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# Combined effects of three crystalline toxins from *Bacillus thuringiensis* with seven proteinase inhibitors on beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae)

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ABSTRACT

*Spodoptera exigua* is a highly polyphagous pest and causes extensive damage to many field and truck crops. Since the larvae are not or only sublethally affected by Cry1Ac, the fused Cry1Ac/Cry1Ab, or the stacked Cry1Ac + CpTI transgenic cottons that are widely planted in China, *S. exigua* has become a major economic pest of cotton across a wide distribution since the commercialization of Bt cottons in 1997 in China. Proteinase inhibitors are potential candidates for enhancing Bt toxicity against, and for expanding control spectrum for insect pests. In the present paper, we first found that *S. exigua* larval midgut fluids could remarkably degrade activated Cry1Ca, and slightly hydrolyze Cry1Ac and Cry1Ab. Subsequently, we investigated interactions between the 3 Cry toxins and 7 proteinase inhibitors, i.e., phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, tannic acid, *N*-α-tosyl-L-phenylalanine chloromethyl ketone, *N*-α-tosyl-L-lysine chloromethyl ketone, elastatinal and ethylenediaminetetraacetic acid (EDTA), by monitoring larval growth and mortality rate. A 6-day dietary exposure of the newly molted 2nd instars to either the inhibitors (3 test concentrations) or the toxins (Cry1Ac, 31.30 ng/cm$^2$; Cry1Ab, 3.2 ng/cm$^2$; Cry1Ca, 0.6 ng/cm$^2$) alone only slightly affected larval growth. In contrast, exposure to the mixtures containing an inhibitor and a toxin, with the exception of those containing EDTA, synergistically reduced larval weight. In general, the synergisms were more obvious at higher inhibitor concentrations. Regarding larval mortality, the inhibitors except EDTA at the highest test concentration showed significant synergism to both Cry1Ab (0.2 μg/cm$^2$) and Cry1Ca (0.04 μg/cm$^2$). As the inhibitor concentration reduced, however, the synergistic effects decreased. These results indicated that trypsin-, chymotrypsin-, and elastase-like proteinases in *S. exigua* larval midgut were involved in proteolytical hydrolyzation of the 3 activated Cry toxins, and protection of Bt Cry toxins from proteinase degradation in the midgut by inhibitors may greatly enhance toxicity against *S. exigua* larvae.



## 1. Introduction

The crystalline protein inclusions formed by *Bacillus thuringiensis* (Bt) during growth consist of toxins with insecticidal activities (Cry toxin) [1,2] against the larvae of many insect species in Lepidoptera, Coleoptera, and Diptera [3]. Transgenic cotton produces active Cry protein(s) throughout its life cycle, is considerably effective against some Lepidopteran pests such as *Helicoverpa armigera* and *Pectinophora gossypiella*, and is highly beneficial to growers and the environment by reducing chemical insecticide applications and preserving arthropod natural enemies [4]. Thus, the transgenic cottons have been widely planted and reached 3.8 million hectares in China in 2007 [5] and 58.4 million hectares worldwide in 2011 [6].

The beet armyworm *Spodoptera exigua* is a common insect pest around the world [7]. The larva is a highly polyphagous herbivore and is known to feed on more than 290 plant species. It causes extensive damage to many field and truck crops. Among the attacked crops are cotton, corn, sugar beet, sunflower, alfalfa, potato, asparagus, pea, tomato, onion, and citrus [8,9]. *S. exigua* larvae have been documented to be sensitive to some Cry toxins such as Cry1Ab and Cry1C [10], and to the stacked Cry1Ac + Cry2Ab [11,12], the stacked Cry1F + Cry1Ac [13], and Vip3a transgenic plants [14]. In contrast, *S. exigua* larvae are not or only sublethally intoxicated by Cry1Ac [15–17], the fused Cry1Ac/Cry1Ab [18–20], or the stacked Cry1Ac + CpTI transgenic cottons [19] that were planted in China. Thus, *S. exigua* was predicted to be one of the outbreaking insect pests in China in 2001 [21]. Consistent with the

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prediction, the survival rate and adult fecundity of *S. exigua* fed on Bt cotton increased significantly during the three successive generations [18]. This indicated that continuous exposure to Bt cotton will increase the adaptation and fitness of *S. exigua* gradually. Consequently, the larva has become a major economic pest of cotton across a wide distribution since the commercialization of Bt cottons in 1997 in China [22,23]. To prolong the benefit of this biotechnology, the transgenic cottons expressing other Cry proteins and alternative control methods should be developed [18–20].

Insect gut proteinases are major enzymes involved in digestion of dietary proteins. According to their mechanism of catalysis, proteinases are classified into four categories: serine proteinases, cysteine proteinases, aspartic proteinases and metalloproteinases [24]. In Lepidopteran insects such as *S. exigua*, serine proteinases dominate larval midgut and contribute to about 95% of total digestive activity [25]. Serine proteinases proteolytically hydrolyze internal peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the enzyme's active site. Serine proteinases fall into three categories in insect species, trypsin-, chymotrypsin- and elastase-like enzymes [24]. Moreover, a metalloproteinase is any proteinase enzyme whose catalytic mechanism involves a metal, such as zinc or cobalt. Metalloproteinases contribute to about 1% of the total digestive activity in Lepidoptera [26]. In Lepidoptera insects, gut proteinases also participate in the proteolytic processing of Bt proteins [27–28]. On one hand, larval midgut trypsins and/or chymotrypsins activate Cry protoxins [29–31]. On the other hand, the proteinases from larval midguts can further degrade activated Cry toxins to form non-toxic segments. In *S. exigua*, larvae feeding on Bt vs. non-Bt cotton for 1, 6 or 24 h significantly increased the trypsin activity in the midgut [32]. Moreover, the trypsin activities were significantly increased after *S. exigua* larvae were subjected to three-generation's selection on transgenic Bt vs. non-Bt cotton [33]. Furthermore, a *S. exigua* strain resistant to Cry1C-producing Bt constitutively over-expressed a trypsin and a serine proteinase, but down-expressed 2 chymotrypsins [34]. These results indicate that larval midgut proteinases are potentially associated with the detoxification of Cry toxins in *S. exigua*.

