Interaction of Cry1Ac toxin (Bacillus thuringiensis) and proteinase inhibitors on the growth, development, and midgut proteinase activities of the bollworm, Helicoverpa zea

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Abstract

Potential resistance development to Bt cotton in certain lepidopterans has prompted research to develop strategies that will preserve this environmental-friendly biotechnology. Proteinase inhibitors are potential candidates for enhancing Bt toxicity against lepidopteran pests and for expanding the spectrum of control for other insects. Interactions of Bt toxin from Bacillus thuringiensis and proteinase inhibitors were investigated by monitoring growth, development, and gut proteinase activities of the bollworm, Helicoverpa zea. Several proteinase inhibitors were combined with Bt protoxin Cry1Ac in artificial diet and fed to newly molted 3rd-instar bollworm larvae to determine effects on larval body weight and length, pupation progress, and mortality rate. Major midgut proteinase activities, including caseinase, trypsin, and chymotrypsin activities, were examined after treatment. A concentration of Bt at a level causing minimal mortality (<10%), was mixed with the following proteinase inhibitors: benzamidine, phenylmethylsulfonyl fluoride (PMSF), and N-α-tosyl-L-lysine chloromethyl ketone (TLCK). When compared with controls, the synergistic effect of Bt toxin and proteinase inhibitors caused significant decreases in mean larval weight and length over time. Midgut samples tested against the substrates azocasein, α-benzoyl-DL-arginine-p-nitroanilide (BApNA), and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPFpNA) showed significant decreases in the protease activity of larvae fed Bt plus inhibitor versus control. Interaction of Bt and proteinase inhibitors significantly retarded larval growth and resulted in developmental delay and up to 20% mortality.

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1. Introduction

The development of transgenic cotton varieties with insecticidal Bt genes (Bt cotton) has been one of the most successful applications of biotechnology research. Since the first introduction into the U.S. in 1995 [1], Bt cotton has been widely used to reduce feeding damage from many lepidopteran pests. Unlike conventional chemical applications, Bt cotton continuously maintains a toxic dose of Bt proteins, and delivers the toxins directly to the most sensitive stage of the target pest, i.e. once the insect hatches and starts to feed. After ingestion, Bt proteins are subjected to proteolytic processes by insect gut proteinases. Bt protoxins are activated mainly by trypsins and/or chymotrypsins. The activated toxins may bind directly to target sites on gut membrane and subsequently cause lysis of the gut and death of the insect. Activated Bt toxins may be subjected to further proteolysis and degradation by gut proteinases to form non-toxic segments. One of the proposed Bt resistance mechanisms indicated that reduced sensitivity to Bt is associated with the absence of proteolytic activity or an activation process that is present in the wild type strain [2–5], or excessive degradation by gut proteinases in resistant strains or less sensitive stages [6–8].
Currently, Bt toxins used for cotton insect control have a narrow range against lepidopteran pests only. Due to large scale adoption of Bt cotton and reduced chemical applications, many originally secondary pests, such as the tarnished plant bug, Lygus lineolaris, and stink bugs, have emerged to cause serious economic loss of cotton production. Because the gut proteinases play an important biochemical role in insect growth and development, these enzymes have been targets for proteinaceous inhibitors whose genes have been incorporated into transformed plants [9]. Jongsma [10] recently proposed use of novel proteinase inhibitor genes to control sucking mouthpart pests.

Because proteins are critical nutrients for insect growth and development, proteinases, such as trypsin, play an essential function for protein digestion and absorption in insects. The introduction of proteinase inhibitors into host plants may substantially suppress protein digestion, and subsequently achieve insect control in a broad range through nutrient deficiency. In addition, introduction of proteinase inhibitors into gut will certainly modify biochemical balance within target insects feeding on Bt cotton. Bt toxins may become stable and more effective against target insects. This study was designed to examine the interaction of Cry1Ac toxin and proteinase inhibitors on larval growth, development, and midgut proteinase activities in Helicoverpa zea.

