Journal of Invertebrate Pathology **75,** 259–266 (2000)
doi:10.1006/jipa.2000.4936, available online at http://www.idealibrary.com on IDEAL®

The Interactions between Soybean Trypsin Inhibitor and δ-Endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* Larva

Ji-hong Zhang, Chen-zhu Wang, and Jun-de Qin

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 19 Zhongguancun Lu, Haidian, Beijing 100080, People's Republic of China

Received April 2, 1999; accepted March 23, 2000

No significant difference in larval mortality was observed when a sublethal dose of *Bacillus thuringiensis* (Bt) var. *kurstaki* HD-1 crystal was supplemented with soybean trypsin inhibitor (STI) in the artificial diet fed to *Helicoverpa armigera* in the laboratory, but supplementing a nonlethal dose of crystal with STI in the diet led to a pronounced reduction of larval growth. This concentration of crystal and two lower concentrations of STI alone had no significant effects on larval growth. The results of substrate–gel electrophoresis demonstrated that the proteases in the *H. armigera* midgut fluid responsible for the degradation of protoxin consisted of at least four proteases with molecular weights of 71, 49, 36, and 30 kDa. All four proteases could utilize casein also as the substrate. When larvae were fed with STI or Bt + STI, the proteolytic activities of the 49-kDa enzyme disappeared, and the activities of the other three enzymes were reduced. Enzyme assays also indicated that feeding larvae with diets containing Bt, STI, or Bt + STI significantly decreased the specific activities of larval general proteases and the trypsin-like enzyme. The protein concentration of midgut fluid was elevated, especially in the larvae fed on the diets containing STI and Bt + STI. Both *in vitro* and *in vivo* studies showed that the degradation of protoxin and toxin could be inhibited by soybean trypsin inhibitors, but when the incubation time was prolonged, the protoxin could be degraded completely, while the degradation of toxin was inhibited further. This suggested that the retention time of toxins in the larval midgut was extended and synergism between insecticidal crystal protein and soybean trypsin inhibitor occurred, which showed as the inhibition of *H. armigera* larval growth. © 2000 Academic Press

Key Words: **soybean trypsin inhibitor; *Bacillus thuringiensis; Helicoverpa armigera;* interaction; toxicity; protease activity; degradation; synergy.**

INTRODUCTION

Helicoverpa armigera is an important pest in China. It has developed resistance to many chemical insecticides, greatly reducing the number of control options (Guo, 1995; Armes *et al.*, 1996). Biological control plays an increasingly significant role in the management of this pest.

Bacillus thuringiensis (Bt), the most effective bioinsecticide, is a gram-positive bacterium that produces crystalline parasporal inclusions containing insecticidal crystal proteins (ICPs), called δ-endotoxins, during sporulation. The crystals themselves have no insecticidal activity. Upon ingestion by susceptible insects, the crystals are solubilized into protoxins with molecular weight around 130 kDa in the alkaline midgut. The protoxins are cleaved to 60 kDa around active insecticidal toxins by the proteolysis of midgut proteases. The toxins have some resistance to the midgut enzymes but can also be further degraded to smaller molecules and thus lose toxicity (Gill *et al.*, 1992). Insect midgut proteases are essential to the production of Bt toxins.

Plant proteinase inhibitors are antinutriental against herbivores (Felton and Gatehouse, 1996). Bioassay tests indicate that proteinase inhibitors reduce insect growth and development and decrease insect survival and biomass (Green and Ryan, 1972; Steffens *et al.*, 1978; Johnston *et al.*, 1993; Burgess *et al.*, 1994; Seldal *et al.*, 1994; Wang *et al.*, 1995). In enzyme assay *in vitro,* the activities of insect midgut proteases were depressed by proteinase inhibitors, while *in vivo,* simple inhibition could not explain the results (Broadway and Duffy, 1986; Larocque and Houseman, 1990; Dymock *et al.*, 1992; Johnston *et al.*, 1993; Wang *et al.*, 1995).

It has been suggested that important interactions can occur among plant chemicals, herbivores, and microbes (Berenbaum, 1988; Reichelderfer, 1991). Proteinase inhibitors are widely distributed in plants

0022-2011/00 $35.00

(Houseman *et al.*, 1991) and have been successfully transformed and expressed in plants (Felton and Gatehouse, 1996). If proteinase inhibitors influence the activities of insect midgut proteases, they may affect the degradation of ICPs and consequently interfere with the toxicity of Bt insecticides. Evaluating the interactions among plant proteinase inhibitors, herbivores, and ICPs could improve the efficacy of Bt used on the plants containing proteinase inhibitor.

MacIntosh *et al.* (1990) reported that several serine proteinase inhibitors enhanced the toxicity of ICPs from Bt var. *kurstaki*, var. *tenebrionis*, and var. *israelensis* against their target insects *Heliothis virescens*, *Leptinotarsa decemlineata*, and *Aedes aegypti*, respectively. They provided additional evidence of potentiation by *in planta* expression of a fused protein, proteinase inhibitor, and ICP, but, they did not determine the mechanism of the potentiation.

