

Original Article

Effect of caffeic acid on feeding, α -amylase and protease activities and allatostatin—A content of Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)—

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Efficiency of caffeic acid (CA) on *Spodoptera littoralis* was investigated. CA was mixed with artificial diet, and feeding indices and allatostatin-A (AST-A) content of the midgut were measured 10 days later. α -Amylase and protease activities were evaluated for 10 days. CA significantly decreased feeding indices. Feeding on an artificial diet containing CA decreased protease and α -amylase activities in the midgut. The incubation of the dissected midgut with AST-A increased α -amylase and protease activities. The injection of AST-A into the hemolymph of larvae also increased protease and α -amylase activities. Competitive ELISA and immunohistochemistry results showed that starvation decreased the AST-A titer and AST-A immunoreactivity (AST-A-ir) cells in the midgut whereas refeeding increased it. Here, for the first time we showed that feeding on an artificial diet containing CA also caused the AST-A level to decrease in the midgut, which itself caused α -amylase and protease activities to decrease.

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Keywords: caffeic acid, Egyptian cotton leafworm, allatostatin-A, digestive enzymes.

Introduction

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is one of the most important pests of several agricultural crops including cotton, eggplant, tomato, and some ornamental products in many countries of the world.¹⁾ *S. littoralis* showed resistance to some organophosphorus, synthetic pyrethroids and insect growth regulators (IGRs),²⁾ including methoxyfenozide.³⁾ Extensive use of synthetic insecticides may have negative effects on humans' health and their environment. Pest outbreak and pest resistance are other negative impacts of synthetic insecticides. To avoid these effects, alternative approaches to pest control must be sought.⁴⁾ It is unlikely that an insect would acquire resistance to natural insecticides because they often contain a mixture of biologically active compounds.⁵⁾ Phenolic compounds are one of the most common groups in plant secondary metabolites. They show toxic activity against insects.⁶⁾ Caffeic acid (CA), a most common phenolic acid, occurs in many plants, such as coffee, vegetables, and fruits. It shows antioxidant and anti-proliferative properties.⁷⁾

CA decreased the relative growth rate (RGR) and efficiency of

conversion of ingested food (ECI) which was dose dependent in *Leptinotarsa decemlineata*.⁷⁾ CA was shown to inhibit protease activity in *Helicoverpa armigera*.⁶⁾

Neuropeptides are involved in many important physiological processes in insects, such as feeding, reproduction, aggression, locomotor activity and circadian rhythm.⁸⁾ The A-type allatostatin family has been reported in Lepidoptera.⁹⁾ Allatostatin A (AST-A) inhibits juvenile hormone biosynthesis.¹⁰⁾ It is also myoinhibitory on the foregut of Lepidoptera.¹⁰⁾ Moreover, AST-A stimulated protease and α -amylase activities in the midgut of *Periplaneta americana*.¹¹⁾

In the present study, the effects of CA on toxicity, feeding indices, enzymatic activities and the titer of AST-A in the midgut of *S. littoralis* have been investigated.

Materials and Methods

1. Insect rearing

S. littoralis specimens were collected in a cotton field in Dezful, Iran (32°22'57"N 48°24'07"E). They were cultured on an artificial diet¹²⁾ at 20°C, 60±2% RH, with a photoperiod of 16:8 L:D. Third instar larvae 24 hr old were used in all tests. Insect rearing and all experiments were done at 20±1°C, 60±2% relative humidity (RH), and with a photoperiod of 16:8 light:dark (L:D) hr.

2. CA

CA was purchased from Sigma Chemical Company.

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3. Survival and feeding index assay

Third instar larvae of *S. littoralis* were fed on an artificial diet containing 50 to 200 ppm/gr of CA. In each experiment, 40 insects were tested with 4 replicates. The survival of insects fed on an artificial diet containing CA was analyzed on alternate days until 10 days.

For feeding index assay, after adding 50 to 200 ppm/gr of CA to the artificial diet, third instar larvae were transferred into plastic containers (diameter: 15 cm, depth: 7 cm) that contained the food. Each container had a hole covered by a mesh net. The experiment was repeated four times (15 larvae per replicate). After 10 days, feeding indices were calculated according to a formula.¹³⁾

4. α -Amylase and protease assay

To prepare a sample for measuring α -amylase and protease activity, the midgut was dissected in 50 mM Tris-HCl (pH 7.4), followed by incubation in the same buffer in the presence or absence of AST-A (ARPYSFGLamide) for 30 min at RT in order to release the enzyme (α -amylase or protease) into the buffer.

