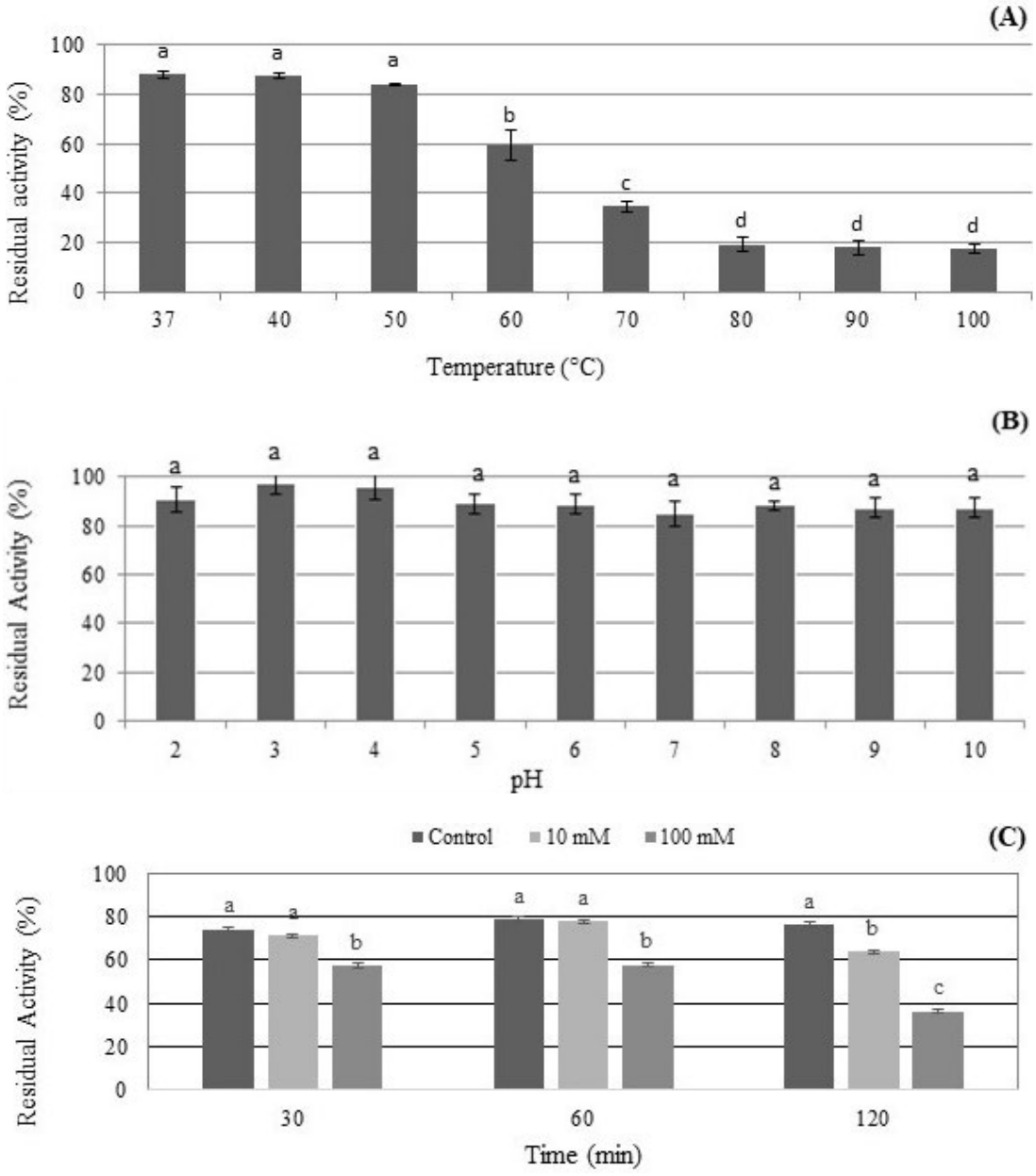

Figure 2: The stability of AmTI. (A) The temperature stability of AmTI after incubation at the indicated temperatures for 30 min; (B) The residual activity of AmTI after incubating at the indicated pH for 60 min at 37 °C; (C) The effect of DTT on the stability of AmTI. The inhibitor was treated with different final concentrations of DTT (10 and 100 mM) for 30–120 min at 37 °C. The residual trypsin inhibitory activity was determined using BApNA as the substrate. The values are represented by means ±SD in triplicate measurements. A significant difference was depicted between the treatment groups using different letters (ANOVA, $p < 0.05$).

