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Cloning eleven midgut trypsin cDNAs and evaluating the interaction of proteinase inhibitors with Cry1Ac against the tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae)

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ABSTRACT

Midgut trypsins are associated with Bt protoxin activation and toxin degradation. Proteinase inhibitors have potential insecticidal toxicity against a wide range of insect species. This study was conducted to evaluate the interaction of proteinase inhibitors with Bt toxin and to examine midgut trypsin gene profile of *Heliothis virescens*. A sublethal dose (15 ppb) of Cry1Ac, 0.75% soybean trypsin inhibitor, and 0.1% and 0.2% N-α-tosyl-L-lysine chloromethyl ketone significantly suppressed midgut proteinase activities, and resulted in reductions in larval and pupal size and mass. The treatment with inhibitor + Bt suppressed approximately 65% more larval body mass and 21% more enzymatic activities than the inhibitor-only or Bt-only. Eleven trypsin-like cDNAs were sequenced from the midgut of *H. virescens*. All trypsins contained three catalytic center residues (H^{73}, D^{153}, and S^{231}), substrate specificity determinant residues (D^{225}, G^{250}, and G^{261}), and six cysteines for disulfide bridges. These putative trypsins were separated into three distinct groups, indicating the diverse proteinases evolved in this polyphagous insect. These results indicated that the insecticidal activity of proteinase inhibitors may be used to enhance Bt toxicity and delay resistance development.

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1. Introduction

The tobacco budworm (TBW), *Heliothis virescens* (Fabricius), is an economically important insect on many field crops. Feeding damage to cotton may incur up to 29% yield loss (Frisvold, 2009). To protect cotton from insect feeding damage, several transgenic varieties have been developed that continuously express 1–2 insecticidal proteins, originally from *Bacillus thuringiensis* (Bt). With tremendous advantages of effective control of major cotton pests without frequent chemical sprays, Bt cotton acreage has increased dramatically since 1996. Wide scale implementation of Bt cotton has placed heavy selection pressure on the target insects. Potential evolution of Bt resistance in lepidopteran cotton pests (Gould et al., 1997) could rapidly decrease the value of Bt plant biotechnology. Another major concern is the shift of pest status. Due to their specificity mainly against lepidopterans and altered chemical control scheme, originally secondary crop pests, such as the tarnished plant bug (*Lygus lineolaris*), stink bugs, and other sucking insects and mites, have increased their pest status in recent years to cause significant economic losses to cotton (Outward et al., 2008). To prolong the benefit of this biotechnology, alternative control measures should be developed to relieve selection pressure and delay potential resistance development among many lepidopteran insects. Another consideration is to make cotton more versatile not only resistant to the lepidopterans via Bt, but also to non-lepidopterans, including emerging sucking insects.

Because natural products are environmentally sound, it is urgent to seek natural gene products that possess potent insecticidal activity. Theoretically, an insect's immediate physiological and biochemical contacts with a plant are enzymes in the midgut or saliva injected into plant tissues by sucking insects for extraoral digestion. A significant portion of midgut and saliva enzymes are proteinases, which are essential for growth and development in all classes of insects.

Trypsins and chymotrypsins are the major proteinases which break down dietary proteins in the midguts of lepidopterans. In addition, trypsin and chymotrypsin-like enzymes are involved in the activation of Bt protoxins, thereby mediating Bt toxicity (Martinez-Ramirez and Real, 1996; Oppert et al., 1996; Karumbaiah et al., 2007). To bind to target tissue receptors, the protoxin must be solubilized and proteolytically cleaved to an activated form by midgut proteinases (Ogiwara et al., 1992). The activated Cry protein then binds to specific receptors located on the midgut epithelium (Bravo et al., 2007). Proteinases also may play a concurrent role in the hydrolytic degradation and subse-

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quent inactivation of the toxic protein. Oppert et al. (1997) indicated that the lack of trypsin-like gut proteinases enabled some insects to adapt to Bt toxins via a mechanism where incomplete or non-activation of the protoxin occurs. In addition to less activation of Bt protoxins, resistant insects may detoxify Bt toxins through excessive hydrolyzing degradation in the midgut.

Proteinase inhibitors can be used to suppress dietary protein digestion and to achieve suppression of insect growth and development. For insect control, Bt toxins coupled with proteinase inhibitors could delay pest adaptation to both groups of insecticidal control proteins (Pannetier et al., 1997). It was suggested that the increase in Bt toxicity observed with protease inhibitors may be due to a reduction in the degradation of toxin in insects with an appropriate adaptive mechanism, such as those with proteases capable of hydrolyzing toxins (Pang and Gringorten, 1998). The introduction of proteinase inhibitors may increase the activity spectrum of Bt plants to insects that degrade the toxin, either by a species-specific trait or an adaptive mechanism (Oppert, 1999).

