

## Proteinase-mediated Insect Resistance to *Bacillus thuringiensis* Toxins\*

(Received for publication, June 4, 1997, and in revised form, July 7, 1997)

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**Two *Bacillus thuringiensis* (Bt)-resistant strains of the Indianmeal moth, *Plodia interpunctella*, lack a major gut proteinase that activates Bt protoxins. The absence of this enzyme is genetically linked to larval survival on Bt-treated diets. When considered with previous data supporting the existence of receptor-mediated insect resistance to Bt, these results provide evidence that insect adaptation to these toxins occurs through multiple physiological mechanisms, which complicate efforts to prevent or manage resistance to Bt toxins in insect control programs.**

Insecticidal proteins of the bacterium *Bacillus thuringiensis* (Bt)<sup>1</sup> are effective for controlling many insect pest species, but insect resistance threatens the long term effectiveness of these toxins (1). With the introduction of transgenic plants expressing Bt toxins in the field (2), insects will likely be under increased selection pressure for resistance. To develop effective toxin resistance management strategies, a full understanding of the physiological and genetic mechanisms by which insects become resistant to these insecticidal proteins is needed.

More than 100 insecticidal crystal protein genes from Bt subspecies have been described.<sup>2</sup> These genes encode protoxins, referred to as Cry proteins, that have different specificity for lepidopteran, coleopteran, or dipteran insects. The Cry1 subclass consists of lepidopteran-active Bt protoxins with apparent molecular masses of approximately 130 kDa, which are solubilized and processed (activated) by gut enzymes to approximately 65-kDa toxins (4). Toxins interact with receptors in the guts of susceptible insects resulting in pore formation in mid-gut cell membranes, ionic imbalance, and consequent septicemia in the insect. Alterations in insect gut physiology or biochemistry could disrupt this sequential process and result in toxin resistance.

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<sup>1</sup> The abbreviations used are: Bt, *Bacillus thuringiensis*; BApNA, *N*- $\alpha$ -benzoyl-L-arginine *p*-nitroanilide; PAGE, polyacrylamide gel electrophoresis.

<sup>2</sup> For an up to date list and explanation of nomenclature, see: [http://epunix.biols.susx.ac.uk/Home/Neil\\_Crickmore/Bt/toxins.html](http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/toxins.html).

Several mechanisms of insect resistance to Bt toxins have been proposed (5). One involves changes in the binding of toxins to gut receptors. In a Bt subspecies *kurstaki*-resistant strain of the Indianmeal moth, *Plodia interpunctella*, reduced binding of Cry1Ab toxin to larval brush border membrane vesicles was associated with increased resistance to the toxin (6). Decreases in toxin binding have also been reported in resistant strains of the diamondback moth, *Plutella xylostella* (7–12), tobacco budworm, *Heliothis virescens* (13, 14), and beet armyworm, *Spodoptera exigua* (15). However, reduced toxin binding is not always associated with resistance to Bt (16, 17), and therefore alternate mechanisms of resistance must exist.

A second mechanism of resistance may involve gut proteinases that interact with Bt toxins. Enzymes from a strain of *H. virescens* resistant to Bt subspecies *kurstaki* (HD-73) were reported to process the protoxin more slowly and to degrade toxin faster than enzymes from a susceptible strain (18). In *Spodoptera littoralis*, an increase in the specific activity of gut proteinases from fifth instar larvae was associated with a loss of sensitivity to Cry1C, possibly due to an increase in the degradation of toxin (19).

Previously, we reported that a strain of *P. interpunctella* resistant to Bt subspecies *entomocidus* has low soluble gut proteinase and Bt protoxin-hydrolyzing activities when compared with the parent-susceptible strain and a strain resistant to Bt subspecies *kurstaki* (20, 21). The slower protoxin hydrolysis observed with gut extracts from the *entomocidus*-resistant strain was an indication that proteinase-mediated mechanisms are involved in resistance to Bt. In this report, we characterize the proteolytic enzyme activity in Bt toxin-resistant insects and demonstrate a genetic linkage between the absence of a major gut proteinase and decreased susceptibility to the toxin.