The Kunitz (STI) family proteins have a molecular mass of about 25 kDa, with one or two disulfide bridges within the most highly conserved region toward the N- terminal domain (Onesti; Brick; Blow, 1991; Amaral et al., 2022). The Kunitz-type soybean trypsin inhibitor (STI) crystal structure is characteristic of disulfide bridges and Kunitz inhibitors. There were two disulfide bridges (Cys64– Cys108 and Cys156–Cys164) in STI and PSPI (Meulenbroek et al., 2012) in COTI, which were well separated from each other in the structure (Zhou et al., 2020). Therefore, the inhibitory effect of the protein is not affected by the break in bonds (DiBella; Liener, 1969).

Stoichiometry and Inhibition Constant [Ki] determination

Figure 3 represents the titration curve of AmTI with bovine trypsin. A stoichiometric study revealed that the AmTI has a 90% inhibition of free bovine trypsin at a 1:1 ratio disulfand having an [Ki] of 2.517×10^{-8} M (data not demonstrated but obtained after analysis using the Enziftter software, Biosoft). PIs from different Leguminosae plants have [Ki] values for trypsin in the 0.1 and 6 nM range (Dantzger et al., 2015). Other inhibitors from the Mimosoideae subfamily, including those in the seeds of *Trigonella foenum-graecum* (TfTI, 3.01×10^{-9} M) (Oddepally; Sriram; Guruprasad, 2013), *Inga laurina* (ILTI, 6×10^{-9}) (Macedo et al., 2007) and *Entada acaciifolia* (EaTI, 1.75×10^{-9}) (Oliveira et al., 2012) had similar values. In contrast, the AmTI could not effectively inhibit bovine chymotrypsin (data not provided), as observed for the EaTI (Oliveira et al., 2012).

Effect of AmTI on the larval development of *Anagasta kuehniella*

We monitored the effect of AmTI on larval development by feeding an artificial diet to the larvae and determining the number and mass of the surviving 3rd, 4th and 5th-instars larvae. The incorporation of 1% AmTI in diets of the larvae of *A. kuehniella* led to a significant difference in mean weight and survival among the 4th-instar larvae (Figures 4 A and B). The 3rd and 5th-instars had no significant difference from the control group.

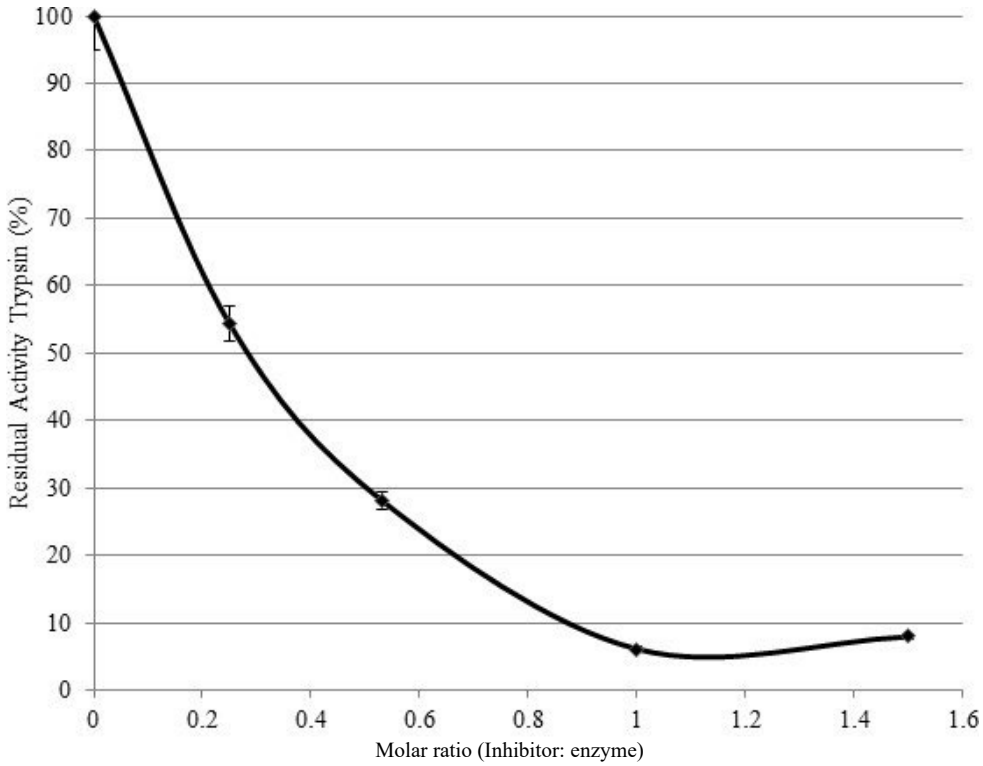

Figure 3: The titration curve of bovine trypsin inhibition using AmTI at 410 nm. The points represent the means of three different assays. Curve equation: $y = -56.321x + 72.722$, $R^2 = 0.9887$.