2. Materials and methods

2.1. Chemicals

Tobacco budworm (Heliothis virescens) diet (#F9915B), USP agar (#7060), and USDA vitamin premix (#6265) were obtained from Bio-Serve (Frenchtown, NJ). Low melting point agarose (Invitrogen, Ultrapure L.M.P Agarose) was used in place of regular agar in diets testing the effects of selected proteinase inhibitors. Benzamidine, phenylmethylsulfonyl fluoride (PMSF), and N-α-tosyl-l-lysine chloromethyl ketone (TLCK), azocasein, α-benzoyl-DL-arginine-p-nitroanilide (BApNA), and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPFpNA) were obtained from Sigma Chemical Company.

2.2. Insect rearing and monitoring

Helicoverpa zea neonates were reared on regular artificial diet in the laboratory at a temperature of 26.5 °C with 40–60% humidity and 12:12 (L:D) light cycle. Newly molted 3rd instar larvae were fed various artificial diets to monitor the effects of added proteinase inhibitors, Bt, and any synergistic effects of proteinase inhibitors with Bt. Inhibitor stock solution was prepared in ethanol for PMSF, and in d-H2O for the remaining inhibitors. Artificial diet, designed to monitor proteinase inhibitor effects, was made by using a low melting point agarose and cooled to 33 °C in a water bath before inhibitors were added to the diet. The concentration of Bt remained constant at 15 ng/ml. The concentrations (w/v) for proteinase inhibitors in diet were as follows: benzamidine (0.5%), PMSF (0.02%), and TLCK (0.04%, 0.08%, 0.16%, and 0.32%). Larvae were regularly measured for changes in weight and length. Three repetitions of six larvae each were monitored until death or pupation occurred.

2.3. Preparation of midgut fluid

After 2, 4, and 6 days of feeding on diets, midguts of larvae were dissected in cold 0.1 M Tris–HCl, pH 8.0, over an ice block. Midguts were homogenized then centrifuged at 5000 rpm for 5 min to remove debris. Supernatant was collected and protein concentration was determined by the Bradford method [11] using Coomassie Plus Protein Assay kit (Pierce, Rockford, IL), with BSA as the protein standard. Midgut enzyme solutions were diluted to 1 mg/ml.

2.4. Proteinase activity assays

Total proteinase activity was measured with azocasein. Enzyme solution (5 µl) was mixed with 20 µl of 200 mM Tris–chloride, pH 8, 5 µl H2O, and 10 µl azocasein solution made in 0.05% SDS (sodium dodecyl sulfate). The reaction was allowed to run for 2.5 h at room temperature. After incubation, 30 µl of 10% TCA (trichloro acetic acid) was added. Reactions were placed on ice for 30 min and centrifuged at 14,000 g for 5 min to remove precipitated protein. After centrifugation, 60 µl of supernatant was added to 40 µl of 1 M NaOH. Absorbance was measured at 405 nm.

To study trypsin-like and chymotrypsin-like proteinase activities, the substrates BApNA and SAAPFpNA were used, respectively. For assays using these substrates, 5 µl enzyme solution was mixed with 45 µl universal pH buffer [12], pH 8. Activities were determined by the addition of 50 µl BApNA (1 mg/ml) or SAAPFpNA (1 mg/ml) in Frugoni Buffer, pH 8.5 (final substrate concentration was 0.5 mg/ml). Absorbance at 405 nm was monitored for 15 min at 37 °C with measurements taken every 15 s.

2.5. Data analysis

Means for each treatment were compared using ANOVA and separated using the Fisher’s protected least significance procedure at the α = 0.05 level (PROC GLM, SAS version 9.1, SAS Institute Inc. Cary, NC). Synergism is defined as the combined effect which is greater than the sum of their individual effects.