### EXPERIMENTAL PROCEDURES

**Insect Dissection**—Late fourth instar larvae were chilled, and the posterior and anterior ends were removed. Guts were excised, immediately submerged in ice-cold 200 mM Tris, pH 8.0, 20 mM CaCl<sub>2</sub> (buffer A), aliquoted 1 gut per 25  $\mu$ l of buffer, and frozen at  $-20^{\circ}\text{C}$  for up to 2 weeks until assayed.

**Proteinase Activity Assays**—For microplate proteinase assays, samples were thawed and spun at 15,000  $\times$  g for 2 min, and the supernatant containing soluble gut enzymes was used in assays. Samples were diluted 1:100 in buffer A, and 50  $\mu$ l were added to a microplate well. *N*- $\alpha$ -Benzoyl-L-arginine *p*-nitroanilide (BApNA, Sigma, 100 mg/ml in dimethyl sulfoxide) was diluted 1:100 in buffer A, and 50  $\mu$ l were added to each well to initiate the reaction (final BApNA concentration was 1.15 mM). After a 30-s incubation at 37  $^{\circ}\text{C}$ , absorbance was monitored at 405 nm at 15-s intervals over a 5-min period. The change in absorbance per min was calculated by the software KinetiCalc3 (Biotek), and the data were converted to micromoles/min/mg of protein in each gut extract. Protein concentration was determined by the method of Bradford (22) using bovine serum albumin as the standard in a microplate assay (Pierce).

For proteinase activity blots, gut extracts from individual larvae or from five pooled individuals were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10–20% Tricine gels (Novex, 0.4 gut eq/lane). The proteinase activity blot procedure was performed as described previously (21).

For zymogram analysis, gut samples were first analyzed for BApNA-hydrolyzing activity by microplate analysis. Volumes of samples were then adjusted for equivalent activity ( $\Delta A_{405\text{ nm}}/\text{min/ml} = 2$ ), and aliquots were subjected to SDS-PAGE on a 10–20% Tricine gel (Novex). Zymogram analysis was performed by incubating the gel with a Cry1Ac protoxin substrate solution (see below) for 2 h at 37  $^{\circ}\text{C}$ , followed by brief

TABLE I

BAPNA-hydrolyzing activity in gut extracts from a Bt-susceptible strain of *P. interpunctella* (688s) and strains resistant to Bt subspecies *aizawai* (133-r), *entomocidus* (198-r), and *kurstaki* (Dpl-r) maintained on treated diets

Extracts were analyzed for the ability to hydrolyze the substrate N- $\alpha$ -benzoyl-L-arginine *p*-nitroanilide (BAPNA).

Strain	Specific activity <sup>a</sup>	Activity relative to strain 688-s
		%
688-s	0.84 $\pm$ 0.09	100
133-r	0.36 $\pm$ 0.06	43
198-r	0.39 $\pm$ 0.06	46
Dpl-r	1.06 $\pm$ 0.10	126

<sup>a</sup> Units are micromoles/min/mg of protein in gut extract ( $\epsilon = 0.87 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , 0.3-cm path length). The number of individual insects used in each sample was 16  $\pm$  S.E.

washes in deionized water and staining with Coomassie Blue, as described previously (21).

Cry1Ac protoxin substrate solution was obtained from Bt subspecies *kurstaki* HD-73 grown on glucose/yeast extract/salts medium at 30 °C for 2–3 days with agitation until sporulation was complete (23). Spores, crystals, and cellular debris were washed by centrifugation three times in phosphate-buffered saline and lyophilized. The lyophilized preparation was resuspended in 100 mM sodium carbonate, pH 10.0, 0.8 M sodium chloride, 10 mM EDTA, 20 mM dithiothreitol. After centrifuging at 1000  $\times$  g for 10 min, the supernatant was diluted 1:1 with deionized water and analyzed for Cry1Ac protoxin concentration by SDS-PAGE, Coomassie Blue staining, and scanning gel densitometry (International Technologies International) using homogenous Cry1Ac protoxin as the standard (data not shown). The concentration of Cry1Ac protoxin in the zymogram substrate solution was determined to be 0.3% and constituted greater than 50% of the total protein in the preparation.