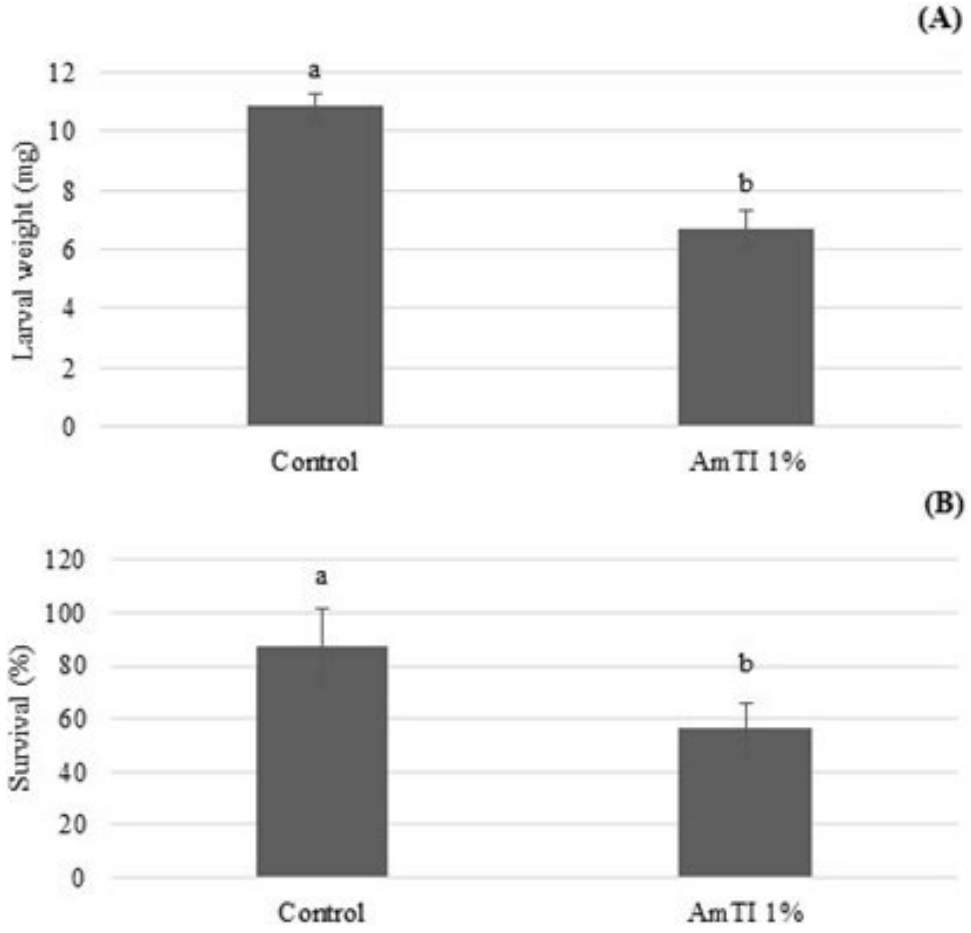

Figure 4: Effects of AmTI on (A) the larval weight and (B) the survival of *Anagasta kuehniella* 4th- instar larvae. Each letter represents a varied form of significant difference between the different treatment means (ANOVA, $p < 0.05$).

The average body weight of 4th-instar larvae on a control diet was 10.85 mg. In contrast, the diet of 1.0% AmTI decreased the average weight by approximately 40%. Similar results were observed in *Anagasta kuehniella* 4th instar larvae served with 1% ApTI (*Anadenanthera pavonina* trypsin inhibitor), showing a 50% reduction in average weight (Macedo et al., 2010). The results also indicate that incorporating 1.0% AmTI into the diet reduced the survival rate of 4th-instar larvae to 36%. The AmTI (1.0%, w/w) concentration corresponded to legume seed levels and was similar to those used by other workers (Oliveira et al., 2019; França et al., 2021).

Nutritional parameters

Several nutritional parameters within the 4th-instar larvae exposed to either 1% AmTI or a control diet were compared. The approximate digestibility (AD) was increased in the *Anagasta kuehniella* 4th-instar larvae fed on the 1% AmTI group (Table 2) than in the control group. AD indicates the amount of the ingested food effectively assimilated by the intestinal walls of insects. The increase in AD within the group fed 1% AmTI demonstrates more excellent food retention in the midgut of the insect for maximum food absorption. It could offset the anti-nutritional effect of AmTI, and similar results were observed by Oliveira et al. (2020).