α -Amylase activity was measured as previously described using a Kikkoman kit (Kikkoman Corporation, Japan).¹⁴⁾ The sample (20 μ L) was incubated with 100 μ L of substrate buffer that contained 2-chloro-4-nitrophenyl 65-azido-beta-malto-pentaoside and 100 μ L of co-working enzyme solution that contained β -glucosidase and glucoamylase. The reaction was stopped by adding 400 μ L of stop solution (sodium carbonate). The absorbance was measured by using a microplate reader (Bio Tek, U.S.A) at 400 nm.

The protease activity was measured by the digestion of azocasein as described previously.¹⁵⁾ Briefly, 60 μ L of the sample was incubated with 60 μ L of 0.5% (w/v) azocasein solution in Tris-HCl (pH 7.4) at 37°C for 30 min. To stop the reaction, 160 μ L of 20% trichloroacetic acid was added. The plate was incubated on ice for 10 min. The sample was centrifuged (4000 $\times g$ at 4°C, 15 min), and the supernatant was used to measure the protease activity. The protein concentration of each sample was measured using a Pierce Protein Assay Reagent Kit (Thermo Fisher Scientific, USA). Bovine serum albumin was used as a standard. The absorbance was measured by microplate reader at 335 nm.

5. Immunohistochemistry

Rabbit anti-AST-A antibody (Genemed Synthesis, South San Francisco, CA, USA) was used as a primary antibody. ARPYSFGL-amide is lepidopteran AST-A.¹⁶⁾

The midgut was dissected from larva in PBS buffer and kept at 4°C overnight in Bouin solution (15 vol. picric acid solution 1.3% in H₂O (saturated), 5 vol. formalin, 1 vol. acetic acid), followed by dehydration, embedding in paraffin, sectioning, dewaxing and finally rehydration as described before.¹⁷⁾ They were rinsed in Tris-buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM Tris-HCl, pH 7.6) for 5 min. Sections were blocked with 5% normal goat serum diluted in TBS at RT for 30 min. AST-A antibody diluted with blocking serum (1:1500)

was added to each section overnight at 4°C. After washing 3 times with TBS (10 min each time), biotinylated anti-rabbit IgG diluted with blocking serum (1:200) was added to each slide. Slides were kept at RT for 1.5 hr. After rinsing 3 times with TBS (each time 10 min), the sections were incubated for 30 min with VECTASTAIN ABC reagent (VECTASTAIN ABC KIT PK-6101). After washing 3 times with TBS, sections were rinsed with 0.1 M Tris-HCl, pH 7.5, for 10 min, followed by development in a diaminobenzidine tetrahydrochloride solution (0.25 mM in 0.1 M Tris-HCl, pH 7.5, 144 mL, 30% H₂O₂, 30 μ L) for 8 min. The slides were dehydrated using an ethanol-xylene series. Later, they were mounted in Bioleut mounting medium (Kouken Rika, Japan). Finally it was visualized using a BX50F4 microscope (Olympus, Japan).

To confirm the specificity of the primary antibodies, instead of the antiserum (anti-AST), normal serum was used for control. The preabsorption test for immunohistochemistry confirmed the antiserum specificity.¹⁶⁾

6. Morphometric analysis

The AST-A-ir cells were quantified using the point-counting method.¹⁸⁾ Immunostained midguts of *S. littoralis* were photographed. Fifteen sections were selected randomly from 150 sections. A transparent grid lattice of 500 (20 \times 25) points was made and put on the image of IHC (digital or printed papers), and the intersecting points covering AST-A-ir cells in the anterior, median and posterior of the midgut were counted. The number of AST-A-endocrine cells per 100 points covering the midgut epithelium were reported.