**Genetic Matings**—Fifteen virgin male adults from Bt-susceptible strain 688-s', in which all individuals displayed the enzyme T1 (see "Results" for explanation of enzyme terminology), were mated with 15 virgin female *entomocidus*-resistant strain 198-r adults, which lacked T1. Gut extracts from approximately 15-day-old larvae from each strain were analyzed for BAPNA hydrolytic patterns by proteinase activity blots. Fifteen female F1 adults were backcrossed to 15 resistant 198-r males, and progeny were analyzed for BAPNA hydrolytic patterns. Female F1 were chosen because meiotic recombination does not occur in female Lepidoptera, and tight linkage would be observed between any pair of traits located on the same chromosome. Insects were reared on untreated diet throughout the matings.

**Proteinase Activity in Backcross Insects**—Ten mg of eggs from the backcrossed progeny were added to an untreated diet, and 15 mg were added to a 125 mg/kg Bt subspecies *entomocidus* (HD-198)-treated diet. Adults were selected from each group ( $n = 27$  from the untreated group and  $n = 12$  from the treated group) and placed on an untreated diet and allowed to mate. Progeny from each group were reared on an untreated diet, and guts were dissected from late fourth instar larvae. Gut extracts were analyzed for proteolytic patterns using proteinase activity blot analysis.

## RESULTS

Because trypsin-like proteinases in *P. interpunctella* guts activate Bt protoxin (21), we examined trypsin-like enzyme activity in Bt-susceptible and resistant strains of the Indian-meal moth. Using the trypsin diagnostic substrate BAPNA in a microplate assay, BAPNA-hydrolyzing activities were detected in a Bt toxin-susceptible strain of *P. interpunctella*, 688-s, and three resistant strains, 133-r, 198-r, and Dpl-r, derived from the susceptible parental strain via selection with spore/crystal preparations of Bt subspecies *aizawai* (HD-133), *entomocidus* (HD-198), or *kurstaki* (HD-1, Dipel®),<sup>3</sup> respectively (24) (Table I). Specific activities of enzymes in the *aizawai*- and *entomocidus*-resistant strains were less than one-half of those in the *kurstaki*-resistant strain and in the parent-susceptible strain. Activity from the *kurstaki*-resistant strain was approximately 30% higher than activity from the parent-susceptible strain.

<sup>3</sup> Dipel® is a registered trademark of Abbott Laboratories.

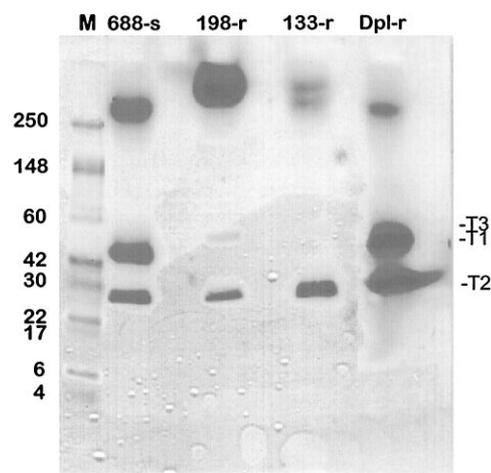


FIG. 1. Proteinase activity blots of gut extracts from a Bt-susceptible (688-s) strain of *P. interpunctella* and strains resistant to Bt subspecies *entomocidus* (198-r), *aizawai* (133-r), and *kurstaki* (Dpl-r). M, molecular mass standards (MultiMark-Novex).

To determine the number and relative size of BAPNA-hydrolyzing enzymes in each strain, larval gut extracts from the *P. interpunctella* strains were analyzed for their ability to hydrolyze BAPNA using activity blots (Fig. 1). Enzymes with relatively high molecular masses (>250 kDa) were observed in extracts from all four strains. Two major BAPNA-hydrolyzing enzymes, designated T1 and T2 (apparent molecular masses ~45 and ~25 kDa, respectively), were observed in the Bt toxin-susceptible strain (688-s) and also in the Dipel-resistant strain (Dpl-r). The enzyme corresponding to T1, however, was not present in gut extracts from the strains resistant to either subspecies *entomocidus* (198-r) or subspecies *aizawai* (133-r). A minor trypsin-like enzyme, T3, was observed in all of these strains, which had an apparent molecular mass of approximately 50 kDa.