In the present work, we used *H. armigera* as the target insect and assessed the effects of supplementing Bt var. *kurstaki* HD-1 crystals with soybean trypsin inhibitor on the mortality and growth of *H. armigera*. In addition, we tested the *in vitro* and *in vivo* effects of soybean trypsin inhibitor on the larval midgut proteases and the proteolysis of ICPs. The role of multiple proteases present in the midgut of *H. armigera* on the degradation of ICPs was also investigated.

MATERIALS AND METHODS

Materials

Dithiothreitol and Coomassie bright blue R-250 were B.D.H products. Acrylamide, *n*,*n*′-methylene-bis-acrylamide, sodium dodecyl sulfate, bovine trypsin, trypsin inhibitor (Type II-S: soybean) (STI), soybean Bowman–Birk inhibitor (SBBI), BApNA, and azocasein were from Sigma. Urografin (76%) was made in XinYi pharmaceutical factory. Other reagents were of analytical grades.

H. armigera were collected from cotton fields in He bei Province and reared on artificial diets at 27°C, 75% R.H., with photoperiod 15L:9D h. The formulation of artificial diet was according to Bot (1966), except that casein and wheat embryo powder were substituted by milk powder and wheat sprout powder, respectively. Adults were fed with 10% honey solution.

B. thuringiensis var. *kurstaki* HD-1 was cultured on a solid medium containing peptone 1%, beef extract 0.5%, and NaCl 0.5% at 30°C.

Preparation of Crystals and Protoxins

Crystals were purified by a biphasic separation in anhydrous sodium sulfate (Na_2SO_4) and carbon tetrachloride (CCl_4). Further purification was performed by isopycnic centrifugation in Urografin gradients as described by Zhang *et al.* (1997).

Crystals were incubated in 0.1 M glycine–NaOH buffer, pH 10.2, and supplemented with 10 mM dithiothreitol at 30°C for 1 h. After centrifugation at 11,200*g* for 15 min, the supernatants were collected as protoxins.

Preparation of Midgut Fluids of H. armigera

Fifth-instar larvae were rapidly dissected at 0–4°C, hemolymph was washed away by precooled 0.15 M NaCl solution, and the midguts and their contents were collected and homogenized in 0.15 M NaCl (3.5 ml/g) in an ice bath. After centrifugation at 11,200*g* for 15 min at 4°C, the midgut supernatant was collected and stored at −20°C.

Protein Quantification

Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

The Effect of Supplementing Bt Crystals with STI on the Mortality of H. armigera

STI was more effective on the inhibition of larval growth and larval midgut protease activities than SBBI (Johnston *et al.*, 1993); so, it was chosen for the bioassay and *in vivo* tests. Different concentrations of crystals were tested to determine their effects on larval mortality. A sublethal dose of crystals (0.0004%, w/w) was used to mix with different concentrations of STI (0.004, 0.04, and 0.4%, w/w) and uniformly added into artificial diets around 40°C. The untreated artificial diets were used as controls. Each diet fed 50 neonates, and each neonate was placed singly in individual tubes. Three repetitions were performed. The mortality of *H. armigera* was assessed on the 5th day. The corrected mortality was determined by the method of Abbott (1925).

The Effect of Supplementing Bt Crystals with STI on the Growth of H. armigera

The 3-day-old *H. armigera* larvae of similar size (larval weight <0.001 g) were fed with untreated artificial diets or treated diets containing Bt crystals alone, STI alone at 0.0004, 0.004, or 0.04% (w/w), or Bt crystals supplemented with STI at 0.0004, 0.004, or 0.04% (w/w). The concentration of Bt crystals in all diets was 8×10^{-7}% (w/w). Each treatment contained 15 larvae. The larvae were weighed after 7 days.

The in Vitro Effect of Soybean Trypsin Inhibitor on the Degradation of ICP

Trypsin-like enzymes are important proteases in the midgut of *H. armigera* larvae (Wang and Qin, 1996). To test the role of trypsin-like enzymes on the degradation of protoxins, bovine trypsin was used for comparison. Larval midgut fluid containing 4 μg proteins or bovine trypsin was incubated with 4 μg STI or SBBI at 30°C for 15 min. This dosage of STI and SBBI could inhibit 80% of trypsin-like enzyme activity of *H. armigera* midgut fluid. The bovine trypsin was adjusted to hydrolyze the same amount of BApNA as that of larval midgut fluid. Protoxin (40 μg) was added separately into the mixture. The incubations were carried out at 30°C for 30 min and 6 h. At the end of the incubation time, an equal volume of sample buffer was added, and samples were heated at 100°C for 3 min to stop the reaction. Protoxins and their proteolytic products without inhibitors were used as controls. Digested and control samples were analyzed by discontinuous SDS–PAGE according to Laemmli (1970). After electrophoresis, the gels were stained with Coomassie blue R-250 (0.001 g/L) for visualization.

To test the effect of soybean trypsin inhibitor on the further degradation of toxin, we prepared toxin by incubating protoxin with *H. armigera* larval midgut fluids in a ratio of 25:1 (w/w of protein) at 30°C for 17 h. *H. armigera* midgut fluid containing 8 μg protein was incubated with 8 μg STI or SBBI at 30°C for 15 min prior to the addition of 40 μg toxin. The incubations were carried out at 30°C for 1 and 24 h. Toxins and their proteolytic products without inhibitors were used as controls.