7. Competitive ELISA

Competitive ELISA was performed as previously described.¹⁹⁾ Briefly, after dissection of the midgut in TBS, homogenization, and centrifugation (4000 $\times g$, 4°C, 15 min), the supernatant was used as an assay sample. After coupling synthetic AST-6 and to BSA with dimethyl suberimidate (Sigma-Aldrich, USA) and preparing an AST-A-BSA conjugate, the plates were coated with AST-6-BSA (0.6 μ g/mL per well) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.0). It was kept at RT for 3 hr. Then, 250 μ L of 2% skimmed milk was added to each well, and the plate was incubated at RT for 1 hr. To each well, 50 μ L of the extract of the midgut or standard peptides (0.01–100 nmol/well) was added, followed by the addition of 50 μ L of the diluted antiserum against AST-6 (1:12000 concentrations in TBS with 2% skimmed milk) to each well. The plate was kept overnight at 4°C. It was rinsed three times with TBS containing 0.5% Tween-20 (TBS-Tw); it was then incubated with 100 μ L of secondary antibody solution in TBS (1:1000) for 1 hr at room temperature. After washing 3 times with TBS-Tw, 100 μ L of the substrate solution (1 mg/mL *p*-nitrophenylphosphate disodium salt hexahydrate [Sigma-Aldrich, USA] in 10 mM diethanolamine buffer [Sigma-Aldrich, USA], pH 9.5) was added to each well and kept for 1 hr at RT. Adding 50 μ L 4 M NaOH to each well stopped the reaction. Finally, the absorbance was read using a microplate

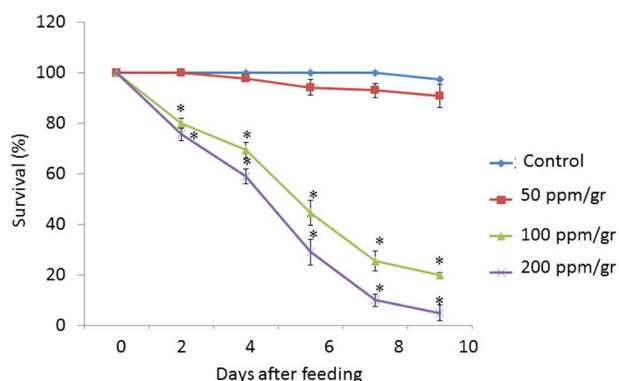


Fig. 1. Survival rate of *S. littoralis*, over 10 days (from the third instar larval stage) after feeding on an artificial diet (from the third instar larva stage for 10 days) after feeding on an artificial diet containing 50, 100 and 200 ppm/gr of CA. CA: caffeic acid.

reader (Bio Tek, USA) at 405 nm.

8. AST-A injection into the hemolymph

A total of 10^{-10} moles of AST-A in $4\mu\text{L}$ of PBS was injected into the insect using a Hamilton syringe (Hamilton, USA), 3 hr before dissection. The puncture was sealed by Aron Alpha instant adhesive (Toagosei, Japan). The injection was used to investigate the effect of AST-A on digestive enzyme activities.

9. Effects of AST-A on α -amylase and protease activities

The dissected midguts of insects fed normally were incubated for 30 min at room temperature in 50 mM Tris-HCl (pH 7.4) in the presence or absence of AST-A. α -Amylase and protease released into the buffer were measured.

10. Statistical analysis

Data were analyzed by one-way ANOVA (Fisher's LSD). For $p < 0.05$, Differences of $p < 0.05$ were accepted as significant.

Results

1. Effect of CA on survival rate and feeding indices

First, it was confirmed that affects the survival rate and feeding indices of *S. littoralis*. CA was showed to have a concentration-dependent impact on the survival of *S. littoralis*. Third instar lar-

vae fed on an artificial diet containing 100 or 200 ppm/gr of CA for 10 days showed reductions in the survival rate by approximately 80 to 95% respectively (Fig. 1).

The feeding indices of larvae feeding on an artificial diet containing CA (100 or 200 ppm/gr) were affected. Approximate digestibility (AD) was increased significantly in larvae fed on an artificial diet containing 100 or 200 ppm/gr of CA. It significantly decreased the efficiency of conversion of ingested food (ECI) and efficiency of digested food (ECD), relative growth rate (RGR) and relative consumption rate (RCR) in larvae fed on an artificial diet containing 100 or 200 ppm/gr of CA. Feeding indices of insects fed on an artificial diet containing 50 ppm/gr of CA were not affected (Table 1).