To determine whether these trypsin-like enzymes hydrolyze protoxin, an experiment was designed to compare the relative hydrolysis of protoxin by gut extracts from Bt-susceptible and -resistant *P. interpunctella* strains. Gut proteinases from the four *P. interpunctella* strains were separated electrophoretically by SDS-PAGE, and the gel was incubated in a solution of Bt subspecies *kurstaki* (HD-73) spores and crystals containing Cry1Ac protoxin as the major protein (Fig. 2). Dark zones in the zymogram demonstrated activity of enzymes from the gut extracts, which are capable of hydrolyzing proteins in the spore/crystal protein preparation. Major hydrolyzing activity corresponded to enzymes T1 and T2 in the susceptible and *kurstaki*-resistant strains but only T2 in the *aizawai*- and *entomocidus*-resistant strains. T1 and T2 have similar levels of activity. However, T2 appears to have a higher level of activity because it comigrates with another protoxin-hydrolyzing enzyme identified as one with chymotrypsin-like specificity (data not shown). No hydrolysis of protoxin by the higher molecular mass enzymes (*i.e.* the >250-kDa BAPNA-hydrolyzing enzymes) was observed.

Until now, BAPNA-hydrolyzing enzymes had only been examined in extracts from pooled insects (Fig. 1). When gut extracts from individual susceptible larvae were used in enzyme activity blots, we observed that the strain consisted of a heterogeneous population of insects with respect to the expression of trypsin-like proteinases. Some insects expressed T1 and T2, whereas others expressed only T2 (data not shown). Therefore, single pair lines of susceptible strain 688-s were established to ensure genetic uniformity for subsequent linkage tests, and progeny were analyzed using proteinase activity

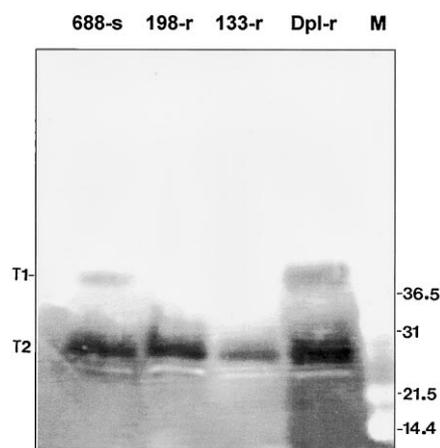


FIG. 2. Zymogram analysis of gut extracts from a Bt-susceptible (688-s) strain of *P. interpunctella* and strains resistant to Bt subspecies *entomocidus* (198-r), *aizawai* (133-r), and *kurstaki* (Dpl-r) using a 0.3% solution of HD-73 spores and crystals as the substrate. M, molecular mass markers (Mark 12-Novex). For better photographic reproduction, the inverse image of the gel is shown. Because this is a gradient gel, diffusion of substrate into the gel is much greater at the top than at the bottom.

blots. Progeny from one of these “isofemale” lines, designated as 688-s’, were analyzed for four generations, and it was determined that all individuals in this population expressed both T1 and T2 trypsin-like enzymes (a representative blot is shown in Fig. 3, 688-s’).

To determine if the T1 allele cosegregates with toxin resistance in *P. interpunctella*, bulked segregant analysis was used. Insects from the homogeneous susceptible strain 688-s’ were mated with those from the *entomocidus*-resistant strain 198-r, and female F1 progeny were subsequently backcrossed to males from the resistant strain. Individual larvae from the parental colonies, the F1 generation, and the backcross were analyzed by proteinase activity blots for BApNA-hydrolyzing enzymes (representative blots are shown in Fig. 3). Based on the analysis of 40 single gut extracts from each strain, the susceptible and resistant strains appeared to be homozygous for the presence and absence, respectively, of the T1 allele. Both parental strains were fixed for T2, since all individuals displayed the T2 enzyme. In the F1 generation, 70 individuals were examined and all exhibited T1 enzyme activity. However, the activity of T1 in the F1 larvae was lower than that in the susceptible strain, consistent with the hypothesis that the F1 were heterozygous for the T1 allele. Of the 70 larvae analyzed for BApNA hydrolytic activity in the backcross, 29 (41%) lacked the T1 enzyme, whereas 41 (59%) expressed both T1 and T2, which is close to the expected 1:1 segregation ratio ( $\chi^2 = 2.86$ , degrees of freedom = 1,  $p = 0.09$ ).