The Effect of Bt Crystals and STI on H. armigera Midgut Proteases and Their Proteolysis of the ICP

Reagents were prepared in 20% fructose solution to stimulate larva feeding as follows: Bt crystals (1.5 mg/ml), STI (86.4 mg/ml), or Bt crystals (1.5 mg/ml) + STI (86.4 mg/ml). Each reagent (5 μl) was applied to the surface of a little cube of artificial diet ($1.5 \times 1.5 \times 1.5$ mm^3 in size). The fructose solution alone was used as control. A 2-day-old fifth-instar larvae deprived of food for 6 h was tested with the diet. The growth and development of larvae fed on differently treated diets was variable. To guarantee the comparability of midgut protease activities of larvae with different treatments, we tested the activities of larval midgut proteases after larvae consumed a relatively high dose of Bt or STI for 1 and 3 h. The dosage of Bt was that which could kill around 50% of the larvae at 2 days, and the dosage of STI was the 2nd-day diet consumption with STI (0.04%, w/w) by fifth-instar larvae. After consuming the diets completely in 1 to 3 h, larvae were placed at −20°C and were dissected to obtain the midgut fluids the next day. Each treatment contained three groups and each group contained five larvae.

The protein concentration of each midgut fluid sample was determined, and each sample was diluted with distilled water to 1 mg/ml to test the activities of general protease and trypsin-like enzyme with the methods of Wang and Qin (1996). The buffer pH used was the optimum pH of the enzyme with the specialized substrate.

General protease activity was assayed using azocasein as the substrate, at a concentration of 20 mg/ml in 0.15 M NaCl. The incubation medium consisted of 0.3 ml substrate and 0.3 ml 0.2 M Gly–NaOH buffer, pH 10.5, containing larval midgut fluids. The reaction was carried out at 30°C for 2 h and stopped by the addition of 0.6 ml trichloroacetic acid (20%, w/v). The mixture was centrifuged at 11,200*g*, 4°C for 15 min, and the absorbance of the supernatant was measured at 366 nm.

Trypsin-like enzyme activity was assayed with BApNA at a concentration of 20 mg/ml in dimethyl sulfoxide. The incubation medium consisted of substrate in 40 μl and 1.5 ml 0.1 M Gly–NaOH buffer, pH 10.5, containing larval midgut fluids. The reaction was carried out at 30°C for 20 min and stopped by the addition of 0.5 ml acetic acid (30%, v/v). The absorbance was measured at 405 nm.

The degradation patterns of protoxin and toxin by larval midgut fluid were demonstrated by SDS–PAGE. After feeding for 3 h, the specific activities of larval midgut protease were lower than those after feeding 1 h, so 3 h was chosen for the following tests. Protoxin (40 μg) was incubated with each diluted larval midgut fluid (4 μl) at 30°C for 8 min and 6 h and analyzed by SDS–PAGE. Toxins were obtained by incubating protoxins with *H. armigera* midgut fluids in a ratio of 10:1 (w/w of protein) at 30°C for 17 h. More larval midgut fluid than in the *in vitro* test was used to ensure that all higher molecular intermediates degraded into toxins. Each group of diluted larval midgut fluid (7 μl) was incubated with toxin (40 μg) at 30°C for 6 h. Then, another quantity of diluted midgut fluid (5 μl) was added to the mixture and the mixture was incubated for another 18 h to maintain high enzyme activities prior to SDS–PAGE analysis.

Substrate–gel electrophoresis was carried out by the method of García-Carreño (1993) to test the proteases responsible for protoxin degradation. Each group of larval midgut fluid (60 μg protein) was loaded onto SDS–PAGE directly. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min at 4°C and then immersed in 1% protoxin or 2% casein simultaneously for 40 min at 4°C.

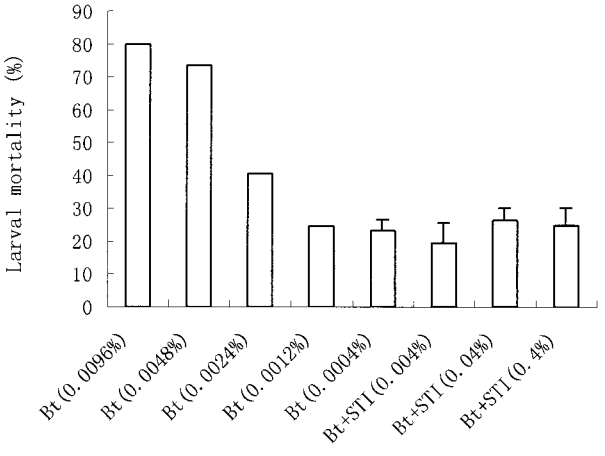

FIG. 1. The effect of supplementing Btk HD-1 crystals with STI on the mortality of *Helicoverpa armigera* larvae. The concentrations of Bt crystals in the treatments combined with STI were all 0.0004% (w/w). Each treatment contained 50 neonates. The treatments with error bars were repeated three times, and the error bars represent SE.