3. Results

3.1. Interaction of Bt and proteinase inhibitors on insect growth

After 2-day feeding on Bt or benzamidine treated diet, both larval body weight and length were significantly
(P < 0.01) reduced compared to those of control (Fig. 1A and B). Bt and benzamidine alone reduced body weight by 62% and 41%, respectively, after feeding for 5 days. Treatment with Bt and benzamidine together significantly reduced body weight by 77% (F(3) = 107.29, P < 0.0001). After feeding for 7 days, Bt and benzamidine alone reduced body weight by 31% and 11%, respectively. Treatment with combination of Bt and benzamidine significantly reduced body weight by 78% (F(3) = 23.48, P < 0.001). The interaction of Bt and benzamidine was significant (F(1) = 6.35, P < 0.05). Larval body length changes were similar to the body weight changes. After feeding for 5 days, Bt or benzamidine treatment alone reduced body length by 26% and 17%, respectively. Treatment with combination of Bt and benzamidine significantly reduced body length by 37% (F(3) = 13.95, P < 0.01). After feeding for 7 days, Bt and benzamidine alone reduced body length by 10% and 5%, respectively. Treatment with combination of Bt and benzamidine also significantly reduced body length by 44% (F(3) = 6.76, P < 0.05).

Results in Fig. 2A and B show that treatments with Bt alone or Bt plus PMSF consistently reduced larval body weight and length after 2, 4 or 6 days of post treatment. Approximately 48% and 66% body weight was reduced by Bt alone and Bt + PMSF, respectively, after 4 days (F(3) = 17.16, P < 0.0001). Body reduction remained at 22% for Bt alone, and 58% for Bt + PMSF treatment after feeding for 6 days (F(3) = 26.08, P < 0.001). Only up to 9% reduction was observed from treatment of PMSF alone. Interaction of Bt and PMSF on body weight at day 6 was significant (F(1) = 6.98, P < 0.05). Bt treatment alone reduced body length by maximal 21% after 4 days (F(3) = 8.02, P < 0.001). No significant difference was observed between PMSF and control after feeding for 2 or 4 days (P > 0.05). Only a minor reduction (8%) was obtained after 6 days. Maximal body length reduction (21%) was observed after treated with Bt for 4 days, and less than 7% reduction was detected after 6 days. Treatment with combination of Bt and PMSF significantly reduced body length by 14%, 34%, and 28%, respectively after 2, 4, and 6 days (P < 0.01).

The larval growth of H. zea was significantly decreased after feeding on diet treated with combination of Bt and TLCK (Fig. 3A and B). Four concentrations of TLCK (0.04–0.32%) were tested with and without Bt. Bt alone and 0.04% TLCK alone treatments had similar body weight with control (Fig. 3A) at day 8 after treatment. Treatment of 0.04% TLCK with 15 ng/ml Bt significantly reduced body weight by 49%. TLCK alone at 0.08%,

0.16%, and 0.32% levels also significantly reduced body weight by 17%, 30%, and 76%, respectively. With the addition of 15 ng/ml Bt, the three highest concentrations of TLCK reduced body weight by 53%, 56% and 83%, respectively. Interaction of TLCK and Bt on larval body weight at day 8 was significant (F(4) = 9.26, P < 0.001). Interaction of TLCK and Bt on larval body length was also significant (F(4) = 3.23, P < 0.05). With addition of 15 ng/ml Bt, 0.04%, 0.08%, 0.16%, and 0.32% TLCK reduced larval body length by 30%, 20%, 20%, and 55%, respectively, at day 8 (Fig. 3B).

3.2. Interaction of Bt TLCK on insect development and mortality

After fed on control diet for 12 days, all larvae pupated (Fig. 4A). TLCK at 0.04% and 0.08% levels did not delay insect development, and 100% of the larvae pupated on day 12 which was the same as for the control. For those larvae feeding on 0.16% TLCK diet, they spent 15 days longer (total 27 days) to reach 100% pupation, but the majority (more than 90%) developed to pupal stage at day 15. Most (approximately 85%) larvae pupated after feeding on 0.32% TLCK diet for 18 days. Bt treatment showed certain effect to delay larval development. Most (97%) larvae pupated at day 15, and 100% larvae developed to pupal stage after feeding on 15 ng/ml Bt diet (Fig. 4B). Combination of Bt and TLCK delayed larval development further. At day 12, less than 30% larvae developed to pupal stage (F(4) = 14.75, P < 0.0001). Interaction of Bt and TLCK was significant. Approximately 90% larvae pupated after feeding on Bt + 0.04% TLCK, Bt + 0.08% TLCK, and Bt + 0.16% TLCK diet. Bt + 0.32% TLCK treatment maintained lower pupation (c.a. 30%) for 30 days, and only 80% larvae pupated after 42 days.