Because of the importance of midgut proteinases on insect growth and development and potential association with Bt toxicity and Bt resistance, the characterization of midgut proteinases will provide critical information needed to develop effective resistance management strategies. To evaluate insecticidal effect of proteinase inhibitors and potential interaction with Bt, we directly incorporated two proteinase inhibitors into artificial diet to study their effect on midgut proteinase activities and larval growth and development in *H. virescens* to evaluate their potential insecticidal effects. We also treated the budworm larvae with a combination of proteinase inhibitor and Cry1Ac to investigate interactions between these bio-active reagents with Bt toxin. To develop an alternative approach, such as RNAi, for suppression of midgut proteinases and to target specific proteinase genes responsible for Bt resistance, we initiated cloning and characterization of midgut trypsin cDNAs.

2. Materials and methods

2.1. Insect and chemicals

Tobacco budworm (*H. virescens*) larvae were obtained from USDA-ARS Rearing Laboratory in Stoneville, MS, and were maintained at 26.5 °C and 40–60% humidity on an artificial diet (containing Tobacco Budworm Diet [#F9915B], USP agar [#7060] and USDA vitamin premix [#6265]) purchased from Bio-Serve (Frenchtown, NJ).

Ultra-pure low melting point agarose was obtained from Invitrogen (Carlsbad, CA). *B. thuringiensis* MVP II (Cry1Ac protoxin) was a gift from Monsanto (St. Louis, MO). Soybean trypsin inhibitor (STI), N-α-tosyl-L-lysine chloromethyl ketone (TLCK), azocasein, N-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA), and N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPFpNA) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Larval growth on the diet supplemented with Cry1Ac and proteinase inhibitors

Stock solutions of STI, TLCK, and Bt were prepared in d-H_2O. To determine the interaction of Bt and proteinase inhibitors on larval growth, each of the two proteinase inhibitors (STI, TLCK) was individually combined with Cry1Ac. The bio-reagent mixtures were incorporated into the artificial diet. A bio-reagent only and a solvent/buffer only (negative control) were included in the treatments. To reduce over heating bio-reagents, ultrapure-low melting point agarose was used at 20 g per litter to replace regular agar. The low melting point agarose allowed the diet to be cooled to 33 °C before the appropriate concentrations of the bio-reagents were added to minimize heat damage to the reagents. Bt protoxin concentration, 15 ppb Cry1Ac, was determined through pre-trial experiments demonstrating low mortality and minor effects on larval weight and length when compared to control larvae.

Tobacco budworm neonates were first reared on an artificial diet containing no Bt or proteinase inhibitors. Newly molted 2nd instar larvae were fed on artificial diets containing selected proteinase inhibitors and Bt protoxin. The concentrations (w/v) for proteinase inhibitors in diet were as follows: STI at 0.75%, and TLCK at 0.1% and 0.2%. Larvae were periodically measured for changes in weight and length. Three repetitions of 6 larvae each (for a total of 18 larvae) were monitored until death or pupation occurred. The treated insects were maintained in a growth chamber at a temperature of 26.5 °C with 40–60% humidity.

2.3. Preparation of midgut fluid and proteinase activity assays

Larval midguts were dissected in cold 0.1 M Tris–HCl (pH 8.0) over an ice block. Midguts were homogenized then centrifuged at 2655*g* for 5 min to remove debris. The supernatant was collected and protein concentration was determined by the Bradford method using Coomassie Plus Protein Assay (Pierce, Rockford, IL) with BSA as the protein standard (Bradford, 1976).

To study trypsin-like and chymotrypsin-like proteinase activities, the substrates N-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA) and N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPFpNA) were used, respectively. Activities were determined by the addition of 50 μl of BApNA or SAAPFpNA (at 1 mg/ml) in Frugoni Buffer (Frugoni, 1957), 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 (Zhu and Baker, 1999).

Total proteinase activity was measured with azocasein. The following were added to each reaction: 20 μl of 200 mM tris-chloride, pH 8, 5 μl H_2O, and 10 μl azocasein solution made in 0.05% SDS. The reaction was allowed to run for 2.5 h at room temperature (21 °C). After incubation, 30 μl of TCA (10%) 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 NaOH (1 M). Absorbance was measured at 405 nm (Zhu et al., 2007).

2.4. Data analysis

Data were statistically analyzed with SAS program (SAS Institute 2003). PROC GLM procedure was used for analysis of variance. Mean separation was conducted using SAS Proc GLM followed by the Fisher's Protected LSD test ($\alpha = 0.05$).

2.5. RNA preparation, cDNA library construction, and sequencing

Fresh midgut tissue, dissected from 4th instar larvae, was immediately ground in TriZol reagent (Invitrogen, Carlsbad, CA). Total RNA was precipitated with isopropanol. mRNA was purified using NucleoTrap mRNA purification kit (BD Bioscience Clontech, Palo Alto, CA). To construct cDNA libraries, approximately 1 μg midgut mRNA was used for reverse transcription. The Creator Smart cDNA Library Construction kit (BD Bioscience Clontech) was used for cDNA library construction. cDNA was ligated into pDNR-LIB vector (Clontech). The ligation was used to transform TOP10 competent cells (Invitrogen), which were then plated on chloramphenicol-agar plates. Approximately 6000 clones were manually picked and sequenced with M13 forward primer on an ABI 3730XL sequencer (Applied Biosystems Inc., Foster City, CA) located at the Genomics and Bioinformatics Research Unit in Stoneville, MS.