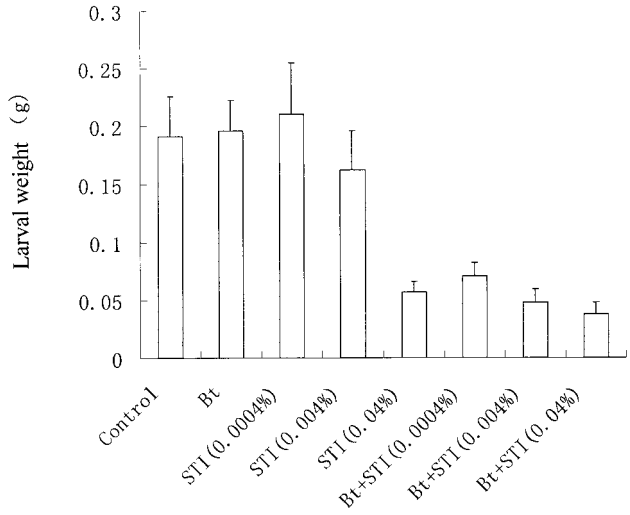

FIG. 2. The effect of supplementing Btk HD-1 crystals with STI on the growth of *Helicoverpa armigera* larvae. The concentrations of Bt crystals in all the treatments were 8×10^{-7}% (w/w). Each treatment contained 3-day-old larvae, 15 and larvae were weighed after 7 days. Error bars represent SE.

Statistical Analysis

Data from the bioassay tests were analyzed with one-way ANOVA using SPSS to determine the significance of differences.

RESULTS

The Effect of Supplementing Bt Crystals with STI on the Mortality of H. armigera

No significant difference was observed in the mortality of *H. armigera* when three different concentrations of STI were added to diets containing a sublethal dose of HD-1 crystals ($P > 0.05$; Fig. 1).

The Effect of Supplementing Bt Crystals with STI on the Growth of H. armigera

Diets containing Bt crystals (8×10^{-7}%, w/w) or STI (0.0004 and 0.004%, w/w) alone caused no significant differences in the growth of *H. armigera*, while diets containing either STI 0.04% (w/w) alone or Bt crystal diets supplemented with concentrations of STI significantly inhibited the growth of *H. armigera* ($P < 0.05$; Fig. 2).

The in Vitro Effect of Soybean Trypsin Inhibitor on the Degradation of ICP

STI and SBBI caused nearly complete inhibition of the degradation of protoxins with *H. armigera* midgut fluid and complete inhibition with bovine trypsin for 30 min. When the incubation time was prolonged to 6 h, the degradation of protoxin by larval midgut fluids approached that of the control without inhibitor, and the degradation of protoxin by bovine trypsin was still greatly inhibited. Furthermore, the rate of degradation of protoxin by bovine trypsin was somewhat slower than that of larval midgut fluid (Fig. 3). These results suggested that other proteases in addition to the tryp-

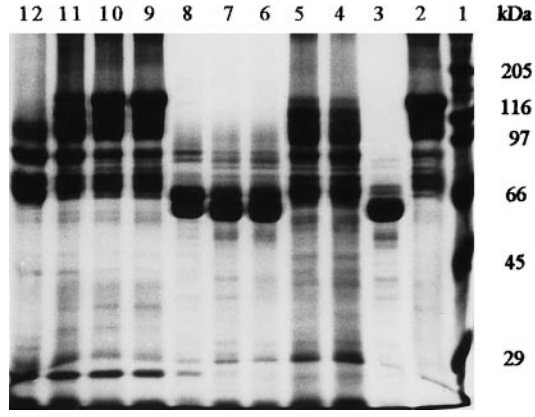

FIG. 3. The *in vitro* effect of soybean proteinase inhibitor on the degradation of Btk HD-1 protoxin. *Helicoverpa armigera* midgut fluids containing 4 μg protein or bovine trypsin were incubated with 4 μg STI or SBBI at 30°C for 15 min. The level of bovine trypsin was equivalent, in terms of the hydrolysis of BApNA, to that of the larval midgut fluids. Then, 40 μg protoxin was added into the mixture separately. The incubations were carried out for 30 min and 6 h. Lane 1, standard protein marker; lane 2, protoxin; lane 3, midgut fluid + protoxin, 30 min; lane 4, midgut fluid + STI + protoxin, 30 min; lane 5, midgut fluid + SBBI + protoxin, 30 min; lane 6, midgut fluid + STI + protoxin, 6 h; lane 7, midgut fluid + SBBI + protoxin, 6 h; lane 8, bovine trypsin + protoxin, 30 min; lane 9, bovine trypsin + STI + protoxin, 30 min; lane 10, bovine trypsin + SBBI + protoxin, 30 min; lane 11, bovine trypsin + STI + protoxin, 6 h; lane 12, bovine trypsin + SBBI + protoxin, 6 h.

