Available online at www.sciencedirect.com
ScienceDirect
Pesticide Biochemistry and Physiology 87 (2007) 39–46
PESTICIDE
Biochemistry & Physiology
www.elsevier.com/locate/ypest

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

Y.C. Zhu [a,*], C.A. Abel [a], M.S. Chen [b]

[a] *Jamie Whitten Delta States Research Center, ARS-USDA, Stoneville, MS 38776, USA*
[b] *ARS-USDA-GMPRC, Manhattan, KS 66502, USA*

Received 13 April 2006; accepted 17 May 2006
Available online 7 June 2006

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, tryptic, 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.

Keywords: Bt; Cry1Ac; *Bacillus thuringiensis*; Proteinase; Inhibitor; Resistance; Cotton; Bollworm; *Helicoverpa zea*

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].

* Corresponding author. Fax: +1 662 686 5421.
E-mail address: yczhu@ars.usda.gov (Y.C. Zhu).

0048-3575/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2006.05.004

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-H_2O 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 H_2O, 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 significant difference procedure at the $\alpha = 0.05$ 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