2. Effects of CA on α -amylase and protease activities

In part 3.1, CA was shown to affect feeding indices. One possible reason is that it may affect digestive enzyme activities. Therefore, it was confirmed here that CA decreased α -amylase and protease activities. The results showed that feeding on an artificial diet containing 100 or 200 ppm/gr of CA decreased α -amylase and protease activities in *S. littoralis*. α -Amylase activity decreased from 132 mU in the control to 62.1 and 55.6, respectively, in insects feeding on food containing 100 or 200 ppm/gr of CA after 10 days (Fig. 2A). It also sharply decreased protease activity in the insect. Protease activity decreased from 120.8 mU in the control to 61.4 and 41.13 mU with treatment (food containing 100 or 200 ppm/gr of CA, respectively, after 10 days) (Fig. 2B).

3. Effect of CA on AST-A content in the midgut of *S. littoralis*

To confirm that affects AST-A content in the midgut, the AST-A titer was measured. First it was confirmed that AST-A-ir occurred in the midgut of *S. littoralis* (Fig. 3). An immunohistochemistry experiment then showed that the number of AST-A-ir cells decreased in the anterior, median, and posterior of the midgut of *S. littoralis* after 72 hr of starvation but increased rapidly 3 hr after refeeding. Feeding on an artificial diet containing 100 or 200 ppm/gr of CA also clearly decreased the number of immunoreactive cells in the midgut (Fig. 4A, B, C). Competitive ELISA results were consistent with the profile of immunohistochemical reactivity, which indicated that the AST-A titer in the midgut extract of insects was decreased after 72 hr of starvation. It increased sharply 3 hr after refeeding. It was also shown that

Table 1. Nutritional indices of 3th instar larvae of *S. littoralis*, 10 days after feeding artificial diet contain 50, 100 and 200 ppm/gr of caffeic acid (CA). An asterisk indicates a significant difference relative to the control treatment^{a)}

Treatment	AD (%)	ECI (%)	ECD (%)	RCR (mg/mg/day)	RGR (mg/mg/day)
Control	80 \pm 0.25	22 \pm 0.2	27 \pm 0.25	0.50 \pm 0.002	0.11 \pm 0.0008
Caffeic acid 50 ppm/gr	81 \pm 0.43	21 \pm 2.66	28 \pm 0.11	0.48 \pm 0.41	0.1 \pm 0.22
Caffeic acid 100 ppm/gr	94 \pm 0.11*	15 \pm 0.97*	15 \pm 0.18*	0.30 \pm 0.01*	0.05 \pm 0.006*
Caffeic acid 200 ppm/gr	95 \pm 0.11*	10 \pm 0.16*	14 \pm 0.55*	0.2 \pm 0.71*	0.044 \pm 0.002*

^{a)} AD: approximate digestibility, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, RCR: relative consumption rate, RGR: relative growth rate. The asterisk (*) indicates that it is significantly different from the corresponding control at $p < 0.05$.

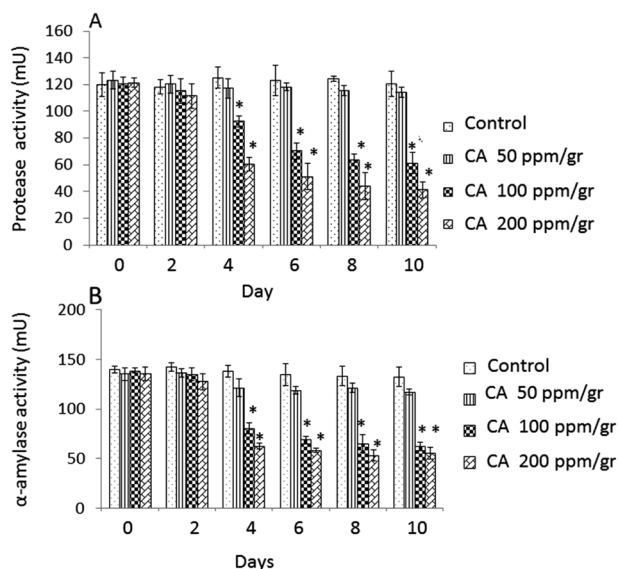


Fig. 2. Protease (A) and α -amylase (B) activities in *S. littoralis* over 10 days (from third instar larval stage) after feeding on an artificial diet containing 50, 100, and 200 ppm/gr of CA. Each point represents the mean \pm SEM. The asterisk (*) indicates that it is significantly different from the corresponding control at $p < 0.05$. CA: caffeic acid.