All larvae survived from 15 ng/ml Bt treatment (Fig. 5). TLCK alone at levels between 0.04–0.16% did not kill any larvae. As much as 10% larvae were killed by 0.32% TLCK.

Fig. 3. Influence of Cry1Ac Bt toxin and four concentrations of proteinase inhibitor TLCK on larval growth of the bollworm. Bars topped with same letters are not statistically different at P = 0.05. (A) Larval body weight, (B) larval body length.

Fig. 4. Interaction of Cry1Ac Bt toxin and proteinase inhibitor TLCK on bollworm development. Result of the same experiment is showing as (A) Influence of different concentrations of TLCK on pupation rate; (B) interaction of Bt toxin and four concentrations of TLCK on pupation rate.

Fig. 5. Effect of Cry1Ac Bt toxin and four concentrations of TLCK on bollworm larval mortality.
treatment. Bt + 0.04% TLCK killed 3% larvae. Bt plus 0.08% TLCK and Bt + 0.16% TLCK killed 6% larvae. Bt combined with 0.32% TLCK resulted in 20% mortality.

3.3. Interaction of Bt and inhibitors on gut proteinase activities

Bt protoxin and three proteinase inhibitors, benzamidine, PMSF, and TLCK, were used to treat diet for feeding bollworm larvae. Gut enzymes were prepared after larvae were fed for 2, 4, and 6 days. Activities were analyzed using azocasein for general proteinase activity, BApNA for trypsin-like proteinase activity, and SAAPFpNA for chymotrypsin-like proteinase activity.

3.3.1. Bt + benzamidine

General proteinase activity increased 2.9-fold from day 2 to day 6 (Fig. 6A). After 6 days, the Bt + benzamidine treatment reduced general proteinase activity by 51%, compared to 26% and 8% reductions by Bt alone or benzamidine alone, respectively. Tryptic activity of control also increased 2.9-fold from day 2 to day 6 (Fig. 6B). Approximately 80% tryptic activity was suppressed by Bt + benzamidine treatment. Bt alone or benzamidine alone reduced activities by 43% or 9%, respectively. Chymotrypsin-like activity in control increased less than 2-fold from day 2 to day 6 (Fig. 6C). Approximately 54% of the activity was suppressed by Bt + benzamidine treatment. Bt alone or benzamidine alone suppressed chymotrypsin activities by 37% or 34%.

3.3.2. Bt + PMSF

From day 2 to day 6, general proteinase activities increased approximately 2-fold for the control, Bt alone, and PMSF alone, but stayed at the same level for Bt + PMSF treatment (Fig. 7A). Up to 60% activity was suppressed by Bt + PMSF. Only 15% or 12% activity was reduced by Bt alone or PMSF alone, respectively. Tryptic-like activity was inhibited by individual or combination of Bt and PMSF (Fig. 7B). Up to 48% or 40% activity were suppressed by Bt or PMSF alone. Activity suppression rate reached 84% in Bt + PMSF treatment. Chymotrypsin-like activity in control increases over time by 1.7-fold (Fig. 7C). Bt or PMSF alone reduced activity by 43% or 15%. Bt and PMSF together suppressed activity by 73%.