Insects from this backcross population were then analyzed for the ability to survive on toxin-treated diets. Fifty eggs were placed on 0, 125, 250, and 500 mg of Bt subspecies *entomocidus* HD-198 per kg of diet, and mortality was determined. A discriminating dose of 125 mg/kg was found to kill all susceptible and heterozygous resistant larvae (data not shown). Mortalities at the three Bt toxin doses, corrected for the control mortality, were 59, 78, and 97%, respectively (25). At the 125 mg/kg dose, 41% of the backcross insects survived, the same proportion of the backcross that lacked the T1 enzyme when reared on untreated diet.

The frequency of the T1 enzyme in descendants of backcross progeny survivors reared on untreated versus toxin-treated diets was determined by proteinase activity blots (Fig. 4). In the untreated group, 20 out of 50 insects (40%) lacked the T1 trypsin-like proteinase, whereas the remainder had both T1

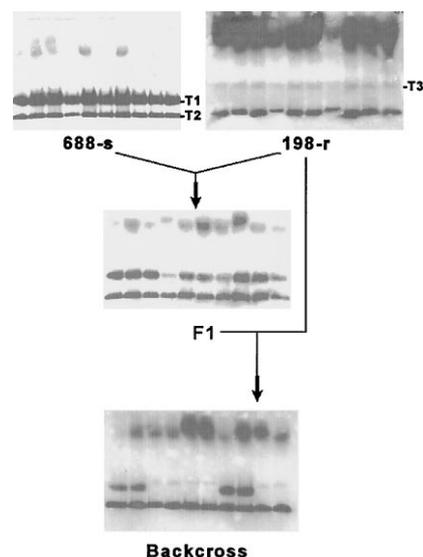


FIG. 3. Representative proteinase activity blots using BApNA and gut extracts from *P. interpunctella* larvae from the mating experiment. Individuals from a Bt-susceptible strain (688-s’) were mated to those from subspecies *entomocidus*-resistant strain (198-r). The F1 generation was then backcrossed to 198-r insects.

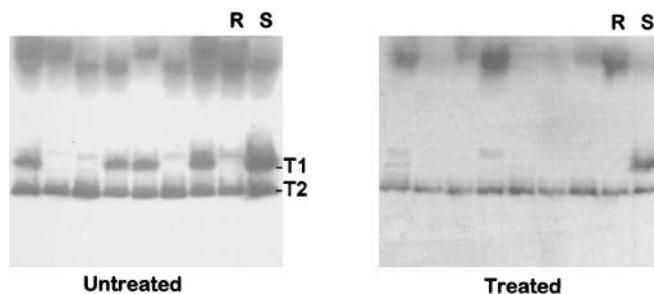


FIG. 4. Representative proteinase activity blots using BApNA. *P. interpunctella* backcross progeny were reared on untreated or treated (125 mg/kg HD-198) diets, adults from each treatment were placed on untreated diet, and gut extracts from individual offspring were analyzed for BApNA-hydrolytic enzymes. Lanes R (gut extract from *entomocidus*-resistant 198-r) and S (gut extract from susceptible 688-s’) are included as controls.

and T2. To eliminate differences in enzyme activity due to dietary toxin influences, adults from backcross progeny surviving on an untreated or HD-198-treated diet (125 mg/kg of diet) were intercrossed *en masse* and placed on untreated diet to lay eggs. Resulting larvae were analyzed for proteinase patterns. In the progeny from untreated diet, 30 out of 50 displayed both T1 and T2 enzymes. All of the progeny from the survivors on the Bt-treated diet (50 out of 50), however, lacked the major trypsin-like proteinase T1.