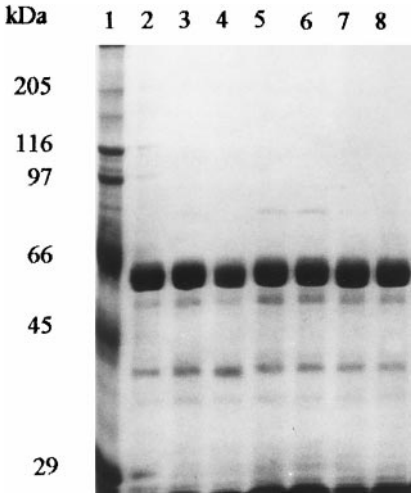

FIG. 4. The *in vitro* effect of proteinase inhibitor on the further degradation of toxin. *Helicoverpa armigera* midgut fluids containing 8 μg protein were incubated with 8 μg STI and SBBI at 30°C for 15 min. Then, 40 μg toxin was added into the mixture separately. The incubations were carried out for 1 and 24 h. Lane 1, standard protein markers; lane 2, toxin; lane 3, midgut fluid + toxin, 1 h; lane 4, midgut fluid + toxin, 24 h; lane 5, midgut fluid + STI + toxin, 1 h; lane 6, midgut fluid + SBBI + toxin, 1 h; lane 7, midgut fluid + STI + toxin, 24 h; lane 8, midgut fluid + SBBI + toxin, 24 h.

sin-like enzyme in *H. armigera* midgut fluid could also participate in the degradation of protoxin. Thus, larval midgut fluid displayed higher efficacy of proteolysis and more resistance to soybean trypsin inhibitors.

Toxin was much more difficult to degrade than protoxin. With the action of larval midgut fluids, no obvious degradation of toxin was observed in 1 h. When the incubation time was prolonged to 24 h, the amount of toxin was reduced slightly, and soybean trypsin inhibitors could inhibit the further degradation of toxin (Fig. 4).

The Effect of Bt Crystals and STI on H. armigera Midgut Proteases and Their Proteolysis of the ICP

The midgut fluid protein concentrations of larvae fed on the Bt and control diets were significantly lower than those of larvae fed on the STI and Bt + STI diets ($P < 0.05$; Fig. 5).

The specific activities of general proteases and trypsin-like enzyme of larvae fed on the Bt and control diets were significantly higher than those of larvae fed on the STI and Bt + STI diets after feeding periods of 1 and 3 h ($P < 0.05$). Furthermore, when larvae had consumed the diets for 3 h, the specific activities of general proteases and trypsin-like enzyme of larvae fed on the Bt diets were significantly lower than those of larvae fed on the control diets ($P < 0.05$; Figs. 6 and 7).

Incubating protoxins with larval midgut fluids for 8 min, the degradations of protoxins by the midgut fluids

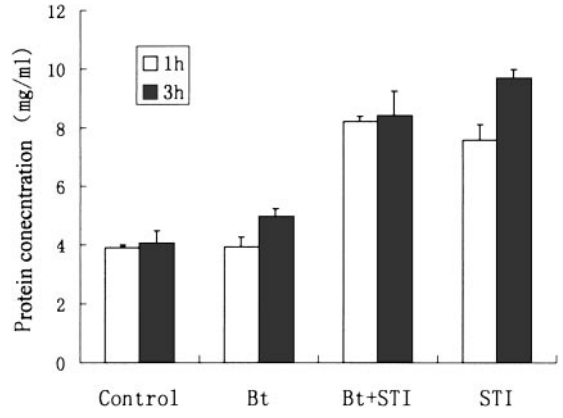

FIG. 5. The protein concentration of midgut fluids of *Helicoverpa armigara* fed on the control diets and the diets containing Bt crystals, Bt + STI, or STI. Larval consumption of Bt crystals or STI was 7.5 μg or 0.432 mg separately. Each treatment contained three groups and each group contained five 2-day-old fifth-instar larvae. After consuming the diets completely in 1 to 3 h, larvae were placed at −20°C and were dissected to obtain the midgut fluids the next day. Error bars represent SE. All the midgut fluids used in the following figures were made in the same way.

of larvae fed on the Bt, Bt + STI, and STI diets were slower than that of larvae fed on the control diets, especially for the larvae fed on the Bt + STI and STI diets. When the incubation time was prolonged to 6 h, the toxin production by midgut fluids of larvae fed on the Bt diets was the same as that of larvae fed on the control diets; all protoxins had been completely degraded. As for larvae fed on the Bt + STI and STI diets, just slightly higher molecular weight intermediates were observed (Fig. 8).

When toxins were incubated with larval midgut fluids for 6 h, no obvious degradation of toxins occurred. When more midgut fluids were added and the incubation time was prolonged to 24 h, significant degradation of toxins could be seen. The toxin degradation

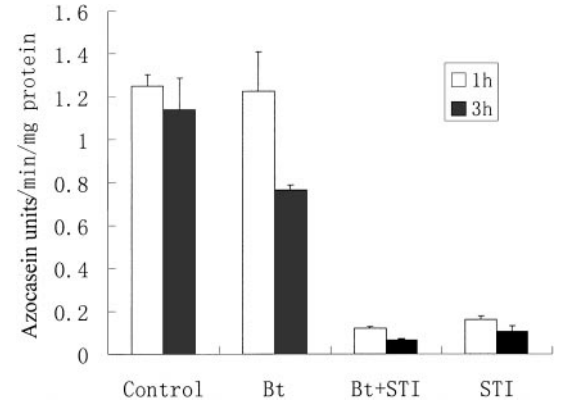

FIG. 6. The specific activities of general proteases of *Helicoverpa armigera* fed on the control diets and the diets containing Bt crystals, Bt + STI, or STI.