Table 2: Nutritional parameters of *Anagasta kuehniella* 4th-instar larvae fed on either 1% AmTI or a control diet.

Nutritional parameters	Control	AmTI (1.0%)
ECI (%)	11.55 ±0.92a	12.14 ±1.11a
ECD (%)	13.29 ±0.81a	13.11 ±1.11a
MC (%)	86.71 ±0.81a	86.89 ±1.11a
AD (%)	86.89 ±0.91a	92.53 ±0.65b

ECI = efficiency of conversion of ingested food; ECD = efficiency of conversion of digested food; MC = metabolic cost; AD = approximate digestibility. Means inside a column after the same letter are not significantly different, $p < 0.05$; the Tukey's test.

Evaluation of trypsin and chymotrypsin activity

Bioactive plant proteins, like protease inhibitors, interfere with insect digestion, reducing nutrient absorption and leading to malnutrition. Trypsin and chymotrypsin are proteases that form the majority of insect digestive activities (Napoleão et al., 2018). Trypsin and chymotrypsin activities were significantly reduced in the midgut lumen of *A. kuehniella* 4th instar larvae after AmTI exposure, suggesting that the larvicidal effect is due to the inhibition caused by both enzymes (Figure 5). A similar phenomenon occurred with the larvae of *Helicoverpa zea, Agrotis ipsilon*, and *Trichoplusia ni* treated with the soybean inhibitor. Interestingly, this inhibition was independent of the concentration of the inhibitor enzyme (Broadway, 1997).

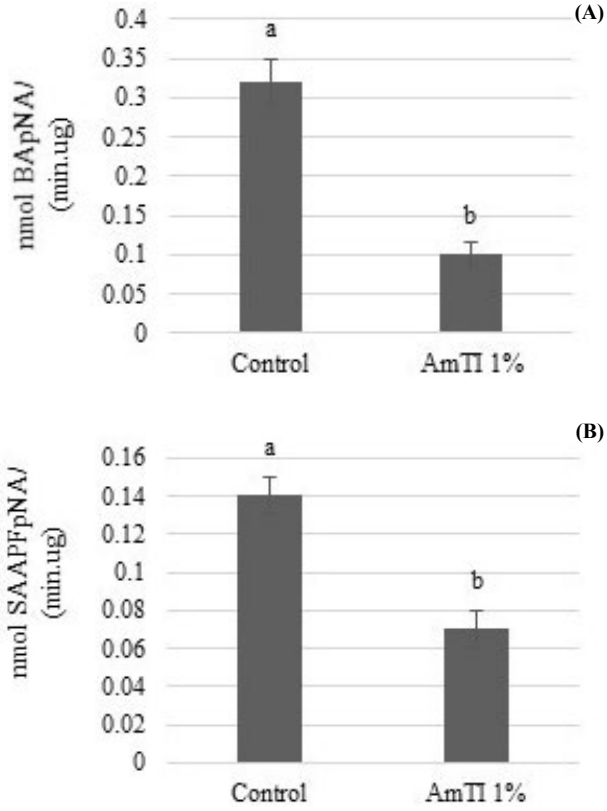

Figure 5: AmTI effects on trypsin (A) and chymotrypsin (B) activities inside the midgut of *Anagasta kuehniella* 4th-instar larvae with BApNA and SAAPF-pNA as substrates, respectively. A significant difference between the treatment means was represented by different letters (ANOVA, $p < 0.05$).

CONCLUSIONS

Therefore, AmTI inhibits the development of *A. kuehniella*, affecting the physiology of nutrition and digestion in this insect while inactivating trypsin activity. *Anagasta kuehniella* cannot adapt to AmTI until the 4th instar, simultaneously affecting larval weight and mortality. The purification of AmTI was successful using fewer purification steps (only two), reaching 10 times higher purification yield than that of classical purification models, suggesting a biotechnological potential to control *Anagasta kuehniella*. Thus, AmTI proved to be an enzyme resistant to denaturing agents and temperature, a trypsin inactivator, and an excellent tool to control pests.

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AUTHOR CONTRIBUTION:

Conceptual Idea: Leite, W.A.; Jacobowski, A.C.; Macedo, M.L.R.; Methodology design: Leite, W.A.; Jacobowski, A.C.; Data collection: Leite, W.A.; Jacobowski, A.C.; Data analysis and interpretation: Leite, W.A.; Jacobowski, A.C.; Macedo, M.L.R.; and Writing and editing: Leite, W.A.; Jacobowski, A.C.

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