Proteinase inhibitors can be used to suppress dietary protein digestion, to reduce insect growth and development, and to decrease insect survival and biomass [27,28,35]. Moreover, proteinase inhibitors can also inhibit proteolytic activation or degradation of Cry toxins to make them less or more effective against insect pests [36,37]. Among proteinase inhibitors, phenylmethylsulfonyl fluoride (PMSF) is a serine proteinase inhibitor that binds specifically to the active site serine residue. Similarly, soybean trypsin inhibitor (SBTI) can form a 1:1 stoichiometric complex with the catalytic site of serine proteinases, including trypsin and to a lesser extent chymotrypsin. Tannic acid is also an inhibitor of serine proteinase. *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) can irreversibly inhibit trypsin-like serine proteinases by alkylating the histidine residue in the active site. *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is the irreversible chymotrypsin inhibitor and alkylates a single histidine residue in the active site of the enzyme. Elastatinal is an effective elastase specific inhibitor. Moreover, ethylenediaminetetraacetic acid (EDTA) is widely used for scavenging metal ions to deactivate metal-dependent enzymes. It is an inhibitor of metalloproteinases [25]. Proteinase inhibitors are potential candidates for enhancing Bt toxicity against and for expanding control spectrum for insect pests. Thus, interactions between Cry toxins and proteinase inhibitors deserve extensive studies in *S. exigua*.

Since transgenic plants express modified, truncated versions of *cry* genes that yield active toxin fragments in China [38], we selected 3 activated Cry toxins Cry1Ac, Cry1Ab and Cry1Ca in the present paper, and confirmed the degradative activities of *S. exigua* larval midgut fluids to the 3 Cry toxins by an *in vitro* experiment. Moreover, we investigated interactions between the 3 Cry toxins and 7 proteinase inhibitors (PMSF, SBTI, tannic acid, TLCK, TPCK, elastatinal, EDTA) by monitoring larval growth and mortality rate. Our results revealed synergism between the Cry toxins and the proteinase inhibitors in *S. exigua* larvae.

## 2. Materials and methods

### 2.1. Insects

The beet armyworm, *S. exigua* was obtained from *Brassica rapa chinensis* at Nanjing (32.0N, 118.5E), Jiangsu Province in China in 2009. The insects were routinely reared on an artificial diet (27.5 g wheat-germ powder, 7.5 g soybean flour, 2.0 g yeast, 2.5 g agar, 93 ml water, 0.2 g Wesson salt mixture, 0.7 g vitamin C, 0.41 mg B1, 0.82 mg B2, 0.41 mg B6, 0.01 mg B12, 0.04 mg biotin 1.63 mg nicotinamide, 0.41 mg folic acid, 1.63 mg calcium pantothenate, 32.64 mg inositol and 0.2 g citric acid) similar to that described previously [39], without exposure to any Cry toxin, in an insectary under controlled temperature (28 ± 1 °C), photoperiod (14 h light/10 h dark) and relative humidity (70–80%).

At laboratory reared by above protocol, *S. exigua* eggs hatched in approximately 5 days. Larvae molted 5 times in around 16 days. Pupae emerged into adults in an average of about 7 days. The life cycle was completed in roughly 30 days.

### 2.2. Chemicals

Cry1Ac, Cry1Ab and Cry1Ca were activated toxins, and were obtained from Envirologix Inc. in the USA. Phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), elastatinal, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), β-mercaptoethanol, glycerol, bromophenol blue, dimethyl sulfoxide, dithiothreitol, coomassie bright blue R-250, acrylamide, *N*,*N*′-methylene-bis-acryl-amide and tetrametheylenediamine were obtained from Sigma–Aldrich (St Louis, MO). Tannic acid, sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)aminomethane (Tris) were acquired from Beijing Chemical Reagent Co. (Beijing, China). All compounds were analytical grade. These chemicals were stored in a refrigerator between the experimental sessions.

To prepare stock solutions, PMSF was dissolved in ethanol; TLCK was in 1 mM HCl; Cry1Ac, Cry1Ab and Cry1Ca were prepared in aqueous phosphate buffer (0.05 M, pH 10); and SBTI, EDTA, TPCK, TLCK and tannic acid were in double distilled water ($ddH_2O$) (Table 1).

### 2.3. Determination of proteinase activity with electrophoresis

After 24 h of starvation, the midguts of the 5th-instar larvae were dissected in cold 0.1 M Tris–HCl (pH 8.0) over an ice block. Midguts were homogenized and then centrifuged at 2655 × g for 5 min to remove debris. Supernatant was collected, and protein concentration was determined by Bradford's method using Coomassie Plus Protein Assay (Pierce, Rockford, IL) with BSA as the protein standard [40].

Eight micrograms of each toxin was mixed with the same quantity of prepared midgut supernatant or $ddH_2O$ (control). The mixtures were kept at 27 °C for 15 min, 30 min, 1 h and 2 h, respectively. After reaction, the samples were treated at 100 °C for 5 min after being mixed with an equal volume of sample buffer, which contained 50 mM Tris–HCl (pH 6.8), 1% SDS, 2% β-mercaptoethanol, 10% glycerol, 2 mM EDTA, and 0.1% bromophenol blue.