3.3.3. Bt + TLCK

General proteinase activity for control, Bt alone, and TLCK alone increased approximate 2-fold from day 2 to day 6 (Fig. 8A). Treatments with Bt alone and 0.04% TLCK alone did not alter general proteinase activity significantly. As much as 53% activity was suppressed by 0.32% TLCK alone. Up to 67% activity was suppressed by Bt + TLCK treatment. Interaction of Bt and TLCK was significant ($F_{(4)} = 7.44, P < 0.001$). Tryptic activity in control increased 2.2-fold from day 2 to day 6, and the other treatments significantly suppressed the activity (Fig. 8B). At day 6, Bt alone, 0.04% TLCK alone, and Bt + 0.04% TLCK showed similar BApNase activity. Up to 78% BApNase activity was inhibited by 0.32% TLCK alone treatment. With addition of 15 ng/ml Bt, 0.32% TLCK treatment could suppress as much as 89% trypsinic activity. Approximately 79% of the activity was inhibited by Bt + 0.08% TLCK and Bt + 0.16% TLCK. Chymotrypsin-like activity in control increased 2.5-fold from day 2 to day 6 (Fig. 8C). The activity was also sensitive to Bt and TLCK treatments. Bt alone suppressed 30% activity. TLCK alone inhibited 24–61% with increased inhibition as TLCK concentration increasing. Combination of Bt and TLCK suppressed 35–69% of the activity. Interaction of Bt and TLCK was significant ($F_{(4)} = 5.77, P < 0.01$).
Although Bt cotton has been successfully adopted to control the major lepidopteran insects on cotton, a major concern is the evolution of resistance by these pests. Many laboratory selection experiments with Bt toxins have indicated that several insect species have developed resistance to Bt toxins [13–18]. Large scale deployment of Bt transgenes and the high efficacy of transgenic Bt plants against target pest insects certainly imposes a selection pressure on naturally existing Bt-resistant genes to increase their frequencies in the populations. Eventually, the effectiveness of this environmentally sound method of pest control could be negated if resistance management strategies are not developed and properly carried out.

The major functions of the gut proteinases include hydrolyzation of ingested protein into peptides during the initial stages of protein catabolism. Serine proteinases are common luminal enzymes in midguts of many Lepidoptera species [19,20]. Serine proteinases in the midgut also activate protoxins, thereby mediating Bt toxicity [21,22], and they also may play a concurrent role in the hydrolytic degradation and subsequent inactivation of the toxic protein. It is also likely that development of Bt resistance is associated with gut proteinase profile and activity changes. Forcada et al. [6] provided evidences that midgut extracts from a Bt-resistant strain of *He. virescens* degraded the Bt toxin in vitro at a rate faster than extracts from a susceptible strain. Shao et al. [8] further proved that the excessive degradation of protoxin in *Heliothis armigera* midgut juice, which contained trypsin-, chymotrypsin-, and elastase-like enzymes, was responsible for the low sensitivity of this species to Bt, with chymotrypsin playing a major role in the degrading process.

4. Discussion

Fig. 7. Midgut proteinase activities of the bollworm on artificial diet treated with Cry1Ac Bt toxin and proteinase inhibitor PMSF. Bars topped with same letters are not statistically different at \( P = 0.05 \). (A) Azocaseinase activity, (B) trypsic activity, and (C) chymotryptic activity.

Fig. 8. Midgut proteinase activities of the bollworm on artificial diet treated with Cry1Ac Bt toxin and four concentrations of proteinase inhibitor TLCK. Bars topped with same letters are not statistically different at \( P = 0.05 \). (A) Azocaseinase activity, (B) trypsic activity, and (C) chymotryptic activity.
Cotton varieties transformed with genes for Bt toxins are designed to disrupt gut epithelial cell physiology of the target insects. However, the potential for gut proteinases to modulate the effectiveness of Bt protoxins in insects is high and was the impetus for more detailed studies on the interaction of Bt toxins and gut proteinase. In this study, we demonstrated the interaction of Bt and proteinase inhibitors. Insect growth was consistently retarded more by Bt + inhibitor treatment than by treatment of Bt alone or inhibitor alone. The synergistic effect was observed in most of our bioassay experiments. Stunted growth was further reflected by suppressed gut proteinase activities in insects treated with Bt + inhibitor.

The mechanisms of the Bt and proteinase inhibitor interaction have not been studied previously. By analyzing growth and gut proteinase activities, we suggest that Bt alone at a sub-lethal dose may damage gut tissues and reduce production of the proteinases. The damage could extend to a change in gut permeability and absorption of nutrients. These limited proteinases still maintain proteolytic activity for providing basic nutrients, and for processing Bt protoxins to toxins and degradation of toxins as well. Limited reduction of growth and proteinase activity are attributed to the damage of gut cell incurred by Bt binding. Most proteinase inhibitors function competitively with the substrate to share the active site of the enzyme to cause nutritional deficiency. Retarded growth is a common symptom of proteinase inhibition in insects. By feeding on diet containing relatively low dose of the proteinase inhibitor, insects were able to live without substantial reduction of body weight and length. The insect still have enough free proteinases to hydrolyze dietary proteins. Proteinase activity data proved that there is no substantial suppression of gut enzyme activity after inhibitor only treatment.