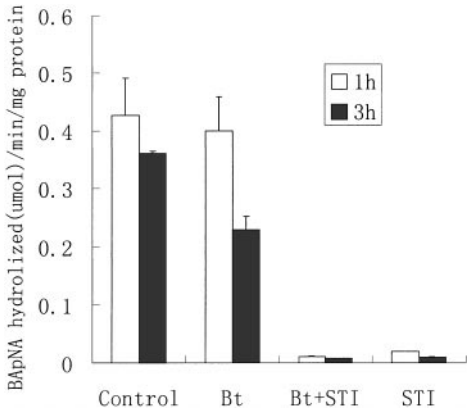

FIG. 7. The specific activities of trypsin-like enzyme of *Helicoverpa armigera* fed on the control diets and the diets containing Bt crystals, Bt + STI, or STI.

rates of larvae fed on the Bt, Bt + STI, and STI diets were slower than that of larvae fed on the control diets, especially for the larvae fed on the Bt + STI and STI diets (Fig. 9).

Substrate–gel electrophoresis indicated that the proteases responsible for the degradation of protoxin and casein were the same, with molecular weights of 71, 49, 36, and 30 kDa. The proteolytic activities of midgut fluid of larvae fed on the Bt diets showed no difference from those of larvae fed on the control diets, but for larvae fed on the Bt + STI and STI diets, the proteolytic activity of the 49-kDa enzyme disappeared, and the other three enzyme activities were depressed (Fig. 10).

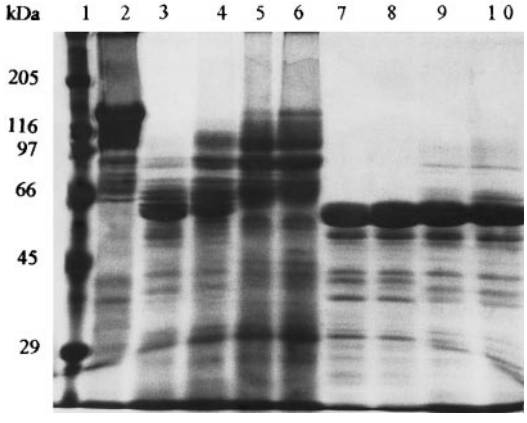

FIG. 8. The degradation of protoxin by midgut fluids of *Helicoverpa armigera* fed on control, Bt, Bt + STI, and STI diets. The larval midgut fluids used in Figs. 8–10 were made when larvae consumed the diets completely in 3 h. Protoxin (40 μg) was incubated with each diluted larval midgut fluid (4 μl) at 30°C for 8 min and 6 h. Lane 1, standard protein markers; lane 2, protoxins; lanes 3–6, protoxins were degraded for 8 min; lanes 7–10, protoxins were degraded for 6 h; lanes 3 and 7, larvae fed on control diet; lanes 4 and 8, larvae fed on Bt diet; lanes 5 and 9, larvae fed on Bt + STI diet; lanes 6 and 10, larvae fed on STI diet.

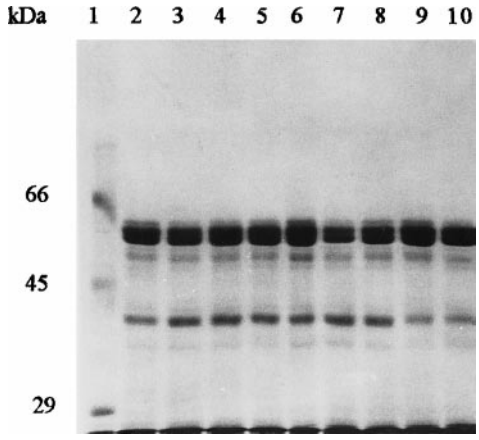

FIG. 9. The further degradation of toxins by midgut fluids of *Helicoverpa armigera* fed on control, Bt, Bt + STI, and STI diets. Each group of diluted larval midgut fluid (7 μl) was incubated with toxin (40 μg) at 30°C for 6 h. Then, another quantity of diluted midgut fluid (5 μl) was added to the mixture and the mixture was incubated for another 18 h. Lane 1, standard protein markers; lane 2, toxins; lanes 3–6, toxins were degraded for 6 h; lanes 7–10, toxins were degraded for 24 h; lanes 3 and 7, larvae fed on control diet; lanes 4 and 8, larvae fed on Bt diet; lanes 5 and 9, larvae fed on Bt + STI diet; lanes 6 and 10, larvae fed on STI diet.