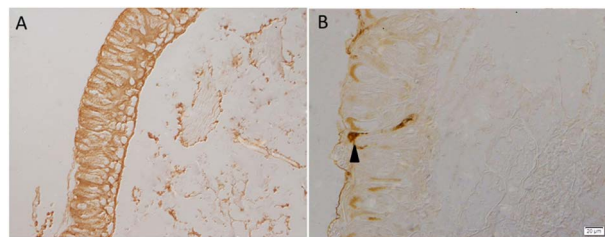


Fig. 3. AST-A-ir cells in the midgut epithelium of third instar larvae of *S. littoralis*. A, control (preabsorption test). B, Alt-ir cells in the midgut epithelium. The arrowhead indicates a cell that became reactive to the AST-A antibody. Scale bars, 50 μ m. AST-A: Allatostatin-A.

feeding on an artificial diet containing 100 or 200 ppm/gr of CA also caused the AST-A titer to decreased (Fig. 5).

4. Effects of AST-A on α -amylase and protease activities

In part 3.3, it was shown that CA affects AST-A content in the midgut. Moreover, in part 3.2, it was confirmed that CA decreased α -amylase and protease activities. It is possible that CA can decrease digestive enzyme activities *via* its effect on AST-A. Therefore, the effect of AST-A on digestive enzyme activities was investigated *in vitro* (part 3.4) and *in vivo* (part 3.5). It was