By applying a combination of Bt and individual proteinase inhibitor, significant reduction of larval body weight and length was obtained. The interaction (measured as growth reduction) of Bt and proteinase inhibitor for most measurements was greater than additive effect (reduction summation) of individual Bt and proteinase inhibitor treatment. Interaction was further reflected by proteinase activity data. Treatment of Bt + inhibitor consistently and significantly suppressed the enzyme activity in gut. The interaction was mostly synergistic, which is consistent with the results of the bioassay. Bt-inhibitor interaction is very likely to be achieved by suppression Bt degradation when proteinase inhibitor is present. Although low dose inhibitor treatment did not critically suppress the hydrolyzation of dietary proteins for nutritional requirements, it might significantly suppress gut proteinases, and subsequently suppress Bt degradation by gut enzymes. More Bt toxin stays actively to bind to targets and create more damage to the gut membrane. Extended gut damage results in further suppression of gut proteinase production, and a subsequent decrease in proteinase activity (Figs. 6–8).

\(N\alpha\text{-tosyl-l-lysine chloromethyl ketone}\) is an alkylating reagent that may react with histidine or cysteine residues at the active site of enzymes. It inactivates serine proteases such as trypsin and prevents proteolytic degradation. TLCK inhibited IL-1beta-induced nitrite formation, an oxidation product of nitric oxide produced by iNOS, in a concentration-dependent manner [23]. TLCK blocks the LPS- or cytokine-induced activation of nuclear factor \(\kappa\)B (NF \(\kappa\)B), which, in turn, blocks the induction of iNOS and COX-2 transcription at the level of mRNA and protein. The transcription factor NF \(\kappa\)B is essential for activation of a number of cytokine-inducible enzymes and was evaluated as a possible site of protease action necessary for IL-1beta-induced coexpression of iNOS and COX-2. In an analogous manner, TLCK inhibited IL-1beta-induced COX-2 enzyme activity (PGE2 formation) and COX-2 gene expression at the level of mRNA and protein. In this study, we found that TLCK is detrimental to growth and development of the bollworm. The biological and physiological effect was concentration-dependent as shown in Figs. 3–5 and 8. TLCK supposedly has no effect on chymotrypsin activity. However, we observed significant suppression (up to 69%) of chymotrypsin activity by treatment of Bt and TLCK. Statistics showed significant interaction of Bt and TLCK, indicating inhibition of trypsin activity by TLCK may enhance Bt toxicity. Increased Bt toxicity may damage the midgut tissue further, and then significantly reduce chymotrypsin production. Our results demonstrated that TLCK is a potent agent for pest management. TLCK, as well as other proteinase inhibitors, could be used directly for suppressing pest population through modifying biochemical and physiological processes. They also can be used for enhancing Bt performance through preventing and delaying resistance development.

In summary, we investigated the interaction between Bt toxin and proteinase inhibitors within the gut of \(H. \text{zea}\). Information from this study may lead to an understanding of how to enhance Bt toxicity and how to slow down tolerance or resistance development in insects. Currently in the Mid-south area, the primary concern is the increasing pest status of plant bugs due to reduced insecticide applications and resistance development since the broad adoption of Bt cotton varieties. Another major concern is the potential threat to Bt cotton as a result of resistance development in lepidopteran pests. A potential solution is the introduction of proteinase inhibitors into the ingestion and digestion systems of target pests. Excessive proteinase inhibitors directly suppress proteolytic processes of not only lepidopteran insects, but also non-lepidopteran insects, such as plant bugs with sucking mouth part. Proteinase inhibitors also synergize Bt toxicity by stabilizing Bt toxins within insect guts.

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