DISCUSSION

Bioassay results showed that a sublethal dose of Btk HD-1 crystals supplemented with STI caused no significant changes in the mortality of larval *H. armigera*. When a nonlethal dose of crystals was added with STI, the growth of larvae was inhibited significantly, though this concentration of crystals and the two lower concentrations of STI alone had no influence on larval growth. The study of MacIntosh *et al.* (1990) indicated

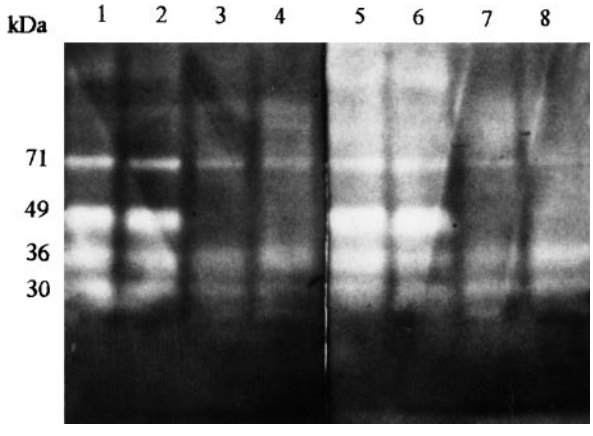

FIG. 10. Substrate–gel electrophoresis showing the proteases responsible for the degradation of protoxin and casein in *Helicoverpa armigera* midgut fluids. Lanes 1–4, substrates were caseins; lanes 5–8, substrates were protoxins; lanes 1 and 5, midgut fluids of larvae fed on control diet; lanes 2 and 6, midgut fluids of larvae fed on Bt diet; lanes 3 and 7, midgut fluids of larvae fed on Bt + STI diet; lanes 4 and 8, midgut fluids of larvae fed on STI diet.

that potentiation occurred in both larval mortality and larval growth inhibition when ICP and serine proteinase inhibitors were combined. *H. armigera* was not tested in their research.

Neither susceptibility nor response of insects to plant proteinase inhibitors were the same (Broadway, 1995). Broadway and Duffy (1986) studied the effects of soybean trypsin inhibitor and potato proteinase inhibitor on the digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. They found that the proteinase inhibitors had no effects on the *in vivo* digestion of protein, and the tryptic activity was significantly elevated. Thus, they concluded that the mode of action of proteinase inhibitors is to cause the hyperproduction of trypsin, which results in the large depletion of the sulfur-containing amino acids required for the synthesis of proteolytic enzymes and causes the antinutrition effects. Broadway and Duffy (1986) tested only the trypsin-like enzyme activity. Larocque (1990) reported that soybean proteinase inhibitors incorporated into the diets of *Ostrinia nubilalis* significantly increased larval trypsin-like enzyme activity, but the chymotrypsin-like enzyme activity declined. A study on *Costelytra zealandica* showed that soybean Kunitze trypsin inhibitor initially depressed larval serine protease activity and then a marked increase occurred after 14 days (Dymock *et al.*, 1992). As for *H. armigera*, Johnston *et al.* (1993) reported that soybean Kunitz trypsin inhibitor caused continual suppression in the activity of trypsin-like enzyme *in vivo*. Wang *et al.* (1995) also suggested that feeding larvae of *H. armigera* on STI reduced protease activities. Our results showed that after ingesting STI or Bt + STI, the specific activities of larval *H. armigera* general proteases and trypsin-like enzyme were inhibited significantly. The reduced protease activities would affect the digestion of dietary proteins and cause the protein concentration of the midgut to be significantly elevated.

The present studies both *in vitro* and *in vivo* showed that the initial degradation of protoxin was inhibited by soybean trypsin inhibitors. When incubation time was extended, the protoxins could be degraded completely, while toxins were more resistant to the proteolysis of larval midgut proteases, and no obvious degradation occurred within 6 h of incubation. When incubation time was prolonged to 24 h, the degraded toxins of treated groups were less than that of the control. This extended the retention time of toxins in the larval midgut, thus increasing the toxicity of Bt. Although it was not sufficient to elevate larval mortality, larval growth inhibition was enhanced.

The results of protoxin and casein–gel electrophoresis indicated that at least four proteases in *H. armigera* midgut fluids were involved in the process of degradation of the protoxin and that the proteases responsible for the proteolysis of protoxin and casein were the same. When the larvae were fed either STI or Bt + STI, the proteolytic activity of the enzyme with the molecular weight of 49 kDa disappeared, and the activities of the other enzymes were depressed. Maybe this is the reason that the midgut fluids of larvae fed on Bt or STI diets could degrade the protoxins to toxins completely with more time, although the initial degradation was inhibited.

The insect midgut is the target organ of Bt δ-endotoxin, but there are a few reports about the effects of δ-endotoxins on the activities of larval midgut proteases. Qiu and Lei (1986) and Wu (1986) studied the effects of Bt var. *israelensis* δ-endotoxins on the activities of mosquito larval midgut proteases and suggested that the protease activities of larvae in the treated group were lower than that of the control group. Our results indicated the same tendency. The proteolytic activity of midgut proteases of *H. armigera* larvae fed on the diets containing Bt for 3 h were significantly lower than those of the larvae fed on control diets. The *in vivo* inhibitory effects of STI, either alone or with Bt, on the proteolytic activities of larval midgut proteases were significantly larger than that of Bt.

Bt δ-endotoxin, a most promising biocontrol agent, and soybean trypsin inhibitor, a potential phytoprotective element, synergized or potentiated the growth inhibition of *H. armigera*, although no additive effect on larval mortality was observed.

ACKNOWLEDGMENTS

We thank S. F. Zhang for providing some experimental equipment and technical advice. Thanks also to X. F. Xiang for help with bioassays. This work was funded by National Natural Science Foundation of China Grant No. 39670492 and a key grant of the Chinese Academy of Science.

REFERENCES

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **18,** 265–267.