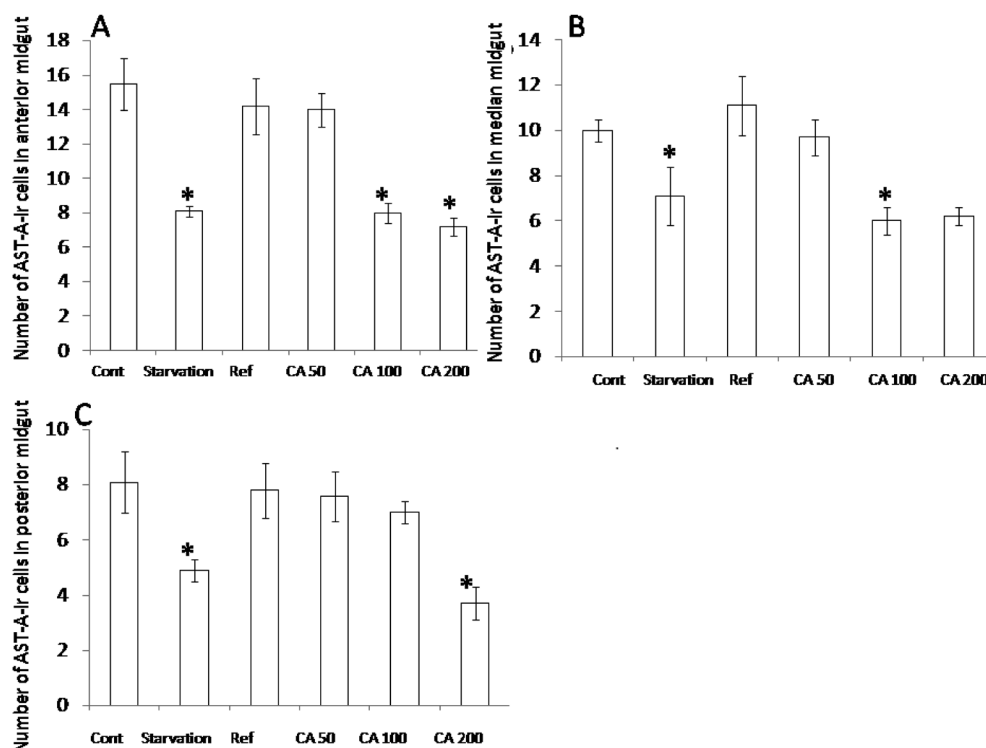


Fig. 4. Distribution of the number of Alt-ir cells in the anterior (A), median (B), and posterior (C) midgut of *S. littoralis* over 10 days (from the third instar larval stage) after feeding on an artificial diet containing 50, 100, and 200 ppm/gr of CA. Each point represents the mean \pm SEM. of ten preparations. * $p < 0.05$, as compared with the number of AST-A-ir cells of larvae fed normally (ANOVA and LSD test). 50 CA: 50 ppm/gr of caffeic acid; 100 CA: 100 ppm/gr of caffeic acid, 200 CA: 200 ppm/gr of caffeic acid, Cont: Control, Ref: Refed.

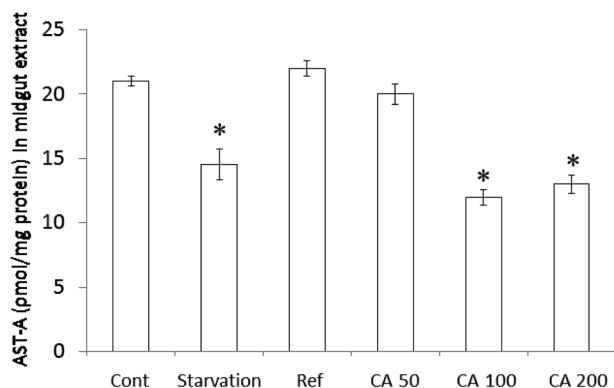


Fig. 5. A competitive ELISA detected the AST-A titer in the midgut of *S. littoralis* after 72 hr of starvation, after being refed 3 hr later and over 10 days (from the third instar larval stage) after feeding on an artificial diet containing 50, 100, and 200 ppm/gr of CA. Each point represents the mean \pm SEM. * $p < 0.05$, as compared with the normal the larvae fed normally as control (LSD test). 50 CA: 50 ppm/gr of caffeic acid; 100 CA: 100 ppm/gr of caffeic acid; 200 CA: 200 ppm/gr of caffeic acid; Cont: Control; Ref: Refed.

also shown that incubation of the dissected midgut of *S. littoralis* with AST-A increased α -amylase and protease activities (Fig. 6A, B).

5. Effect of AST-A injection into the insect's hemocoel on α -amylase and protease activities

AST-A injection of greater than 10^{-10} mol into the insect's hemocoel increased α -amylase and protease activities more than 1.63- and 1.65-fold respectively. PBS injection had no effects on these enzyme activities (Fig. 7A, B).

Discussion

Phenolic compounds show deterrence and toxic activity against

many insects.⁶⁾ CA has a significant negative impact on the survival of *H. armigera*.⁶⁾ Our results also clearly indicated that *S. littoralis* fed on an artificial diet containing CA had reduced survival rate (Fig. 1). At concentrations of 100 and 200 ppm/gr, CA also caused reduced antifeedant activity (Table 1). These results confirm previous studies that showed that CA reduced both ECD and ECI in *H. armigera*.⁶⁾ CA also negatively affects ECI, RGR, and RCR in *L. decemlineata*.⁵⁾

Many factors can affect digestive enzyme activity in the insect midgut, such as starvation, feeding, and some chemicals. Here, we confirmed that feeding *S. littoralis* on an artificial diet containing CA caused protease and α -amylase activities to decrease (Fig. 2A, B). Previous results also showed that CA significantly reduced protease activity in *H. armigera*.⁶⁾

All organisms have different neuropeptides in their nervous system. They are involved in many functions including ecdysis behavior, locomotor activity, learning and feeding.⁸⁾ To the present, many neuropeptides have been reported to be involved in feeding. The short neuropeptide F (sNPF) and crustacean cardioactive peptide (CCAP) are involved in regulating of digestive enzyme activities in *P. americana*.^{14–19)} Although ASTs are well known for gut myoinhibitory, especially in Lepidoptera,²⁰⁾ they also are reported to be a regulator of digestive enzyme activities.¹¹⁾ AST-A was previously detected in endocrine cells of the midgut of *P. americana*.¹¹⁾ Here, we detected AST-A in endocrine cells of the midgut of *S. littoralis* (Fig. 3). For the first time, we showed that feeding on an artificial diet containing CA decreased the number of AST-A-ir cells in the midgut. Moreover, starvation has the same effect on the AST-A level, whereas refeeding increases it (Figs. 4, 5). AST-A increased α -amylase and protease activities *in vitro* (Fig. 6). Moreover, injection into the hemolymph of *S. littoralis* larvae had the same effect on α -amylase and protease activities (Fig. 7). These results confirm the importance of AST-A in regulating digestive enzyme activities. Taken all together it may be possible that feeding on an artificial diet containing CA caused endocrine cells to decrease