#### DISCUSSION

Results from a microplate assay of BApNA hydrolysis by *P. interpunctella* gut extracts demonstrated that activity was reduced in the *aizawai*- and *entomocidus*-resistant larvae when compared with *kurstaki*-resistant or susceptible larvae. We have also observed slower protoxin hydrolysis with enzymes from the *aizawai*-resistant strain, similar to results obtained with the *entomocidus*-resistant strain (20, 21).<sup>4</sup> Therefore, resistance may be a result of reduced proteolytic activity in the *aizawai*- and *entomocidus*-resistant strains, but it is probably unrelated to proteinase activity in the *kurstaki*-resistant strain. The *kurstaki*-resistant strain is probably resistant due

<sup>4</sup> B. Oppert, K. J. Kramer, and W. H. McGaughey, unpublished data.

to a receptor adaptation, like another *kurstaki*-resistant strain of *P. interpunctella* (5).

Results from a proteinase activity blot assay using BApNA as the substrate revealed that *aizawai*- and *entomocidus*-resistant larvae lack a major BApNA-hydrolyzing enzyme, T1. The absence of T1 activity in these strains was also evident in zymogram analysis of Bt toxin-hydrolyzing activity. Since T1 is a protoxin-hydrolyzing enzyme, the lack of an active T1 in the *aizawai*- and *entomocidus*-resistant strains would result in less toxin being generated in the gut. We propose that the absence of this T1 enzyme leads to reduced activation of protoxin and, therefore, provides a survival advantage when insects feed on diets containing Bt toxins.

Matings of susceptible insects that have both T1 and T2 enzymes with resistant insects that have only the T1 enzyme resulted in progeny with comparable T2 activity but only a relatively low T1 activity. A backcross of these insects to the resistant strain resulted in progeny consisting of a mixture of phenotypes, with individuals either displaying both T1 and T2 or only T2. All backcross larvae that survived a discriminating dose of Bt toxin lacked the T1 enzyme. Therefore, when insects do not have an active T1, they are able to survive a dose of toxin that is lethal to insects that are homozygous or heterozygous for T1. These data demonstrate a genetic linkage between resistance and the absence of T1 and are strong evidence for a proteinase-mediated mechanism of resistance to Bt.

Alleles of structural genes that have no product detectable by electrophoresis are called "null" alleles and are usually recessive (26). In other null alleles, the absence of the active enzyme is due to a mutation that results in an inactive protein or prevents its synthesis in some way. Esterase null alleles are the most commonly described. The number of copies of a gene EST-2, coding for esterase B in mosquitoes, is more than 250 times greater in insecticide-resistant mosquitoes than in sensitive strains (3). In that case, the null allele is normal for sensitive insects. In *P. interpunctella*, however, the null allele is a survival advantage for the Bt-resistant insects. This is the first description of a null allele for a trypsin-like proteinase coding gene of which we are aware.

In conclusion, we have demonstrated that some Bt toxin-resistant strains of *P. interpunctella* have lower BApNA-hydrolyzing and protoxin-activating abilities than a susceptible or another resistant strain. These differences are due to the lack of a major gut trypsin-like proteinase in the resistant strains. Results of a genetic analysis demonstrated that insect resistance to Bt toxins segregated with the loss of this major trypsin-like proteinase. The absence of the trypsin-like enzyme apparently results in reduced levels of toxin in the gut, allowing the insects to avoid exposure to high levels of toxin.

This research has led to the understanding that both the genetic variability in the insect population and the composition of the toxin preparation can influence resistance mechanisms. Selection for reduced proteinase activity apparently is possible

with only certain Bt preparations. However, these preparations will not select for a proteinase-deficient phenotype if the genotype is not already present in the population.<sup>5</sup> It is not clear how Bt formulations differ such that they elicit different resistance responses in insects. Understanding the biochemical bases for resistance development in insect populations exposed to different Bt formulations will provide for more effective toxin selection, utilization, and durability in integrated pest management programs that utilize Bt toxins.

*Acknowledgments*—We appreciate the excellent technical assistance provided by Richard Hammel, Roy Speirs, Staci Schmeiser, and Kris Hartzler.

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<sup>5</sup> B. Oppert, K. J. Kramer, and W. H. McGaughey, unpublished observations.