Armes, N. J., Jadhav, D. R., and DeSouza, K. R. 1996. A survey of insecticide resistance in *Helicoverpa armigera* in the Indian subcontinent. *Bull. Entomol. Res.* **86,** 499–514.

Berenbaum, M. R. 1988. Allelochemicals in insect–microbe–plant interactions: Agents provocateurs in coevolutionary arms race. *In* "Novel Aspects of Insect–Plant Interactions" (P. Barbosa and D. K. Letourneau, Eds.), pp. 97–123. Wiley–Interscience, New York.

Bot, J. 1966. Rearing *Heliothis armigera* Hubn. and *Prodenia litura* F. on an artificial diet. *S. Afr. J. Agric. Sci.* **9,** 538–539.

Bradford, M. 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.

Broadway, R. M. 1995. Are insects resistant to plant proteinase inhibitors? *J. Insect Physiol.* **41,** 107–116.

Broadway, R. M., and Duffy, 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.

Burgess, E. P. J., Main, C. A., Stevens, P. S., Christeller, J. T., Gatehouse, A. M. R., and Laing, W. A. 1994. Effects of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Teleogryllus commodus*. *J. Insect Physiol.* **40,** 803–811.

Dymock, J. J., Laing, W. A., Shaw, B. D., Gatehouse, A. M. R., and Christeller, J. T. 1992. Behavioral and physiological responses of grass grub larvae (*Costelytra zealandica*) feeding on protease inhibitors. *N. Z J. Zool.* **19,** 123–131.

Felton, G. W., and Gatehouse, J. A. 1996. Antinutritive plant defense mechanisms. *In* "Biology of the Insect Midgut" (M. J. Lehane and P. F. Billingsley, Eds.), pp. 371–416. Chapman & Hall, London.

García-Carreño, F. L., Dimes, L. E., and Haard, N. F. 1993. Substrate–gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.* **214,** 65–69.

Gill, S. S., Cowles, E. A., and Pietrantonio, P. V. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.* **37,** 615–636.

Green, T. R., and Ryan, C. A. 1972. Wound-induced proteinase inhibitors in plant leaves: A possible defense mechanism against insects. *Science* **175,** 776–777.

Guo, Y. Y. 1995. "IPM on *Helicoverpa armiger*." Golden Shelf, Beijing. [in Chinese]

Houseman, J. G., Larocque, A. M., and Thie, N. M. R. 1991. Insect proteases, plant protease inhibitors, and possible pest control. *Mem. Entomol. Soc. Can.* **159,** 3–11.

Johnston, K. A., Gatehouse, J. A., and Anstee, J. H. 1993. Effects of soybean trypsin inhibitors on the growth and development of larval *Helicoverpa armigera*. *J. Insect Physiol.* **39,** 657–664.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277,** 680–685.

Larocque, A. M., and Houseman, J. G. 1990. Effect of ingested soybean, ovomucoid and corn protease inhibitors on digestive processes of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *J. Insect Physiol.* **37,** 691–697.

MacIntosh, S. C., Kishore, G. M., Perlak, F. J., Marrone, P. G., Stone, T. B., Sims, S. R., and Fuchs, R. L. 1990. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *J. Agric. Food Chem.* **38,** 1145–1152.

Qiu, X. J., and Lei, B. Q. 1986. The crystals of *Bacillus thuringiensis* var. *israelensis* and their effect on the midgut trypsin and chymotrypsin activities of mosquito larvae. *Acta Entomol. Sinica* **29,** 126–130.

Reichelderfer, C. F. 1991. Interactions among allelochemicals, some Lepidoptera, and *Bacillus thuringiensis* Berliner. *In* "Microbial Mediation of Plant–Herbivore Interactions" (P. Barnosa, V. A. Krischik, and C. G. Jones, Eds.), pp. 507–524. Wiley, New York.

Seldal, T., Dybwad, E., Andersen, K. J., and Högstedt, G. 1994. Wound-induced proteinase inhibitors in grey alder (*Alnus incana*): A defence mechanism against attacking insects. *Oikos* **71,** 239–245.

Steffens, R., Fox, F. R., and Kassel, B. 1978. Effect of trypsin inhibitors on growth and metamorphosis of corn borer larvae *Ostrinia nubilalis*. *J. Agric. Food Chem.* **26,** 170–175.

Wang, C. Z., and Qin, J. D. 1996. Partial characterization of protease activity in the midgut of *Helicoverpa armigera* larvae. *Acta Entomol. Sinica* **39,** 7–14.

Wang, C. Z., Xiang, X. F., Zhang, S. F., and Qin, J. D. 1995. Effect of soybean trypsin inhibitor on the growth and digestive physiology of *Helicoverpa armigera* larvae. *Acta Entomol. Sinica* **38,** 272–273.

Wu, Q. Y. 1986. A pathological study on mosquito larvae treated with *Bacillus thuringiensis* var. *israelensis*. *Acta Entomol. Sinica* **29,** 35–39.

Zhang, J. H., Wang, C. Z., Xiang, X. F., and Qin, J. D. 1997. Effect of dissolution and degradation on the toxicity of *Bacillus thuringiensis* delta-endotoxins to cotton bollworm. *Entomol. Sinica* **4,** 357–363.