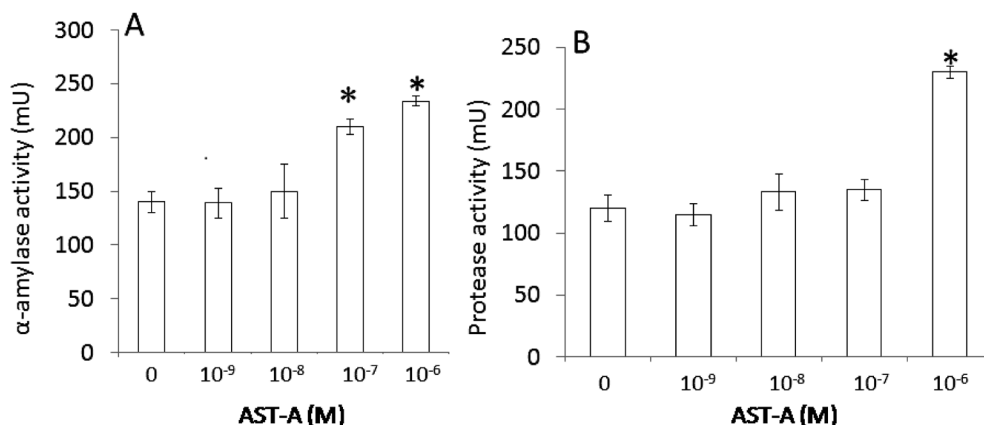


Fig. 6. Effect of AST-A on the midgut α -amylase (A) and protease (B) activities on third instar larvae of *S. littoralis* *in vitro*. Each point represents the mean \pm SEM. of 10 preparations. * $p < 0.05$, as compared with α -amylase or protease activity in the absence of AST-A (LSD test).

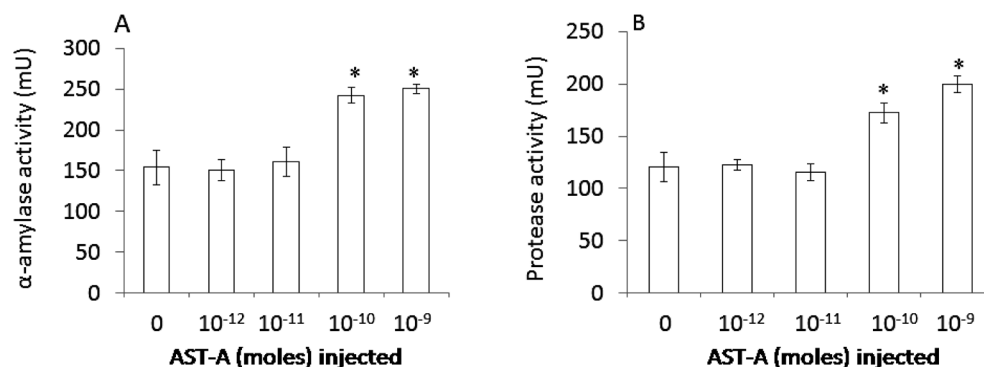


Fig. 7. Change in α -amylase (A) and protease (B) activities in the midgut of third instar larvae of *S. littoralis* 3 hr after the injection of different amounts of AST-A into the hemocoel. Each point represents the mean \pm SEM of 10 preparations. * $p < 0.05$, as compared with α -amylase and protease activities in the absence of AST-A (LSD test). AST-A: Allatostatin-A.

AST-A secretion which itself led to decreased α -amylase and protease activities. To avoid harmful side effects of pesticides, CA can be a useful alternative for controlling this important pest.

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